I. Background and Progress Report (1968 - 9/1/76)

The ability of cells to survive in an environment specifically damaging to its DNA can be attributed to a variety of inherent repair mechanisms.

This is a form of repair in which alterations are directly reversed to their original form. This reversibility is exemplified by the photoreactivation of ultraviolet (uv)-induced pyrimidine dimers. This phenomenon is attributable to the action of an enzyme, photolyase (photoreactivating enzyme), which is able to monomerize the uv-induced pyrimidine dimers in the presence of 320-370 nm light.

Dilution of damage can be effected through a series of sister chromatid exchanges, controlled by recombinational mechanisms as a postreplication event (1). In this form of repair, replication proceeds to the point of damage, stops and resumes at the point of the next initiation site resulting in a gap in the newly synthesized daughter strand. It is presumed that those strands containing damaged regions exchange with undamaged regions of other DNA strands, resulting in the eventual dilution of such damage.

The specific removal of damage from modified DNA was discovered by Boyce and Howard-Flanders (2), Setlow and Carrier (3), and Riklis (4). The ultraviolet photoproducts, pyrimidine-pyrimidine dimers, are excised from a DNA molecule through the sequential action of a variety of nucleases.

Classification of Damage

The varieties of damage imposable on DNA can be classified into three general categories, in order to facilitate enzyme classification related to repair specificities.

Monoadducts are single-base modifications in which an addition reaction, adduct formation, or a chemical transformation leads to the alteration of a single nitrogenous base.

Another class of damage found in DNA exposed to either radiation, intercalating agents and some carcinogens is the formation of diadducts, in which more than one nitrogenous base is involved in the final chemical product. Pyrimidine-pyrimidine dimers arising from ultraviolet light (5), the formation of crosslinks between thymine residues in the presence of psoralen and light (6), or the treatment of DNA with mitomycin C (7) are examples of this form of damage.

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A third class of generic molecular aberration, which is specifically recognized by some nucleases, involves nucleotide sites lacking aglycones. As a secondary chemical reaction arising from alkylation of purines is the base catalyzed N-glycosidic hydrolysis leading to depurination. It is conceivable that mismatched bases may fit into this category of damage by virtue of the distortions arising from lack of hydrogen bonding complementarity.

Classification of Nucleases

Exonucleases are phosphodiesterases which require a terminus for hydrolysis. An endonuclease is a phosphodiesterase which does not require a terminus for hydrolytic activity.

An endonuclease that specifically acts on damaged DNA eventuating in correctional pathways in vivo is a correctional endonuclease or correndonuclease. A correndonuclease I-type endonuclease is defined as one which is specific for damaged DNAs possessing monoadduct derivatives. Those correctional endonucleases which are specific for diadduct modified regions of DNA will be referred to as correndonuclease II.

Much of the current interest and knowledge of repair mechanisms has been confined to uv-induced photoproducts, their effects and subsequent removal. The progress already made is a reflection of a good understanding of the fundamentals of uv-photochemistry which allows for control of photoproduct formation in DNA and ease of analysis (8,9). Consequently, in the ensuing discussions, the removal of the primary uv-photoproducts, pyrimidine-pyrimidine dimers, will be emphasized.

Excision Repair Cycle

1) uvr Genes controlling repair in E. coli

In E. coli, alterations in five genes results in mutants which share the phenotypic properties of being uv-sensitive (uv^-), mitomycin sensitive (M^S), and host cell reactivation negative (hcr^-). These mutants, which map at discrete sites on the chromosome, are classified as uvrA, uvrB, uvrC, uvrD and uvrE. UvrA, uvrB and uvrC are unable to excise pyrimidine dimers from their DNA or from uv-irradiated phage DNA. UvrD and uvrE have been included in this group of excision repair defective mutants chiefly on the basis of their reduced hcr activity.

UvrA, uvrB and uvrC appear very similar in their sensitivity to uv, their resistance to γ-irradiation, sensitivity to host cell reactivation, as well as induction of phage λ . In

addition, efforts to demonstrate a sequential action of these gene products has yielded negative results (10,11). In spite of these phenotypic similarities, we have recently shown that while uvrA and uvrB lack an endonuclease specific for uv-irradiated DNA, uvrC contains wild-type levels of this particular enzyme activity (12). Work reported with DNA extracted from uvrC mutants shows the presence of single-strand breaks after treatment with mitomycin C (13) and uv-irradiation (14). This evidence suggests that in mutants of uvrC the incision of damaged DNA does occur. The uvrC defect possibly affecting a step following incision appears to function in repair by inhibiting the action of ligase at sites generated by the uvrA,B gene product thereby preventing abortive repair.

The mutants classified as uvrD differ from uvrA, uvrB and uvrC, according to their physiological properties. They are somewhat less sensitive to uv-irradiation, sensitive to ir-radiation with γ-rays, show rapid and extensive degradation of DNA following exposure to uv-irradiation and uvrD is dominant over $uvrD^+$.

Unlike uvrA, uvrB and uvrC mutants, which show no enhanced uv-sensitivity as double mutants, the double mutant uvrD uvrB is approximately three times as sensitive as uvrB alone. Since this double mutant shows a significant reduction in DNA degradation, it has been suggested (15) that the uvrD function occurs after the uvrB step.

In the pathway illustrated in Figure 1, uvrA and uvrB genes are involved in the incision step, uvrC gene activity is localized at a post-incision, pre-excision locus, whereas the roles of other uvr genes is unknown.

2) Recombination (rec) genes involved in repair

Another well-studied group of uv-sensitive, mitomycin-sensitive strains of E. coli are those designated as rec. Unlike the uvr mutants, the rec strains are defective in genetic recombination, in addition to being highly sensitive to ionizing radiation. The extreme uv-sensitivity (one dimer per lethal event) of a uvr rec strain and the lack of pyrimidine dimer excision in a uvr rec^+ strain suggests that the uvr and rec gene products mediate separate pathways of DNA repair (2, 16, 17, 18).

Studies of photoproduct excision in vivo in isogenic mutants of recA, recB and recC clearly show that these strains are not excision defective (19, 25). Mutants of E. coli K12, defective in recombination repair, fall into several groups of which recA, recB and recC have been studied in detail (17, 21, 22). Cells lacking the recA function are characterized by

spontaneous DNA degradation, which is enhanced by cellular exposure to uv light (23). recB and recC mutants show reduced recombination, intermediate uv-sensitivity and little DNA breakdown after irradiation. Both strains are inducible for phage λ (24). Double mutants of either recA and a temperature-sensitive polA mutation (polA12) or a recB polA12 show conditional lethality (25).

While the product of the recA gene has not yet been purified, there is some information concerning its regulatory effects on recB recC functioning (26). Definitive progress has been made, however, on the recB recC gene product. These two genes determine a complex nuclease (27, 28, 29) consisting of two subunits (30). The enzyme possesses four activities: an ATP-dependent double-stranded and single-stranded exonuclease; an ATP-stimulated single-stranded DNA endonuclease, and a DNA-dependent ATPase (30, 31).

Identification of recB and recC as the structural genes for this enzyme has been documented (32). The purification of this multifunctional enzyme activity from the respective temperature-sensitive mutants (33) suggests that of the numerous activities associated with the recBC nuclease, only the ATP-dependent, exonuclease hydrolyzing double-stranded DNA, is abnormally thermolabile.

It has been suggested (34, 35) that the product of the recB recC genes are involved in a recombinational pathway (recBC pathway) which accounted for approximately 99% of wild-type recombination. The existence of other, minor pathways is established. The isolation and genetic analysis of new groups of recombination-defective mutants of E. coli K12 (36, 37) substantiated the existence of at least two other recombinational pathways -- recF and recE.

All three pathways are blocked in the absence of wild-type recA gene product.

3) Gene control of repair in mammalian cells

It is apparent from the previous discussions that excision repair in prokaryotes is controlled by several genes. As a reflection of the complexity of biochemical reactions controlling this process, the availability of specific uvr mutants of E. coli has facilitated identification of some of these biochemical steps. The analogous situation in diploid eukaryotic cells is considerably more complicated, making it unfeasible to induce uv-sensitive mutant cell lines. The situation, however, is partially alleviated with the availability of cell lines derived from patients with photosensitive diseases such as Xeroderma pigmentosum and DeSanctis cacchione syndrome.

Xeroderma pigmentosum (XP) is an infrequently occurring skin disorder, which was shown more than 40 years ago to be genetically determined and follow an autosomal recessive pattern of transmission. The biochemical defect in the enzymatic pathway of excision repair has been inferred from indirect experiments. The skin of homozygous affected individuals appears normal at birth, but usually before age three severe changes consequent to sun exposure appear, and then progress relentlessly. Freckles of varying sizes and degrees of brownness appear and are accompanied by increasing dryness, atrophy and a number of keratoses. These changes in the skin characteristically eventuate in some form, sometimes in multiple form, of malignant neoplasia of the skin and metastatic epithelioma, often causing death before the age of 30. Various other forms of benign and malignant tumors of ectodermal and mesodermal origin also occur with a much increased frequency in these affected individuals. All these abnormalities appear to be the consequence of exposure to sunlight.

Measurements of uv-induced pyrimidine dimers in cellular DNA show that normal diploid human skin fibroblasts excise up to 60% of the dimers in 24 hours, but that fibroblasts derived from certain cell lines of Xeroderma pigmentosum epithelial cells excised less than 20% in 48 hours. Alkaline sucrose gradient sedimentation experiments show that during the 24 hours after irradiation of normal cells, large numbers of single-strand breaks appear, and then as repair proceeds, these breaks are mended. Such changes are not seen in the same lines of Xeroderma pigmentosum. Such cells, apparently, fail to start the excision process presumably because they lack the required function of correndonuclease II. These inferential data are supported by the findings of Cleaver, in which X-ray induced phosphodiester bond breaks, or those formed after a uv-irradiation of 5-bromouracil containing DNA, are repairable in both normal and Xeroderma pigmentosum fibroblasts.

Genetic heterogeneity in XP is suggested by the two clinical forms of this disease: the classic form of XP with skin lesions only, and the DeSanctis cacchione form with neurological complications, in addition (38). These mutations have been characterized in different unrelated XP patients by the performance of complementation tests through somatic cell hybridization (39). DNA repair has been studied in binuclear cells resulting from fusions between in vitro cultivated cells from different patients. Following uv exposure, the incorporation of tritiated thymidine is indicative of repair of DNA, and this can be examined by autoradiography. Parental XP cells perform low and neglible levels of this type of thymidine incorporation, where in some combinations, binuclear hybrid cells show repaired DNA synthesis as a result of complementation (40). Three different complementation groups have been found by a group of Dutch workers (41) and four have been identified from patients at the National Institutes of Health in Bethesda (40). Fusion experiments performed using

cells from both laboratories indicate the presence of at least five different complementation groups of Xeroderma pigmentosum. There has also been a sixth class of XP which has been derived from patients which show normal levels of DNA repair. It is apparent from these data that the biochemical control of repair in mammalian cells is at least as complicated, if not more, than is found in E. coli.

4) Gene products

The general mechanism shown in Figure 1 represents our current view of the excision repair cycle present in E. coli and M. luteus in which intrastrand diadduct damaged DNA is sequentially removed, the individual steps of which will be detailed in each section of this application.

The excision event represents a series of enzymatic steps which include incision by rather specific correndonuclaeases under potential control by a subsequent postincision step in which abortive repair is prevented. The concluding step in the excision of damage can occur via two interdependent pathways, which may involve either polymerase-associated exonucleases, or unassociated exonucleases involved in the removal of photoproducts. Mammalian DNA polymerases lack associated exonucleolytic activity of any kind. The excision of photochemical damage in eukaryotes is, therefore, probably catalyzed by unassociated exonucleases. The reinsertion mechanisms may occur either concomitantly in polymerase I-associated excision pathways (short patch repair), or it can proceed as a result of a stepwise mechanism (long patch repair) when single-stranded 5'→3' exonucleases catalyze the removal of photoproduct regions of DNA. Long patch pathways of excision-reinsertion may be controlled in prokaryotes by polymerase II and/or III. The restoration of the strand continuity is under the control of a single polynucleotide ligase step.

INCISION STEP

Correndonucleases - general

Endonucleolytic activities that incise DNA containing damaged bases have been found in extracts derived from bacterial, bacteriophage and mammalian sources. Although many correndonuclease activities have not been fully characterized, a similarity is apparent among most of those activities which have been studied. Correndonucleases, generally, are small proteins, having molecular weights of less than about 30,000 daltons. All act in the absence

of divalent cations, although some enzymes are stimulated by Mg^{++} or NaCl. All appear to incise close to the damage on the DNA strand containing the damage.

M. luteus correndonuclease I (M. luteus gamma [γ endonucleases])

S. Riazuddin, in my laboratory, purified this enzyme from M. luteus (42). It acts on X-irradiated DNA and alkylated DNA as well as on DNA irradiated at doses 1000 times higher than a uv specific endonuclease. Hence it appears to typify the correndonuclease I. The correndonuclease I hydrolyzes 5' to the damage generating 3'-phosphoryl and 5'-hydroxyl groups. The enzyme has a molecular weight of about 17,000 dlatons, incised in the presence of EDTA, but was about three-fold stimulated by 10 mM Mg^{++} (43). Mutants of M. luteus, which lack all endonucleolytic activity on uv-irradiated DNA, are both uv- and X-ray sensitive (45). Thus, it is possible that this γ-specific endonuclease is involved in repair of X-ray and alkylation damage in M. luteus. We have recently shown that this enzyme absent in X-ray sensitive, uv-resistant mutants recognizes the thymine glycol radioproduct specifically.

Correndonuclease II

All known correndonuclease II type enzymes specifically incise uv-irradiated DNA. The enzymes described below are classified as correndonuclease II type enzymes, since it is believed that they are involved in the repair of pyrimidine dimer damage (intrastrand diadduct) as well as crosslink damage (interstrand diadduct).

E. coli correndonuclease II (uvrA, uvrB endonuclease)

The endonuclease involved in the first step of excision repair has recently been isolated and partially characterized by Dr. Andrew Braun in my laboratory (12). The involvement of this enzyme in uv-repair is indicated by its absence in uvrA and uvrB excision defective mutants. The enzyme is small, appearing to have a molecular weight of less than 14,000 daltons. It acts in the presence of 10^{-3} M EDTA and requires an ionic strength of about 50 mM. In an irradiated:unirradiated DNA heteroduplex the enzyme acts only on the damaged strand (46). The incision event occurs 5' to a pyrimidine dimer and generates a 3'-hydroxyl terminus, which renders the break sealable by polynucleotide ligase (47, 48).

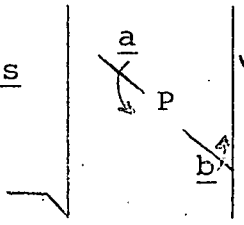
Binding of the enzyme to its substrate can be measured by using the membrane filter technique used for repressors (49). The specific binding of the enzyme to uv-irradiated DNA can be prevented by pretreating the irradiated DNA with yeast photolyase and light (12). The purified enzyme, which has a Km of 1.5×10^{-8}M (pyrimidine dimers) (50), is competitively inhibited by the cocarcinogen caffeine with a Ki of about 10^{-2} M; this is comparable to the inhibitory concentration of this drug *in vivo* (51). The nature of this inhibition is reflected in the specific binding of the caffeine to the enzyme (52). Specific inhibition of the endonuclease by another cocarcinogen, acriflavin, also occurs at concentrations comparable to those found to be inhibitory *in vivo* (52), but in this case it specifically binds to the irradiated DNA substrate.

Since *uvrA* and *uvrB* mutants of *E. coli* are more sensitive than wild type to crosslinking agents such as mitomycin C (53) and psoralen plus light (54), there is reason to believe that the *E. coli* correndonuclease II acts on many diadduct forms of damage.

M. luteus correndonuclease II (uv-endonuclease)

There is an activity, similar in its properties to the *E. coli* uvrA, uvrB correndonuclease, found in *M. luteus* (55). This correndonuclease II incises 5' to the pyrimidine dimers, leaves a 3' hydroxyl terminus (56), requires 50 mM ionic strength and acts in the presence of 10^{-3} M EDTA (12). This enzyme activity is resolved both in DNA cellulose and isoelectric focusing into two separate enzymes having different pI's (4.5, 8.9). These activities function in an additive manner and are both absent in excision defective mutants of *M. luteus* (42). The role of these two enzymes *in vivo* is currently under study.

Preexicision steps



(Incision mechanism a [5'-phosphoryl group])

Although the incision produced by the *E. coli* correndonuclease II provides an initiation site for excision and nucleotide incorporation by polymerase I (56), indirect evidence suggests that an intermediate step between incision and excision exists. This evidence is based on the properties of the excision defective *uvrC* mutant. Such mutants are unable to remove pyrimidine dimers from DNA *in vivo*, although there are indications that single-strand incision events are operative in only a transient manner. Since

exonuclease VII, an excising exonuclease, correndonuclease II and polymerase levels appear to be normal in such mutants, it can be assumed that the capacity for incision and exicision is unaffected. The progression of single-strand molecular weight changes in uvrC mutants is peculiar in that the extent of such breakage is low, whereas the initial rate of single-strand break formation is indistinguishable from wild type cells (57). What is seen is a rapid, partial decrease in single-strand molecular weight, which is quickly restored to that of control chain lengths. DNA isolated from wild type cells under similar conditions, however, exhibits a rapid and more extensive loss in its single-stranded molecular weight, which is fully restored during extended postirradiation time periods.

Double uvrC and temperature-sensitive polynucleotide ligase mutants have been prepared. At temperatures restrictive for ligase, the early rate and extent of postirradiation molecular weight losses are similar to those of wild type cells, followed, however, by the expected accumulation of low molecular weight DNA. At permissive temperatures, however, the molecular weight is rapidly and fully restored. These data, in conjunction with the observations that polynucleotide ligase is capable of resealing the phosphodiester bonds of incised DNA, places polynucleotide ligase in a controlling position during early repair steps. The juxtaposition of a 3'-hydroxyl group and the 5'-phosphoryl group, in association with a pyrimidine dimer containing nucleotide, appears to be sensitive to E. coli ligase.

From these two observations, it can be inferred that the uvrC gene product prevents resealing of the correndonuclease II (incision) before excision of the pyrimidine dimer has occurred. A number of mechanisms, such as a 5'-polynucleotidase, nuclease, or perhaps a binding protein affecting the conformation of the damaged strand can be suggested for its molecular mechanism in restricting ligase activity at this step of repair. This problem is currently under investigation.

PREEXCISION STEP - (Incision mechanism b [3'-phosphoryl group])

The incisions produced by one of the M. luteus correndonuclease I enzyme results in a 3'-phosphoryl terminus, 5'-to a pyrimidine dimer (42). This terminus is not suitable as a nucleophilic site necessary for priming by polymerases. Exonuclease III, because it can act as a phosphomonoesterase at such a 3'-phosphoryl double-stranded terminus, prior to phosphodiester bond hydrolysis, is potentially available for such a function (58).

A 3'-polynucleotidase (59) could conceivably participate at this step in the repair cycle in providing such a nucleophilic site. Exo III mutants (60) show normal uv-sensitivity, implying

that the correndonuclease II enzymes repairing uv-irradiation damage by endonucleolytic activity either do not act by a b type mechanism, or, if 3'-phosphoryl groups were formed, other exonucleases may participate in this step in the cycle.

EXCISION MECHANISMS

It was implicit in earlier observations (61) that excision was, at least, a two-step process in M. luteus. It was found that the purification of a uv-dependent nuclease activity by TEAE cellulose chromatography resulted in the loss of activity, which could be recovered by the reconstitution of two different fractions eluted from such a column. The first fraction, fraction A, did not exhibit any nuclease activity; however, acid-soluble nucleotide release specifically from irradiated DNA required the presence of a second TEAE fraction which contained a non-specific nuclease activity, fraction B. Fraction B was active on unirradiated denatured DNA, and it was assumed that fraction A was an endonuclease.

An analogous multistep excision repair system was demonstrated in phage T4-infected E. coli (62). Correndonuclease II, isolated and purified to homogeneity, is the v-gene product of phage T4. To demonstrate the role of such an endonuclease in excision, crude extracts of cells infected with phage T4 v^- were employed as the source of exonuclease. The exonucleolytic properties associated with such crude extracts were later identified by Japanese workers (63). Similar types of excision systems seem to exist in M. luteus, E. coli and phage T4-infected E. coli.

Two types of exonucleases can execute excision: unassociated exonucleases may act on incised DNA, or DNA polymerase associated exonucleases may react with such incised irradiated DNA intermediates.

DNA polymerase-associate exonucleases - (3'→5' exonucleolytic activity

DNA polymerase I of E. coli is a multifunctional enzyme contained on a single polypeptide chain of molecular weight 109,000 (64). The M. luteus DNA polymerase for all intents and purposes is identical to the E. coli enzyme having a similar molecular weight, nuclease properties and N-ethyl maleimide insensitivity. In preliminary immunological experiments cross-reactivity with antibodies specifically directed against E. coli DNA polymerase (65) is observed.

In addition to their polymerizing properties, these enzymes have two associated exonucleolytic activities, one of which is a 3'→5' nuclease, specific for denatured DNA possessing a 3'-hydroxyl terminus in which 5' nucleotides are the exclusive product of digestion. An important role for this nuclease activity, in conjunction with the enzyme's polymerizing properties, is its editing function , in which this exonuclease activity is capable of digesting in a direction opposite to that of polymerization up to the point of hydrogen bond stability (66).

Polymerase associated 5'→3' exonucleolytic activity

The 5'→3' nuclease activity associated with polymerase I of E. coli and M. luteus is specific for DNA duplexes. This exonuclease activity is stimulated by neighboring 3'-hydroxyl groups provided during simultaneous polymerization of the four deoxynucleoside triphosphates (56, 67-69) included in reaction mixtures in which nicked DNA provides priming and potential templating sites. When synthesis and hydrolysis proceed at comparable rates, there is an extension of the 3'-hydroxyl terminus of a nick and simultaneous removal of nucleotides at the 5'-terminus -- a process referred to as nick translation (69). The translation continues in a 5'→3' direction until the nick is located at the extreme 3'-end of the template strand, at which time both activities cease. This process is probably the dominant reaction in vitro at temperatures below 22° (70). At higher temperatures, however, another process dominates which is expressed by the synthesis of branched, non-denaturable structures. Such aberrant synthetic mechanisms are reflected in an extent of synthesis in excess of one template equivalent (71). Conditions favoring this strand displacement reaction can be limited to nick translation if ligase is present during synthesis (56).

The 5'→3' nuclease initiates hydrolysis of DNA duplexes at internal rather than terminal phosphodiester bonds, regardless of whether such termini are 5' esterified (72).

Irradiated poly (dA:dT) incised nonspecifically with pancreatic DNase was hydrolyzed by polymerase I, liberating dimer-containing fragments. The products of exonucleolytic hydrolysis by the polymerase were oligothymidylates of chain lengths ranging from dinucleotides to heptanucleotides containing photoproducts.

Any assignment of cellular involvement in polymerase mediated repair will be dependent on experiments obtained with those mutants lacking the respective nuclease activities. Those mutant strains of E. coli with negligible levels of DNA polymerase I (pol A), although increasingly sensitive to uv, are considerably more resistant than uvr or rec mutants (73, 74). Pol A strains of E. coli, such as P3478, excise thymine-containing dimers to

the same rate and extent as do wild type cells (75). The inference derived from such results is that the uv-sensitivity of these mutants may be related to the reduced polymerizing capabilities during reinsertion, and that other mechanisms of excision must be operative in these strains of E. coli. Extensive polymerase isolations were carried out in a variety of polA mutants. It was found that polymerase I activity in these E. coli mutants was between 0.5-3% of wild type activity, whereas the associated 5'→3' exonuclease activities remain largely unaffected (76). Although it has been interpreted that the residual 5'→3' exonuclease activity might account for normal dimer excision in such mutants, it can be argued that those endogenous 5'→3' exonucleases not normally associated with pol I can also serve in this capacity.

The size of reinserted regions of DNA from experiments in vivo ranges from 10 nucleotides (short patch repair) to about 3000 nucleotides in length (long patch repair). Pol A1 mutations result in a preponderance of long patch repair synthesis (77). These data may be explicable in terms of the elevated ratio of 5'→3' hydrolysis to polymerizing activity in such mutants. However, the resolved 5'→3' exonuclease fragment, 33S, excises slowly in the absence of concomitant polymerization. The addition of the large (77S) polymerizing fragment markedly stimulates pyrimidine dimer removal (78). These findings are in accord with the known properties of this enzyme in nick translation in vitro.

It is difficult to conclusively assign an in vivo dimer excision role to the 5'→3' exonuclease of polymerase I since temperature sensitive mutants in this exonucleolytic activity have been found to be conditionally lethal by a number of laboratories (79, 80). However, a mutant of E. coli with a substantial defect in the 5'→3' exonuclease, but with normal polymerizing properties, have been described (81, 82). This mutant is marginally uv-sensitive and shows a somewhat reduced pyrimidine dimer excision capability.

Pol III excision capabilities

E. coli polymerase III has associated exonuclease activities (83), in which both 3'→5' and 5'→3' hydrolytic activities have been noted. The 3'→5' activity, like other reported polymerase, prefers single-stranded DNA, releases 5'-mononucleotides, the activity of which is inhibited by a 3'-phosphoryl group. The 5'→3' activity initiates hydrolysis on single strands and can proceed into a duplex region once initiation has occurred. This activity can also initiate hydrolysis of double-strand DNA possessing a single-stranded 5'-terminus. The limit products of hydrolysis may arise from the combined 3'→5' and 5'→3' activities of this polymerase. The structure of uv-irradiated DNA

incised by the M. luteus correndonuclease II is ideally suited for 5'→3' exonucleolytic removal of pyrimidine dimers.

Mammalian DNA polymerases

Three DNA polymerases have been isolated from a variety of mammalian sources. In all cases, these eukaryotic DNA polymerases are uniformly devoid of associated exonuclease activities. The mechanisms of dimer excision in such organisms most likely proceeds as a consequence of the action of unassociated exonucleases exclusively. One of these mammalian excision enzymes, described later in this application, behaves in a manner similar to those isolated from both E. coli and M. luteus.

UNASSOCIATED EXONUCLEASES IN EXCISION

The hydrolysis of the phosphodiester bond 5'- to the photoproduct by the correndonuclease II requires that exonucleases involved in excision initiate hydrolysis in a 5'→3' directional manner. Moreover, an additional requirement for such exonucleases is that they must be able to hydrolyze internal phosphodiester bonds in order to catalyze the removal of pyrimidine dimers. It would appear that those exonucleases involved in the excision of pyrimidine dimers do not hydrolyze the phosphodiester bond linking the pyrimidine nucleotide dimer residues (84).

The catalytic properties of two generally distinguishable, but functionally related exonucleases capable of excising pyrimidine dimers have been identified in M. luteus (43), E. coli (85), T4-infected E. coli (63, 86), rabbit liver nuclei (87, 88), human placenta (89) and mammalian cell lines (90). Both correxonucleases, a uv-exonuclease and exonuclease VII have specificities which are limited to denatured DNA, and are capable of hydrolyzing uv-damaged and undamaged substrates at comparable rates and to the same extent (91). This characteristic property can be used to distinguish those single-stranded exonucleases with potential pyrimidine dimer excision capabilities in vitro.

The correxonuclease from M. luteus can be distinguished from functionally unrelated exonucleases, such as E. coli exonuclease I, venom phosphodiesterase and bovine spleen phosphodiesterase, whose hydrolytic activities are restrained by photoproduct containing DNA. The prokaryotic correxonucleases already characterized have the unusual capacity of initiating hydrolysis from either 3' or 5' termini. In all cases, the products, whether mononucleotides or oligonucleotides, contain 5' phosphorylated termini. The M. luteus correxonuclease is unique in that it re-

quires divalent cation and has no limit hydrolysis product (43). However, E. coli correxonuclease (exonuclease VII) (85) and a similar type of enzyme purified from M. luteus (92) do not require magnesium, thereby allowing for their ability to function optimally in the presence of 10^{-3} M EDTA, permitting its easy detection in crude extracts.

The E. coli correxonuclease can act on denatured DNA and single-stranded regions extending from duplex DNA treated with exonuclease III (85), or λDNA. The M. luteus correxonuclease cleaves part of the twelve nucleotide long single-stranded cohesive ends of λDNA, leaving three to four nucleotides on each end (93). None of these correctional exonucleases are able to use RNA as a substrate, nor are polyribonucleotide:polydeoxyribonucleotide hybrid polymers hydrolyzed by the E. coli correxonuclease.

The correxonuclease activity derived from human placenta, which presumably constitutes the second step in the excision of pyrimidine dimers, has been purified from human placenta by Dr. J. Doniger (89). The enzyme hydrolyzes single-stranded DNA in a 5'→3' and 3'→5' direction, releasing oligonucleotides containing five to eight residues. Furthermore, it is as efficient in dimer removal from incised irradiated duplex DNA as the prokaryotic correxonuclease and pol I. Interestingly, the placental correxonuclease can initiate hydrolysis at nicked and gapped sites on duplex DNA. The exonuclease proceeds in a non-processive manner. A similar activity has been detected in crude extracts of human cell cultures (90). The importance of such exonucleolytic activities in mammalian cells is emphasized by the fact that unlike the bacterial polymerases, none of the mammalian DNA polymerases purified to homogeneity have been found to contain any nuclease activity.

The M. luteus correxonuclease (43) and the enzyme from placenta (89) produce 5'-oligonucleotides during digestion. In general, the limit products of all repair exonuclease action are oligonucleotides bearing 5'-phsophoryl and 3'-hydroxyl termini in which approximately one-third are in the range of dimers to trinucleoside triphosphates and the majority are in the range of tetramers to dodecamers, in which no mononucleotides have been observed as intermediates during hydrolysis.

The correxonucleases and polymerase associated 5'→3' exonucleases yield oligonucleotides as an ultimate product and dimer-containing oligonucleotides from correndo II incised irradiated duplex DNA (44). Since the M. luteus correxonuclease does not hydrolyze the phosphodiester bond located between thymidylate dimer residues, the initial hydrolytic event must have occurred approximately six nucleotides 3'-to the photochemical damage (84). The ratio of nucleotides released per phosphodiester bond originally

hydrolyzed by the correndo II nuclease provides an estimation of the approximate size of the region distorted by the formation of photoproducts. Although such ratios are somehwat dependent on the source of irradiated DNA substrates, they do correlate moderately well with the distortion sizes predicted spectroscopically (94), and from model building (95). From data obtained with the correndonuclease II of M. luteus, the ratio of the number of nucleotides released to the number of phosphodiester bonds broken indicates that the size of the excised region is approximately 6-10 nucleotides in length (44).

The conformational specificities of the polymerase associated and unassociated 5'→3' exonucleases are different, which may influence the ability of these enzymes to excise pyrimidine dimers in regions of differing hydrogen bond stabilities. For example, the combined reaction of correndonuclease II and the single-stranded specific M. luteus correxonuclease seems to function preferentially in AT-rich regions, judging from the distribution of nucleotides 3'- to dimers in excised fragments (44). We are currently engaged in determining not only the nucleotide compositions of fragments excised by polymerase-associated and unassociated exonucleases, but environmental effects as well on the course of action of such nucleases. Parenthetically, it is noteworthy that neither exonuclease VII mutants, nor polymerase mutants affected in their 5'→3' exonuclease activity show severely marked uv-sensitivity, or impaired dimer excision capabilities (81, 96). The construction of double mutants will, therefore, be of considerable value in assessing the involvement in vivo of these various enzymatic activities. That significant increases in uv-sensitivity are not observed in single mutants may reflect either the interdependency or overlapping specificities of the polymerase-associated exonucleases and the unassociated exonucleases.

Reinsertion mechanisms

Following excision of the damaged region, reinsertion of nucleotides is catalyzed by DNA polymerases using the complementary strand as a template. This reinsertion is characterized by non-semiconservative incorporation of nucleotides into the DNA of uv-irradiated E. coli (97).

Reinsertion events in such experiments are distinguishable from semi-conservative replication through the uptake of ^{3}H-BudR into normal isopycnic density regions or intermediate density regions of such gradients. Since 5'BudR is more dense than its homolog thymidine, its incorporation into DNA in a semi-conservative manner (i.e., normal replication) results in the newly synthesized strand of DNA having a greater density than the

template strand. Measurement after shearing to small fragments (0.5×10^6 daltons) of the density of the double-stranded DNA by isopycnic centrifugation in CsCl or NaI reveals radioactivity banding as a "hybrid" area of the gradients. After uv-irradiation, incorporation of nucleotides takes place into relatively short gaps exposed during excision of the damaged bases. Radioactive 5'BrU incorporated into small gaps does not significantly increase the density of the DNA and thus appears in the normal density regions of the gradient (97). This operational definition of short patch repair is restricted to regions of approximately 10-30 nucleotides in length (3, 97). Under conditions in which short gap repair is inhibited, as in pol A mutants, the labeled 5-BrU is associated with intermediate regions of the gradient which are less dense than the hybrid semi-conservatively replicated DNA and more dense than the normal density regions of the gradient. Such regions are calculated to be 1000-3000 nucleotides in length and represent long patch repair (77). Both short and long patch repair occur in wild type cells, but are absent in excision defective cells. Therefore, two branches of reinsertion are available to E. coli cells.

One route of reinsertion is considerably more complicated, more extensive in extent and perhaps, as a consequence, less efficient than the other. This long patch repair (77) requires uvrA (98) and rec genes (77, 99), pol II (100), and/or pol III (101), is ATP dependent in toluenized cells (102) and may require the further support of specific unwinding proteins. An additional pathway involving recF genes (36, 37), which is an exonuclease I sensitive locus (35), can supplement both the pol A and recBC branch of repair and is perhaps similar to recBC post-replicative repair mechanisms (16-18).

It is difficult to assess the specific molecular determinants which influence the direction of excision and reinsertion into any one of the enzymatic branches of repair. Such determinants may be architectural; for example, enzymatic juxtapositions to damaged DNA might govern repair directions. They might also be influenced by the structural conformations of incised DNAs.

Short patch pathway of repair (uvr, pol A, ligase)

There is sufficient evidence that the uvr controlled sequence of events is necessary for excision and reinsertion of nucleotides via this pathway of repair (10, 98). The size of the patches observed in vivo from 5-BrU density labeling is of the same order of magnitude expected from studies in vitro (103). Although it is difficult to determine under normal conditions what proportion of the DNAs is repaired by the pol I system, it

can be deduced (98) that the majority of repaired DNA is in short patches synthesized via the pol I pathway of reinsertion.

Short patch repair has been mimicked under controlled in vitro conditions. Dr. Lester Hamilton, in our laboratory (56), examined the control in vitro of both excision of pyrimidine dimers and the reinsertion of nucleotides catalyzed by DNA polymerase I of M. luteus. Polymerase I type enzymes, unlike pol II and III and phage infected DNA polymerases, specifically bind at nicks, satisfying an important and specific requirement for the short patch excision process (104). It would appear, therefore, that the nick binding and translating properties of these enzymes provide for the necessary concerted mechanisms of reinsertion and excision. Nick translational conditions result in a stoichiometry of equivalence between removal of photoproducts with associated nucleotides and the reinsertion of nucleotides. Furthermore, polymerase is capable of repairing gaps (105). Incubation of this polymerase with excised DNA, for example, at 10° in optimal Mg^{+2} leads to a stoichiometric reinsertion-excision reaction which may be controlled by polynucleotide ligase. Under conditions of strand displacement at 37°, the presence of ligase appears to restrict the stoichiometric ratio to one nucleotide excised per nucleotide polymerized. Therefore, in vitro, the polymerase:correndonuclease II combination, in conjunction with ligase is sufficient for the complete repair of single-strand breaks associated with incision and restoration of biological activities of uv-irradiated transforming DNA.

When transforming DNA is 50% or less inactivated by uv, there is a quantitative restoration of biological activity by these three enzymes in vitro. At higher doses, the maximum in vitro repair capabilities by this enzyme system declines considerably. This is attributed to the formation of double-strand breaks, arising not from irradiation, but as a consequence of the repair process. The undirectionality of repair on strands of opposite polarity leads to double-strand break formation during the excision process. Dr. Hamilton (56) has evidence that polyamines, at concentrations endogenous to E. coli, stabilizes DNA repair intermediates and limits its degradation so as to prevent double-strand breaks. Thus increases in the reactivations of B. subtilis DNA at high uv doses can be faithfully reproduced.

Long patch pathway of repair (uvr, recBC, unwinding protein, pol II/III, ligase)

Delineation of repair pathways is operational and defined according to the density position of ^{3}H-BudR in gradients in which long patches are at the heavy end and short patches are located with the light end of such a gradient among non-replicated DNAs. There are clearly no well separated peaks of label, but rather a spectrum of densities which must be visualized, in

molecular terms, as path average rather than path specific. It was found (77), for example, that the uv-induced non-conservative DNA synthesis, rather than becoming limited in pol A mutants, was in fact considerably stimulated. This stimulated synthesis was dependent on the uvrA gene product (102), indicating a divergent rather than two separate paths of reinsertion. The size of the reinserted patch was at least one hundred times greater than that observed in a pol A dependent short patch pathway of reinsertion and furthermore was dependent on the presence of the rec B, rec C gene products.

Experiments in toluenized systems suggest that both polymerases II and III are involved in uv-induced repair. Since polymerase II lacks a 5'→3' exonuclease function (106), its role in repair must be confined to reinsertion reactions. Repair in toluenized cellular systems semms to be of the long patch type which is missing if both polymerase II and III are inactivated (102).

A controlling feature of long patch repair may be attributable to the size of the fragment excised by the correxonucleases or pol III initiation of hydrolysis of the single-stranded regions of incised DNA. The fragments released by the unassociated exonucleases vary between 6-8 nucleotides in length (44, 83, 85). Once the single-strand fragment is removed, the duplex nature of the excised DNA limits further hydrolysis by the exonucleases. The binding properties, however, of polymerases II and III are limited to much larger regions, such that in order for these polymerases to carry out reinsertion, a gap expansion step is necessary (104). This gap expansion step is most likely catalyzable by the ATP-stimulated double-stranded exonuclease activity, which cannot hydrolyze nicked DNAs but is able, however, to degrade duplex circles containing gaps as short as five nucleotides in length (108). Hydrolysis is bidirectional in both a 5' and 3' direction (109). It is this gap expansion activity which is thermolabile in rec BC temperature sensitive mutants. Furthermore, at restrictive temperatures, it is this rec BC function which confers both uv-sensitivity and reduced viability to such mutant E. coli cells (33).

Preservation of Complementarity

It is at this juncture in the repair process that the DNA is the most vulnerable to hydrolysis by single stranded specific nucleases. Involvement of the rec BC nuclease in a gap expansion process associated with long patch repair mechanisms necessitates some control for preventing the action of the rec BC ATP-dependent single stranded endonuclease from affecting the complementary strand exposed during "gap expansion". There is suggestive data

available implicating certain binding, or unwinding, proteins in preserving the integrity of such exposed complementary strands.

The T4 phage gene 32 protein, required for both recombination and replication of T4 phage, specifically binds in a cooperative manner to single stranded DNA (113). Furthermore, the binding protein forms a ternary complex with DNA and T4 DNA polymerase specifically (114). By virtue of its binding capabilities this protein selectively inhibits the action of single stranded endonucleases at such sites (113). The role of this gene product in repair is supported by data arising with temperature sensitive mutants of "early" T4 genes in which the gene 32 ts mutants are particularly uv sensitive under restrictive conditions (112).

The E. coli binding protein required for the processive polymerization by polymerase II on single-stranded DNA (110) is also potentially able to assume a protective role in the gap expansion process. The E. coli unwinding protein inhibits the single stranded endonucleolytic activity of the rec BC enzyme specifically in vitro without affecting the ATP dependent duplex exonuclease activity. The binding protein of E. coli also participates in preserving the integrity of the strand opposite to that which is being repaired (111).

It may be suggested that the rec A gene product serves similarly in protecting complementary strands exposed at this locus in repair or during postreplication repair in a manner analogous to the T4 gene 32 protein functioning in recombination and replication.

Rec A mutants are uv sensitive and during postirradiation conditions their DNA is extensively degraded. Double rec BC, rec A mutations cure this "recless" state probably by eliminating the enzyme system responsible for such extensive DNA degradation after incision events by the uvr AB gene products. As expected, uvrA or B, rec A double mutants are so exquisitely uv sensitive that a single dimer is lethal (17). From such observations it can be concluded that rec A functions in a protective capacity by specifically inhibiting rec BC functioning at nicks or gaps and furthermore suggests that rec A and uvrA,B gene products represent initial loci in two diveregent pathways of repair. One pathway is through excision and the other through recombinational mechanisms operating during postreplication events.

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