Abstracts of papers presented at the
LVIII Cold Spring Harbor Symposium
on Quantitative Biology

DNA & CHROMOSOMES

June 2–June 9, 1993

Arranged by
Bruce Alberts, *University of California, San Francisco*
Bruce Stillman, *Cold Spring Harbor Laboratory*

Cold Spring Harbor Laboratory
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Cover: Human metaphase chromosomes hybridized with a telomere probe $(AATG_3)_7$ biotin probe-FITC Avidin detection, propidium iodide counterstain. (Courtesy of Dr. David Ward, Yale University School of Medicine.)

PROGRAM

WEDNESDAY, June 2—7:30 PM

Welcoming Remarks: James D. Watson

SESSION 1 INTRODUCTION

Chairperson: B. Alberts, University of California, San Francisco

THURSDAY, June 3—9:00 AM

SESSION 2 CHROMOSOME REPLICATION

Chairperson: B. Brewer, University of Washington, Seattle

THURSDAY, June 3—2:30 PM

SESSION 3 SILENCING AND VARIEGATION

Chairperson: S.C.R. Elgin, Washington University

THURSDAY, June 3—4:30 PM

Wine and Cheese Party

THURSDAY, June 3—7:30 PM

SESSION 4 METHYLATION AND IMPRINTING

Chairperson: **R.J. Roberts,** New England Biolabs
Beverly, Massachusetts

FRIDAY, June 4—9:00 AM

SESSION 5 CHROMOSOME MACHINERY

Chairperson: E. Blackburn, University of California, San Francisco

FRIDAY, June 4—3:00 to 5:00 PM

SESSION 6 POSTER SESSION I

FRIDAY, June 4—7:30 PM

SESSION 7 TRANSCRIPTION AND CHROMATIN

Chairperson: H. Varmus, University of California, San Francisco

SATURDAY, June 5—9:00 AM

SESSION 8 CHROMOSOME DOMAINS

Chairperson: J. Gall, Carnegie Institution, Baltimore

SATURDAY, June 5—3:00 PM

PICNIC

SATURDAY, June 5—7:30 PM

SESSION 9 CENTROMERES, TELOMERES AND REPLICATORS

Chairperson: D. Gottschling, University of Chicago, Illinois

SUNDAY, June 6—9:00 AM

SESSION 10 DNA AND CHROMATIN STRUCTURE

Chairperson: J. Thomas, University of Cambridge, United Kingdom

SUNDAY, June 6—2:00 PM

SESSION 11 CHROMOSOME ORGANIZATION AND STABILITY

Chairperson: R. Kahmann, Universität München, Germany

SUNDAY, June 6—5:30 PM

DORCAS CUMMINGS LECTURE

Eric S. Lander

Whitehead Institute for Biomedical Research and Dept. of Biology
Massachusetts Institute of Technology, Cambridge

"Mapping Genes and Genomes"

MONDAY, June 7—9:00 AM

SESSION 12 TRANSCRIPTION ACTIVATION

Chairperson: T. Maniatis, Harvard University

MONDAY, June 7—3:00 to 5:00 PM

SESSION 13 POSTER SESSION II

MONDAY, June 7—7:30 PM

SESSION 14 RECOMBINATION AND REPAIR

Chairperson: C. Laird, University of Washington, Seattle

TUESDAY, June 8—9:00 AM

SESSION 15 GENOME STRUCTURE

Chairperson: G. Bernardi, Institut Jacques Monod, Paris, France

TUESDAY, June 8—2:30 PM

SESSION 16 GENE REGULATION

Chairperson: **S.M. Tilghman,** Howard Hughes Medical Institute, and Dept. of Molecular Biology, Princeton University

TUESDAY, June 8

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

WEDNESDAY, June 9—9:00 AM

SESSION 17 NUCLEAR STRUCTURE

Chairperson: G. Felsenfeld, NIDDK, National Institutes of Health

Summary: H. Weintraub, Howard Hughes Medical Institute Laboratory, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington.

AUTHOR INDEX

THE REPLICON : THIRTY YEARS LATER

F. Jacob, Institut Pasteur, Paris

In 1963, the Cold Spring Harbor Symposium for Quantitative Biology was devoted to "Synthesis and Structure of Macromolecules". We had there, with Sydney Brenner and François Cuzin, a paper entitled "On the regulation of DNA replication in bacteria". At the beginning of the 60's nothing was known about the mechanisms controlling DNA replication and its coupling with cell growth and division. The enzyme isolated by Arthur Kornberg was able to produce copies of a DNA template in the presence of a template but, in contrast to what was occurring *in vivo*, it did not appear to obey any control *in vitro*. The main theme of the paper we gave at the Cold Spring Harbor meeting of 1963 was the so-called "Replicon model". What I would like to do here is to recall briefly the main points of the model and to summarize the way they have evolved 30 years later.

GENES

By S. Brenner
Department of Medicine
Cambridge University, Clinical School, Cambridge, England and
Scripps Research Institute, La Jolla, California, USA

The molecular biologist's dream is to be able to compute organisms from their DNA sequences. For a long time, geneticists pursued this in microorganisms and simple multicellular organisms using the tools of classical genetics. We isolated mutants and analysed them as deeply as we could. The invention of cloning and sequencing has not only enhanced our ability to analyse the consequences of gene mutation in classical situations but offers radical alternatives to the study of genetics by breeding experiments.

Cloning and sequencing of DNA provide a direct approach to the study of genes and genomes, but obtaining the complete sequence of any organism, even those with small genomes, is still a major task. We have been characterizing the compact 400 Mb genome of the pufferfish, Fugu, and shown that it is eight times smaller than mammalian genomes but is likely to contain all or most of the genes found in higher vertebrates. The genome contains very little repetitive DNA; it is probably a more direct representation of the basic vertebrate genome, unexpanded by the 'junk' that has accumulated in mammalian genomes during the course of evolution. Since the new technologies of molecular genetics allow us to separate the requirements for genome analysis from those for the study of gene functions, we do not have to use the same organism for both. Thus we can use Fugu for gene discovery and gene identification and evaluate the sequence information by recourse to transgenic experiments in mammals or in cells derived from them. In addition, direct comparisons of gene sequences and their function in different organisms might tell us what has happened in genomes during the evolution of complex function.

SPECIFIC ACTIVATION IN REPRESSION OF TRANSCRIPTION

M. Ptashne, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA

Over the past few years many of us have adopted a plausible but highly incomplete view of how gene regulation is effected in higher (as well as lower) organisms. According to that view, proteins bind to DNA and interact with other proteins to activate and repress genes; the mechanisms involved would explain how genes can be expressed in overlapping sets using a minimal number of regulators and extracellular signals. I will review the evolution of certain aspects of that picture and speculate as to how the results of ongoing and future experiments might cause its modification.

FROM SIGMA FACTORS TO TAFs: RELATED FUNCTIONS WITH UNEXPECTED COMPLEXITIES. R. TJIAN, Howard Hughes Medical Institute, Dept. of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

How the thousands of genes encoded by an organism's DNA are transcribed in a temporally regulated and cell-type specific manner is a fundamental question that remains to be fully understood. Much of what we know about transcriptional control was first discovered by studying bacteria and their phages. Elegant biochemical and genetic analysis of RNA polymerases, sigma factors, repressors and activators led to many of the concepts that today continue to influence our interpretation of gene regulatory mechanisms. These early successes suggested that analogous model organisms would also be essential to study the control of gene expression in eukaryotes. In the 1970's, Cold Spring Harbor and a handful of other laboratories made the critical decision to study animal viruses. In the ensuing 20 years, many unexpected findings that are now paradigms for eukaryotic gene regulation were established from the work with animal viruses. For example, experiments with SV40 and adenovirus led to the identification of mammalian site-specific DNA binding proteins, transcriptional enhancers, nuclear oncogenes, interactions with tumor suppressors, and RNA splicing. These early studies also triggered the development of *in vitro* DNA replication reactions and the biochemical dissection of the eukaryotic transcriptional machinery. Rapid progress in defining the components of these enzyme systems in turn heightened our awareness of the intrinsic complexities that govern gene regulation in higher organisms.

As an example of a complex machinery that has evolved to deal with gene regulation, I would like to describe some recent progress in characterizing a novel family of transcription factors we call TAFs, that appear to serve as multi-subunit "sigma factors" in promoter recognition. Studies of both Drosophila and human transcription initiation complexes revealed the presence of seven TAFs that are tightly associated with the TATA binding protein (TBP), and that are required for response to promoter-selective activators (repressors). Based on *in vitro* studies of transcription activation, we propose that TAFs are responsible for directing interactions between sequence-specific regulators and the basal machinery. Recent experiments with recombinant proteins establish that some TAFs indeed make direct contact with site-specific activators to mediate transcriptional enhancement while others are engaged in specific TAF-TAF and TAF-TBP interactions. Similar investigations also provided evidence that distinct TAF-TBP complexes are responsible for differential promoter recognition by RNA polymerases I, II, and III. These studies illustrate the conservation of function and mechanisms governing transcription regulation from bacteria to man, but also underscore the nature of the complex machinery demanded by the regulation of gene expression in higher organisms.

HIV REVERSE TRANSCRIPTASE: STRUCTURE AND SPECIFICITY FOR tRNA

T.A. Steitz, L.A. Kohlstaedt, J. Wang, S. Smerdon, J. Jäger, P.A. Rice and J.M. Friedman, Departments of Molecular Biophysics and Biochemistry and Chemistry, Howard Hughes Medical Institute, Yale University, 260 Whitney Avenue, New Haven, CT USA 06511.

The structure of HIV reverse transcriptase (RT) co-crystallized with a non-competitive inhibitor, Nevirapine, has been determined at 3.2 Å resolution. The polymerase (Pol) domain of the 66 kD subunit has a large cleft while the p51 subunit shows no such cleft. One of the 4 pol subdomains at the base of the cleft has the same structure and conserved catalytic residues as the Klenow fragment suggesting that the catalytic subdomains of polymerases evolved from a common precursor. A-form RNA-DNA hybrid can be model built into the deep cleft that extends between the pol and RNase H active sites. Nevirapine binds in a pocket adjacent to the DNA and at the base of a "thumb-like" protrusion. Mutations in RT that are resistant to Nevirapine have altered protein side-chains contacting the inhibitor. RT side-chains whose mutation reduces the sensitivity of RT to AZT and DDI appear to be largely in contact with the model-built template strand. Experiments on the initiation of RT by $tRNA_3^{Lys}$ and $tRNA_2^{Gln}$ show that both the 3' 18 nt. of the tRNA and the anticodon loop are interacting with the viral template. A 2nd crystal form is being solved by molecular replacement and a 3rd co-crystal form with tRNA is under study.

Sequences and Proteins Controlling Yeast Chromosomal DNA Replication

Stephen P. Bell, York Marahrens, Hai Rao, and Bruce Stillman. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Chromosomal replication in the yeast *Saccharomyces cerevisiae* is controlled by short, well defined origins of DNA replication. Fine structure analysis using a plasmid borne copy of one of these origins of replication, ARS1, indicates that it is composed of four distinct sequence elements; an essential element, **A**, which includes a perfect match to the ARS consensus sequence, and three important elements, **B1**, **B2**, and **B3**. We have now assayed the function of each of these elements at their native chromosomal position using 2-D gel electrophoresis of replication intermediates. These findings indicate that each of the elements also functions in the chromosome. We have also tested the generality of the modular nature of yeast origins of replication by performing a mutational analysis of ARS307. Like ARS1, ARS307 is composed of multiple sequence elements and these elements are interchangeable between the two origins of replication.

In addition to identifying the cis-acting sequences required for ARS1 function we have also identified two trans-acting factors that are likely to mediate the function of one or more of these elements. The yeast transcriptional regulator ABF1 binds to and mediates the function of the B3 element. A second multi-protein complex, the origin recognition complex (ORC), binds specifically to the essential ARS consensus sequence in an ATP dependent manner. ORC is composed of 6 different proteins and the genes encoding each of these proteins have now been cloned. Interestingly, two of these genes have been genetically identified as regulators of transcriptional silencing. Possible roles for ORC in linking DNA replication and transcriptional regulation will be discussed.

INITIATION AT A MAMMALIAN CHROMOSOMAL ORIGIN OF REPLICATION

J.L. Hamlin[1], P.J. Mosca[1,2], and P.A. Dijkwel[1], [1]Department of Biochemistry and [2]Graduate Program in Biophysics, University of Virginia, Charlottesville, VA 22908.

The methotrexate-resistant CHO cell line, CHOC 400, has amplified one allele of the dihydrofolate reductase (DHFR) gene and flanking sequences ~1,000 times. The major amplicon species is 240 kb in length and is analogous to a high copy number plasmid, except that the amplicons are arrayed tandemly in the body of a chromosome. Utilizing *in vivo* labeling strategies, we showed previously that replication initiates somewhere within the 55 kb intergenic region lying between the DHFR and 2BE2121 genes. More recent studies have employed high resolution two-dimensional gel electrophoretic methods to analyze replication intermediates in this locus. This approach has led to the surprising conclusion that, *in vivo*, initiation occurs at multiple sites scattered throughout the intergenic region, rather than at a single, fixed "origin". We have also not been able to detect any altered chromatin structure in the intergenic region in nuclease hypersensitivity studies. Furthermore, we have not been able to detect the autonomous replication of any cloned sequences from this region in mammalian cells. Thus, if there is a *cis*-regulatory element (origin/replicator) in this locus, it will be difficult to identify by the methods that were used to localize origins in microorganisms.

However, we have recently shown that the plant amino acid, mimosine, specifically inhibits initiation of replication in mammalian chromosomes. Based on the properties of this compound, it is likely to act directly on or modify an origin-binding protein. The isolation of the target protein should therefore allow identification of the *bonafide* replicator in the DHFR locus, as well as all other cellular origins that are prevented from firing by this drug. We have been able to cross-link radiolabelled mimosine specifically to a 50 Kda polypeptide in cell extracts, and are in the process of utilizing this assay to identify a cDNA clone that expresses the target protein either in bacterial or animal cell hosts. The protein is short-lived and quite labile to freezing and thawing. It is therefore possible that the inability of *in vitro* extracts to support initiation from cloned chromosomal origins is due to the nature of the initial extract preparation.

DNA REPLICATION IN METAZOAN CHROMOSOMES

M.L. DePamphilis, D.M. Gilbert, H. Miyazawa, F.S. Nallaseth, and J.M. Ortega, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA.

Initiation of DNA replication in metazoan chromosomes does not occur randomly throughout the genome, but is localized to specific sites that may include entire intergenic regions. Techniques that map origins of DNA replication in single-copy sequences of metazoan chromosomes by measuring the distribution of newly synthesized DNA during S-phase in mammalian cells reveal that most replication forks (>80%) emanate from specific origins of bidirectional replication (OBR) that occupy 0.5 to 2 kb. Techniques that detect replication structures such as forks and bubbles by 2-D gel electrophoresis reveal that initiation events are scattered throughout a larger "initiation zone" of 30 to 55 kb, but not within genes. 2-D gel analyses alone reveal that most initiation events at sites of programmed gene amplification in fly chromosomes occur within a 1 kb locus although replication bubbles are found throughout a 6-8 kb initiation zone.

We have undertaken two sets of experiments in an effort to identify those parameters that define a metazoan origin of replication. First we have asked whether or not *Xenopus* eggs or egg extracts, which have previously been shown to replicate essentially any DNA molecule, will preferentially utilize a known mammalian OBR if it is presented either as a circular or linear chromosome or as an intact nucleus. Second, we have asked whether or not bovine papillomavirus DNA, which was previously reported to regulate its copy number through negative as well as positive cis-acting sequences, provides a suitable model for how cellular origins function. Our conclusions are: (1) prior to the blastula stage, *Xenopus* does not preferentially utilize a mammalian origin, regardless of how it is presented. (2) The BPV genome contains cis-acting sequences that suppress either the polyomavirus or SV40 origins of replication, but not the BPV origin. Suppression does not require any BPV proteins. These and other data demonstrate that BPV is not a suitable model for how initiation of cellular DNA replication is limited to once per cell cycle.

The Replication of BPV-1 Chromatin

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Three ideas have been proposed for the mechanism by which enhancer proteins act as auxiliary factors in eukaryote DNA replication. (1) One notion posits that these proteins negate the repressive effects of the core histones by direct or indirect binding to DNA, thus ensuring, in some unknown way, that the actual replication machinery can assemble on the DNA template. (2) This point of view is that transcription factors directly make contact with proteins that are more naturally seen as replication factors, and that this contact stabilizes or promotes a preinitiation complex. (3) Another reasonable position would be that both mechanisms are important, and that one process depends upon the other. For example, competition with the histone octamer for binding to a DNA site may be dependant upon an enhancer protein's physical interaction with other proteins in the replication apparatus.

To address this issue we have exploited an *in vitro* replication system provided by BPV-1 DNA. For papilloma virus replication, the E2 enhancer protein cooperatively assists in binding of the E1 helicase to the viral origin. *In vitro* utilizing naked DNA as template, E2 can stimulate replication by 10–20 fold at limiting E1 levels. This stimulation is not dependant upon an E2 DNA binding site. We suggest that a DNA site for E2 is not critical in this case because of strong protein:protein E1:E2 interactions, and that E2 is an allosteric effector for E1 binding. When BPV-1 chromatin is used as a template, E2 binding sites are required. Thus E2 may serve a dual function in replication enhancement: cooperative binding with E1 and antirepression. Along similar lines we have found that GAL-VP16 can enhance BPV-1 DNA replication when GAL-4 sites are placed beside the origin. We show that GAL-VP16 binds to the cellular factor RPA and that sequestering the SSB to the duplex may promote enhanced replication for conversion from a closed double stranded DNA to an open complex is a critical step in initiation. GAL-VP16 can also effectively counter the repressive effects of the histone octamer, and this requires GAL-4 sites. On these particular templates E2 sites are dispensable for the chromatin replication. We suggest that the interaction with RPA in this latter case is also critical for "anti-repression" as well as activation. Thus, our data support position 3 as summarized above.

DNA REPLICATION, CHROMATIN STRUCTURE, AND TRANSCRIPTIONAL SILENCING

S. Loo, C. Fox, D. Rivier, M. Foss, S. Okamura, P. Laurenson, K. Zito, J. Rine, Dept. of Molec. and Cell Biology, Univ. of California, Berkeley

We have studied the mechanism of transcriptional silencing of HMR and HML by combining in vitro studies of the repressed chromatin with genetic studies of essential genes that have a role in silencing. Nuclei were isolated in which the repressed chromatin at HMR was intact. Using an assay based on HO accessibility and extending it to a battery of bacterial restriction endonucleases, we mapped the boundaries of the SIR-dependent chromatin as being beyond the E and I elements and show that the extent of the repressed region does not shrink even in the absence of I. This provides a serious challenge to all models of silencing requiring looping between E and I. We also show that a 2kb fragment containing HMR can be excised from the chromosome without losing repression, which may make the repressed structure amenable to biochemical analysis.

The HMR-E silencer contains three functional domains: the ARS consensus, the RAP1 site, and the ABF1 site. We show that this silencer is a chromosomal origin of replication that depends upon the ARS and the RAP1 site for firing and for silencing; ABF1 is dispensable for both of these processes. We also used a crippled silencer to find partially defective alleles of essential genes that derepress HMR. To-date we have isolated temperature-sensitive cdc-like mutations in genes encoding two subunits of the origin recognition complex (ORC) of Bell and Stillman, hinting strongly of a role for this complex in both DNA replication and silencing. A topoisomerase active site consensus is present in ORC2, one of the subunits isolated. By mutating the active site tyrosine, we show that this sequence has no essential role in ORC2 function. Conditional alleles of ABF1 complete the missing evidence for ABF1's role in silencing. Additional genes from this screen currently under study should extend these insights.

Finally, we have evidence that SIR1 may act at the HMR-E silencer since LexA-SIR1 and GAL4-SIR1 fusions can functionally replace the RAP1 protein in an appropriately marked silencer. The effect of this fusion protein on replication and its dependence on other SIR genes will be described.

ROLES OF DNA REPLICATION AND GENE SILENCING IN THE MECHANISM OF FISSION YEAST MATING-TYPE SWITCHING

A. Klar, J. Singh, G. Thon, M. Bonaduce, NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Developmental Genetics Section, Laboratory of Eukaryotic Gene Expression, P.O. Box B, Bldg. 539, Frederick, Maryland 21702-1201.

Only one cell of a pair of sister cells switches mating type in *S. pombe*. This developmental asymmetry between sister cells is shown to be the consequence of inheriting specific parental DNA chains at the *mat1* locus. In particular, the process of DNA replication itself produces nonequivalent sister chromatids. *mat1* switching is initiated by a cut at *mat1*. The cut is repaired in the following cell cycle by transposing a copy of the silent donor (*mat2* or *mat3*) locus resulting in a switch of *mat1*. We found that *swi7*, a gene required to generate the cut, encodes the catalytic subunit of DNA polymerase α. We propose that the act of DNA replication itself advances the developmental program such that developmentally nonequivalent sisters result.

Despite the presence of promoter sequences, the *mat2* and *mat3* donor loci remain transcriptionally inactive. We identified the *clr1* (*c*ryptic *l*oci *r*egulator) locus, whose product is required for the donor loci silencing. Mutations in the *clr1* gene also release the meiotic recombination block found between *mat2* and *mat3* loci. Our working model is that *clr1* functions to confer a specific chromatin structure to the mating-type region causing gene silencing and cross-over suppression. Since the sequence-predicted Clr1 protein contains three Zn^{++} fingers, we are testing if it binds to sequences around the silent genes.

Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with ABL.

TELOMERIC POSITION EFFECT: SPREADING THE SILENCE

Hubert Renauld, Oscar M. Aparicio, Paul D. Zierath, Barbara L. Billington, Sanjay K. Chhablani, and Daniel E. Gottschling; Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois.

In *S. cerevisiae*, telomeres repress transcription of genes located nearby. This region-specific gene inactivation is thought to involve the packaging of telomeric domains into silent chromatin. To gain insight into the mechanism of telomeric silencing, a genetic assay to examine the spread of silencing along the distal right arm of chromosome V was developed. The frequency of silencing a telomere-adjacent *URA3* gene decreased with increasing distance of the gene's promoter from the telomere, irrespective of transcriptional orientation. The distance over which telomeric silencing of *URA3* was observed was extended by weakening the gene's promoter; specifically, by deleting PPR1, the transactivator of *URA3*. The silent telomeric domain was extended even further by increasing the gene dosage of *SIR3*. These results suggest that a gene's promoter is a key determinant in controlling silencing on that gene, and that SIR3 is a crucial component of the silent chromatin domain which initiates at the telomere and is assembled inwardly along the yeast chromosome. Lastly, transcription of a telomeric gene blocks further propagation of silencing on its centromeric side, demonstrating the continuous nature of repressed telomeric domains in yeast. Taken together, these data provide a molecular context to study regional gene regulation, and reflect the complex and dynamic organization of eukaryotic genomes into functionally distinct regions.

VARIEGATION PHENOMENA ASSOCIATED WITH SOMATIC PAIRING IN DROSOPHILA

S. Henikoff[1], L. Martin-Morris[1], D. R. Dorer[1], K. Loughney, E. Kershisnik, G. Poortinga and J. Sabl, [1]Howard Hughes Medical Institute and Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington.

Chromosome rearrangements that juxtapose euchromatin and heterochromatin cause mosaic inactivation of neighboring genes, a phenomenon known as position-effect variegation (PEV). PEV alleles are generally recessive; however for the *brown* gene, PEV alleles are dominant. Dominant PEV depends upon somatic pairing of homologs which bring together the heterochromatized *brown* gene region in *cis* to the rearrangement breakpoint and the normal copy of *brown* in *trans*. This *trans*-inactivation of *brown* requires a sequence that maps to the gene itself, most likely to the immediate 5' flanking region. We propose that *trans*-inactivation occurs because a transcription factor necessary for *brown* gene expression forms aberrant heteromultimers with a protein component of heterochromatin.

Both *cis*- and *trans*-inactivation of a transposed copy of *brown* are enhanced by local duplications of the transposon that can be induced by P-transposase. This surprising enhancement might be mediated by paired structures formed by the duplications. In support of this interpretation, we have found that P-transposase-induced local duplications of a sensitive mini-*white* trans-gene show mosaic repression even when they are not in proximity to heterochromatin. Especially striking variegation is seen when 3-4 copies of mini-*white* are in tandem. In such cases, variegation is strongly suppressed by known Suppressors of PEV, even though the trans-genes are quite distant from centromeric heterochromatin. We speculate that paired structures involving repeated sequences initiate heterochromatin formation.

STRUCTURAL CHANGES IN HETEROCHROMATIC CHROMOSOME REGIONS DURING DROSOPHILA DEVELOPMENT

P. Zhang, R. Glaser, L. Keyes, M. Lilly and A. Spradling, Howard Hughes Medical Institute Laboratories, Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21210

Heterochromatin, a ubiquitous but enigmatic component of eukaryotic chromosomes that is rich in middle repetitive, transposable and simple sequence DNAs, comprises nearly 30% of the Drosophila genome. Located predominantly in large pericentromeric blocks and on the Y chromosome, heterochromatic regions contain rRNA genes but few single-copy protein-coding sequences, suppress introduced genes in a characteristic mosaic fashion termed "position-effect variegation" and become drastically underrepresented in polyploid cells during development. How these behaviors are interrelated, their underlying molecular mechanisms, and their biological rationales remain obscure.

We analyzed the structure and behavior of specific heterochromatic chromosome regions using two new approaches. First, the structure and behavior of a particular heterochromatic segment were deduced by virtue of its inclusion on *Dp1187*, a 1300kb minichromosome. Methods were also developed to recover single, genetically marked P transposable element insertions within telomeric and centromeric heterochromatin. These screens revealed and relied on intrinsic preferences of P elements to transpose "locally", and to move from one site to another within heterochromatin. Many specific heterochromatic regions were rendered accessible to molecular cloning and genetic manipulation by this approach.

Studies using these tools revealed greater complexity in the structure and behavior of heterochromatin than previously supposed. Centromeric regions and the Y chromosome contain many "islands" of complex DNA spread along their length, into which P transposons preferentially insert and express detectable levels of marker gene products. Several lines of evidence suggested that some heterochromatic regions undergo structural alterations during development. Insertions near the Y chromosome telomeres, like simple sequence centromeric DNAs, became almost undetectable in salivary gland cells. In contrast, insertions in centromeric islands were hardly underrepresented in the same cells. More rapidly migrating forms of Dp1187 replaced the 1300kb band in ovarian nurse and follicle cells. These derivatives retained the Dp1187 euchromatin but lost most of its centromeric heterochromatin. The copy numbers of other heterochromatic regions were unchanged but they transferred inefficiently on Southern blots due to an unidentified modification. While our studies have yet to definitively determine the molecular mechanisms underlying these changes, we proposed a model in which programmed elimination of certain heterochromatic DNA sequences modulates tissue-specific changes in chromatin organization during development.

GENE SILENCING VIA PROTEINS THAT BIND TO METHYLATED DNA: A YOUNG REGULATORY MECHANISM A. Bird., R. Meehan, X. Nan, S. Cross and P. Tate. Institute of Cell and Molecular Biology, University of Edinburgh, Kings Buildings, Edinburgh EH9 3JR.

In the great majority of animal genomes, DNA methylation is not found in association with genes. Instead it is confined to a relatively minor genomic "compartment" which may contain repetitive sequences. Arguably, the function of methylation in these animals, and in many other eukaryotes, is to neutralise potentially damaging sequences. The situation is different in the vertebrates, where nearly every gene is methylated to some extent. Methylation has spread, and perhaps provides mechanisms of gene regulation that are not available to invertebrate ancestors. There are now several likely examples in the vertebrates of gene silencing by DNA methylation. Evidence will be presented that repression is mediated by methyl-CpG binding proteins (MeCPs). The functions of DNA methylation (in addition to neutralisation of potentially damaging sequences as in invertebrates) appear to be: i) genome-wide repression of weak promoters through low-density methylation; ii) long-term repression of genes by high density methylation of CpG islands, as on the inactive X chromosome and during genomic imprinting. The involvement of DNA methylation in X inactivation and genomic imprinting supports the notion that this is a young regulatory mechanism which participates in regulatory processes that evolved within the vertebrate lineage.

PARENTAL IMPRINTING OF THE INSULIN-LIKE GROWTH FACTOR II AND *H19* GENES IN THE MOUSE

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A long-standing assumption of Mendelian genetics was that autosomal genes, present in two copies in diploid organisms, were functionally equivalent. That this is not always the case in mammals has now been firmly established with the discovery of the phenomenon of parental or genomic imprinting. It is now clear that at least a small subset of mammalian autosomal genes are inherited in differentially active states. The decision to be active or silent is determined by the parent from which the gene is inherited. The laboratory has been studying the molecular mechanism underlying the imprinting of two co-expressed genes which lie within 90 kilobases of each other. One of them, encoding the fetal-specific growth factor, insulin-like growth factor II, is exclusively expressed from the paternal chromosome, while the *H19* gene, which encodes an RNA of unknown function is maternally expressed. We have proposed that the differential imprinting of these two genes is achieved by their competition for a common set of regulatory elements. One or more epigenetic marks, established during gametogenesis when the two parental chromosomes are apart, act to bias the competition in different directions on the paternal and maternal chromosomes after fertilization. Paternal-specific DNA methylation of the *H19* structural gene and its promoter has been proposed as the mark to inhibit *H19* transcription on the paternal chromosome. The maternal chromosome is unmarked by DNA methylation, suggesting that the preferential transcription of the *H19* gene on that chromosome is due to *H19's* inherently stronger promoter, coupled to its proximity to key regulatory elements. This model suggests that the imprinting of *Igf2* is achieved by virtue of its proximity to *H19*. Experiments to test this will be described.

THE ROLE OF DNA METHYLATION IN MAMMALIAN DEVELOPMENT

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Gene targeting in embryonic stem (ES) cells was used to mutate the murine DNA MTase gene. ES cells homozygous for the mutation were viable, but had significantly reduced levels of genomic methylation. The mutation was introduced into the germ line of mice and found to cause a recessive lethal phenotype with a reduction of the genomic DNA methylation level similar to that seen in the homozygous mutant cell lines. These results indicate that while a 3-fold reduction in levels of genomic m^5C has no detectable effect on the viability or proliferation of ES cells in culture, a similar reduction of DNA methylation in embryos causes abnormal development and embryonic lethality.

We have used this mutant mouse strain to investigate whether DNA methylation is involved in the maintenance of gene inactivity of imprinted genes. For this we have measured the transcriptional activity of Igf–2 which is maternally imprinted and of H19 and Igf-R which are paternally imprinted. Contrary to expectation, homozygous mutant embryos did not express the Igf-2 or Igf-R gene but expressed, in addition to the maternal H19 allele, also the paternal H19 allele. This indicates that interference with DNA methylation activates the normally inactive (imprinted) paternal H19 allele. H19 represents, therefore, the first example of a gene whose expression *in vivo* is dependent on DNA methylation. Expression of Igf-2 may not be directly controlled by DNA methylation but might rather be inhibited in cis by an active H19 allele.

ALLELE SPECIFIC STRUCTURE OF IMPRINTED GENE DOMAINS

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The genes Igf2 and H19 are located in one cluster on chromosome 7 in the mouse and chromosome 11 in man. Both genes are imprinted, but Igf2 is inactive on the maternal allele while H19 is repressed on the paternal allele. We have used in situ hybridization to interphase nuclei to examine the replication timing properties of these genes. Both genes are contained within a large structurally imprinted domain which is developmentally conserved and characterized by allele specific replication timing where the paternal copy undergoes DNA synthesis relatively early in S phase. Chromosomal zones of this nature are also characteristic of other imprinted gene loci and in all cases the structural domain includes additional genes which do not have an imprinted expression pattern. In order to understand the mechanisms involved in the regulation of Igf2 and H19 we have analyzed DNA methylation. Regions of paternal specific modification are associated with both genes, but these are established in a secondary manner during embryogenesis rather than being derived directly from the gametes. We have also identified one discrete locus whose methylation pattern is established early in development and this site could therefore serve as an important marker for parental identity and a possible imprinting signal for the entire domain.

MOLECULAR AND GENETIC ANALYSIS OF HUMAN X CHROMOSOME INACTIVATION

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X chromosome inactivation results in the cis-limited inactivation of many, but not all, genes on one of the two X chromosomes in female mammalian cells. Our efforts have focussed on the identification and localization of human genes that "escape" X inactivation, as their analysis should reveal features (either chromosomal or gene-specific) that distinguish active from inactive genes. We have identified at least 10 genes that escape inactivation; their expression from inactive X's ranges from ~20% to ~100% of that from active X's, whereas genes subject to inactivation show no appreciable expression from inactive X's (<0.1% of that from active X's). Genes that escape inactivation are localized widely along the chromosome, suggesting that there are multiple chromosomal domains that can sustain gene expression from an otherwise heterochromatic, inactivated X chromosome.

The process of X inactivation requires, for initiation and/or maintenance of inactivation, a locus called the X inactivation center (XIC). Studies of structurally abnormal, inactive X chromosomes have narrowed the localization of the XIC to ~1.5 megabases in band Xq13. This entire region has been cloned and candidate sequences identified. One gene from this region, XIST, is expressed only from inactive X chromosomes. The product of the XIST gene appears to be a non-coding ~17 kb transcript that is localized within the nucleus, associated with the inactive X heterochromatin. These data, as well as expression data from mouse, indicate that this gene is a strong candidate for a role in X inactivation. Current experiments are directed towards examination of the XIST promoter and evaluation of the effect of XIST expression or XIST deletion on the inactive or active state of neighboring genes.

CONTROL AND FUNCTION OF DNA METHYLATION IN NEUROSPORA CRASSA

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We are investigating the control and functions of DNA methylation in N. crassa, a relatively simple eukaryote amenable to genetic and biochemical analyses. While most of the Neurospora genome is devoid of DNA methylation, a number of chromosomal regions have been identified in which most cytosines are methylated. At least some such regions are relics of Repeat Induced Point Mutation (RIP), a process that detects and mutates sequence duplications during the period between fertilization and nuclear fusion. By investigating how RIP triggers methylation in these regions we have gained insight into the control of DNA methylation. At the same time, we have taken genetic approaches to explore the function of DNA methylation in Neurospora. We have isolated mutants in which DNA methylation is largely or completely absent and have explored the phenotypes of these mutants. So far, we have identified three genes required for DNA methylation in vegetative cells. Mutations in these genes result in several phenotypes, the most striking of which is the production of spores that have extra chromosomes or chromosomal segments. Inhibiting DNA methylation by reducing the synthesis of S-adenosylmethionine mimics this phenotype. We conclude that DNA methylation is of likely importance for normal chromosome behavior.

CRYSTAL STRUCTURE OF THE *Hha*I DNA METHYLTRANSFERASE REVEALS COMMON STRUCTURAL FEATURES FOR ALL DNA (CYTOSINE)-5-METHYLTRANSFERASES

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The first three-dimensional structure description for a DNA methyltransferase is presented. The DNA methyltransferase from the bacterium *Haemophilus haemolyticus* catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to the C-5 position of the internal cytosine in the recognition sequence GCGC. The three-dimensional structure of the M.*Hha*I-AdoMet complex has been determined at 2.5 Å resolution. The core of the structure is dominated by sequence motifs conserved among all DNA (cytosine)-5-methyltransferases (m5C-MTases), and these are responsible for the cofactor binding and methyltransferase function. Due to the conserved nature of m5C-MTases, the information obtained from this structure can be generalized to the entire family, including the mammalian CpG methyltransferase.

The molecule of size 40 x 50 x 60 Å is folded into two domains. A cleft, approximately 25-30 Å wide, 15-20 Å deep and 40 Å long, between the two domains is formed by most of the invariant residues amongst m5C-MTases. Therefore, the cleft is the likely active site where binding of DNA and AdoMet can occur in close proximity to the catalytic center. The large domain is a mixed α/ß structure consisting of the N-terminal two-thirds of the protein. It contains most of the conserved motifs amongst m5C-MTases, and is responsible for providing the catalytic nucleophile Cys81 and binding of the cofactor AdoMet. The AdoMet binding site is located in a cavity in the large domain face of the cleft, adjacent to the active site nucleophile Cys81. The small domain, predominated by ß-strands, contains the "variable region" which is responsible for the specific recognition of target DNA sequence and choosing the cytosine to be methylated.

INITIATION OF BACTERIOPHAGE λ DNA REPLICATION

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Ten purified λ and *Escherichia coli* proteins are required for the initiation of phage λ DNA replication *in vitro*. The λ O and P replication proteins direct the assembly of an ordered series of nucleoprotein preinitiation structures at the phage origin (*oriλ*). Partial disassembly of the complete preinitiation complex through the action of the *E. coli* DnaJ, DnaK and GrpE heat shock proteins results in unwinding of the chromosome, followed by priming and DNA synthesis. Binding of the λ O initiator to negatively supercoiled *oriλ* DNA to form the O-some causes an A+T-rich segment adjacent to the O recognition sequences to adopt a non-B, non-single-stranded configuration, an alteration that may be required for initiation of chromosome unwinding by DnaB helicase. Analysis of O deletion mutant proteins indicates that (i) an amino-terminal region encompassing amino acids 19 - 110 contains all the information necessary for specific recognition and bending of *oriλ* DNA as well as for protein dimerization; and (ii) the C-terminal domain of O (amino acids 183-299) functions in the recruitment of a λ P·*E. coli* DnaB helicase complex to the O-some and has single-stranded-DNA binding activity.

An examination of the template supercoiling density required for initiation of λ DNA replication *in vitro* indicates that the negative superhelical density must be at least – 0.045. In this regard, it is notable that the *E. coli* histonelike protein HU is a potent inhibitor of λ DNA replication. Our analysis indicates that this inhibition results from the capacity of HU to wrap the template DNA and restrain free supercoils. Moreover, we have found that HU manifests a strong preference for binding to supercoiled DNA. HU binds primarily as dimers to relaxed DNA, yet predominantly exists as tetramers and higher order structures on supercoiled DNA templates. In the presence of HU protein, initiation of λ DNA replication becomes strongly dependent on transcription of the *oriλ* template by *E. coli* RNA polymerase. We have found in other studies that the λ O-some blocks propagation of a replication fork apparatus composed of DnaB helicase and DNA polymerase III holoenzyme, but does not interfere with DNA unwinding mediated by DnaB helicase alone.

THE ROLE OF GIN AND FIS IN SITE-SPECIFIC RECOMBINATION

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Gin catalyzed site-specific DNA inversion in phage Mu is stimulated by the *E.coli* FIS protein and a recombinational enhancer sequence. Only negatively supercoiled DNA substrates are recombined and only recombination sites in inverted repeat configuration yield recombination products with high efficiency. Gin and FIS have to assemble the recombination sites in a topologically unique synaptic complex since DNA inversion results in unknotted products and proceeds with a defined change in linking number. The specificity of the synapse is governed by two kinds of contacts between Gin molecules: The intermonomeric interaction within the dimer bound to the recombination site and the interdimeric interaction between the two DNA bound dimers.

We are analyzing different classes of *gin* mutants, which are altered in these interactions. The analysis of FIS-independent Gin mutants has indicated that the respective mutations alter the interaction between the monomers within the DNA-bound dimer. Intragenic mutations suppressing the FIS-independent phenotype cluster in a domain, which is likely to be involved in the interaction between dimers of Gin. Suppression is also observed when the mutations are localized on separate Gin molecules. This finding supports the notion that the requirement for FIS protein is dictated by the character of interactions between monomers in the Gin dimer. Epitope-tagged variants of mutant and wildtype Gin proteins were used to analyze subunit-interactions between Gin molecules. We could show that dimerization of the protein takes place upon binding to the recombination site and that such complexes are rather stable once formed. We are currently using this material to determine whether Gin dimers bound to the recombination sites exchange monomers during recombination.

MECHANISM OF TRANSPOSITIONAL RECOMBINATION: THE PROTEIN-DNA ARCHITECTURE AND COORDINATION OF REACTION STEPS

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Bacteriophage Mu, like other transposons studied in detail, generates a critical transposition intermediate by a process involving two chemical steps. In the first step, two site-specific single strand cleavages expose the 3'-OH ends of the Mu sequence. In the second step, the target DNA is cleaved and covalently joined to the donor DNA 3'-ends (DNA strand transfer) in a concerted reaction. This step generates the strand transfer product containing a pair of forked DNA junctions between the donor and target DNAs. The Mu strand transfer products can be resolved either by DNA replication, or by nucleolytic cleavage and gap repair to generate final transposition products. The basic mechanism of Mu transpositional recombination also applies to other transposition reactions including retroviral DNA integration.

The two chemical steps described above take place within a context of higher order protein-DNA complexes. We have identified a number of protein-DNA complexes as intermediates in Mu transposition. Interactions between the Mu transposase (MuA) and multiple MuA binding sites, including those at the enhancer sequence on the Mu donor DNA are required to achieve a structural transition of MuA to form a stable synaptic complex containing the two Mu ends bound by a tetrameric form of the MuA protein. This process not only assures selection of two properly oriented Mu ends, but it also allows the Mu repressor, which also binds to the enhancer sequence, to regulate Mu transposition at this early reaction step. The assembly and the activities of the protein-DNA complexes are also controlled by MuB protein which binds to the target DNA. The architecture of these complexes not only achieves temporal coordination of the events at the two ends of the element, but also provides mechanisms for selectivity that serve to eliminate physiologically detrimental side reactions.

Using a variety of mutant proteins, we have begun to dissect the architecture of MuA tetramer-donor DNA complex to understand how the reaction steps of Mu transpositional recombination are coordinated. Tetramers containing a mixture of the wild type and mutant monomers with missing C-terminal domain carry out donor cleavage but are unable to promote DNA strand transfer even when a donor DNA with precleaved ends is provided. One dimer within a tetramer appears to carry out donor cleavage while all four monomers cooperate for strand transfer.

ENZYMATIC PROCESSING OF HOLLIDAY JUNCTIONS BY THE *E. COLI* RuvA, RuvB AND RuvC PROTEINS

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The *ruv* region on the *E. coli* chromosome encodes three genes which show similar repair/recombination defective phenotypes. Purified RuvA and RuvB proteins act upon Holliday junctions and promote ATP-dependent branch migration (1). Each protein plays a defined role in the branch migration reaction: RuvA protein binds specifically to the Holliday junction, while RuvB (the ATPase) promotes its movement along DNA. The mechanism of branch migration is likely to involve strand separation since RuvAB protein exhibits DNA helicase activity in vitro.

The product of *ruvC*, RuvC protein, acts independently of RuvAB, and resolves Holliday intermediates to produce recombinant DNA products (2). Resolution occurs by a dual incision process in which symmetrically related nicks are introduced into strands of like polarity close to the junction. Junction-recognition occurs in a structure-specific manner, yet the cleavage reaction is sequence-dependent. The products of resolution, nicked duplex DNA, are repaired by DNA ligase to complete the repair process. DNase I footprinting studies show that RuvC protein protects approx 12 base pairs at the junction point. The DNA within this complex is distorted such that the sugar-phosphate backbones of the two uncut strands become hypersensitive to attack by hydroxyl radicals.

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MOLECULAR AND FUNCTIONAL ANALYSIS OF THE BUDDING YEAST CENTROMERE/KINETOCHORE

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A multisubunit protein complex (CBF3) that binds specifically to the essential CDEIII DNA sequence in the yeast centromere (*CEN*) has previously been shown to contain an activity capable of attaching *CEN* DNA-coated microbeads to taxol-stabilized bovine microtubules, and, in the presence of a suitable energy source, moving the beads in a minus-end oriented direction [Hyman et al., Nature 359, 533-6 (1992)]. This motor activity can also be demonstrated by a standard microtubule gliding assay in the absence of DNA. Thus, the CBF3 complex is a likely candidate for the motor that attaches the replicated chromosomes to the microtubules and drives poleward movement of the chromatids in anaphase A. We have cloned and sequenced the genes specifying two proteins (p110 and p64') purified by *CEN*-DNA affinity chromatography. p110 is one of three dissimilar subunits that make up the core CBF3 complex; whereas p64' is an accessory protein that binds with lower affinity to the *CEN* DNA-CBF3 core complex. The single gene (*NDC10/CBF2*) specifying p110 is essential for viability of yeast, and a temperature-sensitive mutation in that gene produces a pronounced defect in chromosome segregation at the non-permissive temperature (Goh and Kilmartin, JCB, in press; Jiang, Lechner and Carbon, JCB, in press). CBF2p (p110) contains a variant consensus nucleotide binding sequence motif similar to that occurring in various G-proteins. The single gene (*CBF5*) specifying p64' is also essential. Over-expression of *CBF5* in yeast suppresses an *ndc10/cbf2* temperature-sensitive mutation, suggesting direct or indirect interaction of p110 and p64' in vivo. Over-expressed CBF5p binds to microtubules in vitro. This MT binding is dependent upon the presence of a $(KKD/E)_n$ sequence domain similar to known microtubule binding domains in MAPs 1A and 1B. However, neither CBF2p nor CBF5p contains a known consensus motor domain; thus their relationship to the motor activity of CBF3 preparations is unclear.

CHROMOSOME PACKAGING AND ATTACHMENT TO THE MITOTIC SPINDLE IN BUDDING YEAST

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Faithful chromosome segregation requires proper packaging of chromosomes (chromosome condensation and sister chromatid pairing) and attachment of chromosomes to the mitotic spindle. We have studied chromosome packaging in budding yeast using in situ hybridization and mutants affecting the cell division cycle. Our results show that sister chromatids become paired at or near the time of their replication, are held in close juxtaposition at the centromere and multiple positions along their arms, and can become unpaired in the absence of microtubules. The cluster of rDNA repeats on chromosome XII appears to undergo cell cycle dependent changes in structure; from G1 to G2, rDNA appears is an amorphous cap on the nucleus but during M it forms a line like structure strongly reminiscent of condensed chromosomes of higher eukaryotes. We are currently studying the effect of different cyclin mutants on these changes in chromosome structure.

Previously, we showed that the binding of bovine microtubules to minichromosomes isolated from budding yeast is dependent upon the minichromosome's centromere. We have used this assay to gain insight into the properties of centromeres assembled in vivo. Our results suggest that neither chromosomal DNA topology nor proximity of telomeres inhibit the efficient assembly of centromeres with microtubule-binding activity. Minichromosome binding to microtubules can be reversibly inactivated and reactivated by changing the salt concentration. When minichromosomes are inactivated by salt in the presence of microtubules, some centromere factors remain bound to the microtubules while others remain bound to CEN DNA. The association of CEN DNA and its binding proteins has a half-life of greater than 25 minutes. The microtubule-binding activity islike mechanochemical motors, dependent upon the concentration of microtubule polymer and inhibited by ATP but not UTP. Therefore, the yeast centromere appears to consist at least of a core that binds tightly to CEN DNA and a microtubule-binding component(s) with properties like mechanochemical motors. The salt-reversible association of one centromre component necessary for microtubule-binding and the DNA-binding core may mimic an important regulatory step for centromere function in vivo.

THE INCENPs ARE MULTI-DOMAIN PROTEINS SHARING BOTH CHROMOSOMAL AND CYTOSKELETAL FEATURES

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The INCENPs (INner CENtromere Proteins; M_r 133 and 145 kDa) are tightly bound to chromatin until early metaphase, and arrive at the metaphase spindle plate with the chromosomes. However, they leave the chromosomes during late metaphase to become part of the spindle midzone and cleavage furrow during anaphase. This suggests that the INCENPs may be important components of the cell's mitotic machinery, perhaps involved in sister chromatid pairing or in cleavage furrow placement.

By combining library screening with reverse-transcriptase PCR, we have recovered cDNA clones that contain the complete open reading frames (ORFs) of the two chicken INCENPs. Class 1 INCENP cDNA contains an ORF of 839 codons, which includes a predicted central coiled-coil domain of about 200 residues. Class 2 differs primarily in having a 20 aa extension of the coiled coil domain. Both ORFs are hydrophilic and very basic along their lengths. The primary sequence of the INCENPs is completely distinct from those of other known proteins.

To probe INCENP function in vivo, we placed the two classes of INCENP cDNAs into an expression vector that was transiently transfected into HeLa and LLCPK cells. The distribution of the full-length INCENPs in these cells recapitulated that of wild-type INCENPs in chicken cells. Further analysis revealed that the amino-terminal 41 residues are required for INCENP targeting to the spindle midzone. In addition, the coiled-coil region appears to be required for the INCENPs to bind cytoplasmic microtubules. Thus, apparently more than simple microtubule-binding activity is required to permit the INCENPs to move to the spindle during metaphase.

Certain INCENP expression constructs induced dominant negative phenotypes in recipient cells. Full-length wild-type Class 2 INCENP could prevent the progression of cells past telophase. In such cells, mitosis resulted in a single binucleate daughter cell. Deletion of the 57 carboxy-terminal residues also interfered with cell division, producing a range of negative phenotypes. In some cases, newly divided cells rejoined to form a binucleate cell, while other daughters underwent lysis shortly after mitosis. These phenotypes implicate the INCENPs in the process of cytokinesis.

The inability of cells transfected with INCENP deletion constructs to complete cytokinesis lends support the notion that INCENPs and other proteins may be brought by chromosomes to the metaphase plate so that they are correctly positioned to perform essential cytoskeletal functions later in mitosis.

CHROMOSOME TERMINI OF *CAENORHABDITIS ELEGANS* ACT AS CENTROMERES IN MEIOSIS

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The chromosomes of the nematode *Caenorhabditis elegans* are holocentric, that is in mitosis the kinetochores extend along the poleward face of the chromosomes. Typically, in meiosis, holocentric chromosomes do not display extended kinetochores and it is the ends of the chromosomes that appear to be important for meiotic segregation. In *C. elegans* the role of the ends of the chromosomes in meiosis has been investigated by cytological characterization of meiotic segregation of normal and rearranged chromosomes and by characterization of DNA sequences located at the termini of the chromosomes.

In meiotic prophase the homologs of holocentric chromosomes are associated end-to-end, possibly held together through terminalized chiasmata. In *C. elegans* the end-to-end association of the homologs in diakinesis was demonstrated by labeling one end of each chromatid in the bivalent by *in situ* hybridization. Either the left or the right ends of the homologs could be held in association, since probes mapping to either the genetic left or right end of the chromosome were mapped to the center of the bivalent. At metaphase I the bivalents lined up parallel to the long axis of the spindle (axial orientation) and segregated end on towards the spindle pole. The ends of the sister chromatids that led the way to the spindle poles at anaphase I also appeared to hold the sister chromatids together for proper disjunction at metaphase II when the chromosomes were also oriented axially. Thus the ends of these chromosomes adopt the role of the monocentric centromere in holding the chromatids together to ensure proper disjunction and for providing attachment to the spindle. Since either genetic end of the chromosome can provide this function these chromosomes have two potential "meiotic centromeres," only one of which is active at meiosis I. The orientation of the bivalents at metaphase I was modified in certain mutants and strains carrying chromosome rearrangements.

Several classes of repeat sequences have been mapped to the ends of *C. elegans* chromosomes. Some of these have been characterized and include the telomere sequences that cap the ends of the chromosomes and meiotically and mitotically stable chromosome fragments (free duplications). Degenerate telomere repeats, that may be found throughout the genome, appear to be concentrated in the terminal thirds of the autosomes, but are sparse on the X chromosome.

USE OF YEAST ARTIFICIAL CHROMOSOMES TO STUDY DNA REPLICATION AND ITS REGULATION WITHIN THE CELL CYCLE

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A system has been established in *S. cerevisiae* to study DNA replication and its regulation within the cell cycle. This system makes use of yeast artificial chromosomes (YACs) that have origins of replication only on the terminal arms, and a long central region devoid of origin function. Complete replication of the YAC is monitored using a colony color sectoring assay based on maintenance of the *ADE2* gene carried on one end of the YAC. A library of YACs of such a structure were constructed, and a subset were subsequently screened to identify a set of YACs of different lengths.

Characterization of a set of YACs of varying lengths has shown that both the rate of color sectoring and the quantitative rate of loss of the YACs increase in proportion to the length of the YAC, and are a simple function of the length of DNA that has to be replicated from each origin of replication on the YAC. Sectoring and loss are completely suppressed in YACs that have sufficient origins. These results indicate that at least a fraction of the cells in a population are able to enter mitosis with an incompletely replicated YAC. Preliminary characterization of the cell cycle distribution of strains propagating a replicatively unstable YAC indicates that the cell does not detect ongoing replication on the YAC, and respond by delaying the cell cycle until replication is complete.

These replication-defective YACs are currently being used in a variety of genetic screens based on a change in the rate of color sectoring. Both mutant and over-expressed wild-type genes that either increase or decrease the rate of color sectoring are being isolated. These screens are expected to identify genes involved in initiation of DNA replication, regulation of the timing of initiation within the cell cycle, as well as factors required for DNA synthesis.

THE FOOTPRINT OF CHROMOSOMAL PROTEIN HMG-17 ON NUCLEOSOME-CORES AND CHROMATOSOMES.

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Chromosomal proteins HMG-14 and HMG-17 are the only nonhistone proteins known to specifically bind to nucleosomal core particles. These proteins may be involved in the generation or maintenance of structural features specific to active chromatin. Here we propose a model for the location of HMG-17 in nucleosomal core particles and in chromatosomes. The model is based on the use of DNase I and hydroxyl radical cleavage to footprint the protein and its nucleosome binding domain on cores and chromatosomes. Two molecules of HMG-17 can bind specifically to either nucleosome cores or chromatosmes. The protein protects the nucleosomal DNA from hydroxyl radical cleavage at positions 70 and 80 bp from the end (near the dyad axis) and at positions 20-30 bp from the end of the core particle. The protein bridges two adjacent DNA strands at the face of the cores and chromatosomes. Cooperative interactions between the C-termini of two molecules of HMG on the cores are possible. Such a placement explains previous findings indicating that the binding of HMG-14 and -17 increases the stability of nucleosomes and chromatin, without significantly affecting the structure of the nucleosomes. In chromatosomes the sites occupied by HMG-17 are 30-40 base pairs from the end while those occupied by the H1 linker histone are located 20-30 bp from the end. On the other hand, near the dyad axis the binding sites of H1 and HMG-17 overlap. An interplay between histone H1 and HMG-14/-17 proteins may play a role in transcriptional regulation by chromatin.

ROLE OF TTAGGG REPEATS IN THE AMPLIFICATION OF CAD GENES IN CHINESE HAMSTER CELLS

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Chinese hamster cell lines with multiple copies of the CAD gene have been selected *in vitro* for resistance to N-(phosphonacetyl)-L-aspartate (PALA). The chromosomal rearrangements that have occurred during amplification have been analyzed in eight PALA resistant lines by localizing the CAD genes on G-banded chromosomes with fluorescence *in situ* hybridization. The chromosomes of the same cell lines have then been hybridized *in situ* with a synthetic TTAGGG repeat; this sequence is highly repeated at the centromere of most Chinese hamster chromosomes and it is repeated to a lower extent at the telomere of all chromosomes.

The results indicated that gene amplification may be triggered by PALA induced chromosomal aberrations; in particular chromatid breaks would generate ends lacking telomere repeats and participate in the formation of dicentric chromosomes. Successive duplications of extended chromosomal regions would then take place through breakage-fusion-bridge cycles.

In the studied examples, highly repeated TTAGGG sequences, typical of centromeric regions, were always seen at the end of the chromosome arms carrying amplified DNA. This observation suggests that TTAGGG may be frequent sites where the recombination events accompanying gene amplification take place. Moreover, telomere-like structures may be generated from centromeric DNA.

THE STRUCTURE AND FUNCTION OF $d(GA{\cdot}CT)_n$ SEQUENCES.

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Alternating $d(GA{\cdot}CT)_n$ are fairly abundant in eukaryotic genomic DNA. These sequences are often found in or near transcriptional regulatory regions. They are also frequent at recombinatory "hot-spots". Increasing evidence suggest that $d(GA{\cdot}CT)_n$ sequences are likely to participate in processes involving strand exchange. SV40 viruses carrying a $d(GA{\cdot}CT)_{22}$ sequence show an increased genomic instability probably as a consequence of an increased rate of "recombination" (1). Furthermore, a 10-fold increase on the rate of excision of plasmid DNA inserted into SV40 was observed when a copy of this type of sequence was present at either end of the inserted plasmid DNA (2). Alternating $d(GA{\cdot}CT)_n$ sequences show a remarkable degree of structural polymorphism. Several non-B DNA conformations have been described for these sequences. In particular, formation of intramolecular pyr·pur·pur triplexes (*H-DNA) was observed when contained into negatively supercoiled DNA, at neutral pH and in the presence of particular transition metal-ions such as zinc (3). Upon increasing the metal-ion concentration, the pyrimidine strand falls off the triplex an a pur·pur hairpin is formed (4). Most of this conformational flexibility resides in the structural properties of the individual strands. Short oligo[d(CT)] sequences form antiparallel stranded duplexes at acidic pH, stabilized by the formation of C^+C pairs (5). Similarly, short oligo[d(GA)] sequences also form antiparallel stranded duplexes (6). In addition, $d(GA)_n$ sequences were also proposed to form parallel stranded duplexes as well as multistranded complexes (5). Antiparallel stranded $d(GA{\cdot}GA)_n$ is stabilized by alternate A*(anti)*·G*(anti)* and G*(anti)*·A*(syn)*, pairs (6). These results will be discussed in the context of the possible participation of $d(GA{\cdot}CT)_n$ sequences in processes of genetic "recombination".

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IMPROVED INTERPRETATION OF Add(1)(p36) IN NON-HODGKIN'S LYMPHOMAS USING FISH

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Fluorescence *in situ* hybridization (FISH) with biotin labeled chromosome-specific libraries was performed on metaphases from short-term cultures from five cases of non-Hodgkin's lymphoma (NHL). The aberrant chromosomes were often more complex than the banding analysis had led us to believe. Among the rearrangements we found two add(1)(p36) which, in both cases, were by painting shown to be a der(1)t(1;2)(p36;q31). We also found a t(1;19)(p36;p13) where a part of the GTL-negative band 1p36 had been replaced by a similarly GTL-negative band from chromosome 19. This had been overlooked in the banding analysis.
We are currently analysing 5 more cases with additional material at 1p36.

The present study is concentrated on characterizing the breakpoints at 1p36 using specific probes for this region. We believe that this region is of importance in the tumorgenesis of NHL since it is involved in rearrangements in 17% of the NHL and it appeared as a primary rearrangement in two of our cases. A candidate gene could be the leukemia-associated phosphoprotein p18. Furthermore, a cAMP-dependent protein kinase gene is located at 1p36. Protein kinases are frequently involved in signal transductions and have often appeared as potential cancer genes.

This study illustrates the use of chromosome painting in resolving karyotypic uncertainties in NHL, and it shows that new cytogenetic subgroups may emerge when classical banding analysis is supplemented with fluorescence *in situ* hybridization techniques.

The chromosomal site of integration of xenotropic murine leukemia virus Xmv-45 maps on chromosome 5, close to the reeler locus.

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Reeler is an autosomal recessive mutation of the mouse, mapped on proximal chromosome 5, that perturbs cell patterns in the brain. Morphological studies show that the reeler gene controls a key step of brain development and evolution. As the product of the reeler gene is unknown positional cloning is the most obvious strategy for isolating the locus and gain some insight into its function.

Previous work in our laboratory resulted in the mapping of reeler ≈ 7 cM distally from the P-glycoprotein 1 (Pgy-1) gene and ≈ 8 cM proximally from the Engrailed-2 (En-2) locus. In addition, reeler is proximal relative to the reciprocal translocation breakpoint T31H. Both Pgy-1 and En-2 are too remote from reeler to consider walking towards the gene. Therefore work was focused on finding additional probes in this chromosomal region.

We and others showed that the genes for Hepatocyte growth factor (Hgf), Serotonin 5A receptor (5HT5A), Sorcin (Sor) and other components of the Multidrug resistance complex, and the Calcium channel alpha-2 subunit all map in the region of interest, as well as the microsatellites D5Mit1, 61, 3, 66, 13 and 44 and two anonymous DNA probes 3R2 and CD9. None of those markers could be located within walking distance from reeler. Previous studies by Frankel et al. (1989) showed that proviral integration loci Xmv-17 and Xmv-45 are located in proximal chromosome 5. Xmv-45 yields a polymorphism between C and B6 mice and could be mapped using a backcross panel between these strains, incorporating reeler. No recombinant was found among 127 backcross DNAs tested, corresponding to an estimated distance ≤ 0.8±0.8 cM. A DNA fragment flanking the proviral insertion site, called WF, was mapped at the same distance using the same backcross panel.

The position of WF relatively to the T31H translocation breakpoint was determined using a gene dosage assay, namely hybridization to dot blots of DNA from mice carrying respectively 1, 2 and 3 doses of proximal chromosome 5. The WF probe gave a differential signal, showing that the WF locus maps proximal to the T31 translocation breakpoint.

Among the several chromosome 5 markers tested, WF is the closest to the reeler locus and is used to begin a walk towards reeler (Bar et al., this meeting).

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Patterns of DNA Methylation in the Human Genome.

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By using methylation-sensitive restriction enzymes combined with Southern blot hybridization analyses and the technique of genomic sequencing, we have analyzed patterns of DNA methylation in the following segments of the human genome: In 500 kbp of randomly selected human DNA segments (1), in parts of the TNF α and ß genes (2, 3), in the Alu repeat sequences (4), and in part of the gene for the α chain of the interleukin 2 receptor (5). The data indicate that highly specific patterns of DNA methylation exist for each type of primary human cells. These patterns can be very different in established human cell lines (6). Patterns of DNA methylation can be concordant among different individuals (1, 2). In human sperm, the Alu sequences are hypomethylated, and Alu-specific RNA is associated with these cells. The de novo methylation of foreign DNA integrated in established genomes can be interpreted as the consequence of an ancient cellular defense mechanism against the activity of foreign genes (7).

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MECHANISM OF TRANSCRIPTIONAL ACTIVATION BY MYOD PROTEIN *IN VITRO.*

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MyoD is a muscle-specific transcription factor that belongs to the helix-loop-helix (bHLH) family of proteins. MyoD activates transcription by directly binding to a consensus DNA sequence found in the promoters of many muscle-specific genes. It binds DNA as a homodimer or as a heterodimer with proteins encoded by the E2A gene. A question remains as to how MyoD binding to its recognition site effects the basal transcription machinery to activate transcription.

To address this question, we have established an *in vitro* transcription system in which bacterially expressed MyoD proteins activate transcription in a sequence specific manner. Using this system we have demonstrated that MyoD can activate transcription in its homodimeric form, however, the extent of activation is significantly higher when MyoD acts in a heterodimeric complex with E47 protein - the E2A gene product.

To define the domains that are required for transcriptional activation by MyoD, transcriptional extracts were supplemented with different MyoD deletion mutants. A protein containing only the bHLH domain binds the DNA recognition site but does not activate transcription. Previous work has demonstrated that MyoD contains an acidic activation domain within its N-terminal region. However, a protein that is missing the same domain, can activate transcription *in vitro*. This result suggest that the protein contains an additional yet unknown activation domain(s). Mutational analysis is currently underway to define this domain(s).

The *in vitro* system elucidate that MyoD protein activates transcription by facilitating the assembly of pre-initiation complexes at the promoter. Specifically, we can demonstrate that MyoD stabilizes the DAB complex (TFIID,TFIIA,TFIIB) binding to the promoter. However, MyoD does not effect the binding of subsequent factors to the pre-initiation complex (Pol II, TFIIF, TFIIE, TFIIH).

INSECT TELOMERES MAY BE ELONGATED BY THE ADDITION OF A SPECIFIC LINE-LIKE RETROPOSON

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Drosophila and other dipterans apparently lack typical telomeric repeats at the ends of their chromosomes. We propose a telomere elongation model that does not require such repeats or telomerase but is based on frequent transpositions of members of the *Drosophila* HeT-A family, LINE-like retroposons that are only located at telomeres and in centric heterochromatin. Our model is based on the observation that these retroposons frequenly and specifically transpose to broken chromosome ends, attaching themselves with oligo(A) tails. Active HeT-A elements contain a single open reading frame encoding a gag-like polypeptide with three zinc finger motifs, but no open reading frame that could encode a reverse transcriptase. We believe that our element is a typical member of the HeT-A family of retroposons, considering that it had been cloned and sequenced only nine generations after it transposed. The observed frequency of transpositions would be sufficient to counter-balance progressive nucleotide loss that occurs at chromosome ends due to incomplete DNA replication. This model of telomere length maintenance predicts a predominant orientation of HeT-A elements with their oligo(A) tails facing proximally, as well as the existence of irregular tandem arrays of HeT-A elements at chromosome ends. Sequence analysis of HeT-A fragments isolated from directional libraries that were enriched in terminal DNA fragments are consistent with these two predictions and support our model. The proposed role of HeT-A retroposons in chromosome healing and telomere elongation would constitute a genuine cellular function for this unique retroposon.

THE MULTIFUNCTIONAL YEAST DNA BINDING PROTEIN, CENTROMERE AND PROMOTER FACTOR 1 (CPF1) IS A MODULATOR OF CHROMATIN STRUCTURE AND BENDS DNA

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CPF1 is an integral component of the yeast centromere protein complex. It is a Basic-Helix-Loop-Zip protein that binds to DNA via the CDEI motif conserved in yeast centromeres. The CPF1 binding motif is also present in the promoters and UAS regions of numerous genes including many encoding enzymes of the methionine biosynthetic pathway. As cpf1 yeast are phenotypically Met^- (in addition to having sub-optimal centromere function), it has been assumed that CPF1 can act in a positive way as a DNA bound transcriptional regulator via these sites. We however, have shown that a mutant form of the CPF1 protein unable to bind DNA, is still able to confer a Met^+ phenotype despite being unable to restore normal centromere function. We have shown further that CPF1 interacts with the products of the RPD1, RPD3, SPT21 and CCR4 genes to mediate its positive effect on the methionine pathway. What therefore, is CPF1 doing in its DNA bound form at promoters and centromeres?

We show here that DNA bound CPF1 can function to exclude nucleosomes from CDEI motif associated DNA in several gene promoters via a highly acidic domain in the N-terminus of the protein. This function appears to be completely separate to the protein's role in maintaining methionine prototrophy. We show that CPF1 is able to induce a significant DNA bend in the CDEI motif and we discuss the possible consequences that this might have. We also describe preliminary experiments aimed at further dissecting the centromere protein complexes in both Saccharomyces cerevisiae and Schizosaccharomyces pombe.

COMPLETE CHARACTERIZATION OF A LARGE MARKER CHROMOSOME BY REVERSE AND FORWARD CHROMOSOME PAINTING

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Marker chromosomes are small supernumerary chromosomes which are sometimes associated with developmental abnormalities. Hence, the genes involved in such cases are interesting as an approach to understand developmental abnormalities in man. As a first step towards isolating such sequences, marker chromosomes need complete characterization. By combining chromosome isolation by flow sorting and degenerate oligonucleotide primed - polymerase chain reaction (DOP-PCR) we have constructed a DNA library specific for a marker chromosome found in a child with severe developmental abnormalities.
We used fluorescent *in situ* hybridization of the library onto normal metaphase spreads ("reverse chromosome painting") and were thus able to determine that the marker consists of the centromeric part of chromosome 7, the telomeric region of the long arm of chromosome 5 and the telomeric region of the short arm of the X-chromosome. Subsequently, we used normal chromosome-specific libraries of the chromosomes involved, onto metaphases containing the marker chromosome ("forward chromosome painting") and could in this manner establish the precise location of the different chromosome regions on the marker chromosome itself. This is a general approach suitable for outlining marker chromosomes in detail, and will aid in the identification of the genes involved.

STRUCTURAL AND FUNCTIONAL STUDIES OF TWO REPLICATION-INDEPENDENT HISTONE H2A GENES

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The genes for the replication-independent human histone H2A isoprotein species, H2A.X and H2A.Z have been characterized. In addition to being replication-independent, these two histone species have characteristic domains that have been conserved separately throughout evolution. Although rarely more than 15% of the H2A complement, these two histones do appear to be present in all cells of an organism.

As a class, the genes for the replication-dependent histones lack introns and their transcripts terminate in a conserved stem-loop structure rather than a polyA tail. In contrast, the H2A.Z gene contains introns and its transcripts are polyadenylated; these characteristics are sufficient to account for its replication-independence.

However the H2A.X gene presents a paradox. Like the replication-dependent genes, the H2A.X gene lacks introns and its transcripts terminate in the conserved stem-loop. Thus what appears to be a structurally replication-dependent histone gene encodes a replication-independent gene product. (Some H2A.X transcripts are read-through transcripts which are polyadenylated at a downstream site, but this phenomenon does not resolve the paradox.)

Further structural studies show that the H2A.X gene promoter is very similar to other H2A gene promoters with a TATAA box and two divergent CCAAT boxes. These boxes appear to interact with common factors. Despite this apparent similarity, gene specific nuclear runon assays show that the transcription of H2A.X is not linked to replication, while transcription of other H2A genes is downregulated when DNA synthesis is inhibited and upregulated when protein synthesis is inhibited.

The human H2A.X gene is on a different chromosome from those that contain the three known human histone gene clusters. It is suggested that at least part of the replication-dependence of the clustered histone genes is attributable to properties of the clustering of these genes rather than to properties of specific promoters. Thus, the H2A.X gene may be transcribed in a replication-independent manner, because it is not part of a cluster.

REGULATION OF THE EXPRESSION OF THE GENE FOR THE AMYLOID PRECURSOR PROTEIN

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One of the biological hallmarks of Alzheimer's disease (AD) is the accumulation in the brain and blood vessels of ß-amyloid, a peptide of 42-43 amino acids derived from a larger precursor molecule, amyloid precursor protein (APP). In Down's syndrome (trisomy 21), duplication of the APP gene and overexpression of its mRNA leads to neuropathological lesions identical to those observed in AD. The APP promoter is typical of that of housekeeping genes with multiple transcription initiation sites, absence of a TATAA or CAAT box and presence of a GC rich region (85% GC: -90 to -210). In transient transfection assays minimal reporter gene activity can be obtained with the first 96 bp of the promoter. Furthermore, expression of the APP gene can be modulated by environmental factors such as growth factors (NGF, IL-1) or stress (heat shock).

The ubiquitously expressed transcription factor Sp1 binds to a GC rich sequence (GGGCGG) and plays a critical role in the basal level of transcription of numerous genes. To assess the possible role of the GC rich region in transcriptional regulation, we performed DNase I footprinting assays on the proximal 200 bp of the APP promoter with purified Sp1 protein. Four regions were protected from DNase I digestion in presence of Sp1, with two of these binding sites being in the region enriched in GC. All protected areas were on the coding strand and their sequences conformed to the Sp1 consensus binding site. The presence of GC rich regions has been shown to influence chromatin structure while proteins binding to these regions could play a role in the overall regulation of transcription. We are now evaluating the effects of these putative Sp1 binding sites by *in vitro* transcription run-off assays.

Modulation of APP expression has also been shown to involve upstream regulatory elements (URE). In order to identify such elements, we have cloned various portions of the 5' region of the APP gene upstream of the reporter gene chloramphenicol acetyl transferase (CAT). Transient transfection assays performed in NG108, a neuronal cell line, showed that a BamH1 fragment containing 3700 bp of upstream APP genomic sequences had the highest CAT activity. Electrophoresis mobility shift analysis (EMSA) and DNase I footprinting assays on fragments spanning the region -3167 to -3699 demonstrated the presence of a specific DNA binding activity in crude nuclear extracts from NG108 and HeLa cells (-3595 to -3610). No known element described so far corresponds to this sequence. We are currently determining whether this element can serve as an enhancer for a heterologous promoter.

Alphoid DNA is at the functional centromere of the human Y chromosome.

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Alphoid DNA is a set of tandemly repeated DNA sequences with an underlying periodicity of 170bp and is found by cytogenetic techniques to be close to or at the centromeres of all human chromosomes. We have used telomere directed chromosome breakage and sequence targeting techniques to investigate the functional significance of the alphoid DNA at the centromere of the human Y chromosome. We have targeted human telomeric DNA to the Y chromosomal alphoid DNA in each of the two possible orientations and have thereby generated both long arm and short arm acrocentric derivatives of the chromosome. These two derivatives segregate accurately at mitosis and are retained by cells proliferating in the absence of selection. The only major sequence that these acrocentric derivatives hold in common is alphoid DNA. The simplest interpretation of these results is that alphoid DNA is sufficient for mitotic centromere function.

PHYSICAL MAPPING OF THE *W - Ph* AND *Rw* REGION ON MOUSE CHROMOSOME 5

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Our goal is to establish a physical map and characterize the molecular structure of the region surrounding the patch, *Ph*, rump-white, *Rw*, and dominant spotting, *W*, loci on mouse chromosome 5. These three mutant loci are tightly linked to one another with similar pigment defects among other developmental/morphological abnormalities. Two of the mutants (W^{19H}, *Ph*) are associated with deletions of genes encoding tyrosine kinase receptors (Geissler et al., 1988; Tan et al., 1990; Nocka et al., 1989; Stephenson et al., 1991). The third mutation, *Rw*, is associated with a large inversion of the proximal portion of chromosome 5, as shown by *in situ* hybridization of several chromosome 5 genes on *Rw*/+ chromosome spreads (Stephenson et al., submitted).

Probes for *Kit* and *Pdgfra*, candidate genes for *W* and *Ph* respectively, and several other molecular markers genetically mapped to the same region in mouse and humans (*Flk-1*, *D5SC25* and *D5SPhil7*), have been used to establish a physical map. These probes have been used in PFGE analysis and to screen a mouse YAC library. So far a long range restriction map, covering 5 Mb, and a YAC contig spanning 1.5 Mb around *Pdgfra*, *Kit*, and *Flk-1* have been established. The available markers, including YAC end clones have been analyzed on a "deletion panel" containing DNA isolated from the F1 progeny from matings between *Mus spretus* and W^{19H} and *Ph* heterozygous mice. This analysis has allowed us to define the location, the breakpoints and the extent of the deletions associated with W^{19H} (proximal breakpoint) and *Ph*. In addition, PFGE analysis has localized the distal breakpoint of the *Rw* inversion. A 450 kb YAC clone spanning the *Rw* breakpoint is currently being searched for transcribed sequences and the gene disrupted by the inversion.

THE PROBLEM OF DEMONSTRATING "LINKAGE" BETWEEN CHROMATIN CONFORMATION AND ITS VARIOUS MODIFICATIONS.

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During changes in chromatin conformation, chromatin modifications, such as phosphorylation, have long been suspected of comprising the source of energy-drive as well as much of the logic network controlling it.

However, while there is no dearth of published observations of chromatin-protein kinasings, their causal significance in structural changes is commonly unclear. This is largely because of the difficulties in objectively determining or measuring chromatin's "conformation" but is also because there is usually uncertainty about the degree of "nativeness" of the nuclei being observed.

This presentation explores the propositions that *causality* relationships between phosphorylation and conformation change are conveniently studied by observing what is probably the *reversing* of the natural processes and in systems that are still native enough for structure/phosphorylation "linkage" to be demonstrable.

Commonly, one of the first biochemical features of an organelle to be destroyed during its handling is the linkage between the driving and the driven processes within the structure of the organelle. (cf oxidative phosphorylation in mitochondria or energy-dependent transport systems.)

It has been observed that the polyamine-stabilised nucleus changes its phosphorylation status in response to impressed structural changes arising from minor changes in its ionic environment or in response to exo-nuclease damage. Moreover, this latter damage appears to elicit a response that is different for different classes of nucleases.

The polyamine-stabilised nucleus thus appears to have the behaviour expected of an experimental system with enough structural integrity, "nativeness" left to be useful in determining the nature of the linkages between structure and phosphorylation status.

REVERSAL OF TERMINAL DIFFERENTIATION AND CONTROL OF DNA REPLICATION: CYCLIN A AND CDK2 SPECIFICALLY LOCALISE AT SUBNUCLEAR SITES OF DNA REPLICATION

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DNA replication in mammalian cells is known to occur in discrete patterns of nuclear foci, which undergo characteristic changes during S-phase. Here we show that, upon expression of SV40 large T antigen, terminally differentiated myotubes re-enter S-phase and show the same pattern of DNA replication foci as cycling cells. Re-entry into S-phase was correlated with decrease of Rb protein and appearance of cyclin A and cdk2 in the myotube nuclei. Cyclin A and cdk2, but not cyclin B1 and cdc2, are specifically localized at nuclear DNA replication foci and display, in fact, the same characteristic distribution as the DNA replication protein PCNA. This colocalization was observed throughout S-phase in induced myotubes as well as in cycling cells suggesting a direct role of cyclin A and cdk2 in the control of DNA replication. The fact that not only DNA replication but also certain cell cycle proteins are specifically localized to subnuclear DNA replication foci might explain their substrate specificity in vivo. A multiple factor model for the licensing of DNA replication is discussed.

THE MOLECULAR MECHANISM UNDERLYING THE MYOTONIC DYSTROPHY PHENOTYPE AND THE MAINTENANCE OF THE MUTATION IN THE POPULATION.

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Myotonic dystrophy, the most common form of adult muscular dystrophy (average incidence 1 in 8,000), is an autosomal dominant disorder of highly variable presentation. The disease is characterised by the phenomenon of anticipation, in which the phenotype becomes increasingly severe when passing down the generations in a pedigree. In all instances myotonic dystrophy has been found to be an inherited disorder - there have been no confirmed reports of spontaneous isolated cases of the disease.

The mutation underlying the disorder has been identified as the expansion of a CTG trinucleotide repeat in the 3' untranslated region of a putative protein kinase gene. The most common number of repeats is 5 (40% of chromosomes) but in myotonic dystrophy pedigrees this motif becomes highly unstable and may undergo extensive amplification during transmission, resulting in inheritance of several hundred repeats. The severity of the disease symptoms broadly correlates with the repeat number within a pedigree but the mechanisms by which the genotype leads to the phenotype are unknown.

Mild symptoms, for example cataracts, have been reported for patients in whom the expansion is relatively limited (approximately 50 repeats). We report a case of two sisters, the offspring of a consanguineous marriage, each of whom is homozygous for the expansion, having inherited chromosomes with 80 to 100 repeats from each parent. The sisters are currently asymptomatic, despite repeat sizes of this magnitude lying in the range associated with the disease phenotype in other pedigrees. It has also been shown that expansion of the repeat is highly variable on transmission through the two branches of the family and this pedigree may be very valuable in investigating the effects of genotype on phenotype in this disorder.

The more severe forms of myotonic dystrophy have a major adverse effect on reproductive fitness but the mutation appears to be an ancient one. In order to determine the manner in which the mutation is maintained in the population a large study has been initiated to investigate the stability of the CTG repeat when present at the higher end of the normal range, to determine if there is a predisposing minor instability in the repeat size at this level, which may act as a reservoir for the full-blown mutation.

FLANKING SEQUENCES OF HUMAN ALU SOURCE STIMULATE TRANSCRIPTION IN VITRO BY SEQUENCE-SPECIFIC TRANSCRIPTION FACTORS.

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A source gene for a recently expanded human Alu subfamily has been isolated by exploiting a severe speciation bottleneck in the size of this subfamily in the common ancestor of human, chimpanzee and gorilla (Leeflang *et al.*1993)*. The 5′ flanking sequence of this Alu includes several recognizable binding sites for specific transcription factors. As revealed by a progressive series of deletions, there are at least two distinct elements in this flanking sequence which stimulate the *in vitro* RNA polymerase III directed transcription of this Alu. A perfect AP1 site maps within forty nucleotides of this Alu and site specific mutation of the AP1 site abolishes the transcriptional stimulation provided by this region. Competition with specific oligonucleotides using gel mobility shift assays shows that both TATA binding protein and AP1 form specific complexes with this Alu's 5′ flanking sequence. These results indicate that an ancestral Alu source gene acquired positive transcriptional control elements by its fortuitous insertion into this locus.

* Leeflang *et al.* 1993. J. Mol. Evol. (in press).

ROLE OF SIR1 IN ESTABLISHING SILENCING AT YEAST *HM* LOCI AND TELOMERES.

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Previous studies suggest that the yeast SIR1 protein is involved in the establishment of transcriptional silencing at the *HM* mating-type loci. First, *sir1* mutations destabilize repression at the *HM* silent loci by greatly reducing the frequency of re-establishment of the repressed state. Second, overexpression of SIR1 can suppress many different mutants that are partially defective in silencing.

Here we show that a GAL4 DNA binding domain-SIR1 hybrid protein (G_{BD}-SIR1), when targeted to an *HMR* locus containing GAL4 binding sites (UAS_G), can bypass the requirement for the silencer element *HMR-E*. Silencing mediated by G_{BD}-SIR1 requires the trans-acting factors that normally participate in repression, namely SIR2, SIR3, SIR4 and histone H4. Telomeric silencing, which does not require SIR1 and is normally unstable, is enhanced by anchored G_{BD}-SIR1. However, expression of the G_{BD}-SIR1 hybrid in the absence of telomeric UAS_G sites strongly interferes with telomeric silencing. These experiments support a model in which native SIR1 protein is brought to the *HM* loci by proteins bound to the silencers where it acts to assure the efficient establishment of the silenced state. Telomeres appear to lack the ability to recruit SIR1 and that is why telomeric silencing is unstable. The localization of SIR1 to mating-type gene silencers in wild-type cells is probably due to an interaction with the Origin Recognition Complex and may be helped by another silencer binding protein, RAP1.

TELOMERASE ACTIVITY AND STABLE TELOMERES IN HUMAN OVARIAN CARCINOMA.

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Telomeres are composed of highly conserved G-rich repetitive DNA and protein. These structural elements act as a buffer, protecting eukaryotic chromosomes from continual loss of terminal sequences due to incomplete replication of 3' ends of linear molecules by DNA polymerase. Telomeres may also prevent illegitimate recombination of chromosomes and ensure their proper segregation. Loss of telomeric DNA and absence of telomerase, the enzyme which elongates telomeres *de novo*, have been documented in a variety of human somatic tissues *in vivo* and *in vitro*. We have reported decrease in telomere length and lack of telomerase in transformed pre-crisis human cells[1]. In post-crisis immortal cells, on the other hand, we have detected stabilization of telomeres and telomerase activity. Our data support the hypothesis that chromosomes with critically short or no telomeres may contribute to the proliferative crisis of transformed cells, and that activation of telomerase is necessary to arrest the lethal shortening of telomeres, thereby permitting the unlimited proliferation of immortal cells.

Telomerase may play a similar role in the development of human tumours. We hypothesized that metastatic or post-therapy tumours, whose growth might exceed the proliferative potential of non immortal cells, should contain cells expressing telomerase. To test this hypothesis, we measured telomere length and telomerase activity in normal and tumour cells isolated from the ascitic fluid of patients diagnosed with late stage (metastatic) ovarian cancer. We found that in tumour cells telomeres were as short as in cells immortalized *in vitro* (3.7 kbp on average), whereas in normal cells they were approximately 4.5 kbp longer. Furthermore, telomere length in tumour cells was stable. Lastly, we have detected telomerase activity in unfractionated ascitic fluid and shown, upon cell fractionation, that the enzyme is present only in tumour cells. These data indicate that loss of telomeric DNA continues beyond the onset of malignancy, and are consistent with a requirement for telomerase activation in the advanced stages of disease.

[1] Counter *et al.*, EMBO J. 11:1921-1929, 1992.

MAMMALIAN GENE AMPLIFICATION AT EARLY STAGES : THE PRODUCT AND THE SOURCE OF MAJOR CHROMOSOMAL REARRANGEMENTS

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Gene amplification and chromosomal rearrangements are frequent properties of cancer cells but how they are related is unknown. We study these phenomena in an "in vitro" model system : the early stages of adenylate deaminase 2 (AMPD2) gene amplification in Chinese hamster cells. We have performed multi-marker fluorescent "in situ" hybridization (FISH) analysis of several independent clones of amplified mutants.

The major observations are : (a) the extra copies are usually found, like the original one, on a chromosome 1 ; (b) a considerable level of heterogeneity is observed within each clone : the number and the organization of the AMPD2 genes vary from cell to cell ; (c) in about 10 % of the cells, the amplified genes are found on chromosomes with cytologically detectable rearrangements (dicentric and ring chromosomes). Two color FISH with probes for coamplifiable markers has been used to analyze the organization of the amplified chromosomal structures ; it revealed complex megabase-long symetrical inverted repeats. Both the heterogeneity within clonal cell populations and the arrangement of the extracopies in the chromosome are remarkably well accounted for if breakage-fusion-bridge (BFB) cycles involving sister chromatids drive the amplification process at these early stages. Such a mechanism relying on unequal segregation of gene copies at mitosis is expected to generate deleted as well as amplified cells. Moreover, dicentric and ring chromosomes, which are not predicted intermediates of this mechanism, are shown to be frequently generated by secondary events in cells undergoing the chromatid type of BFB cycle. Thus, this mechanism is the major source of both intrachromosomal amplification and genomic rearrangements first limited to a single chromosome, then potentially spread to any additionnal chromosome. Two color FISH also suggests that chromosomal amplification "per se" can trigger chromosomal breakage : during interphase the copies of each amplified marker often cluster in a distinct nuclear domain ; many nuclei have bulges or release micronuclei carrying several copies of one or both markers, indicating that the amplified units destabilize the nuclear organization and may eventually lead to DNA breakage during interphase.

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TOLEDO F., BUTTIN G., DEBATISSE M. (submitted)

CHARACTERIZATION OF SEQUENCES PRIMED WITH A HUMAN CENTROMERE SPECIFIC MOTIF BY THE PCR METHOD TO GENOMIC DNA OF *BOMBYX MORI*.

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Human centromeric antigens are identified by sera from patients (CREST-scleroderma) with anticentromere antibodies. One major protein, CENP-B, binds to a specific 17-mer sequence (CENP-B box) of human alphoid satellite DNA. This sequence is considered to be conserved among mammalian species. In order to look for this sequence in silkworm, *Bombyx mori*, which has a diffuse centromere, we made the probes by PCR method using primers which contain the CENP-B box and flanking region and then carried out fluorescence *in situ* hybridization with the probes to pachytene chsomosomes of the *B. mori* spermatocyte. PCR products from human genomic DNA hybridized to centromeric regions in almost all of human chromosomes and to restricted regions of the *B. mori* chromosomes. Probes amplified by the same primers in *B. mori* genomic DNA hybridized to various positions of human chromosomes including the centromere region, while they hybridized to the restricted region of *B. mori* chromosomes similar to the case of using human centromeric probes. Two major units of 1.3kb and 0.8kb sequences were isolated from the library of PCR products amplified with the *B. mori* genome and hybridized to the centromeric region of several specific human chromosomes. A half sequence of CENP-B box is found out in these clones. In addition, the indirect immunofluorescence method using CREST sera showed to recognize proteins of *B. mori* chromosomes in the expected and similar pattern for detected by the human alphoid sequence probe.

SUBGENOMIC APHIDICOLIN RESISTANT POLYOMA AND CELLULAR DNA SYNTHESIS OCCURS EARLY IN THE DIFFERENTIATION OF CULTURED MYOBLASTS TO MYOTUBES. Nicholas J. DePolo* and Luis P. Villarreal*, Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

Small DNA viruses have been historically used as probes of cellular control mechanisms of DNA replication, gene expression, and differentiation. Polyomavirus (Py) DNA replication is known to be linked to differentiation of many cells including myoblasts. In this report we use this linkage in myoblasts to simultaneously examine (1) cellular differentiation control of Py DNA replication, and (2) an unusual type of cellular and Py DNA synthesis during differentiation. Early proposals that DNA synthesis was involved in the induced differentiation of myoblasts to myotubes were apparently disproved by relying on inhibitors of DNA synthesis (ara-c and aphidicolin), which indicated that mitosis and DNA replication is not necessary for differentiation. Theoretical problems with the accessibility of inactive chromatin to trans-acting factors led us to re-examine possible involvement of DNA replication in myoblasts differentiation. We show here that Py undergoes novel aphidicolin resistant net DNA synthesis under specific conditions early in induced differentiation of myoblasts (following delayed aphidicolin addition.) Under similar conditions, we also examine uninfected myoblast DNA synthesis and show that soon after differentiation induction a period of aphidicolin resistant cellular DNA synthesis can also be observed. This drug resistant DNA synthesis appears to be subgenomic, not contributing to mitosis, and more representative of polyadenylated than non-polyadenylated RNA. These results renew the possibility that DNA synthesis plays a role in myoblast differentiation, and suggest that the linkage of Py DNA synthesis to differentiation may involve a qualitative cellular alteration in Py DNA replication.

DYNAMICS OF CHROMOSOMES DURING MEIOSIS VISUALIZED IN INDIVIDUAL LIVING CELLS OF FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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We have analyzed arrangement and dynamics of meiotic chromosomes in living and fixed cells of fission yeast *Schizosaccharomyces pombe*. First, to visualize chromosome dynamics, living cells were stained with a DNA-specific fluorescent dye, Hoechst 33342. Upon nitrogen starvation to induce meiosis, stained cells mated and went through the process of meiosis on the fluorescence microscope stage. Continuous observation of chromosome dynamics during the entire process of meiosis made it possible to precisely determine the temporal sequence of chromosomal events that had been observed in fixed preparations. In fission yeast meiosis, it had been known that nuclei show characteristic, elongated morphology called "horse tail" before the first meiotic division. In live data, we found a vigorous movement of horse-tail nuclei circulating within a cell. Horse tail nuclei crawl at about 5-6μm/min along the long axis of the cell, keeping a specific nuclear site at the front end of the movement, and make a U-turn at the end of the cell. This movement of horse-tail nuclei begins immediately after karyogamy and continue for several hours. After stopping the movement at the center of the cell, nuclei return to a spherical shape and start meiotic divisions. The period from karyogamy to the second meiotic division took 3-8 hours at 22°C, with a 1-5 hour "horse-tail" period varying from one cell to another.

Second, in fixed meiotic cells, the position of centromeres, telomeres and spindle pole bodies (centrosome equivalent) was determined by fluorescence *in situ* hybridization and indirect immunofluorescence microscopy. In "horse-tail" nuclei, all the telomeres were clustered at a single location at the sharp end of the horse-tail shape. Furthermore, this telomere cluster was found to be closely associated with a spindle pole body located at the front end of the horse-tail movement, while centromeres were on the other side of the nucleus, separated from a spindle pole body. In control experiments, it was confirmed that a spindle pole body is closely associated with centromeres during mitosis as well as the period of meiotic nuclear divisions. Combination of these results suggests that telomeres have yet unknown functions during premeiotic events such as homologous chromosome pairing.

Note: We will present a video movie showing chromosome dynamics in live meiosis.

USE OF SITE-SPECIFIC RECOMBINATION AS A TOPOLOGICAL TRAP OF TRANSCRIPTION-INDUCED DNA SUPERCOILING

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Tracking of proteins along double-stranded DNA can induce torsional strain within a template. This induction is a consequence of the helical structure of DNA and occurs during transcription when a translocating RNA polymerase (RNAP) is prevented from rotating around the imaginary template axis. Localized DNA supercoiling thus contributes significantly to the overall superhelical state of DNA inside a cell and may play an important role in regulatory pathways which employ DNA transactions.

Transcription-induced DNA supercoiling is divided in two domains: a negatively supercoiled domain behind and a positively supercoiled domain in front of an advancing RNAP. The local extent of transient DNA supercoiling, however, and the conformation of superhelical turns remain elusive. I have now used site-specific recombination by γδ resolvase *in vitro* to topologically trap negative (-) supercoils behind an advancing RNAP on a circular, 9 kilo-basepairs (kb) long template. The results show that (-) supercoils are concentrated on a relatively small stretch of < 2.6 kb, and that the maximal superhelical density (σ) therein reaches unexpectedly high values of up to -0.095. The results suggest a model of a mobile DNA superhelix, which contains plectonemic superhelical turns gradually increasing in number along an imaginary superhelix axis. The creation of such a mobile superhelix by protein tracking is likely to have profound effects on the communication between DNA-bound proteins *in vivo*.

HELICASE ACTIVITY OF THE *E. coli* RecBCD PROTEIN: ROLE OF THE INDIVIDUAL SUBUNITS

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The *E. coli* RecBCD, RecB, RecC and RecD proteins have been overexpressed and purified. Mixing of the RecB, RecC and RecD subunits results in a reconstituted RecBCD protein with all of the known activities of the native RecBCD holoenzyme. We have tried to dissect the many different activities of the RecBCD enzyme by studying the properties of the individual subunits alone and in the various combinations. Although both RecB and RecD proteins have consensus "G-K-T" ATP binding sites, and have been shown to bind ATP, only RecB protein on its own has DNA dependent ATPase activity. This ATPase activity is coupled to the ability of the RecB protein to track along single stranded DNA and unwind any annealed oligonucleotides as it does so. Studies with synthetic short oligonucleotides with and without non-complementary "tails" at either end, annealed to ssDNA M13 circles, suggest that the RecB protein tracks along ssDNA in the 3' to 5' direction.

This conclusion is supported by studies with synthetic DNA substrates in which 20-mers are annealed at either the 5' end or the 3' end of a 60-mer. In this case, only the 20-mer at the 5' end is removed. RecD protein does not unwind any of these substrates under the same conditions, but it is possible that it may behave differently when part of the RecBCD complex. As expected, RecC protein shows no helicase activity on its own but it strongly increases the helicase activity of RecB when added to it. Gel retardation studies show that none of the individual subunits binds strongly to the 60/20 substrates, but reconstituted RecBCD binds very strongly, particularly to the tailed ends of a 60/20 substrate in which the 20-mer is annealed to the middle of the 60-mer.

MULTIPLE MODIFIER GENES THAT AFFECT DNA METHYLATION IN THE MOUSE

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A strain-specific modifier gene, *Ssm-1*, has previously been shown to control the methylation of a particular transgene, even when the transgene is integrated at different loci (Cell 65:939). The transgene, which contains the immunoglobulin heavy chain enhancer, metallothionein promoter, E. coli *gpt* coding region, and SV40 splice and poly A signals, is methylated when in a C57BL/6 strain background but becomes unmethylated when bred into DBA/2. Breeding experiments using the BXD recombinant inbred set showed complete concordance, in 26 of 26 strains, between transgene methylation and the C57BL/6 allele of *Fv-1 (Pnd)*, thus localizing the modifier, *Ssm-1*, to distal chromosome 4. New transgenic mice, lacking either the enhancer or promoter, have been made and the methylation of these transgenes is also controlled in a strain-specific fashion. Mapping experiments, using the BXD series, shows that the strain distribution patterns (SDPs) of the modifiers that control methylation of the altered transgenes are similar, but not identical, to the SDP of *Ssm-1*. Moreover the SDPs of the new modifiers are different from each other. These results suggest that methylation of this group of related transgenes may be controlled by multiple functionally related genes. A backcross analysis, using simple sequence repeat markers from chromosome 4 is underway to determine the linkage relationships within this family of modifiers. A working hypothesis, whose test will await the physical isolation of these modifiers, is that these genes encode products that alter the chromatin structure of their targets and might be similar to certain modifiers of position effect variegation in Drosophila.

HOMOLOGOUS RECOMBINATION INTO THE HUMAN β-GLOBIN LCR

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The β-globin Locus Control Region (LCR) is a complex DNA regulatory region that controls the expression of the cis linked β-like globin genes located 50 kb 3' of the LCR. We have initiated investigations of the LCR function by using homologous recombination to mutate the LCR. These studies have been done in MEL cell hybrids that carry a single copy of the human chromosome 11 and transcribe human β-globin mRNA when the cells are induced to differentiate. Our initial studies showed that the insertion of an expressed selectable marker, (driven by the Friend Virus LTR), into the LCR disrupts the LCR mediated regulation of globin transcription. In these cells human β-globin mRNA is no longer expressed when the cells differentiate and instead the selectable marker gene shows an increase in steady state mRNA after differentiation. Present techniques for homologous recombination require the insertion of a selectable marker, therefore, further progress in using homologous recombination to analyze this locus depends on a simple and efficient method for the deletion of the selectable marker and a demonstration that following this deletion the locus functions normally again.

We have used the yeast FLP/FRT recombinase system to achieve precise deletion of the selectable marker gene by expressing FLP both transiently and stably in cells which contain a neo^R gene integrated by HR between sites 1 and 2 of the human β-globin LCR. After FLP mediated deletion, the locus functions as it did before the HR event. We are currently using this system to analyze the function of each LCR HS in its endogenous location with regard to chromatin structure, replication timing, and globin gene transcription and switching. These studies demonstrate the feasibility of using homologous recombination to analyze the LCR and, in general, other complex cis regulatory DNA elements in their normal chromosomal context.

THE IDENTIFICATION AND CHARACTERIZATION OF HUMAN FACTORS INVOLVED IN THE REJOINING OF DNA DOUBLE STRAND BREAKS.

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The DNA double strand break is an important lesion caused by ionizing radiation and other DNA damaging agents, and presents a serious challenge to cellular repair processes. Therefore it is important to know how the repair of this lesion is achieved. Although the ligation of single stranded nicks in synthetic double stranded DNA templates has been well characterized, and three DNA ligases have been described in humans, the enzymes involved in the rejoining of double strand breaks are unknown. An *in vitro* system is described which is highly efficient at the correct rejoining of model DNA double strand breaks, produced by the digestion of a plasmid with a restriction enzyme, both at temperatures which favour ligation (17°C) and also at the physiological temperature of 37°C. This system has subsequently been used as a assay to identify factors which are involved in the process of double strand break rejoining.

By biochemical fractionation of a human cell extract, we have separated multiple activities which are capable of rejoining a *Sal* I restriction cut plasmid. We have also extensively purified another protein (REP I) which contains no break joining activity on its own, but will stimulate the break joining activity 4 fold. This protein appears to be of very low abundance and we currently trying to examine its biochemical properties.

Surprisingly, we have shown that fractions containing ligase I, as demonstrated by Western blotting, are totally inactive on this template unless REP is also present. The further purification of the various break joining factors and elucidation of the interactions between these is in progress.

TELOMERE-ASSOCIATED CHROMOSOME FRAGMENTATION

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A YAC-cloned human telomeric fragment is functional when reintroduced into mammalian cells. A vector containing a correctly orientated 2kb stretch of human terminal repeat sequence was constructed based on pSV2*his*, linearised and electroporated into a hamster-human hybrid cell line. 20% of transformants generated by random integration of the construct have a chromosome in which one telomere is provided by the introduced DNA sequences. Analysis of the integration sites by plasmid rescue indicates that breakage is random and does not involve targeting to existing terminal repeat arrays.

It has been shown previously that by simultaneously applying both positive and negative selection pressures the chromosomal content of somatic cell hybrids can be modified. Combining this approach with the introduction of cloned telomeres we have demonstrated that it is possible to generate stable truncated mammalian chromosomes. The model system used to test this scheme was a somatic cell hybrid containing a human X chromosome with the his^r marker targeted to *MIC2* (Xp22.32). A telomere-containing construct based on pSV2*hygro* has been electroporated into this cell line. The cells, which are routinely maintained in histidinol, were selected for $hygro^r$ and simultaneously back selected against *HPRT* using 6-TG. Using this approach a panel of cell lines has been generated carrying nested terminal deletions of the human X chromosome extending from Xq24-q26 to the centromere. In 90% of these lines telomere-healing has been accompanied by duplication of the terminal DNA sequences suggesting that the broken chromosomes may have undergone breakage-fusion-bridge cycles before rescue.

Several derivative human X chromosomes have been generated which have been broken within the centromere and consist only of the short arm of the chromosome. In these lines the introduced telomere is juxtaposed with the alpha satellite DNA. These telocentric X chromosomes will be fragmented into smaller chromosomes using TACF.

THE *REC2* GENE OF *USTILAGO MAYDIS* CONTROLS HOMOLOGOUS TARGETING OF LINEAR PLASMID DNA

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Ustilago maydis cells bearing the *rec2-1* mutation are hypersensitive to DNA damage, completely blocked in meiosis, and deficient in damage induced mitotic recombination. To investigate the function of *REC2*, we designed assays for homologous targeting in which plasmid-borne mutant alleles of the *LEU1* gene must recombine with a chromosomal *leu1* allele to confer leucine prototrophy. Results from these experiments indicate that *REC2* plays a direct and critical role in pairing and strand invasion during homologous recombination.

In *REC2* strains, the introduction of a double-strand break or gap in plasmid *leu1* sequences stimulated homologous targeting 50-fold compared to circular plasmids. In a *rec2-1* background, the strong stimulation induced by double strand breaks was completely absent. Recombination events in these studies resulted in either integration of the vector by a crossover or by replacement of the genomic *leu1-1* mutation by corresponding wild-type sequence, presumably via gene conversion. To examine if the *rec2-1* mutation affects the distribution of these two types of events, we performed Southern hybridization analysis on genomic DNA from individual recombinant colonies. In both *rec2-1* and *REC* 2 strains, integration of the targeting vector occurred 35-50% of the time, regardless of the conformation of transforming DNA. These results provide evidence that *REC2* acts before the resolution step of recombination.

We conclude that the *REC2* gene product is a critical component of an apparatus that repairs DNA double-strand breaks or gaps in mitotic cells through recombination with the intact homologue or sister chromatid. The complete block in meiosis is likely due to an inability to process double-strand breaks into recombinant chromosomes. We hypothesize that the *REC2* gene product interacts with single-stranded termini formed at sites of double-strand breaks by an exonuclease and is involved in the search for homology and/or the catalysis of strand invasion and D-loop formation. In support of this hypothesis, the sequence of *REC2* reveals a region of significant homology to bacterial *RecA*, and preliminary biochemical experiments on partially purified *REC2* protein demonstrate RecA-like activities.

MUTATIONAL ANALYSIS OF A TYPE II TOPOISOMERASE CLEAVAGE SITE: BASE PREFERENCES FOR THE ENZYME AND FOR INHIBITORS

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The bacteriophage T4 topoisomerase is inhibited by various antitumor agents (e.g. *m*-AMSA), each of which traps the enzyme in a DNA cleavage complex. We have analyzed the sequence preferences for cleavage of a strong *m*-AMSA-induced T4 topoisomerase cleavage site contained on a 30-bp oligonucleotide. Oligonucleotide substrates that contained each of the four nucleotides at 14 positions surrounding the cleavage site, including the positions within the 4-bp stagger created by cleavage, were tested for cleavage in the absence or presence of several type II topoisomerase inhibitors (*m*-AMSA, mitoxantrone diacetate, a modified ellipticine, VP-16, and oxolinic acid). In addition, cleavage was tested with an *m*-AMSA-resistant T4 topoisomerase that has altered sensitivity to several of the inhibitors (Huff and Kreuzer (1990) J. Biol. Chem. 265, 20496-20505).

Strong base preferences were detected at eight positions flanking the cleavage site. At these positions, the same base preferences were found regardless of which inhibitor or which enzyme was used to induce cleavage, indicating that these positions are important in recognition of the cleavage site by the enzyme. The preferred bases showed dyad symmetry with respect to the cleavage site, indicating that the two protomers of the topoisomerase dimer interact with DNA in an analogous manner.

In contrast, the preferred bases immediately adjacent to the cleaved phosphodiester bonds were highly specific to the inhibitor used, with the position to the 5' side of each cleaved bond showing the greatest specificity. These results strongly suggest that the inhibitors interact directly with the DNA bases at the cleavage site, placing the inhibitor binding site precisely at the site of DNA cleavage. The base preferences we found at these inhibitor-specific positions are similar to those found with the eukaryotic enzyme for the inhibitors which have been used in published consensus results (Fosse *et al.* (1991) Nucl. Acids Res. 19, 2861-2868; Pommier *et al.* (1991) Nucl. Acids Res. 19, 5973-5980), suggesting that the same rules of inhibitor-DNA interactions may influence cleavage complex formation by diverse type II topoisomerases.

HUMAN GENOME ORGANIZATION: INSIGHTS FROM ANALYSIS OF CHROMOSOME 21

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Most information on human genome organization has been obtained by cytogenetic techniques, with reference to Giemsa and Reverse bands. Now, with the construction of long range physical maps and the availability of yeast artificial chromosome (YAC) contigs, it is possible to analyze features of genome organization directly at the molecular level. Chromosome 21 is an ideal model for such studies. The long arm spans approximately 40 Mb, and both a detailed physical map and an essentially complete YAC contig are available. These are being used to examine the distribution of, and the correlations among, several features of genome organization: GC content, CpG frequency, CpG island frequency (as determined by rare restriction sites), Alu and Line density, and gene density. In analysis of >10 Mb (approximately 25% of the long arm) of YAC DNA, it has been observed that distributions of the first 4 of these features often differ from that expected from accepted pictures of G and R bands. In addition, correlations among these features are not clear, e.g. as CG content increases, CpG island frequency does not necessarily increase, even in noted gene rich regions; some R band segments appear Line rich and most G band segments appear Alu rich.

Organizational features may be more recognizably related to transcriptional characteristics. Therefore, a complete gene map of the chromosome is under construction. The technique of cDNA hybrid selection is being applied to all clones from the minimal YAC contig. This rapid and efficient technique has been very effective in identification of coding sequences from YACs spanning 3 Mb of the MHC region: in addition to all known genes, 50 new coding sequences have been selected. Preliminary data from chromosome 21 YACs indicate that known genes are also being retrieved, in addition to new genes, including novel Zinc fingers. By using cDNAs from a wide variety of tissues, developmental times, and cells lines, it should be possible to isolate most or all genes from each YAC. This will allow comprehensive determination of gene density and gene size relative to chromosomal location.

IDENTIFICATION AND PURIFICATION OF A PROTEIN THAT BINDS TO THE INITIATOR REGION OF THE RIBOSOMAL PROTEIN S16 GENE

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Two DNA elements, the TATA box, located approximately 30 nucleotides upstream from the start site, and the initiator element, encompassing the start site, play a pivotal role in RNA polymerase II transcription. The molecular mechanisms by which the TATA box directs transcription has been well studied, however, little is known about how initiator elements direct transcription. Trans-acting proteins that interact with initiator elements are beginning to be identified.

Previous studies of the mouse ribosomal protein S16 gene demonstrated that transcription is initiated at a discrete site within a polypyrimidine tract. Point mutations in this tract altered the start site of transcription both in vivo and in vitro. In contrast, mutations in the TATA box region influenced the efficiency of transcription but did not change the location of the start site of transcription. Recently, we have identified and purified to apparent homogeneity a protein that binds to the rpS16 initiator sequence. The protein appears to be a heterodimer with subunits of 45 kD and 55 kD. Methylation interference and DNAase I footprinting studies with both wild-type and mutated rpS16 initiator sequences were used to identify DNA-protein contacts. Studies in progress are aimed at understanding how the rpS16 initiator protein directs the general RNA polymerase II transcription machinery to initiate transcription with such precision.

INVERTED DNA REPEATS: A SOURCE OF EUKARYOTIC GENOMIC INSTABILITY

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While inverted DNA repeats are generally acknowledged to be an important source of genetic instability in prokaryotes, relatively little is known about their consequences in eukaryotes. Using bacterial transposon Tn*5* and its derivatives we have demonstrated that long inverted repeats also cause genetic instability in the yeast *Saccharomyces cerevisiae* leading to deletion. Furthermore they induce homologous recombination. Replication plays a major role in the deletion formation. Deletions are stimulated by a mutation in the DNA polymerase δ gene (*pol3*). The majority of deletions result from imprecise excision between small (4-6 bp) repeats in a polar fashion often generating quasipalendrome structures that subsequently may be highly unstable. Breakpoints are clustered near the ends of the long inverted repeats (<150 bp). The repeats have both an intra- and interchromosomal effect in that they also create a hotspot for mitotic interchromosomal recombination. Intragenic recombination is 5-18 times more frequent for heteroalleles involving the long inverted repeats as compared to heteroalleles without the repeats. We propose that both deletion and recombination are the result of altered replication at the basal part of the stem formed by the inverted repeat.

AFFINITY PURIFICATION OF MOLONEY MURINE LEUKEMIA VIRUS CHROMATIN FROM INFECTED CELLS

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Our goal is to develop a system to study proteins that associate in vivo with the Moloney Murine Leukemia virus (M-MuLV) enhancer elements by the isolation of intact proviral chromatin. The moloney murine leukemia virus (M-MuLV) long terminal repeats (LTR's) contain tandem repeated transcriptional enhancer sequences that consist of smaller motifs, that bind cellular DNA-binding proteins implicated in transcriptional regulation. The M-MuLV enhancers are also important for disease specificity and latency of disease induction. To enrich for proviral chromatin containing M-MuLV LTR sequences, an affinity purification scheme has been employed which relies on bacterial lac repressor affinity for lac operator sequences. An infectious M-MuLV recombinant has been constructed that contains bacterial lac operator sequences inserted into a non-essential region downstream from the 5' LTR of the virus (M-MuLV-lacO). Nuclei from M-MuLV-lacO infected cells were digested with Pvu II (which will liberate an LTR fragment containing lac O sequences), and digested chromatin was leached from the nuclei in hypotonic buffer. M-MuLV-lac O chromatin was then recovered by binding to an affinity matrix consisting of a beta-galactosidase/lac repressor fusion protein anchored to acrylamide beads by an anti-betagalactosidase monoclonal antibody (Levens and Howley,(1985)*Mol. Cell. Biol.* 5:2307-2315). Specifically bound chromatin was eluted under physiological conditions by incubation with the galactose analog IPTG. Southern blot analysis confirmed the specific enrichment of M-MuLV proviral chromatin by this method. Efforts to identify proteins associated with the M-MuLV chromatin are in progress.

IDENTIFICATION OF TWO REPLICATION TIMING DOMAINS IN THE MURINE ADA GENE REGION

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DNA replication in mammalian cells is under strict spatial and temporal control with certain regions of the genome being replicated early in S-phase and others replicated late. The replication timing of a specific chromosomal region appears to be related to chromatin structure and transcription. In order to understand the mechanisms underlying the coordination of replication and transcription, we are studying the replication timing topography of the murine ADA gene region.

Cosmids spanning 250 kb of DNA have been isolated from B1/150, a murine cell line containing the ADA gene region amplified on double minute chromosomes (DMs). We have found that this region contains at least two origins of DNA replication, each initiating replication at distinctly different times in S-phase. One origin, the 377 origin, isolated and characterized in our laboratory localizes approximately 160 Kb 3' of the ADA gene in the DMs. PCR analyses of newly replicated nascent strand DNA from B1/50 cells retrosynchronized by FACS indicate that the 377 origin and the ADA gene replicate in distinctly different S-phase windows in the amplified cell line. The ADA gene therefore probably replicates under the control of another origin on the DM chromosome.

We have used fluorescence *in situ* hybridization (FISH) on random populations of unsynchronized interphase nuclei to determine the replication timing and genomic order of a series of cosmids spanning the ADA-377 region. Replication timing of each of these cosmids in their normal chromosomal context was evaluated by performing FISH on aneuploid (CL-1D) and diploid (YAC-1) mouse cell lines. Using bi-color FISH we have identified two distinct chromosomal replication timing domains and their boundary in the murine ADA gene region. Three color FISH experiments indicate that the chromosomal order of the cosmids is different from the DM order indicating the ADA region has been significantly rearranged during gene amplification. Comparative studies of the replication timing topographies in single copy and amplified lines are in progress to see whether or not the same domains and boundaries are maintained outside the chromosomal context. The replication timing of selected cosmids from the ADA-377 contig has also been compared between two different cell types (fibroblasts and lymphocytes) to determine if replication timing of a particular region is tissue dependent.

TRANSFECTION-MEDIATED REGENERATION OF FUNCTIONAL TELOMERES IN HUMAN CELLS. John Hanish, Judy Yanowitz, Henrik Tommerup, and Titia de Lange. The Rockefeller University, New York.

We have observed that transfecting human cells with telomere-containing neo constructs results in efficient positioning of the transfected sequences at chromosome ends. These telomere regeneration events are demonstrated by the sensitivity of the terminal neo fragments to Bal31 exonuclease and by localization of the transfected DNA to chromosome ends by FISH. Similar observations were made in hamster cells by Farr et al. (PNAS (1991) 88, 7006). We have developed conditions under which a telomere regeneration event occurs in as many as 80 % of the transfected cells. Efficient telomere regeneration in human cells allows the rapid detection of relative telomere regeneration frequency from pools of colonies. Using this assay in HeLa cells, we are determining the cis-acting elements required for telomere regeneration and the mechanism by which it occurs.

Cis-acting requirements: We have determined that optimal telomere regeneration is obtained with constructs that carry the cloned telomere at, or within 50 bp of, a DNA end. Circular plasmids are 2-3 fold less effective. Telomere regeneration is strongly dependent on the length of the TTAGGG repeat array as 0.8-1.6 kb of telomeric DNA result in 80 % telomere regeneration while 0.1-0.4 kb of TTAGGG repeats display marginal or no activity. Finally, telomere regeneration is sequence dependent, as 1.1-1.6 kb of either TAGGG or TTGGGG repeats are not active.

Chromosome breakage: We cloned the genomic DNA proximal to two telomere regeneration sites and determined that both loci are originally chromosome-internal, suggesting that the mechanism of telomere regeneration includes chromosome breakage. This argues against homologous recombination with a pre-existing telomere as the primary mechanism of telomere regeneration.

Telomere healing: The new telomeres are often several kb longer than the transfected DNA, indicating that telomere regeneration includes a telomere healing event. Two mechanisms could account for this: homologous recombination with an endogenous telomere or telomerase-mediated addition of telomeric repeats. We are now assaying long TTAGGG repeat arrays that are interrupted by point mutations every 160 bp. These interrupted telomere tracts are expected to be incapable of homologous recombination and should allow us to discriminate between the models for telomere healing.

Our results corroborate the belief that TTAGGG repeats are sufficient for telomere function in human cells and show that genes placed at human telomeres can be properly expressed. Analysis of the telomere regeneration capacity of heterologous repeat sequences may reveal characteristics of the cellular machinery responsible for recognition of telomeric DNA.

VISUALIZATION OF TRANSCRIPTION AND REPLICATION SITES IN HUMAN CELLS

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We have developed a permeabilized cell system that retains most of the RNA and DNA polymerizing activities of the cell. HeLa cells were encapsulated in agarose microbeads, permeabilized with streptolysin O, and incubated with triphosphates in a 'physiological buffer'.

Focal sites of transcription were visualized after incubation with Br-UTP and immunolabelling with an antibody that reacts with Br-RNA. After extending nascent RNA chains by <400 nucleotides *in vitro*, ~400 focal synthetic sites can be seen by fluorescence microscopy. Most foci contain the Sm antigen-component of the splicing apparatus. α-amanatin prevented incorporation into these foci and allowed visualization of 25 nucleolar foci. Both nucleolar and extra-nucleolar foci remain after nucleolytic removal of 90% of chromatin.

Sites of DNA replication have also been shown to be focally concentrated, however, such focal concentrations could arise by aggregation induced by fixation. Sites of replication were directly labelled by incubation with fluorochrome-dUTP conjugates; ~150 sites of fluorescence were seen in unfixed cells by conventional and digital fluorescence microscopy. They are unlikely to be artifacts as physiological buffer is used throughout and all the replication activity found *in vivo* is retained. These foci also appeared to be attached to an underlying structure as they remained when 90% of chromatin was removed, even in this entirely unfixed preparation.

Replication sites have also been analysed by electron microscopy. Early S-phase cells were incubated with biotin-11-dUTP, then 90% of the chromatin was removed before electron microscopy of thick resinless sections. Dense morphologically discrete ovoid structures were immunolabelled with gold conjugated anti-biotin antibodies after 2.5 min incubation; after 5 min the label began to spread into adjacent chromatin which became extensively labelled after 1 hour. This provides evidence for fixed polymerization factories attached to a skeleton, with replication occurring as the template moves through them.

We conclude that replication and transcription sites occur in factories attached to an underlying skeleton.

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Free chromatin structure and its application in genome analysis

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After conventional chromosome slide preparation, a small proportion of elongated chromatin structure may be obtained in addition to the typical condensed mitotic chromosomes and interphase nuclei. Those structures were ignored for years because of their low frequency among metaphase chromosomes and interphase nuclei. One particular form of structure, which we named free chromatin, could be enriched by chemical treatment at the G2 phase of the cell cycle, possibly through inhibiting or interfering with the process of chromosome condensation. We have exploited these extended, 300 Å chromatin fibers in combination with fluorescence *in situ* hybridization for gene mapping studies (PNAS 89:9509-9513, 1992). In this report, we present data to describe the nature of free chromatin. Through cell cycle analysis, we show that the efficiency of free chromatin generation is cell cycle related, and that it appears to be related to the stability of nuclear envelope during the interphase to metaphase or metaphase to interphase transition. Based on the latter assumption, we have also investigated alternative means to generate free chromatin. One simple method based on alkaline buffer treatment is devised. As an example of chromosomal structure study, we demonstrated that the mouse major and minor satellite sequences are not interspersed with each other but separated as two units, which can be directly visualized as two colored string like patterns. We have also studied the order of three repetitive sequence elements (PTRS-25, PTRA-20 and PTRA25) at the centromere regions of human chromosome 15. By multicolor FISH analysis on free chromatin their apparent order was found to be PTRS-25 (short arm)-PTRA-20-PTRA-25. We then applied the technique to study the order and genomic organization of YAC clones isolated from the long arm of human chromosome 7. Although the order of the clones could not be determined by metaphase mapping, their relative position could be visualized directly on free chromatin. For example, three closely mapped clones, HSC7E515, HSC7E803 and HSC7E571, were localized to 7q21 by metaphase FISH analysis; their order was established by the pattern of three different colors on free chromatin. We suggest that FISH detection with free chromatin is a powerful technique not only in the construction of physical maps for the human genome but also in chromosomal structure studies.

A Z-DNA BINDING PROTEIN FROM CHICKEN BLOOD NUCLEI

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A protein (Zα) that appears to be highly specific for the left-handed Z-DNA conformer has been identified in chicken red blood cell nuclear extracts. Zα activity is measured in a bandshift assay using a radioactive probe consisting of a $(dC\text{-}dG)_{35}$ oligomer that has 50% of the deoxycytosines substituted with 5 bromodeoxycytosine. In the presence of 10 mM Mg^{2+}, the probe converts to the Z-DNA conformation and is bound by Zα. The binding of Zα to the radioactive probe is specifically competed for by linear poly(dC-dG) stabilized in the Z-DNA form by chemical bromination but not be B-form poly(dC-dG) or boiled salmon-sperm DNA. In addition, the binding activity of Zα is competed for by supercoiled plasmids containing a Z-DNA insert but not by either the linearized plasmid or by an equivalent amount of the parental supercoiled plasmid without the Z-DNA forming insert. Zα can be crosslinked to the ^{32}P labeled brominated probe using UV light, allowing us to estimate that the minimal molecular weight of Zα is 39 kd.

AN UNUSUAL REPEAT SEQUENCE MAPPING TO HUMAN CHROMOSOME 4q35-qter IS IMPLICATED IN FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder affecting primarily the facial, shoulder and upper arm muscles. Linkage of FSHD to chromosome 4q35 has been established. In a search for homeobox genes, using a degenerate oligonucleotide for the Helix-3 region of the homeodomain, we isolated a cosmid (13E) mapping to human chromosome 4q35-qter. A probe, p13E-11, isolated from this cosmid detects abnormalities on Southern blots of FSHD patients, in sporadic cases this appears to be due to *de novo* DNA rearrangements (Wijmenga *et al.* Nature Genetics 2:26-30, 1992). Cosmid 13E has a very unusual structure, consisting primarily of 3 very similar, repeated 3.2kb KpnI fragments. Overlapping cosmids containing up to eight copies of these repeat units have been isolated. Sequence analysis of the repeats shows that they contain a homeobox motif (which appears to be conserved in other species), however, additional sequences with homology to known human repetitive DNA are also present and it is not clear whether the sequences in this region encode functional homeobox genes. The DNA rearrangements observed in FSHD appear to be a deletion of a variable number of these repeat units. It is likely that FSHD is caused either by deletion of a functional copy of this repeat or by a 'position effect' on a nearby gene.

IDENTIFICATION OF ESSENTIAL KINETOCHORE COMPONENTS IN SACCHAROMYCES CEREVISIAE

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To find mutants in potential kinetochore components, we have designed and utilized two in vivo assays of kinetochore integrity to screen an existing large set of S. cerevisiae mitotic chromosome segregation mutants, the *ctf* collection. One assay detects relaxation of a transcription block formed at centromeres in yeast. The other assay is designed to detect weakened attachment of centromere DNA to microtubules as an increase in the mitotic stability of a dicentric test chromosome. Two strains containing the mutations *ctf13-30* and *ctf14-42* were identified as putative kinetochore mutants by both assays. We show that *CTF14* is identical to *NDC10/CBF2*, a recently identified essential gene which encodes a 110 kD kinetochore component. *CTF13* is a previously uncharacterized gene essential for mitotic growth. The temperature sensitive *ctf13-30* mutation confers an increase in the rate of mitotic missegregation at permissive temperature, and upon shift to nonpermissive temperature, an accumulation of large budded cells with a G2 DNA content, a single chromosomal mass at the mother-bud neck, and a very short spindle. At permissive temperature, *ctf13-30* strains exhibit a detectable increase in H1 kinase activity relative to *CTF13* controls, and at nonpermissive temperature, H1 kinase activity levels in *ctf13-30* strains are equivalent to nocodazole arrested strains, suggesting that the *ctf13-30* mutation causes an accumulation in M phase. DNA sequence analysis predicts a 478 amino acid protein with no significant overall homology to known proteins. We show that antibodies recognizing epitope-tagged CTF13 protein decrease the electrophoretic mobility of a CEN DNA-protein complex formed in vitro. Together, the genetic and biochemical data indicate that CTF13 is an essential kinetochore protein.

ANAPHASE IS INITIATED BY PROTEOLYSIS RATHER THAN THE INACTIVATION OF MPF

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We have used frog egg extracts that assemble mitotic spindles to identify the event that triggers sister chromatid separation. Adding a non-degradable form of cyclin B prevents MPF (maturation promoting factor) inactivation but does not block sister chromatid separation, showing that MPF inactivation is not needed to initiate anaphase. In contrast, adding an N-terminal fragment of cyclin, which acts as a specific competitor for cyclin degradation, produces a dose-dependent delay in MPF inactivation and sister chromatid separation. Methylated ubiquitin, which inhibits ubiquitin-mediated proteolysis, also delays sister chromatid separation, suggesting that ubiquitin-mediated proteolysis is necessary to initiate anaphase. The N-terminal cyclin fragment inhibits chromosome separation even in extracts that contain only non-degradable forms of cyclin, suggesting that proteins other than the known cyclins must be degraded to dissolve the linkage between sister chromatids.

EVIDENCE THAT THE DNA REPLICATION FORK BLOCKED AT THE DNA TERMINATION SITE IS AN ENTRANCE FOR THE RECBCD ENZYME INTO DUPLEX DNA

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In both procaryotes and eucaryotes, there are specific regions or sites, named "hotspots", where homologous recombination occurs at a higher rate. DNA replication origin in procaryotes (phageT4) is one example and another example is "*HOT1*" site in yeast. Molecular mechanisms enhancing homologous recombination are not well understood. Microscopically, there is a site "Chi", the homologous recombination of the surrounding region of which is stimulated. In various analyses of the Chi sites only the lambda phage system has been extensively used, because in the *E. coli* system there are numerous Chi sites and an entrance for the Chi responsible enzyme (RecBCD) has not been identified on the circular chromosome. We have identified eight kinds of DNA fragments (named Hot DNA), exhibiting homologous recombinational hotspots activity, derived from *E. coli* chromosome. The Hot activities of all Hot DNAs were enhanced extensively, under RNase H-defective (*rnh*$^-$) conditions. Seven Hot DNAs were clustered at the DNA replication terminus region on the *E. coli* chromosome and had Chi activities. Hot activities of HotA, B and C, the locations of which were close to three DNA replication terminus sites *TerB*, *A* and *C* sites, respectively, disappeared when terminus-binding (*tau* or *tus*) protein was defective, thereby suggesting that their Hot activities are termination event dependent. Other Hot groups showed termination-independent Hot activities. In addition, at least HotA activity proved to be dependent on a Chi sequence, because mutational destruction of the Chi sequence on the HotA DNA fragment resulted in disappearance of the HotA activity. The disappeared HotA activity was reactivated by insertion of a new properly oriented Chi sequence at a position between the HotA DNA and the *TerB* site. From these observations and positional and orientational relationships between the Chi and the *Ter* sequences, we propose a model in which the DNA replication fork blocked at the *Ter* site provides an entrance for the RecBCD enzyme into duplex DNA.

DE NOVO AMPLIFICATION OF THE *DFR1* GENE OF YEAST

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Selection conditions were found which allowed the growth of yeast cells carrying two or more copies of *DFR1,* the gene encoding dihydrofolate reductase. These conditions were used to select for methotrexate resistant strains. Southern hybridizations show that about one quarter of these carry extra copies of *DFR1.*

Four of five of the amplified strains were shown by pulsed-field gel electrophoresis to carry the extra copies of *DFR1* on chromosome XV: the native site of the gene. Aneuploidy for chromosome XV was ruled out by finding that *ADH1,* on the opposite arm of chromosome XV, was not present in additional copies. Three to four copies of *DFR1* were present in each of these strains. The size of the amplicons is not sufficient to change the migration rate of chromosome XV on a pulsed field gel.

The fifth amplified strain was found to contain an autonomously replicating circular DNA molecule which appears to carry two copies of *DFR1* on a molecule with monomer unit size of about 9 kilobases. This strain became resistant to 1.2 mg/ml methotrexate, under which conditions there were more than 100 monomer unit copies of the episome per cell. The episome was isolated by alkaline preparation and used to transform a *dfr1* mutant strain to wild-type.

Thus we have seen both chromosomal and extrachromosomal amplicons, similar to the observations of amplification in mammalian cells.

REPLICATION ORIGINS AND STRUCTURAL ORGANISATION IN PLASMID AND CHROMOSOMAL REPLICONS : A DEVELOPMENTAL STUDY IN XENOPUS

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Origins of DNA replication in metazoan chromosomes have remained elusive and their establishment may rely on nuclear organization and chromatin structure rather than on rigidly defined sequences. Furthermore metazoan replication origins may vary according to the transcriptional program and differentiation state of the cell. To investigate these questions we are examining the replication patterns of Xenopus rDNA sequences on plasmids and in their normal chromosomal context at various stages of embryonic development by 2D gel electrophoresis of replication-fork-containing restriction fragments.

We found that initiation and termination occur on plasmids without detectable sequence specificity in Xenopus eggs or egg extracts. A single, randomly situated initiation event takes place on each plasmid molecule despite the abundance of potential origins.

Upon fertilization, Xenopus embryos undergo a series of very rapid cell cycles in which active chromosome replication occurs in the absence of interfering transcription. We found that replication at this stage involves classical replication forks that are affixed to the nuclear matrix, as described for differentiated cells. There was no evidence for atypical single-stranded replication intermediates. Again, the 2D gel patterns showed that initiation and termination occur at multiple, apparently random sites in the chromosomal rDNA cluster at this developmental stage. All replication intermediates as well as linear (non-replicating) restriction fragments were quantified by storage phosphor technology. A mathematical analysis of these measurements allowed to derive that the mean rDNA replicon replicates in 7.5 min, and is 8-12 kbp in size. This time is close to the total S phase duration, and this replicon size is close to the maximum length of DNA which can be replicated by two simultaneously active replication forks within the short span of time of the S phase. We infer that i) most rDNA origins must be synchronously activated at S phase onset and that ii) origins must be evenly spaced along chromosomal DNA, in order that no stretch of chromosomal DNA is left unreplicated at the end of this very short S phase. Since initiation does not rely on the use of specific origins, it is suggested that this spatially and temporally concerted pattern of initiation matches some periodic architectural feature of the chromatin fiber, which itself need not rely on any special DNA sequence. We are investigating the possibility that permanent nuclear matrix attachment sites may occur at this stage at evenly spaced but random sequences rather than at classical MAR/SAR sequences.

At the midblastula transition, the cell cycles slow down and transcription resumes. Preliminary results on origin spacing and specificity after this developmental transition will also be presented.

TFIIB AND TFIIA CAN INDEPENDENTLY INCREASE THE AFFINITY OF THE TATA BINDING PROTEIN FOR NAKED DNA, BUT PRE-INITIATION COMPLEX FORMATION IS COMPLETELY INHIBITED BY NUCLEOSOME FORMATION

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The general transcription factors TFIIB and TFIIA are the first to associate with the TATA binding protein (TBP) during formation of a transcription initiation complex on RNA polymerase II promoters, yet their functional roles during pre-initiation complex assembly are unclear. DNAse I footprint titration was used to measure the effects of TFIIB and TFIIA on binding of TBP to a consensus TATA box. Under reaction conditions optimized for TBP:DNA complex formation, the presence of TFIIB increased affinity of TBP for the TATA box by 2.5 fold, while TFIIA had no effect. When TBP binding conditions were sub-optimal, both TFIIB and TFIIA independently increased TBP affinity by approximately 10-fold. This function could also be seen by an increased requirement for TFIIA in TBP-mediated transcription reactions. Therefore both TFIIB and TFIIA have the intrinsic ability to regulate pre-initiation complex formation by directly increasing the affinity of TBP for the TATA box.

To examine the ability of TBP, TFIIB, and TFIIA to interact at a TATA box incorporated into a nucleosome, a 155 bp DNA fragment containing two rotational phasing sequences and a consensus TATA box was constructed such that the TATA box would be located at the dyad axis of symmetry on the nucleosome. Variants of the template in which that TATA box was positioned 3 or 6 bp closer to the phasing sequence were also constructed to rotate the TATA relative to the surface of the core histones. End-labelled templates were mixed with histone octamers isolated from HeLa cells and assembled into mononucleosomes by salt dilution. The resulting monoucleosomes were resistant to micrococcal nuclease and exhibited a characteristic 10 bp repeat upon cleavage with DNAse I. TBP was unable to bind to any of the three nucleosomal templates, even at concentrations 1000-fold higher than that required to footprint naked DNA. The presence of TFIIB and/or TFIIA did not facilitate TBP binding. This suggests that the ability of these factors to initiate preinitiation complex formation is inhibited by nucleosomes. The ability of upstream activators and histone modifications to facilitate TBP binding on nucleosomes is currently being investigated.

NUCLEAR PROTEINS THAT BIND THE PRE-mRNA 3' SPLICE SITE SEQUENCE r(UUAG/G) AND THE HUMAN TELOMERIC DNA SEQUENCE d(TTAGGG)n

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Telomeric DNA is composed of multiple repeats of a short sequence, one strand of which is usually guanosine-rich (T_2AG_3 in vertebrates, T_2G_4 in *Tetrahymena*, and T_4G_4 in *Oxytricha*). In organisms where the structure of this DNA has been examined, the G-rich strands protrudes at the 3' end as a 12 - 16 nucleotide single-stranded extension. We identified and purified HeLa cell nuclear proteins that bind to single-stranded d(TTAGGG)n, the human telomeric DNA repeat, using a gel retardation assay. Immunological data and peptide sequencing experiments, as well as two-dimensional gel electrophoresis, indicated that the purified proteins were identical or closely related to the hnRNP proteins A1, A2/B1, D, and E and to nucleolin. These proteins bound to double-stranded d(TTAGGG)n much less efficiently than to single-stranded form. They also bound to RNA oligonucleotides having r(UUAGGG) repeats more tightly than to DNA of the same sequence. The binding was sequence specific, as point mutation of any of the first four bases [r(UUAG)] abolished it. The fraction containing hnRNP D and E proteins was shown to bind specifically to a synthetic ribo-oligonucleotide having the 3' splice site sequence of the human β-globin IVS 1, which includes the sequence r(UUAGG). Intriguingly, the members of the hnRNP D and E proteins identified here (D01, D02, D1*, and E0) were distinct from those derived from hnRNP complexes on two-dimensional gel. Thus, they are nuclear proteins that are not stably associated with hnRNP complexes. These studies establish the RNA-binding specificity of the hnRNP D and E proteins. Furthermore, they suggest the possibility that these hnRNP proteins have the potential to bind to chromosome telomeres, in addition to having a role in pre-mRNA metabolism.

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GENE IDENTIFICATION AND MAPPING IN THE CLASS II REGION OF THE MHC

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The MHC spans about 4mbp of DNA on the short arm of chromosome 6 and encompasses over 80 genes which are divided into three main classes: I, II and III. Some of the genes are involved in antigen processing and presentation, and gene identification is a useful strategy to gain insight into these functions. We have now cloned much of the MHC in cosmids or Yeast Artificial Chromosomes. By screening cDNA libraries, we have identified 12 new genes in the class II region which belong to one of three groups:

1. Genes with non-immunological functions. These include *RING3*, a human gene highly homologous to the *Drosophila female sterile homeotic* gene, *fsh*.

2. Novel class II genes, *DMA* and *DMB*. These genes appear to encode a new member of the Ig gene superfamily, intermediate between class I and class II in its sequence.

3. Genes involved in antigen processing. Two of these genes, *TAP1* and *TAP2* are members of the ABC transporter family and encode proteins which could have a role in transporting peptides across the ER membrane for antigen presentation. A second pair of genes (*LMPs*) share sequence homology with a large multicatalytic protease complex, called proteasome. We believe that the LMPs influence cleavage of proteins into peptides before transport through the ER.

We are now systematically screening the MHC class II region to identify all the genes it encodes. There is evidence from deletion mutants of a gene involved in class II peptide processing which we are specifically searching for. In order to complete the analysis we are aiming to sequence the class II region. So far, we have sequenced the regions around the TAP/LMP genes (66kb) as well as the DMA and DMB loci. These gene clusters are flanked by long stretches of DNA with no identifiable genes, some of which contain long stretches of repetitive DNA.

ISOLATION OF GENES FROM SCHIZOSACCHAROMYCES POMBE WHICH INTERFERE WITH CHROMOSOME SEGREGATION WHEN OVEREXPRESSED

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We have set up a selection scheme in the fission yeast *S. pombe* to rapidly identify and clone genes involved in chromosome segregation, and more specifically those encoding centromere/kinetochore proteins. Rather than screening for traditional conditional mutants our strategy has been to use an interference assay by overexpressing random *S. pombe* cDNA's. The rationale being that the kinetochore is likely to consist of a number of interacting polypeptides which must be present in a precise amount in order to achieve assembly. Overexpression of one of these components should disrupt the kinetochore structure, leading to cell death via a high rate of chromosome loss and non-disjunction events.

Random *S. pombe* cDNA's have been overexpressed from the inducible *nmt* promoter in a strain of *S. pombe* carrying an additional, non-essential, minichromosome. The strain and minichromosome are marked to allow the identification of colonies undergoing chromosome loss. Transformants which were not able to grow upon prolonged full induction of the expression have been collected, and those showing an increase in ploidy have been selected by cytofluorometry. From 15,000 transformants, 235 have been found to be lethal upon prolonged induction of the expression. Among these, 20 have been selected which show a high rate of minichromosome loss together with an abnormal DNA content. These cDNA's are in the process of being further characterised by cytological analyses. In addition, the ends of the cDNA inserts are being sequenced in order to search for homologies in data bases. The results of these analyses will be presented.

ROLES OF THE HUMAN TOPOISOMERASE II ISOZYMES IN CELL DIVISION

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Topisomerase II (topo II) is an essential enzyme required for most, if not all, DNA metabolic functions. The enzyme transiently cleaves and religates double-stranded DNA thereby altering its topology. In addition to an important role in cell division, topo II has also been shown to be a constituent of the nuclear scaffold. Most of the functional studies on topo II have been carried out in lower eukaryotes, particularly yeast, in which only one topo II gene appears to be expressed. In contrast, there are at least two different isozymes of the type II topoisomerase enzyme in human cells. The isozymes described so far are designated α (170 kDa) and β (180 kDa). To try to identify the respective functions of the α and β isozymes in human cells, we have cloned full-length cDNAs encoding these enzymes. Analysis of sequence data shows that each is equally homologous to the lower eukaryotic form of topo II and thus neither can be viewed as the generic topo II in human cells. We have subcloned the human topo IIα and β genes into a yeast expression vector and studied complementation of a yeast conditional lethal topo II mutant which arrests at the G2/M boundary due to a failure to segregate replicated chromosomes. Surprisingly, both the human α and β genes complement the yeast top2(*ts*) mutant and thus permit mitotic cell division. This yeast background is being used to further dissect the individual functions of the two human isozymes. In particular, we aim to identify the roles that they may play in meiosis and in genetic recombination.

SV40 EARLY REGION DNA CONTAINS PERIODIC SIGNALS THAT PROMOTE LONG RANGE NUCLEOSOME ORDERING IN CHROMATIN ASSEMBLED IN VITRO AND IN TRANSFECTED COS-1 CELLS

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Nucleosome alignment on replicating plasmids assembled into chromatin in Cos-1 cells was studied by micrococcal nuclease digestion of isolated nuclei and Southern hybridization using three short SV40 DNA probes in addition to plasmid or plasmid insert probes. The constructs examined all contained the SV40 replication origin (ori), but varied in the other SV40 sequences that were included. In some constructs, particular regions of SV40 DNA were simply deleted. It was found that the extent of nucleosome alignment into an ordered array was low in constructs that contained only the SV40 ori region. Generally, no more than three well defined multiples of a unit nucleosome repeat could be detected upon probing either plasmid DNA or a 2.3 kbp anonymous chicken DNA insert that was contained in one of the plasmids. The positions of the three nucleosome oligomer bands detected closely resembled the first three bands of the cellular chromatin ladder, detected by ethidium bromide staining, which revealed 8-9 multiples of 185±5 bp. In contrast with these results, constructs that contained the whole SV40 early region exhibited a significantly greater degree of nucleosome alignment. In some cases, 8-9 multiples of a 200±bp repeat could be resolved. Moreover, in transfected cells, the presence of the SV40 early region was necessary for extended nucleosome alignment over the SV40 late and ori regions, or on adjacent pBR327 DNA.

In an in vitro chromatin assembly system, using purified chicken erythrocyte histones plus polyglutamic acid, the SV40 early region became packaged into a 200±5 bp nucleosome array that extended for more than 2800 bp. Nucleosome ordering was considerably poorer in the late region. Although the extended nucleosome alignment in the early region required the addition of linker histone, a 200 bp periodicity was clearly evident in the absence of linker histone, indicating that core histone octamers prefer certain SV40 sequences and distribute themselves, to a large extent, at 200 bp intervals throughout much of the early region. Despite the high degree of regularity in relative nucleosome spacings, positioning with respect to the DNA sequence was not consistent with a unique phasing frame in vitro or in transfected cells. These data strongly suggest that signals with a periodicity of 200 bp exist in the SV40 early region, with two or more positioning frames, and that nucleosome ordering on SV40 DNA in the nucleus spreads from the early region, probably as a consequence of histone H1-nucleosome interactions.

IDENTIFICATION OF A LOCUS CONTROL REGION FOR THE HUMAN GROWTH HORMONE GENE CLUSTER.

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Transcriptional activation of the human growth hormone (hGH)/ chorionic somatommatropin (hCS) gene cluster results in expression of hGH-N in the pituitary and hGH-V, hCS-L, hCS-A, and hCS-B in the placenta. 500 bp of 5' flanking sequence are sufficient to direct expression of hGH-N in transfected pituitary cells but not in transgenic mice. This suggests that critical regulatory sequences, necessary for expression of hGH-N in the pituitary, are not present in the promoter region. We had previously identified two distal DNase I hypersensitive sites (hss), located 27 and 33 kb 5' of hGH-N, that are selectively present in chromatin from placental and pituitary nuclei. A cosmid insert containing hGH-N and 41kb of contiguous 5' flanking DNA was used to produce 5 transgenic mouse lines. Analysis of F_1 offspring established that all 5 lines produced hGH-N mRNA in the pituitary at levels in proportion to transgene copy number and comparable to the endogenous mouse GH mRNA, suggesting the presence of a locus control region. A series of transgenic founder lines containing 5' deletions of the cosmid insert extending to -22, -7, -5 and -0.5 kb were generated. Analysis of pituitary RNA from these lines showed that sequences between -7 and -22 kb are essential for high level transcriptional activation of hGH-N in the pituitary. Examination of other tissues reveals no hGH-N expression. 3 additional DNase I hss have been located in the -7 to -22 kb region in chromatin from transgenic pituitaries. The sequences underlying these sites are conserved in the mouse genome, and one sequence crosshybridizes to additional human sequences. 3 transgenic founder lines were generated with a construct juxtaposing the two distal 5' hss at -33 and -27 kb directly to hGH-N. All 3 lines consistently produced hGH-N mRNA in the pituitary, but at low levels, only 1% of those observed in mice containing $\geq$ 22 kb of 5' flanking DNA. All 3 lines also expressed high levels of hGH-N in the kidney and low levels in the brain. We conclude that the establishment of transcriptional competence of the hGH-N gene during the development and differentiation of the pituitary requires the participation of upstream regulatory elements in addition to the proximal promoter sequences, and that these elements have properties of a locus control region.

CONTROL OF MEIOTIC RECOMBINATION AND SEGREGATION OF THE SMALLEST YEAST CHROMOSOME

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During meiosis I, homologous chromosomes pair, undergo reciprocal recombination and segregate from each other to reduce the chromosome number by half. To understand the mechanism of these processes, we have studied the meiotic behavior of chromosome *I* from *Saccharomyces cerevisiae*. Chromosome *I* is the smallest yeast chromosome and contains a DNA molecule that is only ~230 kb. 95% of this DNA has been cloned and the majority of it has been sequenced and mapped for transcribed regions.

I. All genetically mapped genes were localized to specific transcribed regions. Several markers were introduced in regions that lacked classically defined genes. Additional genetic mapping was performed and the genetic and physical maps compared, calculating recombination rates (cM/kb) over the entire chromosome. Rates were relatively constant over most of the physical map but varied by an order of magnitude at several locations. Recombination was suppressed on both sides of the centromere and in regions adjacent to both telomeres. Translocation of a telomere to a region that normally undergoes high levels of recombination lowered the recombination rate, suggesting that telomeres can suppress recombination.

II. Recombination rates on this and other small chromosomes were on average higher than on larger yeast chromosomes and rates responded directly to the size of the chromosome. This chromosome-size dependent control appears to be due to crossover interference which was found to be more efficient on larger chromosomes than on smaller ones. Models that explain how interference is responsible for chromosome-size dependent control of meiotic recombination will be presented.

III. Either two univalent nonhomologous chromosomes or two nonrecombinant homologous chromosomes segregate from each other at meiosis I by a process termed distributive disjunction. This process is obligatory for some small chromosomes and is occasionally required when there are nonrecombinant homologues. Several models for distributive disjunction have been suggested that are either dependent or independent of a physical interaction between the segregating chromosomes. To better understand distributive disjunction, we have investigated whether two univalent nonhomologous chromosomes physically interact during meiosis. Yeast strains that are monosomic for both chromosomes *I* and *III* and diploid for all other chromosomes undergo distributive disjunction of the two monosomic chromosomes. Electron microscopy of Ag stained nuclei and fluorescence *in situ* hybridization with chromosome-specific probes showed that the two nonhomologous chromosomes paired at meiotic pachytene forming a novel structure. Thus, distributive disjunction is preceded by an interaction between segregating chromosomes.

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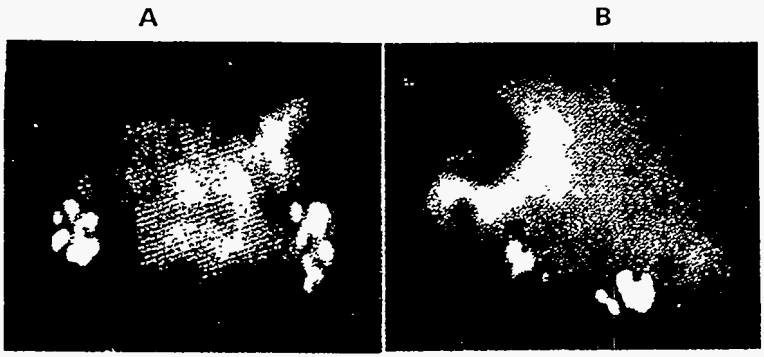
CONTROL OF MEIOTIC RECOMBINATION AND SEGREGATION OF THE SMALLEST YEAST CHROMOSOME

A physical association between nonhomologous chromosomes precedes distributive disjunction in yeast. Fluorescence *in situ* hybridization of meiotic pachytene nuclei showing chromosome *I* (red), chromosome *III* (green) and chromosome *V* (orange).

A. Nucleus from a normal diploid strain showing single hybridization signals due to meiotic pairing of homologous chromosomes.

B. Nucleus from a strain that is monosomic for chromosomes *I* and *III* and diploid for all other chromosomes. Adjacent red and green signals are due to nonhomologous pairing of monosomic chromosomes *I* and *III* and single orange signal is due to homologous pairing of chromosome *V*. Chromosomes *I* and *III* segregate from each other by distributive disjunction (Loidl, Scherthan and Kaback, submitted to *Nature)*.



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POSITION INDEPENDENT EXPRESSION OF STABLY TRANSEFCTED TRANSGENES BY NUCLEAR MATRIX BINDING SEQUENCES

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Matrix attachment regions (MARs) are DNA sequences that bind the nuclear matrix. Although limited data is available on the biological functions of MARs, they are thought to define the structural and functional boundaries of chromatin domains; in stable transfection assays they appear to buffer transgenes from position effects and allow for elevated levels of transgene expression. Recently, 5' and 3' sequences of the human apolipoprotein B gene (apoB) that bind the nuclear matrix have been described.

We show that stably transfected apoB promoter-driven transgenes flanked by the apoB 5' and 3' nuclear matrix-binding sequences show position-independent expression in both human (Hep-G2) and rat (Fao-1) hepatoma cell lines; transgenes which lack the matrix binding sequences show position-dependent expression. Furthermore, stably transfected transgenes which contain the 5' and 3' apoB matrix binding sequences are expressed at elevated levels in both Hep-G2 and Fao-1 cells. Stably transfected heterologous promoter-driven constructs flanked by the apoB 5' and 3' matrix-binding sequences are expressed in a position-independent manner in Fao-1 cells; transgenes without the 5' matrix-binding sequences show position-dependent expression. These data provide evidence that apoB sequences that bind the nuclear matrix function as MARs to buffer both homologous and heterologous promoter-driven constructs from position effects in stably transfected cells.

MOLECULAR-GENETICS OF CHROMOSOME INHERITANCE AND HETEROCHROMATIN STRUCTURE IN DROSOPHILA

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We are studying the molecular-genetics of chromosomal inheritance using a model eukaryotic system, the Drosophila minichromosome *Dp(1;f)1187 (Dp1187)*. Our recent genetic and cell biological analyses indicate that this 1300 kb minichromosome (Karpen and Spradling, 1990, Cell 63, 97) is transmitted normally through meiosis and mitosis. In addition, two differently marked *Dp1187* minichromosomes pair and disjoin normally in ~97% of female meioses, using an achiasmate pairing system. A *Dp1187* derivative with two easily scorable marker genes (y^+ and ry^+; Karpen and Spradling, 1992, Genetics 132, 737; Tower, Karpen, Craig and Spradling, 1993, Genetics 133, 347) has been γ-irradiated to generate minichromosomes with gross alterations in structure. Potentially interesting derivatives were identified initially by phenotypic criteria, then characterized by molecular (PCR and pulsed-field analyses), genetic (mitotic and meiotic transmission, Y suppression), and cytological (neuroblast squashes) methods. Analyses of the structure and transmission of derivatives with large deletions or inversions have allowed us to localize the centromere to a specific (<430 kb) region of the *Dp1187* heterochromatin. Surprisingly, this region contains a large block of complex (single copy or middle-repetitive) DNA, in addition to satellite sequences. Studies of the meiotic segregation of *Dp1187* and its derivatives provide evidence for the importance of similar chromosome size to proper segregation of achiasmately-paired chromosomes; however, our results indicate that normal disjunction also requires either specific pairing site(s), or a minimal chromosome size. Furthermore, experiments using *Dp1187* derivatives reveal an interaction between chromosomal elements and the genes that act in *trans* to promote transmission and segregation.

The isolation of *Dp1187* deletion derivatives also has allowed us to characterize the molecular structure of previously inaccessible parts of the heterochromatin, uncovering a surprising amount of substructure. We will present the progress of experiments that utilize this detailed structural information to clone portions of *Dp1187* heterochromatin into yeast artificial chromosomes (YACs), which will facilitate direct structure-function analyses of the centromere and other components of chromosome pairing and transmission. The practical applications of this research includes the development of a minichromosome vector for genetic transformation of large genes, and for further analyses of chromosomal and genomic functions.

SPERM DECONDENSATION IN *DROSOPHILA* EMBRYO EXTRACTS

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During fertilization, highly condensed sperm chromatin is decondensed as a first step to transfer paternal genetic information to the zygote. *Drosophila* embryonic extracts form nuclei from demembranated sperm chromatin at relatively low efficiency. However, sperm decondensation in *Drosophila* embryo extracts was very efficient, rapid and synchronous where 100% of input sperm chromatin was decondensed; 3 min at 25 °C. The *Drosophila* decondensation factor(s) was(were) soluble, heat-stable and N-ethyl maleimide resistant. Two sperm-specific basic proteins, X and Y, were removed from *Xenopus* sperm coincident with morphological decondensation.

Previously we purified a heat-stable nucleoplasmin-like protein from *Drosophila* embryos extracts. This protein promotessperm decondensation, either alone or when added to a *Xenopus* egg extract immunodepleted of endogenous nucleoplasmin. *Drosophila* extracts immunodepleted of the *Drosophila* nucleoplasmin-like protein still catalyzed sperm decondensation effectively. Antibodies directed against *Xenopus* nucleoplasmin did not recognize any protein in extracts. These observations suggest that *Drosophila* embryo extracts contain another heat-stable sperm decondensation factor distinct from the nucleoplasmin-like protein.

Drosophila embryo extracts decondensed sperm more efficiently than oocyte extracts and decondensed metaphase chromosomes from cultured cells. These data suggested that sperm decondensation in *Drosophila* was similar to that in *Xenopus* but not identical. Because *Drosophila* is amenable to systematic genetic manipulation, the *in vitro Drosophila* nuclear assembly system will provide a powerful tool for analysis of nuclear structure as well as nuclear function.

ROLE OF DNA BENDING IN TRANSCRIPTION ACTIVATION BY FOS AND JUN FAMILY PROTEINS

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Fos and Jun induce opposite orientations of DNA bending at the AP-1 site. To investigate the functional role of DNA bending by these proteins, we have mapped the protein domains that induce DNA bending. A 33 amino acid peptide encompassing the basic region was sufficient to induce a DNA bend. However, additional regions outside the dimerization and DNA binding domains were found to modulate DNA bending. These regions were localized to proline-rich domains that coincide with the transcription activation domains of Fos and Jun. Fos and Jun chimeras that contain these domains in a heterologous context also induce DNA bending. These domains induce the same DNA bend angle change regardless of their sequence context. The orientation of DNA bending is determined by the relative positions of the DNA binding and DNA bending domains in the protein. These domains are modular with respect to both their transcription activation and DNA bending functions. Thus, DNA bending is likely to be intimately involved in transcription activation by Fos and Jun.

The AP-1 site is recognized by a large number of bZIP family proteins. To investigate if DNA bending could confer functional specificity to members of this family, we determined the DNA bends induced by homo- and heterodimeric complexes formed among twelve bZIP family proteins. DNA bending was common, although not universal, among members of this family. Each protein induced a DNA bend of distinct orientation and magnitude. The variety of structures adopted by the AP-1 site when bound by different bZIP family proteins suggests that it represents a sequence of unusual flexibility. This structural flexibility may facilitate recognition of the AP-1 site by bZIP proteins that induce changes in DNA structure. The diversity of DNA structures induced at the AP-1 site may contribute to regulatory specificity among the large family of proteins that can bind to this site.

To directly visualize DNA bending by Fos and Jun alone, and in the context of other components of the transcription complex, we have examined complexes formed at the AP-1 site by transmission electron microscopy. Fos and Jun induced a directed DNA bend at the AP-1 site, consistent with the electrophoretic mobility analysis. Studies of the properties of this DNA bend, and its role in transcription complex assembly are underway.

CLONING AND EXPRESSION OF THE CATALYTIC POLYPEPTIDE OF HUMAN DNA POLYMERASE ε

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Studies of Simian virus 40 DNA replication in vitro have revealed that DNA polymerases α and δ are involved in eukaryotic DNA replication. In yeast Saccharomyces cerevisiae, also a third polymerase, DNA polymerase II, or ε, has been shown to be essential for chromosomal DNA replication (1). Its putative counterpart in humans, DNA polymerase ε is so far known as a DNA repair enzyme (2). We have cloned the cDNA encoding the catalytic polypeptide of human DNA polymerase ε (3). Its deduced primary structure is highly similar to yeast DNA polymerase II, confirming that they are indeed analogous enzymes, and suggesting a replicative role for DNA polymerase ε also in mammalian cells.

To elucidate the biological function of human DNA polymerase ε, we have begun raising monoclonal antibodies against the catalytic polypeptide and studying its expression. We have also started producing the catalytic polypeptide in baculovirus and Semliki Forest virus systems.

1. Araki, H., Ropp, P.A., Johnson, A.L., Johnston, L.H., Morrison, A. and A. Sugino (1992) EMBO J. 11, 733-740.
2. Nishida, C., Reinhard, P. and S. Linn (1988) J. Biol. Chem. 263, 501-510.
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CELL CYCLE DEPENDENT EXPRESSION AND LOCALIZATION OF TYPE II DNA TOPOISOMERASES

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In mammalian cells, two isoforms of type II DNA topoisomerase are present. Although their structures and enzymatic activities are similar, their roles and sites for actions appeared to be quite distinct. Using highly specific antibodies recognizing either α-isoform alone or both α-and β-isoforms, we identified each and studied its localization in various stages of the cell cycle.

By immunoblotting analysis of total cell extracts, the β-isoform was detected throughout the cell cycle as a 180 kDa protein, while the α-isoform of 170 kDa transiently appeared in quantities during G2-M phase and was almost undetectable in the interphase cell extracts. Thus the α-isoform was present only in proliferating cells and tissues, such as thymus, spleen and testis. It is noticed that remarkable amounts of the α-isoform was often found in the transformated cells. Upon the fractionation of cell extracts, the α-isoform was solely found in the chromosome scaffold fraction prepared from the metaphase arrested cells. In contrast, the β-isoform was enriched in the nuclear matrix fraction, prepared from the interphase nuclei. Thus, their concentrated localizations were very distinct.

By indirect immunofluorescent technique, the α-isoform appeared as several strong staining spots in the nuclei at the stage prior to the onset of chromosome condensation. The location of spots was coincident with the kinetochore region of the condensed chromosomes. In addition, scaffold-like structures, were stained along the condensed chromosome axis. The dot staining at the kinetochore region remained until the two chromatids were aplit. Then the spots vanished completely and seemed to be dispersed along the whole chromatin fibers. The β-isoform was localized evenly in the interphase nuclei except nucleoli.

In conclusion, we suggest that the α-isoform functions to disentangle and decatenate DNA strands for the chromosome condensation and segregation during metaphase and that the β-isoform removes the topological constraints of DNA caused by actively ongoing transcription and replication in the interphase cells.

THE BC1 RNA GENE IS A MASTER GENE FOR AMPLIFICATION OF THE RODENT ID FAMILY OF REPEATS.

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There is growing evidence that very few copies of the short, interspersed repeatd DNA sequences (SINEs) found in mammalian genomes are capable of making copies. Instead, the amplification and evolution of the SINEs appears to be dominated by a very small number of master genes.

There are about 130,000 ID repeats in the rat genome and yet the major small transcript related to ID repeats is transcribed from the single copy BC1 RNA gene. Its abundant transcription and gene structure suggest the possibility that it could be a major master gene for the rodent ID repeats. We have sequenced the BC1 gene through a number of divergent rodent species, including rat, mouse, hamster and guinea pig. We have also analyzed ID repeats from the rat mouse and guinea pig genomes. The appearance of the BC1 gene in the rodent lineage has predated the insertion of all, or almost all of the ID copies. The evolution of the BC1 gene very closely parallels that of the ID repeats throughout the rodents, with the exception of several specific subfamilies in rat. Its early placement in the rodent genome, its strong transcription pattern and its close parallel with ID repeat evolution demonstrate that the BC1 RNA gene has been the dominant master gene for rodent ID repeat amplification and evolution. It is clear that several subfamilies of ID repeats in the rat, however, must have been generated from a different master gene.

We identified a gene duplicate copy of the BC1 gene in guinea pig and found ID copies that match sequence changes in both of those BC1 genes. This demonstrates that one way of creating a new, active master gene is through a gene duplication event (rather than the RNA mediated retroposition which generates the SINE copies normally).

The BC1 gene is ancestrally derived from either a Phe or Ala tRNA gene. SINEs have been found to generally mutate at a neutral evolution rate, suggesting that individual copies are not under significant selection. In contrast, the BC1 gene is well conserved throughout rodents, much more so than the flanking gene sequences. This suggests that the BC1 gene may have 'exapted' into a new function which is under selection, in contrast to the bulk of the other ID-repeat related sequences.

CENTROMERE STRUCTURE AND KINETOCHORE ASSEMBLY SITE IN *MUS SPRETUS*

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How does the DNA sequence of a mammalian centromere correlate with its biological behaviour? A reasonable hypothesis is that an array of binding sites for the centromere-associated CENP-B protein, such as those found in human alpha satellite or mouse minor satellite, is sufficient in itself to direct kinetochore assembly. However, there are examples where neither CENP-B protein binding nor minor satellite sequences at a chromosomal location leads to a functional centromere. Conversely, in certain cases a centromere can form in the absence of any detectable CENP-B binding or minor satellite sequences.

In the familiar lab mouse *Mus musculus* the sites of kinetochore assembly (i.e. CREST staining) coincide with arrays of minor satellite sequence. The situation is strikingly different in *M. spretus*, which separated from *M. musculus* some 4Myr ago. We have cloned its centromeric satellite and find that its sequence is very similar to the minor satellite of *M. musculus*, and every example we have contains a perfect match to the 17bp mouse CENP-B binding site. The satellite appears very homogeneous, and PFG analysis using a CENP-B box oligo suggests it is present in arrays corresponding to many megabases of sequence per chromosome. *In situ* hybridisation using this oligo results in labelling of the entire heterochromatic domain of the centromere. Visually the signal with the CENP-B box oligo covers some 5-10% of the linear length of the chromosome, but more importantly it is much larger than the discrete 'double dot' of signal produced by CREST.

Double labelling produces the usual 'dot' of CREST signal, always within a much larger satellite array. Using a polyclonal antisera which detects the mouse protein (a kind gift of Bill Earnshaw) we find that CENP-B is found only at the site of kinetochore assembly. It is not associated with those parts of the satellite arrays away from the kinetochore, suggesting that a CENP-B binding site does not necessarily result in protein binding *in vivo*. Furthermore, for any particular homologue pair (identified by banding) the position of CREST staining appears to be at a fixed site within the array.

The minor satellite is heavily methylated in *M. musculus*, with the only CpG dinucleotides being within the CENP-B box. We are currently investigating whether methylation prevents CENP-B binding to those regions of the satellite arrays away from the kinetochore. Epigenetic control of CENP-B binding by methylation may be particularly relevant in examples of inactive centromeres (such as isodicentrics) with no apparent change in DNA sequence.

MEIOTIC DOUBLE-STRAND BREAKS IN NATURAL AND ARTIFICIAL SACCHAROMYCES CEREVISIAE CHROMOSOMES

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During meiosis, homologous chromosomes pair, undergo recombination and then segregate from each other to reduce the chromosome number by half. Double-strand breaks (DSBs) in DNA are recombinogenic in yeast, and occur in meiosis near several sites of elevated meiotic recombination.

We have developed methodology for identifying meiotic DSBs in full-length chromosomal DNA of S. cerevisiae rad50S mutants, using pulse-field gels. We have found that meiosis-specific DSBs are a general feature of S. cerevisiae chromosomes, occurring preferentially at specific sites. We have mapped the sites on chromosomes I, III and VI. Several of the DSBs occur at previously identified regions of elevated meiotic reciprocal recombination and/or gene conversion. We found on average one preferred meiotic DSB site per 25-30 kb. Certain DSB sites consistently give stronger signals than others. On chromosome III, the strongest signal (5′ to THR4) is at a site of elevated meiotic recombination that has also been implicated in meiotic chromosome segregation.

We have also looked for meiotic DSBs in YACs (Yeast Artificial Chromosomes) comprised mainly of human DNA. The YACs, which have yeast centromeres, telomeres and selectable markers, vary with regard to genomic source, length and mitotic stability, and some are from genomic regions with a propensity to deletions or other rearrangements. The YACs behave like native S. cerevisiae chromosomes with respect to double-strand breakage in meiosis. Each YAC examined has a characteristic pattern of breakage. This pattern depends on the human DNA: the same human DNA in different contexts has the same pattern of breakage. Furthermore, the breaks are seen when the YACs are present as monosomes or disomes, indicating that pairing of homologous chromosomes is not a prerequisite for double-strand breakage.

BIOCHEMICAL AND STRUCTURAL ANALYSIS OF ACIDIC TRANSCRIPTIONAL ACTIVATION DOMAINS

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Acidic activation domains are found in a number of gene-specific transcription factors from yeast to man. The structure of these regions, and the mechanism by which they act are currently unknown. We report here studies of peptides corresponding to the activation domains of the yeast GCN4 and GAL4 proteins. Circular dichroism spectroscopy demonstrates that both peptides have very low helix-forming potential, but readily form β-sheet-containing structures at slightly acidic pH. These and other data suggest that acidic activation domains are neither "acid blobs" or amphipathic α-helices.

We also show that both peptides bind to yeast TBP in a specific manner, as do the analogous domains from several other proteins. One possible mechanism for transcriptional activation is that this contact enhances the ability of TBP to bind to the TATA box. Quantitative experiments using cloned factors are reported that test this hypothesis.

AN ATYPICAL CHROMATIN ORGANIZING DOMAIN IN THE WHEY ACIDIC PROTEIN GENE

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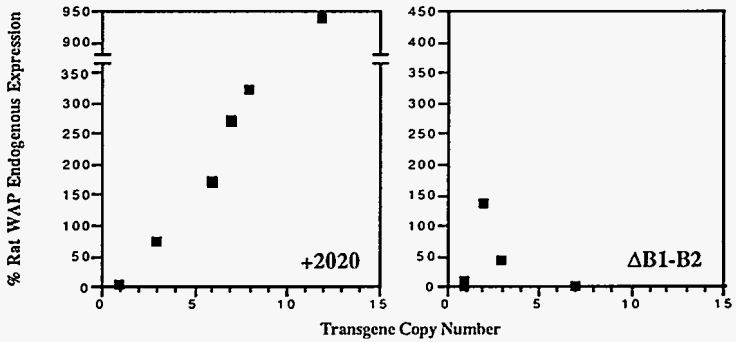
The whey acidic protein (WAP) gene encodes the major whey protein in rodent milk. WAP is the product of a small, single gene locus. We have identified a GC-rich element in the rat WAP 3' untranslated region (3'UTR) which confers integration site-independent expression to several transgenes, a role associated with locus control and/or matrix attachment regions. This region is more highly conserved than the WAP gene coding region. However, despite the apparent functional similarity, the rWAP 3'UTR has no homology with previously identified LCRs or MARs.

In transgenic mice, a 3kb rat WAP genomic fragment (+2020), composed of 949bp of 5' flanking sequences, intragenic sequences, and 70bp of 3' flanking DNA, was expressed in the lactating mammary gland at, or above, endogenous levels in a copy number-dependent manner (1). However, a 55 base pair internal deletion within +2020 3'UTR (ΔB1-B2) resulted in position-dependent expression.



Addition of the rWAP 3'UTR to heterologous transgenes resulted in expression in all lines (n =14), while a control transgene containing the mouse ß-casein 3'UTR was not always expressed. Copy number-dependence of heterologous constructs was enhanced for transgenes which had both the rWAP 3'UTR and a rWAP promoter, suggesting a possible interaction between factors which associate with these elements. While the rWAP 3' UTR does not contain a DNase I hypersensitive (HS) site, two HS sites exist in the 949 bp promoter region. Deletion of the distal HS site leads to the total loss of rWAP expression.

We have taken two approaches to study the mechanism by which these elements may establish an active chromatin domain. First, electrophoretic mobility shift assays have demonstrated that at least two distinct nuclear factors associate specifically with the rWAP 3'UTR DNA (but not RNA) *in vitro*, possibly in a cooperative manner. In addition, we have established that the distal HS site in the rWAP promoter contains at least one NF1 binding site. Second, we have used an *in vivo* nuclear matrix association assay to demonstrate that the +2020 transgene is localized to the nuclear matrix in a tissue-specific manner. By analysis of deletion mutants, we are attempting to correlate nuclear matrix association with the presence of a functional rWAP 3'UTR element. Based on these data, we hypothesize that the rWAP 3'UTR acts to establish a region of active chromatin around a transgene. This may occur by a protein-mediated interaction between the 5' and 3' boundaries of the gene or, alternatively, via rWAP 3'UTR directed localization to the nuclear matrix. (Supported by NIH grant CA 16303)

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A NOVEL NUCLEAR TRANSPORT DOMAIN IN THE RSV INTEGRASE

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The early events of the retroviral replication cycle culminate with the integration of a complete DNA copy of the viral genome into the host cell chromosome. Following cytoplasmic entry and reverse transcription of the viral RNA genome to a linear double stranded DNA, the virion-supplied integrase (IN) protein remains associated with the viral DNA in a high molecular weight nucleoprotein preintegration complex. Although the structure and composition of this cytoplasmic preintegration complex is ill-defined and may vary between different retroviruses, all such complexes minimally contain IN as a common component.

The manner in which the viral preintegration complexes are transported to the nucleus of infected cells remains unknown. One possibility is that an element of the nucleoprotein complex facilitates active nuclear localization. Transient expression of the avian (RSV) and human (HIV-1) IN proteins in COS cells revealed that both of these viral proteins accumulate in the nucleus. In order to identify the determinants governing IN nuclear localization, various domains of RSV IN were transiently expressed in COS cells as ß-galactosidase fusion proteins. The use of ß-galactosidase serves two purposes: (i) unaltered bacterial protein when expressed in eukaryotic cells localizes to the cytoplasm, and its large size precludes nuclear entry by simple diffusion; and (ii) the free C-terminal segment of ß-galactosidase is enzymatically active in the fusions, permitting a direct cytochemical assay of chimeric protein location with an X-gal staining technique. Our analyses show that the RSV IN protein possesses a functional nuclear localization domain spanning amino acids 206-242 that displays no homology with canonical nuclear transport signals.

DNA-PROTEIN INTERACTIONS AT KAPPA-B ENHANCERS INVOLVING NF-KB/REL FAMILY AND HMG-I (Y)

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We study the interactions of NF-kB/rel family of transcription factors with synthetic kB enhancers in vitro. We apply high resolution EMSA in combination with UV-crosslinking to resolve and characterize distinct DNA-protein complexes. A panel of monospecific antibodies to the members of NF-kB/rel family was instrumental in determining the protein composition of complexes both by "supershifting" EMSA bands and by immunoprecipitation of cross-linked proteins. These analyses demonstrated that the complexes are **(i)** kB site-specific, i.e. kB probes with different DNA sequence context preferentially bind different complexes in a given nuclear extract; **(ii)** tissue-specific, i.e. the same probe forms different complexes in different nuclear extracts. These results were supplemented with cotransfection data on the activity of *CAT* constructs driven by distinct kB sites, with combinations of expression vectors for distinct NF-kB/rel proteins. A *subset* of kB probes in the natural sequence context of several kB enhancers that are characterized by distinct pattern of NF-kB complex formation, also strongly binds HMG-I(Y), a protein recently implied as a co-factor in the activation via NF-kB. The effects of HMG-I(Y) on the formation of different NF-kB/rel complexes and its additional sequence requirements for binding are underway. Finally, we studied effects of NF-kB/rel binding on the structure of DNA at the kB site. Previously, permutation analysis suggested a strong localized distortion, presumably due to DNA bending. We now supplement these data with phasing analysis to reveal that the *major* contribution to the additional gel retardation is not due to directed bend. Cyclization kinetics and circular probe binding analyses are in progress to resolve the discrepancy between the results of these two commonly used gel electrophoresis techniques.

HOMOLOGY-CONSCIOUS NON-HOMOLOGOUS RECOMBINATION AND GENE EVOLUTION

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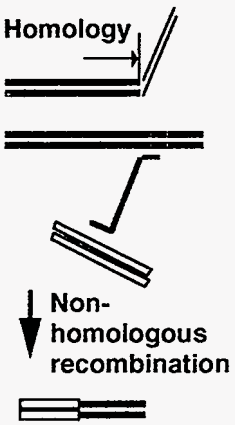
Two types of DNA recombination, homologous recombination, involving long homology, and non-homologous recombination, involving only few base pairs of homology, have been regarded quite different. We found novel recombination mechanism in which homologous interaction of two DNAs apparently causes non-homologous recombination with another DNA. This recombination was detected in *E. coli* cells where homology-dependent double-strand break repair was blocked by multiple mutations including *ruvC* and *recG* (Genetics 119:751). The non-homologous recombination depends on *recE* gene product, an enzyme for homologous recombination, and on homology in two DNAs. Some non-homologous recombination events took place precisely at the point where this homology ends (Figure).

This mechanism would search non-homology among homologous DNAs and eliminate it. We hypothesize that it protects genes from gross alteration like intron loss during gene evolution. Interaction between a pair of genes with homologous exon sequences but with different intron sequences would be aborted. Thus it may help speciation at the gene level. It may occasionally generate a novel gene by exon shuffling.



THE GENE FOR CBF3B A COMPONENT OF THE S. CEREVISIAE KINETOCHORE RESEMBLES A TRANSCRIPTIONAL ACTIVATOR

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Purification of CBF3 a multi-subunit protein complex that binds specifically to the CDE III element of wild type S. cerevisiae centromere DNA (CEN) but not to centromere DNA with a point mutation that inactivates centromere function in vivo was described in (1). CBF3 preparations are able to mediate plus to minus directed movement of CEN DNA along microtubules in vitro and CBF3 was shown to be a necessary component for this movement to occur (2). CBF3 consists of three proteins (CBF3A, CBF3B and CBF3C). The gene for CBF3A (CBF2) was recently isolated, characterized and shown to be identical to NDC 10, a ts mutant that exhibits severe chromosome non-disjunction at the non-permissive temperature (3,4) supporting the role of CBF3A for chromosome segregation.

To clone the CBF3B gene tryptic peptides of CBF3B were subjected to automated Edman degradation. Oligonucleotides designed according to the peptide sequence data were used to amplify a CBF3B gene fragment by PCR. This DNA fragment was used to isolate the CBF3B gene from a yeast genomic library in Dash II. Sequencing the CBF3B gene revealed CBF3B to contain a N-terminal Zn_2Cys_6 zinkfinger motive and a C-terminal acidic region resembling yeast transcriptional activators like Gal 4 with the zinkfinger domain conferring protein - DNA and the acidic region protein - protein interactions. X ray crystallography data recently had revealed that Gal4 makes direct base contacts only to a highly conserved GGC triplet at each end of the Gal4 binding site (5). The fact that the CDE III element of yeast CEN DNA also contains a highly conserved GGC triplet that is absolutely essential for centromere function and CBF3 binding supports the idea that specific CEN binding of CBF3 is achieved by CBF3B - CDE III interaction.

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CONDITIONAL SILENCING: THE *HMRE* MATING-TYPE SILENCER EXERTS A RAPIDLY REVERSIBLE POSITION EFFECT ON THE YEAST *HSP82* HEAT SHOCK GENE.

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Chromosomal position effects are derived from the spread of heterochromatin or the action of regulatory elements. While the yeast *Saccharomyces cerevisiae* lacks heterochromatin at the cytological level, it possesses two loci on chromosome III, the silent mating cassettes *HML* and *HMR*, which have been shown to exert a position effect on the transcription of heterologous genes. Position effects at the *HM* loci are mediated by *cis*-acting sequences, E and I, termed "silencers", and numerous *trans*-acting factors, including Sir1-4. To gain insight into the molecular determinants underlying chromosomal position effects, we have introduced *HMRE*-bearing fragments into the upstream region of the yeast heat shock gene, *HSP82*, which is located on the left arm of chromosome XVI. We have found that an ectopic *HMRE* silencer can in fact exert *SIR4*-dependent repression of *HSP82* expression. However, it represses only basal, not induced, transcription. It rapidly losses its ability to repress the expression of *HSP82* following heat induction, and rapidly regains its function when cells are returned to nonstressful conditions. In contrast to this flexibility in function, no discernible changes in the protein/DNA interactions are detected within the upstream region of the *HMRE/HSP82* allele irrespective of stress or *SIR4* background. ABF1, RAP1, and ARS factor binding sites within the silencer and the heat shock factor binding site upstream of *HSP82* are constitutively occupied. The rapidity in reestablishing silencing that we see is in apparent contrast to an earlier observation that DNA replication is obligatory for the onset of *SIR* repression at the silent mating-type loci. We are currently testing whether reestablishment of silencing at the *HMRE/HSP82* locus can occur in G1-arrested cells, by using strains supersensitive to α-mating pheromone.

DNA AMPLIFICATION AND TRANSCRIPTION IN DNA PUFF II/9A OF *SCIARA COPROPHILA* .

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DNA puffs that form in salivary gland polytene chromosomes of the fungus fly, *Sciara coprophila,* are sites of DNA amplification and enhanced transcription. Both processes can be precociously induced by ecdysone in cultured salivary glands.

To investigate the molecular basis of ecdysone induction, we focused on a 35 kb area from DNA puff II/9A that contains two transcription units, genes II/9-1 and 2. A 718 bp promoter fragment of gene II/9-1 was sufficient to maintain tissue and temporal specificity of the ecdysone response of a reporter gene in transgenic *Drosophila.* The sequence of the promoter area revealed three matches to the ecdysone receptor response element (EcRE). Electromobility shift assays using antibody against the ecdysone receptor (EcR) showed that EcR can bind to these elements; DNase I hypersensitivity and *in vivo* footprinting experiments support that notion. *In vitro* footprinting studies using mutated probes suggest a role for other proteins such as ultraspiracle in establishing the ecdysone response and point to a functional redundancy between the EcREs.

Two-dimensional (2D) gels identified an origin of DNA amplification spanning 6 kb with the majority of initiation events confined to a 1 kb area upstream of gene II/9-1 (Liang et al., *Genes Dev.,* in press). An array of DNase I hypersensitive sites is observed in the origin region during, but not after DNA amplification. To investigate origin structure in greater detail we designed a novel 3-dimensional gel method that involves running a neutral/neutral 2D gel followed by cutting out vertical slices from the area containing replication intermediates, rotating these slices 90°, and running an alkaline gel for each of the gel slices. Using this method we confirmed our 2D gel data on the location of the major initiation zone and also showed that only one initiation event seems to occur on a given DNA molecule in the 6 kb zone. Furthermore, the fork arc seen in the 6 kb zone as part of a mixed fork-bubble pattern is not derived from broken bubbles.

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RNA POLYMERASE II TRANSCRIPTION POTENTIATION BY SEQUENCE-SPECIFIC FACTORS DURING DNA REPLICATION AND CHROMATIN ASSEMBLY

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Gene activation by RNA polymerase II is a multistep process that involves interactions between sequence-specific gene activators and basal transcription factors. The sequence-specific factors function to facilitate the transcription reaction and to counteract chromatin-mediated repression. We have been studying the mechanisms of gene activation by sequence-specific factors with chromatin templates containing histone H1.

By using chromatin reconstituted from purified components, we had previously demonstrated that nucleosomal core-mediated repression of RNA polymerase II transcription occurred when nuclesomes were located over the start site of transcription. Histone H1 repressed transcription when the start sites were located in the linker DNA. We found that sequence-specific transcription factors were able to relieve H1-mediated transcription inhibition in a process referred to as antirepression. The ability of GAL4 derivatives to counteract chromatin-mediated repression was dependent upon a transcriptional activation region in the activator protein in addition to presence of a negatively charged species, which appeared to act as a histone acceptor, in the reaction medium.

We have developed a cell-free system from *Drosophila* embryos that assembles regularly spaced nucleosomes onto double-stranded DNA. This extract also replicates single-stranded DNA and concomitantly assembles it into chromatin. Histone H1 can be incorporated into the chromatin during assembly. H1-containing chromatin was more transcriptionally repressed than chromatin containing only nucleosomal cores, and selective removal of H1 from H1-containing chromatin partially relieved the chromatin-mediated repression. We have found that the order of addition of transcription factors relative to chromatin assembly was critical in gene activation. Binding of transcription factors prior to nucleosome assembly on double-stranded DNA leads to the generation of a nuclease hypersensitive site at the TATA box and to the establishment of a transcriptionally competent state for the template.

We have also used the *Drosophila* extract to show that a competition exists between the binding of transcription factors and the assembly of nucleosomes on replicating DNA. This competition results in the binding of the transcription factors to the DNA and the potentiation of the template for transcription. The data provide direct biochemical evidence that support models of gene activation during the process of DNA replication.

FACILITATED BINDING OF REGULATORY FACTORS TO NUCLEOSOMES AND HISTONE DISPLACEMENT BY NUCLEOSOME DISASSEMBLY.

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In vitro studies indicate that replacement of nucleosomes with transcriptional regulatory factors at promoter and enhancer elements proceeds through the binding of upstream factors to nucleosomes followed by displacement of the underlying core histones. The ability to bind nucleosomal DNA is a property of only a subset of upstream factors. The human heat-shock factor is unable to bind to nucleosomal DNA. By contrast, derivatives of the yeast factor, GAL4, and the human factor, Sp1, are able to bind nucleosomal DNA with a small penalty in affinity relative to binding naked DNA. Moreover, the binding of histone H1 to nucleosome cores does not significantly further reduce the affinity of GAL4 for nucleosomal DNA. The binding of GAL4 and Sp1 to nucleosomes is "facilitated" by the presence of multiple binding sites. This results in the filling of 5 GAL4 sites on nucleosome cores in a cooperative manner, jumping from 1 to 5 bound dimers. However, the cooperative nature of GAL4 binding is lost upon tryptic removal of the core histone amino-terminal tails suggesting that "facilitated" binding overcomes inhibition mediated in part by the core histone amino termini.

The binding of GAL4 or Sp1 to nucleosome cores results in the formation of ternary complexes containing the transcription factor, core histones and DNA. Addition of non-specific DNA to GAL4/nucleosome complexes dissociates the histone octamers from approximately half of the ternary complexes generating GAL4/DNA complexes. This suggests that histone binding components are required for nucleosome displacement. Addition of the histone-binding protein nucleoplasmin to these reactions stimulates the initial binding of GAL4 to nucleosomes and the subsequent displacement of histones onto non-specific DNA, resulting in 100% nucleosome displacement. Analysis of the protein composition of ternary GAL4/nucleosome complexes formed in the presence of nucleoplasmin revealed that nucleoplasmin depleted the nucleosome cores of H2A/H2B dimers upon the binding of GAL4. Thus the binding of the regulatory factor, GAL4, induced nucleosome disassembly: by displacing H2A/H2B dimers onto nucleoplasmin followed by displacement of the H3/H4 tetramer onto non-specific DNA.

TRANSCRIPTION FACTOR ACCESS TO DNA IN THE NUCLEOSOME

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We have determined the structure of DNA in a nucleosome core and the contributions of individual histones to this structure (1, 2). The histone core has been found to exert a dominant and highly stable constraint on DNA structure (3, 4). Xenopus borealis 5S rRNA genes will assemble into translationally positioned nucleosome cores as determined by hydroxyl radical cleavage, micrococcal nuclease mapping of histone-DNA contacts and histone-DNA cross-linking. We have examined the consequences for transcription factor TFIIIA association with the 5S rRNA gene of modifying 5S nucleosome core structure by histone acetylation or histone H2A/H2B depletion (5, 6). Both of these modifications facilitate TFIIIA recognition of the 5S DNA-histone complex. The consequences for transcriptional activation have also been examined (7-9). Linker histones (H1 and H5) interact with 5S DNA in the nucleosome preferentially and asymmetrically. Linker histones repress 5S RNA gene transcription and interfere with the association of transcription factors with the gene (10, 11).

These observations lead to a model where sub nucleosomal structures are accessible to trans-acting factors and transcriptionally competent, whereas mature nucleosomes, H1 plus (H2A, H2B, H3, $H4)_2$, are inaccessible and transcriptionally repressed (12). Such sub nucleosomal structures are present in nascent chromatin immediately following replication or in transcriptionally active chromatin domains.

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REMODELLING OF CHROMATIN STRUCTURE AND TRANSCRIPTIONAL ACTIVATION BY STEROID RECEPTORS

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Regulatory sequences of the steroid inducible MMTV promoter are organized over six positioned nucleosomes. Steroid receptors bind on the surface of the second phased nucleosome, nuc-B. Hormone activation of the promoter leads to modulation of nuc-B structure in vivo and recruitment of a series of transcription factors into an initiation complex. In vivo crosslinking experiments indicate that the structural transition at nuc-B is global in nature. DNA-histone interactions are altered across the complete octamer core. H1 is also depleted from the induced promoter, indicating the nucleoprotein transition is more pronounced than simple core alteration. NF1/CTF, a component of the MMTV initiation complex, is excluded from uninduced stable chromatin but binds constitutively to transiently introduced DNA. We suggest that the chromatin transition is necessary and responsible for NF1 loading. NF1 is excluded from an A/B disome reconstituted in vitro, whereas the glucocorticoid receptor can bind, partially recapitulating the in vivo observations.

We also find that transiently introduced progesterone receptor is unable to activate stable replicated MMTV chromatin, although it functions efficiently on transient templates, whereas constitutively expressed endogenous receptors function on both templates. These results suggest either, 1) steroid receptors are subject to functional modifications required for chromatin interaction, or 2) chromatin can be differentially organized in open or closed states depending on the presence of a given activator.

Our results indicate that transcription factors enjoy selective access to specifically organized MMTV chromatin and modulation of this nucleoprotein structure is necessary during transcription activation to permit binding of the initiation complex. Thus, a chromatin template containing positioned nucleosomes is an active participant in transcriptional activation, and modulation of this template structure is an important feature of steroid hormone action.

NUCLEOSOME POSITIONING AND TRANSCRIPTION

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In yeast, **a**-cell specific genes (e.g. *STE6*) are repressed in α-cells by the α2 repressor and MCM1 bound to the α2 operator. In α- but not in **a**-cells, positioned nucleosomes abut the α2 operator, occluding the TATA box, and extend into the *STE6* structural gene. These nucleosomes form a highly organized chromatin domain; they are positioned with base pair precision and separated by defined length linkers. The role of this organized chromatin structure in repression of transcription is under investigation in studies which are designed to move the TATA box through the positioned nucleosomes. The linker and adjacent 20 bp of core particle DNA in a nucleosome positioned next to the α2 operator in a different DNA context are accessible to another protein, dam methylase.

Certain deletions and point mutants in the amino terminal region of histone H4 disrupt the stable positioned nucleosomes adjacent to the α2 operator. Mutations in two other genes, *SSN6* and *TUP1*, which are necessary for repression also lead to altered stability of the nucleosomes with micrococcal nuclease digestion patterns like those observed in **a**-cells. There is a perfect concordance of absence of the stable nucleosome and absence of full repression of a reporter gene under control of an **a**-cell specific promoter. We are currently investigating whether chromatin structure is also important for a second function of α2, repression of haploid specific genes in diploid cells by the **a**1/α2 complex.

In contrast to the dominance of chromatin organization over transcription of the **a**-cell specific genes, a tRNA gene is transcribed by RNA polymerase III even when it is placed in the middle of a predicted nucleosome; the nucleosome is not present. Similarly, the GAL4 activator protein can bind to UAS_G, in chromatin, disrupting a positioned nucleosome which contains the *cis*-acting element.

These results demonstrate hierarchies in the competition between histones and *trans*-acting factors for sites on DNA. Together with other studies, they emphasize the importance of chromatin in control of DNA function in replication, repair and transcription.

THE REGULATION OF TRANSCRIPTION IN YEAST BY HISTONES H3 AND H4

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Our laboratory has used the genetic tools of the yeast, *Saccharomyces cerevisiae*, to probe nucleosome and histone function *in vivo*. Our previous work has argued that nucleosomes repress transcription *in vivo* but that the nucleosome is also a complex regulator of gene activity. Histone H4 contains different overlapping N terminal domains involved in the activation of transcription at GAL1 and PHO5 genes (residues 4-23) and repression of gene activity at the silent mating loci and adjacent telomeres (residues 16-29). In addition histone H3 contains an N terminal region (residues 4-15) required for the repression of GAL1. More recently we have mapped new regions at the H4 N terminus required for the repression of unactivated, basal transcription. In addition, we have found new H3 N-terminal sequences required for GAL1 activation and yet other regions required for the repression of genetic information adjacent to telomeric sequences. In an attempt to determine the mechanisms by which these diverse regulatory phenomena take place we have a) defined GAL1 and PHO5 promoter elements required for H4 and H3 N terminal function; b) made functional hybrid fusions between histone H3 and H4 domains; c) attempted to identify regulatory proteins which are required for H4 N terminal function *in vivo*; d) identified factors which interact directly with histones *in vitro*; and finally e) determined the effect of histone H3 and H4 N-termini on DNA topology and chromatin structure. The results of these approaches will be presented.

ROLE OF YEAST SNF AND SWI PROTEINS IN TRANSCRIPTIONAL ACTIVATION

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In the budding yeast *S. cerevisiae* five functionally related proteins, SNF2/SWI2, SNF5, SNF6, SWI1 and SWI3, are important for transcription of many genes regulated by diverse signals. Each of the SNF proteins, when bound to DNA as a LexA-SNF fusion, functions as a transcriptional activator. Work from our lab and others (Peterson and Herskowitz, 1992, Cell 68: 573; Yoshinaga et al, 1992, Science 258: 1598) indicates that in vivo SNF and SWI proteins function coordinately with various gene-specific transcriptional activators. Several lines of evidence suggest that SNF and SWI proteins may alter chromatin to alleviate repression. SNF2/SWI2 has sequence homologs in many eukaryotes and resembles helicases. We have shown that SNF2/SWI2 has dsDNA-stimulated ATPase activity required for transcriptional activation. Using the two-hybrid system, we are identifying proteins that interact with SNF2/SWI2 in an effort to elucidate the role of this ATPase in transcriptional activation.

CHROMATIN STRUCTURE AND THE REGULATION OF GLOBIN GENE EXPRESSION. G. Felsenfeld, J. Boyes, E. Bresnick, J. Chung, D. Clark, J. Knezetic, M. Minie, V. Studitsky, C. Trainor, T. Vandergon, G. M. Clore, A. Gronenborn, & J. Omichinski, National Institutes of Health, Bethesda, MD 20892, U.S.A.

We have investigated the mechanisms that determine tissue- and stage-specific expression in the chicken globin gene family. Tissue-specific expression of globin genes is regulated both by factors restricted to hematopoietic lineages, such as GATA-1 (Eryf1), and by ubiquitous factors. Stage-specific expression of two embryonic globin genes that we have studied does not appear to depend on strictly stage-specific factors, but may be accounted for by variation during development in the abundance of GATA-1 and other regulatory factors present at all developmental stages.

Chromatin structure also plays an important part in the transcription process. A sequence element with the properties of a locus control region has been identified within the chicken β-globin cluster. A quite different kind of regulatory element, which behaves as an insulator, has been found far upstream of the cluster. This element, when placed between promoter and enhancer, blocks the activating effect of the enhancer, and may well serve as a boundary of the chromatin 'domain'. The presence of histone octamers on the coding regions of transcriptionally active genes poses a different kind of problem: How can RNA polymerases make their way through such chromatin structures? We have devised experiments suggesting how histone octamers can transfer out of the path of an advancing polymerase.

REGULATION OF THE HUMAN β-GLOBIN DOMAIN

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The different parameters and interactions involved in the developmental regulation of the human β-globin gene domain will be discussed. The most important regulatory region is the Locus Control Region (LCR) and is situated at the 5' end of the domain. It consists of four DNaseI hypersensitive regions, each of which consists of a combination of binding sites for erythroid specific and ubiquitously expressed transcription factors. Linkage of each of these regions to a combination of globin genes results in a specific developmental pattern of the genes, indicating that at least part of the developmental programme is coded for by the LCR. The second level of regulation is coded for by the sequences immediately flanking the genes, e.g. mutations in the γ globin promoter can alter its developmental pattern of expression. The third level of regulation is specified by the competitive interactions between the genes and the LCR which is largely dependent on the position of the genes relative to the LCR.

HISTONES H1/H5 AND CHROMATIN FOLDING IN THE TRANSCRIPTIONALLY COMPETENT CHICKEN β-GLOBIN CHROMOSOMAL DOMAIN

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Transcriptionally competent genes are DNase I sensitive and this sensitivity extends to large chromosomal domains in which the transcription units are embedded. One possible explanation for the sensitivity is that chromatin folding is disrupted or somehow altered, and that this might be caused by changes in linker histone interactions or content. Using UV cross-linking, immunoprecipitation with anti-H1/H5 antibodies and probing of the DNA for the presence of specific sequences, we have examined ~ 6 kb of DNA containing the chicken β^A-globin gene and flanking sequence for association with histones H5 and H1 in adult chicken erythrocytes. This extends an earlier study of linker histone distribution on various transcriptionally repressed and competent sequences (including ~ 2 kb of the β^A globin gene) in these cells. We have also examined the ability of micrococcal nuclease-generated chromatin fragments from the same region to fold into "pseudo higher-order structures", and the general sensitivity of the sequences to DNase I. We detect alterations in linker histone content (or mode of binding) and chromatin folding throughout the ~ 6 kb region, which is uniformly DNase I-sensitive. Strikingly, the altered folding and DNase I sensitivity extend throughout the β-globin gene cluster, and both upstream and downstream of it, covering altogether a region of ~ 40 kb. It seems likely that altered H1/H5 contacts or H1/H5 depletion also occur throughout this region. Thus, even in mature erythrocytes, in which H5 has largely replaced H1 during a general shut-down of transcription during maturation, the entire domain containing the tissue-specific β-globin genes appears to be "open".

Destabilization of the entire domain may be a consequence of the breakdown of cooperative interactions between H1/H5 molecules, assuming that these occur in chromatin as they do on naked DNA. The interactions are likely to be between the globular domains of the linker histones, and indeed the isolated globular domains of both H1 and H5 bind to DNA in a highly cooperative manner.

STRUCTURAL-FUNCTIONAL ORGANIZATION OF CHROMOSOMAL DNA DOMAINS

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Large DNA domains (ca. 100 kb long) attached by the ends to the nuclear matrix represent the basic structural-functional units of eukaryotic chromosomes. At the first approximation, they correspond to replicons.

The following questions concerning DNA domain organization will be discussed.

1. Isolation of DNA domains using *in vivo* or *in vitro* cutting by topoisomerase II inhibitor VM26.
2. Properties of some DNA domains containing single copy or repetitive genes.
3. Characterization of putative replication origins located at the ends of DNA domains.
4. The role of DNA domain integrity for maintaining transcription within the domain.
5. Novel genes whose protein products are involved in long distance interactions within or between DNA domains.

CHROMATIN DOMAINS CONSTITUTE REGULATORY UNITS FOR THE CONTROL OF EUKARYOTIC GENES

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Nuclear DNA is organized in topologically constrained loop domains. In order to investigate the relevance of loop domains for the control of gene activity, we mapped a specific eukaryotic gene locus in respect to its chromatin organization. The active chicken lysozyme gene in oviduct cells and macrophages is located within the same 20 kb domain of elevated DNase sensitivity of DNA, terminating at both ends in nuclear matrix attachment regions (MARs). Up to 9 DNaseI hypersensitive sites are located within the chromatin domain, marking the positions of multifactorial regulatory elements, each responsible for a specific sub-aspect of the total control of the gene. The transfer of the entire chicken gene domain into transgenic mice consistently led to high level and macrophage specific activity of the transgene, independent of its random position in the genome(1). This shows that the structurally defined chromatin domain is functionally equivalent to the complete regulatory unit for transcription. We now have mapped by deletion analysis the cis-element constituting the effective locus control function(2) and we find: (a) that it can not be mapped to a single locus control region (LCR), (b) that individual enhancers can direct expression in the right cell but can't consistently suppress position effect mediated expression in wrong cells, (c) that the deletion of domain border regions increases the incidence of ectopic expression with incomplete transgene domains, and (d) that cis-regulatory elements interact not in a hierarchical, but in a cooperative fashion with the promoter region ("concerted action model"). We could also show that transfer of the lysozyme gene domain into mouse embryonal stem cells leads to position independent gene activation when transgenic ES cell clones were *in vitro* differentiated to macrophages(3). Our results imply that "gene domain transfer" can be used to ensure consistently correct transgene expression in transgenic organisms and in somatic gene therapy without the need for homologous recombination.

(1) Bonifer, C., Vidal, M., Grosveld, F. and Sippel, A.E. (1990) Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice EMBO J. 9, 2843-2848

(2) Bonifer, C., Yannoutsos, N., Krüger, G., Grosveld, F. and Sippel, A.E. (1993) Dissection of the locus control function located on the chicken lysozyme gene domain in transgenic mice (submitted)

(3) Faust, N., Bonifer, C., Wiles, M.V. and Sippel, A.E. (1993) Differences between adult and early fetal macrophage cells as revealed by transgene expression in macrophages derived from transgenic mice and *in vitro* differentiated mouse embryonic stem cells (submitted)

DOMAIN BOUNDARIES

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Eukaryotic chromosomes are thought to be organized into a series of topologically independent domains which function not only in the packaging of DNA into the nucleus, but also for the proper regulation of gene expression. Critical to this model of chromosome organization are specialized elements that define the boundaries of chromatin domains. Two different types of elements in *Drosophila* which appear to function as domain boundaries will be discussed.

The first are the scs-like elements from the 87A7 heat shock locus. The 87A7 heat shock puff spans ~15 kb and contains two divergent hsp70 genes. Flanking the puff are two elements, scs and scs', that are good candidates for boundaries of chromatin domains. Each structure consists of a central, nuclease-resistant core flanked by two major and several minor nuclease hypersensitive sites. We used an enhancer-blocking assay to define the DNA sequences involved in boundary function. When placed between the *white* upstream regulatory elements and a *white* reporter gene, scs was able to prevent the enhancer dependent activation of the *white* gene in the eye and testis. Deletion analysis suggests that sequences closely associated with the nuclease hypersensitive sites are required for boundary function.

The second are the *Mcp* and *Fab-7* elements from the *Drosophila* bithorax complex (BX-C). An extremely large *cis*-regulatory region generates the parasegment-specific expression patterns of the homeotic BX-C genes, *Ubx*, *abd-A*, & *Abd-B* . This *cis*-regulatory region appears to be organized into a series of independent chromatin domains that specify parasegment specific patterns of expression. Two small deletions in BX-C, *Mcp* & *Fab-7*, are dominant gain-of-function mutations. Both remove sequences that separate neighboring *cis*-regulatory domains (*Mcp*: *iab-4* & *iab-5*; *Fab-7*: *iab-6* & *iab-7*). Our analysis suggests that the *Mcp* & *Fab-7* elements function as domain boundaries. Screens for new deletions that disrupt *Mcp* or *Fab-7* boundary function localize the critical sequences to DNA segments of ~0.5 kb. Moreover, both the *Mcp* and *Fab-7* boundaries have chromatin structures that closely resemble the scs-like elements. Analysis of flies transgenic for a construct containing the *white* gene of *Drosophila* and sequences from *Mcp* have revealed an unexpected *Mcp*-mediated activity. Flies homozygous for P[*Mcp*, mini-*white*] show a pairing-sensitive inactivation of the mini-*white* gene present in the transgene.

A YEAST ARTIFICIAL CHROMOSOME (YAC) CARRYING THE MOUSE TYROSINASE GENE COMPLEMENTS FULLY THE ALBINO PHENOTYPE IN TRANSGENIC MICE

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Transgenic mice have become invaluable for the analysis of gene function. Expression of transgenes in mice, however, often fails to follow the temporal and spatial pattern and to attain the level of the endogenous copies. Only in exceptional cases has position-independent and copy number dependent expression been reproduced. Regulatory elements that control expression of a gene can be far away from the start site of transcription. The size constraint of standard constructs may, however, prevent the inclusion of important remote regulatory elements. Yeast artificial chromosomes provide a means of cloning large DNA fragments. Therefore, we attempted to generate transgenic mice by transfer of YACs carrying long fragments of genomic DNA. We succeeded to transfer a 250 kb YAC covering the mouse tyrosinase gene into mice by pronuclear injection of gel-purified YAC DNA. The YAC was found to be inserted into the mouse genome without major rearrangements and expression of the YAC-borne tyrosinase gene resulted in complete rescue to the albino phenotype of the recipient mice. Expression form the transgene reached levels comparable to that of the endogenous gene and showed copy number dependence and position independence. Introduction of large segments of DNA has great potential for identification of genes by complementation of mutations, analysis of complex genetic units and study of long-range regulatory mechanisms such as genomic imprinting and X-inactivation.

THE MECHANISM OF TELOMERASE: KINETIC AND MUTAGENIC ANALYSES

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Telomeres, the ends of eukaryotic chromosomes, are essential for chromosomal stability. In evolutionary diverse eukaryotes telomeric DNA consists of very simple, tandemly repeated sequences characterized by a typically G-rich strand which is synthesized by the ribonucleoprotein enzyme telomerase. However the telomeric repeat sequences of several budding yeasts were recently found to be much more complex than typical telomeric sequences, yet all retain a short G-rich motif.

A specialized RNA moiety of telomerase contains the template which specifies the telomeric DNA sequence. Telomerase adds telomeric DNA repeats to the 3' end of a DNA primer, which normally base pairs with the template region within the RNA moiety of telomerase. Using synthetic oligonucleotides and extensively purified telomerase from Tetrahymena, we found that the sequence of the primer 5' to the template-paired region affects the kinetic parameters of the telomerase reaction: kcat of polymerization was increased by the presence of 5', especially G, residues in the primer. We propose that this inductive effect contributes to telomere recognition and chromosome healing specificity in vivo.

Previously we showed that in *Tetrahymena* specific site-directed mutations of the telomerase RNA template sequence caused cellular senescence, implying that telomerase action is necessary for telomere maintenance and nuclear function. We have extended these experiments to test additional telomerase RNA mutations in vivo. Based on these findings we will discuss possible explanations for the evolutionary conservation of telomeric DNA.

TELOMERASE CLEAVAGE, RECONSTITUTION AND REGULATION

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Telomeres are essential for chromosome stability and replication. Telomere replication involves the *de novo* addition of tandemly repeated simple telomeric sequences, TTGGGG in *Tetrahymena* and TTAGGG in mammals, onto chromosome ends by the enzyme telomerase. Telomerase is an RNP in which the internal RNA component templates the addition of telomeric repeats. In recent studies we found that, in addition to elongation, *Tetrahymena* telomerase will cleave nucleotides from the 3' ends of specific telomeric primers. This cleavage is similar to cleavage catalyzed by RNA polymerases; cleavage is stimulated at pause sites in the elongation reaction. To dissect the mechanism of telomerase we have developed a functional reconstitution assay. When endogenous RNA is digested with micrococcal nuclease, telomerase is inactivated. Activity was restored by the addition of synthetic T7 *Tetrahymena* telomerase RNA. Synthetic RNAs mutant in the template region altered the specificity of nucleotides added in vitro. The reconstitution assay will allow us to characterize the template and other functional regions of the telomerase RNA.

Telomere length regulation in mammals is complex. We and others have proposed a model in which telomerase is active in the germline, inactive in many somatic cells and reactivated in immortalized cell lines. We found that, like human cells, mouse primary fibroblast telomeres shortened during in vitro growth and became stabilized after immortalization. Stabilization was associated with the reactivation of telomerase. In vivo telomerase activity was detected in mouse testes but was absent in mutants lacking primary spermatocytes. In newborn mice, telomere lengths were similar in many tissues while length differences were very apparent in adult tissues, suggesting developmental regulation of telomerase. Telomerase activity was detected in embryonic stem cells, and experiments are underway to determine if activity is altered after differentiation. To further investigate the role of telomerase in germline cells, we have identified activity in *Xenopus* oocytes. Telomerase is present at all stages of oocyte development and after maturation with progesterone. These animal studies in mice and *Xenopus* will allow us to define the role that telomerase may play in normal development as well as in cellular senescence and immortalization.

TELOMERE REPLICATION IN SACCHAROMYCES: A ROLE IN CELL CYCLE PROGRESSION?

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Saccharomyces telomeres end in ~ 350 bps of $C_{1-3}A/TG_{1-3}$ DNA. The predicted intermediate in telomere replication by either a telomerase or gene conversion mechanism is a transient extension of the TG_{1-3} strand. We recently demonstrated that the ends of yeast chromosomes and linear plasmids acquire TG_{1-3} tails of $\gtrsim$ 30 bases late in the S phase; the TG_{1-3} tails on linear plasmids can support telomere-telomere interactions by non Watson-Crick base pairing (Wellinger, et al., 1993 Cell 72:51). Two-dimensional gel electrophoresis and density transfer experiments were used to demonstrate that linear plasmids, like the ends of natural chromosomes, replicate in late S phase. Density transfer experiments also established that the acquisition of the TG_{1-3} tails occurred immediately after conventional replication forks approached the ends of the linear plasmid. Thus, telomere replication may be the very last step in S phase. Prior to mitosis, all telomere-telomere interactions are resolved and the TG_{1-3} tails are processed to yield completely duplex DNA molecules. We speculate that the TG_{1-3} tails have two functions: they serve as replication intermediates and they mark the completion of S phase by allowing telomeres to interact with each other thereby masking chromosome ends from the RAD9 check point. Several approaches are being used to test some of the predictions of this model. First, two genes that encode TG_{1-3} single-strand DNA binding proteins have been cloned and sequenced; their roles in telomere structure and replication are under investigation. Second, we have developed a genetic screen to identify genes involved in telomere replication. The first gene characterized in this screen is PIF1, a gene shown by others to encode a DNA helicase required for maintenance of mitochondrial DNA. The effects of PIF1 on mitochondria and on telomeres can be separated by mutation. In the nucleus, the PIF1 protein appears to inhibit telomere elongation and de novo telomere formation.

STRUCTURE AND FUNCTION OF *SCHIZOSACCHAROMYES POMBE* CENTROMERES

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Centromeric DNAs in common laboratory strains of the fission yeast *Schizosaccharomyces pombe* span approximately 40 to 100 kilobase pairs (kb) in length, are characterized by several classes of centromere-specific repeated DNA sequences, and are organized on each chromosome into a large inverted repeat that flanks a 5-7 kb non-homologous central core. The centromere of a particular chromosome is not a defined genetic locus, but can contain significant variability with respect to organization of repeated sequences.

Functional analyses indicate that both central core sequences and centromeric repeat K sequences are necessary for centromere activity. Appreciable mitotic and meiotic centromere function is retained with circular minichromosomes that contain only a part of a single K repeat and the central core and small portions of the core associated repeats from either *cen1* or *cen2*. In addition, neither centromeric L repeats, B repeats, nor the inverted repeat motif appear to be absolutely necessary for function.

While most of the centromere-specific repeated sequences are packaged into nucleosomes typical of bulk chromatin, central core sequences show, both in parental chromosomes and in functional minichromosome derivatives, an unusual chromatin structure that is essentially devoid of regular nucleosomal packaging. This structure is facilitated by the presence of K repeat sequences located several kb away, and is necessary, but not sufficient, for centromere function. A model for centromere function in fission yeast would likely involve a physical interaction, possibly through DNA looping, between K repeat and central core sequences that is mediated by specific proteins. Several DNA fragments from within the centromeric K repeat exhibit electrophoretic fragment mobility shifts after incubation with *S. pombe* chromatin extracts and excess amounts of non-specific competitor DNA, indicating the presence of specific centromere DNA binding activities in the fission yeast nucleus.

REPLICATION OF *SACCHAROMYCES CEREVISIAE* CHROMOSOME ///

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The 200 kb region of chromosome /// between the left telomere and the *MAT* locus contains fourteen *ARS* elements. We have mapped origins and termini of replication in this region using two dimensional agarose gel electrophoresis. The five highly active origins of replication identified map to the positions of five *ARS* elements: *ARS305*, *ARS306*, *ARS307*, *ARS309* and *ARS310*. The DNA sequences required for origin function coincide with the sequences required for *ARS* function on plasmids. Deletion from the chromosome of small fragments carrying any one of these *ARS* elements removes the associated replication origin. In addition, point mutations in the consensus sequence of *ARS307* previously shown to abolish *ARS* function abolish origin function. *ARS308*, which is associated with the centromere, is active as an origin in approximately 20% of cell cycles. The extreme instability of a 61 kb circular derivative of the chromosome from which both of its highly active origins were deleted demonstrates that there are no other DNA sequences within this circular chromosome capable of efficient origin function.

Six other *ARS* elements do not function as replication origins at detectable levels: *ARS300*, *ARS301*, *ARS302*, *ARS303*, *ARS304* and *ARS313*. The reason(s) for their apparent lack of chromosomal origin function is under investigation. One possibility, that *ARS302* and *ARS303* fail to function because of their proximity to the telomere, has been tested by examining their activity in a circular derivative of chromosome /// that lacks telomeres. Neither *ARS* shows origin activity in the circular chromosome, demonstrating that their failure to function as chromosomal origins is a property of these *ARS* elements or their chromosomal context which is not related to telomere proximity. In addition, when a new telomere is placed within 1 kb of *ARS307*, origin function is not repressed. A second possibility, that origin function is repressed by nearby active origins, has been tested by deleting active origins from the chromosome. In a full length (350 kb) derivative of chromosome /// from which all active origins in the left 200 kb were deleted, no origin activity was detected in the 200 kb region, demonstrating that repression by active origins cannot explain the inactivity of the remaining *ARS*s. A third possibility, that some of these "silent" *ARS* elements are used as meiotic replication origins, has been examined by analyzing DNA from premeiotic S phase. The nine *ARS* elements that have been examined to date show the same pattern of usage in meiosis as mitosis.

DNA replication terminates in relatively broad (10 to 12 kb) intervals between active origins, suggesting that converging replication forks move at similar rates and that termination occurs where converging forks happen to meet and not at specific sites. The observations that deleting an origin causes a shift in the position of termination and that the the 200 kb of DNA on the left end of chromosome /// can be efficiently replicated by a single fork from the right arm demonstrate that this region of chromosome /// does not contain any specific termini of replication.

THE TOPOGRAPHY OF YEAST CHROMOSOME REPLICATION.

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To replicate a chromosome seems such a simple thing: each molecule needs to be replicated once but only once per cell cycle. In *E. coli* both the mechanics and the regulation of the process is simplified by having to replicate only a single chromosomal DNA molecule from a single origin. The segmented genome of eukaryotes with replication beginning at multiple sites per chromosome adds additional levels of complexity to the problem. For the past twenty years our lab has been interested in understanding the topography of replication--how the different parts of a chromosome influence the process of replication. By studying the chromosomes of yeast, we have shown that initiation of replication at different origins on a single chromosome can begin at different times within the S phase, that not all chromosomal origins are used in every S phase, and that replication forks proceed at variable rates through different regions of the chromosome.

The position of a chromosomal origin plays a major role in determining its time of initiation in S phase. Origins located near a telomere, either in a chromosome or on a linear plasmid, initiate replication later in S phase than they do when located at more internal sites on a chromosome or on a circular plasmid. Telomere sequences at the physical end of the DNA molecule can influence an origin that is more than 30 kb away. Telomere sequences at internal sites also influence timing, but to a much lesser extent--both the delay in initiation time and the distance over which the influence is exerted are less. Recently, we have identified a late chromosomal origin that is more than 200 kb from a telomere. It is surrounded by earlier replicating regions. This origin has the unique property that on a circular plasmid it maintains its late time of activation. These findings suggest that some sequence, other than telomeres, is also capable of influencing origin activation time.

The efficiency of initiation at an origin depends upon its context. If two ARSs are located near one another (less than 5 kb), either in the chromosome or on a plasmid, the efficiency of initiation at each ARS is reduced. Only one or the other origin, but seldom both, initiates replication during a cell cycle. In addition, the preference for which origin is used can be significantly influenced by adjacent sequences.

Replication forks face various impediments to their movement. One site of fork arrest is found within the rDNA locus. A sequence at the 3' end of the 35S transcription unit prevents replication forks from entering the transcribed portion of the rDNA repeat. This arrest of replication forks is polar in that replication forks proceeding in the same direction as RNA polymerase I pass the barrier unimpeded. While transcription per se is not essential for the arrest of replication forks, recent results suggest that the promoter and enhancer play a role in fork arrest.

IDENTIFICATION AND ISOLATION OF DNA REPLICATION ORIGINS FROM THE SYRIAN HAMSTER CAD AND MOUSE ADA LOCI

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Mammalian gene amplification initiates, in some cases, by the production of submicroscopic circular extrachromosomal molecules, "episomes", that replicate with the timing and stringent copy number control of the corresponding chromosomal sequences. We dissected episomes mediating amplification of the Syrian hamster CAD gene and the mouse adenosine deaminase (ADA) gene into molecular clones to ascertain whether replication initiates within the same sequences and by the same mechanisms in extrachromosomal structures and in the corresponding single copy loci from which they are derived. Putative initiation regions were initially regionalized by determining which restriction fragments were labeled earliest after entry of synchronized cells into S-phase, and then localized more precisely by determining where leading strands diverge and lagging strands, Okazaki fragments, converge. Divergence of Okazaki fragments indicated regions within which two adjacent replication bubbles terminate.

All three methods revealed that DNA synthesis originates within the CAD structural gene in CAD episomes and in the single copy native locus. Elongation proceeds bidirectionally , but the rate appears faster towards the 3'-end of the gene. Hybridization of Okazaki fragments to targets of approximately the same size produced dramatically different hybridization signals. This may reflect the existence of regions within which Okazaki fragments are either not produced, or that they are ligated at different rates in different regions. We are using leading strand analyses to explore the possibility that additional OBR's exist within regions that do not hybridize to Okazaki strands.

Similar analyses performed in the ADA locus showed that replication initiation commences within a region ~28.5kb upstream of the ADA structural gene in the 480kb ADA episome present in mouse B1-50 cells and in the single copy locus. Initially, only a single initiation locus was detected using broadly spaced targets for Okazaki fragment analysis, but a finer subdivision revealed multiple adjacent regions within which replication initiates, as found in the CHO DHFR initiation zone. ADA initiation loci, like those in CAD, contained subregions exhibiting strong differences in Okazaki fragment hybridization. Intense Okazaki fragment hybridization commencing at the earliest times measurable in S-phase and continuing for at least 90 min was observed in regions corresponding to the collision of forks from adjacent bubbles, suggesting that barriers to replication fork progression may define the termini of some initiation regions. The existence of replication fork barriers between adjacent bubbles in the CHO DHFR locus may help to reconcile the previously reported 2D gel patterns and Okazaki fragment and leading strand analyses.

DNA BENDS AND TRANSCRIPTION ACTIVATION

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The DNA helix axis is straight only under idealized conditions. Bending of the molecule can result from flexing under thermal fluctuations, from intrinsic regularities in the sequence, and from stress induced by binding proteins and other ligands. Intrinsic bending or curvature of DNA results when certain base sequences, such as runs of oligo dA•dT about half a helical turn long, are repeated in phase with the DNA helical repeat. The resulting bent DNA molecules can be characterized by comparing their electrophoretic mobility with that of normal DNA. Using this technique, and varying the phasing between different bends to assess their relative direction, we have concluded that A tracts cause DNA to bend in a direction which is toward the minor groove in a coordinate frame located near the center of an A_{5-6} tract. In addition, we have measured the extent of DNA curvature by determining the relative rate of cyclizing molecules containing phased A tracts, with the conclusion that each tract bends the helix axis by $18 \pm 2°$.

The bendability of DNA when interacting with proteins depends on base sequence, with similar rules for *E..coli* CAP protein and nucleosomes: A•T rich sequences are preferred when the minor groove is compressed, whereas G•C rich regions are better if the major groove is compressed. Cyclization kinetic measurements suggest a value of 90° to 100° for the CAP-induced DNA bend angle in solution. In studies of transcription complexes we have found that the bend induced in DNA by CAP protein is maintained and perhaps enhanced in the ternary open complex containing CAP and RNA polymerase.

Intrinsic DNA bends can sometimes replace the function of protein-induced bends. In the *E. coli lac* promoter the rate of forming open promoter complexes is enhanced as much as ten fold in promoter constructs having the CAP binding site replaced by phased A tracts, provided that the intrinsic DNA bend is in the same direction as the natural bend produced by CAP. These results suggest a role in gene activation for the bend itself, in addition to possible effects due to contacts between CAP and polymerase.

Two proteins can act synergistically in binding to DNA if they both prefer that DNA be bent in the same direction. We have explored the potential magnitude of this effect by measuring the binding affinity of CAP protein for its binding site in small circles, compared to the affinity for linear fragments. Effects of up to two orders of magnitude and more are observed. These results suggest a mechanism by which CAP-induced bends can assist in transcription initiation.

STEREOCHEMICAL PRINCIPLES OF DNA TARGET SELECTION BY THE STEROID/NUCLEAR RECEPTOR

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Transcriptional regulatory proteins interact with one another, and with the basal transcription apparatus, to produce a near continuum of responses. These processes require specific high affinity interactions between proteins that occur only after one or more of the proteins bind to the DNA regulatory element. The steroid/nuclear receptor family is a well studied system that exhibits such DNA-dependent specific protein-protein interactions including those responsible for DNA target selection.

The DNA targets of the steroid/nuclear receptor family are distinguished by: (1) the base sequence of the 6-basepair 'half-sites'; and, (2) the orientation of the half-sites as well as the number of basepairs between them. We have studied the crystal structure of the glucocorticoid receptor's DNA-binding domain (GR-DBD) in complexes with a variety of DNA targets. We have also studied DNA complexes of mutational variants of the GR-DBD designed to recognize alternative half-site sequences and alternative half-site arrangements. These studies reveal; (1) the stereochemistry of half-site recognition, and (2) structural features influenced by DNA-binding that select for targets with appropriate orientation and spacing of half-sites.

CRYSTALLOGRAPHIC ANALYSES OF THE COMPLEXES OF TWO ZINC-FINGER CONTAINING DNA-BINDING DOMAINS WITH THEIR TARGET DNA-BINDING SITES: THE HUMAN OESTROGEN RECEPTOR AND THE DROSOPHILA TRANSCRIPTION FACTOR *tramtrack*.

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The steroid hormone receptors bind via a highly conserved DNA-binding domain to related DNA target sites. Mutation of just three amino acids has been shown to switch the specificity of the glucocorticoid receptor (GR) to that of the oestrogen receptor (ER). We have solved the structure of the DNA-binding domain from the oestrogen receptor in complex with DNA to 2.4Å. Comparison of this structure with the published structure of the related glucocorticoid receptor (at 2.9Å) reveals that whilst in the GR structure conserved amino acids make contacts to base pairs common to both DNA target sites, these contacts are rearranged in the ER structure. Hence, the discrimination of the receptor binding sites involves a general rearrangement of DNA-binding amino acids rather than slotting different amino acids into a common scaffold.

We have solved the structure of the two zinc-finger domains from the Drosophila transcription factor *tramtrack*. in complex with DNA (to 2.8Å). Whilst this structure reveals many of the features expected from comparison of biochemical data with the published structure of the three zinc-fingers from Zif268, a number of novel features are observed that illustrate that the repertoire of zinc-finger DNA interactions may be more complex than initially anticipated.

X-RAY STRUCTURE OF THE NUCLEOSOME CORE PARTICLE

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Crystals of nucleosome core particle containing DNA of a 5S RNA gene regulatory sequence and histone octamers were prepared. They contain two particles or 412 kD per asymmetric unit and diffract to 3.5-4 Å resolution on average. Information from heavy atom derivatives and molecular replacement are being combined to solve the X-ray structure. New heavy atom derivative crystals have been prepared from cysteine containing, recombinant histone proteins (see abstract: Luger *et. al.*)

Further information with respect to the structure of the histone octamer has allowed a detailed reassignment of the electron density corresponding to the histone proteins in the 7 Å nucleosome core particle structure containing mixed sequence DNA. Virtually all parts of the histone proteins including most of the tail regions can be assigned their position in the core particle structure.

Crystals of a nucleosome have been obtained via reconstitution using the globular domain of histone H1 expressed in bacteria, the four core histones, and a 179 bp DNA fragment (see abstract: Rechsteiner and Richmond). The properties of this particle and its crystals are currently under investigation.

STRUCTURE AND DYNAMICS OF THE HISTONE CORE OF THE NUCLEOSOME.

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The structure of the octameric histone core of the nucleosome has recently been determined by single crystal x-ray crystallography to a resolution of 3.1 Ångstroms (Arents et al., *Proc. Natl. Acad. Sci., USA, vol 88, p. 10148, 1991*). The histone octamer is a tripartite protein assembly in which a centrally located $(H3\text{-}H4)_2$ tetramer is flanked by two H2A-H2B dimers, in agreement with the results of earlier solution physicochemical studies (*Biochemistry, vol. 17, p. 4955, 1978; ibid. vol. 19, p. 1339, 1980*). The outer dimensions of this wedge-shaped particle (65 Å diameter, 60 Å and ca. 10 Å maximum and minimum length, respectively) are in agreement with those reported earlier by Klug et al. (*Nature, vol. 287, p. 509, 1980*). The mass distribution within this protein assembly generates a left-handed protein superhelix with a pitch *ca.* 28 Å, by the spiral arrangement of the following elements: (H2A-H2B) (H4-H3•H3-H4) (H2B-H2A). This protein superhelix complements the space defined by the left-handed supercoil formed spontaneously *in vitro* by double-stranded DNA when the water activity in its immediate microenvironment is lowered below a critical value (*Cell, vol. 13, p. 295, 1978*).

Novel features of the recently determined structure are: The structured domains of each of the four types of protein chains exhibit a common folding pattern, i.e., the *histone fold*, which consists of one long central helix, flanked on either end by a loop segment and a shorter helix. The rest of the sequences in each chain have differing organizations, and the amino termini are mostly disordered in the crystal. The folded chains do not pack as eight globular entities inside the octamer, but instead, associate in pairs, roughly lengthwise, in a characteristic "handshake" motif and thus generate the H2A-H2B and the H3-H4 dimers. The two H3-H4 dimers form a tightly interlocked assembly (the tetramer) via limited contacts, whereas the two H2A-H2B dimers interact rather loosely with each other as well as with the tetramer. We will present further results on the analysis of this structure and the progress of our ongoing work towards its refinement.

RETROVIRAL INTEGRATION MACHINERY AS A PROBE FOR DNA STRUCTURE AND ASSOCIATED PROTEINS. H.-P. Muller, P.M. Pryciak, and H.E. Varmus, Department of Microbiology and Immunology, University of California, San Francisco, CA 94143.

During the retrovirus life cycle, a nucleoprotein complex (NPC)containing a newly-synthesized, linear duplex of viral DNA and integration (IN) protein inserts viral DNA into host chromosomes. Minichromosomes, as well as naked DNA, can serve as a target for cell-free integration reactions using NPCs (1) or purified IN proteins (2) as a source of integration activity. By using a PCR-based assay to monitor the frequency with which individual positions in the nucleotide sequence of a target molecule are selected as insertion sites, we have shown (i) that integration occurs at many positions, but not randomly, into naked DNA; (ii) that assembly of DNA into nucleosomes does not inhibit, and often augments, integration; (iii) that some sites, often spaced approximately 10 nucleotides apart, are used more efficiently when present within a rotationally-positioned nucleosome; and (iv) that preferential insertions appear to result from attacks by the integration machinery within the major groove where the DNA is facing away from the histone octamer (2). Furthermore, integration is blocked by proteins, such as the yeast alpha-2 protein, that bind in the major groove (2). Preferential use of integration sites is also observed after retroviral infection, when insertions occur into SV40 minichromosomes in vivo(3).

These findings suggest that the retroviral integration reaction may provide a means to survey chromatin in vivo or in vitro for factors that correlate with physiological activities of chromosomes. In efforts to understand the significance of the altered use of integration sites, we are following insertion site selection after bending DNA by various means or after binding DNA to proteins. We have found that selection of sites can be altered by binding DNA to the catabolite activator protein (CAP), which is known to induce a sharp bend at its binding site (4), and by bending naked DNA that lies between two lac operator sites brought together by lac repressor (5). We are attempting to explain these changes in relation to known structures of the DNA-protein complexes and in relation to the different abilities of NPCs and purified IN proteins to respond to altered DNA conformations.

(1)Pryciak et al, EMBO *11*:291,1992. (2)Pryciak and Varmus, Cell *69*:769,1992. (3)Pryciak et al, PNAS *89*:9237,1992. (4)Schultz et al, Science *253*:1001,1991 (5)Kramer et al, EMBO *6*:1481 1987.

THE STRUCTURE OF METAPHASE CHROMOSOME BANDS:

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The chromatin fiber of metaphase chromosomes is organized into topological loops thought to be anchored by highly A+T-rich regions of about 1 kb termed SARs (scaffold-associated regions). Loops are held together by a network of proteins that form the scaffolding of the chromatid which is mirror symmetrically coiled in sister chromatids to achieve final packing. The major component of the scaffolding, topoisomerase II, binds SARs specifically and is directly implicated in chromosome condensation.

What is the structural relationship of the loop model and the classical banding pattern? Bands can be observed in acetic acid-methanol fixed chromosomes with DNA base-specific fluorochromes. A+T base-specific dyes are known to generate the dominant Q-bands (quinacrine-, Hoechst-bright); Q-bands represent the late replicating compartments enriched in repressed genes. They are flanked by the more GC-rich R-bands (quinacrine-, Hoechst-dull); they represent the early replicating compartments containing many active and activatable genes. The bulk DNA concentration of R and Q bands is quite similar. The reported difference in base pair content (3-5%) between these compartments and the available models do not satisfactorily explain the banding phenomena.

We have developed Q and R-like banding techniques on native, aqueous chromosomes suitable for 3D analysis by confocal microscopy. Our structural analysis identifies a highly A+T rich axis, thought to be the scaffolding, in the body of native chromatids. This scaffolding is differently positioned in the Q and R-bands relative to the main axis of the chromatid. The large Q bands are found to consist of tightly wound coils; this positions the A+T rich scaffolding toward the periphery of the chromatid. In contrast, the major R-bands contain a more axially elongated (streched coil) scaffolding; this positions the highly A+T rich sequences of the scaffolding more centrally along the main chromosome axis.

Our observations suggest that the alternating bands generated with base-specifc fluorochromes is due to a visual "pattern formation" resulting from the alternating position of the A+T rich scaffolding during its progression from telomere to telomere. In the Q-band regions, the closely juxtaposed (tight) coiling of the scaffolding, transversely across the chromatid, generates a bright "A+T" signal. In contrast, the longitudinal position of the A+T rich scaffold in the R-band yields a duller "A+T" signal (no visual reinforcement from other A+T rich neighbors). In line with this interpretation are immuno-fluorescence studies of topo II. This protein co-localizes to the A+T rich scaffolding in the native chromosome yielding a Q-type banding pattern. In summary, chromosomal bands can be explained by the loop-scaffolding model.

FLUORESCENCE IN SITU HYBRIDIZATION STUDIES OF INTERPHASE CHROMATIN ORGANIZATION

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Some aspects of interphase chromatin organization can be studied by fluorescence in situ hybridization (FISH) experiments. FISH is used to fluorescently tag the sites of specific DNA sequences. The proximity of tagged sites can be measured simply and rapidly. Chromosome painting experiments reveal that chromosomes occupy discrete, but variably sized domains in interphase nuclei. We have studied the organization of chromatin within these domains by measuring the physical distance between the hybridization sites of well-mapped DNA sequences from human chromosome 4p16.3 and other chromosome regions. For distances from ~50 kbp to 2 Mbp, chromatin behaves according to the predictions of a random walk model: e.g., the square of mean interphase distance is linearly related to known genomic distance. Thus, the molecules behave over this range as flexible chains, which are free to assume a large number of random configurations. Striking deviation from random walk behavior occurs at large distances (>2 Mbp on 4p16, >1 Mbp on 6p21). Preliminary experiments with probes from different bands on chromosome 19 show no further increase in mean interphase distance from 2 Mbp to 60 Mbp. These observations suggest that a constraining higher order organization dominates random behavior at these large distances. Our measurements show that the proximity of specific DNA sequences in interphase chromatin can be used to construct genomic maps with ~100-kbp resolution. In a test of a mapping strategy based on interphase proximity measurements, a map of 13 probes from a 4-Mbp region of chromosome 4 could be reconstructed from a matrix consisting of 56 pair-wise distance measurements. The order and relative spacing of markers in this map were consistent with published maps. Our findings also help to define the sensitivity of interphase FISH in detecting small (sub-band) chromosome rearrangements.

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MOLECULAR GENETICS AND GENOMIC STABILITY OF CHECKPOINT CONTROLS IN BUDDING YEAST.

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The eucaryotic cell cycle is regulated in part by checkpoints that inhibit entry into mitosis if DNA damage is unrepaired or if DNA replication is incomplete. We have identified 6 checkpoint genes in the budding yeast Saccharomyces cerevisiae, we call collectively MEC genes (mitosis entry checkpoint); all 6 genes are required for arrest in the G2 phase after DNA damage, and in addition 2 of the 6 are also required for arrest in S phase when DNA replication is inhibited. These genes likely may play a role in signal transduction, and analysis of the conceptual protein sequences indicates one gene encodes a GTPase (*MEC3*) and a second a protein kinase (*MEC2*), proteins with roles in signal transduction in other systems. From null mutants we conclude that the S phase control has an essential constitutive role in cell division whereas the G2 checkpoint is essential only when there is DNA damage.

We are interested in identifying all forms of genomic instability that the wildtype checkpoint normally act to minimize. To this end we will describe a complex chromosome event which appears checkpoint-related because it occurs spontaneously at a 10X higher frequency in *rad9* that in *RAD+* and is DNA damage-inducible, and may occur by a breakage fusion bridge cycle, an hypothesis that we are currently testing.

S-PHASE IS COUPLED TO START AND MITOSIS IN FISSION YEAST BY THE *cdc18*$^{+}$ GENE PRODUCT

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In the fission yeast *Schizosaccharomyces pombe* two gene functions, *cdc2*$^{+}$ and *cdc10*$^{+}$, are required for commitment to the cell cycle at Start. The *cdc10*$^{+}$ gene encodes an 85kDa protein which is an essential component of a recently identified transcription factor complex that activates specific genes at the beginning of S-phase. As an approach to understanding how S-phase is coupled to passage though Start, we initiated experiments to identify critical downstream target(s) of p85^{cdc10}. A fission yeast cDNA library expressed under the control of the thiamine repressible *nmt1*$^{+}$ promoter was introduced into the temperature sensitive *cdc10-129* mutant, and transformants were screened for growth at the restrictive temperature under conditions that allowed high level expression of the cloned cDNAs. This approach resulted in the identification of a cDNA which suppressed the lethality of the *cdc10-129* mutation and which was subsequently shown to be derived from the *S. pombe cdc18*$^{+}$ gene. Analysis of synchronized cell populations demonstrated that transcription of the *cdc18*$^{+}$ gene is periodic in the cell cycle, reaching a maximum near the G1/S boundary. Moreover, the expression of *cdc18*$^{+}$ mRNA is dependent upon functional p85^{cdc10}. The *cdc18*$^{+}$ gene contains an open reading frame of 577 amino acids, preceded by a promoter region containing MCB-like sequence elements characteristic of yeast genes expressed at the G1/S transition. The predicted amino acid sequence of *cdc18*$^{+}$ is about 28% identical to the budding yeast *CDC6* protein which has been implicated in the intitation of S-phase, and the two proteins share a conserved purine nucleotide binding motif. Repression of *cdc18*$^{+}$ gene expression completely prevents entry into S-phase, resulting in the accumulation of cells with a 1C DNA content. In spite of this failure to initiate chromosomal DNA replication, such cells rapidly divide resulting in the appearance of inviable cells with less than a 1C DNA content. Based upon these results, we propose that the *cdc18*$^{+}$ gene is a essential down stream target of the *cdc10* transcriptional control at Start and is required for initiation of DNA replication. In addition, it is essential for the checkpoint mechanism which coordinates the completion of S-phase with mitosis.

GENETIC REGULATION OF GENOMIC FLUIDITY IN NORMAL AND NEOPLASTIC CELLS

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Chromosomal abnormalities are routinely seen not only in spontaneously formed tumors, but also in chemically and virally transformed cells. It has been hypothesized these changes are important components of tumorigenesis, yet little information is available on the molecular basis of these rearrangements that are found in tumor cells. We are interested in the control of genomic rearrangements especially as it pertains to the neoplastic cell. We have approached this question by examining one marker for genomic instability, gene amplification. Gene amplification is frequently observed in tumors and transformed cell lines. This phenomenon is known to contribute to the generation of drug-resistant tumor cells and quantitation of the event is believed to have prognostic value in several types of neoplasia.

Previously we have reported a correlation between the ability of a cell to amplify endogenous genes and their ability to form a tumor when injected in the appropriate animal. In these types of experiments we exposed cells to N-(phosphonoacetyl)-L-aspartate (PALA), a drug which specifically inhibits the aspartate transcarbamylase activity of the multifunctional CAD enzyme and selects for amplification of the CAD gene, and observed a striking parallel between the ability of these cell lines to become resistant to this drug and the ability of these same cells to form tumors after injection into day-old syngeneic rats. Molecular analyses of independent PALA-resistant subclones confirmed that, in each case, this resistance was due to amplification of the CAD gene. In stark contrast, we find that gene amplification is undetectable ($<10^{-9}$) in primary, diploid cell populations from both rodent and human lines. These results demonstrate that a dramatic difference exists between primary diploid cell populations and transformed populations in their ability to amplify genomic sequences and suggests a significant difference in genetic stability between these two cell types.

To identify the genetic elements that might be involved in the regulation of gene amplification we asked if the ability to amplify was a dominant or recessive genetic property. We found that the ability to amplify an endogenous gene behaved as a recessive genetic trait. Amplification frequency in somatic cell hybrids were suppressed by over five orders of magnitude (10^{-3} to $<10^{-8}$). The control of gene amplification potential segregated independently of tumorigenicity and immortality. Our results suggest that one type of genomic fluidity, gene amplification, is suppressed in normal, diploid cells and the loss of this suppression could underlie the generation of the genotypic changes noted in multistep carcinogenesis.

In more recent work, we have identified the tumor suppressor gene, p53 as one of the genetic elements that modulate gene amplification and aneuploidy.

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GENETIC REGULATION OF GENOMIC FLUIDITY IN NORMAL AND NEOPLASTIC CELLS

Manipulation of p53 gene expression can effect amplification rate thru effects on cell cycle progression. The genetic control of these processes in human cells and their eventual consequences for neoplasia will be discussed.

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ROLE OF TFIID AND GENERAL COFACTORS IN TRANSCRIPTIONAL ACTIVATION

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The TATA-binding subunit (TBP) of TFIID and a number of other general initiation factors (TFIIA, B, E, F, G/J and H) suffice for basal transcription by RNA polymerase II from most core promoters. In contrast, high levels of induction by transcriptional activators require, in addition, both the multisubunit TFIID and a general cofactor designated USA. Purification of human TFIID by affinity methods has led to the identification and partial cloning of approximately 12 TBP-associated factors (TAFs). Collaborative studies with Y. Nakatani, T. Kokubo and D.-W. Gong (NIH) have identified apparent homologs (based on sequence similarities) to many of these in Drosophila TFIID. Identification of the human p250 subunit as the cell cycle regulatory protein CCG1 provides support for the idea that individual TFIID subunits (or domains thereof) may have selective functions for specific activators or regulatory pathways.

The general cofactor USA represses basal promoter activity but effects a large net increase in promoter activity in the presence of activators. Further purification has led to the identification of both a negative cofactor (NC1), which appears to function by competition with TFIIA for binding to TBP/TFIID, and two distinct positive cofactors (PC1 and PC2), which in isolation can also stimulate basal transcription. One of these (PC2) appears to be a large complex of circa 500 kDa.

Further studies of the structure and function of the TFIID subunits and the USA-derived components will be discussed.

X-RAY CRYSTALLOGRAPHIC STUDIES OF EUKARYOTIC TRANSCRIPTION FACTORS

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My laboratory is working on the development of a detailed understanding of the physical principles governing molecular recognition in biological systems. We are examining model systems derived from the transcriptional control machinery responsible for regulating eukaryotic gene expression. Our approach is to use X-ray crystallography and other biophysical methods to determine and characterize the three-dimensional structures of biological macromolecules and their complexes with DNA or other ligands.

Our studies of protein-DNA recognition traverse the entire length of a typical eukaryotic gene promoter, including basal class II transcription factors, promoter proximal binding factors and distal enhancer binding factors. Recently, we solved the crystal structures of TFIID TATA-box binding protein, Max homodimer complexed with the E-box sequence CACGTG, upstream stimulatory factor complexed with the same E-box, and hepatocyte nuclear factor-3γ complexed with its DNA enhancer binding site.

REGULATION OF THE INITIATION OF TRANSCRIPTION BY RNA POLYMERASE II.

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The formation of a transcription competent complex on class II promoters is a complex process requiring seven factors and RNA polymerase II. Using antibodies directed against the different general transcription factors we have analyzed whether all seven factors are an integral part of the preinitiation complex. In the presence of ribonucleoside triphosphates, the complete preinitiation complex can readily start synthesis of an RNA chain from a specific start site. As RNA synthesis proceeds, the transcription complex undergoes a transition from the initiation to the elongation mode which is accompained by structural changes. These changes have been studied by probing the transcription complex assembled on immobilized templates with antibodies directed against individual factors as well as by template competition studies. We have further analyzed isolated elongation complexes (ternary complexes) paused at different distances from the transcription start site. This analysis has allowed us to determine the factors travelling with RNA polymerase II during elongation.

We have also analyze what cellular components are essential for mediating activation of RNAPII transcription. We have found that the addition of different activators to in vitro transcription reactions reconstituted with highly purified general transcription factors, in addition to TFIID and RNA polymerase II, resulted in low, non-physiolgical, levels of activated transcription. Real activation, approaching levels similar to what is observed in vivo, was found to require the addition of two protein fractions, namely ACF and Dr2. ACF (activating cofactor) appears to be a mediator. Dr2 is a 95 kDa polypeptide that displays two novel activities. In the absence of an activator, Dr2 represses basal transcription; in the presence of activators (acidic-rich, glutamine-rich or proline-rich), Dr2 repression was overcome and high levels of activated transcription were observed. Transcription reactions containing TBP, in lieu of TFIID, were also repressed by Dr2; however, TFIIA could overcome Dr2-mediated repression resulting in basal levels of transcription. Gel mobility shift assays together with immunoprecipitation experiments indicate that Dr2 interacts specifically with TBP suggesting that Dr2 inhibits the association of TFIIA with TBP. Partial amino acid sequence of Dr2, obtained from trypsin digestion of the 95 kDa polypeptide, lead to the discovery that Dr2 was identical to a previously isolated cDNA clone.

CELLULAR AND VIRAL TRANSCRIPTIONAL ACTIVATORS

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Expression of eukaryotic structural genes is primarily regulated at the transcriptional level by promoter-specific activator proteins. These activators function through a common set of general or basic transcription factors. Cellular activators are typically sequence-specific DNA binding proteins whose sites are present within target promoters. We have been using in vitro systems to analyze how activators communicate with the general transcription machinery to stimulate transcription. We have been performing experiments aimed at addressing the following questions: (i) What step (or steps) in the transcription process do activators affect?, (ii) Which general transcription factor (or factors) is the direct target of activators?, (iii) Do different classes of activators work by similar or different mechanisms?, and (iv) How do multiple activators cooperate with one another to activate transcription synergistically?

Many animal viruses encode activator proteins that modulate the transcription rates of viral and in some cases cellular genes. Viral activators often have atypical features; for example, many viral activators are not sequence-specific DNA binding proteins. Because viral activators must act through the same basic factors we have asked whether their idiosyncratic appearances reflect underlying differences in mechanism of action. This question is being examined drawing on studies of the adenovirus E1a protein, human T-Cell leukemia virus Tax protein and human immunodeficiency virus Tat protein.

HYDRATION OF BIOLOGICAL MACROMOLECULES IN SOLUTION, SURFACE STRUCTURE AND MOLECULAR RECOGNITION

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Nuclear magnetic resonance techniques have been developed for investigations of the hydration of proteins and nucleic acids in aqueous solution (G. Otting and K. Wüthrich, J. Amer. Chem. Soc. **111**, 1871-1875 (1989), G. Otting, E. Liepinsh and K. Wüthrich, Science **254**, 974-980 (1991)). All individual hydration water molecules observed so far in solution are dynamic in the sense that they exchange rapidly with the solvent water. For internal hydration water the life-times with respect to exchange in and out of the hydration sites are in the range $\sim 1 \cdot 10^{-2} - \sim 1 \cdot 10^{-8}$s, indicating that the biomacromolecules undergo high amplitude structure fluctuations on this time scale. Surface hydration waters are typically characterized by exchange life times in the range 20–500ps, indicating that the interface between biological macromolecules and hydration water is highly mobile. A small number of hydration water molecules have been observed in the protein–DNA interface of a complex formed between a 14-base pair DNA duplex and the *Antennapedia(C39S)* homeodomain. Implications for the structural basis of transcriptional control by homeodomains will be discussed.

STRUCTURAL STUDIES OF THE YEAST MATα2 HOMEODOMAIN PROTEIN

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The MATα2 repressor is a homeodomain protein that regulates transcription of cell-type-specific genes in yeast. In order to gain insight into the molecular basis for the specific binding of α2 to DNA, we have determined the crystal structure of the homeodomain of α2 bound to DNA, at a resolution of 2.7 Å. The atomic model of the complex reveals that specific contacts between α2 and DNA are mediated primarily via the third of three α-helices that comprise the compact homeodomain fold. A large network of phosphate contacts presumably serves to stabilize complex formation. The interactions seen in the crystal structure are consistent with the results of studies of mutant α2 proteins. Comparison with the Drosophila engrailed homeodomain, as well as other homeodomain proteins, reveals that the homeodomain fold is highly conserved. Moreover, the docking of the homeodomain recognition helix is also highly similar due to contacts with the DNA formed by a set of highly conserved homeodomain residues. This suggests that all homeodomain proteins interact with DNA in a similar manner.

In the cell, α2 represses haploid-specific genes by binding DNA as a heterodimer formed by α2 with another homeodomain protein, a1. To investigate how these homeodomains interact with one another and bind DNA, we have crystallized an a1/α2/DNA complex and are proceeding with the x-ray crystallographic analysis of these crystals.

ASSEMBLY OF A MULTICOMPONENT TRANSCRIPTIONAL REGULATORY COMPLEX: THE VP16-INDUCED COMPLEX.

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Transcriptional regulatory proteins are frequently members of large families of related proteins that display similar if not identical DNA-binding specificities. The cis-regulatory specificity of such family members can be differentiated by the selective assembly of multiprotein complexes templated by the DNA and specific protein--protein contacts. Through such mechanisms, the closely related human POU-homeodomain proteins Oct-1 and Oct-2 can differentially regulate transcription as a result of selective association with the herpes simplex virus (HSV) activator protein VP16.

After HSV infects mammalian cells, the virally encoded virion protein VP16 (also called Vmw65 or α-TIF) forms a heteromeric complex with a host cell factor called HCF (also referred to as C1, VCAF, CFF). HCF is a family of related polypeptides that upon association with VP16 apparently primes VP16 for association with Oct-1 on the cis-regulatory target of VP16 activation, the TAATGARAT (R = purine) motif in the HSV immediate early promoters. VP16 fails to associate effectively with Oct-2 because of a single amino acid difference on the exposed surface of the DNA-bound homeodomain. Association with VP16 has two important effects on the activity of Oct-1: First, a very potent VP16 activation domain enhances the ability of Oct-1 to activate transcription of mRNA-type promoters and, second, VP16 stabilizes Oct-1 binding and thus recruits Oct-1 to a class of TAATGARAT motifs for which Oct-1 (and Oct-2) has little affinity on its own. By this latter recruitment mechanism, Oct-1, through its ability to associate with VP16, is able to activate promoters that are not responsive to Oct-2 even though Oct-1 and Oct-2 display the same DNA binding specificity on their own.

The Oct-1 POU domain bound to DNA serves as the receptor for formation of the VP16-induced complex. In addition to the homeodomain, the POU domain contains a second DNA-binding domain called the POU-specific domain. Both the POU-specific and POU homeo domains are helix-turn-helix containing DNA-binding domains. Indeed, the POU-specific domain is structurally similar to the λ repressor DNA-binding domain (Assa-Munt et al., Cell 73, 193-205 [1993]). Analysis of a human dwarfism mutation indicates that like the homeodomain, the POU-specific domain also apparently controls transcription through protein interactions with the exposed portion of the DNA-bound POU-specific domain. Here, we will describe these and other features of the components of the VP16-induced complex and how they come together to form this multicomponent regulatory complex.

NUCLEAR LOCALIZATION OF GENES, TRANSCRIPTS AND RNA SPLICING COMPONENTS: EVIDENCE FOR A NUCLEAR COMPARTMENT AT CHROMOSOME DOMAIN BOUNDARIES

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The nuclear topography of splicing snRNPs, mRNA transcripts and individual genes was analyzed with regard to the relation of these nuclear entities to chromosome domains in various mammalian cell types. Indirect immunofluorescence using anti-Sm antibodies revealed several specific distribution patterns of splicing snRNPs in the analyzed cell types, which also depend on physiological parameters such as the temperature of cell culture. While focal accumulations of Sm antigen were generally observed in coiled bodies distributed in the nucleoplasm, there was no apparent Sm staining in coiled bodies located within the periphery of nucleoli. The combination of fluorescence in situ hybridization and immunodetection protocols allowed to simultaneously visualize individual chromosome domains and Sm antigen or the transcript of an integrated human papilloma virus genome. Three-dimensional analysis of the fluorescently stained target regions was performed by confocal laser scanning microscopy. We demonstrate, that RNA transcripts and components of the splicing machinery are generally excluded from the interior of the territories occupied by the individual chromosomes. Furthermore we applied dual color in situ hybridization and confocal laser scanning microscopy to analyze the spatial arrangement of active and inactive genes regarding the corresponding chromosome domains. The analyzed genes were found preferentially located in the periphery of the chromosome domains. Based on these findings we present a model for the functional compartmentalization of the cell nucleus. According to this model the space between chromosome domains including the surface areas of these domains defines a three-dimensional network-like compartment in which gene transcription and splicing of mRNA occurs. We propose to call this space the inter-chromosome domain compartment.

INITIATION AND TERMINATION OF DNA REPLICATION IN VIRAL GENOMES AND MULTIGENE FAMILIES AS MODELS FOR MAMMALIAN GENOME REPLICATION

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We have investigated the replication of the Epstein-Barr virus (EBV) genome and the human ribosomal RNA (rRNA) genes as model systems for mammalian chromosomal replication. Two complementary two-dimensional (2D) agarose gel electrophoresis methods were used to determine sites of replication initiation and termination. We have found that the replication of the intact 172 kb extrachromosomal EBV genome in latent infection is similar to that observed for smaller plasmids that contain its genetically defined origin of replication, *oriP*. In the EBV genome, initiation of replication occurs specifically at or near a dyad symmetry element within *oriP*. Replication forks proceed in both directions, but a barrier to the progression of one replication fork at a family of repeated sequences within *oriP* results in an asymmetric, bidirectional mode of replication. We have also detected two additional strong barriers at or near two highly expressed viral genes transcribed by RNA polymerase III (the *EBER* genes). Forks are stalled in the same direction in which these genes are transcribed.

We have also studied the human rRNA gene family as a model for the replication of mammalian chromosomes. This multigene family encodes the 18S, 5.8S and 28S rRNAs and consists of several hundred tandemly repeated copies of a 44 kb repeat unit distributed among five acrocentric autosomes. 2D gel patterns consistent with replication initiation were observed spanning most of the 31 kb non-transcribed spacer (NTS) but not within the 13 kb transcription unit or adjacent regulatory elements. Termination of replication occurs throughout the rDNA repeat units, and, in some repeats, specifically at or near the junction of the 3' end of the transcription unit and the NTS. This site-specific termination of replication is the result of barriers to fork movement in the region where termination of transcription occurs. We have also investigated the temporal pattern of replication of rDNA during S phase. Two distinct classes of rRNA genes were observed, one replicates predominantly in the first half of S phase while the other replicates predominantly in the second half of S phase.

The site-specific initiation observed at the EBV *oriP* differs from the broad initiation zone found within the rDNA chromosomal loci. Site-specific termination of replication, however, is observed in both systems.

STEPS IN CHROMOSOME FORMATION AS SHOWN BY THE DNA-SPECIFIC OSMIUM-AMMINE STAINING TECHNIQUE

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The formation of metaphase chromosomes is generally thought to take place during the brief prophase that follows the G2 phase. Chromosome formation was reexamined by electron microscopy in the cycling cells of mouse duodenal crypts stained by the DNA-specific osmium-ammine technique. This technique shows changes in nuclear DNA that make it possible to divide the cell cycle into eleven stages (El-Alfy et al. Anat. Rec. 1993, Suppt, p.48).

During the four stages that precede karyokinesis, there are virtually no changes in the DNA accumulations along nucleoli and nuclear envelope, but the rest of the nucleoplasm shows extensive modifications as follows. At stage I there are mainly fine nucleofilaments dispersed in the nucleoplasm. At stage II, many of the nucleofilaments assemble into small (57-114 nm wide) clusters, which constitute the main feature of that stage. At stage III, further assembly yields midsized (114-258 nm wide) clusters. Continued assembly leads to the appearance of some large (>258 nm wide) clusters at stage IV. By the end of this stage, besides the large clusters, there remain a few dispersed nucleofilaments, small and midsized clusters, as well as the DNA accumulations along nucleoli and nuclear envelope. At stage V which corresponds approximately to prophase, all these structures somehow give rise to the large clusters (about 560 nm wide) which virtually constitute all the nuclear DNA. The assembly of nuclear DNA from the stage I nucleofilaments to the stage V clusters is a slow process extending over 8 hours.

The next step in chromosome formation occurs when the nuclear envelope breaks down and thus initiates stage VI or metaphase. About 6 of the large clusters line up and coalesce to yield the elongated, about 0.6μm wide, chromatids paired into chromosomes. This phenomenon occurs in about 15 min. In summary, chromosome formation includes two steps: a slow assembly of nucleofilaments into clusters and a rapid coalescence of the clusters into chromatids.

THE ROLE OF RAP1 AND TELOMERE STRUCTURE IN TELOMERE POSITION EFFECTS

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Genes located adjacent to telomeric sequences undergo reversible cycles of activation and repression, each state being maintained for multiple generations. To investigate the role of the yeast telomere-, silencing-, and UAS-binding protein RAP1 in telomere position effects, we have characterized two sets of mutant cells: a) a set of *rap1* alleles (termed the *rap1^t* alleles) that produce truncated RAP1 proteins missing the C-terminal 144-165 amino acids, and b) null mutants of the *RIF1* gene, encoding a protein capable of interaction with the C-terminus of RAP1. Our results indicate that loss of the C-terminus of RAP1 abolishes position effects at yeast telomeres and diminishes silencing at the *HML* locus. Elimination of position effects in these cells is associated with increased accessibility to the E. coli *dam* methylase in vivo. Thus, the C-terminal domain of RAP1 is required for telomere position effects. In contrast, *rif1* deletion alleles increase the frequency of repressed cells, suggesting that RIF1 may normally act to antagonize, rather than to facilitate, the formation or maintenance of the repressed state.

To further define the C-terminal domain involved in telomere position effects, we have mutagenized the C-terminus of RAP1 in vitro and have identified both nonsense and missense mutations that, while dispensible for the essential functions of RAP1, result in partial or complete loss of telomere position effects. These studies have revealed a 25 amino acid lysine and arginine-rich C-terminal tail region that is an absolute requirement for this process. We are currently investigating the factors that associate with this region and testing the possibility that the function of the C-terminal tail lysine residues is mediated through acetylatation in vivo.

Using the *rap1^t* alleles to generate wild type cells differing only in telomere tract lengths, we have also demonstrated that telomere position effects are highly sensitive to changes in the size (or structure) of the telomeric tract. Longer poly($G_{1-3}T$) tracts can increase the frequency of transcriptional repression at the telomere, suggesting that telomeric poly($G_{1-3}T$) tracts play an active role in the formation or stability of subtelomeric transcriptional states.

MAPPING DNA TORSIONAL TENSION WITHIN THE *DHFR* GENE DOMAIN IN CHINESE HAMSTER CELLS

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Torsional tension in DNA has been suggested to play an important role in the initiation of transcription of many genes both in prokaryotic and eukaryotic cells. We recently developed a technique to examine the topological status of specific DNA sequences in intact cells using photoactivated psoralen as a probe for torsional tension (Ljungman & Hanawalt, PNAS 89; 6055-6059, 1992). The assay is based upon the observation that the intercalation of psoralen into DNA is favored if the DNA is underwound by negative torsional tension and discriminated against by positive tension. By studying the effect of nicking cellular DNA by X-rays prior to the treatment with psoralen, the topological status of any sequence of interest can be determined. Using this technique, we found that the level of psoralen-induced DNA cross-links in the promoter region of the amplified dihydrofolate reductase (*DHFR*) gene in Chinese hamster ovary B11 cells was significantly reduced by prior X-irradiation indicating the presence of negative torsional tension. No significant effect of prior nicking was observed for any of the other five sequences tested in the *DHFR* gene domain nor for the genome overall. The combined treatment with the topo-isomerase I & II inhibitors camptothecin and VP-16 resulted in a marked build up of positive torsional tension downstream of the *DHFR* gene. Furthermore, the negative tension observed in the promoter DNA disappeared when the transcription of the *DHFR* gene was down regulated by serum starvation but not after the cells had been treated with the RNA polymerase II inhibitor DRB. These results suggest to us that the active *DHFR* gene domain contains negative torsional tension specifically in its promoter DNA and that this tension is not directly the result of transcription elongation but rather may be dependent upon poising of the gene for transcription.

NUCLEOSOMAL ORGANIZATION OF INTERPHASE TELOMERE-SPECIFIC CHROMATIN IN HIGHER EUKARYOTES

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Eukaryotic chromosomes are terminated with specialized structures called telomeres, which have many important structural and functional roles in interphase, mitotic and meiotic chromosomes. Telomeres in lower eukaryotes such as Oxytricha, Euplotes, Tetrahymena and yeast were demonstrated previously to have non-nucleosomal organization, suggesting that telomeres are specialized structures, unlike the nucleosomal substructure characteristic of the bulk of the eukaryotic genome.

This study addresses the question of the nucleoprotein structure in rat and other vertebrate telomeres. Using standard electrophoretic and sedimentation analyses of nuclease-treated telomeres, we have unexpectedly found that the bulk of the telomeric rat DNA is organized into nucleoprotein subunits that are nearly indistinguishable from nucleosomes. The subunits are cleaved with a 10.4 bp periodicity by DNAse I, have a sedimentation coefficient of 11.2 S, and are digested with MNase to form a stable 146 bp core particle that comigrates with nucleosome core particles on nucleoprotein gels. Each of these properties is characteristic of nucleosome core particles. Oligomers of the telomeric subunits sediment identically to oligomers of bulk chromatin, and are less condensed after treatment of the chromatin with Bio-Rex 70, which removes histone H1 from bulk chromatin. The unique features of telomeres in rat nuclei are an unusually short and regular DNA repeat length of 157 bp, altered MNase sensitivity in nuclei, and an absence of demonstrable binding of histone H1 to the monomer subunit. We conclude that the telomeres of rat are comprised of closely-spaced nucleosomes, in contrast to the telomeres of lower eukaryotes, which show no evidence of nucleosomal structure. We have also found very short repeat lengths in telomeres of other vertebrate organisms such as mouse, human, chicken, turtle, salamander and trout, isolated both from tissue and culture cells. These results suggest a conserved, unique chromatin structure for telomeres in higher eukaryotes, that is probably important for telomere function.

LINEAR PLASMID N15: POSSIBLE ROLE OF Z-DNA IN THE FORMATION OF THE TERMINAL HAIRPIN LOOPS

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Prophage of the temperate coliphage N15 is a linear plasmid with covalently closed ends (Svarchevsky and Rybchin, 1984). Previously we established that a 56bp nonideal palindrome containing 14bp of alternating pyrimidine/purine sequence is involved in terminal hairpin-loops formation (Malinin et al. 1992)

<---56bp------YRYRYRY RYRYRYR------MunI---->
5'.T..CAATaGtCCATTATACGC/GCGTATaATGGgCaATTG..A.3'
3'.A..GTTAtCaGGTAATATGCG/CGCATATTACCcGtTAAC..T.5'

The availability of the segment that is capable of converting to Z-DNA conformation may play a key role in recognizing and cutting of the palindrome by plasmid encoded enzyme(s)

To elucidate the biological role and secondary structure of the palindrome two plasmids have been constructed. One of them contains original 56bp nonideal palindrome from the N15 genome and the other one has the same palindrome in which two central CG residues are deleted to produce a new site for MluI. Constructed plasmids have the sites for MunI and MluI only in the palindromic regions, as verified by sequencing.

<-54bp----------MluI----------MunI---->
5'..T..CAATaGtCCATTATACG/CGTATAATGGgCaATTG..A..3'
3'..A..GTTAtCaGGTAATATGC/GCATATTACCcGtTAAC..T..5'
YRYRYR: YRYRYR

On the basis of restriction analysis of the supercoiled and relaxed plasmids we made a conclusion that cruciform and Z-DNA structures exist in examined plasmid preparations in the physiological range of negative superhelical densities and that the conversion of cruciform structure to Z-DNA is favoured by four unpaired residues. We also suppose that nicks necessary for the terminal hairpins formation occur adjacent to the B-Z DNA junctions.

CLEAVAGE STAGE HISTONES ARE THE FIRST HISTONES IN SEA URCHIN DEVELOPMENT

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CS histones (CS=cleavage stage) are the first histones to appear in the chromatin of a developing sea urchin embryo. They are synthesized during oogenesis to give a maternal store in the egg and also during the first cell divisions in the cleaving embryo, where they were first detected. CS histones condense the maternal chromatin in the oocyte and they replace sperm-specific histones in the male pronucleus immediately after fertilization, thus leading to extensive chromatin remodeling and subsequent activation of the paternal genome (Poccia et al., 1992).
So far, CS histones have resisted all cloning attempts based on screening with hèterologous histone gene probes, suggesting that they differ considerably from other sea urchin histone variants.
Starting from partial protein sequence information, we isolated cDNAs for histones H1, H2A, H2B and H4 from eggs of the sea urchin *Psammechinus miliaris*. These novel histone genes are expressed only in oocytes, eggs and very early embryos. Thus they can be defined as CS histone genes.
These CS histones are transcribed from replacement variant histone genes which encode exceptionally long polyadenylated mRNAs that are expressed throughout the cell cycle.
Histone CSH1 turned out to be exceptional in that it is the longest H1 protein ever described and shows low sequence homology (~40%) with other sea urchin H1 histones. Interestingly, the CSH1 core region shows 60% homology with B4 (H1m), the early embryonic histone H1 protein of *Xenopus laevis* (Smith et al., 1988). Another feature unique for CSH1 is an unexpected stretch of acidic aminoacids in the very N-terminal region. The function and evolutionary conservation of this region is now being investigated.

MOLECULAR STUDIES OF GAMETIC IMPRINTING IN THE MOUSE

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Gametic imprinting in the mouse may represent a special case of genomic imprinting, a class of biological phenomena effecting epigenetic modifications of chromosomes. Studies of the mechanisms involved will be facilitated by identification of imprinted genes.

The rationale behind our approach is that parthenogenetic embryos will be deficient in transcripts from genes which must be expressed from the paternal genome. In an earlier study involving parthenogenetic embryos, we demonstated that three stages suffered major embryonic loss; implantation, pregastrulation (day 5/6) and postgastrulation (after day 7.5). This suggested that we focus our search for imprinted genes on blastocysts and early postimplantation embryos (day 5). We performed differential hybridization of mouse embryo cDNA libraries with probes from fertilized (plus) and parthenogenetic (minus) embryos. Probes were generated from single embryos by PCR amplification of cDNA. Several clones were identified. None showed an all-or-nothing signal. Northern blot analysis revealed that one gene is expressed strongly in the placenta, two are expressed strongly in the embryo, and the last failed to hybridize with adult or prenatal tissue (day 7.5 and later). We believe that this latter gene is expressed very early and in extraembryonic tissues.

To confirm the results of the differential screen, dot blots of a panel of cDNAs from (1) androgenetic, fertilized and gynogenetic blastocyst stage embryos and (2) parthenogenetic and fertilized day 5 embryos were hybridized with our clones. Four clones revealed differential expression in the day 5 embryos. Roughly equivalent expression was seen in blastocyst stage embryos, however. Interestingly, no difference was seen in the expression of two known imprinted genes at either the blastocyst stage or day 5 embryos.

REPLICATION OF A PLASMID DNA CONTAINING AUTONOMOUSLY REPLICATING SEQUENCE 1 FROM SACCHAROMYCES CEREVISIAE WITH PURIFIED PROTEINS

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Understanding of eukaryotic DNA replication has been advanced rapidly with studies on the cell-free SV40 DNA replication system. However, the fact that SV40 T antigen acts not only as the initiator protein which binds to the origin but also as the DNA helicase may give to this system a limitation as a model of initiation of chromosomal DNA replication. Among eukaryotes, the autonomously replicating sequence (ARS) of S. cerevisiae is the only example of well-defined chromosomal replication origins that functions in plasmid. We developed a model system for ARS DNA replication. In this system, negatively supercoiled plasmid DNA containing ARS1 was replicated with proteins composed of SV40 T antigen as a DNA helicase, DNA polymerase α/primase, the single-stranded DNA binding protein from human cells and E. coli DNA gyrase. DNA replication started from ARS region and proceeded bidirectionally with the synthesis of leading and lagging strands.

The addition of human topoisomerase I, which relaxes both positive and negative supercoiling, to this system inhibited DNA replication. In the absence of DNA gyrase, however, only the 3'-flanking region (B domain of ARS1) of core consensus sequence was replicated and replication was extended after the addition of human topoisomerase I. These results suggest that DNA replication starts from ARS region where the DNA duplex is unwound by torsional stress; this unwound region can be recognized by a DNA helicase (T antigen) with the assistance of the single-stranded DNA binding protein and DNA gyrase is mainly involved in the elongation step as a swivelase.

Ishimi and Matsumoto (1993) Proc. Natl. Acad. Sci. U.S.A., in press.

CELLS FROM COCKAYNE'S SYNDROME GROUP A BUT NOT GROUP B ARE DEFECTIVE IN GENE SPECIFIC REPAIR OF 6-4 PHOTOPRODUCTS

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Cockayne's syndrome's (CS) is an autosomally recessive disorder characterised by severe photosensitivity, neurological defects, growth retardation and premature ageing. At the cellular level, CS cells are hypersensitive to the killing effects of uv-light but unlike cells from the uv-sensitive disorder, xeroderma pigmentosum (XP) this sensitivity does not correlate with the overall ability to excise uv-induced DNA damage. We have previously demonstrated (Venema *et al* Proc. Natl. Acad. Sci. USA, 87:4707 1990) that cells from CS complementation groups A and B are able to remove pyrimidine dimers from the bulk of chromatin but are not able to preferentially repair pyrimidine dimers from transcriptionally active regions of DNA. We have extended these studies to a second DNA adduct, the 6-4 photoproduct.

Normal cells rapidly remove 6-4 photoproducts from transcriptionally active genes while repair in the bulk of the chromatin proceeds at a slower rate. CS cells from complementation group A show a normal ability to preferentially repair transcriptionally active genes. In contrast, cells from CS complementation group B are defective in the repair of 6-4 photoproducts in active genes. These results have important implications for understanding the role of chromatin structure in DNA repair and the role of the CS A and B gene products.

INTERACTION OF THE τ_1 TRANSACTIVATION DOMAIN OF THE HUMAN GLUCOCORTICOID RECEPTOR WITH THE GENERAL TRANSCRIPTIONAL APPARATUS

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The glucocorticoid receptor (GR) is a member of a large family of ligand dependent sequence-specific transcription factors. After the binding of it's cognate ligand and DNA response element the subsequent events whereby the GR effects the rate of target gene expression remain unclear. However, protein-protein interactions are likely to play a major part in this process. We have studied the role of protein-protein interactions in the mechanism of action of the τ_1 transactivation domain, using recombinant receptor proteins expressed in bacteria and yeast cells. Previously we have shown that over expression of the τ_1 transactivation domain *in vivo* inhibited both gene expression (squelching) and cell growth. The effect on gene expression preceded that on cell growth, consistent with specific inhibition of transcription by τ_1, and could be observed using a basal promoter[1]. These studies have subsequently been extended using a yeast cell free transcription system. Purified τ_1 specifically inhibited transcription from a basal promoter derived from the *CYC1* gene and from the adenovirus 2 major late core promoter in a concentration dependent manner[2]. This inhibition was correlated with the transactivation activity of τ_1, and was not specifically reversed by recombinant yeast TATA-binding protein (TBP). Taken together, the above studies indicate that the τ_1 transactivation domain of the GR interacts directly with the general transcriptional apparatus through some target protein(s) that is distint from TBP. Current studies, aimed at identifying the target factor(s), involve the analysis *in vitro* of cloned basal factors and fractionated yeast extracts.

1. Wright et al. (1991) *Mol.Endocr.* **5**: 1366-1372.
2. McEwan et al. (1993) *Mol.Cell.Biol.* **13**: 399-407.

A LIVER-SPECIFIC TRANSCRIPTIONAL ENHANCER AND BOUND TRANSCRIPTION FACTORS EXISTING IN A PRECISELY POSITIONED NUCLEOSOME ARRAY

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We have been investigating the hierarchies by which liver transcription factors bind to DNA and function in a chromatin context. The mouse serum albumin gene is controlled in part by an enhancer element 10 kb upstream which binds liver-enriched and ubiquitous transcription factors *in vitro*. We used *in vivo* footprinting protocols with DNaseI to show that 6 central factor binding sites of the enhancer are occupied in liver chromatin, but not in chromatin of tissues in which the gene is not expressed. Two of the occupied sites are essential for enhancer activity and bind the hepatic transcription factor HNF3. Binding of the factors in intact hepatocytes was confirmed in a methylation protection experiment where a DMS-containing solution was used to perfuse the liver *in situ*. The HNF3 sites and other occupied sites span a 160 bp segment of DNA that corresponds precisely to the region where the albumin enhancer is DNaseI hypersensitive in liver chromatin. To examine the nucleosomal arrangement of the enhancer, the positions of micrococcal nuclease cleavage in liver nuclei were mapped at the nucleotide level of resolution, using a modification of the LM-PCR protocol. Our results suggest that the enhancer binding factors form a higher-order particle, designated N1, either with a nucleosome or in place of a nucleosome that is translationally positioned along the DNA. Three apparent nucleosomes, N2-N4, are positioned immediately upstream of N1. The adjacent nucleosome N2 appears to be rotationally positioned and contains two factors bound in liver nuclei. The phased array occurs in liver chromatin and not in chromatin from non-expressing tissues, nor does it occur when albumin enhancer sequences are assembled into nucleosomes *in vitro*, implying that specific binding factors maintain the array. The precise positioning of the N1 particle and the concomitant positioning of factor binding sites upstream of N1 into linker and nucleosomal segments suggests a specific hierarchy by which liver transcription factors act in chromatin. *In vitro* chromatin assembly systems are being used to understand the role of HNF3 and other liver-enriched factors in nucleosome positioning.

AN RNASE-SENSITIVE PARTICLE CONTAINING *DROSOPHILA MELANOGASTER* DNA TOPOISOMERASE II

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Most DNA topoisomerase II (topo II) in cell-free extracts of 0-2-h-old *Drosophila* embryos remains in the supernatant after low-speed centrifugation. Virtually all of this apparently soluble topo II is actually particulate with a sedimentation coefficient of about 67 S. Topo II-containing particles of similar size were detected in *Drosophila* K_c tissue culture cells, 16-19-h-old embryos and *Xenopus* egg extracts. *Drosophila* topo II-containing particles were insensitive to Triton X-100 and DNAse I but could be disrupted by incubation with 0.3 M NaCl or RNase A. After either disruptive treatment, topo II sedimented at 9 S. RNase sensitive nonnuclear topo II-containing particles were also sensitive to micrococcal nuclease. Results of chemical crosslinking corroborated those obtained by centrifugation.

IDENTIFICATION, CLONING AND CHARACTERIZATION OF AMPLIFIED DNA IN TUMOR CELLS BY CHROMOSOME MICRODISSECTION

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Cancer cells have the propensity to develop regions of DNA sequence amplification. Clonal variants carrying amplification of specific genes such as oncogenes or drug resistance genes may become predominant in the cell population because their overexpression confers a selective advantage. It is therefore of interest to identify and characterize those genes which are the biological targets of amplification events in tumor cells. A number of general methods have been described for the detection of amplified DNA which do not require the use of previously cloned amplification unit probes. These include techniques based on DNA electrophoresis (in-gel renaturation and restriction landmark genomic scanning), and a recently reported molecular cytogenetic technique, comparative genome hybridization (CGH). All of these techniques are primarily analytical and do not lead directly to the generation of high complexity amplification unit libraries. Because amplified genes are frequently associated with recognizable cytologic alterations, homogeneously staining regions (HSRs) or double minutes (dms), we sought to apply our recently developed chromosome microdissection technique to the analysis of these abnormalities (Meltzer et al., Nature Genetics 1:24-28, 1992). This approach, combining microdissection, polymerase chain reaction, fluorescence in situ hybridization (FISH) and microclone library construction was modeled on known amplification units located either extrachromosomally (dms containing MYCN in NCI-H69) or intrachromosomally (HSRs containing MDM2/SAS/GLI in OsaCL and NGP127). Amplification unit microclone libraries have been generated from as few as five cells. Additionally, by using the microdissection PCR product for FISH to normal metaphases, it was possible to determine the chromosomal derivation of each amplification unit. Using this strategy, the melanoma cell line HAA carrying an unknown HSR (Trent et al., Cancer Research 44:233-237, 1984) was shown to encode amplified and over expressed copies of the IGF1R gene. In this HSR, the amplification unit was derived from a single recognizable band, but in the other examples studied, the amplification units juxtaposed sequences from distant chromosomal sites. Because of its rapidity, minimal sample size requirements and direct generation of amplification unit probes, chromosome microdissection technology can significantly facilitate the analysis of amplified DNA.

REPRESSION OF THE HERPES SIMPLEX VIRUS-1 (HSV-1) α4 GENE CONTAINED IN THE VIRAL GENOME IS MEDIATED BY THE BINDING OF ITS GENE PRODUCT TO THREE SITES.

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The α genes, the first set of genes expressed after infection, are induced by the virion α trans-inducing factor, VP16. The viral regulatory protein ICP4, the product of the α4 gene, has been shown to repress a subset of α genes and to induce the transcription of β and γ genes, expressed later in infection. ICP4 binds directly to two types of DNA sequences. Type A sites have a high affinity for ICP4 and share a consensus sequence whereas no consensus has been derived for the lower affinity, type B sites. In the α4 gene, mapping studies have identified a type A site between -4 and +12 and two type B sites between -197 and -135. A major problem in the analyses of viral DNA response elements is that authentic regulation of gene expression is observed primarily in infected cells; transient expression systems frequently do not yield results consistent with the events that occur during infection. The objective of the studies described here was to determine the role of these three ICP4 binding sites on transcription. A panel of mutant viruses was made that contained a chimeric reporter gene consisting of the 5' untranscribed and transcribed, non coding domain of the α4 gene fused to the coding domain of the thymidine kinase gene. The transcription of this chimeric gene was regulated by response elements in the α4 domain. Each of the ICP4 binding sites in the chimeric gene was mutated singly and in combination, and the effect of these mutations was determined during the course of viral infection by RNase protection assays with reference to an internal control. The results indicated that (a) the mutations did not affect the transactivation of the chimeric gene or the stability of the transcript, (b) the A site at -4 to +12 mediates repression early in infection and (c) repression measured at 8 hours post infection was alleviated by mutagenesis of all three binding sites. The role of the B sites in the repression of α4 gene occurs late in infection and in cooperation with the A site.

Association of intrinsically bent DNA elements with different types of recombination events in mammalian cells.

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DNA structural elements are thought to be involved in numerous biological process. In particular bent DNA, either intrinsic or protein induced, has been associated with transcriptional regulatory elements, replication origins, chromosomal structural elements and prokaryotic site specific recombination. Recently, we reported that mammalian illegitimate recombination events such as integration, excision and spontaneous chromosomal deletions, were also associated with intrinsically bent DNA elements. We have extended our study to other types of recombination events in mammalian cells. Here, we report the association of intrinsically bent DNA elements with chromosomal retroviral insertion sites and also with preferential sites of rearrangement at the *hprt* locus. Localisation of bent DNA elements was determined by electrophoresis in polyacrylamide at low temperature. Under such conditions bent DNA demonstrates an abnormal electrophoretic mobility. The results were that out of 10 distinct retroviral insertion sites, nine were associated with bent DNA elements. In the case of the *hprt* rearrangements, which occur frequently between exon 2 and 3, a bent DNA element was also found to be present at that site. This association of bent DNA elements with recombination events might be a general feature of eukaryotes since preliminary results indicate the presence of intrinsically bent DNA at the site of a yeast chromosomal recombination hot-spot (ARG4 locus).

DIFFERENTIATION AND MAINTENANCE OF CHROMATIN STRUCTURE

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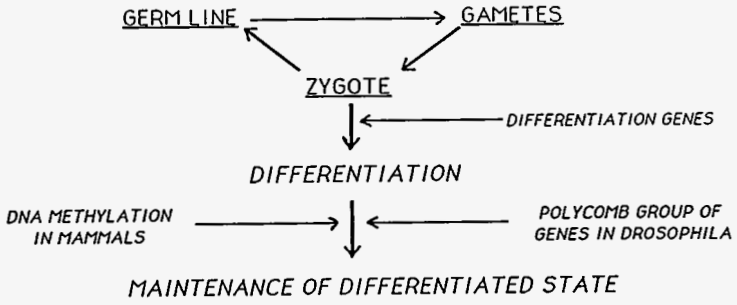
Level of DNA methylation varies in a temporal and region specific manner in murine embryonic, extraembryonic and germ cell lineages. Methyl transferase (MTase) mutant mouse embryos fail to develop beyond 11-day stage. MTase has been found to localise in the replication foci in nucleus during S-phase. These observations suggest that the structure of chromatin established by early differentiation genes may be inherited through cell divisions by marking specific stretches of DNA by methylation. Lack of DNA methylation in some well studied organisms neccessiates, however, another parallel mean for the maintenance of such structures.

Genetic and molecular studies on early development and modifiers of position effect variegation (PEV) in drosophila suggest that non-histone chromosomal prot ns (like polycomb group, PcG, genes and some modifiers of PEV) may be involved in such a process. Also, like MTase, Pc is present as maternal protein in large amounts and produced zygotically subsequently. As shown below in the figure, these observations suggest a functional equiva lence between methylation in mammals and PcG genes in lower organisms. Possible mechanisms leading to the establishment and maintenance of chromatin structure during development in various organisms (yeasts, nematodes, insects and mammals), contribution of organisation of coding and non-coding genome to it and the testable predictions of such proposed mechanisms will be discussed.



PROTEIN-PROTEIN INTERACTIONS MEDIATING THE SILENCING AND ACTIVATION FUNCTIONS OF A YEAST REGULATORY PROTEIN: THE SILENCING FACTORS SIR3 AND SIR4 BIND TO THE RAP1 C-TERMINUS

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RAP1 (Repressor/Activator Protein 1) is a sequence-specific DNA-binding protein in yeast that regulates transcription in a context-dependent manner. RAP1 binds to the promoters of a large number of genes, where it activates transcription. RAP1 also binds to the mating-type gene silencer elements where it participates in repression, and to the poly($C_{1-3}A$) sequences at telomeres where it regulates telomere structure and, possibly, telomeric position effect. We have identified activation and silencing domains of RAP1 that map to adjacent regions in the C-terminus of the protein [1, 2]. In addition, we have isolated mutations in this C-terminal domain that specifically affect silencing, demonstrating that at least some repression functions of the protein are genetically separable from its (essential) activation functions [3].

Using a LexA-RAP1 hybrid protein in the two-hybrid system we have identified two new putative RAP1-interacting proteins, SIR3 and SIR4. Both *SIR3* and *SIR4* are essential for mating-type gene silencing and telomeric position effect. To ask whether either interaction (RAP1-SIR3 or RAP1-SIR4) is dependent upon *SIR* gene function, we have mutated each of the four *SIR* genes in the two-hybrid reporter strain. Neither interaction is dependent upon native *SIR* gene function.

Surprisingly, mutation of *SIR3*, *SIR4*, and to a lesser extent *SIR2*, converts a non-activating LexA-RAP1 hybrid into a strong activator. These data suggest that SIR proteins can bind to the RAP1 C-terminus and mask the nearby activation domain. Together with other genetic studies of silencing-defective *rap1*^{s} mutants, these data suggest a complex interaction between SIR proteins and the RAP1 C-terminus, perhaps in conjunction with competing co-activators, that modulates the function of RAP1. Biochemical studies are underway to examine these interactions in vitro with the aim of understanding how protein-protein and protein-DNA interactions determine the specificity of RAP1 action at particular chromosomal loci.

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BINDING OF GAL4 TO DNA IN POSITIONED NUCLEOSOMES IN YEAST. Randall H. Morse, Laboratory of Cellular and Developmental Biology, National Institutes of Health, Bethesda, MD 20892

Binding of the transcriptional activator GAL4 to DNA in positioned nucleosomes has been investigated in yeast cells. In cells grown in galactose, transcriptional activation of a *lacZ* reporter gene by GAL4 is seen whether or not its binding site is in a predicted positioned nucleosome. To monitor changes in chromatin structure due to GAL4 binding, a single 17 bp binding site was separately introduced into two positioned nucleosomes: the strongly positioned nucleosome I of TRP1ARS1 (Thoma *et al.* (1984) **J. Mol. Biol.** 177, 715), and nucleosome IV in the plasmid TALS, which is positioned adjacent to the α2/MCM1 operator in yeast α cells (Roth *et al.* (1990) **Mol. Cell Biol.** 10, 2247). In both cases, nucleosomes are positioned as predicted in cells grown under conditions (+glucose) such that GAL4 is not bound. In contrast, in either inducing (+galactose) or non-inducing (-glucose, -galactose) conditions which allow GAL4 binding, chromatin structure near the GAL4 binding site is perturbed. A derivative of GAL4 possessing the DNA-binding domain but lacking the activation domain is able to bind to its site and alter nucleosome positioning when expressed at very high levels. A similar derivative expressed at lower levels is unable to bind to the GAL4 binding site in a positioned nucleosome, but does bind and perturb local chromatin structure when the binding site is not occluded by a strongly positioned nucleosome. These bindings indicate that at least some transcription factors can bind and activate transcription *in vivo* in the face of what would appear to be a repressive chromatin structure. The outcome of the competition between a transcription factor and histones for a particular binding site may depend on both the amount of factor present and on its particular type of activation domain.

DROSOPHILA HMG1 PROTEINS ARE ASSOCIATED WITH HIGHLY COMPACTED DNA

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Non-histone proteins of the High Mobility Group (HMG) have been implicated in all processes associated with DNA from replication to chromosome segregation, but their exact functions remain elusive. We are investigating the role of the HMG1 group using the *Drosophila* system. The fly contains two such proteins (dHMG-D and dHMG-Z) that are expressed throughout embryogenesis. dHMG-D is present in the syncytium and dHMG-Z is expressed only in the zygote. DNA binding analysis shows that the HMG-box, the N-terminal 74 residues, is the DNA recognition motif and binds DNA sequence-selectively, preferring certain A/T rich sequences, as shown by methylation interference protection and DNaseI cleavage. These sequences suggest the proteins bind through minor groove interactions. There are several such sequences in the original probe used to identify the protein in a λgt11 expression library screen.

Immunochemistry using antisera against *E. coli* expressed dHMG-D and dHMG-Z polypeptides reveals both proteins are detected associated with DNA undergoing mitosis as observed in synchronized cleavages of the syncytial blastoderm, and later in individual mitotic domains arising during the three postblastoderm mitoses. Neither protein is detected during interphase in the blastoderm and postblastoderm stage embryos. However, by western analysis the protein for HMG-D is observed throughout embryogenesis, suggesting the epitopes are masked in some way in the DNA of interpahse nuclei. The antibodies also detect additional species by western analysis which probably correspond to post-translationally modified HMGs.

STRUCTURAL ANALYSIS OF THE CANDIDATE 'IMPRINTING BOX' OF THE MOUSE *Igf2 RECEPTOR* - COMPARISONS TO OTHER SEQUENCES KNOWN TO BE IMPRINTED

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Recent reports have substantiated the finding that specific sequences in the vicinity or within imprinted genes show parental origin dependent modifications associated with the imprinting phenotype[1,2]. The methylation pattern of such genomic regions show a marked difference depending on the parental germ line (paternal or maternal) they originated from.
Analysis of a region - situated in the second intron of the *Igf2 receptor* gene - has revealed a CpG island containing several iterated repeats. Comparison of this sequence with those of other known imprinted genes and transgenes, like the *Igf2* gene[3], the *Xist* locus neccessary for the X chromosome inactivation[4] or the TG.A transgene[5] did not detect any direct sequence homology but did demonstrate a conserved organization in that all contain iterated repeats and had a high GC density (albeit not all of them are identical with CpG islands).
The relevance of repeats for epigenetic modifications leading to the imprinting phenotype will be discussed.

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A NOVEL MITOCHONDRIAL "MULTI-SITE SPECIFIC ENDONUCLEASE" ACTIVITY AT EARLY STAGE OF MEIOSIS IN THE YEAST *SACCHAROMYCES CEREVISIAE*.

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Sequence specific endonucleases in eukaryotic cells have been shown to initiate genetic recombination near the cleavage sites *in vivo*. Double strand breaks of DNA introduced by these endonucleases offer a good substrate for recombination. Each endonuclease can be classified into two major categories :1) Endonucleases having strict sequence recognition, and 2) endonucleases with multiple specific cutting sites ("multi-site specific endonucleases"). The endonucleases in the former group includes HO endonuclease and "intron-encoded endonucleases" that trigger site-specific recombination events. This type of endonucleases usually exhibit very strict recognition, so that only a few cleavage sites are allowed even in whole genome. Endonucleases categorized into the second group introduce multiple double strand breaks in various DNA substrates *in vitro*. Endo. *Sce*I in yeast *S. cerevisiae*, identified by biochemical *in vitro* assay, is a typical example of multi-site specific endonuclease. This enzyme is localized in mitochondria, and generates multiple DNA double strand breaks at specific sites in mitochondrial DNA *in vivo*. Endo. *Sce*I was shown to efficiently initiate gene conversion at *oli2* locus which includes a major Endo. *Sce*I cleavage site *in vivo* [Nakagawa, Morishima & T.S. (1992) EMBO J 11:2707].

In addition to Endo. *Sce*I, using an *in vitro* assay, we have identified a nobel sequence specific endonuclease activity in a meiotic cell-free extracts from synchronously-sporulating strain of *S. cerevisiae* (SK1) that lacks a gene for Endo. *Sce*I. DNA ends are generated in 23 bases consensus regions and have 4 bases 3'-overhang, a common feature of all recombinational sequence specific endonucleases in yeast. Genetic evidences, together with the result of subcellular fractionation, revealed that the endonuclease activity is mitochondrial, and a gene for the endonuclease is encoded in mitochondrial genome. Interestingly, the activity reached a maximum (~40-fold higher compared to exponential growing phase) 3 hours after transferring cells into sporulation medium, and thereafter the endonoclease activity went away before the appearance of asci. The data imply cell-cycle regulation of a multi-site specific endonuclease and activation of unknown recombination event in mitochondrial genome during early stage of meiosis in yeast cells.

MOLECULAR CLONING AND ANALYSIS OF DNA SUPERCOILING FACTOR REVEAL ITS ABILITY TO BIND CALCIUM

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A protein factor with an estimated molecular mass of 50KD has been purified to homogeneity from the silk gland of the silk worm Bombyx mori. The factor, termed DNA supercoiling factor, introduces unconstrained negative supercoils into relaxed closed circular DNA in conjunction with eukaryotic DNA topoisomerase II. DNA topoisomerase I cannot substitute for eukaryotic DNA topoisomerase II in the supercoiling reaction. The reaction is dependent on ATP and inhibited by etoposide, a specific inhibitor of eukaryotic DNA topisomerase II. When DNA topoisomerase I is subsequently added to the supercoiling mixture, the supercoiled DNA becomes relaxed.

Complementary DNAs coding for the factor have been cloned from the silkworm B. mori and the fly Drosophila. Analyses of these clones reveal that both factors share homologies to a central part of bacterial DNA gyrase A subunit and Ca-binding domains of calmodulin and troponin C. When the factor was subjected to an SDS/PAGE, transferred to a nitrocellulose filter and, after renaturation, allowed to bind radioactive calcium, it gave a radioactive band. These results suggest that the factor is a Ca-binding protein. We are now studying the effect of Ca on the supercoiling reaction.

Expression of the factor in Drosophila embryos was analyzed by staining with antibody against the factor. In early division stage embryos, we observed the antibody staining on the nuclei.

ALLELIC SILENCING IS RESPONSIBLE FOR THE LOSS OF ABO BLOOD GROUP ANTIGENS IN MALIGNANCY

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Loss of ABO blood group antigens is found on the surface of carcinomas and on the red blood cells of some patients with haematological malignancies. As the ABO locus *per se* is unlikely to be important in cancer progression, the loss of antigenic expression is a signpost to other genetic changes in the malignant stem cell population. Selection against a syntenic tumour suppressor gene may be the underlying cause of these changes.

We have used PCR to determine the genotype and to assess loss of heterozygosity at the ABO locus in four patients with haematological malignancy showing loss of red cell ABO antigens. All patients were heterozygous (AO) and PCR genotyping showed no difference in allele dosage. Thus, loss of ABO is not due to loss of heterozygosity. Reverse transcription PCR showed that these four patients had markedly decreased transcription of the A allele relative to the O allele. Therefore, in these cases, loss of ABO antigens results from the silencing of a single allele of the ABO gene. Similar loss of phenotypic but not genotypic heterozygosity was obtained with colon carcinoma biopsies and with colon carcinoma cell lines. Allelic silencing represents a type of (epi)genetic event which may be under-recognised in human malignancy and genetic disease.

HISTONE H4 ACETYLATION DEFINES THE TRANSCRIPTIONAL POTENTIAL OF CHROMATIN

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Although it has long been accepted that the level of histone acetylation is higher in transcriptionally active than inactive chromatin, many questions about the role of acetylation remain unanswered. For example, is it part of the transcription mechanism itself or does it serve to mark potentially active and quiescent regions of the genome? We have used two approaches to address these questions, both based on the use of antibodies to acetylated H4. In the first, we have used indirect immunofluorescence microscopy to examine the distribution of acetylated H4 in interphase and metaphase chromosomes. In both, the level of H4 acetylation in constitutive, centric heterochromatin is low (Turner et al.Cell 69, 374, Jeppesen et al. Chromosoma 101, 322). We have now extended these studies to show that regions of increased H4 acetylation along the arms of mammalian metaphase chromosomes correspond to conventional R-bands, regions known to be enriched in coding DNA, while H4 in the facultative heterochromatin of the inactive X chromosome in female cells is underacetylated. Sharply-defined bands of acetylated H4 are present on the Xi in human cells, at least some of which correspond to the location of genes whose expression persists on Xi. In the second approach, antibodies are used to immunoprecipitate chromatin fragments based on their level of H4 acetylation. By testing DNA isolated from bound (i.e. acetylated) and unbound fractions with a variety of DNA probes, we have been able to determine the acetylation status of H4 associated with different, closely defined regions of the genome. In agreement with the immunofluorescence results, DNA from heterochromatic regions is found almost exclusively in the unbound fractions. In contrast, DNA within or adjacent to coding regions is found distributed between the bound and unbound fractions, irrespective of the transcriptional status of the gene in question. We suggest that H4 acetylation levels are higher in coding regions of the genome and that turnover of acetate groups is relatively high, hence the distribution of coding DNA between antibody-bound and unbound fractions. Permanent silencing (as in Xi) is associated with reduced H4 acetylation.

ACTH CAUSES RAPID CHANGE IN HIGHER-ORDER CHROMATIN STRUCTURE OF MOUSE ADRENOCORTICAL CELLS.

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Nuclear organization is apparently necessary for transcription and replication. Both processes seem to take place in discrete foci organized throughout the nucleus, what may be a consequence of association between enzymatic complexes and a nucleoskeleton. Sequences that mediate the attachment of DNA to the nuclear scaffold usually cohabit with regulatory elements. It became interesting to investigate whether the higher-order chromatin structure, represented by the array of supercoiled DNA attached to the nuclear matrix, is alterated when mammalian cells are induced to modify their pattern of replication and transcription. For this purpose we chose Y-1 mouse adrenocortical cells, that display a classical steroidogenic response to ACTH (adrenocorticotropic hormone) and in addition, respond to this hormone with a temporary late G1 block and induction of early-immediate genes (c-fos, fosB, c-jun, junB, junD and NGFI-B), as well as genes encoding steroidogenic enzymes. The effects of ACTH and drugs that mimic its intracellular messengers, dcAMP (dibutyryl cyclic AMP) and PMA (phorbol-13-myrystate-12-acetate) on the higher-order chromatin structure of Y-1 mouse adrenocortical cells were analyzed using the nucleoid sedimentation technique. ACTH treatment causes a rapid (40 min) increase (65%) in the nucleoids' sedimentation rate. This ACTH response is specific for adrenal cells. dcAMP mimics this effect of ACTH but to a lower extent. PMA initially decreases nucleoids' migration during the first 3 hrs of treatment and increases the migration rate thereafter; by 17 hrs the rate is equivalent to that obtained with ACTH treatment. These results suggest that reorganization of the nuclear structure may be relevant to the mechanism of action of ACTH. They also describe a useful system for study of the relationship between nuclear structure and DNA functions, since the alteration of nuclear structure is rapid and both conformations can be compared within a single cell line.

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SIR3 AND SIR4 LOCALIZE TO THE NUCLEAR PERIPHERY AND INFLUENCE BOTH TELOMERE LENGTH AND CHROMOSOME STABILITY

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The Silent Information Regulator genes of *S. cerevisiae*, SIR3 and SIR4, are required for transcriptional repression at the silent mating type loci and at yeast telomeres. Using affinity purified antibodies recognizing SIR3 and SIR4 (L. Pillus, Colorado) we have shown that the SIR3 and SIR4 gene products are localized in aggregates positioned at the nuclear periphery, much like those observed with antibodies recognizing the telomere-binding protein RAP1. In both sir3 and sir4 mutant strains, telomeres lose their peripheral localization, as monitored by RAP1 immunofluorescence. This suggests a direct interaction of SIR3 and SIR4 with telomeric structures.

We have examined the effects of sir3 and sir4 mutations of the length of the $C_{1\text{-}3}A$ telomeric repeats and on chromosome stability. The $C_{1\text{-}3}A$ repeat of yeast chromosomes, but not of an artificial minichromosome, is approximately 120bp shorter in sir4 mutant strains, and about 50bp shorter in sir3 mutant strains. The effect is cumulative in the sir3sir4 double mutant. The mitotic stability of chromosome V is also reduced in sir4 mutant strains, while that of a minichromosome is not, suggesting that sequence elements other than the $C_{1\text{-}3}A$ repeats are influenced by the loss of SIR4.

Our data implicate the SIR3 and SIR4 gene products in maintenance of the perinuclear localization of telomeres in yeast and suggest that this localization is important for the telomere-associated position effect on transcription. Consistent with this interpretation, we find that mutations that do not disrupt telomeric silencing, such as rif1 and rap1^{S}, do not influence the subnuclear positioning of telomeric complexes, while the rap1-17 mutation, which interferes with telomeric silencing, does. Our results suggest a model in which association with the nuclear periphery mediates chromatin-dependent gene repression and influences chromosome stability. This is consistent with the high degree of homology between SIR4 and the nuclear lamins, which are implicated in maintenance of heterochromatin at the nuclear periphery of higher eukaryotic cells.

REVERSIBLE INACTIVATION OF A FOREIGN GENE DURING THE ASEXUAL CYCLE OF *N.crassa* TRANSFORMANTS.

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This study was initiated in order to understand better the genetic transformation of the coenocytic fungus *N. crassa*. A plasmid construct with the hygromycin phosphotransferase (*hph*) gene fused to the expression elements of the *trpC* gene of *A. nidulans* was used to obtain hygromycin B (Hyg) resistant transformants of *N. crassa*. We demonstrate that most of the transformants arise from integration of the transforming DNA in only one of the nuclei present in the protoplasts. The *hph* genes are heavily methylated in the transformants harboring multiple copies of the plasmid DNA. In the genome of *N. crassa* only 0.4% of cytosine residues are methylated (Russell *et al.* 1987. *J. Bacteriol.* **169:** 2902-2905) whereas the *hph* transgenes have about 80%. Interestingly, about 40% of such transformants also show reversible inactivation of the *hph* genes. In contrast, the transformants containing only a single insertion of the transforming plasmid have stable Hyg resistance and do not contain detectable methylcytosine residues in the *hph* genes. The integrated transforming DNA is physically stable in the absence of Hyg selection pressure in spite of being present in multiple copies (Pandit and Russo. 1992. *Mol. Gen. Genet.* **234:** 412-422).

The strong correlation between methylation of the cytosine residues and the presence of multiple copies of the plasmid DNA suggests a possible role of the presence of DNA sequence homology in the mechanism of the *de novo* methylation of the *hph* genes during the asexual cycle of *N. crassa*. We have therefore constructed strains harboring two copies of the *hph* gene located either on the same chromosome or on different chromosomes, from the transformants known to harbor a single insert. However, neither methylation nor reversible inactivation of the *hph* genes is observed in these strains. One possibility is that the multiple copies need to be located very close to each other, as seems to be the case in many transformants.

The phenotypic expression of the inactive *hph* genes can be restored by growing the transformants either with Hyg selection pressure or in the presence of 5-azacytidine. In the first case the *hph* genes are inactivated again in the absence of Hyg selection pressure while the activation of the *hph* gene by 5-azacytidine gives stable Hyg^r strains. The derivatives obtained in the presence of 5-azacytidine show extensive DNA rearrangements.Our data suggest that the presence of methylcytosine residues primarily blocks recombination within the transgenes. We propose a model suggesting that the formation of heterochromatin like structure leads to the reversible inactivation of the *hph* genes and that the presence of the homologous DNA sequences and the methylated cytosine residues is necessary but not sufficient for this phenomenon.

THE ASSOCIATION OF TELOMERES WITH CHROMOSOME STICKINESS IN IRRADIATED ATAXIA TELANGIECTASIA CELLS

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Cells derived from individuals with ataxia telangiectasia (AT) exhibit increased baseline frequencies of chromosome aberrations, often involving telomere-telomere associations. In addition, AT cells are highly sensitive to ionizing radiation, as manifested by decreased cell survival and increased frequencies of chromosome aberrations at mitosis. Using the technique of premature chromosome condensation to visualize chromosome events in interphase cells, we have previously shown that AT cells show increased levels of initial chromosome breaks and a partial defect in the repair of chromosome aberrations (Pandita and Hittelman, Rad. Res. 130: 94-103, 1992). One unique component of the chromosome aberrations visualized immediately after irradiation was a form of chromosome stickiness whereby aberrations appeared to physically occur due to an inability of chromatin regions to dissociate from each other during chromosome condensation. Interestingly, telomere regions appeared to be frequently involved in such interactions. To determine whether telomere regions are preferentially involved in sticky chromosome associations after irradiation in AT cells, telomere repeat sequence probes were utilized to visualize chromosome telomeres using the fluorescence in situ hybridization technique. Metaphases from three AT lymphoblastoid cell lines exhibited 20-30% fewer telomere signals as compared to metaphases from two normal control lymphoblastoid cell lines, and no interstitial signals were visualized. When AT cells were irradiated with gamma rays, allowed to accumulate in mitosis , and probed with telomere repeat sequences, telomeres were demonstrated to be frequently associated with regions of chromosome stickiness. In some cases, telomeres appeared to be associated with interstitial chromosome regions, and the chromosome associations were so strong as to induce chromatid breaks. Interestingly, the chromosome lesions observed in irradiated AT cells resembled those in normal cells treated with topoisomerase inhibitors, suggesting that the lesions might result from a defect in the nuclear envelope breakdown and chromatin condensation processes at mitosis. Studies are ongoing to address this hypothesis. Supported by NIH grants CA 6294 and CA 27931.

A DYNAMIC STUDY OF SPONTANEOUS DNA BRANCH MIGRATION.

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DNA branch migration, a process whereby two homologous DNA duplexes exchange strands, is an essential component of genetic recombination. In the simplest model, the elementary step of branch migration is movement of the Holliday junction one base pair in either direction with equal probabilities. According to this model, spontaneous DNA branch migration proceeds as a random walk. We are studying the dynamics of spontaneous branch migration using homologous DNA duplexes of varying length containing complementary single stranded tails. Annealing of these duplex substrates gives rise to a mobile Holliday junction. Migration of the Holliday junction resulting in complete strand exchange yields heteroduplex products. We have shown that a single base mismatch is sufficient to block the strand exchange reaction. Following the time course of the strand exchange reaction, we have obtained an estimate of the step time for branch migration as a function of temperature and ionic conditions.

MECHANISMS OF STABLE GENE REPRESSION ACTING AT THE HIGHER ORDER CHROMATIN LEVEL

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To ensure a correct transition between early determinative events and processes of differentiation, developmental decisions need to be maintained over many cell generations. Very little is known about the molecular basis of such mechanisms of 'cellular memory'. We are analyzing the Polycomb-Group (Pc-G), which in concert with the trithorax-Group (trx-G), is responsible for maintaining the spatially restricted expression patterns of developmental regulators (i.e. homeotic genes) after the early patterning system ceases to act. The Pc-G is involved in securing a stably repressed state of regulatory genes, whose ectopic expression would result in aberrant body patterns. Several pieces of evidence suggest that the Pc-G acts at the higher order chromatin level, by compacting inactive genes in a "heterochromatic" fashion. We found that the Polycomb (Pc) protein is associated with at least three other members of the Pc-G in a multimeric protein complex. Using immunofluorescence we could demonstrate that the four proteins have overlapping binding sites on larval polytene chromosomes. We made structure-function analysis of the Pc protein. Mutations in the evolutionarily conserved chromo domain prevent the protein from binding to its target genes and seem also to disrupt the Pc-G multimeric complex. Interestingly, we found that a particular modification of the Pc protein is involved in complex formation. This modification becomes apparent in late embryogenesis at a time when the Pc-G is starting to act. In order to study the effect of the Pc-G on the chromatin structure we have generated constructs containing a GAL-4 activatable LacZ reporter gene flanked by Pc-G regulated sequences (i.e. presumptive border elements of the BX-C). In transgenic fly lines the Pc-G regulated sequences can either completely suppress the activation of GAL-4 or give rise to a variegated tissue pattern. A similar effect is seen on the eye-color white gene used as a marker on the constructs. The effects we observe with the Pc-G elements are reminiscent of the phenomena observed in position effect variegation where euchromatic genes are affected by heterochromatin. We are currently analyzing the *in vivo* accessibility of DNA for methyltransferases to directly test the compaction state of the Pc-G repressed chromatin. Our results give additional support to the idea that the stable maintenance of gene repression is achieved through a regulation at the higher order chromatin structure.

DNA WRAPPING AND NUCLEOPROTEIN COMPLEX ASSEMBLY BY THE CHROMATIN-ASSOCIATED HIGH MOBILITY GROUP (HMG) PROTEINS 1 AND 2

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The mammalian high mobility group proteins HMG1 and HMG2 are abundant, chromatin-associated proteins that have been implicated in numerous activities including transcription, replication, and nucleosome assembly, but whose exact cellular function is not known. We have found that these proteins can substitute for the prokaryotic DNA bending protein HU in facilitating the assembly of the Hin invertasome, an intermediate structure in the Hin-mediated site-specific DNA inversion reaction. Accessory proteins like HU and HMG's are necessary when the length of the DNA between the recombinational enhancer and one of the Hin protein binding sites is less than ~100 bp. HMG1 and 2 stimulate the inversion reaction with higher specific activity than HU and promote assembly of the invertasome complex on substrates containing just 51 bp between these cis-acting sites. Using a ligase-mediated circularization assay we demonstrate that HMG1 and 2 can bend DNA extremely efficiently, forming circles of 59-99 bp. The helical repeat of the substrate DNA, calculated by Fourier analysis of the circularization data, was similar to estimations of the helical repeat of linear DNA in vitro. Analysis of different domains of HMG1 generated by partial proteolytic digestion indicated that DNA binding domain B is sufficient for both bending and invertasome assembly. We suggest that an important biological function of HMG1 and 2 is to facilitate cooperative interactions between cis-acting proteins by promoting DNA flexibility. A general architectural role for HMG1 and 2 in chromatin structure is also suggested by their ability to wrap DNA duplexes into highly compact forms.

A PROTEIN THAT BINDS G-STRAND TELOMERE DNA FROM CHLAMYDOMONAS REINHARDTII.

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Telomeres are the DNA/protein complexes at the ends of linear chromosomes that function to ensure chromosome integrity. We have identified a polypeptide in Chlamydomonas cell extracts that specifically binds to single stranded (ss) DNA that has the sequence $(TTTTAGGG)_n$ (Chlamydomonas G-strand telomere sequence), and can also bind to a ss 3' overhang structure thought to be present at the 3'-ends of Chlamydomonas telomeres. We have isolated a Chlamydomonas cDNA clone that encodes a fusion protein that has this ss G-strand binding activity and a predicted molecular weight of 26 kDa. Antibodies raised against a peptide from the predicted amino acid sequence recognize the native Chlamydomonas ss-G-strand binding protein. The predicted amino acid sequence of this G-strand Binding Protein (GBP) includes two RNA recognition motif domains.

CHROMATIN STRUCTURE AND THE COMPUTATIONAL IDENTIFICATION OF TRANSCRIPTIONAL REGULATORY DOMAINS

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We are developing computational methods for the detection of informational signals in DNA sequence, that may reflect previously unrecognized functional domains within chromatin. These domains are likely to contain both sequence-specific protein factor binding sites and diffuse DNA conformational information. Furthermore, all domains of a particular type (eg. promoters) need not necessarily contain the same type of informative signals. The successful application of computational methods to this type of DNA sequence analysis, requires consideration of a diverse range of biological factors and chromatin context.

This approach was used to identify a purine/pyrimidine motif whose distribution, using a 200bp sliding window, correlates with the location of transcriptional regulatory domains. Both distal enhancer domains and clusters of this motif were found to be nonrandomly distributed in relation to promoter regions, consistent with a periodicity of ~1.2KB. This would align the major clusters of this motif and >90% of distal transcriptional regulatory domains vertically along the same face of a 30nm chromatin fiber. The motif is unlikely to be a transcription factor binding site and may reflect a conformational signal. This computationally derived information suggests novel mechanisms involved in the function of these domains *in vivo*, not readily detectable using current experimental model systems.

We are using several techniques to computationally determine the precise periodic distribution of this motif around transcriptional regulatory domains. Applications for signal decomposition and frequency analysis techniques to primary sequence composition data will also be presented.

EVENTS LEADING TO DE NOVO TELOMERE SYNTHESIS IN EUPLOTES ARE MARKED BY CHANGES IN TELOMERASE RNA, TELOMERE PROTEIN, AND TELOMERE PROTEIN TRANSCRIPT ABUNDANCE.

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Literally millions of telomeres are synthesized *de novo* during macronuclear development in the ciliate *Euplotes crassus*. To learn more about the role played by the *Euplotes* telomere protein, the telomere protein homolog, and telomerase during telomere synthesis, we have correlated changes in the corresponding transcript abundance with specific developmental events. We have shown that rapid increases and decreases in the abundance of individual transcripts occur during specific stages of *Euplotes* macronuclear development. This is the first clear demonstration that transcript levels are regulated in hypotrichous ciliates. Our findings suggest that in *Euplotes*, as in other ciliates, transcript abundance regulates the synthesis of proteins needed during development.

Both the telomere protein and the telomere protein homolog transcripts increased in abundance at unexpected times during macronuclear development suggesting that these proteins are involved in previously uncharacterized steps in telomere synthesis. The telomere protein transcript peaked twice during conjugation. As both peaks paralleled micronuclear division, it appears that the protein may have a micronuclear function. Perhaps it binds micronuclear as well as macronuclear telomeres. The telomere protein transcripts also peaked during telomere addition suggesting that the protein binds to the newly synthesized oversized telomeres. Telomerase RNA levels increased ~ 12 fold during development. The increase started surprisingly early and peak levels were reached just after telomere addition. The telomere protein homolog transcripts showed a single peak at 80-90 hours of development. This is the time when the oversized telomeres are trimmed to the mature size and the anlagen DNA is replicated. Thus, the homolog may play a role in either trimming or replication of the newly synthesized telomeres.

CLONING, MAPPING AND SEQUENCING OF SUBTELOMERIC REGIONS OF SACCHAROMYCES SPECIES: VARIATION AND MOSAICISM OF REPEATS.

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The subtelomeric regions of yeast, as well as many organisms, are composed of several different repeated elements which vary between chromosome ends and strains. This structure can make conventional mapping and cloning difficult. Using gene transplacement to mark individual copies of a repeat for mapping and subsequent cloning of adjacent sequences alleviates the difficulties. The use of marked Y's (the most distal subtelomeric repeat) has allowed for the cloning of several new subtelomeric repeats from the ends of chromosomes IX, X, XIII and XV. Analysis of these clones provides interesting details about the structure and evolution of telomere regions.

The end of XV that was cloned has non-telomere, non-Y' homology with one end each of XIII and XVI in all strains of S. cerevisiae for as much as 20 kb. This element contains an enolase like sequence (70% DNA homology with Enolase A but with frameshifts and other disruptions). This repeat is specific to S. cerevisiae as it is not found in S. paradoxus or S. bayanus strains. The left ends of IX and X (identical in S288C) have at least three different novel repeats that vary in location between strains. Two of these are specific to S. cerevisiae while the third is found in all strains of S. paradoxus as well. The clone from the end of XIII has sequence homology in two constant locations in all strains of S. cerevisiae and S. paradoxus. It is also found in other variable locations.

These structures indicate a dynamic evolutionary nature for subtelomeric regions.

MOSAIC EXPRESSION OF A CYTOKERATIN GENE IN TRANSGENIC MICE. A. Ramirez[1], A. Bravo[2], J. Jorcano[1], and M. Vidal[3]; [1]CIEMAT, Madrid; [2]Universidad de leon, Leon; [3]Centro de Investigaciones Biologicas, Madrid (Spain).

Keratins are a family of epithelial-specific intermediate filaments. They have been divided into subfamilies of type I (acidic) and type II (basic). Filament formation requires one polypeptide of each group. The different epithelia are characterized by a defined pair of keratins. In stratified epithelia, the proliferating basal cell layer expresses the K5/K14 pair, whereas the terminally differentiating cells of the suprabasal layer express other specific pairs depending on each type of epithelium. To study the cis-control elements of basal layer-specific keratins we are using the bovine cytokeratin III gene, which is the orthologous bovine to the human and mouse keratin 5 gene. We have introduced into mice constructions in which genomic fragments of the keratin III gene have been hooked up to the *E. coli lac Z* gene. We have found that 5.5 kb of 5' flanking and promoter sequences of the cytokeratin III gene drive expression to the basal layer of stratified epithelia such as those of skin, hair follicles, tongue, oral cavity, esophagus, stomach, etc. β-galactosidase activity was seen also in the suprabasal layer of some epithelia. The expression of the transgene was, however, mosaic in every 4-8 week old animal of the three expressing lines obtained. The expression of the transgene in embryos showed the correct developmental pattern but, surprisingly, differed from that of adult animals in that it was no mosaic (or very little). It is not clear whether this mosaic expression is related or not to the chromatin packaging events involved in silencing chromosomal domains such as those associated with *Drosophila's* possition effect variegation. What seems unusual in these transgenic mice is that the onset of inactivation takes place very late in development, since newborn animals also have a non-mosaic expression. Moreover, the fact that every expressing line showed a mosaic expression suggests that it is the transgene itself rather than the integration site what is responsible for such expression pattern.

FULL CHI ACTIVITY REQUIRES THE PRESENCE OF A 3' SINGLE-STRAND-SPECIFIC EXONUCLEASE

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Chi sites are hotspots for homologous recombination in *E. coli.* Chi enhances recombination *via* direct contact with RecBCD recombinase. We proposed a model in which RecBCD unwinds the DNA at Chi, creating a recombinagenic split-end of DNA there[1].

To test for the presence of single-strand DNA ends at Chi, we are examining whether DNA in this region is sensitive to single-strand-specific exonucleases expressed *in vivo.* We report that a partial function mutation, *sbcB15*, in the gene encoding a 3' single-strand-dependent exonuclease, Exo I, reduces the ability of Chi to promote recombination. This effect is not due to swamping-out of Chi-promoted events by another recombination pathway, first, because the overall levels of recombination are not increased in the *sbcB* strains. Second, although *sbcB* mutations can help activate a *recJ*-dependent recombination system in *E. coli*, called the RecF pathway, the decrease in Chi activity seen in *sbcB* cells is *recJ*-independent. Because the mutation debilitating Exo I 3' single-strand-dependent exonuclease appears to remove recombination events only at Chi, we infer that the substrate for this enzyme is present at Chi. This is evidence that a 3' single-strand DNA end is formed at the Chi site *in vivo.*

[1]Rosenberg, SM and PJ Hastings 1991 The split-end model for homologous recombination at double-strand breaks and at Chi. *Biochimie* 73, 385-399.

Isolation of cDNAs coding for cellular factors involved in chromosomal DNA replication with the two hybrid system

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Chromosomal DNA replication is a tightly regulated process which involves a highly elaborated network of protein-DNA and protein-protein interactions. DNA polymerase α/primase (Polα) has been shown to be the key enzyme responsible for initiation of DNA replication. To begin to identify the components of the multi-protein complex participating in the initiation reaction of DNA replication in mammalian cells, we adopted the expression cloning strategy by using the two hybrid system (Fields and Song (1989) Nature 340: 245-246). We fused the catalytic subunit (p180) of the human DNA polymerase α/primase complex to the DNA binding domain of the yeast GAL4 protein. Unexpectedly, this GAL4-p180 fusion protein activated transcription from a *GAL1* promoter *in vivo*. The domain responsible for transcriptional activation has been mapped to the amino-terminal part of the p180 molecule by fusing five overlapping portions of the p180 cDNA to the GAL4 DNA binding domain. We are currently analysing the function of this transactivating domain which is located in close vicinity to the domain binding to SV40 large T antigen (Dornreiter et al. (1993) Mol. Cell. Biol. 13: 809-820).
With the two hybrid system, we have isolated several cDNA clones encoding cellular proteins that bind to the catalytic subunit of Polα by screening a human cDNA library (kindly provided by Dr. S. Elledge). The sequence analysis of one of these cDNAs showed that it encodes the 70 kDa subunit (p70) of human Polα. This subunit had not beeen cloned before from mammalian cells. We are currently mapping the binding site of p70 on the p180 molecule and are analysing the biochemical function of this p70 subunit. Work is also in progress to establish a protein linkage map of the DNA polymerase α/primase complex with the two hybrid system by fusing the cDNAs encoding the four subunits of Polα to the DNA binding or transactivating domain of the GAL4 protein. In addition, we are analysing the identity of the other cDNA clones whose gene products interact with the p180 molecule. This work is supported by grants from the NIH to T.W. and a HFSP fellowship to L.R..

A NOVEL SEQUENCE FAMILY LOCATED ADJACENT TO MANY TELOMERES OF CHIMPANZEE CHROMOSOMES IS ABSENT FROM THE HUMAN GENOME.

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A number of distinct sequence families, found in subterminal regions and immediately adjacent to telomeres, have been isolated from the human genome by a variety of strategies. One of these sequence families is present at the ends of many chromosome arms and Southern analysis shows these sequences are also present in the chimpanzee and gorilla genomes. However, we have used an anchored PCR strategy to isolate sequences adjacent to chimpanzee telomeres and found that the sequence families isolated from the chimpanzee genome are novel and distinct from and those found adjacent to telomeres of human chromosomes. The major novel class of sequence adjacent to chimpanzee telomeres is composed of long arrays of a 32bp repeat unit which cross-hybridizes in Southern blot analysis, to the gorilla but is absent from the human and orang-utan genomes. *In situ* hybridization of a probe composed of the 32bp repeat unit to chimpanzee chromosomes shows that it hybridizes predominantly to subterminal regions of many but not to all chromosome arms and it constitutes a subterminal satellite sequence. The rapid turnover of subterminal sequences in primate genomes will be discussed.

THE GENETICALLY UNIQUE PSEUDOAUTOSOMAL REGION ON Xp/Yp INCLUDES AN ADP/ATP TRANSLOCASE GENE WHICH ESCAPES X-INACTIVATION, WHEREAS A HOMOLOGUE ON Xq IS SUBJECT TO X-INACTIVATION.

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Pairing and recombination of the sex chromosomes in man takes place between two small regions of sequence identity: the pseudoautosomal regions. These regions are located at both distal ends of the X and Y chromosomes, adjacent to the telomere. Genes in the pseudoautosomal region on the short arm escape X-inactivation.

In male meiosis, an obligatory chiasma is restricted to these unique regions of 2.6 and 0.4 Mbp extension, leading to the tenfold differences in sex-specific recombination rates. Markers adjacent to the Xp/Yp telomere exhibit 50% recombination in males, which is probably the most highly recombinogenic in the human genome.

In the region with a 27 % recombination frequency between the X and Y chromosome, a gene member from the ADP/ATP translocase family, active on the X and Y chromosome, was isolated and characterised. This pseudoautosomal gene, ANT3, plays a fundamental role in the energy metabolism of the eukaryotic cell, catalysing the exchange of ATP and ADP across the mitochondrial membrane.

The position of a highly conserved gene found in yeast, plants and mammals within the pseudoautosomal region is interesting for a number of reasons. First, the existence of an ANT gene on the short and on the long arm of the X chromosome may shed light on the evolution of this multigene family and on the differentiation and evolution of the sex chromosomes. Secondly, ANT3 escapes X inactivation, whereas its isoform ANT2 on the long arm of the X chromosome is subject to X-inactivation. Thirdly, the corresponding genes, ANT3 and ANT2, provide first evidence of two closely related X-chromosomal genes, which show striking differences in their X-inactivation behavior, one being inactivated and the other not. We are currently using these two corresponding genes as a system for examining mechanisms of X-inactivation by comparing their regulatory regions.

GENOMIC DISTRIBUTION OF UNUSUAL DNA STRUCTURES

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During the last decade we have learned a great deal about the structural polymorphism of DNA. Numerous studies have shown that sequence-specific non-B-DNA conformations exist, often times in response to environmental conditions, especially negative supercoiling. However, in spite of the wealth of biophysical data available for these structures, we have as yet been unable to demonstrate a well defined biological function for any of the non-B-DNA structures. In an effort to gain some insight into the possible biological significance of unusual DNA structures, we have been using computer assisted methods to study the occurrence and distribution of potential Z-DNA and cruciform forming sequences in genomic DNA from both humans and *E. coli*. Previously we have shown that potential Z-DNA forming sequences have a distinctly non-random distribution across human genes and, most notably, that such sequences are more commonly found near the 5′ ends of genes [Schroth, G.P., Chou, P.-J., and Ho, P.S., *J. Biol. Chem.* 267, 11846-11855 (1992)]. The twin-domain model of transcription-induced supercoiling suggests that DNA near the 5′ ends of a gene would be in a dynamic negatively supercoiled state during the transcription of the gene, which could potentially drive the formation of Z-DNA in many of the sequences which we have mapped. The concentration of Z-DNA forming sequences in regions of genes which are most likely to be negatively supercoiled, suggested to us that these sequences might have a function in the regulation of gene expression in eukaryotes.

We have now studied the distribution of potential Z-DNA forming sequences in more than 200 genes from *E. coli* (over 250,000 total bp of DNA). In marked contrast with the human gene results, potential Z-DNA forming sequences are randomly distributed across *E. coli* gene sequences. Indeed, upon re-analysis of our previous data on human genes, we realized that the distribution of Z-DNA forming sequences found in the exons of human genes was also random. Therefore in the protein-coding regions of both human genes (exons) and *E. coli* genes (the entire transcribed region), the distribution of potential Z-DNA forming sequences is random. The differences between the distribution of potential Z-DNA forming sequences in human and *E. coli* genes is probably due to fundamental differences in the structure, organization and regulation of eukaryotic versus prokaryotic genes. Furthermore, the random nature of the occurrence of Z-DNA forming sequences in *E. coli* suggests that Z-DNA may have no general role in the regulation of gene expression in prokaryotes. We will also be presenting results of our analysis of both the human and *E. coli* gene data sets for the occurrence and distribution of strong cruciform-forming sequences using a search algorithm which is currently in development.

MOLECULAR LOCALIZATION OF CHROMOSOMAL TRANSLOCATION BREAKPOINTS INVOLVING HUMAN CHROMOSOME 11q23-q24 BY HIGH RESOLUTION FLUORESCENT *IN SITU* HYBRIDIZATION (FISH).

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FISH allows specific visualization of whole genomes, entire chromosomes, chromosomal regions and unique sequences in metaphase and in interphase cells. The chromosomal location of specific genes can be determined and chromosomal abnormalities can be highlighted.

To isolate translocation breakpoints of chromosome 11q, a physical landmark map was constructed using chromosome 11-specific cosmids from somatic cell hybrids or flow sorted chromosomes and mapping them on 11q by FISH. The t(4;11) (q21;q23) breakpoint associated with Acute Lymphocytic Leukemias (ALLs) was found to be located between the CD3 and Thy-1 genes on 11q23. One YAC, B22B2L, was isolated by us and other laboratories and shown to contain the translocation breakpoint and to hybridize to both derivative chromosomes in cell lines and leukemic patients carrying the t(4;11) translocation. A contig map spanning more than 360 kb on 11q23 was constructed by subcloning YAC B22B2L in cosmids. By FISH the breakpoint was localized to the region covered by cosmids c108 and c116 which showed hybridization signal on both derivative chromosomes 4 and 11, while cosmids c62 and c4 were localized centromeric and telomeric, respectively, to the 11q23 breakpoint. After identification of the cosmids spanning the t(4;11) breakpoint by FISH, the gene spanning the translocation breakpoint on 11q23 was identified as a Trithorax-like gene (M. Djabali, L. Selleri et al., Nature Genetics 2:113, 1992).

By using FISH to metaphase chromosomes and interphase nuclei, localization of the Ewing's Sarcoma (ES) translocation breakpoint on 11q24 was determined within a 1.5 Mb chromosomal interval, between two closely spaced cosmid markers, which bracket the breakpoint on 11q24 (L.Selleri, G Hermanson et al., P.N.A.S. 88:887, 1991). This enabled detection of the t(11;22) (q24;q12) in metaphase chromosomes and interphase nuclei of cell lines and of fresh tumors (M.Giovannini, L. Selleri et al., J. Clin. Invest. 90:1911, 1992) and proved that interphase cytogenetics is a rapid alternative to chromosomal analysis for the detection of the t(11;22) translocation in small round cell tumors. We have now isolated, characterized and mapped a 250 kb colinear YAC which contains the entire Fli-1 gene, which is disrupted by the ES translocation breakpoint on 11q24 (O. Delattre, J. Zucman et al., Nature 359:162-165, 1992). This YAC spans the ES breakpoint on 11q24, as shown by FISH to metaphase chromosomes and interphase nuclei of ES cell lines and patients samples.

DISTAL AND PROXIMAL CONTROL REGIONS FOR MAMMALIAN α- AND β-GLOBIN GENES

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A detailed study of the evolution of the developmentally regulated α-like and β-like globin gene clusters provides a unique guide to their control mechanisms. Regulation of these gene clusters involves both distal locus control regions (LCR) and proximal control sequences. The β LCR extends over 14 kb at the 5' end of the gene cluster, has four DNase-hypersensitive sites (HSs), and is required for efficient expression of β-like globin genes in a position-independent, copy-number-dependent manner. Comparisons among different mammals shows that spacing between the HSs is particularly well-conserved. Furthermore, the orthologous sequences show strong interspecific matches far beyond the known protein-binding sites. This suggests that a very long block of conserved DNA may be required for the full range of regulation. We have used a novel hybrid ε-globin-luciferase reporter gene to investigate some regulatory properties of the LCR segments. Both HS2 and HS3 are capable of enhancing the transient expression of the ε-globin-luciferase gene in unintegrated constructs, but a much greater increase in expression is seen after integration into a chromosome, presumably reflecting insulation from position effects. HS4 will increase expression only after integration. Further studies are aimed at discriminating between sequences required for enhancement versus those required for insulation or domain opening. The distal major control region (MCR or HS -40) from the human α-globin gene cluster is being evaluated in similar experiments, using a hybrid α-globin-luciferase reporter gene construct. Both 5' flanking and internal sequences of the rabbit α-globin gene are needed for full expression; this segment includes binding sites for YY1 and Sp1. In contrast to experiments in which only 5' flanking regions of the human α-globin gene are used to drive a reporter gene, constructs including both 5' and internal sequences are readily expressed in transient assays, and no effect of the MCR is seen. We propose that the 5' flank and internal sequences of the α-globin gene comprise an extended promoter that does not require an enhancer for transient expression from unintegrated constructs.

THE LOOP SEQUENCE PLAYS CRUCIAL ROLES FOR ISOMERIZATION OF INTRAMOLECULAR DNA TRIPLEXES IN SUPERCOILED PLASMIDS

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An intramolecular DNA triplex, which consists of a triple-stranded stem and a single-stranded region is formed at a polypurine•polypyrimidine (Pur•Pyr) tract under torsional stress. Pur•Pyr sequences are overrepresented in eukaryotic genomes and often are proximal to regulatory regions and recombination hot-spots. Recently, the *in vivo* existence of triplexes, isolation of a triplex DNA binding protein from human cells, and their function in gene expression processes have been reported, suggesting potential biological functions of DNA triplexes.

Two isomers exist for an intramolecular Pyr•Pur•Pyr triplex: one with the 3'-half of Pyr strand as the third strand (H-y3), and the other with the 5'-half of Pyr strand as the third strand (H-y5). Interestingly, most of Pur•Pyr sequences analyzed to date form the H-y3 isomer. To clarify this preferential formation, we cloned alternating G and A repeats (total 32 bp) with various base composition in the central 4 bp into pUC19, and characterized the triplex formation by chemical probes (OsO_4 and diethyl pyrocarbonate (DEPC)) and 2-D agarose gel electrophoresis.

Two isomers can be clearly distinguished each other by DEPC modification pattern, since 5'-half and 3'-half of Pur strand are modified (i.e. single-stranded) in H-y3 and H-y5, respectively. It was shown that the Pur•Pyr sequences with high G+C content in the center dominantly form the H-y5 isomer, and that Mg^{2+} stabilizes the H-y5 conformation. The chemical probing also showed that the first nucleotide involved in Hoogsteen base-pairing from the loop into the triplex stem is strongly modified in both H-y3 and H-y5, indicating that the base triad is unstable.

The formation of H-y3 relaxes about one more superhelical turn than that of H-y5 does, as revealed by 2D-gel analyses. Thus, H-y3 and H-y5 conformations are topologically, thereby energetically not equivalent. This implies that the nucleation event in the triplex formation differs between H-y3 and H-y5. The nucleation of the H-y5 needs less unwinding of the duplex, and it can proceed by the bending of the Pyr strand towards major groove. In contrast, the formation of the H-y3 needs more unwinding of the primary helix and the Pyr strand folds back from the minor groove to the major groove. Furthermore, as the G+C content in the center increases, the triplex requires more supercoil energy for formation, indicating that the opening of the central region in Pur•Pyr tracts is the initial step in the pathway for the triplex formation.

In summary, the content and position of G+C residues in the loop are crucial determinants for the triplex formation as well as its isomerization. A model for the mechanism will be presented.

DYNAMICS OF DNA AND CHROMOSOMES IN THE PRESENCE OF TYPE II DNA TOPOISOMERASES

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Type II DNA topoisomerases catalyze the concerted breakage and rejoining of the two strands of double-stranded DNA molecules, enabling double-stranded DNA segments to pass through one another in an enzyme-mediated reaction (1). How do the catalytic properties of type II DNA topoisomerase affect the dynamical properties of linear DNA molecules? We have hypothesized that these enzymes can release the topological constraints which give rise to the reptation motion (2).

In a first part, we consider the aqueous solution of linear DNA as a viscoelastic system, and we determine transport coefficients. In presence of enzyme, the solution responds as a living polymeric system (3). A sizeable reduction in viscosity is predicted for DNA molcules with 10^6 or more base pairs (4). More precisely, the relation between the longest relaxation time and the number N of base pairs is changed from a reptation type ($\sim N^3$) to a Rouse type ($\sim N^2$) behavior.

In a second part we examine the effect of the enzyme on chromosome condensation which is known to require the presence of type II DNA topoisomerase both *in vivo* and *in vitro* (5). Using a model describing the collapse of a homopolymer (6), we estimate the time required for chromosome condensation in the absence of enzyme: it is a reptation time which can overestimate the actual time by several orders of magnitude. This explains the requirement for the catalytic properties of the enzyme. We propose a phantom chain model of chromosome condensation which allows to calculate the amount of enzyme required for this process, and discuss its implications for the architecture of eukaryotic chromosomes.

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A YEAST MUTATION THAT CAUSES OVER-REPLICATION AND AMPLIFICATION OF A PORTION OF THE GENOME

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We are investigating several mutations of the yeast *S. cerevisiae* that cause rapid cell death only if the cells have a competent DNA Polymerase α enzyme. We have found that one conditionally lethal mutation of this type, called *dos1*, causes cells to accumulate excess genomic DNA when they are placed at a restrictive temperature. The excess DNA is partly due to the amplification of specific genomic fragments. Mutations of this sort should provide clues about how cells confine DNA replication to precisely one round per cell cycle.

We have isolated "polymerase sensitive" mutants reasoning that a mutation that causes a cell to replicate its genome at an inappropriate time should not cause unmanageable levels of damage if it is paired with a second mutation that blocks the cell's ability to perform DNA synthesis. The mutations that we have found are rescued from making a lethal error during a brief incubation at the restrictive temperature by the presence of a temperature sensitive mutation in the *POL1* gene that encodes DNA Polymerase α.

Most of the excess DNA in *dos1* cells appears to be composed of scattered genomic sequences, but a portion of this over-replication is due to an approximately 100-fold amplification of a specific region of chromosome VII. The accumulation of excess DNA occurs in the absence of cell division. Over-replication does not continue indefinitely, but rather appears to be limited to about 50% of the cell's DNA content. As expected from our original screen, *dos1* cells are rescued from lethality by the presence of a conditional mutation in *POL1*. To our surprise, they are sensitive to increasing levels of the wild type *POL1* gene. Mutant *dos1* cells that also lack the S-phase checkpoint provided by the *MEC1* gene (T. Weinert, personal communication) die more rapidly, indicating that the error made in *dos1* cells is monitored by *MEC1*.

The excessive accumulation of DNA and the amplification of part of the genome in cells lacking fully functional *DOS1* indicate that this gene plays an important role in maintaining the stability of the yeast genome.

COORDINATION OF TRANSCRIPTION AND PRE-mRNA SPLICING WITHIN THE CELL NUCLEUS

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In this study we have examined how the processes of transcription and pre-mRNA splicing are spatially coordinated within the cell nucleus. In actively transcribing cells, splicing factors are localized to both perichromatin fibrils and interchromatin granule clusters (Fakan et al., 1984 J. Cell Biol. 98:358-363; Puvion et al. 1984 J. Ultrastruct. Res. 87:180-189; Spector et al. 1991 EMBO J. 10:3467-3481) while hnRNP C-proteins (Fakan et al., 1984 J. Cell Biol. 98:358-363) are localized to perichromatin fibrils. In the present study, we have localized RNA polymerase II to perichromatin fibrils supporting the concept that these fibrils represent nascent transcripts. Upon inhibition of RNA polymerase II function, the number of perichromatin fibrils appeared greatly reduced and splicing factors were shuttled to sites of interchromatin granules. When transcription was reinitiated, perichromatin fibrils reformed and splicing factors returned to these fibrils as well as being localized to interchromatin granule clusters. These data suggest that splicing factors are present at sites of active transcription (perichromatin fibrils) and at storage and/or assembly sites (interchromatin granule clusters). In order to further demonstrate that splicing factors are recruited to sites of active transcription, we introduced exogeneous DNA templates into cell nuclei and examined the response of the transcription and splicing factors to the introduction of these new transcription sites. We have found that upon initiation of transcription from either viral or non-viral transfected DNA templates, both transcription and splicing factors are shuttled from their normal distribution in the host cell to the new sites of active transcription. These findings provide "in vivo" evidence that the processes of transcription and pre-mRNA splicing are both spatially and temporally associated within the interphase nucleus. Therefore, the organization of splicing factors in a speckled distribution in actively transcribing cell nuclei is a reflection of transcription and pre-mRNA splicing activities. In addition, our findings suggest that there are signals generated in the nucleus that regulate the compartmentalization of factors to nuclear regions where they will be functioning.

A MOLECULAR ANALYSIS OF RECOMBINATION IN *ARABIDOPSIS THALIANA*.

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There is a large international collaboration to generate an overlapping YAC library covering the *Arabidopsis* genome. Chromosome 4 is being studied in detail by this group, and currently 35 YAC contigs cover approximately 80% of this chromosome.

A preliminary analysis of recombination within a 26cM region of chromosome 4 covered by YAC contigs, has been achieved. Genetic distance generated from analysing the segregation of 28 RFLP markers in 300 recombinant inbred lines has been compared to physical distance of the DNA contained in the YAC clones. The range of frequencies of recombination have been seen to vary 10 fold, and relative "hot" and "cold" spots of recombination have been identified.

In order to position these hotspots more accurately 1000 recombinants are being generated using flanking visible markers to aid selection. A high density of PCR based RFLP markers are being identified in the region in order to fine map the recombination sites.

Additionally, YAC clones in the location of putative hotspots are being studied in the double strand break repair deficient, rad50s yeast strain. The position of the double strand breaks on native yeast chromosomes has been shown by other workers to correspond to recombination hotspots. We are interested to see whether foreign DNA carried in YAC clones behaves in a similar way.

CHARACTERIZATION OF CHD-1: A MAMMALIAN DNA BINDING PROTEIN THAT CONTAINS A CHROMODOMAIN AND A SNF2/SWI2-LIKE HELICASE DOMAIN.

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Regional modifications of chromatin structure can selectively alter the accessibility of certain genes to the transcriptional apparatus, and in doing so, play a major role in the regulation of gene expression. At the present very little is known about the proteins that participate in this type of mechanism. We have recently discovered a mammalian protein with attributes that suggest its involvement in chromatin modification.

Two overlapping cDNAs that encode a 197 kD sequence-selective DNA binding protein were isolated from libraries derived from mouse lymphoid cell mRNA. In addition to a DNA-binding domain, the protein contains both a chromodomain, which occurs in proteins that are implicated in chromatin compaction, and an SNF2/SWI2-like helicase domain, which occurs in proteins that are believed to activate transcription by counteracting the repressive effects of chromatin structure. Because of this interesting combination of motifs, we have named this protein CHD-1 (for chromodomain-helicase-DNA).

Northern blot analyses have revealed multiple CHD-1 mRNA components which differ both qualitatively and quantitatively among various cell and tissue types. Furthermore, these analyses indicate that at least some of the different sized CHD-1 mRNAs are apparently the result of inclusion or exclusion of the region encoding the DNA-binding domain. The various mRNAs could conceivably encode tissue-specific and developmental stage-specific isoforms of the protein.

Studies aimed at better defining the region of the protein that exhibits DNA-binding activity and its specificity indicate that CHD-1 preferentially binds to AT-rich sequences. Both SouthWestern and mobility shift experiments with maltose binding fusion proteins containing various regions encompassing the carboxy-terminal portion of the protein have implicated a 428 amino acid segment as responsible for the DNA-binding activity. Close inspection of the protein sequence in this region revealed two small motifs (KRPKK and RGRPR) which have previously been shown to be important for the AT-rich minor groove DNA-binding activity of proteins such as HMG-I, D1 and Engrailed.

CHROMATIN STRUCTURAL MARKERS OF HUMAN X CHROMOSOME INACTIVATION

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The inactive X chromosome is maintained in a largely heterochromatic state that is transcriptionally inactive and late-replicating. We are interested in defining elements that may be responsible for the establishment and/or maintenance of active and inactive X chromatin. To this end, we have been examining both global and local chromatin structural properties of active and inactive human X chromosomes. Nuclear matrix binding properties and local chromatin conformation may play a role in the maintenance or establishment of a given activity state. In a number of systems, actively transcribed sequences have been observed to associate with the nuclear matrix while inactive sequences do not. Two matrix attachment regions (MARs) have been identified previously in the human HPRT gene. In hamster-human hybrid cell lines, we observe matrix binding at a MAR upstream of the HPRT transcriptional start site that appears to occur preferentially in the active, but not the inactive, state. A similar binding pattern appears at the ARS/MAR located in the first intron of the gene. Work is in progress to identify additional MARs in the HPRT region and determine their utilitzation in different X-inactivation states.

To examine closely the chromatin structure at the HPRT promoter and first intron ARS/MAR, DNase I footprinting in conjunction with ligation-mediated PCR is being employed in a modified genomic footprinting scheme to identify protected sequences and chromatin structural alterations at these sites. Chromatin poening has been predicted to initiate at MARs, and increased DNase sensitivity may result. Functional characterization of the MARs is being pursued through an assay by which the ability of the MAR to confer plasmid autonomy is tested in human erythroleukemia cells. Matrix binding properties at other loci also is of interest; in particular, the X inactivation centre (XIC) will be studied in light of data that show unusual transcriptional activity (solely from the inactive X) in this region.

DETERMINATION OF THE MECHANISM OF NUCLEOSOME DISRUPTION DURING ACTIVATION OF THE PHO5 PROMOTER.

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Previous work has indicated that PHO4 is primarily responsible for triggering a disruption of four nucleosomes in the *S. cerevisiae* PHO5 promoter. Binding of PHO4 to its sites in the PHO5 promoter can trigger disruption of not only a nucleosome bearing an internal PHO4 binding site, but also adjacent nucleosomes which lack internal PHO4 binding sites. PHO4 is a basic helix-loop-helix protein that contains an acidic domain which is required for transcriptional activation (Ogawa and Oshima, Mol. Cell. Biol. 10:2224). We are currently defining the role of this acidic domain in the disruption of nucleosomes *in vivo*. Deletion mutagenesis was used to create various derivatives of PHO4 which contain either a minimal DNA-binding domain or a deletion of the acidic domain itself. These are being employed to test which domains are required to disrupt nucleosomes with either internal or adjacent PHO4 binding sites. Finally, data will be presented regarding the participation of pleiotropic yeast activators in the PHO5 chromatin transition.

5-AZACYTIDINE TREATMENT OF THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE* LEADS TO CELL CYCLE ARREST

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Cytosine methylation has been implicated in a number of biological phenomena in higher eukaryotes and bacteria, but attempts to demonstrate its existence in lower eukaryotes such as *Drosophila* and yeast have proved unsuccessful. The recent isolation of the *cnd1*$^+$ gene from *S. pombe*, which shares considerable sequence homology with cytosine-specific methyltransferases from other organisms, has led us to reinvestigate the existence of cytosine methylation in fission yeast using the cytidine analogue 5-azacytidine. 5-Azacytidine is known to inhibit cytosine methylation due to the formation of stable covalent complexes between cytosine methyltransferases and 5-azacytidine-containing DNA. Here we demonstrate that 5-azacytidine treatment of *S. pombe* leads to cell cycle arrest in G2, and that this arrest is dependent on the cell cycle checkpoint mechanisms which act to prevent the onset of mitosis in the presence of damaged or unreplicated DNA. Furthermore 5-azacytidine treatment of the budding yeast *S. cerevisiae* also results in cell cycle arrest in G2, upon heterologous expression of a bacterial cytosine methyltransferase. These results suggest that cytosine methylation does occur in fission yeast. We propose that the effects of 5-azacytidine on *S. pombe* are mediated through the *cnd1*$^+$ gene product and our current efforts are directed toward establishing this link. Our observations demonstrate the 5-azacytidine, a widely used chemotherapeutic agent has no general cytotoxicity in either budding or fission yeast and suggest that the cytotoxic effects which 5-azacytidine exerts in higher eukaryotes are, at least in part, a result of 5-azacytidine induced DNA damage.

HEPADNAVIRAL COVALENTLY CLOSED CIRCULAR (ccc) DNA FORMS DIMERIC MOLECULES, PRESUMABLY BY BREAKAGE AND JOINING OF MONOMERS WITHIN THE NUCLEUS AND INDEPENDENT OF VIRAL DNA SYNTHESIS.

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In hepadnavirus infections, the parental virion DNA is converted into a ccc molecule. The viral ccc DNA is localized to the nucleus of the infected cell where it is believed to act as the sole template for hepadnavirus transcription. The ccc DNA is isolated from infected cells both as supercoiled (SC) and relaxed circular (RC) molecules. Furthermore, it is established that the bulk hepadnaviral ccc DNA does not replicate semi-conservatively by a DNA dependent DNA synthesis scheme. Rather, it is synthesized by a pathway which involves reverse transcription of a viral "pregenomic" RNA within cytoplasmic nucleocapsid particles. (J. Tuttleman, C. Pourcel and J. Summers, Cell 47:451, 1986.)

Ultracentrifugation analyses of ccc DNA isolated from the livers of geese infected with the duck hepatitis B virus indicate the presence of a small fraction (<1%) of both SC and RC dimeric ccc DNAs; both circular and catenated dimers are inferred to be present. It is unlikely that the viral ccc dimers arise by an aberrant DNA synthesis mechanism. It is much more probable that they are generated by "breakage and joining" of monomer ccc DNAs, and thus represent intermediates in viral chromosome repair or recombination.

DNA-PROTEIN INTERACTIONS IN THE REPLICATION ORIGIN REGION OF THE rRNA GENES IN *TETRAHYMENA*

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We are investigating the molecular mechanisms that regulate DNA replication in eukaryotes by studying the rRNA genes (rDNA) of *Tetrahymena thermophila.* In this organism, the rDNA is amplified to 10,000 copies in the form of 21 kb linear palindromic molecules. An origin of replication is located near the center of the palindrome within the 5' non-transcribed spacer (5'NTS) and DNA fragments containing this region promote autonomous replication of plasmids in the macronucleus. Specific cis-acting sequences that appear to modulate origin function have been identified by analysis of rDNA mutations induced *in vivo.* Several of these mutations lie in copies of an AT-rich conserved sequence element, the Type I repeat, present in four copies within the 5'NTS.

We have characterized two distinct DNA-binding proteins that interact with the Type I repeat using Fe(II)EDTA cleavage footprinting and gel-mobility shift assays. One of these, designated ds-TIBF, binds preferentially to duplex DNA whereas the other, A-TIBF, binds preferentially to single-stranded DNA. Binding competition studies revealed that ds-TIBF binds with high affinity but only moderate specificity to Type I sequences. It also forms complexes with other duplex oligonucleotides containing long stretches of dA/dT. In contrast, A-TIBF specifically recognizes single-stranded oligonucleotides corresponding to the Type I repeat sequence and does not bind to generally A-rich sequences. A-TIBF has greatly reduced affinity for altered versions of the Type I repeat in which sequences that form the 5' or 3' borders of the conserved element are deleted.

One copy of the Type I repeat is located 22 nt upstream from the rRNA transcription initiation site and has been shown to be an essential component of the rRNA gene promoter. Although this repeat differs slightly in sequence from the other three, it is bound by A-TIBF with an affinity equal to that of the Type I elements in the replication origin region. These findings suggest that A-TIBF could play a role in transcription as well as DNA replication. Current efforts are directed at developing *Tetrahymena* cell-free extracts in which the functional roles of the Type I repeats and proteins that interact with them can be investigated.

CHARACTERIZATION OF A HUMAN AUTOANTIGEN ASSOCIATED TO MITOTIC NUCLEOLUS ORGANIZER REGIONS (NORs)

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A human autoimmune serum from a rheumatoid arthritis patient has been found to stain nucleolus organizing regions of metaphase chromosomes from a variety of mammalian cells. In interphase the staining was restricted to some area of the nucleolus, presumably to the fibrillar centers and the dense fibrillar material. This rare serum reacts with a 90-92 kDa polypeptides in all the cell lines tested. Actinomycin D treatments demonstrated changes in the distribution of these autoantigens after inhibition of transcription. To analyze these components of NORs, several cDNA clones were selected from a CHO cell lambda-ZAP library by using the human autoimmune serum. Sequencing analysis indicated that we have selected cDNAs for the ubiquitous ribosomal transcription factor UBF. Hamster UBF shows a very high homology to others UBF DNAs already described in mammalian (human, rat, mouse) and non mammalian (xenopus) species. A chromatography steps procedure is presented to purify this NOR component from mouse liver extracts.

CENTROMERIC DODECASATELLITE DNA SEQUENCES FORM NON-WATSON-CRICK FOLD-BACK STRUCTURES

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Although it is now well documented that highly repetitive satellite DNA sequences are located primarily in the centromeric heterochromatin, all the data available concerning the relationship between satellite DNAs and centromere structure and/or function are largely circumstantial. Recently we have been able to isolate a G+C rich satellite, the *dodecasatellite*, from the centromeric region of *Drosophila melanogaster* chromosomes that has revealed the existence of similar sequences in different species through evolution. This result suggest that G+C rich satellites could have specific structural characteristics involved in centromeric functions. To explore this we have been investigating the possible structures of *dodecasatellite* DNA as a first step in addresing the relationship of structure to function. *Dodecasatellite* has a repeat unit length of 11-12 bp and its consensus sequences are ACCAGTACGGG-ACCGAGTACGGG. The repeats have an asymetric distribution of guanine and cytosine resulting in one strand being relatively G-rich in comparison with the other. Guanine-rich sequences occur in telomeric repeats and have the ability to form, under appropiate *in vitro* conditions, a variety of novel DNA structures. The versatility of such guanine-rich single strands appears to depend on the exact sequence. Using non-denaturing gel electrophoresis, chemical modification and thermal denaturation analysis we have been able to show that *dodecasatellite* G-strand oligonucleotides can associate to form stable non-Watson-Crick fold-back structures containing G·G and G·A base pairs. If this behaviour result to be a property of centromeric satellite DNAs, these sequences could utilize this potential to participate in the assembly of the kinetochore of higher eukaryotes.

NUCLEIC ACID BINDING PROPERTIES OF THE EST1 PROTEIN

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Telomerase is responsible for replicating the G-rich strand of the telomere. This enzyme is a novel reverse transcriptase which carries an internal RNA responsible for templating newly synthesized telomeric DNA. Although the RNA components from two ciliate telomerases have been cloned, no protein component has yet been identified from the biochemically defined telomerase activities. A candidate for a protein component is the *EST1* gene product of yeast (from which telomerase activity has not yet been identified). Yeast cells deleted for the *EST1* gene show the phenotypes predicted for a defect in telomerase (1); in addition, the Est1 protein shares very limited sequence similarity with reverse transcriptases (2).

We have examined the ability of a recombinant Est1 protein to bind either telomeric DNA substrates or a ciliate telomerase RNA, using gel mobility shift assays. Est1 protein tagged with six histidine residues and expressed in *E. coli* was purified to ~80% homogeneity using Ni-agarose affinity chromatography. This Est1 preparation exhibits specific binding to two single-stranded G-rich DNA oligo-mers corresponding to two variants of the yeast telomeric sequence, but does not bind to a C-rich oligo (which is an exact complement to one of the G-rich yeast oligos), a human G-rich telomeric oligomer or five different non-telomeric oligomers. In addition, Est1 does not bind duplex yeast telomeric DNA, nor is binding to a duplex with a protruding G-rich overhang enhanced relative to binding to single-stranded yeast telomeric oligomers. These data show that the Est1 protein binds with high specificity to yeast single-stranded G-rich telomeric oligos and imply a direct role for Est1 at the telomere, either as a component of telomerase or as a telomere end binding protein.

Est1 also exhibits binding to both the *Euplotes* telomerase RNA and two other unrelated RNAs of similar size. Although competition experiments indicate a slight preference for binding to the *Euplotes* RNA, there is both significant competition and binding activity by the non-telomerase RNAs. However, protein titration experiments show that RNA binding by Est1 is considerably stronger than the telomeric DNA binding described above, consistent with a role for Est1 as part of an RNP. The lack of specificity may be due to the fact that we have not tested the correct RNA sequence in this assay; we are currently examining whether Est1 is associated with an RNA (the predicted yeast telomerase RNA) in vivo.

(1) Lundblad and Szostak (1989). Cell *57*, 633-643
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CELL CYCLE-DEPENDENT DISTRIBUTION OF TELOMERES, CENTROMERES, AND CHROMOSOME-SPECIFIC SUBSATELLITE DOMAINS IN THE INTERPHASE NUCLEUS OF MOUSE LYMPHOCYTES.

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Fluorescence *in situ* hybridization and 3-D image analysis were combined to study the distribution of specific chromosome subdomains (centromeres, telomeres, or subsatellite regions) through the course of the cell cycle in cultured mouse lymphocytes whose shape is spherical.

The centromeres and proximal telomeres, which are preferentially distributed in the exterior volume in G1 nuclei, move toward the center between G1 and G2, while distal telomeres move progressively toward the nuclear periphery, in a direction opposite to that of proximal telomeres. A similar movement of the centromeres has been described in human lymphocytes, but no information concerning the distribution of telomeric sequences during the course of the cell cyle is yet available. A comparison with non-acrocentric human chromosomes would be valuable since telomeres and centromeres may segregate independently.

Quite surprizingly, the subsatellite sequences which are located just below the major satellite region on metaphase chromosomes seem to behave as independent domains, unrelated to the centromeric regions.The absence of an apparent spatial relationship between the homologous signals of the subsatellite region specific for chromosomes 8, 14, and X, and their apparent autonomy in regard to their interior/exterior distribution, also reinforce the idea that, in the interphase nuclei,the chromatin is not organized into rigid structural domains.

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SYSTEMATIC DETECTION OF DNA METHYLATION DURING THE DEVELOPMENT OF THE MOUSE CENTRAL NERVOUS SYSTEM (CNS) USING RLGS-M METHOD.

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We have reported a new approach for the systematic detection of genomic DNA methylation that is based on Restriction Landmark Genomic Scanning using methylation-sensitive endo-nuclease (RLGS-M). In this report, we tried to scan the mouse C3H/HeN genomic DNA to detect the transcriptionally active regions during the development of the mouse central nervous system. This approach is based upon the assumption that DNA methylation, especially methylation of CpG islands, will account for a significant status of gene expression. RLGS patterns of genomic DNA were produced from telencephalons from either mouse embryos at 9.5-, 13.5-, 16.5- days; postnatal mice at 1- and 10- days or adult mice. These were compared with each other and with the RLGS profiles of adult liver. We used NotI as a restriction landmark to survey the development-associated genes (loci), because almost all NotI sites are located near transcripts (6 out of 7 NotI-linking clones). We identified 44 spot loci (1.7%) out of 2600 spots which show appearance and disappearance, and the remaining spots appeared consistently during various stages of the CNS development. Several spots (genes) showed liver- or brain-specific methylation patterns. Thus, the status of DNA methylation is reproducibly changed as programmed. These findings suggest that RLGS-M is a widely applicable method for detecting developmental changes in the methylation status of loci throughout the genome.

PERTURBATION OF TELOMERES AND CHROMOSOME STABILITY.

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Telomerase is the enzyme responsible for synthesizing the telomeric DNA sequence, TTAGGG, which, in long direct tandem repeats, protects the ends of mammalian chromosomes. Without these protective telomeric caps, the chromosomes theoretically would fuse together, resulting in cell death. Telomerase is a ribonucleoprotein that synthesizes the telomeric DNA sequence by using the RNA it carries as a template. Telomerase is thus the only known reverse transcriptase endogenous to mammalian cells. Telomerase activity, which has not been found in normal somatic cells, appears to be activated as a critical step in the immortalization of tumor cells.

We are interested in perturbing telomere maintenance to specifically address what role telomeres play in maintaining chromosome stability. Because of telomerase's reverse transcriptase-like activity, we are studying the effects of a variety of inhibitors of reverse transcriptase on telomerase activity, telomere maintenance, and chromosome stability in cell lines. One promising candidate was found to greatly decrease chromosome stability as judged by a high incidence of dicentric chromosome formation. Under non-toxic growth conditions we have observed as many as 45% of the cells in a population containing one or more dicentric chromosomes. The incidence of cells with dicentric chromosomes and the rate of accumulation increases with drug concentration. At the highest dose used, the rate of accumulation sharply diminishes at approximately 8 population doublings at which time the incidence reaches a plateau. This is consistent with a gradual loss of telomeric sequence giving rise to the increased chromosome instability. The plateau effect could be the result of the cells reaching a steady state in telomere gain and loss. In addition, we observe multicentric chromosomes, some with up to 10 centromeres. This is consistent with some chromosomes loosing their telomeres from both of their ends. We are now in the process of exploring what effect some reverse transcriptase inhibitors have on radiation induced chromosome break repair as well as attempting the difficult task of monitoring telomere lengths shorter than 1Kb.

CHARACTERIZATION OF THE HUMAN SINGLE-STRANDED DNA-BINDING PROTEIN: REPLICATION PROTEIN A

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Replication Protein A (RPA) is a multisubunit single-stranded DNA-binding protein that is absolutely required for SV40 DNA replication and is probably involved in chromosomal DNA replication. In addition to interacting with DNA, RPA is involved in specific protein-protein interactions with SV40 large T antigen, DNA polymerase alpha and possibly with other replication proteins. These interactions seem to be essential for RPA function. Human RPA is composed of subunits of 70, 32, and 14 kDa. We have characterized the biochemical and functional properties of RP-A in order to understand its role(s) in DNA replication. RPA binds to single-stranded DNA as a heterotrimer with an affinity constant of approximately $10^9\ M^{-1}$, covering approximately 30 nucleotides. The affinity of RPA for single-stranded DNA varied over several orders of magnitude depending upon both the length and the sequence of the DNA being bound.

The 32 kDa subunit of RPA becomes phosphorylated at the beginning of S-phase *in vivo* and when incubated under SV40 replication conditions *in vitro*. We are examining the effect of phosphorylation on the activities of RPA to determine the role of phosphorylation in modulating DNA replication. Phosphorylation of the 32 kDa subunit *in vitro* causes a small increase in the DNA binding activity of the RPA complex.

We have expressed RPA in *E. coli* and purified the resulting soluble RPA complex to homogeneity. This recombinant complex is able to bind DNA and support SV40 DNA replication. In addition, the recombinant complex becomes phosphorylated *in vitro*.

CHROMATIN FOLDING

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A model for the folding of chromatin has been developed utilizing the properties of the nucleosome-linker DNA unit, without the imposition of long-range symmetry via specific nucleosome-nucleosome contacts or linker paths. A principal feature of the model is the relative rotation between consecutive nucleosomes that is based on the length of the linker segment and results from the helical twist of DNA. When consecutive linkers are kept constant, the model generates a family of symmetrical fiber- and ribbon-like structures according to the actual linker length employed. More interestingly, when consecutive linkers are allowed to vary, as occurs in nature, structures that show many of the irregularities of native chromatin fibers are obtained. These include a complex trajectory, an approximately cylindrical cross-section, regions with local periodicity in nucleosome position, nucleosomes positioned toward the periphery of the fibers, and an internal location of linker DNA.

The general principles of the model are being tested through the 3D reconstruction of nucleosome oligomers embedded and imaged in vitrified low salt buffers (Dustin et al, 1991. J. Struct. Biol. 107, 15-21). This method allows the folding conformation to be determined without the normal requirement for fixation and adhesion to a flat substrate. From random sequence oligonucleosomes it is possible to determine the range of entry-exit angles of linker DNA, the influence of histone H1 on chromatin conformation, and the changes that accompany salt-induced compaction. Extension of the method to chromatin reconstituted on DNA containing nucleosome position sequences provides an approach for establishing the rotation betweeen nucleosomes for a given length of linker DNA, a parameter that is difficult to measure by other means.

A GROUP OF SMALL BASIC PROTEINS ARE ASSOCIATED WITH KINETOPLAST DNA OF TRYPANOSOMES

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In eukaryotes, nuclear DNA is organized into nucleosomes and supra-nucleosomal structures mainly by histones. In striking contrast, the *in vivo* structure and organization of DNA-protein complexes in mitochondria are not known. The mitochondrial DNA of trypanosomes (kinetoplast DNA or kDNA) consists of a massive network of catenated DNA maxicircles and minicircles. In the trypanosomatid *Crithidia fasciculata* , each kDNA network contains 5000 copies of 2.5-kb minicircles and about 25 copies of 37-kb maxicircles. Electron microscopic studies indicate that kDNA networks exist in the kinetoplast as a highly organized disc-like structure in which the DNA appears to be aligned as a regular stack of fibers. In contrast, kDNA networks purified and spread for electron microscopy are at least 10-fold larger in diameter (similar to that of the cell) and show little organization.

In order to isolate protein components of condensed kDNA *in vivo*, we have crosslinked *C. fasciculata* cells with formaldehyde. Crosslinked kDNA-protein networks purified by differential sedimentation retain a condensed structure similar to that observed in cells. Treatment with proteinase K causes the networks to expand to a size close to that of whole cells and suggests that the associated proteins may play a role in maintaining the condensed structure of the kDNA *in vivo*. Crosslinked proteins are released from the networks by reversal of the crosslinks by heating. Amino-terminal sequences were determined for five small proteins (p15, p16, p17, p18 and p21) released from crosslinked networks and the complete gene sequences encoding p16, p17 and p18 have been obtained. All three proteins contain very similar cleavable presequences nine amino acids in length which contain hydrophobic and basic residues and lack acidic residues. Calculated isoelectric points for the three proteins are 10.5, 11.1 and 11.4 respectively. The three proteins have similar amino acid compositions and are rich in alanine, lysine and arginine residues. The predicted amino acid sequences of p17 and p18 have 57% homology and 43% identity. The N-terminal half of p16 also shows significant homology to p17 and p18, suggesting that these proteins may represent members of a novel family of mitochondrial DNA binding proteins. Purification of the kDNA associated proteins identified here by overexpression of the cloned genes and preparation of specific antesera against these proteins should provide necessary means for characterizing the interaction of each of the proteins with kDNA and with each other.

The Detection of Unwound DNA Sequences in a Yeast Centromere (CEN4) and in a Gene Control Region (CYC1).

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Several experimental methods for the detection and characterization of putative unwound regions in DNA are available today, including: a. Application of single strand specific nucleases, like S1, P1 or mung bean nuclease, which can localize unwound regions. b. Two dimensional topoisomer analysis, which formally determines the extent of unwinding. c. Conformation specific DNA reagents which can distinguish between the various paranemic (unwound) structures DNA may assume (cf. Yagil, Crit. Revs. Bioch. and Mol. Biol., 26: 475-559, 1991).

We have applied these three methods to characterize unwound DNA regions inserted into negatively supercoiled plasmids. The extent of unwinding of a region in the promoter of the chicken beta globin gene, previously identified by Nickol and Felsenfeld (Cell, 35:467), has been determined by 2d topoisomer analysis. The degree of unwinding is pH and spermine dependent. P1 analysis at physiological pH shows that spermine directs unwinding towards the two main cruciforms present in the vector. Topoisomer analysis by the minicircle technique shows that at physiological pH and under sufficient torsional strain four primary turns in the globin promoter region can be unwound.

The same techniques were applied to two plasmids with inserted yeast sequences. Both inserts are sensitive to P1 at neutral pH. One plasmid contains the centromere region of chromosome 4 (CEN4). Application of dimethyl sulfate and $KMnO_4$ reveals unpaired bases at sub elements CDE III and CDE I of the centromere, but, under the conditions so far studied, not in the long AT rich CDE II element in between. The existence of a paranemic structure unseparated into single strands is considered.

The second plasmid contains the promoter region of the iso-1-cytochrome c gene (CYC1). The principal P1 cleavage point maps in the promoter region within a sequence containing a 96% purine tract of 22 bases, with only limited mirror symmetry. The limited symmetry of the P1 sensitive region favors the formation of a paranemic duplex at the higher torsional strains. A role for the unwound region in activation of the CYC1 gene is suggested.

PARENTAL METHYLATION PATTERNS OF A TRANSGENIC LOCUS IN EARLY DEVELPOMENT

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In mammals and a few other organisms, some genes are expressed only when they are paternally inherited and others when they are maternally inherited; a phenomenon known as genomic imprinting. Likewise, in some transgenic mouse strains, the methylation status of a transgenic locus was found to alternate depending upon the sex of the parent from which it was tranmitted. In the adult tissues of transgenic mouse MPA434, which harbors a single copy of a mouse metellothionein-I (MT-I) promoter/human transthyretin fusion gene on chromosome 11, transgenic MT-I promoter region is methylated when it is maternally inherited while undermethylated when it is paternally inherited (Sasaki et al., 1991). We have previously shown that the maternally transmitted methylation is erased in fetal germ cells and re-established only in the process of oogenesis (Ueda et al., 1992). However, whether these patterns persist after fertilization and how early in germ cell lineage does the methylation disappear remain to be answered.

Blastocysts were isolated from a female homozygous for transgenic locus mated with a wild-type male (maternal transmission) and from a wild-type female mated with a male homozygous for transgenic locus (paternal transmission). Methylation status was assayed by digesting the blastocyst DNA with a methylation-sensitive restriction enzyme HhaI followed by PCR amplification of the targeted region. Endogenous MT-I promoter on chromosome 8, which is constitutively undermethylated, was simultaneously analyzed as a control for DNA purity and enzyme digestion. The results showed that the transgenic MT-I promoter region was undermethylated upon paternal transmission while it was methylated upon maternal transmission.

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MULTIGENE FAMILY OF MNB1a THAT MAY BE CLASSIFIED INTO A NOVEL DNA-BINDING PROTEIN OF MAIZE

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A cDNA clone encoding DNA-binding protein, which may be classified into a novel DNA-binding protein, was isolated from a cDNA library, which was constructed in λgt11 expression vector with mRNA from maize leaves. This cDNA was obtained by screening with synthetic oligonucleotides corresponding to a segment (-276 to -252 relative to the transcription start site) of the cauliflower mosaic virus (CaMV) 35S promoter. This region was shown to be a target site of maize nuclear factor, MNF1, which binds to both the CaMV 35S promoter and the C4 photosynthetic phosphoenolpyruvate carboxylase (PEPC) gene promoter of maize[1,2]. MNF1 had been suggested to be a putative transcription factor for regulation of expression of several genes in leaves[1,2]. The protein coded by the isolated clone was termed MNB1a. MNB1a recognized two copies of AAGG motif in the MNF1-binding site on the CaMV 35S promoter as the important bases, as does MNF1 in maize nuclear extracts from leaves. However, MNB1a did not bind to the MNF1-binding site on the PEPC gene promoter. These observations suggest that binding-specifities of MNB1a and MNF1 are similar but not identical. The amino acid sequence deduced from the DNA sequence of the MNB1a cDNA demonstrated that MNB1a had three regions rich in basic amino acids and one region rich in acidic amino acids. Since no known protein with significant homology with MNB1a was found in a survey of the DNA database, MNB1a may be classified as a new class of DNA-binding protein. Genomic Southern blot analysis suggest that MNB1a gene belongs to a multigene family and that members of this gene family have a highly homologous domain[3]. MNF1 might be another member of this gene family.

Two cDNA clones whose restriction maps were different from the MNB1a cDNA were also isolated from a maize cDNA library by plaque hybridization using the MNB1a cDNA as a probe. Nucleotide sequence analysis of these clones revealed that a domain was highly conserved in each N-terminal region. These cDNA clones and the MNB1a cDNA were shown to be originated from different genes by genomic Southern blot analysis. The expression pattern of these genes will be presented.

Finally, several genomic DNA fragments of *Arabidopsis thaliana* were shown to be hybridizable to the maize MNB1a cDNA. This observation suggests that the structure of MNB1a is conserved in genes of the plant kingdom, that is, among monocots (maize) and dicots (*Arabidopsis thaliana*).

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ORGANIZATION OF THE CHROMOCENTER IN THE NUCLEUS OF MATURE HUMAN SPERM

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The localization of centromeres in mature human sperm nuclei has been shown by immunofluorescent labelling and FISH. Structural elements formed by interactions between nonhomologous centromeres (dimers, tetramers, linear arrays and "V" shape structures) were observed. They organize the compact chromocenter buried inside the nucleus. 3D structure of the chromocenter was studied by confocal microscopy.

Telomere regions of chromosomes were localized by FISH on the nucleus periphery. A model describing gross nuclear architecture (which can influence selective unpackaging of the paternal genome upon fertilization) is proposed.

MOUSE p53 ACTIVATES THE RAT *MUSCLE* CREATINE KINASE (***CKM***) GENE BUT REPRESSES THE RAT *BRAIN* CREATINE KINASE (***CKB***) GENE.

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Creatine kinases (CK) catalyze the reversible transfer of a high-energy phosphate group from ATP to creatine, thus regenerating ATP in cell types which expend large amounts of ATP. While CKM is expressed almost exclusively in skeletal and cardiac muscle, CKB expression is more diverse being highest in brain glia, much lower in cardiac muscle, kidney and undetectable in some other tissues. Interestingly, CKB expression is also high in tumors from different cell types many of which contain mutations in p53 alleles (Minna et al. Cancer Res. 41:2773 [1981]; Science 246:491 [1989]). We have also detected high levels of CKB mRNA in HeLa cells and have tested whether this is due to the extremely low amounts of p53 in HeLa. HeLa are transformed by Human Papilloma Virus (HPV) whose E6 protein binds to and destabilizes p53. Indeed, transient transfection into HeLa of a CKB promoter-CAT hybrid construct (**pCKB-CAT**) resulted in high expression. Interestingly, cotransfection of pCKB-CAT with a plasmid expressing wild type mouse p53 (**p53 wt**) *repressed* **pCKB-CAT** by 10-20-fold in HeLa but *not* in CV-1 monkey kidney cells. This suggests p53-mediated repression of pCKB-CAT in HeLa may require a corepressor not found in CV-1. Conversely, in a cotransfection of **p53 wt** with a *CKM* promoter-CAT construct (pCKM-CAT) into CV-1, p53 *activated* pCKM-CAT by 18-40-fold. Since no activation of pCKM-CAT by p53 occurred in HeLa, this suggests p53-mediated activation of pCKM-CAT in CV-1 requires a coactivator not present in HeLa. These results may also suggest possible involvement of p53 in development regulating the CKB to CKM isozyme switch when myoblasts fuse into myotubes and activate muscle-specific expression. In addition, we analyzed plasmids with substitution mutations in p53 for trans-activation and trans-repression.

NUCLEAR MATRIX AND TRANSCRIPTION REGULATION: INTERACTION OF SOLUBLE NUCLEAR MATRIX PROTEINS WITH THE IMMUNOLOBULIN HEAVY CHAIN ENHANCER

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The immunoglobulin heavy chain (IgH) gene is an excellent model system for studying tissue-specific gene expression since it is only expressed in B lymphocytes. One intriguing finding has been that the IgH intronic transcriptional enhancer contains segments which specifically interact with the nuclear matrix, so called nuclear matrix association regions (MARs), suggesting that nuclear matrix attachment might play some role in enhancer function. Another interesting aspect is that two of the binding sites of the IgH enhancer suppressor protein, NF-μNR, overlap the putative MAR recognition sequences of the enhancer. The potential overlap between the MARs and the NF-μNR binding sites suggests that there is a functional relationships between NF-μNR and the nuclear matrix.

In order to investigate the consequences of this overlap we sought to precisely map the MARs of the IgH enhancer. Since footprinting with the standard insoluble matrix preparation has proved technically difficult, nuclear matrix proteins were solubilized with urea and then examined for MAR-binding capabilities. This solubilized nuclear matrix preparation was found to stimulate the binding of MAR-containing DNA fragments to insoluble nuclear matrix preparations; presumably the soluble matrix proteins first bind to the DNA fragment, and then this complex binds to the insoluble matrix through protein-protein interactions. This activity is specific for MAR sites since binding of the IgH enhancer MAR, the Ig kappa MAR and a Drosophila MAR to insoluble matrix was stimulated by the soluble preparation, whereas residual binding of a control fragment from Cμ was not. Further purification of nuclear matrix by DEAE chromatography is being used to identify the matrix protein responsible for this stimulation.

Finally, in support of the proposal that NF-μNR is distinct from the nuclear matrix, purified NF-μNR inhibits MAR binding, rather than stimulates. Further experiments analyzing the interaction between NF-μNR and soluble matrix proteins will be presented.

CODING SEQUENCES OF DNA CONTAIN FRAME SPECIFIC **INTERNAL** ANTISENSE. James E. Zull[1], Ronald C. Taylor[2], George S. Michaels[2], and Norman B. Rushforth[1]; [1] Case Western Reserve University, Cleveland, OH, and [2]Division of Computer Research and Technology, NIH, Bethesda, MD.

We have examined whether coding sequences in DNA represent antisense to other sequences in the same strand. This study was prompted by prior postulates that there may be structural relationships between proteins coded at the same locus in the complementary strands. We developed a data base of 138 proteins from more than 20 species which contained wide represenation from the known protein folding classes. These proteins were examined for internal antisense at the amino acid level, in all three frames. For statistical evaluation of the results, 1000 DNA chains were randomly assembled for each protein, and the mean and standard deviation for the number of internal antisense sequences determined. Numerous short segments which represent sense-antisense pairs, "pairons", were found in all the proteins. These ranged in length from two to ten amino acids. Overall, the number of "pairons" in frame three was similar to the random chains, but there were many fewer in frame one and many more in frame two. This pattern was also apparent at the individual protein level: in the correct reading frame the number of "pairons" was usually less than in the random chains; in frame two, the number of "pairons" was frequently much higher than the random chains; and in frame three, the number of "pairons" was similar to the random chains. In frame two, for 30% of the proteins the number of "pairons" of all lengths was significant ($P < 0.01$) and for 20% the number was very highly significant ($P < 0.0001$). Although the lesser number of "pairons" in frame one was not significant for the individual proteins, cumulative frequency distribution analysis showed that the differences between the frames was still highly significant ($P < 0.01$). The number of base pairs in the "pairons" was also highly significant when compared to the random model ($P < 0.001$). The differences in antisense content were also related to the GC content; high GC correlated with high numbers of frame two "pairons", and low GC content with low "pairon" numbers. There was no correlation with length of the sequences. We conclude that DNA contains frame specific antisense sequences, but that this is not related to protein folding. A model describing a role for antisense in evolution will be presented.

CHIASMA INTERFERENCE AND THE DISTRIBUTION OF EXCHANGES IN *DROSOPHILA MELANOGASTER*

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In many eukaryotes, meiotic exchanges interfere with each other. The coefficient of coincidence ($S4$), an inverse measure of interference, is zero for intervals that are close together and is about unity when the distance (X) between the intervals is about 0.4 Morgans. The relationship between $S4$ and X is similar for organisms in which the rates of exchange per kb are highly dissimilar. Thus, inteference is likely to be directly dependent on linkage map distance rather then on physical distance. Foss et al. (1993, *Genetics* **133**: 681-691) proposed that recombinational intermediates (C's) are distributed without interference. C's can be resolved with exchange (Cx) or without exchange (Co). Interference of Cx's with each other results from a requirement for a fixed number of Co's between adjacent Cx's. These assumptions lead directly to an expression for $S4$ as a function of X. The only adjustable parameter in the expression is the number (m) of Co's that must fall between adjacent Cx's. This number can be estimated from the fraction of gene conversions that are observed to be crossovers for flanking markers. For Neurospora, $m = 2$; for Drosophila, $m = 4$. The model evaluated at these values fits the data for these organisms. The model also successfully predicts the frequencies of Drosophila X chromosome tetrads with zero, one, two, or three exchanges as well as the distributions of exchanges along the linkage map in the tetrads of different rank. The success of the model argues for a device that travels along a meiotic bivalent (on the central element of the synaptonemal complex?) and counts Co's.

INTERPLAY OF DNA/DNA INTERACTIONS AND CHROMOSOME TOPOGRAPHY DURING MEIOSIS

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During meiosis, chromosomes condense, pair, form synaptonemal complex (SC) and recombine. New information about these events and their interrelationships (see below) and a general model that accounts economically for the events of meiotic prophase will be presented.

I. DNA events. (1) Mapping ends of meiosis-specific double strand breaks (DSBs) suggests that they are formed by a sequence non-specific nuclease that is sensitive to DNA/chromatin structure. (2) A prominent non-linear recombination intermediate present for $\geq$60 minutes between DSBs and reciprocal recombinants has been identified. At a DSB hot spot, the intermediate is tightly localized to the DSB sites and contains only full length strands. (3) Suppression of recombination between sister chromatids occurs prior to formation of the intermediate in (2). (4) Preliminary results are suggestive of DNA/DNA interactions without DSBs.

II. Chromosome pairing. We have developed an *in situ* hybridization analysis that detects pairing at individual chromosomal loci without regard to the nature of interactions elsewhere along the chromosome. This permits analysis of chromosome pairing at early times and in mutants where pairing is incomplete. A specific early pairing intermediate is revealed that we interpret to represent connections between chromosome loops on the two homologs and to be the feature underlying a "300nm juxtaposition" of chromosome cores observed in many organisms by classical cytology. A similar stage occurs during exit from pachytene. Mutant analysis suggests that the early pairing step can occur without DSBs and that (DNA) homology searching may occur in two stages.

III. "DNA" events are required for SC formation. We have argued that events in the DNA pathway are required for events in the SC pathway. Mutations in presumptive "DNA" genes, a DNA repair function (*rad50S*) and a meiosis-specific RecA-like function (*dmc1*), block DSB processing and conversion to the non-linear intermediate respectively; both mutations also block SC formation. (1) A search for related mutations has revealed five more, in different genes, all of which confer one of the two previously observed phenotypes; these must represent important "breakpoints" in prophase. (2) Anti-Dmc1 antibodies reveal multiple (up to 50) strong foci of staining spaced along chromosomes at stages prior to pachytene, and Dmc1 protein binds DNA *in vitro*. We infer the existence of a stage-specific protein/DNA complex (N.K. and D.K. Bishop, Dept. of Radiation Oncology, U.of Chicago). (3) Structural aspects of meiosis are not rate-limiting for recombination: without axial cores or SCs (*red1*), the timing of meotic recombination is the same as in a wild type strain.

RECOMBINATION GENES OF EUKARYOTIC CELLS HMOLOGOUS TO *S. CEREVISIAE RAD51* AND *E. COLI RECA.*

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The *RAD51* gene of *S. cerevisiae* (*ScRAD51*) is a homologue of the *E. coli recA* gene (*EcrecA*) and plays crucial roles in both mitotic and meiotic recombination and in repair of double strand breaks of DNA.

We have found that ScRad51 and EcRecA proteins form strikingly similar structures together with dsDNA and ATP. Their right handed helical nucleoprotein filaments extend the B-form DNA double helixes to 1.5 times in length and wind the helix. The similarity and uniqueness of their structures must reflect functional homologies between these proteins. Therefore, it is highly probable that similar recombination proteins are present in various organisms of different evolutional states.

We have succeeded to clone genes from human (*HsRAD51*), mouse (*MmRAD51*) and fission yeast (*SpRAD51*) homologues of the *ScRAD51* gene, and found that the homologues are widely distributed in eukaryotes. The predicted MmRad51 and HsRad51 proteins are almost identical, differing only by four amino acids and highly homologous to both yeast proteins, but less so to ScDmc1. All of these proteins are homologous to EcRecA, but in the region from residues 33 to 240 of RecA which was named "homologous core". All of Rad51 homologues are longer in the N-terminal region than the RecA protein, while they are shorter in the C-terminal region. The homologous core is likely to be responsible for functions common for all of them, such as the formation of a helical nucleoprotein filament that is considered to be involved in homologous pairing in the recombination reaction.

The mouse gene is transcribed at a high level in thymus, spleen, testis, and ovary, at a lower level in brain and at a further lower level in some other tissues. It is transcribed efficiently in recombination active tissues.

A clear functional difference of Rad51 homologues from RecA was suggested by the failure of heterologous genes to complement the deficiency of *Scrad51* mutants. This failure seems to reflect the absence of a specific compatible partner, such as ScRad52 protein in the case of ScRad51 protein, between different species.

Thus, these discoveries play a role of the starting point to understand the fundamental mechanisms of recombination in higher eukaryotes and find a way to improve the frequency of gene targeting in mammalian cells and in gene therapy.

MISMATCH REPAIR AND GENETIC STABILITY IN *E. coli* AND HUMAN CELLS. Karin Au, Deani Cooper, Woei-horng Fang, Jude Holmes, Michelle Grilley, Robert Lahue, Guo-min Li, Matthew Longley, John Taylor, Leroy Worth, and Paul Modrich, Department of Biochemistry, Duke University Medical Center, Durham, NC 27710

In *E. coli* methyl-directed mismatch repair eliminates pre-mutagenic lesions that arise via replication errors, and components of this repair system also block ectopic recombination events. We have reconstituted an excision repair reaction dependent on 10 activities (MutH, MutL, MutS, DNA helicase II, SSB, exonuclease I, RecJ exonuclease (or exonuclease VII), DNA polymerase III holoenzyme, and DNA ligase) that can account for function of the methyl-directed system in replication fidelity. Repair is initiated by the mismatch-provoked, MutHLS- and ATP-dependent incision of the unmodified strand at a hemimethylated d(GATC) sequence. The resulting strand break can occur either 3' or 5' to the mismatch on the unmethylated strand. In the ensuing excision reaction, exonucleolytic hydrolysis initiates at the strand break and removes that portion of the unmodified strand spanning the d(GATC) site and the mismatch. The repair system thus possesses a bidirectional excision capability.

We have also shown that MutS and MutL proteins modulate RecA-catalyzed heteroduplex formation in response to mismatched base pairs. Although MutS and MutL are without effect on RecA-mediated M13-M13 or fd-fd strand transfer, the Mut proteins abolish heteroduplex formation between M13 and fd DNAs, which are 97% homologous. Inhibition of strand transfer between these "homeologous" DNAs can be observed in the presence of MutS alone, but this effect is dramatically potentiated by MutL.

Human cell nuclear extracts support strand-specific mismatch correction in a reaction that is similar to bacterial repair with respect to both mismatch specificity and mechanism. We have also identified a mutant human cell line that is defective in this reaction. This MT1 line was isolated by Thilly and colleagues (Goldmacher et al. (1986) **J. Biol. Chem.** *261*, 12462-12471.) by virtue of its ability to survive the cytotoxic effects of MNNG. MT1 cells nevertheless remain sensitive to mutagenesis by MNNG, are hypermutable in the absence of alkylating agents, and as we have shown, are deficient in repair of the 8 base-base mismatches. This finding implicates mismatch repair in cytotoxicity of DNA alkylators and suggests that the reaction contributes to genetic stability in man.

MOLECULAR MECHANISM OF EXCISION REPAIR

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Nucleotide excision repair is the major molecular mechanism for eliminating modified bases from DNA and is the sole repair mechanism for excising bulky adducts from a DNA duplex. We have elucidated the action mechanisms of human and *E. coli* excision nucleases (excinucleases).

The human excinuclease is made up of 8 subunits, XPAC through XPGC and ERCC1. It removes DNA damage in 27-29 nt-long oligomers by hydrolyzing the 22nd-24th phosphodiester bond 5' and the 5th phosphodiester bond 3' to the damaged nucleotide(s) in an ATP-dependent reaction. The enzyme requires Pol δ/ε, PCNA and dNTPs for catalytic turnover but not for a single round of excision. The human excinuclease appears to be the prototype eukaryotic excision nuclease as other eukaryotes ranging from yeast to frog apparently employ the same incision pattern.

The *E. coli* (A)BC excinuclease is the ATP-dependent nuclease activity resulting from partly overlapping functions of UvrA, UvrB, and UvrC proteins, and removes DNA lesions in 12-13 nt-long oligomers. UvrA is a molecular matchmaker, makes an A_2B_1 complex with UvrB, delivers this subunit to the damage site, and dissociates from the stable UvrB-DNA complex in which the DNA is severely kinked and locally melted. UvrC recognizes the UvrB-DNA complex, binds to it and induces a conformational change in UvrB enabling it to hydrolyze the 4th or 5th phosphodiester bond 3' to the lesion; then, UvrC incises the 8th phosphodiester bond 5' to the lesion. The dodecanucleotide carrying the lesion is released by helicase II and the repair gap is filled in by PolI. All prokaryotes tested carry out excision repair by an identical mechanism.

DNA JOINING IN MAMMALIAN CELLS

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Three different ATP-dependent DNA ligases are present in mammalian cell nuclei. The enzymes are distinguished by catalytic specificity, molecular weight, peptide mapping, and immunological analyses. Partial functional assignments have been made possible by the identification of a human cell line, 46BR, with altered DNA ligase I. The line was derived from an individual with clinical symptoms of stunted growth, severe immuno-deficiency, sun sensitivity, and a predisposition to cancer. The condition was generated by an inactivating and a partly inactivating mutation in the two alleles of the structural gene for DNA ligase I. The latter mutation was inherited from the mother of the patient and is retained in an SV40-transformed hemizygous subline of 46BR with the same cellular phenotype. 46BR cells are hypersensitive to a wide range of DNA-damaging agents. DNA ligases II and III are normal in 46BR cells. The malfunctioning DNA ligase I from 46BR has a strongly reduced ability to convert DNA-adenylate to joined DNA, leading to an accumulation of reaction intermediates. This may account for defective DNA excision repair in 46BR cells. However, joining of Okazaki fragments is not greatly impaired in 46BR, indicating that DNA sealing during lagging-strand DNA replication may be performed by another enzyme, probably DNA ligase III. The activity of DNA ligase I is dependent on phosphorylation by casein kinase II in an N-terminal region of the protein far removed from the active site. Rejoining of strand breaks in cellular DNA is modulated also by transient binding of the abundant nuclear factor, poly (ADP-ribose) polymerase, at strand interruptions.

NUCLEOTIDE EXCISION REPAIR OF DNA BY MAMMALIAN CELL EXTRACTS AND PURIFIED PROTEINS

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Nucleotide excision repair is the main pathway that cells use to remove damage caused to DNA by the ultraviolet component of sunlight. In humans, a deficiency in this process is associated with the heritable disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and trichothiodystrophy (TTD). Individuals with XP are affected with skin lesions (including tumours) at a dramatically high rate.

During nucleotide excision repair, lesions are located, DNA is incised on the damaged strand, an oligonucleotide containing the damage is excised, and a repair patch of ~ 30 nucleotides is synthesised. Genetic and biochemical studies tell us that more than 20 protein components are involved in this process. There is good evidence that these factors are functionally similar in different eukaryotes. The mechanism can remove many chemical adducts as well as UV damage, and seems to recognise a class of structural distortions in the double helix, rather than the specific molecular structure of lesions. We are using a cell-free system to dissect this process. The DNA-binding properties of the XPAC (xeroderma pigmentosum group A complementing) protein suggest that it is involved in the recognition of damage (1). Other XP/CS/TTD complementing proteins participate in this and subsequent steps, in cooperation with distinct ERCC (excision repair cross-complementing) proteins identified from studies of rodent cells. Some factors that participate in semi-conservative DNA replication also function during nucleotide excision repair. These include the single-stranded DNA-binding protein RPA, and PCNA (2). *In vitro* analysis is beginning to reveal specific roles for the different components in the reaction mechanism. In addition, studies of the repair of defined, site-specific DNA damage can provide information on the basis for the effectiveness of chemotherapeutic drugs (3).

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2. Shivji *et al.* (1992) *Cell* 69:367
3. Szymkowski *et al.* (1992) *Proc. natl. Acad. Sci. USA* 89:10772

DNA SEQUENCE ANALYSES OF THE HUMAN AND MOUSE T CELL RECEPTOR LOCI

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The T cell receptors of mouse and human are encoded by three multigene families for each species - α/δ, β and γ. We have sequenced contiguous stretches of about 100 kb of DNA from the joining and constant regions of the human and mouse α/δ loci. The analysis of these sequence data has revealed a striking homology conservation extending over the entire ~100 kb stretch. For the human T cell receptor β locus, we have determined the longest contiguous stretch of human genomic sequence (around 450 kb). Analysis of these data revealed the surprising presence of a trypsinogen gene family between the Vβ and Dβ gene segments. Sequence data we have obtained have also allowed us to approach the molecular biology of the T cell receptor loci with powerful new tools.

CHROMOSOMAL ORGANIZATION AND MOLECULAR EVOLUTION OF C_2H_2 ZINC FINGER PROTEIN GENES

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We have mapped over 100 different clones containing zinc finger sequence motifs on human chromosomes and over 50 on murine chromosomes. Many of these zinc finger protein (ZFP) genes are clustered in telomeric regions or in interstitial bands known to exhibit chromosome fragility (P. Lichter, et al., Genomics, 13, 307, 1992). One gene cluster, containing over 40 ZFP genes (or pseudogenes) of the KRAB (Krüppel-associated box) ZFP subtype, has been identified in the chromosome 19p12-13.1 region (E. Bellefroid, et al., EMBO J., in press). All members of this cluster share sequence homology with ZNF91, a gene that encodes 35 finger repeats at its c-terminus, and have a highly conserved 276 nucleotide "spacer" sequence between the KRAB domain and the first finger repeat. Transcription of members of this gene subgroup is detectable in many human tissues, although their expression is significantly higher in T-lymphoid cells. In contrast to the KRAB and ZNF motifs, the "spacer" motif cannot be detected by hybridization or PCR analysis in murine, rat or prosimian genomes (Tarsier, Galago and Lemur). This ZFP subfamily is detectable by PCR analysis in all species of great apes, old world monkeys and new world monkeys examined. The spacer sequence of ZNF91 orthologs in chimp, gorilla and orangutan is identical to that in human while the orthologs from new world monkeys exibit 80% homology. The evolution of this gene subfamily is being examined by hybridization of a pool of YAC clones containing the human 19p ZFP gene cluster on primate chromosomes. The YAC pool hybridizes to syntenic chromosomes of great apes and orangutans; analysis of other primates is in progress. These data indicate that the ZNF91 ZFP gene family is absent from rodents and prosimians but appeared at a later stage of primate evolution.

A CRITERION FOR THE COMPLETENESS OF LARGE-SCALE PHYSICAL MAPS

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One of the most vexing issues surrounding the large-scale physical mapping of DNA is the question of when done is done, or for that matter half done. It is commonplace for maps to be described as 90% done for years while mapping continues with full vigor. This experience suggests that existing measures of completeness are poorly formulated.

A good measure of completeness should meet several criteria: 1.) Increases in the measure should correlate strongly with increased utility of the map; 2.) The measure should be readily adjustable to different map resolutions; 3.) The measure should be a useful guide to resource allocation during a large project (i.e. it should indicate when to shift resources from one type of activity to another in order to move toward completion).

We propose that the ideal measure is one that focuses on the ordering of mapping landmarks such as sequence-tagged sites (STSs). An ideal map of a 100 Mbp chromosome at a resolution of 100 kbp would have 1000 equally spaced, ordered STSs. Approach to this ideal could simply involve listing the uniquely ordered STSs starting at one end of the chromosome and proceeding to the other. The list would have to be filtered to deal sensibly with closely spaced markers. Once filtered, a set of 800 ordered markers would imply 80% completion.

This simple concept has major practical ramifications that will be discussed with examples from actual physical mapping projects. It can also be readily extended to provide a quantitative measure of the accuracy of physical maps.

Mapping Genes and Genomes: Genetic Dissection of Complex Traits

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Although geneticists have traditionally studied single-gene traits, most variation in natural populations is polygenic in its basic. Such polygenic traits include many human diseases and most agronomically important variation. Analyzing such complex inheritance requires the ability to follow the inheritance patterns of entire genomes simultaneously, through the use of detailed genomic maps.

To facilitate such studies, we have been developing detailed genomic maps in the mouse, the rat and the human. We will report our progress toward these goals, describe streamlined methods for constructing such chromosomal maps, and discuss large-scale genomic features apparent from such maps.

We have also been applying these maps, together with new analytical techniques, to a variety of problems of complex inheritance, including: genetic analysis of a major modifier of inherited colon cancer in the mouse; type I diabetes in the rat; hypertension in the rat; loss-of-heterozygosity in a variety of mouse tumors; and a human chrondrodysplasia common in Finland that serves as a model for the application of population genetics to the mapping of disease genes. We will describe these projects as paradigms for the genetic dissection of complex traits.

MAPPING AND SEQUENCING THE NUCLEAR GENOME OF THE YEAST *SACCHAROMYCES CEREVISIÆ*: RESULTS FROM THE EUROPEAN ENTERPRISE.

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The baker's yeast *Saccharomyces cerevisiæ* is one of the most appealing cases among lower eucaryotes for systematic sequencing programs. It combines the advantages of having a small genome (ca. 14 megabases in total, distributed in 16 chromosomes), remarkably compact (one gene every 2 kb, on the average), and of offering unique possibilities for reverse genetics, thanks to its precise and efficient homologous recombination system. The importance of *S. cerevisiæ* for bioindustries as well as for modern genetics (ca. 18 % of its 7000 genes have already been characterized) further fosters such a program.

A year ago, a consortium of more than 30 european laboratories has published the sequence of chromosome III of *S. cerevisiæ*, the first eucaryotic chromosome entirely sequenced, as the result of the first phase of the european program (1989-91) that was initiated by André Goffeau (Louvain-la-Neuve). The work was coordinated by Steve Oliver (Manchester). The second phase of the european program has begun in january 1991 with the systematic sequencing of chromosome II (820 kb) under the coordination of Horst Feldmann (Munich), and of chromomsome XI (665 kb), that I coordinate.

New cosmid libraries and maps have been constructed for this purpose and a new mapping procedure has been developped. Simple rules for efficient operation of a large consortium of laboratories have been established, and quality controls have been imagined and applied to all sequences prior to their assembly.

The results of the complete sequencing of chromosome XI will be presented in details [1]. More than 300 protein coding genes have been discovered, the products of which are systematically analyzed with the newest versions of protein databases at MIPS (Martinsried). Homology searches are also carried out at the nucleotide level to detect tRNAs, delta sequences, Ty elements or putative regulatory sequences. Global aspects of the entire chromosomal organization as deduced from its sequence will also be presented.

Propects for subsequent phases of the european program, with four new chromosomes started in january 1993, will be summarized.

[1] In addition to the present author, the sequencing work on chromosome XI was carried out in the laboratories of the following contractors: W. Ansorge, M. Bolotin-Fukuhara, F. Foury, H. Fukuhara, F. Hilger, C. P. Hollenberg, J. C. Jauniaux, A. Jimenez, S. Oliver, P. Philippsen, R.J. Planta, F.M. Pohl, M. Rieger, C. Rodrigues-Pousada, G. Thireos and D. von Wettstein.

SEQUENCING THE GENOME OF *C. elegans*

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The haploid genome of *C. elegans* contains about 100Mb of DNA in six chromosomes. The project to map the entire genome has resulted so far in a high-resolution map covering more than 95% of the genome, consisting of some 17,500 cosmid and 3,500 YAC clones. Closure is now being approached with the use of STSs developed at the ends of contigs and through construction of large insert YACs (average insert size approx. 800kb).

The *C. elegans* sequencing project was initiated in 1990, using the extensive map resources as a starting point. The first three years constituted a pilot phase undertaken by a consortium of our two labs in St. Louis and Cambridge, England. The aim of the pilot project was to sequence 3Mb, a goal we are on target to meet. In this period we also aimed to develop strategies for genome sequencing that would be applicable to the whole genome.

Our sequencing method is an initial shotgun stage using a combination of M13 (1-2kb) and phagemid (6-9) clones, followed by a primer-based walking phase. Since 1990, we have increased our sequencing efficiency through automation of several stages in the procedure. Template preparation and sequencing reactions can now be routinely carried out by a robot; the editing process has been facilitated by using the program XDAP, which assembles data collected by ABI 373A fluorescent gel readers; and continued development of our assembly software has now led to a fully automated package, which clips the raw data and performs shotgun assembly without operator intervention. At present, all editing is done interactively, but the development of a new basecalling algorithm should allow reliable quality estimates of individual base calls, making possible the implementation of an automated editor. The operator will then only have to deal with failed clones and compressions and devise strategies for contiguation and double-stranding.

The primary repository for the *C. elegans* data is the database management program ACEDB. All data is then released to the EMBL databases or to GenBank. To date, 1208kb have been submitted to public databases. 239 genes have been predicted in the completed regions by analysis using BLAST and GENEFINDER, giving a density of 1 in 5kb. More than 1/3 of the genes have significant matches to previous database entries. Comparison with the cDNA matches (49) in the region versus the total identified (3491) permits an estimation of total gene number (16,000).

A FIRST GENERATION PHYSICAL MAP OF THE HUMAN GENOME

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33,500 YAC (900kb mean size) from a whole human genome library were individually fingerprinted by producing patterns of restriction fragments carrying human repeats. Three enzymes were used and 10, 000 Southern blots were hybridized with 2 probes , one for an L1 sequence, the other for a THE consensus sequence giving, respectively, 6 and 11 fragments per megabase of genome on the average. Preliminary data analysis indicates that approximately 700 contigs have been assembled, most of the chimeric clones and the small insert YACs being eliminated. Validation of these contigs is being obtained by 3 methods :

i) by metaphase chromosome FISH where YAC DNA from individual contigs are pooled and labelled. A single location is found when the contig is not chimeric.

ii) by hybridizing the Alu PCR products of individual YACs from a given contig to other members of that contig.

iii) by genetically ordered polymorphic STS content mapping.

More than 1000 such markers distributed along the genome have already been used to screen the 33,500 YAC. This validation will also allow precise mapping of most of the contigs (by FISH and STS). Moreover the order of YACs within a given contig is roughly obtained by a specific computer algorithm. We estimate that approximately 90% of the human genome has been covered, 50-70% with contigs larger than 5 Megabases.

THE HIGH MOBILITY GROUP PROTEIN HMG I(Y) IS REQUIRED FOR THE SYNERGISTIC ACTIVITIES OF TWO DISTINCT VIRUS-INDUCIBLE ELEMENTS OF THE HUMAN INTERFERON-β GENE PROMOTER

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Virus induction of the human interferon-β (huIFN-β) gene requires synergistic interactions between distinct virus inducible promoter elements (positive regulatory domains, PRDI through PRDIV). Activation of PRDII requires both NF-κB and the high mobility group protein HMG I(Y). The two proteins bind to different regions of PRDII, and HMG I(Y) stimulates the binding of NF-κB. We now report that HMG I(Y) directly interacts with the p50 and p65 subunits of NF-κB, and the p50 dimerization domain is required for this interaction. Functional synergy between NF-κB and HMG I(Y) at PRDII therefore appears to involve both protein-DNA and protein-protein interactions.

We have recently found that a strikingly similar mechanism is involved in virus induction of PRDIV. However, in this case HMG I(Y) synergizes with the transcription factor ATF-2. PRDIV is also a composite element, consisting of an ATF-2 binding site immediately flanked by two HMG I(Y) binding sites. Mutations that affect binding of either protein to PRDIV adversely affect virus induction. In addition, antisense ATF-2 and HMG I(Y) experiments indicate that both proteins are required for virus induction. HMG I(Y) appears to act, at least in part, by stimulating the binding of ATF-2 to PRDIV. The functional significance of these observations is supported by the identification of two inducible protein-PRDIV DNA complexes. One complex contains an ATF-2 homodimer plus HMG I(Y), and the other contains an ATF-2/c-Jun heterodimer plus HMG I(Y). The observation that HMG I(Y) directly interacts with both ATF-2 and c-Jun suggests that these inducible complexes involve both protein-DNA and protein-protein interactions.

The transcriptional synergism between PRDII and PRDIV in the intact huIFN-β promoter appears to involve direct interactions between ATF-2 and NF-κB, since the two proteins specifically interact in vitro in the absence of DNA. Significantly, this interaction is enhanced by HMG I(Y). Interestingly alterations in the relative positions of PRDII and PRDIV in the IFN-β promoter profoundly affect virus induction. For example, insertion of a half helical turn between these elements dramatically reduces the level of virus induction. However, insertion of an additional half helical turn fully restores the activity.

These results strongly suggest that ATF-2, NF-κB and HMG I(Y) specifically interact with the huIFN-β promoter and with each other to form a three dimensional complex that is required for virus induction. We propose that HMG I(Y) plays an essential role in the assembly and stability of this complex.

CHROMATIN: STRUCTURAL DETERMINANTS IN THE REGULATED EXPRESSION OF *DROSOPHILA hsp26.*

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Previous analysis of the *hsp26* gene of *Drosophila melanogaster* has demonstrated that the chromatin structure of this gene is highly defined, both before and after gene activation by heat shock. While the body of the gene is incorporated into a specific nucleosome array, the upstream regulatory region is organized so that the two DNase I hypersensitive sites (DH sites), nucleosome-free regions, are located over the critical heat shock elements (HSEs) (centered at -59 and -340 relative to the transcription start site). We wished to determine the functional significance of this structure, and to identify the elements that dictate this organization. *hsp26* constructs containing site-directed base substitutions, deletions, or rearrangements of the upstream regulatory elements have been fused in frame to the *E. coli lac* Z gene and reintroduced into the *D. melanogaster* genome by P-element-mediated germ line transformation. Chromatin structure of the transgenes was analyzed (prior to gene activation) by DNase I, MNase, or restriction enzyme treatment of isolated nuclei, and heat-inducible expression was monitored by measuring β-galactosidase activity. The results indicate that correct positioning of the DH sites is essential for heat-shock inducible gene expression; a shift in the positions of upstream nucleosomes (caused by replacement of nucleosomal DNA by a repetitious sequence) blocks gene activation. Two $(CT)_n$ repeats (located at -85 to -135, and -341 to -347) are the primary determinants in establishing the chromatin structure; they appear to act by binding the GAGA factor. Neither the HSEs nor the TATA box are essential for formation of the DH sites, although they play a critical role in transcription.

Gene expression is also regulated by the packaging of blocks of chromatin. Euchromatic genes juxtaposed to heterochromatin are often down regulated (position effect variegation, PEV); such down regulation is suppressed by a lack of HP1 (heterochromatin protein 1). To investigate the basis of this down regulation, we have placed a marked *hsp26* gene into heterochromatic regions using P-element transposition. The chromatin structure of these repressed transgenes is being analyzed in terms of the nucleosome array (DH sites). Exploring the effects of HP1 gene dosage on the chromatin structure of such transgenes should provide insights into the mechanism of down regulation.

MECHANISMS REGULATING FACTOR ACCESS IN NUCLEAR CHROMATIN

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The organization of eukaryotic DNA in chromatin limits the accessibility of regulatory sequences for the interaction with nuclear proteins. Mechanisms regulating factor access in nuclear chromatin therefore play a crucial role in controlling the establishment of an active state of a gene locus. We analyzed the potential of various cis-regulatory sequences to "program" nuclear chromatin for factor access during B-cell development. Gene constructs consisting of a synthetic T7 promoter and flanking reporter sequences were introduced into the germ line of mice. The accessibility of the T7 promoter in nuclear chromatin of transgenic pre-B cells was subsequently examined by incubating nuclei with T7 RNA polymerase and analyzing the synthesis of nascent T7 transcripts.

Our data demonstrate that a 95bp immunoglobulin heavy chain (μ) enhancer core element was necessary and sufficient to confer sequence-specific accessibility upon the cis-linked T7 promoter independent of its chromosomal position. This potential of the μ enhancer core did not require the presence of matrix-attachment regions nor lead to formation of DNase I hypersensitive sites and could be mechanistically uncoupled from an active transcriptional state of the transgene. By comparison, the SV40 enhancer exhibited a reduced ability to govern factor access. Thus, a specific nucleotide sequence comprising several factor binding sites is sufficient to generate a local accessibility in transcriptionally inactive nuclear chromatin. We are currently extending our analysis to the mechanisms regulating long-range factor access in nuclear chromatin.

CONTROL OF MyoD ACTIVATION DURING DEVELOPMENT

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When MyoD Is expressed in a large number of cell types it converts those cells to muscle. MyoD is a bHLH transcription factor and only this region (68 amino acids) is required for stable myogenic conversion. Experiments addressing how MyoD becomes activated in mice, worms, and frogs will be presented.

(1) In frogs, a major observation is that MyoD produced from an injected RNA is cytoplasmic until mesoderm induction occurs and then it enters the nucleus but only in the induced cells (with Ralph Rupp).

(2) In worms, we have studied an upstream activator of MyoD, skn-1, first described by J. Priess and B. Bowerman as a maternal-effect mutant. skn-1 is a very unusual DNA binding protein.

(3) In mice, we have focused on how MyoD activates its own transcription as well as the transcription of downstream myogenic structural genes and the other members of the MyoD family of myogenic regulators (i.e., myogenin, Myf-5, and Mrf-4). We show that additional cellular factors (?chaperonins) can control the homo- or heterodimer state as well as the DNA binding capacity of bHLH proteins. Using a hormone inducible MyoD chimeric protein, we show that MyoD is a primary activator of myogenin and myogenin is a primary activator of downstream myogenic structural genes.

ESTABLISHMENT OF CELL-TYPE IN *B. SUBTILIS*

R. Losick, The Biological Laboratories, Harvard Univ.

How does a cell of one type gives rise to dissimilar cell types? During sporulation in *B. subtilis* the formation of an asymmetrically-positioned septum partitions the sporangium into unequal-sized cellular compartments called the forespore and mother-cell. The cells each receive a chromosome from the parent cell but follow different programs of gene expression. What are the earliest events that establish differential gene expression? Evidence indicates that a transcription factor known as σ^F is produced shortly before the formation of the sporulation septum but does not become active in directing gene expression until after septation when its activity is restricted to the forespore. σ^F-directed gene expression in the forespore then sets in motion a chain of events that accounts for differential gene transcription in both compartments of the sporangium. Evidence indicates that σ^F is held in an inactive state in the predivisional cell and in the mother cell by SpoIIAB, an anti-sigma factor that binds to σ^F and inhibits its capacity to direct transcription. Three proteins have been identified that enable σ^F to escape from the inhibitory action of SpoIIAB in the forespore. These are FtsZ, a cell division protein that is required for the formation of the sporulation septum, SpoIIE, an integral membrane that is also required for the formation of a normal sporulation septum, and SpoIIAA, a regulatory protein that is believed to antagonize the action of SpoIIAB. We propose that some unidentified aspect of septum formation drives SpoIIAA into a complex with SpoIIAB selectively in the forespore, thereby unleashing σ^F-directed gene expression in one compartment of the sporangium.

CONTROL OF SURFACE COAT GENE EXPRESSION IN AFRICAN TRYPANOSOMES.

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African trypanosomes are unicellular eukaryotic parasites transmitted by tse-tse flies. In their mammalian host trypanosomes are entirely covered by a dense surface coat. By drastically changing the composition of the coat - i.e. antigenic variation - a sub-fraction of the trypanosomes escapes immune lysis.

The coat of bloodstream trypanosomes consists of a single protein species, the Variant-specific Surface Glycoprotein (VSG). A trypanosome has some 10^3 different genes for VSGs, but usually only one of these is expressed at any time. The expressed gene is invariably located near the end of a chromosome. The trypanosome can change its coat in three ways:

1. The VSG gene in the active telomeric expression site (ES) is replaced by a copy of a different VSG gene. Replacement may be partial and limited to gene segments encoding exposed VSG epitopes.
2. Another VSG gene ES is turned on and the previously active one turned off.
3. When a trypanosome enters the tse-tse fly, it shuts off VSG gene transcription all together and replaces the VSG coat with a procyclin (PARP) coat.

In my talk I shall discuss our present knowledge of the mechanism of VSG gene ES switching in bloodstream trypanosomes and of ES inactivation during the transition from mammal to fly. Specific questions to be addressed are: What is the relative contribution of transcription initiation and transcription attenuation control in determining VSG gene transcription? What is the nature (recently solved by us) and function of the small amounts of the novel modified nucleotide pdJ enriched in sub-telomeric chromosome regions and what role does it play in silencing VSG gene ES? How can a trypanosome combine antigenic variation with specific uptake of host macromolecules, such as transferrin?

SNURPOSOMES

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Because of its large size, the amphibian oocyte nucleus or germinal vesicle (GV) is a favorable object for studying the intranuclear localization of small nuclear ribonucleoproteins (snRNPs). We have studied the distribution of all the major and several minor snRNPs by in situ hybridization and immunofluorescence in GVs of the newt Notophthalmus and the toad Xenopus. As expected from their known function in pre-mRNA splicing, U1, U2, U4, U5, and U6 snRNAs are associated with the nascent transcripts on the loops of the lampbrush chromosomes. They also occur in hundreds to thousands of extrachromosomal bodies (about 1-4 μm diameter) that we call B snurposomes ("snurp" for snRNP and "some" from the Greek for body). U7 snRNA, which is involved in processing the 3' end of histone pre-mRNA, is found in morphologically distinct bodies that we call C snurposomes. C snurposomes are much less abundant than Bs. Most of them are free in the nucleoplasm, but a few are attached to the chromosomes at the histone gene loci. A third type of extrachromosomal body, the A snurposome, contains U1 but not other tested snRNAs; so far it has been found in GVs of the newt but not the toad. Finally, U3 and U8 snRNAs occur in the multiple nucleoli, where they presumably function in pre-rRNA processing, and also in hundreds of previously unrecognized smaller granules throughout the nucleoplasm. We have not yet determined whether these smaller granules are minute nucleoli that contain rDNA and synthesize rRNA, or are a new type of snurposome that contains only the snRNPs involved in pre-rRNA processing. We propose that snurposomes are sites for preassembly and storage of snRNP complexes used elsewhere in the nucleus. In its simplest form this hypothesis suggests that there could be as many different types of snurposomes as there are processes in which snRNP complexes are involved. We are currently examining the localization of U11, U12, 7SK and other snRNAs in the GV.

DYNAMIC CHROMOSOME STRUCTURE: FOUR-DIMENSIONAL APPROACH

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Four-dimensional imaging approaches -- three-dimensions as a function of time -- are used to investigate dynamic aspects of chromosome architecture. We typically micro-inject labeled functional protein(s), or antibody fragments, to highlight a chromosomal event within *Drosophila* embryos. Wavelength multiplexing allows several components to be followed. The collection of four-dimensional image data avoids problems with fixation artifacts, antibody penetration, exclusion qualifications, and other problems usually present even in high-quality, high-resolution three-dimensional subcellular structure work. We specifically see chromosomal and nuclear structures that are changing, with characteristic motions, as a function of the cell cycle and development. We require that the embryo develop and hatch on time, indicating that minimal perturbation has taken place. This powerful approach provides a direct link between structure and function, principally because one is forced to consider and analyze the temporal variations in the structures. Progress has been made in providing a quantitative analysis of the motion components within the structures.

This methodology has been used to study the *in vivo* distribution and dynamics of topoisomerase II, an enzyme required for chromosome condensation and segregation. Topoisomerase II is localized spatially and temporally, correlating with its role in chromosome condensation and segregation. No axial core is seen along mitotic chromosome lengths.

THE ROLE OF CHROMOSOME TERRITORIES IN THREE-DIMENSIONAL GENOME ORGANIZATION AND FUNCTION

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A model for the functional compartmentalization of the eukaryotic cell nucleus is proposed. It is based on evidence (i) that chromosomes occupy discrete territories, called chromosome domains, and (ii) that the splicing machinery is located at the periphery of the chromosome domains. Firstly, we postulate that the space between adjacent chromosome domains together with the surface area of the chromosome domains defines a three-dimensional compartment for gene expression, mRNA splicing and transport to the nuclear pores, termed interchromosome domain (ICD) compartment (for supporting evidence see Lichter et al., this conference). Genes which are permanently expressed and genes whose expression needs to be regulated rapidly in a given cell type may be located at or close to the surface area of each chromosome domain. Diffusible factors controlling gene expression and mRNA splicing may be concentrated in the ICD compartment due to chromatin packaging and charge distribution effects which prevent them from penetration into the interior parts of the chromosome domains. Since access times required for diffusible factors in order to reach their specific chromosome targets depend not only on their numbers and dimensions but also strongly on the total search volume, such a compartmentalization should help to speed up gene regulation. Secondly, we postulate that the expression of genes may be permanently shut off by chromatin packaging mechanisms which move such genes away from the ICD compartment into an interior part of the respective chromosome domains. Model calculations indicate that relatively small changes of the volume and shape of a given chromosome domain can have considerable effects on the number of genes which may become exposed at its surface. Such considerations are consistent with recent findings indicating that the active X-domain (Xa) shows a more extended shape than the inactive X-domain (Xi), while volume ratios < 2 were generally determined for Xa/Xi. Charge distributions of chromosome domains may be under genetic control and not only have an effect on compartmentalization of diffusible factors but may also contribute to chromatin/chromosome domain movements in the cell nucleus. The two central postulates of this model have numerous consequences for the function of the genomes in eukaryotic cells and provide many experimental opportunities for falsification.

INTRACELLULAR TRANSCRIPT LOCALISATION AND TRANSCRIPT EXPORT IN EARLY *DROSOPHILA* EMBRYOS

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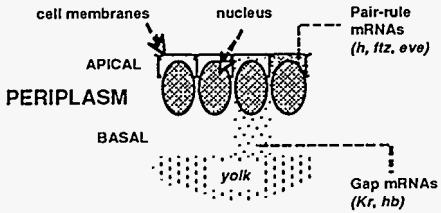
mRNAs are frequently asymmetrically localised within the cell cytoplasm to specific sub-compartments. The *Drosophila* blastoderm embryo is particularly well-suited for studying this phenomenon because initial development takes place in a large, multi-nucleate cell. During this time, transcripts from the pair-rule segmentation genes specifically accumulate apically of the layer of blastoderm nuclei (apical periplasm – see Figure). This destination is gene-specific as other transcripts are basally localised (*string*) or unlocalised (apical **and** basal; gap-genes).



We have previously studied the mechanism of apical transcript localisation by analysing protein and transcript localisation from hybrid genes. We used a *lacZ* reporter gene encoding basal transcripts to define sequences responsible for apical localisation (Davis and Ish-Horowicz, Cell *67*, 927-940: 1991). For all three pair-rule transcripts analysed, localisation depends on sequences 3' of the protein-coding sequences. A transcript retaining only 125bp of *even-skipped* 3'-sequences is apically localised. Apical localisation of such transcripts targets β-galactosidase protein apically, suggesting that protein localisation depends, in part, on transcript localisation.

We have analysed transcript localisation in several mutant genotypes that disrupt the cytoarchitecture of blastoderm embryos. These and other results argue against cytoplasmic mechanisms of transcript localisation, including selective transcript degradation, and diffusion of basal transcripts to apical binding-sites. We also exclude "gene-gating" models of transcript localisation depending on chromatin organisation (Blobel, PNAS. *82*, 8527-8529: 1985) by showing that adjacent transcripts within the same chromatin domain localise independently, according to their 3'-sequences. We propose a model in which pair-rule transcripts include 3' signals that direct them into apical pathways of nuclear export.

PROBING FUNCTIONAL ORGANIZATION WITHIN THE NUCLEUS: VISUALIZING GENES AND THEIR RNAS

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Recent studies will be summarized which provide evidence for the higher level organization of pol II gene transcription and RNA splicing in mammalian cells. The subnuclear site of transcription for the fibronectin gene was determined by simultaneous hybridization of the gene and its RNA, detected in two different colors. In 88% of nuclei, the gene was conincident with the RNA "track" or focus, and was usually localized towards one end, indicating a polarity to the track. Simultaneous visualization of introns and exons showed that the specific intron studied was present throughout only part of the RNA track defined by the cDNA probe, demonstrating that splicing occured along the track, possibly in "assembly line" fashion. The transcription/splicing site for the fibronectin gene exhibited a specific higher-level organization, such that in the majority of cells it associated with larger "transcript domains" enriched in poly A RNA, snRNPs, and SC-35. It was most frequently postioned just at the periphery of these domains. The three-dimensional topography of the 20-40 transcript domains enriched in RNA and processing components was studied using digital imaging microscopy. These regions were shown to represent non-contiguous centers which lie in a single plane just below the midline of the nucleus in three different cultured cell types. These domains, implicated as regions of increased RNA metabolic activity, are within the interior of the nucleus, some distance from the nuclear envelope. Ongoing studies concerning the position of other genes and primary transcripts with respect to these regions will be summarized. In addition, other work will be described which utilizes a new approach for studying the arrangement of specific DNA sequences with respect to chromatin loop domains. Results thus far provide direct visual evidence for the differential packaging of replicating DNA and transcriptionally active genes. Finally, results will be presented describing the nuclear distribution of XIST RNA, which is implicated as the product of the X-inactivation center and exhibits a nuclear distribution consistent with its stable association with the inactive X chromosome in interphase nuclei.