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MASTER

# CHARACTERIZATION OF MUNTJAC DNA

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Characterization of Muntjac DNA

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# CHARACTERIZATION OF MUNTJAC DNA

## ABSTRACT

Richard C. Davis

Sister chromatid exchange (SCE) in muntjac chromosomes is generally proportional to the chromosomal DNA content, but the SCE frequency is reduced in the heterochromatic neck region of the X chromosome (Carrano and Wolff 1975, Carrano and Johnston 1977). The physical properties of muntjac DNA and the kinetics of repair of UV damage in muntjac heterochromatin and euchromatin were examined and compared with the distribution of sister chromatid exchange.

Examination of muntjac DNA showed that: (1) Muntjac DNA has two major CsCl density components. The main band with a density of 1.700 constitutes about 86% of the genome and the remaining heavy shoulder DNA consists of a mixture of density classes from 1.705 to 1.719. (2) Muntjac DNA which renatures with a Cot of less than 0.1 is almost exclusively heavy shoulder, but this rapidly renaturing fraction consitutes less than 8% of the DNA. (3) This rapidly renaturing DNA has a broad melting range around 83°C. (4) Late replicating muntjac DNA is enriched in main band density sequences. (5) The heavy shoulder DNA is uniformly distributed among the muntjac chromosomes despite the fact that the muntjac X-chromosome has a very long heterochromatic centromere region. These results support the conclusions that muntjac heavy shoulder DNA consists of moderately repeated sequences which are widely interspersed throughout the genome and that DNA in muntjac heterochromatin consists of more than simple sequences.

Study of unscheduled DNA synthesis (UDS) in muntjac showed that: (1) While mitomycin C is a strong inducer of SCE, it fails to induce any significant UDS. (2) Ultraviolet light induces both SCE and UDS in muntjac chromosomes. (3) UV induced UDS is generally proportional to DNA content in muntjac chromosomes but is reduced in the heterochromatin of the X chromosome of cells irradiated in plateau. (4) UV induced SCE are similarly distributed in chromosomes of cells irradiated after release from plateau. (5) In cells irradiated after release from plateau, the repair interval between irradiation and entry of the cells into S fails to significantly influence the level of SCE ultimately expressed in euchromatin or heterochromatin. During the same interval a substantial fraction of the lesions leading to UDS are removed. These results are consistent with the conclusions that the UV-induced SCE-producing lesion is long lived compared to the UDS-inducing lesion and that the production of SCE in the heterochromatin and euchromatin of muntjac is independent of any repair process that both takes place on the time scale of unscheduled DNA synthesis, and is blocked by entry of the cells into S phase.

Arthur A. Tobey March 17, 81
Chairman Date

Anthony V. Carrano 16 March 1981
Co-Chairman Date

# ACKNOWLEGMENTS

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At the risk of offending all others who have worked heroically in my behalf, I would like give special thanks to two people:

Anthony Carrano has provided much encouragement, many helpful ideas, unwavering support, and has always been there when things needed to be discussed. I thank you for a smooth and pleasant graduate-studenthood.

Marianne, my wife, has been the fount of enthusiasm and support from the day I received my acceptance to graduate school, through the midnite study sessions and 24 hour experiments, and through months of writing and "paper explosions" in the living room. At the risk of becoming a computer-widow as well as a thesis-widow, she unreservedly endorsed the purchase of the Apple which did most of the text-editing and drew most of the figures in this thesis. My very special thanks to you.

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INTRODUCTION:

The phenomenon of sister chromatid exchange (SCE) was demonstrated by Taylor in 1957 (Ta57) using autoradiography of cells cultured for one round of DNA synthesis in tritiated thymidine followed by one round of synthesis in unlabled medium. Latt (La73) introduced a technique for direct observation of SCE by fluorescent microscopy of cells which had been grown for two rounds of replication in the presence of the thymidine analog 5-bromo-deoxyuridine (BrdUrd). Wolff and Perry (Wo74a, Pe74) extended this procedure so that the sister chromatid exchanges could be observed in Giemsa stained cells without the use of fluorescence microscopy. This technique, called the fluoresence plus Giemsa (FPG) method, is currently the most common technique for observing SCE's. These procedures have been used to determine a few basic features of chromsome structure and sister chromatid exchange:

1) SCE's involve four strand breaks in the DNA of the affected sister chromatids (Wo64).

2) The 5'-3' polarity of the rejoined strands is conserved (Ta58), (Ti75).

3) The baseline levels of SCE which are observed may be at least partially induced by the incorporation of tritiated thymidine (Wo64) or BrdUrd used for SCE detection (St77, Wo74a).

4) The number of SCE observed may be markedly increased above the baseline levels by prior exposure of the cells to DNA damaging agents such as ultraviolet light (Ka73) or certain chemicals which chemically react with the DNA (Pe75, Ca77, Ca78) or which intercalate between the stacked base pairs (Ca78).

5) The lesions engendered in the DNA by chemicals or radiation must persist to a succeeding S-phase of the cell if they are to result in SCE formation (Wo74).

6) For a number of chemicals and for UV, both the number of SCE's formed and the number of mutations induced in exposed cells is a linear function of the dose (Ca78), though the relative efficiencies for each agent and response may be quite different.

7) The number of indiv CE's per chromosome is proportional to the DNA content of the chromosome (Ca77).

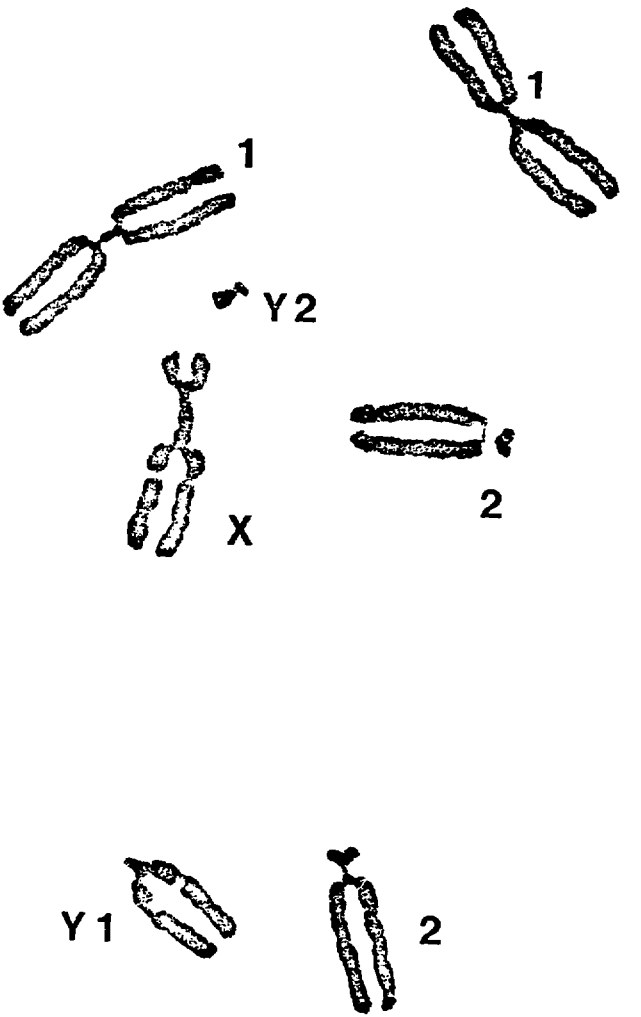
8) The induced SCE's per unit DNA are less prevalent in the constitutive heterochromatin than in the euchromatin or boundaries between heterochromatin and euchromatin (Ca77, Ca75).

The relationship between the nature of heterochromatin and the mechanism of reduced SCE formation is unclear. There have been some authors who have failed to find suppressed sister chromatid exchange formation in heterochromatic regions (Ki75, Sc76, Na75) in human and Microtus agrestis, but investigators working with muntjac (Ca75, Ca77), Chinese hamster and Microtus montanus (Hs76), kangaroo rat (Bo76a), and Allium cepa (Sc77) have observed this suppression.

Carrano et al. (Ca75, Ca77) chose to work with muntjac because of its unique karyotype. The chromosome complement of the Indian muntjac (Muntiacus muntjac vaginalis) as described by Wurster and Benirschke (Wu70, Wu72) contains seven chromosomes in the male and six in the female. Each of the chromosomes is readily identified without special banding procedures. In the male (Fig. 1) the number one autosome-pair consists of large metacentric chromosomes with secondary constrictions on the distal region of one homolog. The number two autosome-pair are acrocentrics about two thirds the length of the number one chromosome. The X-chromosome consists of a euchromatic long arm about the size of the number two chromosome, containing a secondary constriction in the proximal region and connected by a very long

Figure 1. Metaphase chromosomes of the male muntjac; Muntiacus muntjak vaginalis.

FIGURE 1



heterochromatic neck or stalk to a small arm. The Y1 chromosome is a telocentric similar in size, banding pattern, and secondary constriction to the euchromatic portion of the X. The Y2 is the small sex determining chromosome often called the "dot" chromosome. It was the neck region of the X-chromosome that was so important in Carrano's studies of sister chromatid exchange frequency in heterochromatin. The great length of this heterochromatic region made it possible to count SCE's and compare their frequencies with those in euchromatin. The Y2 chromosome in the muntjac and the centromere regions of the other muntjac chromosomes are also C-band positive (Ka74a) (Fig. 2) but these heterochromatic re not long enough to measure internal SCE frequencies.

The lesions to the DNA which promote sister chromatid exchange and the cellular mechanisms which lead to their production are not well understood. There is no accepted model which explains why the production of SCE would be different in heterochromatin than in euchromatin. This lack of understanding may be expected in light of the fact that SCE's have only been observable by the FPG technique for less than ten years. However, the nature of DNA in heterochromatin and the production and repair of DNA lesions have a much longer history. It was from these vantage points that the work reported here was undertaken. First, the general properties of muntjac DNA and its components were measured and compared with the DNA in the heterochromatin and euchromatin of other species. Secondly, the DNA of the muntjac X-chromosome was isolated and compared with that of the other muntjac chromosomes. Finally, the rate of repair of well characterized lesions was compared in muntjac euchromatin and heterochromatin with the rate of production of SCE's in this repaired DNA. The results provide some information related to currently proposed models of sister chromatid exchange.

Figure 2. Metaphase chromosomes of the male muntjac treated by the C-band technique (from Ca75).

# FIGURE 2

BULK DNA OF THE MUNTJAC

Constitutive heterochromatin is, by definition, that part of the chromatin that remains relatively more condensed or is more densely stained by nucleophilic dyes throughout the cell cycle (e.g., Su72). This heterochromatin is most frequently associated with the centromeric regions of chromosomes and there is some evidence that it is distributed toward the periphery of the interphase nucleus (Ha74). The DNA in heterochromatin has often been found to contain highly repetitive DNA sequences, that is, the sequence of specific deoxyribonucleotide bases along the DNA strand is found to be non-random and in fact repeats itself or nearly repeats itself every several to several hundred bases. Because of the simplicity, the brevity and the extreme repetitiousness of these sequences, it is generally assumed that they cannot code for the sequence of amino acids in a protein. For example, in Drosophila virilis it has been found (Wa76) that there are thousands of the tandem repeat units of the sequence 5'-AXAAAXT-3' where "X" denotes a variable base. In fact, variants of this simple sequence constitute about 25% of the genome. There is no agreed upon theory explaining the usefulness of this repetitious DNA to the eucaryotic cell, but there are a number of standard techniques for characterizing the number, size, and base composition of repeated DNA sequences. Several of these were used in this research and have been used by others to characterize the DNA in the muntjac.

Three main questions were asked in studying the bulk DNA of the muntjac:

1) What are the general physical properties of muntjac DNA?

2) Is there any readily identified subcomponent of muntjac DNA which can be associated with heterochromatin in general or the heterochromatic neck of the X-chromosome in particular?

3) Is muntjac heterochromatin late replicating? The major approaches used to study bulk DNA of the muntjac were: (a) cesium chloride equilibrium-density-gradient profiles, (b) reassociation kinetics of thermally denatured DNA, and (c) hyperchromicity of thermally denatured DNA.

CESIUM CHLORIDE DENSITY GRADIENTS:

Theory

A short sequence of DNA bases can easily have a base composition which is slightly different than the average DNA of the cell. If this sequence is repeated many times within the genome then there exists a significant subpopulation of the DNA which has an unusual base composition. It has been found that DNA's with different base compositions have corresponding differences in bouyant densities. Schildkraut (Sc62) presents the formula:

$$\% GC = \frac{\rho - 1.660}{0.098}$$

and %AT = 1 - %GC

where ($\rho$) is the bouyant density of the double-stranded DNA in CsCl and %AT is the percentage of the nucleotide pairs in the double helix consisting of adenine and thymine. Many, though not all, of the repeated sequences in eucaryotic DNA have bouyant densities which differ from that of the remainder of the DNA. These DNA components with differing density are historically called satellite DNA's while repeated sequences having the same bouyant density as the bulk of the DNA (Ce76) are referred to as "cryptic satellites".

Hinton and Dobrata (*Hi76*) present a detailed development of the theory of the distribution of biological molecules in equilibrium density gradients. A uniform aqueous solution of cesium chloride when placed in a centrifuge will become nonuniform under the influence of the centrifugal force field until the flux of atoms displaced by the centrifugal force is balanced by the flux of diffusion which tends to restore a uniform solution. The resulting concentration gradient of cesium chloride also represents a density gradient, where the gradient takes the form:

$$dc/dr = k(T)r\omega^2$$

where c is the concentration of the solute (CsCl), r is the distance from the center of the rotor, k(T) is a temperature dependent constant and $\omega$ is the angular velocity of the rotor. The density of the gradient will be the same as the initial uniform solution at a distance $r_o$ from the center of the rotor where:

$$r_o^2 = \frac{r_t^2 + r_b^2}{2}$$

where $r_t$ and $r_b$ are the distances of the top and bottom of the gradient, respectively, from the center of the rotor (F167).

DNA molecules in the solution will tend to migrate in the density gradient until they reach their own bouyant density. Subpopulations of the DNA which have lower densities in CsCl will reach equilibrium at a point in the centrifuge tube closer to the center of the rotor, while higher density fractions will tend to equilibrate further from the axis of rotation. The DNA density

subpopulations which are separated in cesium chloride equilibrium gradients can be measured either via direct absorption spectroscopy at 254 nm in analytical ultracentrifuges or they can be physically separated by fractionating preparative gradients.

MATERIALS AND METHODS:

Preparative gradients:

All gradients were formed in polyallomer tubes (5/8 inch X 2-1/2 inch) using 3.95 grams cesium chloride, Harshaw Chemical Co. and 3.11 to 3.14 grams liquid including sample DNA. Water or 0.06 M KCl - 0.5 mM EDTA was used as the liquid to bring the sample to its final weight and density of 1.77 g/ml. The mixture was agitated gently to dissolve the cesium chloride and the remainder of the tube's volume was filled with mineral oil. The tube was capped and all air bubbles were displaced with mineral oil before the tube was sealed. The samples were spun at 35,000 RPM for 3 days at 20°C in a Beckman 50 fixed-angle rotor.

The gradients were fractionated by puncturing the bottom of the tube. The gradient was displaced from the tube by injecting measured aliquots of mineral oil through the tube's cap. Normally, 40 fractions of 0.1 ml were collected from each tube.

If the DNA content was to be measured by UV absorbtion, then each fraction was diluted with 0.1 ml of 0.06 M KCl-0.5 mM EDTA. The absorbtion of each fraction at 254 nm was then measured against a similar aliquot of KCl-EDTA.

If the DNA content of the fractions was to be measured by scintillation counting then the fractions were collected in 25 ml glass scintillation vials.

Each fraction was diluted with 0.5 ml water or KCl-EDTA and 10 ml of Insta-Gel scintillation counting solution (Packard) was added to each vial. The samples were counted in a Packard scintillation counter with the gain and energy windows set such that less than 0.5% of the tritium decays were detected in the carbon-14 channel and about 8% of the $^{14}C$ decays were detected in the tritium channel. Background for each channel was 10-20 CPM. Scintilation counts were analyzed by: (a) subtracting background from each channel, (b) correcting for spillover of the $^{14}C$ counts into the tritium counts for each fraction and (c) normalizing the counts for each nuclide in each fraction to the sum of the counts in all the fractions for that nuclide. The data were then plotted with the fraction of the counts for each nuclide as a function of the fraction number.

Analytical gradients:

Analytical gradients were carried out with dual sector cells. The second sector was used as a absorbtion reference and carried all ingredients of the sample sector except the DNA. The capacity of each sector was about 0.6 ml and the path length was 1.1 cm. When purified DNA was analyzed for each sample, a mixture of 0.645 gm CsCl (Harshaw, optical grade) and 0.5 ml liquid including about 3 micrograms sample DNA and 3 micrograms poly dAT as a density marker was prepared for each sample sector. The bulk of the liquid was 0.06 M KCl-EDTA. The samples were run in a Beckman 40 titanium analytical rotor for 60 h at 45,000 RPM in a model E ultracentrifuge and then scanned for relative absorbtion at 254 nm. The poly-dAT marker DNA was assigned a cesium chloride density of 1.679(Sz68) when analyzing the absorbtion profiles.

The bouyant densities of other points on the gradient were assigned on the basis of the formula (FI67):

$$\rho_s = \frac{\rho_m \omega^2}{2\beta_o} (r_s^2 - r_m)^2$$

where

$\rho_s$ is the density of the unknown

$\rho_m$ is the marker density (1.679)

$\omega$ is the angular velocity of the rotor in radians/sec

$r_s$ and $r_m$ are the radial displacements of the unknown and marker DNA's respectively, and

$\beta_o$ is a constant dependent on the mean density of the gradient, in this case: $1.190 \times 10^9$.

ISOLATION OF BULK DNA FROM CULTURED CELLS.

Muntjac cells were grown to near confluency in Falcon plastic roller bottles (490 $cm^2$). One to 12 rollers, each containing about $2 \times 10^7$ cells were used. The medium was removed and the cells harvested by treatment with trypsin. The cells were removed from trypsin by centrifugation at 800rpm for 5 m and resuspended in 0.06M KCl-0.5 mM EDTA at approximately 1 ml per $10^7$ cells. To each ml of cellular suspension, 0.1 ml of sarcosine in 45% ethanol and 0.1 ml of pronase (2mg/ml in 0.1M NaCl, 0.01M

Tris buffer, pH 7.5) was added to lyse cell membranes and digest proteins associated with the DNA. The solutions were gently mixed with the cellular suspension and allowed to digest at room temperature for 3-4 h. An equal volume of cold 100% ethanol (EtOH) was then layered on top of the lysis mixture and the DNA was gently wound out on a glass stir rod. The DNA was rinsed twice in cold 70% EtOH and stored refrigerated at 4°C in 100% EtOH. When needed, the DNA was dried in air, weighed, clipped into small pieces with fine sissors and dissolved by gentle agitation in 0.01 M KCl, 1 mM EDTA at a concentration of 0.5 to 1.0 mg/ml. Further purification of the DNA from contaminating protein and RNA was achieved by isolation of the DNA containing fractions from preparative CsCl gradients. The ultraviolet absorption spectrum of such DNA is shown in Fig. 3. The cesium chloride was removed and the DNA concentrated by negative pressure dialysis. The absorption of the DNA at 230 nm and 280 nm (as indicators of RNA and protein contamination) was not improved by isoamyl alcohol-chloroform or phenol extraction.

Density Profile of Muntjac DNA

A cesium chloride gradient of tritium labeled muntjac DNA is shown in Fig. 4. The individual points represent the measured activity in each fraction. The solid line represents a mathematical fit to the points. The density profile is a single asymmetric peak with an extended "tail" on the high density (low fraction number) side of the peak. In this paper the high density fraction will be called heavy shoulder DNA though it is not resolvable as a distinct peak. DNA with the modal density will be called main band DNA (Co71). A single density component would be represented in a cesium chloride gradient as simple gaussian with a coefficient of variation dependent on the molecular weight of

Figure 3. Ultraviolet absorbtion profile for purified muntjac DNA in 0.06 m KCl, 0.5 mM EDTA measured against a blank cuvette containing the same buffer.

**FIGURE 3**

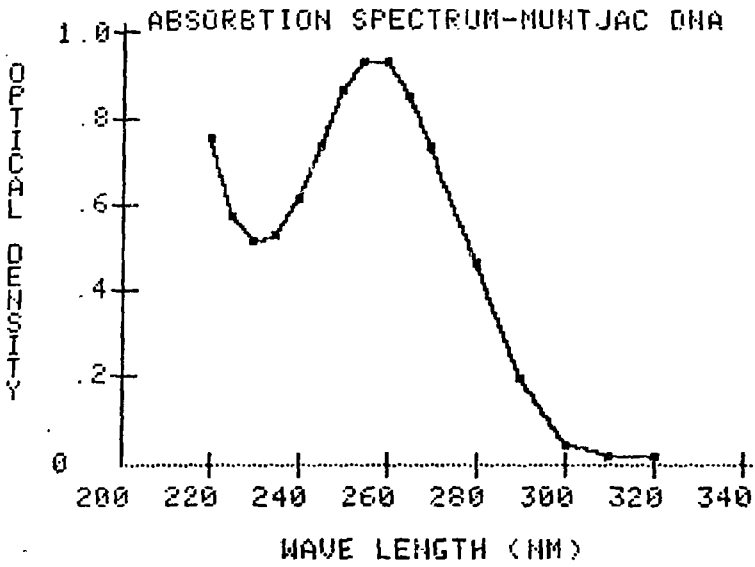
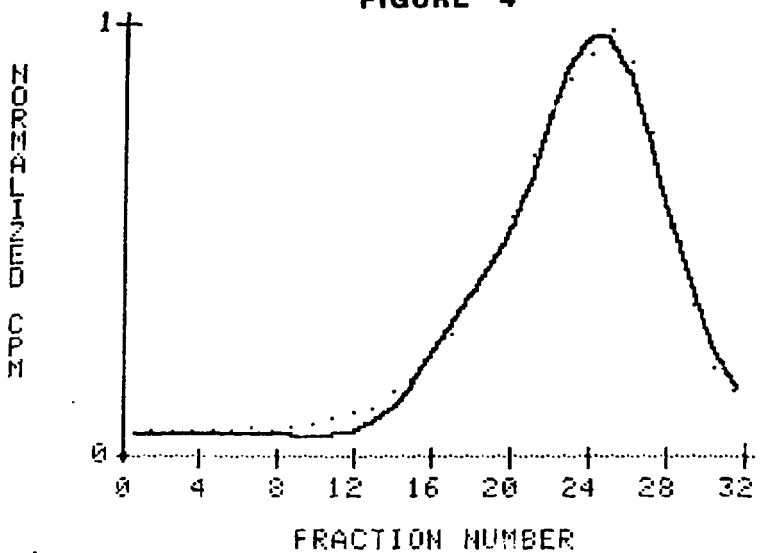

ABSORBTION SPECTRUM-MUNTJAC DNA
OPTICAL DENSITY
1.0
.8
.6
.4
.2
0
200
220
240
260
280
300
320
340
WAVE LENGTH (NM)

Figure 4. Preparative CsCl gradient of tritium *labeled muntjac* DNA. Higher fraction number corresponds to lower density. The data points indicate the normalized radioactivity in each fraction. The solid curve represents the least squares fit of two gaussians to the data.

**FIGURE 4**



the DNA. If it is assumed that the muntjac DNA consists of two density components, the heavy shoulder and the main band, then the cesium chloride density profile would be represented as the sum of two gaussians. It was assumed that the heavy shoulder and main band DNA have the same molecular weight so that the two gaussians would have equal coefficients of variation (Fl67). Fig. 5 shows the computer generated fit to the data points in Fig. 4. A least squares approximation was used to determine the position of the means and the areas of two gaussians with equal coefficients of variation (D. Moore, personal communication). The sum of these two curves is shown as the solid line superimposed on Fig. 4. The smaller gaussian, representing the heavy shoulder DNA, comprises about 14% of the area of this summed curve.

Physical Isolation of Muntjac Heavy Shoulder DNA

In order to more closely examine the "heavy shoulder" density profile of muntjac, DNA isolated from cultured cells was fractionated by means of a preparative cesium sulphate gradient. In this experiment, the gradient included mercury chloride to enhance the density difference between the GC-rich DNA and the main-band and AT-rich DNA's. The absorption profile of the resulting gradient is shown in Fig. 6. Fraction number one corresponds to the highest density and fraction 40 to the lowest. The mercury acts to make GC-rich DNA appear in the low density region of the gradient with a wider separation from the main band than it would have in normal equilibrium CsCl gradients (Sz68, Wi76). As shown in the figure, fractions 13 through 28 were pooled for "MAIN BAND" and fractions 29 through 40 were pooled for "HEAVY SHOULDER".

These two DNA fractions (main band and heavy shoulder) were dialyzed to remove the cesium sulphate and the mercury chloride. The density profiles of these two fractions were then examined in analytical cesium chloride

Figure 5. Two gaussians with equal coefficients of variation found by least squares approximation to describe distribution of activity in CsCl density gradient of muntjac DNA. The sum of these two curves is the distribution shown in Figure 4. Again, the data points are the measured activity in each fraction. The smaller heavy shoulder peak is about 14% of the DNA. The means given in the figure are the fraction numbers for the means of the fitted gaussians.

**FIGURE 5**

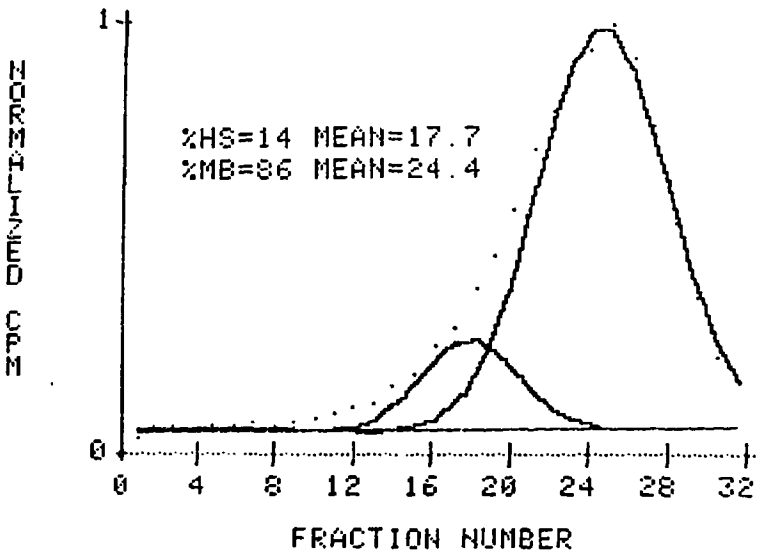
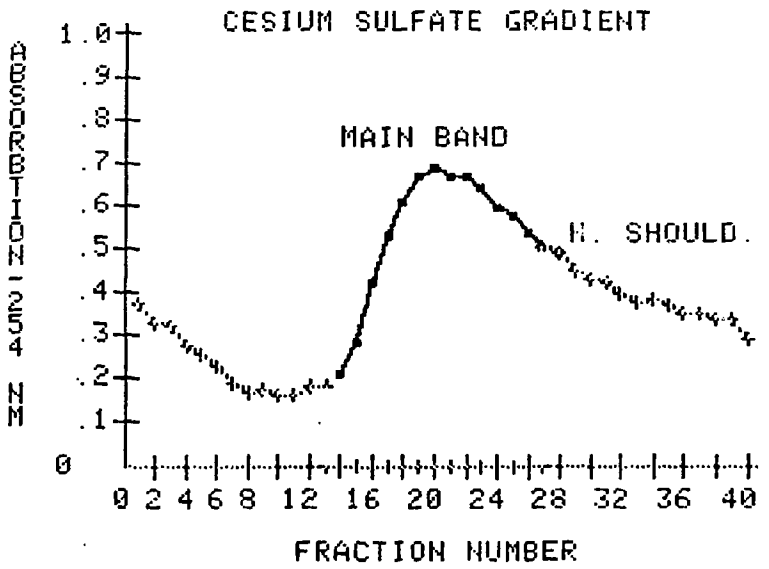

1
0
NORMALIZED CPM
%HS=14 MEAN=17.7
%MB=86 MEAN=24.4
0
4
8
12
16
20
24
28
32
FRACTION NUMBER

Figure 6. Preparative mercury-cesium sulphate gradient of muntjac DNA, showing cuts taken for main band and heavy shoulder DNA. Density decreases with increasing fraction number. The mercury shifts the G-C rich DNA to the less dense region of the gradient, and provides greater separation for deviant density components. Even with increased separation, the heavy shoulder does not form a separate peak.

**FIGURE 6**


CESIUM SULFATE GRADIENT
MAIN BAND
N. SHOULD.
ABSORBTION-254 NM
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0 2 4 6 8 12 16 20 24 28 32 36 40
FRACTION NUMBER

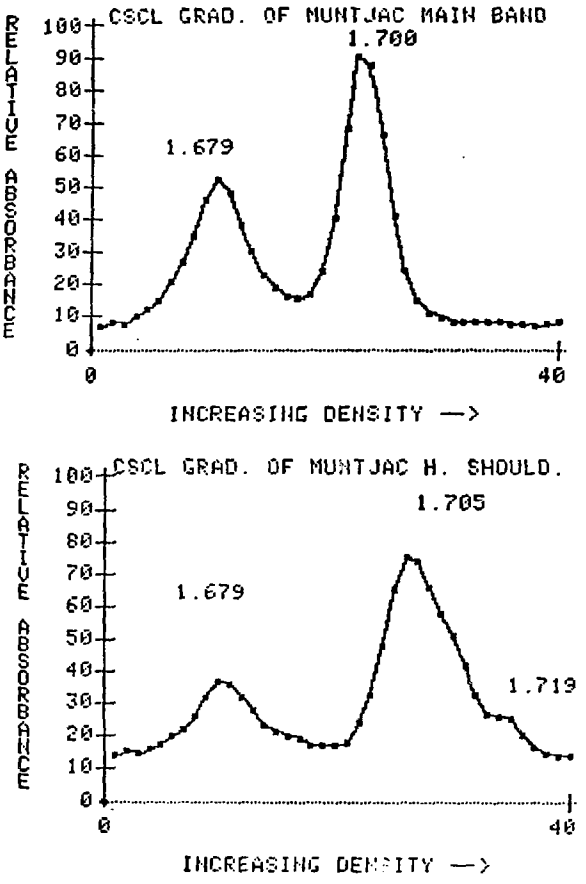
gradients. The absorption profiles of these two analytical gradients are shown in Fig. 7a and 7b. For analytical gradients, the highest density region is to the right on the graph. The peak corresponding to the poly-dAT density marker is labeled with its density of 1.679. The densities of the other peaks are marked on the graph as they were calculated from their relative positions in the gradient by the mathematical relationship given in materials and methods. The original data was a continuous-line absorption profile and the data points on the graph represent 40 evenly spaced points on the original data which were digitized for computerized plotting.

Technically, the distance along the ordinate does not represent a linear increase in the density of the gradient, but the relationship does not diverge significantly from linearity between marked points so that rough visual interpolations may be made.

The density profile of the purified main band (Fig. 7a) appears to consist of a single gaussian with mean CsCl density of 1.700. This density corresponds to a G-C base pair content of 41%. There probably are other minor subcomponents having slightly deviant densities on either side of the mean but this peak is indicative of a homogeneous density class comprising the main band. The profile of the heavy shoulder (Fig. 7b), by contrast, does not obviously consist of any single density component or components. Rather this DNA consists of a heterogeneous mix of density classes with defined density peaks at 1.705 (46%GC) and 1.719 (60%GC). There appear to be a number of subcomponents between these densities that are not resolvable by this technique.

Figure 7. Analytical CsCl gradients of the main band (above) and heavy shoulder (below) DNA obtained from the cesium sulphate gradient of figure 6. Density increases from left to right in these figures. The poly dAT density marker is assigned a density of 1.679. The heavy shoulder consists of a mixture of density components from 1.705 to 1.719. The main band is a symetrical peak with density 1.700.

**FIGURE 7**


CSCL GRAD. OF MUNTJAC MAIN BAND
1.700
1.679
RELATIVE ABSORBANCE
100
90
80
70
60
50
40
30
20
10
0
0
40
INCREASING DENSITY —>
CSCL GRAD. OF MUNTJAC H. SHOULD.
1.705
1.679
1.719
RELATIVE ABSORBANCE
INCREASING DENSITY —>

Density of Rapidly Renaturing DNA

DNA associated with heterochromatin often has highly repeated nucleotide sequences. When DNA is thermally denatured and then held under suitable conditions, these repeated sequences have the most rapid kinetics of reassociation (We68, Br74a). In these experiments, cesium chloride equilibrium gradient analysis was used to determine what density class or classes is associated with the most highly repeated sequences in the muntjac genome.

In this experiment:

1) Muntjac DNA was labeled with either tritium or carbon-14. Muntjac cells were maintained in exponential growth for at least three days in the presence of either $^{3}$H-TdR, 1 μCurie/ml, 42 Ci/mM, or $^{14}$C-TdR, .01 μCi/ml, 60 mCi/mM.

2) The labled DNA was isolated as described previously.

3) The tritium labled DNA was fractionated on a preparative CsCl gradient into "main band" and "heavy shoulder" components. See Fig. 8.

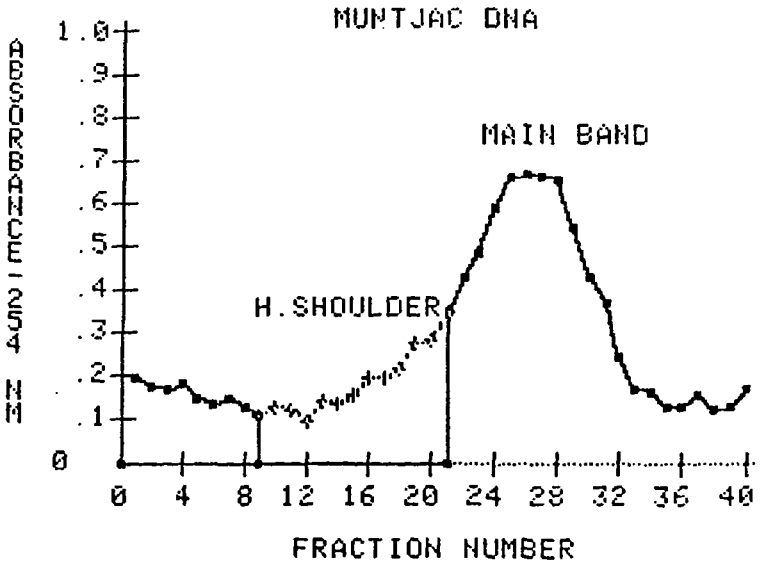
4) The two tritium labled components were dialyzed against KCl-EDTA to remove the CsCl, concentrated by evaporation, and dialyzed against phosphate buffer, pH 7, 0.05M.

5) All three DNA's (the two tritium labled components and the $^{14}$C labeled bulk DNA) were sheared by sonication for three 10 second intervals separated by 1 minute on ice. Under these conditions sonication results in 400 to 800 bp DNA fragments (Sc62).

6) Both tritium labeled components were brought to 0.12M phosphate buffer, thermally denatured for 10 minutes at 100°C and allowed to reassociate to a Cot of 0.1 at 60°C.

Figure 8. Preparative CsCl gradient showing the cuts taken for heavy shoulder and main band density components in experiment to determine density of rapidly renaturing DNA. Density decreases with increasing fraction number.

**FIGURE 8**


MUNTJAC DNA
ABSORBANCE 254 NM
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
MAIN BAND
H.SHOULDER
0 4 8 12 16 20 24 28 32 36 40
FRACTION NUMBER

7) The reassociated fraction of each component was recovered by HAP chromatography. The chromatography was carried out by a modification of the methods of Britten (Br67, Br68, Ko71, Be71). Bio-Rad DNA-grade hydroxyapatite was prepared in pasteur pipet columns blocked with siliconized glass wool. Fig. 9 shows the retention of such columns for thermally denatured and native bulk DNA. At phosphate buffer concentrations of greater than 0.125 M almost all single strand DNA has been eluted while double stranded DNA elutes between 0.15 and 0.25 M.

Tritium labeled main band and heavy shoulder muntjac DNAs were separately denatured, reassociated to a Cot of 0.1 and placed on such columns. The columns were rinsed with 0.125 M phosphate until counts in the eluent contained only background levels of radioactivty. The columns were then rinsed with 0.5 molar phosphate to collect the reassociated double stranded DNA.

8) The reassociated, tritium-labled DNA from heavy shouler and main band DNA were separately combined with bulk $^{14}C$ labeled control DNA, and analyzed on preparative CsCl gradients.

The resulting gradient for reassociated heavy shoulder DNA is shown in Fig. 10. The $^{14}C$ labled bulk DNA (native DNA) shows the normal muntjac profile with an asymmetry extending the heavy shoulder on the dense portion (lower fraction number) of the gradient. The tritium counts from the reassociated heavy shoulder DNA are concentrated in the region corresponding to heavy shoulder in the control ($^{14}C$) DNA.

The reassociated counts from main band DNA were too few to form a smooth peak but most of these counts were also in the heavy shoulder region (data not shown).

Figure 9. Retention of hydroxyapatite for native and thermally denatured DNA as a function of phospate buffer concentration.

**FIGURE 9**

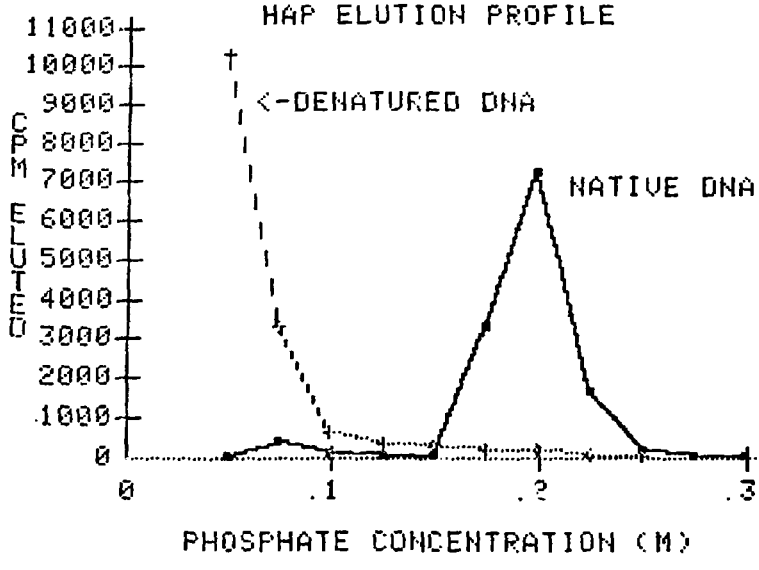
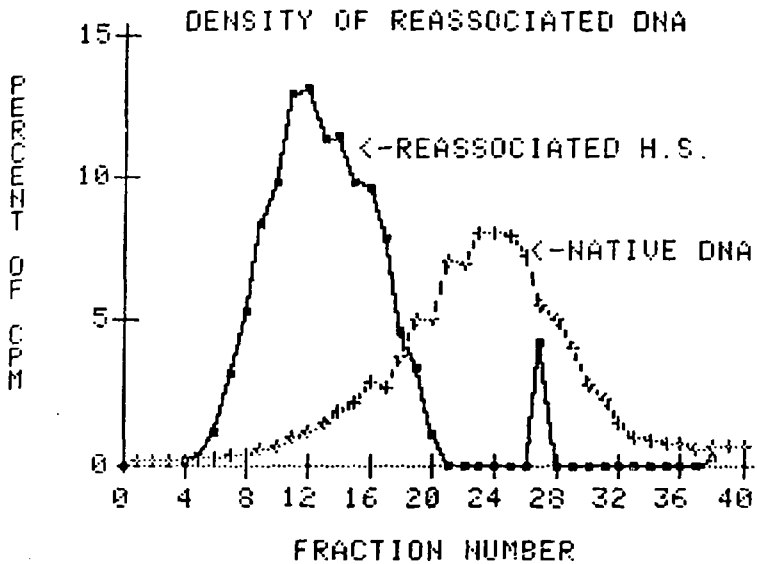

HAP ELUTION PROFILE
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CPM ELUTED
<-DENATURED DNA
NATIVE DNA
0
.1
.2
.3
PHOSPHATE CONCENTRATION (M)

Figure 10. CsCl density gradient profile of rapidly renaturing fraction recovered from heavy shoulder DNA. The reassociated DNA was found in the same fractions as heavy shoulder of native DNA on the same gradient. Density decreases with increasing fraction number.

**FIGURE 10**

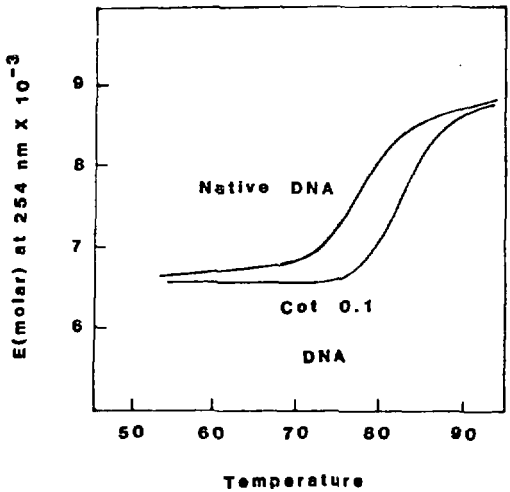

DENSITY OF REASSOCIATED DNA
PERCENT OF CPM
15
10
5
0
<-REASSOCIATED H.S.
<-NATIVE DNA
0
4
8
12
16
20
24
28
32
36
40
FRACTION NUMBER

Thermal Denaturation of Rapidly Renaturing DNA

The rapidly renaturing component of muntjac DNA was futher characterized by thermal denaturation hyperchromicity profile. The melting profile of DNA gives an indication of its average base composition, the homogeneity of base composition and the fraction of bases which are in stable base paired configuration (Ha74). Muntjac DNA reassociated to a Cot of 0.1 was isolated as described above and dialized against 0.06 M KCl, 0.5 mM EDTA. This DNA and similarly sonicated bulk DNA were placed in quartz cuvettes in a temperature controlled Beckman UV absorption spectrometer. Absorbtion at 260 nm was measured as a function of temperature for both samples. The data as fit and analyzed by the methods of Hatch et al (Ha74) are shown in Fig. 11. The optical density of the DNA at 260 nm increases as it denatures. The UV absorption of the DNA solution was corrected for thermal expansion and converted to molar extinction coefficient. The melting temperature was taken as the point where the increase in the extinction coefficient reached 50% of the total increase observed during the denaturation. The melting range ($\sigma$) is the temperature range over which the extinction coefficient rose from 25% to 75% of its full range (Hatch, personal communication). The melting temperature of the rapidly reassociating DNA was found to be about 83°C ($\sigma$= 6 degrees) while bulk muntjac DNA melted at 78°C ($\sigma$= 7.2 degrees). The relatively high melting temperature of the rapidly reassociating fraction is to be expected for DNA having a higher G-C base pair content (Ma68). However the relatively large melting range is not indicative of a single homogeneous satellite-like DNA but is more consistent with a mixture of several repeated DNA sequences with similar but not identical melting points. Hyperchromicity is the percentage increase in the optical density from

Figure 11. Molar extinction coefficients of native muntjac DNA and the rapidly renaturing fraction shown as a funtion of temperature. The hyperchromicity of both DNAs is about 35%. The native muntjac DNA melts at 78°C in 0.06M KCl, 0.5mM EDTA. Melting range (sigma) = 7.2 The rapidly renaturing fraction melts at 83 C with melting range = 6.0.

**FIGURE 11**


E(molar) at 254 nm X $10^{-3}$
9
8
7
6
Native DNA
Cot 0.1
DNA
50
60
70
80
90
Temperature

native DNA below the melting point to completely denatured DNA. The hyperchromicity of both DNA's was about 35%, indicating that the renatured DNA was relatively well base paired.

Comings (Co71) studied the density profile and some reassociation kinetics of munjac DNA. He reports that muntjac DNA contains a main band density satellite constituting 4% of the genome and that the shoulder is not satellite. However, there are some difficulties with the evaluation of this work. Comings does not specify the reassociation Cot used. No attempt was made to separate the reassociated DNA from the single strand material and so the putative satellite is a very small bump on the tail of the single stranded material. Finally, the density difference between heavy shoulder and main band is not much greater than Comings' gradient to gradient variation in peak position for single strand DNA.

Density of Late Replicating Muntjac DNA

Several authors have looked at the time of replication of the heterochromatic neck of the X-chromosome of muntjac (Co71, Ka74, Sh74, Br79, Ki80). Comings (Co71) reported that heterochromatin of the muntjac replicated most intensively during the 5th to 7th hours of an 8 hour S-phase. Similarly, Kimura (Ki80) found that thymidine incorporation in the neck began in the second hour of S and rose steadily to peak during hours 4 through 6, but then declined sharply during the 7th and 8th hours. The following experiments measured the density distribution of muntjac DNA replicated late in S.

Length of G2

To determine the length of the G2 phase, exponentially growing muntjac cells in tissue culture rollers were continuously labeled with $^{14}C$ thymidine

60 mCi/mMole, 0.01 μCi/ml for 1 week. The cells were then pulse labeled for 1 hour with tritiated thymidine 42 Ci/mMole, 1 μCi/ml. The labeled medium was removed, the cells were rinsed with PBS and returned to unlabeled medium. Each hour, starting 2 hours later, sample rollers were treated with Colcemid for 1 hour. Mitotic cells were harvested by shakeoff and the incorporated label in the DNA was measured by TCA precipitation and scintillation counting. The recovered $^{14}C$ was taken as a measure of the number of cells harvested and the tritium to $^{14}C$ ratio was interpreted as a measure of the level of S-phase synthesis which was taking place in the harvested cells at the time of the pulse. The results are shown in Fig. 12. Pulse labeled cells begin to be recovered by mitotic shakeoff about 3.5 hours after the removal of the labeled medium.

Density of Late Label

The CsCl density profile of late replicating muntjac DNA was determined in cells obtained by mitotic shake-off at various times after the pulse label. Cells were labeled as above. At 3.5 and 5 hours after the pulse, sample rollers were treated with Colcemid for 1.5 hours. The cells recovered by mitotic shakeoff were pelleted by centrifugation and lysed for cesium chloride gradient analysis. The results for both Colcemid treatments were the same. Cells treated with Colcemid at 5 to 6.5 hours had the DNA density distribution shown in Fig. 13. The continuous label has the appropriate density distribution for bulk muntjac DNA with a slight shoulder or asymmetry on the high density side of the main band peak. The pulse labeled DNA is enriched in main band and depleted in heavy shoulder activity.

The samples were treated with Colcemid from 3.5 to 5 hours and from 5 to 6.5 hours after the pulse labeling period. Taking the length of G2 as 3.5 hours,

Figure 12. Length of G2 is indicated as time required after pulse for label to appear in cells recovered in mitotic shakeoff. Cells are uniformly labeled with $^{14}C$ and pulse labeled with tritium. The tritium/$^{14}C$ ratio shown is taken as the pulse label incorporated per cell in the mitotic population. Tritium labeled mitotic cells first appear 3 to 4 hours after pulse.

**FIGURE 12**

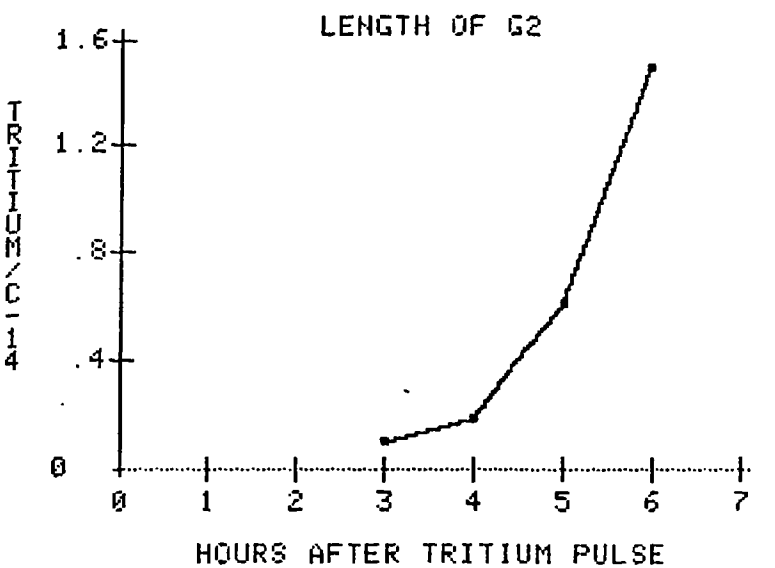
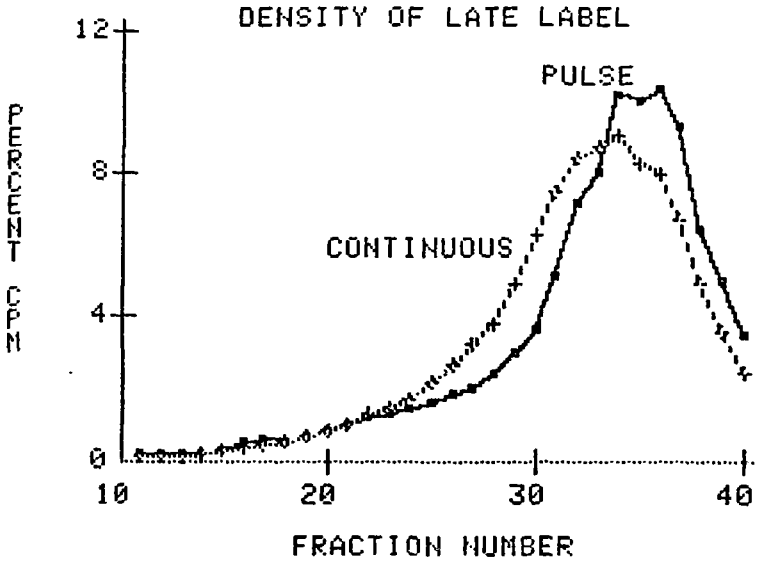

LENGTH OF G2
TRITIUM/C-14
1.6
1.2
.8
.4
0
0
1
2
3
4
5
6
7
HOURS AFTER TRITIUM PULSE

Figure 13. CsCl density gradient distribution of tritium label in mitotic cells collected 6 hours after pulse. The cells had also been continously labeled with $^{14}C$. The pulse label is concentrated in the main band fractions and depleted in the heavy shoulder fractions. Similar label distribution was seen for cells collected 4.5 hours after pulse. Density decreases with increasing fraction number.

**FIGURE 13**


DENSITY OF LATE LABEL
PULSE
CONTINUOUS
PERCENT CPM
12
8
4
0
10
20
30
40
FRACTION NUMBER

then the cells collected by mitotic shakeoff were correspondingly labeled in hours 6.5 to 8 and hours 5 to 6.5 of an eight hour S-phase.

At the time of peak labeling of heterochromatin, there is a reduced labeling of heavy shoulder DNA. This result shows that at least some of the heavy shoulder DNA is replicated at a different time in S than the heterochromatic neck.

These results may merely reflect a general tendency for the replication of G-C rich sequences early in S and the late replication of A-T rich sequences (Bo71, Fl71, Sc80). Recently R. Langlois (personal communication) has used flow studies to follow the average base composition of cells proceeding through S. Hoechst 33258 and chromomycin A3 are both DNA stains with fluoresence roughly proportional to DNA content. However, in cells stained with both dyes, the hoechst fluoresence is enhanced for A-T rich DNA (La75) while the chromomycin fluoresence is enhanced by G-C rich DNA (La80). All cells studied so far, including muntjac, appear to replicate more G-C DNA early in S and more A-T DNA late in S.

Interestingly, direct microscopic examination of muntjac chromosomes using the same fluoresence ratio test indicates that the neck of the X-chromosome contains G-C rich sequences. Comings (Co71) found reduced quinicrine fluorescence in the muntjac heterochromatin. This result is also consistent with the conclusion that muntjac heterochromatin is GC rich (Ko78).

## ISOLATION AND PURIFICATION OF DNA FROM SORTED CHROMOSOMES.

### Introduction

The experiments above indicated that muntjac DNA contained a subpopulation of rapidly renaturing sequences with a higher G-C content than main band DNA. Satellite DNA's in many species, for instance mouse (Wa76) and kangaroo rat (Ha74, Ha76) are concentrated in the heterochromatin. If the G-C rich DNA of the muntjac is concentrated in the heterochromatin then it could be expected that the X-chromosome might contain a high fraction of that high-density DNA in its long heterochromatic neck region. Carrano et al., (Ca76, Ca79) have demonstrated purification of the each chromosome of the muntjac using the cell sorter. This technique was used to determine the CsCl gradient profile of the X chromosome and to compare that profile with the profile of bulk muntjac DNA and other chromosomes of the cell.

This required:

1) Preparation, staining and sorting of muntjac chromosomes.
2) Development of a method to recover very small quantities of DNA from the sorted chromosomes.
3) Development of a method to remove the DNA fluorochrome ethidium bromide used in sorting the chromosomes and,
4) Characterization of the density profile and molecular weight of the isolated DNA.

### Materials and Methods

Preparation of the chromosomes for sorting was carried out by a modification of the procedure described by Carrano et al (Ca76). Colcemid 0.32 micrograms/ml was added to rollers of muntjac cells in exponential growth 8 to 15 hours before shakeoff. The suspended cells were pelleted, resuspended

in cold 0.075 M KCl with Colcemid and placed on ice. After 30 minutes the cells were again pelleted and resuspended in 0.75 M hexylene glycol containing 25 mM Tris, pH 7.5, 0.5 mM $CaCl_2$, and 1 mM $MgCl_2$. The resuspended cells were placed in a 37°C bath for 10 minutes and then returned to ice. The chromosomes were released from the cells by brief shearing in an ice-cooled Virtis homogenizer. The chromosomes were stained by 1:1 dilution with $2 \times 10^{-4}$ M ethidium bromide in hexylene glycol-Tris buffer.

Recovery of DNA from Sorted Chromosomes

The chromosome preparation was kept on ice during the sorting procedure and the chromosomes were maintained in suspension by a slowly-rotating miniature stir-bar. The sorted droplets containing the desired chromosomes were collected in polyallomer ultra-centrifuge tubes on ice. Tubes containing $2 \times 10^5$ to $2 \times 10^6$ sorted chromosomes were balanced by adding cold chromosome suspension buffer to a uniform height about 3 mm below the rim. The tubes were placed in a swinging bucket centrifuge which had been precooled to 4°C. Centrifugation at 20,000 RPM, 4°C, was begun immediately and continued for a minimum of 2 h but usually overnight or about 16 h. The rotor was allowed to stop without the brake. The supernatant was poured off and the walls of the centrifuge tube wiped dry before righting the tube.

To each tube was added:

(a) 1ml KCl (0.06M) EDTA (0.5 mM)

(b) 0.1 ml sarcosine (5% in ethanol)

(c) 0.05 ml autodigested pronase (2mg/ml)

The chromosomes were allowed to digest for at least 1 h at room temperature and a small stir-bar was added in experiments where high molecular weight was not a consideration. Any carrier or control DNA was added at this point.

Removal of Ethidium Bromide

Because ethidium bromide alters the density of DNA (Le67) it had to be removed before density gradient analysis. This adaptation of a procedure described by LePecq (Le67) was used to restore stained DNA to native density. The lysis solution was passed over Dowex 50 ion exchange resin (20-50 mesh) which had been converted to the sodium-ion form. The resin column was contained in a pasteur pipet, the tip of which had been blocked with a sparing amount of siliconized glass wool. The column height was about 65 mm and the beads were pre-rinsed with the KCl-EDTA solution above. After the meniscus was lowered to the surface of the beads at the top of the column, 0.5 ml of sample was added and allowed to flow into the column and the eluent from the base of the column discarded. The remainder of the sample was then allowed to flow into the column and the eluent from that and a subsequent 2 ml rinse with KCl-EDTA were collected for CsCl gradient or molecular weight analysis.

Molecular Weight Measurements

To determine the effect of sorting on chromosomal DNA, the single and double strand molecular weights of the recovered DNA were measured using alkaline or neutral 5 to 20% sucrose sedimentation gradients (Ap77). A standard Beckman mixing chamber and peristaltic pump were used to form linear gradients under the samples in the bottom of the tube. Alkaline gradients were 0.5 M NaOH and samples to be measured for single strand molecular weight were made alkaline with 1/10 volume 5 M NaOH at least 1 hour before formation of the gradient. The samples were pumped out of the tubes by layering 40% sucrose under the gradient and collecting 40 fractions in scintillation vials. The counts were analyzed as described for equilibrium

gradients. The rate of sedimentation of a DNA molecule per unit centrifugal force is the S-value and is related to its molecular weight by the formula:

$$S = k1\ M^{k2}$$

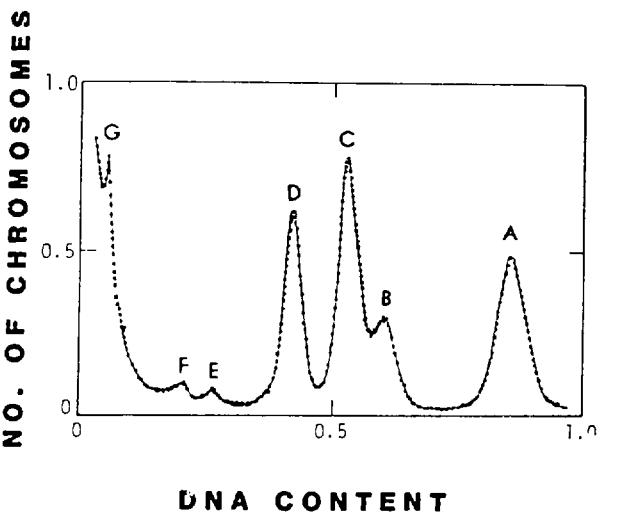
where M is the molecular weight and the k1 and k2 are constants related to the type of molecule and sedimentation coefficients (St65). The S values were determined using the method and tables of McEwen (Mc67) and the molecular weights were determined from the S-values using the constants given by Studier (St65). A $^{14}C$ labeled DNA was sedimented with each sample as a molecular weight standard and had a double strand molecular weight of 20 million daltons and a single strand molecular weight of 1 million as determined by velocity sedimentation in the analytical centrifuge using the calculations as given in Studier (St65).

*Results*

The flow histogram of isolated muntjac chromosomes stained with ethidium bromide is shown in Fig. 14. The abscissa of each peak corresponds to the fluorescent intensity of the chromosome. Since the fluorescent dye ethidium bromide is a DNA specific and quantitative stain (Le67, Le71) the abscissa is also proportional to the DNA content of the chromosome. The area under each peak is proportional to the fraction of the population represented by that chromosome. The exponential curve which underlies the peaks consists of chromosome fragments and non-specifically stained debris. Fragments or debris having the same fluoresence as a chromosome will be sorted with that chromosome so that very small chromosomes and chromosome fragments which represent a very small proportion of the population can be sorted but will be be less pure due to the debris contamination.

Figure 14. Flow distribution of muntjac chromosome fluoresence. The abscissa is the ethidium bromide fluoresence or DNA content of the chromosome. The ordinate is the relative number of chromosomes. Peaks A, B, C, D, and G are the 1, X, 2, Y1, and Y2 (or dot) chromosomes repectively. From Carrano et al., (Ca76).

# FIGURE 14



The double strand molecular weight gradients for sorted chromosomes and similarly treated unsorted chromosomes are shown in Fig. 15. The tritium labeled chromosomes are shown as the solid curve and the $^{14}C$ labeled bulk DNA standard as the broken curve. The numbers above the curves represent the molecular weight in millions of daltons for the DNA in that fraction of the gradient. In both cases the standard is a broad peak with a maximum at 20 million daltons. The DNA from the chromosomes is bimodal. Later more extensive experments with identically prepared chromosomes from much more rapidly growing hamster cells were unimodal with the same molecular weights as found for the lower weight peaks shown here. The unsorted chromosomes have a low molecular weight peak at about 7 million daltons and a very broad high molecular weight peak which has a maximum at about 190 million. The double stranded molecular weight for the DNA from sorted chromosomes has a very similar profile but with much reduced molecular weight. The low molecular weight peak is at about 1.8 million daltons and the very broad high molecular weight peak is centered about 30 million.

The single strand molecular weight profile for sorted chromosomes is shown in Fig. 16. The standard is a broad single peak with a maximum at about 1.14 million daltons, the same as that for the main peak of the sorted chromosomes. The single strand molecular weight reduction on sorting was about a factor of 2 for both hamster and muntjac.

It appears that preparation of the chromosomes for sorting introduces a large number of breaks in the DNA. Sorting of the chromosomes reduces the molecular weight further by a factor of 3 to 6 in the double stranded measurement and a factor of two or so for single stranded DNA. Recently, Blumenthal (Bl79) and Yu (Yu81) have developed methods of chromosome preparation which produce much higher molecular weights.

Figure 15. Double strand molecular weight of DNA recovered from sorted chromosomes (above) and from chromosome suspension prior to sort. Curves show the distribution of activity on a 5 to 20% neutral sucrose sedimentation gradient. Fraction number 1 is from the top of the tube and increasing fraction number corresponds to increasing molecular weight. The broken curve is the 20 million dalton molecular weight standard. The solid curve is the activity recovered from the chromosomes.

**FIGURE 15**

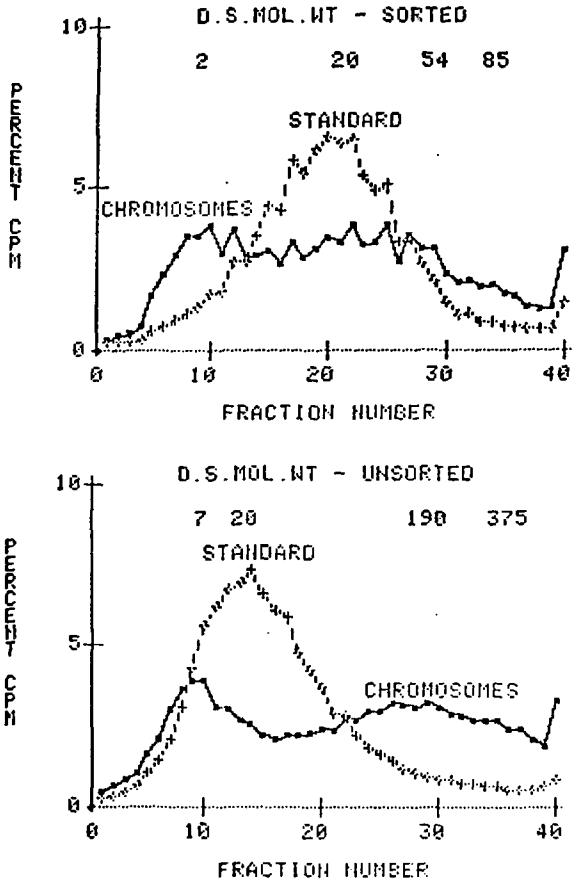
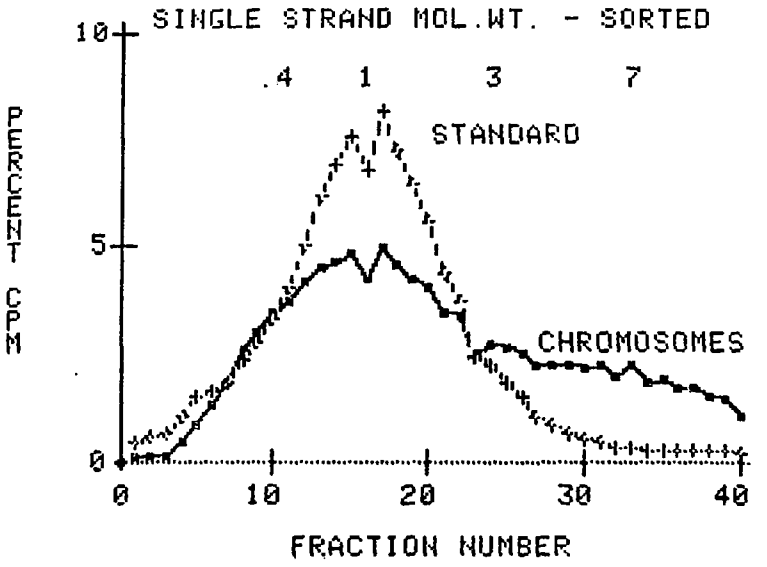

D.S.MOL.WT - SORTED
2
20
54
85
STANDARD
CHROMOSOMES
PERCENT CPM
10
5
0
0
10
20
30
40
FRACTION NUMBER
D.S.MOL.WT - UNSORTED
7
20
190
375
STANDARD
CHROMOSOMES
PERCENT CPM
10
5
0
0
10
20
30
40
FRACTION NUMBER

Figure 16. Single strand molecular weight of DNA recovered from sorted chromosomes. Curves show the distribution of activity on a 5 to 20% alkaline sucrose sedimentation gradient. Fraction number 1 is from the top of the tube and increasing fraction number corresponds to increasing molecular weight. The broken curve is the 1 million dalton molecular weight standard. The solid curve is the activity recovered from the chromosomes.

**FIGURE 16**

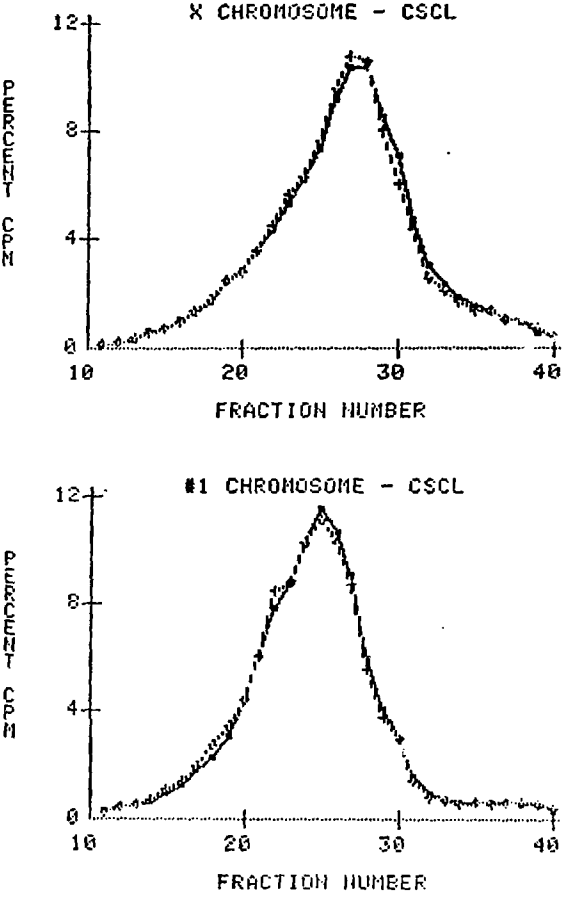

SINGLE STRAND MOL.WT. - SORTED
.4
1
3
7
STANDARD
CHROMOSOMES
PERCENT CPM
10
5
0
0
10
20
30
40
FRACTION NUMBER

CsCl density gradient analysis of sorted chromosomes is shown in Fig. 17. For both the X chromosome and number one autosome shown here and for all the other muntjac chromosomes except the dot Y2 chromosome, the density profile of the tritium from the sorted chromosomes exactly coincides with the density profile of the $^{14}C$ labeled bulk DNA standard. The sorting of the dot or Y2 chromosome was not attempted due to limitations on purity and quantity. Fragments from other chromosomes have DNA contents in the same range as the Y2 chromosome and any sorted population containing the Y2 is likely to be contaminated with DNA from other chromosomes.

The data of Carrano and Johnston (Ca77) indicate that the heterochromatic neck of the X chromosome in muntjac constitutes about 13% of the DNA of that chromosome. The DNA content of the centromere regions of the other chromosomes has not been measured. However, the C-band lengths of each of the other chromosomes as measured in Fig.2 are roughly 10 to 15% of the length of the respective whole chromosome. Thus there are at least two possible interpretations of the CsCl gradients of the sorted chromosomes: first, that the G-C rich DNA of muntjac is concentrated in the heterochromatin of the chromosomes and each of the chromosomes has a proportionate fraction of its DNA in heterochromatin, or second, the G-C rich DNA is scattered proportionately among the chromosomes of the muntjac independently of the heterochromatin.

Figure 17. Cesium chloride equlibrium density distribution of activity recovered from sorted chromosmes. The X-chromomsome (top) and chromosome 1 (bottom) have density profiles identical to that of bulk DNA. For each graph, the solid curve is the tritium recovered from the sorted chromosomes while the superimposing broken curve is $^{14}C$ labeled bulk DNA. Increasing fraction number corresponds to decreasing density.

**FIGURE 17**


X CHROMOSOME - CSCL
PERCENT CPM
12
8
4
0
10
20
30
40
FRACTION NUMBER
#1 CHROMOSOME - CSCL
PERCENT CPM
12
8
4
0
10
20
30
40
FRACTION NUMBER

Muntjac DNA Overview

These experiments have shown that:

1) Muntjac DNA has two major CsCl density components. The main band with a density of 1.700 constitutes about 86% of the genome. The remaining 14% of the DNA, the heavy shoulder, is probably a mixture of several subfractions with densities in the range of 1.705 to 1.719. This broad range of heavy shoulder densities is more characteristic of a heterogeneous mix of moderately repeated DNA sequences than of a true satellite DNA.

2) DNA which renatures at a Cot of less than 0.1 is almost exclusively heavy shoulder. This rapidly renaturing fraction constitutes less than 8% of the DNA. True satellite DNA should be close to complete reassociation at Cot 0.1 (Ha74a).

3) The rapidly renaturing fraction has a high melting point (83°C) and broad melting range. These are the properties expected of a G-C rich DNA with sequence heterogenity.

4) Late replicating DNA is enriched in main band density components and depleted in heavy shoulder components. This may reflect general late replication of A-T rich sequences throughout the DNA and does not rule out late replication of specific G-C rich sequences.

5) The heavy shoulder DNA is uniformly distributed among the chromosomes of the muntjac. This result leaves two possibilities for the intra-chromosomal distribution of heavy shoulder DNA:

1) Blocks of heavy shoulder DNA are proportionately distributed among the chromosomes, or

2) Widely interspersed heavy shoulder DNA is is uniformly spread throughout the genome.

In general, moderately repeated DNA's are widely interspersed (Ha74a, Ce75, Be78) but some small subcomponent of the heavy shoulder such as the rapidly renaturing fraction may be localized to the heterochromatin. The methods used in this work were not sufficiently sensitive to determine this.

## SCE AND UDS IN EUCHROMATIN AND HETEROCHROMATIN

### INTRODUCTION

Carrano and Johnston (Ca77) reported a reduced level of sister chromatid exchange in the heterochromatin of muntjac cells exposed to mitomycin C. This response may be due to a variety of causes. Two possibilities are:

1) Altered levels of damage in heterochromatin relative to euchromatin or;

2) Altered levels of repair of lesions leading to sister chromatid exchange in the heterochromatin relative to euchromatin.

In this section I present data from experiments designed to compare levels DNA repair as measured by unscheduled DNA synthesis with the level of sister chromatid exchange in muntjac euchromatin and heterochromatin.

Unscheduled DNA synthesis (UDS) is the name given to cellular repair of damaged DNA as detected by counting autoradiographic grains over cells exposed to a DNA damaging agent.(e.g., Vi80, Jo78). The damaged cells are provided radioactive DNA precursors, usually tritiated thymidine, with which to repair this damage. After several hours exposure to label, the cells are washed and placed on slides, and the slides are dipped in nuclear track emulsion. The slides are developed by the procedures normally used with photographic film. The silver grains over the cells are taken as an indication that the cell incorporated the labeled thymidine while repairing its DNA. Cells in S-phase will also incorporate the labeled precursors and this scheduled synthesis must either be suppressed by S-phase blocking agents, or the S-phase cells must be excluded from the observations.

Certainly not all cellular repair is detectable by UDS. Thymine dimers repaired by photoreactivation, for instance, will not result in the incorportation of any new atoms into the DNA. If some other repair mechanism is invoked and just the damaged nucleotide is replaced then only about 25% of the repairs will result in the incorporation of label assuming that 25% of the bases are thymines and that the damage is evenly distributed among the bases. Clearly, damage repaired by short-patch repair in which only a few bases surrounding the site of damage are replaced is much less detectable by UDS than damage repaired by long-patch repair in which up to several hundred bases are replaced along the double-helix.

In a normal UDS experiment, one grain probably corresponds to the incorporation of about 10,000 to 100,000 new bases in an existing double helix. This is calculated as follows:

Assuming a normal autoradiographic efficiency of about 10% (Fe67), then one grain corresponds to 10 decays.

If we use the equation:

$$dN/dt = kN$$

where

dN/dt is the disintegration rate

N is the number of tritium atoms present and

$$k = \frac{\ell n 2}{t_{\frac{1}{2}}(sec)}$$

then we can solve for the number of tritium atoms required to give 10 decays in 3 to 30 days, the normal photographic exposure period in a UDS experiment. Solving for N gives an answer of about 20,000 tritium atoms for a 3 day exposure or 2000 atoms for a 30 day exposure. The numbers at the beginning of the paragraph are derived by further assuming that 25% of the replaced bases are thymidines and 80% of the replaced thymidines are labeled.

This type of repair, which results in the massive replacement of DNA bases, was used to compare cellular repair rates with the cellular production of SCE. In the following sections of the thesis I describe experiments and the design of supporting protocols centered on answering the questions:

1) How does the production of unscheduled DNA synthesis relate to the production of sister chromatid exchange?

2) Is reduced SCE in the heterochromatin of the muntjac an indication of increased repair of lesions leading to SCE in this region? In other words, is UDS enhanced in muntjac heterochromatin?

3) In response to a DNA damaging agent how does the time scale of UDS compare to rate of reduction of lesions leading to SCE? In other words, in a cell with damaged DNA, do lesions that cause the cell to carry out unscheduled synthesis disappear at the same rate as lesions that lead to the production of sister chromatid exchange?

THEORY OF THE BASIC EXPERIMENT

Wolff et al., (Wo74) have shown that lesions to the DNA will result in SCE only if the damaged cell has carried out normal DNA synthesis subsequent to the damage. This has been interpreted to mean that some cellular activity during S-phase encounters a lesion in the DNA and, in response, causes the recombination of DNA strands which results in an observable exchange in following mitoses. We can interpret this to mean that S-phase "fixes" or expresses lesions which might otherwise be repaired or removed without the production of a SCE.

If we continue with these assumptions;

1) That the formation of a SCE is a direct response to a DNA lesion;
2) That the lesion or class of lesions that result in a sister chromatid exchange can be repaired by the cell;
3) That this repair can occur without the formation of SCE and prevents the subsequent formation of a SCE that otherwise would have occured and,
4) That the repair can occur prior to and outside of S-phase,

then the following experiment will allow the comparison of unscheduled DNA synthesis with the repair of lesions leading to sister chromatid exchange.

1) Block muntjac cells in cycle outside of S-phase. In these experiments, the cells were held in plateau phase and in some experiments additional suppression of DNA synthesis was attempted with the use of hydroxyurea or sodium butyrate.

2) Release the cells from cycle-block (before or after damaging the DNA.)

3) Use a DNA damaging agent to create lesions that will lead to both UDS and SCE. First experiments were carried out with mitomycin C and later ultraviolet light was used.

4) Monitor the relative intensity of UDS in heterochromatin and euchromatin and compare with the relative frequency of sister chromatid exchange.

5) Measure UDS as a function of time after DNA damage. In other words, measure the rate of removal of lesions repairable by unscheduled synthesis.

6) Measure the level of induced SCE as a function of the time interval between damage to the DNA and entry of the cells into S-phase synthesis. If SCE producing lesions are repaired outside of S, then allowing more time between DNA damage and entry of the cells into DNA synthesis should result in fewer sister chromatid exchanges.

## UNSCHEDULED DNA SYNTHESIS AND MITOMYCIN C

### Introduction

Carrano and Wolff (Ca75) first showed reduced levels of spontaneously occuring SCE in muntjac heterochromatin. Later, Carrano and Johnston (Ca77) demonstrated the reduced level of induced sister chromatid exchange in muntjac heterochromatin using mitomycin C as the inducing agent. Mitomycin C (MMC) is a potent inducer of SCE which is known to act as a bifunctional alkylating agent to produce interstrand DNA crosslinks (Iy63, Iy64). MMC also alkylates a single strand of the DNA without producing an interstrand crosslink(To74). The ratio of these two lesions appears to be about 10 MMC adducts to 1 crosslink (To74). In these experiments, survival of muntjac cells to MMC as a function MMC concentration was measured and compared to levels of unscheduled DNA synthesis induced at the same dose.

### Materials and Methods

Muntjac cells were seeded at $1.3 \times 10^6$ in T-25 Falcon flasks 7 days before exposure to MMC. At this density, confluency is reached 2 days before treatment, with a final population of about $5 \times 10^6$ cells per flask. Scheduled DNA synthesis was further suppressed by adding hydroxyurea (HU) (final concentration 10 mM) 15 minutes before treatment with MMC. This concentration of HU was maintained throughout the MMC treatment and subsequent labeling period.

A concentration of MMC of $3 \times 10^8$ molecules/cell, or in this case, $5 \times 10^{-7}$ molar will induce large numbers of SCE without significant toxicity to the cells. The cells in this experiment were treated at 0X, 1X, 3X, 10X, or 30X this level. The mitomycin was added with sufficient high specific activity (40-60 Ci/mmole) tritiated thymidine to bring the final concentration to 10 .nicrocuries/ml. After 1 hour, the MMC was removed and replaced with

medium containing only HU and $^{3}$H-TdR. Four hours later, the cells were rinsed three times with sterile PBS, treated with trypsin and processed either for autoradiography or cell survival colony counts.

For autoradiography, the cells were dropped on slides following treatment with the standard 15-20 minutes in hypotonic and fixation in 3:1 methanol:acetic acid. The slides were dipped in NTB-2 Kodak nuclear track emulsion and developed after 7 days.

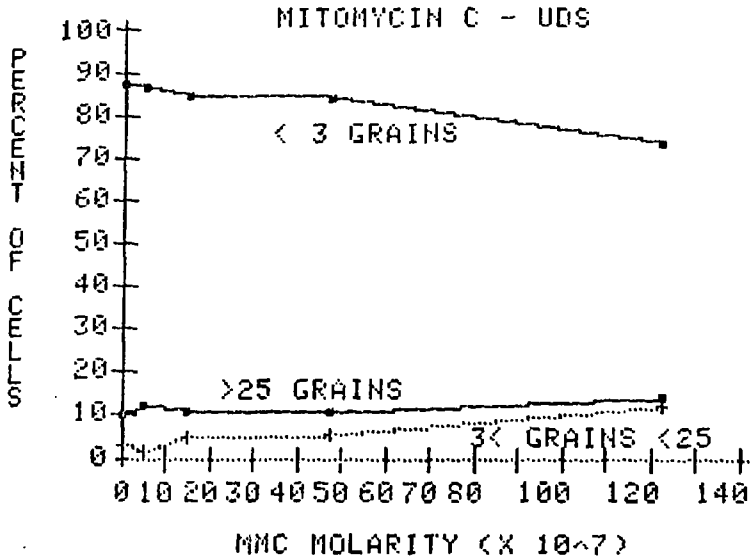
For cell survival, the cells were plated in T-25 plastic flasks at 300, 600, 1000, 3000, or 10,000 cells per flask. Five replicates at each of two or three concentrations were plated. After 14 days, the medium was gently poured out, and the cells carefully fixed twice for 30 minutes with 5ml of 10% formalin. The colonies were stained with 5ml of 10% Giemsa for 15-30 minutes, rinsed with distilled water and dried. Colonies containing 50 or more cells were counted as survivors.

Results

Unscheduled DNA synthesis was measured by counting the grains over 300 cells from each treatment group. The results are shown in Fig. 18. Cells with more than 25 grains were considered residual S-phase cells. Cells having more than 3 grains but less than 25 were considered to be cells displaying UDS. Cells having less than 3 grains constituted more than 80% of the cells in groups except the highest dose sample. MMC induces little to no UDS. The "unscheduled synthesis cells" constituted less than 12% of the population for all treatments and MMC failed to significantly increase either the average grain count of the UDS population or the fraction of the cells scored in the UDS group. At the highest MMC concentration there does appear to be a slight

Figure 18. Unscheduled DNA synthesis grain counts for muntjac cells treated with mitomycin C. The fractions of unlabeled cells ( < 3 grains), S-phase cells ( > 25 grains), and UDS cells did not significantly change from control values for a wide range of MMC doses.

**FIGURE 18**


MITOMYCIN C - UDS
PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
< 3 GRAINS
>25 GRAINS
3< GRAINS <25
0 10 20 30 40 50 60 70 80 100 120 140
MMC MOLARITY (X 10^7)

increase in cells displaying more than 3 grains. However, MMC never induces a major fraction of the population to incorporate thymidine as would be expected for true UDS.

Survival of muntjac cells is shown in Fig. 19. The survival curve is a straight exponential with little or no shoulder. The multiple points represent the averages of the flasks seeded at different cell concentrations. Survival at the highest dose (about $10^{10}$ MMC molecules per cell) is 2 to 3%.

Discussion

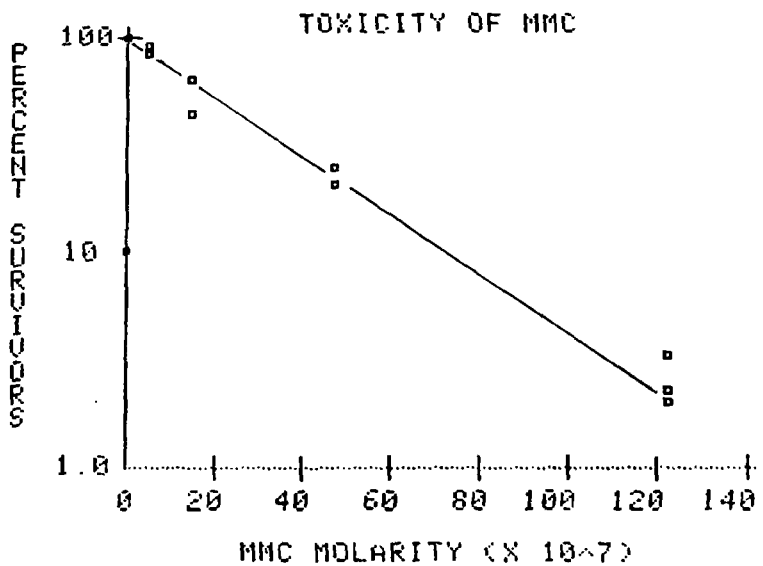
From these experiments it is clear that MMC is a very weak inducer of unscheduled synthesis. This does not mean, of course, that MMC induced DNA lesions are not repaired. Repair which does not result in the massive incorporation of new bases into the repaired DNA, or repair which is restricted to S-phase will not be detected by these protocols. Similarly, although the lack of a shoulder on the survival curve might be cited as evidence of lack of repair of MMC lesions, it seems clear from the high number of SCE induced at low doses where survival is also high, that a few lesions are tolerated in some fashion by the cell.

These experiments show that lesions which lead to SCE are not necessarily lesions which lead to unscheduled synthesis. There may be, however, lesions induced by other agents which are repaired by unscheduled DNA synthesis and which lead to the formation of sister chromatid exchanges.

At all MMC doses, 10 to 15% of the cells showed more than 25 grains indicating residual S-phase activity in spite of 10 mM hydroxyurea and confluent growth conditions. In the absence of induced unscheduled DNA synthesis, these cells were easily identified. However, the measurement of heavy UDS in future experiments could easily be confused by the presence of

Figure 19. Survival of muntjac cells to MMC. The ordinate is percent survivors shown on a logarithimc scale. The multip!. points at each dose represent mean survivals observed for different cell seed numbers in the assay plates.

**FIGURE 19**


TOXICITY OF MMC
PERCENT SURVIVORS
100
10
1.0
0
20
40
60
80
100
120
140
MMC MOLARITY (X 10^7)

cells with 25 to 100 grains. Also, in the central experiment in which we wish to measure cellular repair as the population approaches S-phase, it is desirable to know the cell cycle distribution of the treated population and the effectiveness of the synthesis suppressors. In the following section, experiments are presented which characterize muntjac cells in plateau phase and also describe the effects of hydroxyurea and another S-phase suppressor, sodium butyrate.

CHARACTERIZATION OF MUNTJAC PLATEAU PHASE:

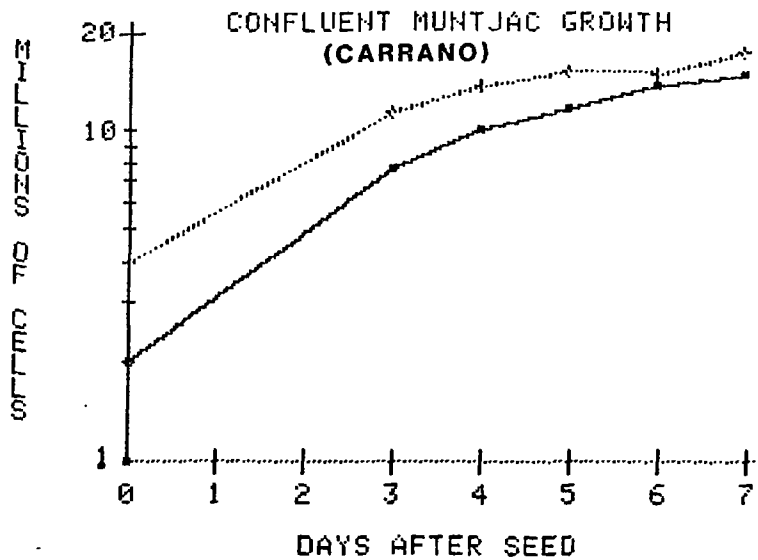
Effects of Confluency and S-phase Suppressors.

The experiment with MMC was carried out using cell densities based on the results of an experiment previously carried out by Carrano (personal communication). These data are shown in Fig. 20. Muntjac cells were seeded into 75 $cm^2$ plastic flasks with seed numbers of 2 or 4 million cells. On days 3 through 7 after subculture, sample flasks were treated with trypsin, and the number of cells harvested measured by Coulter counter. For both seed numbers, the population increased to a maximum value of 15 to 17 million cells per flask. For the higher seed number (4 million cells) this population was achieved 5 days after subculture. In the MMC experiment, and all plateau phase experiments reported here, cells were seeded at densities equal to or greater than 4 million cells per 75 $cm^2$ and minimum of 5 days growth was allowed.

Hydroxyurea is extensively used to block S-phase cells(Mi71) especially in experiments where it is necessary to monitor unscheduled synthesis. HU blocks the entry of cells from G1 into S and either kills or blocks further incorporation by those cells already in S-phase(Mi71). The concentration of hydroxyurea used in the mitomycin C experiment (10mM) was modeled on the experiments of

Figure 20. Muntjac cell numbers in 75 square centimeter platic culture flasks seeded with 2 million (solid curve) or 4 million cells (broken curve). Note logaritnmic scale on ordinate. Data of Carrano.

**FIGURE 20**


CONFLUENT MUNTJAC GROWTH
(CARRANO)
MILLIONS OF CELLS
20
10
1
0
1
2
3
4
5
6
7
DAYS AFTER SEED

Ikushima (Ik77). The toxicity of hydroxyurea at significantly higher concentrations could complicate measurements of cellular repair (Si67, Fo77) but 10 mM HU failed to completely inhibit DNA synthesis in muntjac.

Further clarification of the experimental conditions best suited to measuring unscheduled DNA synthesis in synchronized muntjac cells was needed. In this section three basic questions are asked:

1) What is the cell cycle distribution of muntjac cells in extended confluency?
2) How do the DNA synthesis inhibitors, hydroxyurea and sodium butyrate influence this distribution?
3) How do these inhibitors influence the ability to measure unscheduled DNA synthesis in the blocked cells?

Confluency and the Effects of Hydroxyurea on Residual S-phase Cells

Materials and Methods

Measurments of the cell cycle distribution of muntjac cells in extended confluency were carried out by flow cytometry. Plastic 100 mm culture dishes with an actual growth area of about 57 $cm^2$ were seeded with 4 X $10^6$ muntjac cells from exponentially growing cultures. Cells were harvested after varying periods of time by treatment with 0.5% trypsin and fixation in cold 70% ethanol. Fixed cells were refrigerated until shortly before flow analysis when the ethanol was removed and the cells resuspended at room temperature in Chromomycin A3 at a concentration of 20 μ g/ml and a cell density of 6 X $10^5$ cells per ml. Chromomycin fluorescence of the cells was measured by flow cytometry and taken to indicate DNA content. Analysis of the resulting DNA histograms (De74) was used to obtain the fraction of each population in G1, S, and G2+M. Each sample was also examined directly by fluoresence microscopy to determine the number of doublets.

To measure the effects of hydroxyurea on residual S-phase incorporation of plateau cultures of muntjac, cells were seeded with 1.38 X $10^6$ cells per 60mm culture dish(20 $cm^2$). Thymidine incorporation with and without HU was measured at 1, 5, 6, 7, and 8 days after plating. At 0.25, 0.5 or 1.0 hours before addition of the tritiated thymidine, the medium was replaced with medium containing 10 mM hydroxyurea. Control plates received no HU. After 20 minutes in 10 μ Curie/ml medium, the cells were rinsed 3 times with PBS, treated with trypsin and placed on slides for autoradiography as previouly described. After 7 days exposure, the slides were developed at 4°C.

Results

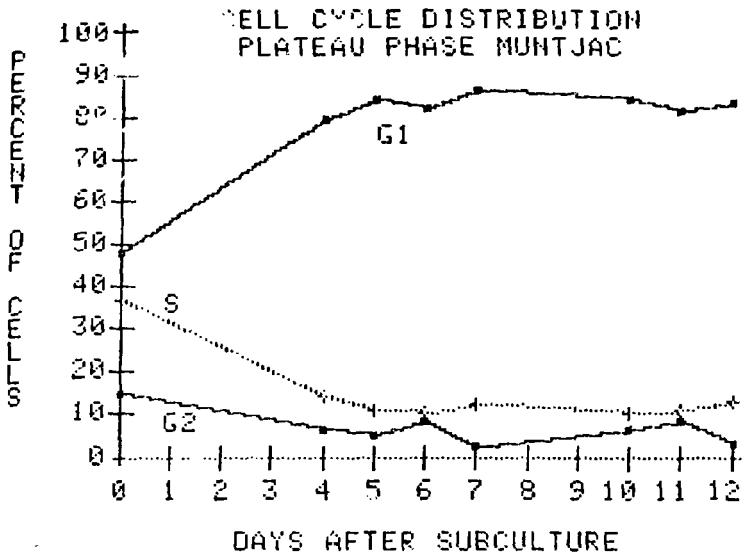
Flow analysis of the DNA content of muntjac cells after varying periods of time in confluency is shown in Fig. 21. The percent of the cells in G1, S, or G2+M is shown as a function of time after seeding of the cells. The values at 0 days are those associated with cells in exponential growth, for example, see Fig. 28b. After 5 days, the G1 cells represent 84% of the population, G2 and M combined are 5% and S is 11%. These percentages remain roughly the same out to 12 days when the last measurement was made.

Flow cytometry of DNA content will register a doublet of two G1 cells as a single G2 cell so the G2 population is artificially high in these measurements. Direct microscopic examination showed that all samples contained 3 to 6% doublets, accounting for most of the observed G2 population.

The 10 to 14% S-phase population observed here by flow cytometry coincides very well with the fraction of S cells detected by elevated grain counts in the MMC experiment.

Figure 21. Cell cycle distribution of muntjac cells in extended plateau as determined by flow cytometry. Much of G2 population may be doublets.

**FIGURE 21**


CELL CYCLE DISTRIBUTION
PLATEAU PHASE MUNTJAC
PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
G1
S
G2
0 1 2 3 4 5 6 7 8 9 10 11 12
DAYS AFTER SUBCULTURE

The effect of HU and confluency on DNA synthesis was measured by thymidine incorporation experiments. On the basis of autoradiographic grain counts, cells were considered as unlabeled (0 to 5 grains), lightly labled (6 to 20 grains), heavily labeled (20+ grains), or uncountable if the cells were so thickly covered with grains, or if the grains were densely clustered so as to be unresolvable. The fraction of the cells which were unlabeled as a function of time after subculture is shown in Fig. 22. After 5 days, the fraction of unlabeled cells is about 93% and rises to about 96% after 8 days, irrespective of the presence of HU. One day after subculture, 1 hour of HU actually seemed to decrease the number of unlabeled cells. Thymidine incorporation for cells 1 day after subculture is shown in Fig. 23 and for 8 days after subculture in Fig. 24. Three hundred cells were scored for each sample. In each figure the top histogram shows the distribution of grains in cells without hydroxyurea while the lower histogram is for cells treated for 1 hour with 10 mM HU prior to addition of tritiated thymidine. At all times, the effect of the hydroxyurea is to shift cells from the uncountable category into the heavily labeled category and somewhat into the lightly labeled category. This is true not only of the 1 hour HU treatment shown but also for the 0.5 and 0.25 hour treatments (data not shown). Thus, while hydroxyurea drastically reduces thymidine incorporation, it appears to change a cell that is readily identifiable as an S-phase cell into one that could easily be confused with a cell carrying out a high level of unscheduled DNA synthesis.

Conclusions

While confluent muntjac cultures maintain a stable number of cells attached to the surface of the culture dishes, there is a residual level of DNA

Figure 22. Suppression of S-phase cells by confluent growth and 10 mM hydroxyurea.

**FIGURE 22**

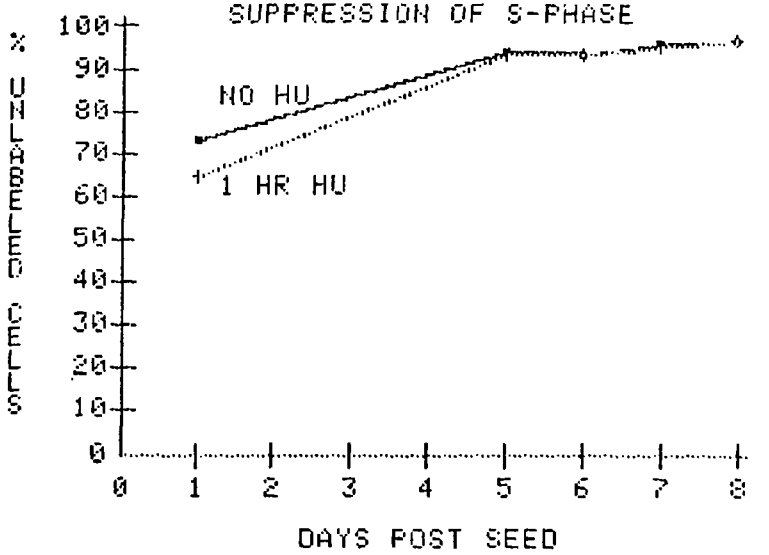
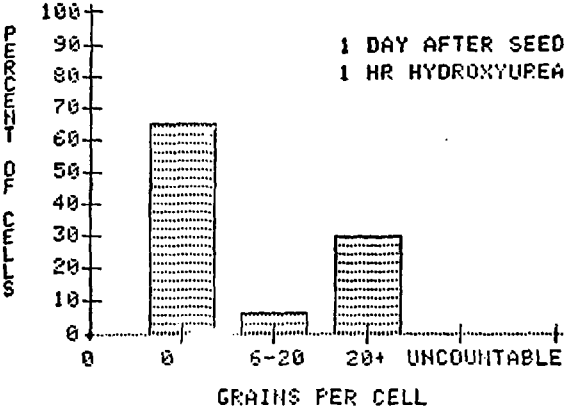

SUPPRESSION OF S-PHASE
% UNLABELED CELLS
100
90
80
70
60
50
40
30
20
10
0
NO HU
1 HR HU
0
1
2
3
4
5
6
7
8
DAYS POST SEED

Figure 23. Distribution of thymidine uptake one day after subculture as determined by autoradiography. Cells were either pretreated with 10 mM hydroxyurea (top) or untreated (bottom).

**FIGURE 23**


PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
1 DAY AFTER SEED
1 HR HYDROXYUREA
0
0
6-20
20+
UNCOUNTABLE
GRAINS PER CELL

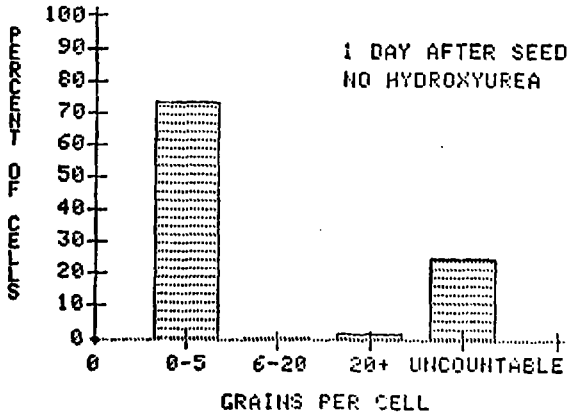
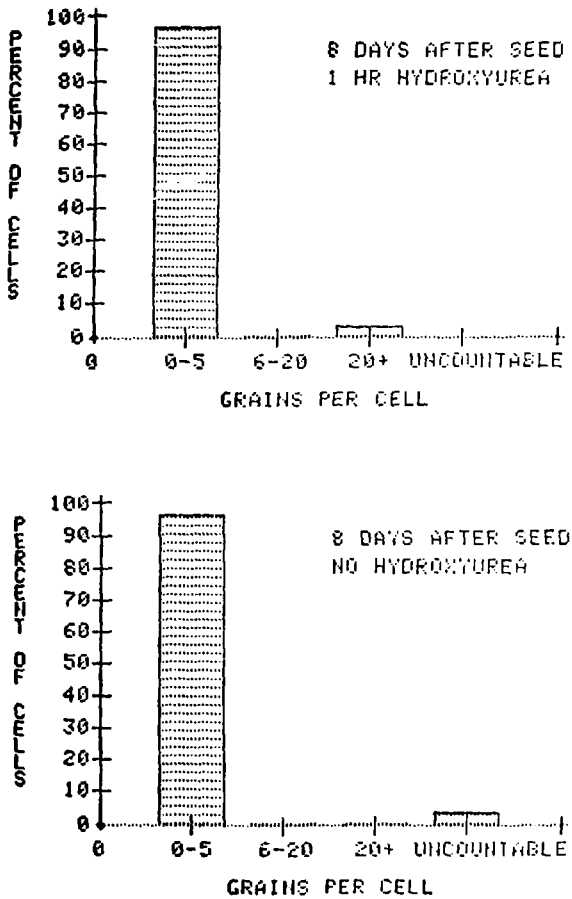

PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
1 DAY AFTER SEED
NO HYDROXYUREA
0
0-5
6-20
20+
UNCOUNTABLE
GRAINS PER CELL

Figure 24. Distribution of thymidine uptake eight days after subculture as determined by autoradiography. Cells were either pretreated with 10mM hydroxyurea (top) or untreated (bottom).

**FIGURE 24**


PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
8 DAYS AFTER SEED
1 HR HYDROXYUREA
0
0-5
6-20
20+ UNCOUNTABLE
GRAINS PER CELL
PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
8 DAYS AFTER SEED
NO HYDROXYUREA
0
0-5
6-20
20+ UNCOUNTABLE
GRAINS PER CELL

synthesis in about 10% of the cells. Flow cytometry detects a similar percentage of the population with S-phase DNA content indicating that these cells are progressing through the cell cycle as opposed to "turning over" their DNA without cycle progression. Muntjac cells grown on surfaces will shed some cells into the culture medium. Since only the cells attached to the surface are detected by these experiments, it is possible that some low level cell growth is occuring and is balanced by loss of cells into the medium or by some degradative process.

Hydroxyurea (10 mM) will markedly reduce this residual S-phase synthesis but it appears that attempts to measure unscheduled DNA synthesis would not be improved by HU. Rather than entirely eliminating scheduled synthesis in these cells, hydroxyurea seems to reduce thymidine incorporation to a level where it might readily be confused with unscheduled DNA synthesis.

## SUPPRESSION OF DNA SYNTHESIS WITH SODIUM BUTYRATE

### INTRODUCTION

Though the effect is not well characterized, sodium butyrate will, when used at low concentrations for short periods of time, block cell cycle progression without toxicity to the cells. As such, butyrate seemed worth investigating and characterizing as a potential cell synchronizing agent for use in the proposed experiment comparing DNA repair and the production of sister chromatid exchange.

Sodium butyrate treatment induces a number of effects in cultured cells. In HeLa cells, for instance, butyrate induces the biosynthesis of alkaline phosphatase (De77, Gr74), follicle-stimulating, hormone (Gh77), human chorionic gonadotropin (Gh76, Gh77a), a sialyltransferase (Fi76), and β -andrenergic receptors (Ta77). Butyrate also induces morphological changes

in cultured cells that result in smooth protrusions of the cellular membrane producing a jagged cellular appearance (Al76, De76, Fi76, Gi73, Sc76a, Si75). Butyrate exerts its effects by some unknown regulatory pathway. Although the cyclic AMP analog; di-butyryl-cyclic AMP induces similar morphological changes and although butyrate induces changes (Gh75, He75) in the cellular concentration of cyclic AMP (Pr76), butyrate probably does not exert its influence by modulating the cellular concentrations of cyclic AMP, since the effects observed with di-butyryl cyclic AMP seem to be independent of those induced by butyrate (Gi73, He75, Si75). Also the calcium ionophore A23187 (He75) can suppress butyrate-induced changes in cellular morphology, suggesting a regulatory role of the calcium ion. Butyrate can also induce the enzyme CMP-sialic acid:lactosylceramide sialyltransferase, whose activity is correlated with the production of jagged membrane processes (Fi74, He76, Si77).

Another major response of cultured cells to butyrate treatment is a cessation of cellular proliferation and DNA synthesis (Ha77). Studies of the chromatin of treated cells showed large increases in the hyperacetylated forms of histones H3 and H4 (Ne78, Ri77, Si78) apparently resulting from decreased activity of a histone deacetylase (Se78, Vi78). The DNA associated with the acetylated histones has increased sensitivity to DNase I (Vi78), implying alteration of chromatin structure if not modified transcriptional regulation. The relation between the suppression of DNA synthesis and other cellular responses to butyrate is not clear, and no data are available on the cell-cycle distributions induced by butyrate.

In this section we present data on the butyrate-induced cell-cycle effects and the kinetics of recovery to the exponential growth of cells. The results

suggest that sodium butyrate produces at least two blocks in the cell cycle, one at the G1-S border and one that results in an accumulation in cells having a DNA content characteristic of the G-2 phase.

## MATERIALS AND METHODS

### Cell Culture

Cells of the male Indian muntjac (Muntiacus muntjac vaginalis), obtained from the American Type Culture Collection, Rockville, Md., U.S.A., were cultured in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum and penicillin-streptomycin. The doubling time of the muntjac cells was about 40 h. The CHO cells were clone AA-8 isolated by Thompson et al., (Th80) and were grown in α-MEM supplemented with 10% fetal calf serum and penicillin-streptomycin. The doubling time of CHO cells was about 12 h. All cells were grown in 100-mm Corning petri dishes containing 10 ml of medium in a humidified atmosphere of 5% $CO_2$. For treatment with sodium butyrate, the medium was removed from exponentially growing cells and replaced with similar medium containing 5, 7, or 10 mM sodium butyrate (Baker, butyric acid, sodium salt).

Thymidine incorporation in butyrate-treated muntjac cells was measured after a 1.25 h pulse of 10 μ curies/ml tritiated (methyl) thymidine ($^3$H-TdR) (specific acitivity, 42 Ci/mmole). The labeled cells were rinsed with phosphate-buffered saline (PBS), treated with trypsin and centrifuged, and the pellet was digested in 1 ml of lysis solution containing 0.5 M NaOH, 0.1% NP-40, and 0.02 M EDTA. This solution was neutralized using 1 ml of 1 M Tris-HCl (no pH adjustment) and the DNA precipitated using 2 ml of 20% TCA. The precipitated DNA was collected on filter paper, rinsed with 5% TCA and chloroform, and placed in 10 ml Insta-Gel scintillation fluid (Packard) to measure radioactivity.

Analysis of Recovery from Butyrate

Exponentially growing CHO cells were treated with medium containing 10 mM sodium butyrate. After 24 or 72 h, the culture medium was replaced by fresh medium containing 0.1 μ Ci/ml of $^{3}$H-TdR. At successive 6-h intervals, cell samples were treated with trypsin, fixed in cold 70% ethanol, and placed on slides for autoradiography or stained for flow cytometry. For autoradiography, slides were dipped in Kodak emulsion NTB-3, held at 5°C, and developed at 5°C in Kodak developer D-19.

Preparation for Flow Cytometry and Sorting

Cells were treated with trypsin, counted using a Coulter counter, and fixed in cold 70% ethanol. For staining, the cells were suspended in 17 μ M Chromomycin A-3 (CA3) in 14.8 mM magnesium chloride for at least 20 min before the flow measurements. The cells were then analyzed in a flow cytometer for DNA content on the basis of CA3 fluorescence intensity after laser-light excitation at 457 nm (Gr79). Cells were sorted using a modified version of the Becton-Dickinson FACS-II.

Survival of Butyrate-Treated Cells

After various periods of butyrate treatment, cells were treated with trypsin and replated in 60-mm petri dishes containing 5 ml of fresh medium at approximately 300 cells/dish. After 1 wk (CHO cells) or 2 wks (muntiac cells), the medium was removed, and the cells were fixed in 10% formaldehyde and stained with crystal violet. Survival was calculated as the number of colonies containing 50 or more cells divided by the number of cells plated and the plating efficiency. The plating efficiency of CHO cells was 76% and muntjac cells 44%.

In all treatments the cell-culture medium was removed before analysis, and all conclusions refer only to the remaining cells attached to the plates.

UV Survival

Survival of muntjac cells to ultraviolet light was measured by exposing plateau phase cells to varying doses of 254 nm light. Cells were seeded at a density of 4 X $10^6$ per 100 mm plastic culture dish (57 $cm^2$) 5 days before irradiation. Immediately before UV treatment, the culture medium was removed and plates were rinsed with sterile PBS. Irradiation was carried out as described by Thompson(Th80) at an exposure rate of about 0.13 Joules/$m^2$/sec as measured by a digital radiometer calibrated at 254 nm (Ultra-Violet Products model J-260, sensor J-2601A). After irradiation, the cells were treated with trypsin and replated in 60 mm culture dishes (20 $cm^2$) at 300, 600, 1000, 3000, or 10,000 cells per plate. After 2 weeks growth, the cells were fixed, stained, and counted as described in the MMC survival experiment.

Unscheduled DNA Synthesis

For analysis of the effects of butyrate on unscheduled DNA synthesis, muntjac cells were treated with 10 mM butyrate for varying lengths of time or at varying concentrations for 70 hours. The cells were rinsed twice with PBS containing the appropriate concentration of sodium butyrate and then irradiated with 8 joules per square meter ultraviolet light at 254 nm. Control cells were mock irradiated by placing them in the irradiation chamber at the same time with their plastic lids left on. The cells were then covered with medium containing 10 μ Ci/ml high specific activity tritiated thymidine and butyrate at the same concentration which the cells had previously seen. After three hours, the medium was removed, the cells were rinsed twice with PBS, detached with trypsin and dropped on slides after the normal hypotonic

treatment and fixation in 3:1 methanol:acetic acid. When the slides were dry, unbound tritium was removed by rinsing the slides for 5 minutes each in 5% trichloro-acetic acid with 1% sodium pyrophosphate, two changes of 70% ethanol, and once in 100% ethanol. Autoradiography was carried out as previously described and unscheduled DNA synthesis was measured by counting the grains over 50 non-S-phase cells for each treatment. Cells were considered to be S-phase if the number of grains was uncountably large, or if the grains occured in dense clusters rather than being randomly distributed over the cell.

## RESULTS

### Suppression of Thymidine Incorporation

As shown in Fig. 25, thymidine incorporation in exponentially growing muntjac cells treated continuously with 5, 7, or 10 mM butyrate decreased exponentially during the first 20 h. The rate of incorporation began to stabilize after about 30 h.

### Cellular Proliferation and Survival

Cell growth following butyrate treatment is shown for muntjac cells in Fig. 26a and CHO cells in Figs. 26b and 26c. The number of cells per plate of both muntjac and CHO cells treated with 10 mM butyrate continued to increase for the first 12 to 24 h. After this, the number of cells attached to the plates declined, and at 72 h the number of CHO cells stabilized at a level slightly below that present before butyrate addition. During the same time period, the

Figure 25. Thymidine incorporation in butyrate-treated muntjac cells measured after TCA precipitation of the labeled DNA. The butyrate concentrations were ( ) 5 mM, ( Δ) 7 mM, and ( □) 10 mM.

**FIGURE 25**

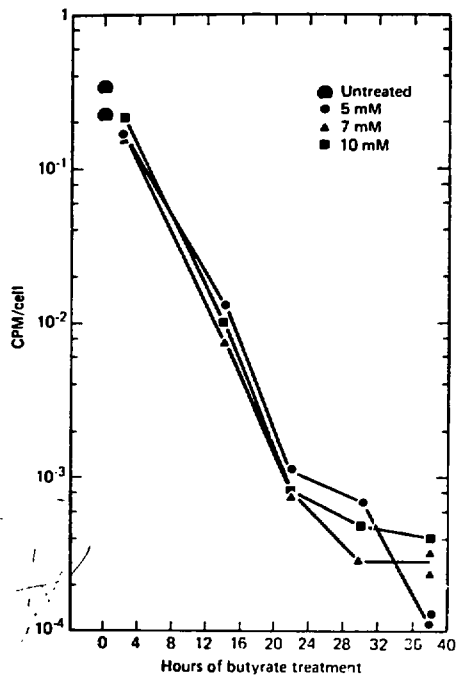
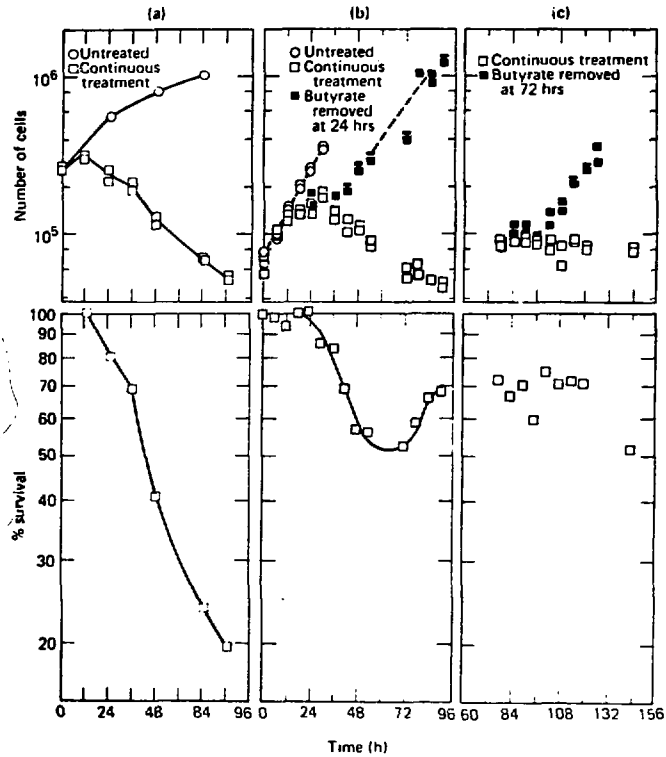

Untreated
5 mM
7 mM
10 mM
CPM/cell
1
$10^{-1}$
$10^{-2}$
$10^{-3}$
$10^{-4}$
0
4
8
12
16
20
24
28
32
36
40
Hours of butyrate treatment

Figure 26. Growth of butyrate-treated muntjac (a) and CHO (b and c) cells. Exponentially growing cells were either untreated ( ○ ), treated continuously with 10 mM butyrate ( □ ) or treated and then placed in medium without butyrate ( ■ ) after 24 h (b) and 72 h (c). Plates from each category were treated with trypsin and the number of cells in a sample counted by Coulter counter.

Bottom Survival of muntjac (a) and CHO (b and c) cells continuously treated with 10 mM butyrate.

**FIGURE 26**


(a)
(b)
(c)
Untreated
Continuous treatment
Untreated
Continuous treatment
Butyrate removed at 24 hrs
Continuous treatment
Butyrate removed at 72 hrs
Number of cells
% survival
Time (h)

muntjac cell population decreased to about one quarter the initial population. Survival of the attached muntjac and CHO cells (Fig. 26, bottom) remained high for the first 12 to 24 h of treatment in 10 mM butyrate. After this, survival of the remaining attached cells declined almost exponentially so that at 48 h about 40% of the muntjac cells and about 55% of the CHO cells survived. At later times, muntjac survival continued to decline but the fraction of the attached CHO cells which would form colonies abruptly increased.

Recovery of CHO From Butyrate Treatment

CHO cells placed in nonbutyrated medium after a 24 h treatment began log-phase growth after a delay of about 24 h (Fig. 26c). During the interval between removal of the butyrate and initiation of exponential growth, the number of attached cells remained roughly constant. Thymidine incorporation, as detected by autoradiography, in CHO cells released from a 24-h butyrate treatment began about 18 h after butyrate removal (Fig. 27). The percentage of cells that incorporated label after continuous exposure to tritiated thymidine then increased gradually over a period of 24 h to about 95%.

Cell-Cycle Distribution

Analysis of untreated, exponentially growing cells by flow cytometry showed a typical DNA histogram (Figs. 28a and b). The peak at the lower fluorescence intensity represents cells having a DNA content characteristic of the G1 phase; cells having a DNA content corresponding to G2 phase are included in the peak at the higher fluorescence intensity. Cells in S phase have intermediate DNA contents and are represented in the histogram by the low a curve connecting the two peaks. Analysis (De74) of CHO cell distributions showed the cells to be about 25% G1, 15% G2, and 60% S phase; muntjac cells were about 48% G1, 15% G2, and 37% S phase.

Figure 27. Thymidine incorporation of CHO cells after release from 72 h of butyrate block.

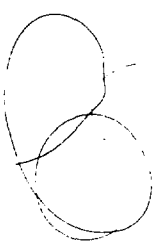


**FIGURE 27**

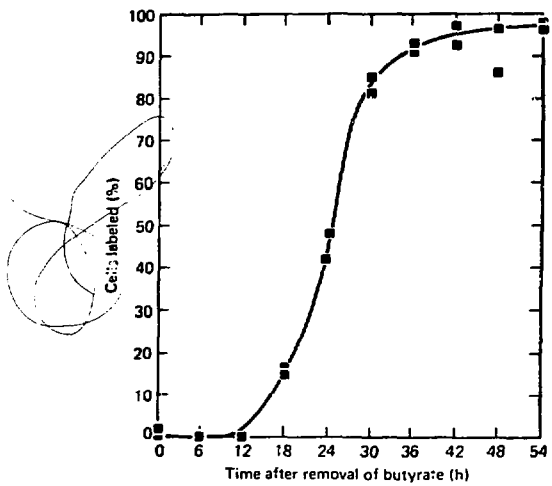
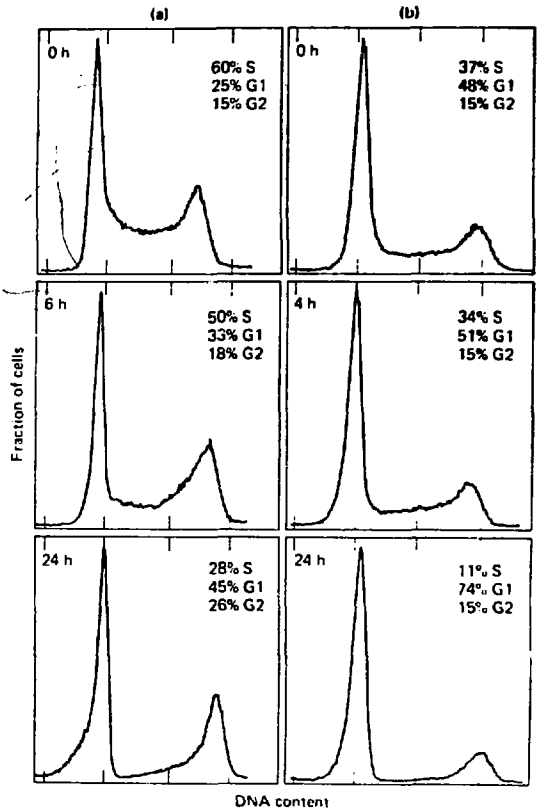

100
90
80
70
60
50
40
30
20
10
0
Cells labeled (%)
0
6
12
18
24
30
36
42
48
54
Time after removal of butyrate (h)

Figure 28. Flow cytometric analysis of CHO (a) and muntjac cells (b) after treatment with 10 mM butyrate.

**FIGURE 28**


(a)
(b)
0 h
60% S
25% G1
15% G2
0 h
37% S
48% G1
15% G2
6 h
50% S
33% G1
18% G2
4 h
34% S
51% G1
15% G2
24 h
28% S
45% G1
26% G2
24 h
11% S
74% G1
15% G2
Fraction of cells
DNA content

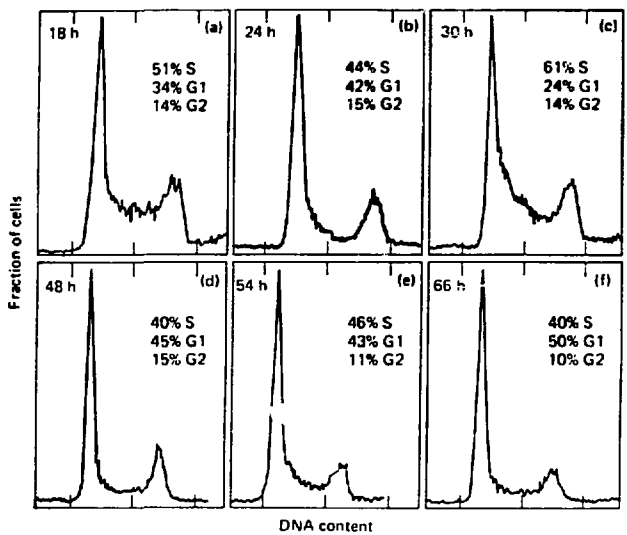
Addition of 10 mM butyrate rapidly led to changes in the cell-cycle distribution as evidenced by the flow histogram of cellular DNA content (Fig. 28). Within 6 h, the S-phase population of CHO cells, particularly those in early S (Fig. 28a), decreased significantly. After 24 h, the S-phase region of the DNA distribution is almost completely absent, having a very sharp transition at the G1-S interface. The fraction of the population that appears in G2 remains constant or increases gradually during this period of butyrate treatment. Even after 72 h, the fraction of the population that has the DNA content of G2 cells has not decreased (data not shown). The G2 peak, even after a very long time, has a tail of cells with the DNA content of late S-phase cells. It is unlikely that the DNA of these cells is actually replicating since thymidine incorporation after long butyrate treatment is negligible. The muntjac cells (Fig. 28b) responded similarly, but more slowly. At 4 h the early S phase population decreased less dramatically than that of the CHO cells in Fig. 28a, but at 24 h the cells in S phase were depleted. This was accompanied by a corresponding accumulation of cells in G1 and G2.

CHO cells sorted from the G2 peak onto slides and examined directly by fluorescence microscopy contained 2 to 16% doublets (two attached cells) regardless of the period of butyrate treatment, indicating that the G2 peak indeed represents G2 cells and not doublets of G1 cells.

The DNA histograms of CHO cells released from 24- and 72-h butyrate treatments are shown in Figs. 29 and 30. The cell-cycle distribution remained unchanged for several hours. After 24 h in 10 mM butyrate (Fig. 29), the recovering CHO cells were partially synchronized. At 18 h post-treatment, a large fraction of the population entered S phase. For the next 24 to 30 h the

Figure 29. Cell-cycle distribution of CHO cells after release from 24 h exposure to butyrate. Cells were fixed and stained for flow cytometry at 18 h (a), 24 h (b), 30 h (c), 48 h (d), 54 h (e), and 66 h (f) after release from the buryrate block.

**FIGURE 29**


18 h
(a)
51% S
34% G1
14% G2
24 h
(b)
44% S
42% G1
15% G2
30 h
(c)
61% S
24% G1
14% G2
48 h
(d)
40% S
45% G1
15% G2
54 h
(e)
46% S
43% G1
11% G2
66 h
(f)
40% S
50% G1
10% G2
Fraction of cells
DNA content

population alternated between one having cells predominately in the G1 and G2 phases and one having a much larger than normal proportion of cells in the S phase. About 66 h after butyrate removal, the DNA histogram was characteristic of normal exponentially growing cells. In cells treated with Butyrate for 72 h (Fig. 30), no significant S-phase population was evident until 18 h after the butyrate was removed. Then the S and G2 portions of the histograms gradually increased, but the cell-cycle distributiuon remained disturbed even at 54 h post treatment.

Survival of UV-Treated Muntjac

The survival curve for muntjac cells in confluent culture is shown in Fig. 31. The cells have a $D_{37}$ of about 7 joules/meter$^2$ and a $D_o$ of about 5 joules/meter$^2$ (n = 1.4). This sensitivity to killing by UV is a little higher than would be expected for other cell lines in exponential growth (Th80) but some of this observed sensitivity may be due to the synergistic effects of ultraviolet irradiation in plateau followed by immediate treatment with trypsin. Later experiments (see Fig. 36) showed that cells irradiated in plateau and treated with trypsin immediately after UV were much slower to recover than those irradiated 17 hours after trypsin treatment.

Unscheduled DNA Synthesis

Unscheduled DNA synthesis was measured in plateau phase muntjac cells treated with 8 joules per square meter 254 nm ultraviolet light. Autoradiographic grain counts of cells treated for varying lengths of time with 10 mM butyrate before irradiation are shown in Fig. 32. After 12 hours of butyrate, the grain count distribution does not appear to be substantially affected when compared to cells receiving no butyrate. The level of unscheduled synthesis at longer treatment times, however, was progressively

Figure 30. Cell-cycle distribution of CHO cells after release from 72 h butyrate block. Cells were fixed and stained for flow cytometry at 6 h (a), 12 h (b), 18 h (c), 24 h (d), 30 h (e), 36 h (f), 42 h (g), 48 h (h), and 54 h (i).

**FIGURE 30**

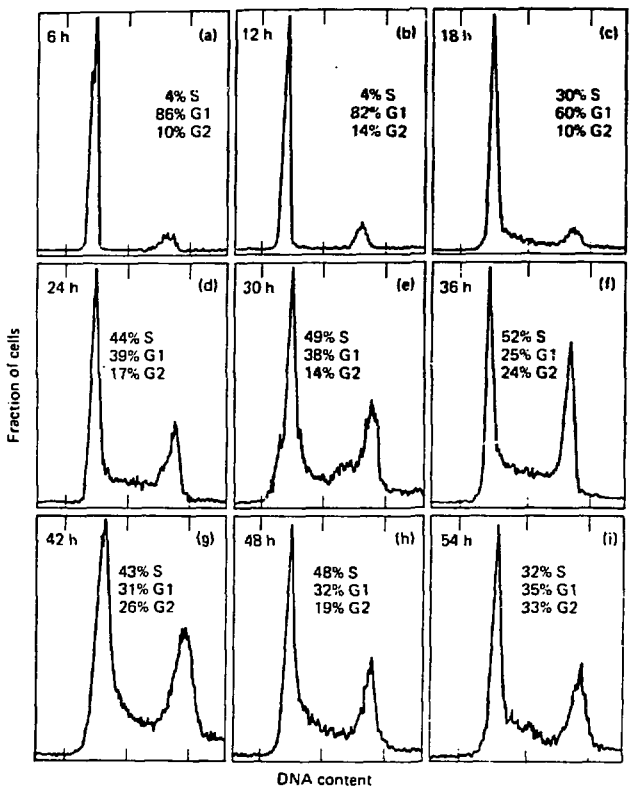

6 h
(a)
4% S
86% G1
10% G2
12 h
(b)
4% S
82% G1
14% G2
18 h
(c)
30% S
60% G1
10% G2
24 h
(d)
44% S
39% G1
17% G2
30 h
(e)
49% S
38% G1
14% G2
36 h
(f)
52% S
25% G1
24% G2
42 h
(g)
43% S
31% G1
26% G2
48 h
(h)
48% S
32% G1
19% G2
54 h
(i)
32% S
35% G1
33% G2
Fraction of cells
DNA content

Figure 31. Survival of muntjac cells to UV light. The ordinate is percent survivors shown on a logarithimc scale. The multiple points at each dose represent mean survivals observed for different cell seed numbers in the assay plates.

**FIGURE 31**

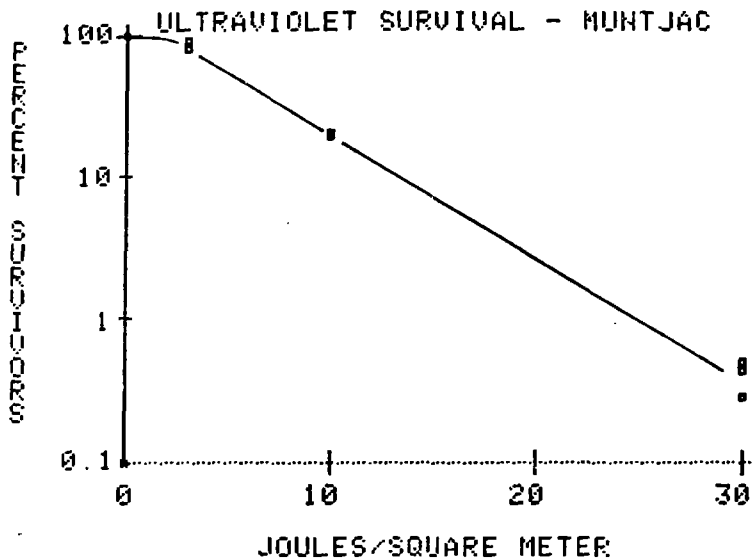
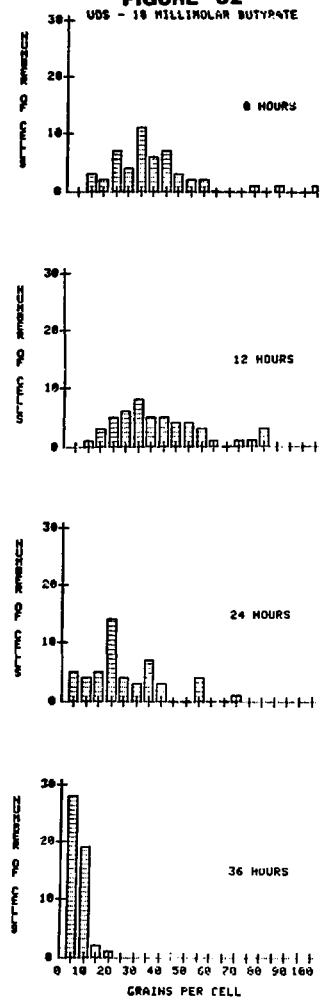

ULTRAVIOLET SURVIVAL - MUNTJAC
PERCENT SURVIVORS
100
10
1
0.1
0
10
20
30
JOULES/SQUARE METER

Figure 32. Distribution of unscheduled DNA synthesis grains in muntjac cells treated with 10 mM sodium butyrate for 0, 12, 24, or 36 hours prior to irradiation with 8 joules per square meter ultraviolet light.

**FIGURE 32**

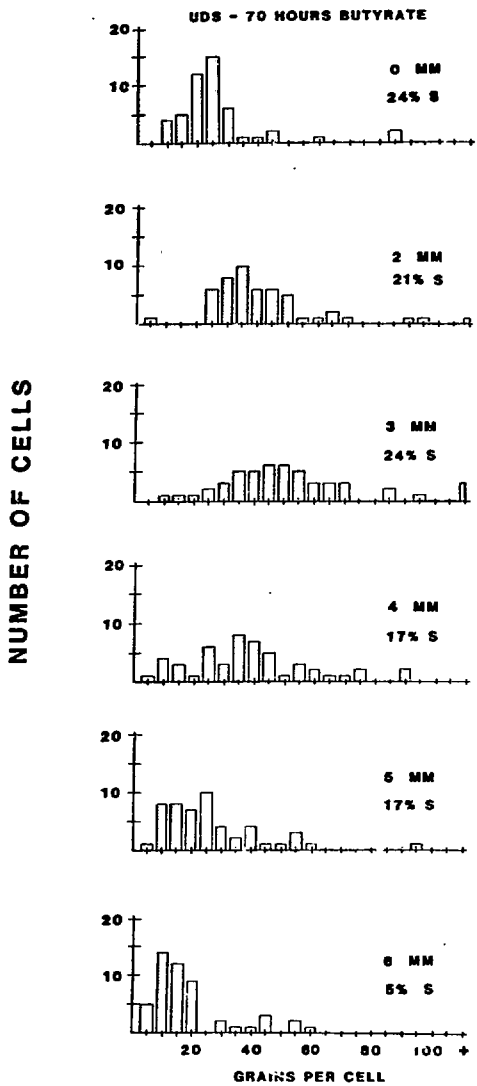

UOS - 10 MILLIMOLAR BUTYRATE
0 HOURS
12 HOURS
24 HOURS
36 HOURS
0 10 20 30 40 50 60 70 80 90 100
GRAINS PER CELL

lower. In all cases, control cells which received no UV had fewer than 10 grains per cell, and greater than 90% of these cells had fewer than 5 grains per cell (data not shown). The results of this experiment suggest that measurement of long term UDS rates are reduced in the presence of 10 mM butyrate and therefore it would be unsuitable to use long treatments with butyrate for reducing the S-phase population.

To determine if lower butyrate concentrations would block S-phase without affecting unscheduled synthesis in long exposures, exponentially growing muntjac cells were treated with varying concentrations of butyrate for 70 hours and then irradiated with 8 joules/$m^2$ of 254 nm UV. UDS was measured by autoradiography as before. Cells with uncountable or dense clusters of grains were considered to be S-phase cells and were excluded from the grain count distributions. The results of this experiment are shown in Fig. 33. Each panel shows the grain count distribution in irradiated cells. The butyrate concentration and the percentage of the population which appeared to be in S-phase are shown in numbers at the right. At low doses, UDS does not appear to be affected, but neither is the S-phase population reduced. At intermediate doses, UDS grain counts actually increase, but the S-phase population is still not substantially affected. At the highest tested doses (5 and 6 mM) the S-phase population is lowered and so are UDS grain counts. At all concentrations, non-S-phase cells in the unirradiated controls had fewer than 10 grains per cell and greater than 90% of these cells had fewer than 5 grains.

Figure 33. Distribution of unscheduled DNA synthesis grains in muntjac cells treated for 70 hours with either 0 mM, 2 mM, 3 mM, 4 mM, 5 mM, or 6 mM sodium butyrate prior to irradiation with 8 joules per square meter ultraviolet light.

# FIGURE 33


UDS - 70 HOURS BUTYRATE
NUMBER OF CELLS
0 MM
24% S
2 MM
21% S
3 MM
24% S
4 MM
17% S
5 MM
17% S
6 MM
5% S
20
10
20
40
60
80
100 +
GRAINS PER CELL

## DISCUSSION

Butyrate blocks cell-cycle progression, resulting in a depletion of the S phase an an accumulation of cells having DNA contents characteristic of the G1 and G2 phases. Experiments with muntjac cells showed that 10 mM butyrate reduces the rate of DNA synthesis by more than two orders of magnitude in 24 h. Muntjac cells continue to divide early in this treatment, but after 12 h the fraction of cells remaining attached decreases exponentially. Survival of the muntjac cells attached to the plates decreases exponentially after 12 h in 10 mM butyrate.

More extensive experiments were possible with the rapidly-cycling and easily-cultured CHO cells. CHO cells treated with 10 mM butyrate continued to divide for about 24 h and then cells gradually became detached, apparently leaving a small resistant, nondividing subpopulation after about 84 h. Survival of the attached CHO cells contrasted sharply with that of the muntjac. CHO cells are not killed for 24 h and those remaining attached after 84 h are resistant to further killing. CHO cells released from 24 or 72 h in butyrate begin to cycle again after about 18 h. Those blocked for 24 h demonstrate cell-cycle synchrony for up to 48 h after removal of the block. The data from these experiments indicate that butyrate exposure blocks entry of G1 cells into the S phase and that most of those cells already in S phase will progress into G2. We have little basis for deciding whether the G1-S block is established immediately or develops gradually, but the selective depeletion of early S-phase cells is noticeable within 4 h. Other investigators have found evidence of an increase of G1 cells in butyrate-treated cell cultures (Si75).

The fact that cell numbers continue to increase for 12 to 24 h after butyrate addition indicates that either butyrate does not block cells at the mitosis-G1 interface or that such a block is slow to be established. The block that results in the increase of cells having G2-M DNA content probably occurs earlier in the cell cycle at the end of the G2 phase, for example, since we have observed very few mitotic cells in treated cell populations. Such a block would allow mitotic cells to divide and would, if the block develops slowly, account for cell numbers continuing to increase after butyrate addition.

Our evidence for the accumulation of cells in G2 is the appearance of cells having a DNA content typical of cells in the G2 phase in histograms generated by flow cytometry. Some objects having an apparent G2 DNA content appear in flow histograms because of doublets consisting of two attached G1 cells. The percentage of G1 doublets can be measured directly using the cell sorter. Microscopic examination of the cells sorted from the G2-M peak reveals that between 2 and 16% of these objects could be G1 doublets. This percentage does not change with butyrate treatment, which reinforces the evidence that butyrate produces an accumulation of cells having G2 DNA content.

Our data cannot entirely rule out the possibility that the cells having DNA content typical of the G2 phase are butyrate-induced tetraploids also blocked in G1. Upon release from butyrate however, tetraploid G1 cells should replicate to produce a peak having twice the normal DNA content of cells in G2 phase. No such peak was observed.

Ten millimolar butyrate inhibits unscheduled DNA synthesis in muntjac cells after 24 to 36 hour exposures. However, the decreased grain counts may well be attributable to the high toxicity of the butyrate at these same

exposures. In 70 hour exposures at 2 to 6 millimolar where the grain counts are higher than in unirradiated control cells, it is not clear whether the butyrate actually influences DNA repair, or whether there is an indirect effect such as an alteration of thymidine transport. In any case, it is not apparent from these experiments that there are experimental conditions under which butyrate would suppress S-phase incorporation of thymidine while allowing the long term observation of unaffected unscheduled DNA synthesis.

It is not clear which of butyrate's effects is primary (i.e., the result of direct and immediate molecular interaction of cellular components with buryrate) and which are secondary (i.e., the biochemical or regulational consequences of previous butyrate reactions). Most of the biochemical effects observed require several hours to occur and are reversible, implying that they are subsequent events in a regulatory chain as opposed to a direct binding or reactions involving butyrate. The inhibition of DNA synthesis, for example, takes place over nearly 24 h. Thus, although it is unlikely that butyrate is the molecule that interferes directly with the advance of the DNA polymerase, one might consider whether the other butyrate effects are a feature of the inhibition of DNA synthesis. Deutsch et al., (De77) present evidence that other inhibitors of DNA synthesis induce alkaline phosphatase production in HeLa cells and are synergistic with butyrate in causing morphological changes of the cell to a spindle-like shape. On the other hand, Rubenstein et al., (Ru79) found that marked hyperacetylation of the histones could be induced by other closely related short chain fatty acids without marked inhibition of cellular proliferation. These results seem inconsistent with any general model in which butyrate-induced modification of chromatin structure by hyperacetylation of

histones suppresses DNA synthesis. Alteration of the cellular concentration of regulatory molecules may be another mechanism by which butyrate might exert its effects. Prasad and Sinha (Pr76) found that butyrate doubled the intracellular concentration of cyclic AMP and suggested that some of the effects of butyrate might be mediated by cyclic AMP. Investigators examining the effects of exogenously supplied cyclic AMP often use the dibutyryl form to facilitate transport into the cell, and some caution has been advised because butyric acid is used in the derivitization of cyclic AMP (Le75, Pr76).

Coffino et al., (Co75, Co78) used flow cytometry to measure the cell-cycle distribution of S49 lymphoma cells treated with dibutyryl cyclic AMP. They found an accumulation of cells in G1 and a rapid disappearance of early S-phase cells. In contrast to the results reported here for butyrate, their work with 0.1 mM dibutyryl cyclic AMP showed an almost complete disappearance of G2- and S-phase cells within 18 h. The difference in response times may merely reflect differences in cell-cycle times but the lack of accumulation of G2 cells implies that the effect of dibutyryl cyclic AMP differs from that of butyrate, assuming the cell lines are comparable. Also in contrast to our results with butyrate, they observed that the duration of the block induced by dibutyryl cyclic AMP does not affect re-entry into the cell cycle after release of the block (Co81).

Similarly, the morphological effects of butyrate seem to be distinct from those induced by dibutyryl cyclic AMP. Dibutyryl cyclic AMP induces morphological changes in XC (rat sarcoma) but not in HeLa cells (Gi73, Si75), while butyrate is an effective inducer of morphological changes in HeLa (He75). These data imply at least some independent determinants of the morphological effects of butyrate and dibutyryl cyclic AMP.

Other regulatory molecules could be involved. The morphological effects of butyrate and dibutyryl cyclic AMP are reversed by the calcium ionophore A23187 if calcium is included in the medium (He75). A regulatory role of cell-membrane permeability to calcium ions is implied in butyrate-induced changes in morphology and butyrate-enhanced activity of the CMP-sialic acid:lactosylceramide sialtransferase. This sialyltransferase is involved in the production of the glycophingolipid GM3 (He76, Vi80). Furthermore, Brady and Fishman (Br74) speculate that the production of specific cell-surface glycolipids represents a switch point in the progression of the cell cycle, but at this time we have no sound evidence of a deterministic link between butyrate-induced membrane permeability to calcium and regulation of cell-cycle progression.

Of course, more than one primary effect of butyrate may be present. Cousens et al., (Co79) reported that histone deacetylases from hepatoma tissue culture (HTC) cells are noncompetitively inhibited by butyrate in vitro. It is presumably the butyrate inhibition of these deacetylases that leads to the hyperacetylation of histones H3 and H4 in vivo. And yet, as was noted earlier, it is difficult to attribute butyrate-induced suppression of cellular proliferation to generalized histone hyperacetylation. Rubenstein et al., (Ru79), using a general label for proteins, found that only 16 of the 500 proteins in HTC cells detected by two-dimensional gel electrophoresis had altered rates of synthesis or breakdown after butyrate treatment, implying that the regulational changes induced by butyrate are somewhat selective.

Until we know more about all of the effects induced by butyrate and their interrelationships, it will be difficult to assess the mechanisms that are common to these effects.

Butyrate blocks cell cycle progression but the variety of butyrate-induced effects with poorly understood mechanisms make it seem a risky experimental tool. In particular, the distortion of unscheduled DNA synthesis by butyrate makes it presently unsuitable as a cell cycle blocking agent in the investigation of DNA repair and the production of sister chromatid exchange.

## LOCALIZATION OF UDS AND SCE VS RATES OF REPAIR OF LESIONS LEADING TO SCE AND UDS

### Introduction

We previously proposed to compare the localization of UDS with that of SCE and to compare the rates of repair of lesions leading to SCE with the rate of disappearance of lesions that induce UDS. Ultraviolet light is a potent inducer of unscheduled DNA synthesis, as demonstrated above. Others have shown that UV induces increased sister chromatid exchange (Wo74, Ka74). UV has been shown to damage DNA primarily by the induction of pyrimidine dimers (Cl75) and these lesions are substantially if not completely repaired. These properties make UV a suitable DNA damaging agent for comparing the induction and repair of lesions leading to SCE and UDS.

Blocking cells in G1 prior to carrying out UV irradiation helps solve three experimental problems:

1) Lesions to the DNA are converted to SCE during S so cells at different cell cycle stages have different times for repair. For a synchronized population there is a more clearly defined time for the repair of SCE producing lesions: the time between irradiation and entry of the cells in S. By contrast, an exponentially growing population has a variety of elapsed times for entry of cells in S. This range extends from those cells that are already synthesizing DNA at

the time of treatment, to those cells that have just completed S and have the whole of G2, mitosis, and G1 in which to carry out repair before any lesions are "fixed" in S.

2) Cells with different cell cycle progression rates have different opportunities for repair. Starting with a synchronized, non-S population before irradiation and collecting samples at second division after irradiation decreases ambiguity caused by the fact that cells cycle at different rates. A heterogeneous distribution of cell cycle times will mix cells with different elapsed cell cycle times in one sample. An initially synchronized population that is measured only at second division ensures equivalent cell cycle times and that absolute times have elapsed for each of the cells measured for SCE.

3) Cells at different stages in the cell cycle may have differing sensitivity to DNA damaging agents. Treating a synchronized population should reduce heterogeneity of response.

Unfortunately, use of the cycle blocking agents hydroxyurea and sodium butyrate confuse the measurement of unscheduled DNA synthesis. However, in the course of characterizing these agents, it was found that the residual S-phase cells in a plateau population could easily be eliminated observationally in UDS experiments and that residual G2 cells probably constitute no more than 2% of the population. Therefore, in the remainder of these experiments cells are held in plateau but no chemical blocking agent is used.

In the final set of experiments, three questions are asked:

1) What is the distribution of UDS and SCE in the chromosomes of muntjac cells irradiated with UV in plateau phase?

2) In cells released from plateau and irradiated at varying intervals before entry into S, how does the level of SCE induced relate to the "repair interval" allowed?

3) In these cells irradiated after release from plateau, what is the time course of unscheduled DNA synthesis after each of these irradiations, and how does the rate of removal of UDS producing lesions compare with the rate of removal of SCE inducing lesions?

Materials and Methods

For the determination of UDS and SCE in cell irradiated in plateau, 4 X $10^6$ cells were seeded into 100 mm plastic culture dishes (57 $cm^2$) and grown for 6 days. The cells were rinsed twice with PBS, irradiated with 8 joules/$m^2$ and cultured for 4 hours in 10 μ Curie/ml tritiated thymidine. After three PBS rinses, the cells were treated with trypsin and recultured in 15 ml of 30 μM BrdUrd in 75 $cm^2$ plastic culture flasks, each with 1.4 X $10^6$ cells. From 80 to 96 hours after UV, samples were collected with 8 hr of Colcemid treatment and dropped on slides after the normal treatment with hypotonic and fixation in 3:1 methanol acetic acid. The slides were then either dipped for autoradiography or processed for the scoring of SCE.

The time of entry into S-phase for cells released from plateau was measured for three different treatments: unirradiated cells, cells irradiated immediately before release from plateau, and for cells irradiated 18 hours after release. Cells were seeded at 5 X $10^6$ per 100 mm culture dish (57 $cm^2$) five days before release. One set of plates was rinsed twice with PBS, irradiated with 8 joules/$m^2$ and then treated with trypsin for subculture.

Other cells were subcultured without UV. Subcultured cells were plated at $5 \times 10^5$ cells per 60 mm culture dish and 5 ml medium. Medium containing 0.1 μ Ci/ml high specific activity tritiated thymidine was added to the irradiated cells and half the unirradiated cells. The remaining half were given unlabeled medium until 18 hours after subculture when they were rinsed with PBS, irradiated and placed in similar labeled medium. At 4 hour intervals, sample plates from each group were treated with 1 ml lysis solution and thymidine incorporation of the cells was measured by TCA precipitation of tritium labeled DNA. The lysis solution and TCA precipitation procedures were those previously described for measurement of butyrate inhibition of DNA synthesis.

In this experiment cells were released from plateau and irradiated at either 12, 18, or 21 hours after release.

For each irradiation time two measurements were made: (1) the level of SCE induction in heterochromatin and euchromatin and, (2) the level of UDS at 1 hour intervals after irradiation. For measurement of UDS and SCE in cells irradiated at intervals after release from plateau, $4 \times 10^6$ cells per 100 mm culture dish (57 $cm^2$) were seeded and grown for 8 days prior to treatment with trypsin and subculture to a cell density of $10^6$ per 100 mm culture dish. At 12, 18, 21 hours after subculture sample plates were rinsed, irradiated and re-covered with medium. For analysis of SCE, some plates were given medium containing 30 μ M BrdUrd and five hour Colcemid collections of second division cells were harvested 65 and 70 hours after release from plateau. For analysis of one hour UDS rates, the remaining plates were labeled during the period: 0 to hour-1, hour-1 to hour-2, or hour-3 to hour-4 after each irradiation with medium containing 10 μ Ci/ml tritiated thymidine. Unlabeled medium was used between irradiation and the labeling period.

After labeling, UDS plates were rinsed with PBS and treated with trypsin. Cells were then treated in hypotonic, fixed in 3:1 methanol:acetic acid and dropped on slides. For autoradiography, the slides were rinsed in TCA-pyrophosphate solution and an ethanol series, dipped in NTB-2, and held and developed at 4°C as previously described.

Long term UDS was also measured after the irradiation at 12 hours post release. Sample plates were labeled from 0 to hour-4, hour-4 to hour-8, hour-8 to hour-12, and hour-12 to hour-16 after the irradiation.

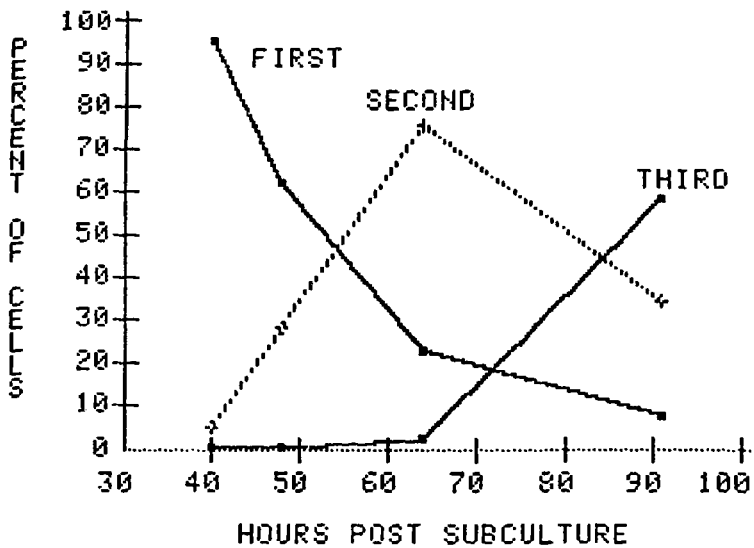
## Results

### Distribution of UDS in Cells Irradiated in Plateau

In order to measure UDS and SCE levels and localization in the chromsomes of muntjac cells irradiated in plateau,the cells were given tritiated thymidine for four hours after UV and then subcultured into medium containing BrdUrd. Samples were collected for analysis at intervals 40 to 91 hours after subculture. The cells that were treated for analysis of SCE were measured to determine the fraction of metaphases that had completed 1, 2, or 3 rounds DNA replication in BrdUrd. The results for unirradiated control cells is shown in Fig.34. The fraction of metaphases having completed one round of synthesis is represented by the curve labeled "FIRST". The curve representing metaphases having completed 2 and 3 rounds are labeled "SECOND" and "THIRD", respectively. Second division metaphases peak at about 64 hours after subculture. By contrast, in irradiated cultures, cell progression is greatly delayed and at 91 hours first division cells still constituted 67% of the metaphases and a significant fraction of the metaphases were in their third

Figure 34. Metaphase number of unirradiated cells as a function of time after release from plateau. Cells were grown in BrdUrd from the time of release. At various times cells were harvested, stained by the FPG technique and for each metaphase, microscopic determination was made of the number of rounds of DNA replication carried out since release. The curves represent the fraction of cells in their "FIRST", "SECOND" or "THIRD" metaphase since release from plateau.

**FIGURE 34**


PERCENT OF CELLS
100
90
80
70
60
50
40
30
20
10
0
FIRST
SECOND
THIRD
30
40
50
60
70
80
90
100
HOURS POST SUBCULTURE

division (data not shown). Repeat experiments showed that second division cells never became a major fraction of the metaphase population even at much longer times. As will be shown, later experiments determined that irradiation in plateau followed closely by treatment with trypsin causes a much delayed and prolonged entry of the cells into S (see Fig. 36). Such an effect would account for the delay observed here.

This synergistic effect between UV and trypsin made the measurement of SCE in this experiment difficult to carry out or interpret. However, the labeling of the cells for UDS was carried out before the trypsin treatment and the first division cells were utilized for autoradiography.

Grains were counted over metaphase muntjac cells collected 88 hours after subculture. The grains were counted for each of the muntjac's seven chromosomes except for the dot Y2 chromosome. The grain counts on the X-chromosome were further broken down into those associated with the long arm, the "neck" region, the short arm, and the two junctions between the neck and the adjacent arms. The resulting counts for each chromosome and region were divided by the fraction of the whole-cell DNA content represented by that chromosome or region as given by Carrano and Johnston (Ca77). This figure will be refered to here as the "adjusted grain count". It is the number of grains per unit DNA where the unit is the DNA complement of the muntjac cell. This adjusted grain count is the number of grains to be expected over a whole cell assuming the counted chromosome or region is proportionally representative. If the UDS grain counts are proportional to DNA content, then each chromosome and region will have the same adjusted grain count. These adjusted counts are

shown in Fig.35. The standard errors were adjusted by the same factor, and the error bars in the figure represent two standard errors on either side of the mean.

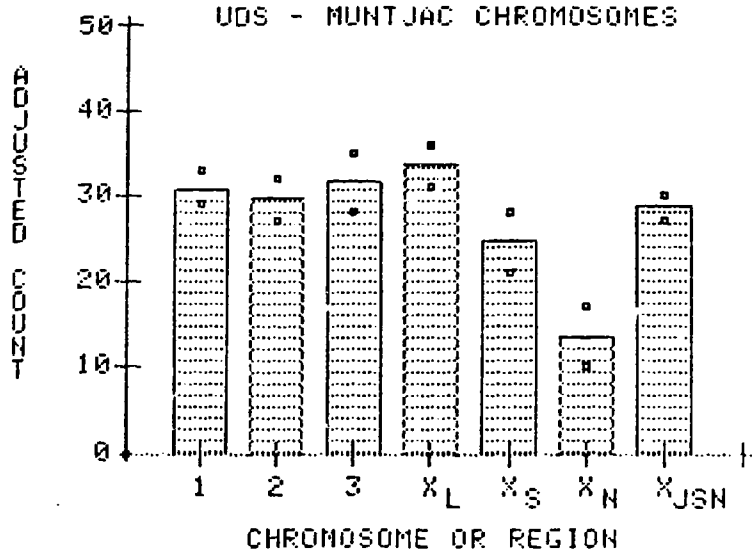
The data for chromosomes 1, 2, and 3 are labeled by number, while $X_L$, $X_S$ and $X_N$ refer to the euchromatic long arm, the short arm and the heterochromatic neck region of the X chromosome, respectively. The grain counts for the junctions are included in the bar marked $X_{JSN}$. The data for the short arm of the X, the neck of the X and the two junction regions were summed and the adjusted count was calculated on the basis of the DNA content of the whole region. These data are labeled $X_{JSN}$. The standard error for these summed data is the root mean square of the individual standard errors subsequently adjusted to the total DNA content of the region. The grain counts for the euchromatic regions of cell, i.e., chromosomes 1, 2, 3, and the long arm of the X, are not significantly different. The heterochromatic neck region has slightly less than half the grains expected for its DNA content and is significantly decreased.

The adjusted grain count for the $X_{JSN}$ region including the neck, the short arm and both junctions, is almost the same as that of the euchromatin. The adjusted grain count for the short arm of the X is slightly lower than that for euchromatin (see data in Table 2 in the Appendix). The deficit of grains in the short arm and in the heterochromatic neck is balanced by the large number of grains at the junctions.

The distribution of unscheduled DNA synthesis grains observed in muntjac chromosomes from cells exposed to UV in plateau phase is very similar to the baseline distribution of SCE seen by Carrano and Wolff (Ca75) and to the SCE induced by mitomycin C as reported by Carrano and Johnston (Ca77).

Figure 35. UV induced unscheduled DNA synthesis in muntjac chromosomes. The "adjusted count" or grains per unit DNA is given for chromosomes 1, 2, and 3 and regions of the X chromosome: long arm ($X_N$), short arm ($X_S$), and neck ($X_N$) The bar marked "$X_{JSN}$" is the adjusted count for the combined regions of the neck, the short arm, and the junctions of the neck with the long and short arms. The error bars are at two standard errors on either side of the mean.

**FIGURE 35**


UDS - MUNTJAC CHROMOSOMES
ADJUSTED COUNT
50
40
30
20
10
0
1
2
3
X L
X S
X N
X JSN
CHROMOSOME OR REGION

Entry into S-phase After Plateau

Counts from the TCA precipitated DNA of cells continously labeled after release from plateau are shown in Fig. 36. Unirradiated control cells have a slightly elevated baseline incorporation of thymidine but demonstrate increased incorporation between 21 and 24 hours after subculture. Cells which were irradiated in plateau immediately before trypsinization (UV-BEFORE) show reduced background incorporation and did not achieve significant S-phase incorporation by the end of the experiment, more than 40 hours after subculture. Cells which were irradiated 17 hours after release from plateau (UV-AFTER) have suppressed background counts but begin S-phase incorporation about the same time as unirradiated controls and any UV induced delay is small. The incorporation of these irradiated cells is at least as intense as that of controls, and UV toxicity is not apparent in these results.

The strong synergism between UV and trypsin helps explain the long delay described above and made it necessary to carry out further experiments with as much time between subculture and irradiation as possible.

UDS vs Repair Interval

Unscheduled DNA synthesis was measured in whole cell nuclei as a function of time after irradiation. Cells irradiated 12 hours after subculture and labeled for 4-hour intervals had the grain distributions shown in Fig. 37. For each interval, grains were counted on 50 cells judged not to be in S-phase. The cells were given 4 days autoradiographic exposure and the fraction of cells which were considered in S rose from about 30% in the first time interval to about 50% in the last. The UDS during the first four hours is clearly more intense than during the later periods. In the second, third, and fourth 4-hour

Figure 36. Thymidine incorporation for muntjac cells as a function of time after release from plateau. Determined by TCA precipitation of DNA of cells grown continuously in tritiated thymidine (0.1 microCurie/ml) "UV-BEFORE" cells received 8 joules/square meter immediately before subculture while "UV-AFTER" were irradiated 17 hours after subculture. "CONTROL" cells received no UV.

**FIGURE 36**

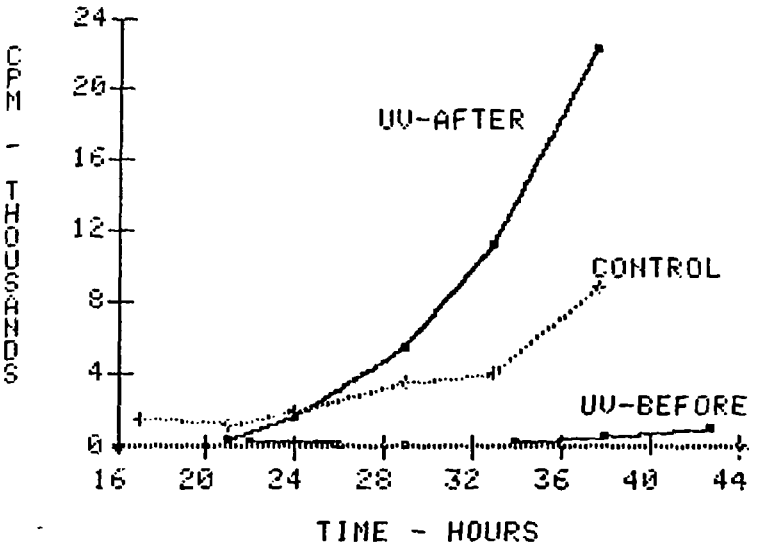
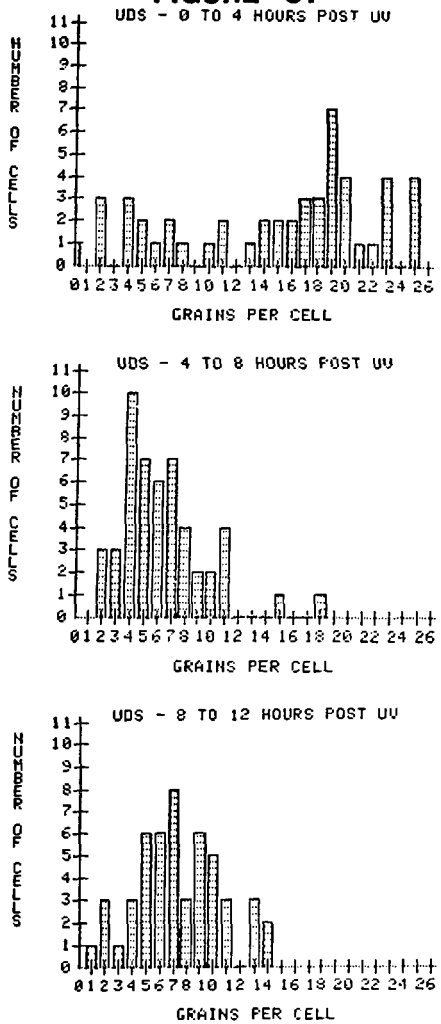

CPM - THOUSANDS
24
20
16
12
8
4
0
UV-AFTER
CONTROL
UV-BEFORE
16
20
24
28
32
36
40
44
TIME - HOURS

Figure 37. Distribution of unscheduled DNA synthesis grains on cells irradiated 12 hours after release from plateau. Cells were grown in 10 microCurie/ml tritiated thymidine for 4-hour intervals starting 0, 4, 8 or 12 (not shown) hours after irradiation. Four day autoradiographic exposure. Excludes cells judged to be S-phase.

# FIGURE 37


UDS - 0 TO 4 HOURS POST UV
NUMBER OF CELLS
0 1 2 3 4 5 6 7 8 10 12 14 16 18 20 22 24 26
GRAINS PER CELL
UDS - 4 TO 8 HOURS POST UV
NUMBER OF CELLS
0 1 2 3 4 5 6 7 8 10 12 14 16 18 20 22 24 26
GRAINS PER CELL
UDS - 8 TO 12 HOURS POST UV
NUMBER OF CELLS
0 1 2 3 4 5 6 7 8 10 12 14 16 18 20 22 24 26
GRAINS PER CELL

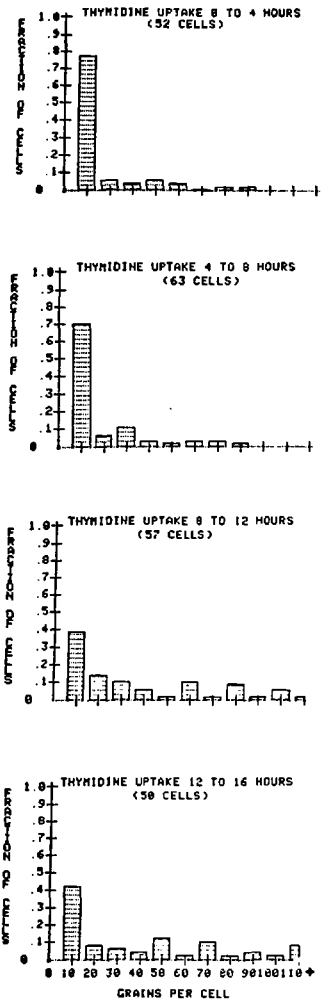
periods, the distributions appear as a single peak with a mean of about 6 grains per cell, about twice that of unirradiated control cells. During the first 4 hour interval, there is a broad peak of cells having 13 to 26 grains with a "tail" on this peak of cells having grain counts similar to those observed during later labeling periods. In summary, the greatest UDS occurs during the first four hours post irradiation This unscheduled synthesis involves the vast majority of the cells. Less than 10% have counts in the 0 to 3 grain background range. At later times there is much reduced unscheduled synthesis, but still most cells have higher numbers of grains than unirradiated cells.

Fraction of Cells in S

The observation at 12 hours after subculture of about 30% S-phase cells as determined by autoradiography needs to be reconciled with the previous experiment where cells did not seem to begin significant thymidine incorporation until about 20 hours after subculture. To further examine this issue, replicate slides from the above experiment were given a 3 hour autoradiographic exposure. Very few UDS grains will be seen under these circumstances, and the grain distributions observed were assumed to be mostly due to S-phase incorporation. Again, 50 (or more) cells were scored but no cells were excluded as uncountable. The results are shown in Fig. 38. The data are now grouped in 10 grain intervals, the lowest of which is assumed to correspond to the non-S-phase population. Again, the S population rises from about 25% to about 55% over the course of the experiment. However, there is also a large increase in the average number of grains for those cells that are incorporating label. For example in the first interval, less than 8% of the cells had more than 40 grains whereas in the last 4 hours 32% of the cells had more than 40 grains.

Figure 38. S-phase incorporation at 12 to 16, 16 to 20, 20 to 24, and 24 to 28 hours after release from plateau. Distribution of thymidine incorporation in cells irradiated 12 hours after release from plateau. Same cells as in figure 37 except the autoradiographic exposure was 3 hours and no cells were excluded.

## FIGURE 38


THYMIDINE UPTAKE 8 TO 4 HOURS
(52 CELLS)
THYMIDINE UPTAKE 4 TO 8 HOURS
(63 CELLS)
THYMIDINE UPTAKE 8 TO 12 HOURS
(57 CELLS)
THYMIDINE UPTAKE 12 TO 16 HOURS
(50 CELLS)
FRACTION OF CELLS
0 10 20 30 40 50 60 70 80 90100110+
GRAINS PER CELL

So the "residual S-phase cells" in confluent or recently confluent muntjac cultures have suppressed DNA synthesis levels compared to true S populations and the transition to S-phase by these synchronized cells involves an increase both in the fraction of cells carrying out synthesis and the level of that synthesis.

Short Term UDS

To determine the rate of removal of UDS inducing lesions, unscheduled DNA synthesis was measured during 1 hour intervals after each exposure. The slides received a 23 day autoradiographic exposure and grains were counted for 50 non-S-phase cells at each time point, for each dose. The mean grain counts for each interval are shown in Fig. 39. For each dose-time the mean grain counts decrease roughly linearly with time to a background level of 3 to 5 grains per cell. The grain count distributions as a function of time post-irradiation are shown for the irradiation at 21 hours after subculture in Fig. 40. From both these figures it is clear that the most rapid UDS occurs during the first hour after UV but quite significant repair is occuring during the second and third hour as well.

The data for the mean grain counts for all three exposures is given in Table 3 in the Appendix.

The course of UDS for the doses given at 12 and 21 hours are quite similar but the initial hour of unscheduled synthesis for cells irradiated at 18 hours appears slightly elevated. The standard errors on the measurements of first-hour unscheduled synthesis are 1.2 to 1.4 grains per cell. The mean first-hour UDS rate for the cells irradiated at 18 hours is therefore 3.5 to 4 standard errors above mean first-hour rates for cells receiving UV at 12 and 21

Figure 39. Unscheduled DNA synthesis for cells irradiated at 12, 18, and 21 hours after release from plateau. For each irradiation time, the curve gives the mean grain count per cell for cells labled for 1 hour starting 0, 1, 2, or 3 hours after irradiation. The points are plotted at the end of each labeling period. Autoradiographic exposure was 23 days.

**FIGURE 39**

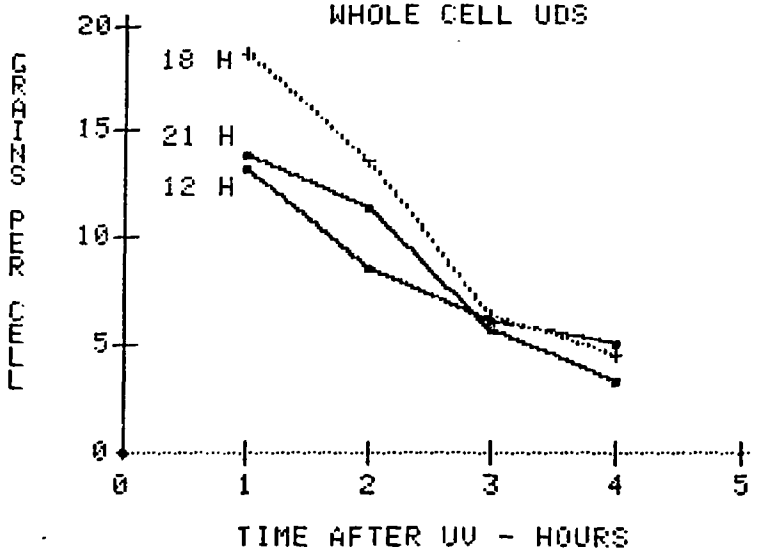
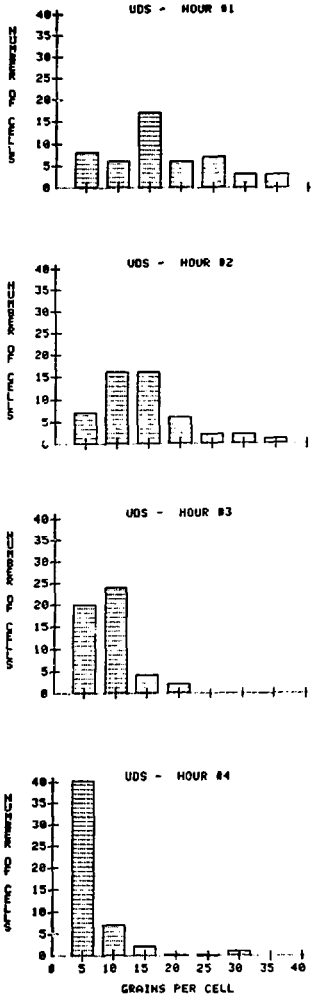

WHOLE CELL UDS
GRAINS PER CELL
20
15
10
5
0
18 H
21 H
12 H
0
1
2
3
4
5
TIME AFTER UV - HOURS

Figure 40. Distribution of short term UDS grain counts for cells irradiated at 21 hours after release from plateau.Cells were labeled for 1 hour starting 0, 1, 2, or 3 hours after irradiation. Autoradiographic exposure was 23 days.

**FIGURE 40**


UDS - HOUR #1
UDS - HOUR #2
UDS - HOUR #3
UDS - HOUR #4
NUMBER OF CELLS
0 5 10 15 20 25 30 35 40
GRAINS PER CELL

hours. There is no apparent biological reason for unscheduled DNA synthesis to be elevated at 18 hours. However, this elevation may reflect difficulties with UV dosimetry. This problem will be addressed further in the discussion section.

In summary, 8 joules/$m^2$ ultraviolet irradiation at 254 nm is a potent inducer of unscheduled DNA synthesis in muntjac cells released from plateau. The level of UDS induced is roughly equivalent at 12, 18, and 21 hours. The level of induced synthesis is highest during the first hour after irradiation and declines approximately linearly over the next four hours. By the fourth hour after UV the level of induced synthesis is reduced by a factor of 2 to 4 below the first-hour levels.

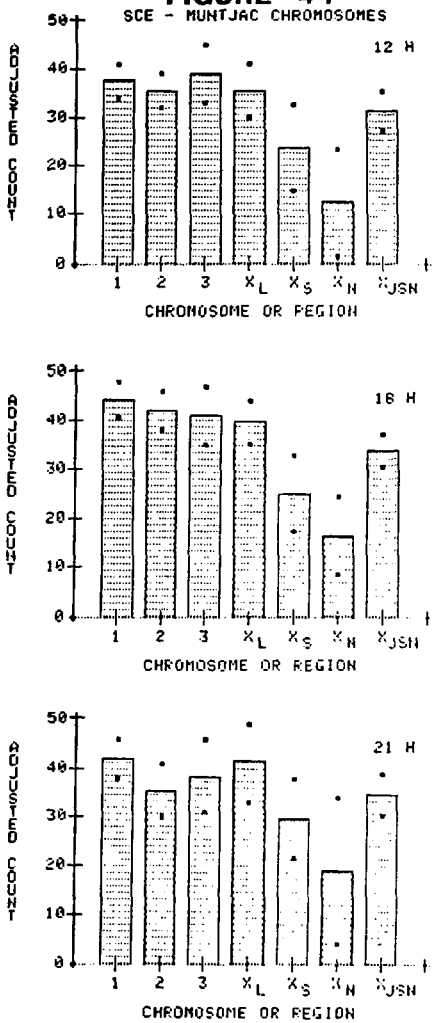
Slor and Cleaver (SI78) report significantly higher repair replication close to S, but this effect was not observed here.

SCE vs Repair Interval

The distribution of SCE on muntjac chromosomes irradiated at 12, 18, and 21 hours after release from plateau is shown in Fig. 41. Separate SCE frequencies are shown for chromosomes 1, 2, 3, the euchromatic long arm of the X ($X_L$), the short arm of the X ($X_S$), and the heterochromatic neck region of the X ($X_N$). The SCE at the junctions joining the neck to the long and short arms are not included in the measurement for $X_N$, $X_S$ or $X_L$. The SCE frequency for the combined neck and short arm regions of the X including exchanges occuring at the two junctions is indicated by the bar marked $X_{JSN}$. The height of the bars indicate the measured level of sister chromatid exchange adjusted to the DNA content of the chromosome or region of the X-chromosome. The error bars are at two adjusted standard errors on either side of the mean.

Figure 41. Distribution of UV-induced SCE in muntjac chromosomes for cells irradiated at 12, 18, and 21 hours after release from plateau. The values given are the adjusted SCE count or the SCE per unit DNA. The error bars are at two standard errors on either side of the mean.

# FIGURE 41


SCE - MUNTJAC CHROMOSOMES
12 H
16 H
21 H
ADJUSTED COUNT
0
10
20
30
40
50
1
2
3
$X_L$
$X_S$
$X_N$
$X_{JSN}$
CHROMOSOME OR REGION

The confidence intervals overlap for chromosomes 1, 2, 3, and the long arm of the X, at each time point, implying that these euchromatic regions have SCE frequencies in proportion to their DNA content. By contrast, the mean SCE frequency is slightly reduced in the short arm of the X and significantly reduced in the heterochromatic neck region of the X-chromosome (except at 21 hr). The combined region including the X-neck, short arm and the two junctions ($X_{JSN}$) has a mean SCE frequency higher than the means in either $X_N$ or $X_S$. the high SCE frequencies at the junctions cause the average frequency for the region to approach that of the euchromatic chromosomes.

The combined adjusted SCE frequency for the euchromatic chromosomes 1, 2, 3 and the long arm of the X are shown as the upper curve in Fig. 42. The lower curve shows the adjusted SCE frequency in the neck of the X and the error bars indicate two standard errors on either side of the mean. The SCE frequency is significantly reduced in the neck region for all exposure times. The mean SCE frequency in the euchromatin is about 8% higher at 18 hours and about 7% lower at 12 hours than at 21 hours. Both differences are statistically significant. Fig. 43 compares mean euchromatic grain counts with $X_N$, $X_S$ and $X_{JSN}$.

The elevation in both UDS and SCE for the cells irradiated 18 hours after subculture may be the result of a problem with the UV dosimetry. In all previous experiments, the cells for different protocols have been irradiated simultaneously so that all cells received the same dose independently of any inaccuracies or instabilities in the dosimeter or light source. In this experiment however, there were 3 hour periods between exposures when the light source and dosimeter were off. Before each irradiation, the light source and dosimeter

Figure 42. Adjusted SCE levels in total euchromatin and the neck of the X as a function of the irradiation time in hours after release from plateau.

**FIGURE 42**

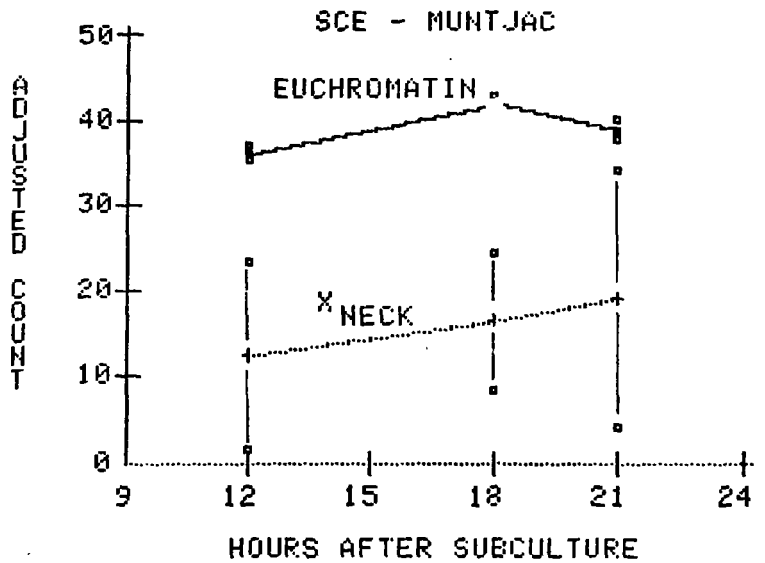
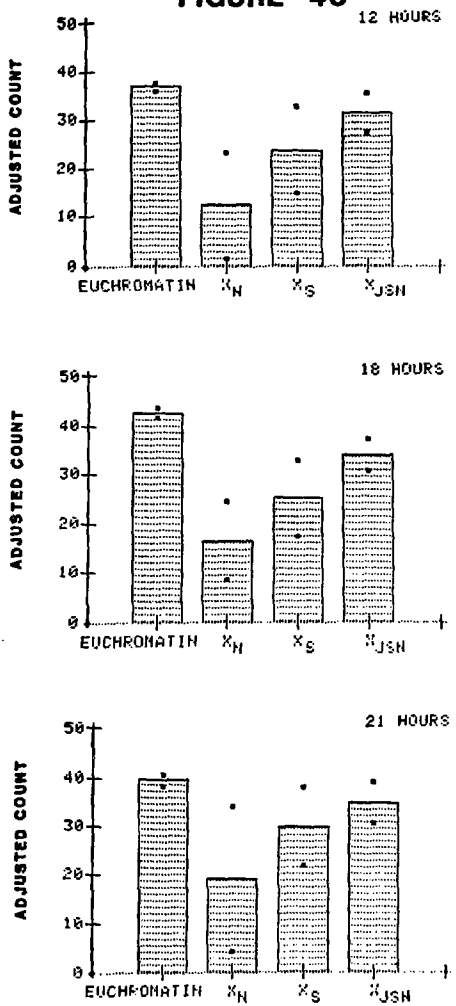

SCE - MUNTJAC
EUCHROMATIN
NECK
ADJUSTED COUNT
50
40
30
20
10
0
9
12
15
18
21
24
HOURS AFTER SUBCULTURE

Figure 43. Distribution of adjusted SCE levels in total euchromatin and the neck of the X ($X_N$), the short arm of the X ($X_S$), and the combined neck short arms plus junctions ($X_{JSN}$).

# FIGURE 43

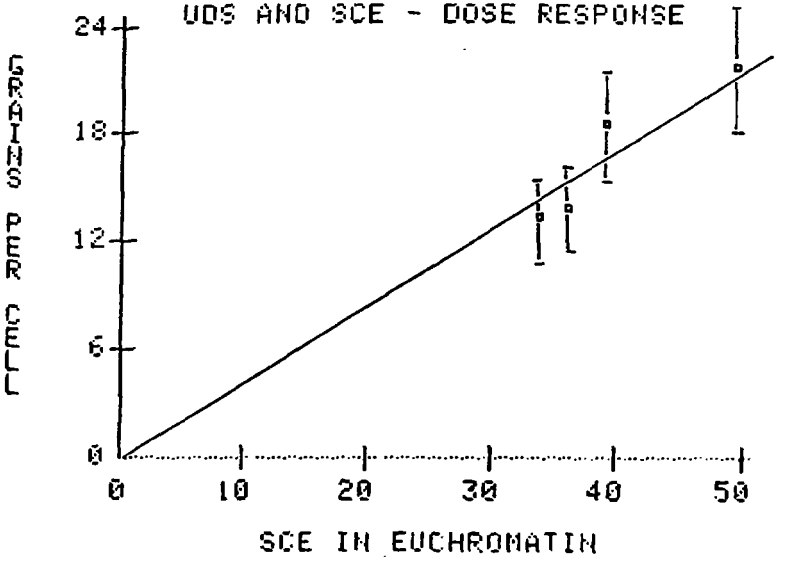

12 HOURS
18 HOURS
21 HOURS
ADJUSTED COUNT
50
40
30
20
10
0
EUCHROMATIN
$X_N$
$X_S$
$X_{JSN}$

were allowed to stabilize. The output of the dosimeter is digital and the smallest increments are equivalent to 8% of the usual output of the light source (8 joule/$m^2$/minute). The dosimeter reading did vary, by one increment, from one exposure to the next or even occasionally from before the exposure to after. The assumption had to be that any instability was in the light source so that changes in the dosimeter readings were matched by changes in the exposure time. Assuming a perfectly stable dosimeter, there is an uncertainty of +/- 8% in the dose from one exposure to the next.

The anomalously high SCE and first-hour UDS counts for the irradiation at 18 hours may therefore be due to a higher UV dose. There is some evidence to support this hypothesis. There was originally to be another irradiation at 15 hours, but the shutter on the UV light source jammed and the cells received an estimated extra 20 to 25% UV dose. Obviously, these cells weren't included in the analysis but they were measured for UDS and SCE. In Fig. 44 the first-hour UDS counts are plotted against the euchromatin SCE frequencies. The error bars are the usual two standard errors on either side of the mean. (The SCE error bars are not shown but are about 1.5 times the dimension of the data points.) The high dose point is in the upper right hand corner of the graph while the data for the irradiations at 12, 18, and 21 hours are clustered near the middle. A straight line can be readily drawn through the origin and the data points. The first-hour UDS levels are reasonable predictors of the euchromatin SCE frequencies, independently of the UV dose and the SCE repair interval. If it is accepted that the variations in first-hour UDS and euchromatin-SCE frequencies are reflections of UV dosimetry then, within the error of UV dose, the SCE frequencies are constant and there is no significant repair of lesions leading to SCE in the interval between plateau and entry of the cells into S.

Figure 44. Correlation of first-hour UDS grains with total euchromatin SCE for four UV exposures.

**FIGURE 44**


UDS AND SCE - DOSE RESPONSE
GRAINS PER CELL
24
18
12
6
0
0
10
20
30
40
50
SCE IN EUCHROMATIN

Neglecting any dosimetry problems, if the SCE frequencies are taken as indicators of cellular repair of lesions leading to sister chromatid exchange then there is a maximum of 16% repair during the 9 hour period of the experiment.

The rate of repair in the heterochromatin is not as well defined statistically. The SCE per unit DNA is lower than in euchromatin at all time points in the the experiment (Fig. 42). The ratio of mean euchromatic SCE frequency to mean heterochromatic SCE is in the range of 2 to 3 for all time points (see data in Table II in Appendix). So there is no dramatic evidence that repailr processes are at work selectively removing heterochromatic lesions.

It is clear that unscheduled DNA synthesis is not selectively at work removing DNA lesions in the heterochromatin. In fact, these experiments with UV induced UDS have shown that the level of unscheduled synthesis is reduced in heterocromatin relative to its DNA content.

The fact that UV induced UDS is reduced in heterochromatin to about the same degree as SCE might be taken as evidence the unscheduled synthesis causes SCE or that it "fixes" generalized lesions and produces lesions which lead to SCE. There are a number of authors who have argued that the repair process itself leads to the production of sister chromatid exchange (De77a). The mitomycin C experiments reported here, however, show that if DNA repair is responsible for the induction of SCE it is not exclusively unscheduled DNA synthesis. The failure of MMC to induce any UDS while inducing copius SCE indicates that if any repair process is involved in the MMC-incuded sister chromatid exchange, it is likely some repair system which does not replace long stretches on nucleotides in the DNA.

The present experiments show that if there is any role for repair processes in the removal of DNA lesions which would otherwise lead to the formation of SCE then this repair takes place on a different time scale and to a less massive extent than is characteristic of UDS. UV induced UDS lesions are reduced by a factor of 2 to 4 over a period of 4 hours while the reduction in SCE producing lesions over the same period was somewhere between zero and a few percent. Of course, this is only a fair comparison if several underlying assumptions are correct.

Some of these assumptions are:

1) Repair of SCE forming lesions takes place on a time scale reasonably comparable to the data sampling time. If SCE inducing lesions are selectively repaired very rapidly or very slowly then changes in 3 hours or 12 hours may be unmeasureable (Wolff et al., (Wo74) found that the UV-induced SCE-forming lesion was long lived.).

2) Repair of SCE forming lesions can take place in transit from plateau to S. If repair is restricted to some other portion of the cell cycle, S-phase for instance, then measurements restricted to pre-S or very early S will conclude that there is no repair.

3) Repair of SCE forming lesions is substantially blocked once the cells enter S. If significant repair continues well after the cells begin DNA synthesis, then differences in damage levels extant when the cells enter S-phase are likely to be reduced as long as repair continues.

Overview: SCE and UDS

It is clear from these experiments and those of others that UDS is not critically related to the formation of sister chromatid exchange. The fact that MMC fails to induce UDS shows that unscheduled synthesis is not necessary for the conversion of primary DNA damage to the observable SCE.

Conversely, DNA damage which leads to UDS need not lead to increased SCE. For example, xeroderma pigmentosum complementation groups have markedly varied excision repair capabilities but the level of induced UDS has no clear relationship to the observed increase in SCE (De77a). Similarly Chinese hamster cells with varying excision repair capabilities have the same level of induced SCE in response to UV (Wo74).

Several authors have reported repair of UV-induced lesions leading to SCE. Kato (Ka74), Natarajan (Na80), and Ishizaki (Is80) have claimed on the basis of photoreactivation experiments that the pyrimidine dimer is the lesion responsible for the SCE. This conclusion is difficult to reconcile with several pieces of experimental evidence:

1) There is the lack of correlation between excision repair capability and the level of SCE produced as noted above in the studies of Wolff (Wo74) and DeWeerd-Kastelein (De77a).

2) Only one SCE is produced for each 10,000 dimers (Na80). To invoke the dimer as the inducing lesion requires explanation of why such a limited subset of the dimers actually leads to an observed exchange. Natarajan suggested, for example, that the inducing lesion might lie in some unusual location within the chromatin (Na80).

3) The rate of repair of lesions leading to SCE is very small compared to the rate of dimer repair. As shown in the experiments reported here, unscheduled DNA synthesis is most intensive in the first hour or two after irradiation and continues only at low levels after four hours. During the same time period, SCE appeared to decline only a few percent if at all. Similarly MacRae et al., (Ma79) found that CHO cells arrested by growth in arginine deficient medium reported a 45% decrease in SCE over a period of 48 hours. Even this moderate repair may be artificially high since it was only c served at the highest doses where decreased survival was associated with increasing repair time. Under such circumstances, much of the SCE decrease may be attributable to death of the more highly damaged cells.

The observation of reduced sister chromatid exchange in muntjac heterochromatin was a central motivation in carrying out the experiments reported here. Perhaps this reduction is a direct result of some property of the DNA in the heterochromatin. These experiments looked for DNA of aberrant density or repeated sequence DNA which might be associated uniquely with muntjac heterochromatin. Properties of muntjac heavy shoulder DNA and distribution of that DNA among the chromosomes showed that a simply observed subcomponent of muntjac DNA is an unlikely cause for reduced heterochromatic SCE.

Reduced levels of damage to DNA in the heterochromatin is another possible cause of reduced SCE. For bulky chemical mutagens, this is a plausible theory. The closer association of DNA in the heterochromatin with itself and with associated proteins makes restricted access for large chemical moieties a

nice heuristic explanation of reduced damage. For example, Moyer et al., (Mo77) claim a 4 to 5 fold higher binding of N-hydroxy acetylaminofluorene to euchromatin over heterochromatin in rat liver. (These authors were defining heterochromatin in terms of chromatin sedimentation velocity rather than cytogenetic observation.) On the other hand, nonuniform distribution of UV lesions is less plausible. Since the action spectrum for ultraviolet damage to DNA closely corresponds to the DNA-UV absorption spectrum it is assumed that the UV light interacts directly with the DNA molecule. This makes it difficult to claim reduced UV damage to the DNA in heterochromatin on the basis of physically restricted access.

A third explanation of reduced SCE in heterochromatin is altered repair of SCE inducing lesions in heterochromatin (Bo79, Bo77). There is no concensus on what constitutes the SCE inducing lesion, so there are no direct measurements of its repair. A number of authors have examined the distribution of repair incorporated label but no consistent picture arises from their results:

Meltz and Painter (Me73) and Lieberman and Poirier (Li74) found that the reassociation kinetics of UV-induced repair-incorporated label was the same as that of label incorporated by semiconservative synthesis, implying that repair synthesis was uniformly distributed.

Harris et al., (Ha74a) using chemical mutagens found higher autoradiographic grain counts from repair incorporated label over the interphase nuclear core (which they assumed to be euchromatin) than over the nuclear periphery (heterochromatin).

Berliner et.al.(Be75) carried out similar studies with UV and 6 MeV electrons but found that the UDS grains were concentrated on the nuclear periphery whether or not the underlying chromatin was heterochromatic by electron density standards.

Johnston and Sperling (Jo78) found enhanced UDS incorporation in centromere and telomere regions of prematurely condensed chromosomes of HeLa cells exposed to UV.

Finally, Bodell and Banerjee (Bo76) found less repair label incorporated into mouse satellite DNA after MMS or MNU treatments.

The finding reported here of reduced repair in the heterochromatin is consistent with the findings of Bodell and Banerjee (Bo76) and Harris et.al (Ha74a) and contradictory to those of Johnston and Sperling (Jo78).

SUMMARY: UDS and SCE .

These experiments have shown that:

1) Mitomycin C is a very weak inducer of unscheduled DNA synthesis in muntjac cells. This drug, which produces marked increases in sister chromatid exchange at low concentrations, fails to significantly increase incorporation of thymidine even at concentrations that are lethal to more than 95% of the cells.

2) Ultraviolet light induces both UDS and SCE in muntjac chromosomes.

3) Muntjac cultures grown to confluency have a residual population of S-phase cells that may be readily recoginzed by their intense incorporation of labeled thymidine.

4) Hydroxyurea (10mM) partially suppresses thymidine incorporation by these residual S-phase cells but the low level incorporation that remains may confuse the measurement of unscheduled synthesis.

5) Sodium butyrate (10mM) effectively suppresses thymidine incorporation and results in accumulations of cells with G1 and G2 DNA contents. However, butyrate concentrations which are sufficient to block cell-cycle progression also suppress unscheduled DNA synthesis.

6) The level of UV-induced unscheduled DNA synthesis in chromosomes of muntjac cells irradiated in plateau is generally proportional to the chromosomal DNA content. However, UDS in the short arm and the heterochromatic neck of the X-chromosome are reduced. This distribution of UDS is very similar to the previously reported distribution of baseline and MMC-induced SCE in muntjac chromosomes.

7) UV-induced SCE are similarly distributed in the chromosomes of muntjac cells irradiated after release from plateau.

8) In cells irradiated after release from plateau, the repair interval between irradiation and entry of the cells into S fails to significantly influence the level of SCE ultimately expressed in euchromatin or heterochromatin. During the same interval a substantial fraction of the lesions leading to UDS are removed. These results are consistent with the conclusions that the UV-induced SCE-producing lesion is long lived compared to the UDS-inducing lesion and that the production of SCE in the heterochromatin and euchromatin of the muntjac is independent of any repair process that both takes place on the time scale of unscheduled DNA synthesis and is blocked by entry oi the cells into S-phase.

# APPENDIX - TABLES

Table 1. Mean grain counts for unscheduled DNA synthesis grains over metaphase chomosomes of muntjac cells irradiated in plateau. The grains on the X-chromosome were either over the long euchromatic arm ($X_L$), the junction of the long arm with the neck ($X_{JLA}$), the heterochromatic neck ($X_N$), the junction of the neck with the short arm ($X_{JSA}$) or the short arm ($X_S$). Grains were also scored over the autosomes 1, 2, and 3 (or Y1). The number of regions or chromosomes scored is given as N.

TABLE 1

UDS GRAINS ON CHROMOSOMES

| | $X_L$ | $X_{JLA}$ | $X_N$ | $X_{JSA}$ | $X_S$ | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|
| N | 222 | 224 | 221 | 225 | 224 | 223 | 227 | 123 |
| MEAN | 3.36 | 0.26 | 0.27 | 0.21 | 0.99 | 6.77 | 4.01 | 3.47 |
| VAR. | 4.41 | 0.27 | 0.33 | 0.19 | 0.99 | 13.13 | 5.86 | 4.71 |
| S.D. | 2.10 | 0.52 | 0.57 | 0.44 | 1.00 | 3.62 | 2.42 | 2.17 |
| S.E. | 0.14 | 0.03 | 0.04 | 0.03 | 0.07 | 0.24 | 0.16 | 0.20 |

Table 2. Adjusted grain counts for unscheduled DNA synthesis over metaphase chromosomes of muntjac cells irradiated in plateau. To obtain the adjusted count, the mean grain count for each region has been divided by the fractional DNA content for each region or chromosome as given by Carrano and Johnston (Ca77). The standard errors were adjusted by the same factor. Adjusted counts are given for chromosomes 1, 2, 3 (Y1), the long arm of the X ($X_L$), the short arm of the X ($X_S$), and the combined region including the junctions, the short arm and the neck ($X_{JSN}$). The adjusted count for total euchromatin is also shown and represents the computed frequency for the euchromatic portion of the cell. This was found by adding the frequencies for two chromsome #1's, two chromsome #2's, a chromosome #3 (Y1), and an X-long-arm ($X_L$). This summed frequency was adjusted to to the euchromatic DNA content (92%). The standard error for combined regions is the weighted root-mean-square average standard error for the included regions subsequently adjusted for DNA content.

TABLE 2

ADJUSTED GRAIN COUNTS

| | 1 | 2 | 3 | $X_L$ | $X_S$ | $X_N$ | $X_{JSN}$ | TOTAL EUCHR. |
|---|---|---|---|---|---|---|---|---|
| MEAN | 30.77 | 29.70 | 31.55 | 33.60 | 24.68 | 13.55 | 28.77 | 30.86 |
| S.E. | 1.10 | 1.19 | 1.78 | 1.41 | 1.68 | 1.90 | 0.74 | 0.28 |

Table 3. Whole cell UDS grain counts for muntjac cells irradiated at 12, 18, or 21 hours after release from plateau. Thymidine incorporation was measured during the first, second, third, and fourth hours after each irradiation.

TABLE 3

WHOLE CELL UDS

| | | 12 H | 18 H | 21H |
|---|---|---|---|---|
| HOUR 1 | MEAN | 13.22 | 18.56 | 13.82 |
| | S.E. | 1.18 | 1.44 | 1.19 |
| HOUR 2 | MEAN | 8.52 | 13.54 | 11.26 |
| | S.E. | 0.77 | 1.60 | 0.93 |
| HOUR 3 | MEAN | 6.00 | 6.26 | 5.62 |
| | S.E. | 0.73 | 0.55 | 0.52 |
| HOUR 4 | MEAN | 5.02 | 4.50 | 3.30 |
| | S.E. | 0.70 | 0.55 | 0.59 |

Table 4. Sister chomatid exchange frequencies for muntjac cells irradiated at 12, 18, or 21 hours after release from plateau. Exchange frequencies were measured for the long arm of the X ($X_L$), the junction of the long arm with the neck ($X_{JLA}$), the heterochromatic neck ($X_N$), the junction of the neck with the short arm ($X_{JSA}$), the short arm ($X_S$), and the autosomes 1, 2, and 3 (Y1).

TABLE 4

12H SCE

| | $X_L$ | $X_{JLA}$ | $X_N$ | $X_{JSA}$ | $X_S$ | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|
| N | 36 | 33 | 32 | 34 | 37 | 81 | 78 | 37 |
| MEAN | 3.53 | 0.33 | 0.25 | 0.35 | 0.95 | 8.27 | 4.76 | 4.27 |
| VAR. | 3.06 | 0.23 | 0.38 | 0.24 | 1.21 | 13.62 | 4.93 | 4.16 |
| S.D. | 1.75 | 0.48 | 0.62 | 0.49 | 1.10 | 3.69 | 2.22 | 2.04 |
| S.E. | 0.29 | 0.08 | 0.11 | 0.08 | 0.18 | 0.41 | 0.25 | 0.33 |

18H SCE

| | $X_L$ | $X_{JLA}$ | $X_N$ | $X_{JSA}$ | $X_S$ | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|
| N | 53 | 44 | 40 | 45 | 52 | 87 | 92 | 44 |
| MEAN | 3.96 | 0.39 | 0.33 | 0.29 | 1.00 | 9.72 | 5.66 | 4.48 |
| VAR. | 3.10 | 0.24 | 0.22 | 0.21 | 1.17 | 14.75 | 7.24 | 4.71 |
| S.D. | 1.76 | 0.49 | 0.47 | 0.46 | 1.08 | 3.84 | 2.69 | 2.17 |
| S.E. | 0.24 | 0.07 | 0.08 | 0.07 | 0.15 | 0.41 | 0.28 | 0.33 |

21H SCE

| | $X_L$ | $X_{JLA}$ | $X_N$ | $X_{JSA}$ | $X_S$ | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|
| N | 29 | 25 | 21 | 26 | 27 | 53 | 55 | 30 |
| MEAN | 4.14 | 0.32 | 0.38 | 0.19 | 1.19 | 9.21 | 4.76 | 4.20 |
| VAR. | 4.71 | 0.23 | 0.45 | 0.16 | 0.69 | 12.04 | 7.08 | 5.15 |
| S.D. | 2.17 | 0.48 | 0.67 | 0.40 | 0.83 | 3.47 | 2.66 | 2.27 |
| S.E. | 0.40 | 0.10 | 0.15 | 0.08 | 0.16 | 0.48 | 0.36 | 0.41 |

Table 5. Adjusted sister chromatid exchange frequencies for metaphase chromosomes of muntjac cells irradiated at 12, 18, or 21 hours after release from plateau. To obtain the adjusted count, the mean SCE frequency for each region has been divided by the fractional DNA content for each region or chromosome as given by Carrano and Johnston (Ca77). The standard errors were adjusted by the same factor. Adjusted counts are given for chromosomes 1, 2, 3 (Y1), the long arm of the X ($X_L$), the short arm of the X ($X_S$), and the combined region including the junctions, the short arm and the neck ($X_{JSN}$). The adjusted count for total euchromatin is also shown and represents the computed frequency for the euchromatic portion of the cell. This was found by adding the frequencies for two chromsome #1's, two chromsome #2's, a chromosome #3 (Y1), and an X-long-arm ($X_L$). This summed frequency was adjusted to to the euchromatic DNA content (92%). The standard error for combined regions is the weighted root-mean-square average standard error for the included regions subsequently adjusted for DNA content.

TABLE 5

ADJUSTED SCE COUNTS

| | 1 | 2 | 3 | $X_L$ | $X_S$ | $X_N$ | $X_{JSN}$ | TOTAL EUCHR. |
|---|---|---|---|---|---|---|---|---|
| 12 HOUR | | | | | | | | |
| MEAN | 37.59 | 35.26 | 38.82 | 35.30 | 23.75 | 12.50 | 31.33 | 36.80 |
| S.E. | 1.86 | 1.85 | 3.00 | 2.90 | 4.50 | 5.50 | 1.99 | 0.47 |
| 18 HOUR | | | | | | | | |
| MEAN | 44.18 | 41.93 | 40.73 | 39.60 | 25.00 | 16.50 | 33.50 | 42.61 |
| S.E. | 1.86 | 2.07 | 3.00 | 2.40 | 3.75 | 4.00 | 1.64 | 0.48 |
| 21 HOUR | | | | | | | | |
| MEAN | 41.86 | 35.26 | 38.18 | 41.40 | 29.75 | 19.00 | 34.67 | 39.43 |
| S.E. | 2.18 | 2.67 | 3.73 | 4.00 | 4.00 | 7.50 | 2.2 | 0.59 |

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