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RIBOSOMAL PROTEIN S3: A MULTIFUNCTIONAL EUKARYOTIC
DNA REPAIR ENZYME ACTIVE ON ULTRAVIOLET AND
OXIDATIVE DNA DAMAGE

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
PROGRAM IN MOLECULAR BIOLOGY

BY
DAVID M. WILSON III

CHICAGO, ILLINOIS

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LIST OF ABBREVIATIONS

μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
AP	apurinic/apyrimidinic
AP3	Drosophila AP endonuclease/ribosomal protein
AT	<u>ataxia telangiectasia</u>
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BS	Bloom's syndrome
Ci	curie
CIAP	calf intestinal alkaline phosphate
CPD	cyclobutane pyrimidine dimer
CS	Cockayne's syndrome
D	daltons
DDBP	damage-specific DNA binding protein
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate

DTT	dithiolthreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(<i>B</i> -aminoethyl ether) tetracetic acid
ERCC	excision repair cross-complementing
FA	Fanconi's anemia
FaPy	formamidopyrimidine-DNA glycosylase
g	gram
GIT	guanidinium isothiocyanate
GST	glutathione S-transferase
HEPES	N-[2-hydroxyethyl]piperazine N'-[2- ethansulfonic acid]
IPTG	isopropyl- <i>B</i> -D-thio-galactosidase
kb	kilobases
kD	kilodaltons
LB	Luria broth
M	molar
mg	milligram
MGMT	O6-methylguanine DNA methyltransferase
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger RNA

MMS	methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
MOPS	3-[N-morpholino] propanesulfonic acid
NBT	nitro blue tetrazolium chloride
O.D.	optical density
PBS	phosphate buffered saline
PEG	polyethylene glycol
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PPP	pyrimidine (6-4) pyrimidone photoproduct
RNA	ribonucleic acid
Rrp1	recombination repair protein 1
rRNA	ribosomal RNA
SAM	S-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
TBE	tris-boric acid-EDTA-electrophoresis buffer
TE	tris-EDTA buffer
TEA	triethanolamine
TEMED	tetramethylethylenediamine
tRNA	transfer RNA
UDG	uracil-DNA glycosylase
UV	ultraviolet
X g	times gravity
XP	<u>xeroderma pigmentosum</u>

CHAPTER I

INTRODUCTION

The physical and chemical structure of DNA is continually threatened by endogenous and exogenous agents that cause a wide spectrum of DNA damage. In addition, alterations in the genetic information of cells can arise from the inherent chemical instability of DNA as well as by spontaneous errors in recombination and replication. Unrepaired lesions in DNA can result in mutations which disrupt the controlled and integrated actions of critical gene products either by stimulating unregulated gene expression or by causing the synthesis of defective proteins. In either case, DNA damage has been shown to be associated with genetic defects, carcinogenesis, aging, and cell death.

To defend against the numerous factors that alter DNA structure or base sequence, cells have evolved an elaborate array of repair systems. These systems maintain the integrity of genetic material by correcting DNA damage. In general, DNA repair processes can be viewed as the complex cellular responses associated with the restoration of the normal nucleotide sequence of DNA after damage.

The involvement of DNA repair systems in preventing carcinogenesis is clearly demonstrated by the high incidence of cancer found associated with several well-recognized DNA-

repair-defective human diseases [i.e. xeroderma pigmentosum (XP), Bloom's syndrome (BS), Fanconi's anemia (FA), Cockayne's syndrome (CS), and ataxia telangiectasia (AT)]. Thus, studies attempting to elucidate the molecular and biochemical processes which regulate DNA repair mechanisms in both normal and DNA-repair-defective cells are ongoing. In this dissertation, I describe the *Drosophila melanogaster* ribosomal protein S3 and its role as a multifunctional DNA repair protein that is active on DNA containing abasic sites or 8-oxoguanine lesions, two potentially mutagenic DNA alterations.

Interestingly, S3 was originally classified as a core component of the 40S subunit of ribosomes. The focus of much of the research on ribosomes has been to determine the relationship between ribosomal structure and function; thus, there has been an ongoing effort to isolate and characterize all the ribonucleoproteins of eukaryotic (rat) ribosomes. These proteins have undergone extensive analysis and, for simplicity, have received nomenclature based on their two-dimensional electrophoretic patterns. S3 is one of the 30 to 40 proteins identified as a constituent of the 40S subparticle.

Ribosomal protein S3 is of particular interest because it was found to crosslink with eukaryotic translation initiation factors eIF-3 and eIF-2 as well as to mRNA. These data suggest that S3 is a component of the preinitiation complex and participates in the initiation of protein synthesis.

Using a cDNA fragment that encodes *Xenopus laevis* ribosomal protein S1, the homolog to mammalian S3, a cDNA encoding rat ribosomal protein S3 was isolated. The protein specified by the reading frame of the rat cDNA was identified as S3 by comparing the deduced amino acid sequence with peptide sequence derived from purified S3; these proved to be identical. In addition, *in vitro* transcribed/translated rat S3 protein yielded a product which migrated in two-dimensional gel electrophoresis to a position that had the coordinates of S3.

The cDNA encoding the human S3 ribosomal protein was isolated during a search for genes whose level of expression changed during tumorigenesis. Utilizing the technique of subtractive screening, several clones whose level of expression is elevated in adenocarcinoma of the colon were isolated. One of these clones showed significant identity to the cDNA encoding rat ribosomal protein S3. Subsequent experiments found that the human S3 transcript level, as well as several other mRNAs encoding ribosomal proteins, were elevated in colorectal tumors and polyps. Of the six ribosomal proteins identified as being overexpressed in human colorectal cancers, three (S3, P0, and S6) have been found to perform other cellular functions. S3 (as will be described in this dissertation) and P0 are multifunctional DNA repair proteins, and S6 is a tumor-suppressor gene in *Drosophila* involved in the hematopoietic system. While increased levels

of ribosomal proteins may be a casual result of cellular proliferation, these discoveries suggest that these ribosomal proteins may play a more active role in transformation.

Recently, several studies have implicated other DNA repair proteins as being multifunctional. The yeast homolog (*SSL2/Rad25*) to the human DNA repair enzyme *ERCC3* has been found to participate in translation initiation of specific mRNAs containing inhibitory stem-loop structures upstream of the initiation codon. On the other hand, an *SSL2* allele made to resemble the defective *ERCC-3* gene present in individuals suffering from XP and CS confers ultraviolet (UV) light hypersensitivity to yeast cells, but does not affect *SSL2* ribosomal function or cell viability. Moreover, the *Drosophila* homolog to *ERCC-3*, *haywire*, displays a central nervous system mutant phenotype, a characteristic affiliated with XP and CS patients. Thus, *SSL2* performs two cellular functions: one involved in protein synthesis and another related to DNA repair. In addition, the major human DNA repair AP endonuclease (*APE*), an enzyme which initiates the process of excision repair of abasic sites, also regulates the DNA binding activity of several transcription factors by a posttranslational mechanism entailing reduction-oxidation. Thus, a class of proteins is beginning to emerge as transcriptional and translational regulators that are encoded by genes involved in DNA repair, and may therefore begin to explain the pleiotropic nature of human diseases with

compromised systems of DNA repair.

We decided to pursue studies of the *Drosophila* ribosomal protein S3 with the knowledge that another ribosomal protein P0 (one of six ribosomal proteins overexpressed in colorectal cancers, others being S6 and S3) displays AP endonuclease activity, and that several other DNA repair proteins are multifunctional. In addition, results from Dr. Stuart Linn's laboratory at University of California, Berkeley, identified human ribosomal protein S3 as a defective AP endonuclease activity in XP complementation group D (XPD) cells. XP is a rare genetic disease which renders the afflicted individual sensitive to ultraviolet irradiation.

Initial experiments analyzing the endonuclease activity present in unfractionated extracts from human fibroblasts of XP patients showed complementation groups A, B, C, and E to have slightly reduced levels of endonuclease activity, whereas group D displayed one-sixth of the normal activity. This endonuclease activity from cultured human fibroblasts was subsequently resolved into two species by phosphocellulose chromatography -- a flowthrough fraction and an eluted fraction. Upon further investigation of the XPD cell line extracts, the flowthrough species was found to lack endonuclease activity. Furthermore, XPD line extracts were defective in reactivation of UV-irradiated DNA, supporting the idea that XPD cells are unable to repair UV-induced DNA damage. In an attempt to identify the altered endonuclease

activity in XPD, a protein was purified (utilizing the same protocol designed to purify the flowthrough species from XPD cells) from normal cells that demonstrated glycosylase and endonuclease activity. This protein was microsequenced and determined to be the human ribosomal protein S3. Subsequent experiments revealed that purified fractions of the S3 protein from XPD extracts showed no DNA repair activity.

The focus of my dissertation research has been to analyze mechanisms of DNA repair in *Drosophila*, a model genetic, molecular, and biochemical eukaryotic organism. In particular, these studies have revolved around ribosomal protein S3 and its role as a DNA repair enzyme on UV-induced DNA damage and the lesion 8-oxoguanine. The formation of 8-oxoguanine occurs through the activity of reactive oxygen species produced during normal cellular processes as well as from exposure to high doses of UV irradiation (10,000 J/m²) and other environmental mutagens (i.e. ionizing radiation). The presence of 8-oxoguanine in DNA has been demonstrated to cause G to T and A to C transversions, which may be the source of mutations responsible for the increased incidence of tumor promotion and carcinogenesis associated with this DNA adduct. These findings illustrate the importance that organisms retain the capacity to repair this lesion. In *Escherichia coli*, it has been shown that the removal of 8-oxoguanine is mediated through the action of formamidopyrimidine-DNA glycosylase (FaPy). I describe in this dissertation that a protein

present in *Drosophila* capable of acting on 8-oxoguanine residues is 80% identical to the rat ribosomal protein S3, suggesting that S3 is part of a newly emerging class of multifunctional proteins encoded by genes involved in DNA repair.

In this dissertation, data describing the molecular and biochemical characteristics of *Drosophila* S3 ribosomal/DNA repair protein will be presented. Briefly, I used a rat cDNA encoding ribosomal protein S3 to clone the *Drosophila* homolog from a 5.5 to 7.5 day pupal lambda gt10 cDNA library. The *Drosophila* clone includes a single open reading frame of 738 nucleotides (246 amino acids) which predicts a protein with a molecular weight of 27,470 daltons. The *Drosophila* encoded S3 protein is 80% identical to the rat and human ribosomal proteins. The motif K K/R X K/R has emerged as a common feature in many nuclear localization sequences described to date and is found in the N-terminal part of the *Drosophila* S3 protein (KKRK). In addition, there exist three potential phosphorylation sites and a single potential glycosylation site. These consensus domains may serve as modes for regulating S3 localization and/or activity. Genomic clones encompassing the S3 gene were also isolated and the organization of the gene determined by sequence analysis. The S3 gene spans less than 2 kb and consists of two exons interrupted by a single 263 bp intron. Additionally, the 5' transcriptional start site was resolved. *In situ*

hybridization of polytene chromosomes as well as southern blot analysis of *Drosophila* genomic DNA indicate that the S3 gene is likely a single copy gene. The S3 cDNA maps to a region on the third chromosome in the vicinity of region 95A, a previously identified *Minute* ribosomal gene. *Minutes* are a class of genes that, when present in the hemizygous condition, produce a characteristic phenotype consisting of short slender bristles and delayed development, whereas, homozygotes are late larval lethals. It is believed that most, if not all, these mutations affect ribosomal protein genes. Developmental analysis of both S3 mRNA and protein indicates that the gene is constitutively expressed. However, the localization of the *Drosophila* S3 gene product between the cytoplasm and the nucleus varies and is dependent on the stage of development. During the life cycle, the S3 protein is dispersed predominantly in the cytoplasm (70% cytoplasmic and 30% nuclear), except during the pupal stage where it is distributed 50%-50%. The S3 protein was detected in purified preparations of ribosomes, nuclear matrix (where topoisomerase II was also found), and chromatin by Western blot analysis using affinity purified antibody produced against a *Drosophila* S3 fusion protein. This nuclear localization suggests a possible role for S3 in DNA metabolism and is consistent with the presence of the nuclear targeting sequence in the N-terminal domain. S3 fusion protein purified from DNA-repair-deficient bacteria demonstrates class I AP lyase activity on

AP DNA; that is, S3 cleaves on the 3' side of AP sites by a beta-elimination reaction mechanism. Furthermore, this protein was shown to be active on heavily UV-irradiated DNA and cut at certain guanine photoproducts. Using an oligonucleotide containing a single 8-oxoguanine residue, S3 specifically recognized and incised 3' to this adduct via beta,delta-elimination reaction. These results suggest that S3 demonstrates both N-glycosylase and AP lyase activities specific for 8-oxoguanine. S3 appears to be a multifunctional protein with roles in ribosomal function and DNA repair.

CHAPTER II

REVIEW OF RELATED LITERATURE

The inherent chemical instability of DNA, the production of reactive oxygen species during normal cellular metabolism, and the continuous exposure to environmental mutagens, all represent a potential threat to the integrity of the genetic information of cells (for review of DNA damage and repair see Friedberg, 1985). If left unrepaired, damage to DNA can have deleterious biological consequences for the organism, such as transformation, aging, or cell death, through the introduction of mutations, which may cause the impairment of normal cellular functions. Thus, to protect against potentially detrimental insults to DNA, organisms have evolved an elaborate array of DNA repair mechanisms that permit the removal/tolerance of DNA damage. In this chapter, types of DNA damage, mechanisms utilized by organisms to repair DNA damage, and a new class of multifunctional proteins will be discussed.

A. DNA Damage

DNA is continuously being modified by a number of environmental agents as well as several endogenous agents which are formed during normal cellular reactions (Halliwell

and Aruoma, 1991). Furthermore, spontaneous alterations in DNA composition can arise due to the intrinsic chemical instability of DNA or from the infidelity of DNA replication, recombination, and repair synthesis (Loeb and Reyland, 1987). DNA base modifications, strand breaks, depurinations, inter or intrastrand cross-links, and rearrangements are several types of alterations reported to occur either spontaneously and/or after exposure to DNA-damaging agents (Figure 1). It is this damage to genetic material which can ultimately, if unrepaired, result in aging, cell death, and/or transformation (Bohr et al., 1989). These events are manifested through the formation of mutations, which may cause the impairment of normal cellular functions.

It is important to recognize the difference between damage and mutation. DNA damage is an alteration in DNA structure, which often cannot be replicated and is eventually repaired. Mutations are permanent changes in the polynucleotide sequence that resulted from erroneous repair/replication of DNA damage and appear as normal DNA components. These alterations in nucleotide sequence are believed to play a significant role in aging, carcinogenesis, cell death, and/or several human diseases.

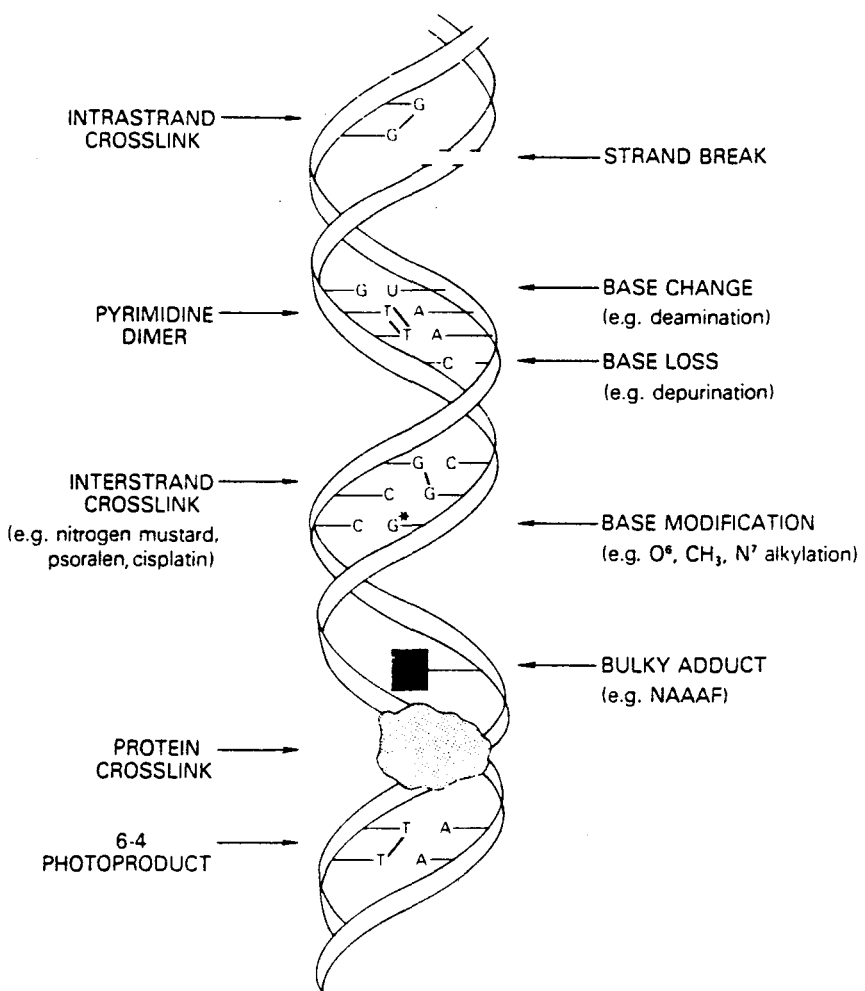


Figure 1: Common forms of DNA damage [Reprinted with permission from Williams and Wilkins Company (Bohr et al., 1989)].

1. Endogenous Insults

DNA is unstable under normal physiological conditions (for review see Lindahl, 1993). Four important endogenous processes leading to significant DNA damage are depurination, deamination, methylation, and oxidation (Figure 2; Totter, 1980; Ames, 1983; Saul et al., 1987).

a. DNA Hydrolysis

Lindahl (1993) proposed that depurination, depyrimidination, single-strand break generation, and deamination are all frequent forms of spontaneous DNA damage. It has been estimated that a mammalian cell at 37°C loses approximately 12,000 to 14,000 purines (Guanine-G and Adenine-A) and 600 to 700 pyrimidines (Cytosine-C and Thymine-T) from its DNA per day by spontaneous hydrolytic cleavage of the base from the deoxyribose phosphate backbone (Lindahl, 1977; Tice and Setlow, 1985). These apurinic damages at neutral pH and physiological solvent conditions have a half-life of 100 hours at 37°C before the chain breaks by a beta-elimination reaction, generating a single-strand break (Lindahl and Andersson, 1972). During an individual's lifetime, in a single mammalian cell, approximately 3% of the total purines in the cell's DNA will undergo spontaneous depurination caused by hydrolysis (Lindahl and Nyberg, 1972). Sites of base loss have been demonstrated to block DNA replication machinery leading to cell death (Loeb and Preston, 1986). In addition,

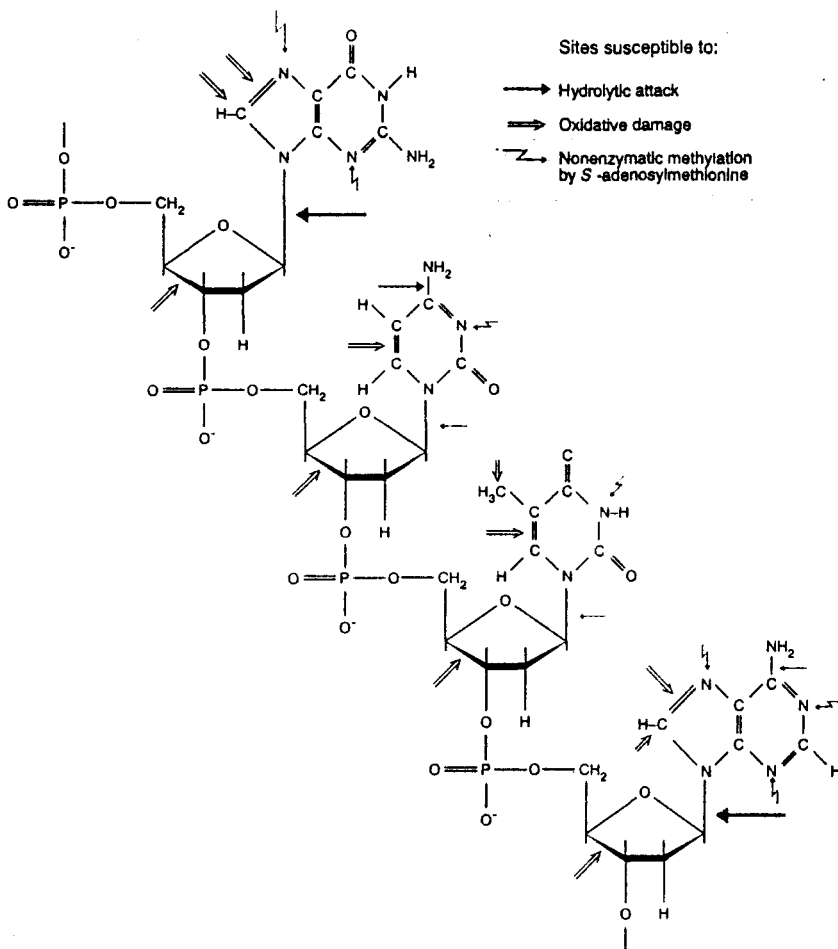


Figure 2: Target sites for intracellular DNA decay. A short segment of one strand of the DNA double-helix is shown with the four common bases (from top: guanine, cytosine, thymine, adenine). Sites susceptible to hydrolytic attack are indicated by solid arrows, oxidative damage by open arrows, and nonenzymatic methylation by *S*-adenosylmethionine as zig-zagged arrows. Major sites of damage are indicated by the large arrows [Reprinted with permission from *Nature* (Lindahl, 1993), copyright (1993) Macmillan Magazines Limited].

since apurinic/apyrimidinic (AP) sites represent a loss of genetic information, they are potentially mutagenic through the action of error-prone DNA repair. For single-strand breaks, the spontaneous incidence has been estimated to be 55,200/cell/day (Tice and Setlow, 1985). Strand breaks leaving behind 3' termini which are not substrates for DNA polymerase(s) can likewise lead to cell death unless removed.

In addition to the problem of the intrinsic lability of the N-glycosyl bonds, DNA base residues are susceptible to hydrolytic deamination (Lindahl, 1993). The rate of spontaneous deamination of cytosine to uracil is estimated to be approximately 192/cell/day (Shapiro, 1981). 5-methylcytosine moieties are produced through a catalytic mechanism carried out by a DNA methyltransferase. These lesions deaminate to thymine three to four times more rapidly than cytosine to uracil (Lindahl and Nyberg, 1974). Both the conversion of cytosine to uracil and 5-methylcytosine to thymine are potential sites for point mutations (Duncan and Miller, 1980). Interestingly, such GC to AT transitions account for one-third of single-site mutations observed in inherited human disease (Cooper and Youssoufian, 1988). Currently, the enzymatic methylation of cytosine to 5-methylcytosine has only been shown to occur in mammalian cells (Selker, 1990). Thus, deamination of 5-methylcytosine would not pose a problem in lower eukaryotes, such as *Drosophila melanogaster*.

Deamination of DNA purines is a rare event, occurring at about 2-3% the rate of cytosine deamination (Karran and Lindahl, 1980). Still, the conversion of adenine to hypoxanthine, which preferentially base-pairs with cytosine, results in the generation of a premutagenic lesion. Xanthine, the product of deamination of guanine, base pairs with cytosine, the correct base, and, therefore, is not directly mutagenic and is not deleterious.

b. Nonenzymatic DNA Methylation

In addition to the spontaneous forms of DNA damage which can arise under normal physiological conditions, there exist several endogenous mutagenic factors which react with DNA to produce marked structural alterations (Lindahl, 1993). One of the best characterized and most important of these factors is S-adenosylmethionine (SAM). SAM normally acts as an efficient methyl group donor in most cellular transmethylation reactions. However, methylation of DNA which yields primarily N7-methylguanine and N3-methyladenine occurs as a minor side reaction of the necessary methyl transfer function of SAM (Barrows and Magee, 1982). Thus, SAM has the same effect as a weak alkylating agent. N7-methylguanine, which does not alter the coding specificity of the base, appears relatively harmless. N3-methyladenine, on the other hand, is a cytotoxic lesion that blocks DNA replication (Karran et al., 1982). SAM induces the formation of the highly mutagenic lesion O6-

methylguanine only in trace amounts. Instead, the production of this miscoding base derivative accounts for the very strong mutagenic activity of several other alkylating agents. Recently, *in vivo* evidence was reported which clearly demonstrates that in *Saccharomyces cerevisiae* endogenous DNA alkylation damage is a source of spontaneous mutation (Xiao and Samson, 1993).

Other cellular compounds represent potential threats to the integrity of genetic material because of their ability to form covalent adducts with DNA. For instance, glucose, glucose-6-phosphate, and possibly other sugars present in a cell have been demonstrated to modify DNA and have been shown to be mutagenic (Bucala *et al.*, 1984; Lorenzi *et al.*, 1986; Lee and Cerami, 1987).

c. DNA Oxidation

DNA damages produced by free radicals are probably the most frequent lesions that the cell encounters (Ames, 1987). In fact, Ames (1987) estimated that each human cell sustains an average of 10^3 "oxidative hits" per day. This phenomenon is the result of reactive oxygen species that are generated during normal aerobic metabolism (Halliwell, 1991) or from exposure to exogenous agents such as ionizing radiation (Hutchinson, 1985). These metabolic by-products include a short list of chemicals such as the superoxide radical, the hydroxyl radical and hydrogen peroxide, which have been

demonstrated to induce DNA damage and may contribute to a variety of human disorders, tumor promotion and aging (Halliwell and Gutteridge, 1990; Fischer et al., 1988; Saul et al., 1987). The most reactive species, the hydroxy radical, produces a broad spectrum of DNA damage (Teoule, 1987), the predominant forms being modified bases, apurinic/apyrimidinic (abasic or AP) sites and single-strand breaks with 3' termini blocked by nucleotide fragments (Hutchinson, 1985; Giloni et al., 1981; von Sonntag, 1991). AP sites may also originate by spontaneous or mutagen-induced hydrolysis or by the action of DNA glycosylases that remove various altered bases (Wallace, 1988). These abasic sites have been shown in *Escherichia coli* to block the progress of the DNA replication apparatus and cause mutation (Loeb and Preston, 1986). Strand breaks produced by free radical attack on DNA are refractive to DNA polymerase, and therefore, repair synthesis (Henner et al., 1983; Demple et al., 1986; Johnson and Demple, 1988a, b; Ramotar et al., 1991b). Thus, these 3' termini are an important class of lethal oxidative DNA damage and must be removed to maintain genetic integrity.

The major mutagenic base lesion generated by hydroxyl radicals is 8-hydroxyguanine (Grollman and Moriya, 1993). 8-hydroxyguanine base-pairs preferentially with adenine rather than cytosine and, thus, causes transversion mutations after replication. The oxidation of guanine is thought to be one of the major spontaneous events which results in a directly

premutagenic lesion. Other well studied DNA adducts generated by free radical attack are the ring-saturated derivatives of a pyrimidine (i.e. thymine and cytosine glycols and pyrimidine hydrates; Halliwell and Aruoma, 1991). These derivatives are non-coding bases and are, therefore, primarily cytotoxic lesions. However, under certain circumstances they can be mutagenic (Basu *et al.*, 1989). Recently, DNA damage by oxygen free radicals was definitively shown to cause mutations by modifying nucleotide bases, which results in miscoding when the DNA is copied by DNA polymerase, and by altering the conformation of the DNA template (Feig and Loeb, 1993).

d. DNA Replication

When considering semiconservative DNA synthesis as a source of DNA damage, it has been estimated that the error frequency in newly replicated DNA is about 10^{-6} to 10^{-9} per nucleotide (Loeb and Reyland, 1987). However, the actual mutation frequency per nucleotide is even lower than this by about another three orders of magnitude due to the repair of mispaired bases in DNA by mismatch repair machinery (Drake, 1969).

2. Exogenous Insults

Some of the most common environmental insults which can alter the chemical structure of DNA are ultraviolet light from the sun, alkylating agents, ionizing radiation, and a variety

of dietary chemicals (Brash, 1988; Saffhill et al., 1985; Hutchinson, 1985; Ames, 1983). These chemical and physical agents react directly with DNA to introduce modifications in base composition.

a. Ionizing Radiation

Ionizing radiation is capable of inducing hundreds of products in DNA (Hutchinson, 1985). These DNA damaging capabilities are a result of both direct and indirect effects of ionizing radiation. The so-called direct effects result from direct interaction of the radiation energy with DNA. The indirect effects are due to the interaction of reactive oxygen species, formed by the radiation, with DNA. In view of the similarities between some endogenous mutagens produced by normal aerobic metabolism and those (such as hydroxyl radicals) produced by radiation, one might expect spontaneous and radiation-induced point mutations to have some mechanisms in common. The hydroxyl radical is the major product formed by the action of ionizing radiation on water. Thus, many of the DNA damages identified as products of ionizing radiation are similar to those adducts which result from attack of DNA by reactive oxygen species.

The rate of destruction of purines by hydroxyl radicals occurs at a much slower rate than with pyrimidines (Hutchinson, 1985). However, the destruction of the imadazole ring of purines has been found to yield 5-formamidopyrimidine

compounds after exposure to ionizing radiation in the presence or absence of oxygen. Furthermore, radiolysis products of thymine that have been detected include thymine glycols, formylpyruvylurea, and hydroxybarbituric acid. Another important biological consequence of ionizing irradiation of DNA is the formation of single strand breaks. Moreover, most radiation-induced mutations are either inferred or demonstrated to be DNA deletions (Sankaranarayanan, 1993). In any case, ionizing radiation has been shown to be both mutagenic and carcinogenic.

b. Alkylating Agents

Alkylating agents are potent environmental mutagens which are capable of forming a considerable number of different adducts by reacting with cellular DNA (for review see Lindahl *et al.*, 1988). These agents can alkylate all four bases of DNA at the nitrogens or oxygens as well as the sugar phosphates of the DNA backbone. However, the distribution of the adducts at the various sites depends on both the chemical structure of the alkylating agent and the alkyl group itself. Although none of these DNA lesions can be entirely ruled out as a potential source of mutations, there is extensive literature suggesting that adducts at the O6-position of guanine and the O4-position of thymine may be of particular importance in this respect. O6-methylguanine preferentially pairs with thymine rather than cytosine resulting in a GC to

AT transition (Loveless, 1969). O4-methylthymine, also a miscoding base, induces AT to GC transitions (Preston *et al.*, 1986). Another important product of attack on DNA by alkylating agents is N3-methyladenine, which causes cell-killing (Karran *et al.*, 1982). N3-methyladenine blocks the progress of DNA polymerases during replication (Larson *et al.*, 1985). However, the principle target of alkylating agents is the N7 position of guanine (Saffhill *et al.*, 1985). Although N7-alkylguanine itself is not a deleterious lesion, it can undergo a rearrangement to yield a ring-opened imidazole form (formamidopyrimidine). Formamidopyrimidine residues have been shown to inhibit DNA polymerase synthesis (Boiteux and Laval, 1983) and several observations suggest that this adduct may play a significant role in processes leading to mutagenesis and/or cell death by alkylating agents. In addition, N-alkylpurines, such as those just mentioned, are indirectly mutagenic because their removal, either in a spontaneous chemical reaction or by the action of DNA glycosylases results in the formation of AP sites. While AP sites normally prevent DNA replication, under special circumstances they can also lead to mutations (Loeb and Preston, 1986). N-alkylpurines may also contribute, simply by accumulating, to other biological effects such as induction of chromosomal aberrations as well as the aging process (Vogel and Natarajan, 1979; Gensler and Bernstein, 1981). These conclusions were based upon circumstantial evidence. Finally, some alkylating

agents are bifunctional; that is, they have two reactive groups. Each molecule is, therefore, capable of reacting with two sites in DNA, which potentially can result in the formation of an inter- or intra-strand crosslink, which appear to block both DNA replication and transcription (Friedberg, 1985).

c. Dietary Chemicals

The normal diet of an organism contains many potentially mutagenic and carcinogenic agents such as alcohol, nitrosamines, and phorbol esters. In fact, Ames (1983) describes 16 examples of DNA-damaging chemicals found in plants that are part of the human diet. Many of these mutagens and carcinogens exert their effect through the generation of oxygen radicals. In addition, these chemicals are capable of reacting with DNA to generate single-strand breaks, double-strand breaks, and bulky DNA adducts.

d. Ultraviolet Radiation

One of the most common deleterious environmental agents that organisms are exposed to is ultraviolet (UV) light (Friedberg, 1985). Although proteins and cellular membranes are targets of UV irradiation, the major target is DNA, which absorbs short wavelength UV most efficiently (254nm/UVC). In particular, UV irradiation induces the formation of numerous DNA photoproducts, the most prominent ones being the

cyclobutane pyrimidine dimer (CPD) and pyrimidine (6-4) pyrimidone photoproduct (PPP) (Setlow, 1968). Induction frequencies of these two lesions vary between organisms and is sequence dependent. It has been reported that the preferred di-pyrimidine order for CPD or PPP formation is TT > TC > CC (Haseltine, 1983).

Both CPDs and PPPs are thought to be non-instructional bulky lesions (Setlow, 1968). However, little is known as to how these UV-induced photoproducts contribute to transformation of a UV-exposed cell into a malignant cancerous cell. Though TT is by far the most frequent putative site for a UV-induced lesion, base-pair changes at these sites were observed to be infrequent. Instead mutations are primarily seen at the C of TC, CT, or CC cyclobutane dimers, and to a lesser extent, at the C of TC and CC (6-4) photoproducts (Brash, 1988). This finding supports the hypothesis that DNA polymerases preferentially insert an A opposite a modified, non-coding nucleotide (The A-rule).

B. Consequences of DNA Damage

Adducts in DNA have been shown to block transcription and replication, which can lead to cell death (Bernstein and Berstein, 1991). Alternatively, the erroneous repair (i.e. SOS response, which is a bacterial error-prone repair mechanism) or replication of DNA that contains a lesion can give rise to mutations. In addition, several of the DNA

adducts described are highly mutagenic miscoding bases which preferentially pair with the incorrect nucleotide causing transitions or transversions. Such a permanent alteration in the DNA sequence may alter the expression of genes, including those involved in controlling cell proliferation and differentiation. In this way, routes in the multi-step process of cancer can be initiated by DNA damage. Clearly, the role of DNA repair in the prevention of carcinogenesis is demonstrated by the several well-known human diseases associated with DNA repair defects, which are associated with an increased risk for developing cancers (Bohr *et al.*, 1989). Besides a predisposition to cancer, individuals afflicted with a DNA-repair disorder display a bewildering array of clinical symptoms, including immunodeficiencies, neurological problems, skeletal abnormalities and altered growth.

The fact that reactive oxygen species have been implicated as the cause of over fifty genetic diseases (Halliwell, 1991) indicates that prevention and repair of DNA oxidation are critical events in protecting cells against free radical attack. In fact, it was recently reported that the paralysis of individuals afflicted with amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, results, in part, from the accumulation of superoxide radicals which causes the death of motor neurons (Rosen *et al.*, 1993). Though this disease is associated with a mutation in superoxide dismutase, a scavenger of superoxide, similar

diseases may exist in which DNA repair of oxidative DNA damage has been compromised. Certainly, the evolutionary retention of DNA repair functions indicates that they fulfill necessary biological roles.

The accumulation of DNA damage is thought to initiate the process of aging (Saul *et al.*, 1987; Ames, 1989). One view of the somatic damage theory of aging is that the amount of maintenance and repair of somatic tissues is always less than that required for indefinite survival. Thus, some DNA damage will amass resulting in the impairment of normal cellular functions which leads to aging, and ultimately, cell death.

C. DNA Damage/Repair and the Role of Chromatin Structure and Transcription

Thus far, DNA damage has been discussed without regard to the higher levels of organization of DNA molecules in the genomes of living cells. In eukaryotes, DNA is associated with histone and non-histone chromosomal proteins to form chromatin (for review see Smerdon, 1991). In addition, chromosomal DNA is further packaged and organized into a structural hierarchy. It has been demonstrated that certain forms of DNA damage are not random in their distribution. For instance, base damage induced by some chemicals occurs at a higher frequency in the linker (unbound DNA) region, while other types of damage such as ultraviolet are uniformly distributed in DNA (Lieberman *et al.*, 1979). Therefore,

chromatin structure has a significant influence on the concentration and distribution of various forms of DNA damage.

Sites of DNA base damage in chromatin are not all equally accessible to DNA repair enzymes (for review see Hanawalt, 1991). In fact, studies have revealed that, for some types of DNA damage such as cyclobutane pyrimidine dimers produced in DNA by UV light, preferential repair occurs at transcriptionally active regions of chromatin. Along these lines, the transcribed strand of a gene is repaired at a higher rate than the non-transcribed strand (Bohr, 1991). Thus, repair may be directly coupled to the transcription apparatus (Bootsma and Hoeijmakers, 1993). Presumably, a stalled RNA polymerase, which has encountered a non-coding base derivative or bulky DNA adduct, would represent a cellular signal for the recruitment of DNA repair proteins. Recently, the transcription-repair coupling factor from both *E. coli* (Mfd) and humans (ERCC6) has been identified (Selby et al., 1991; Troelstra et al., 1992).

Studies in *S. cerevisiae* on repair of UV-induced cyclobutyl pyrimidine dimers indicate that a) nontranscribed regions are repaired at low, basal rates and the actual repair rate is modulated by the stability of nucleosomes, b) nucleosome stability may determine how transcription and repair are coupled, and c) increased repair is seen, though not necessarily in direct correlation, with increased rates of transcription (Bedoyan et al., 1992). Similar results were

seen in human cell extracts where assembly of DNA into nucleosomes is associated with marked suppression of nucleotide excision repair (Wang *et al.*, 1991). This assembly apparently must occur at steps that precede repair synthesis.

D. DNA Repair Mechanisms

Cells have evolved the capacity to remove or tolerate lesions in their DNA (Friedberg, 1985). The most direct mechanisms for repairing DNA are those that simply reverse damage and restore DNA to its normal structure in a single step. A more complex mechanism, excision repair, involves incision of the DNA at the lesion site, removal of the damaged or inappropriate base(s), and resynthesis of DNA using the undamaged complementary strand as a template. This system of repair can be further categorized into base and nucleotide excision repair as will be discussed.

1. Direct Reversal of Damage

The most direct mechanism for repairing DNA involves simple reversal of the damage (Mitra and Kaina, 1993; Sancar, 1990). The most widely understood enzymes that carry out this form of repair are O⁶-methylguanine-DNA methyltransferase (MGMT) and photolyase. This process of direct repair of premutagenic lesions stands in marked contrast to alternative strategies for DNA repair that are observed more frequently, such as excision repair.

a. Methyltransferases

The repair protein MGMT irreversibly transfers the alkyl group from the O6 position of guanine to a cysteine residue contained within its active site, restoring the DNA to its natural state in a single step (Olsson and Lindahl, 1980; Demple *et al.*, 1982). This process is a stoichiometric reaction and leads to the inactivation of the protein molecule, also referred to as a "suicide enzyme." MGMT activity has been detected in all organisms tested so far, and although the overall amino acid sequences of the various MGMT molecules are very different, the sequence surrounding the alkyl acceptor cysteine is highly conserved (PCHRV; Mitra and Kaina, 1993).

Methyltransferase activity has been characterized most extensively in *E. coli* (for review see Lindahl *et al.*, 1988). *E. coli* contains two genes encoding MGMT proteins. The *ada* gene, which encodes a 39 kDa protein, has been cloned and the protein purified to physical homogeneity (Demple *et al.*, 1985). This protein has two receptor domains, one which acts on the premutagenic lesions O6-alkylguanine and O4-alkylthymine and the other which acts on alkylphosphotriesters. *Ada* is expressed constitutively at very low levels but is inducible by brief treatment of cells with alkylating agents, a phenomenon known as the adaptive response (Samson and Cairns, 1977). This process is regulated by the *Ada* protein itself which is converted to a

transcriptional activator after transferring a methyl group to the acceptor site within the protein (Nakabeppu and Sekiguchi, 1986). In addition to the Ada protein, a smaller MGMT (19 kDa), encoded by the *ogt* gene (Potter *et al.*, 1987), is present. This protein is constitutively expressed and is non-inducible.

The genes encoding MGMTs have also been cloned from yeast (Xiao *et al.*, 1991), rat (Potter, 1991), mouse (Santibanez-Koref *et al.*, 1992) and human (Rydberg *et al.*, 1990; Tano *et al.*, 1990; Hayakawa *et al.*, 1990). Unlike the dramatic induction seen with the *E. coli ada* gene, expression of mammalian MGMT genes is only slightly enhanced by exposure to DNA-damaging agents and only in specific cells or tissues (for review see Pegg, 1990a; Wilson *et al.*, 1993). Moreover, there does not appear to be an adaptive response in higher eukaryotes (Lindahl *et al.*, 1988). *E. coli* and mammalian MGMTs (for review see Mitra and Kaina, 1993) have also been shown to display different rates of reaction with different alkyl residues. For instance, MGMT repairs O6-methylguanine in DNA at a much slower rate than Ada. Furthermore, mammalian MGMT reacts with ethyl and butyl derivatives of guanine at a significant rate, whereas the *E. coli* Ada protein reacts poorly with O6-ethylguanine and is nearly inactive with butyl derivatives (Morimoto *et al.*, 1985; Pegg and Dolan, 1987; Pegg, 1990b). *Ogt*, however, is more efficient at removing longer alkyl chains and may be responsible for their repair *in*

vivo (Wilkinson *et al.*, 1989). As an alternative, the removal of large alkyl DNA adducts may occur by the action of the nucleotide excision repair pathway in *E. coli* (Samson *et al.*, 1988). Lastly, while O4-alkylthymines are substrates, although poor ones, for bacterial (Ada and Ogt) and yeast MGMTs, they are acted on by human MGMT at extremely slow rates, if at all (Sassanfar *et al.*, 1991). Recent studies have found that the 28 amino acid carboxy tail present only in mammalian MGMTs plays a role in determining MGMT substrate specificity (Morgan *et al.*, in press).

To date, the gene encoding the *Drosophila* MGMT has not been isolated. However, methyltransferase activity has been identified in crude extracts of *Drosophila* from all developmental stages except for embryos (Green and Deutsch, 1983; Guzder *et al.*, 1991). Some investigators believe that *Drosophila* do not possess methylpurine-DNA glycosylase activity, and therefore, it has been proposed that *Drosophila* MGMT protein molecules may act on a broader spectrum of alkylation DNA damage to compensate for the lack of DNA glycosylase repair enzymes (Guzder *et al.*, 1991).

b. Photolyases

Another enzyme which carries out direct reversal of DNA damage is photolyase (for review see Sancar, 1990). This enzyme binds to the site of a pyrimidine dimer, a potentially lethal lesion, and utilizing energy provided by visible light,

reduces the cyclobutane ring to restore DNA to its native state. Binding of photolyase to the pyrimidine dimer is light independent, whereas, photoreactivation requires the absorption of a photon from near ultraviolet or visible light (300-500 nm). Moreover, these enzymes, because they are expressed at low constitutive levels, are extremely efficient at recognizing pyrimidine dimers in the midst of a large excess of nondimerized pyrimidines. While photolyase activity has been detected in over 50 organisms including bacteria, plants, yeast and marsupial mammals, it has never been found conclusively in placental mammals (Ley, 1993). The most recent study regarding this issue was unable to detect the presence of a DNA photoreactivating enzyme in cell-free extracts of HeLa cells or human white blood cells, suggesting that humans most likely do not have DNA photolyase (Li et al., 1993).

The genes encoding photolyases have been cloned from *E. coli*, cyanobacterium *Anacystis nidulans*, and yeast (*PHR1*) (Sancar et al., 1984c; Sancar, 1985; Yasui and Langeveld, 1985; Yasui et al., 1988). Alignment of these various photolyases reveals the evolutionarily conserved nature of this protein. Moreover, there exists weak homology to a helix-turn-helix motif within the carboxy-terminal region of the protein (Kobayashi et al., 1989), which may represent the DNA-binding domain. Recent spectroscopic studies indicate that each photolyase contains two chromophores (Jorns et al.,

1987; Payne *et al.*, 1987; Sancar and Sancar, 1988). In the photolyases of yeast and *E. coli* the chromophores are 1,5-dihydroflavin and 5,10-methenyltetrahydrofolate, which appear to be required for photolysis by this folate class of enzymes. Recently, the first report of photoreactivation of pyrimidine (6-4) pyrimidone photoproducts was published (Todo *et al.*, 1993). This activity was found in extracts from *Drosophila*.

Binding of photolyase to pyrimidine dimers *in vitro* enhances both the recognition of dimers by the UvrABC excision nuclease and the rate at which turnover occurs (Sancar *et al.*, 1984a). Furthermore, expression of photolyase in an excision proficient strain of either yeast or *Drosophila* increases UV survival in the absence of photoreactivating light (Sancar and Smith, 1989; Boyd and Harris, 1987). Overall, these results indicate that photolyase increases the efficiency of nucleotide excision repair in the dark.

2. DNA Damage Recognition

Recognition of DNA lesions by cellular components is the first step in the process of DNA repair. In the bacterium *E. coli*, several damage recognition proteins have been identified and, in some cases, extensively characterized (Van Houten, 1990). For example, the UvrABC excision repair system is capable of recognizing and repairing a large variety of bulky DNA adducts. In addition, both constitutive and inducible DNA damage binding activities have been identified in mammalian

nuclear extracts, as well as yeast extracts. These eukaryotic damage-specific DNA binding proteins (DDBP) display a high affinity for UV-irradiated double-stranded DNA (Feldberg and Grossman, 1976; Chu and Chang, 1988a; Chu *et al.*, 1990; Patterson and Chu, 1989; Hirschfeld *et al.*, 1990; Abramic *et al.*, 1991; Robins *et al.*, 1991; Hwang and Chu, 1993) and/or for cisplatin-modified DNA (Chu and Chang, 1988b, 1990; Chao *et al.*, 1991; Hughes *et al.*, 1992). An additional protein has been partially purified which demonstrates high affinity for *N*-acetoxy-2-acetylaminofluorene-modified double-stranded DNA (Moranelli and Lieberman, 1980). Comparisons of these various protein factors indicate that they appear to represent distinct activities. However, further characterization of these DDBPs is required to determine their relationship. Moreover, whether or not these binding proteins are involved in other aspects of DNA repair, such as nucleotide excision repair, needs to be addressed. For instance, these proteins may be responsible for initiating the excision repair pathway by binding to the site of DNA damage and recruiting the repair machinery.

cDNAs encoding a human protein (SSRP1) that binds selectively to DNA modified by cisplatin recently have been isolated (Toney *et al.*, 1989; Bruhn *et al.*, 1992). Moreover, two distinct human cDNAs were identified that encode proteins which bind AP DNA preferentially over undamaged, methylated, or UV-irradiated DNA (Lenz *et al.*, 1990). These findings

represent steps towards ultimately elucidating the roles of these proteins in various aspects of DNA repair processes.

The human counterpart of the *E. coli* UvrABC mutants may be the autosomal recessive disease XP. XP is a genetic disease which renders the afflicted individual sensitive to sunlight and is characterized by defective repair of DNA damaged by ultraviolet radiation or agents that produce bulky DNA adducts (Cleaver, 1990). Human cells contain a factor (XPE-BF), which binds to DNA damaged by UV, cisplatin, or denaturation, that appears to be absent in a subset of patients from XP complementation group E, one of eight complementation groups (Chu and Chung, 1988a; Chu *et al.*, 1990; Keeney *et al.*, 1992; Hwang and Chu, 1993). Demonstration that the primary defect in XP group E involves XPE-BF awaits cloning of the gene.

3. Excision Repair

Excision repair is a complex biochemical process which requires a battery of proteins (Figure 3; Sancar and Sancar, 1988). This process was first thought to involve recognition of a damaged nucleotide by an excision endonuclease, cleavage of the phosphodiester bond in the vicinity of the DNA damage, removal of the damaged region, and the resynthesis of the resulting gap by DNA polymerase and ligase (Van Houten, 1990). However, the discovery of DNA glycosylases identified an alternative mode for initiating the excision repair response,

referred to as base excision repair (Figure 3; Sakumi and Sekiguchi, 1990). In this situation, modified or nonconventional bases are released by a specific DNA glycosylase which cleaves the bond between the base and its deoxyribose. The DNA is subsequently incised at the resulting apurinic/apyrimidinic (AP) site by an AP endonuclease and the damaged base removed and replaced. Thus, base excision repair is a general mechanism in which ordinarily a single damaged or inappropriate base is excised from the genome and replaced with the correct nucleotide. In nucleotide excision repair, the other pathway (Figure 3), DNA damage such as bulky chemical adducts and pyrimidine dimers are incised directly by the operation of a complex endonuclease. This pathway is initiated by the binding of specific DNA-damage recognition proteins to the damaged-induced distortion. This complex serves as a binding site for an endonuclease, which incises the DNA on both sides of the altered nucleotide(s). The damaged area, which, unlike base excision repair, consists of several nucleotides, is removed and the resulting gap filled in by the action of DNA polymerase and DNA ligase.

a. Base Excision Repair

Base excision repair involves two major classes of repair enzymes, namely, N-glycosylases and AP endonucleases (Wallace, 1988; Sakumi and Sekiguchi, 1990; Doetsch and Cunningham, 1990). DNA N-glycosylases are enzymes that hydrolyze the N-

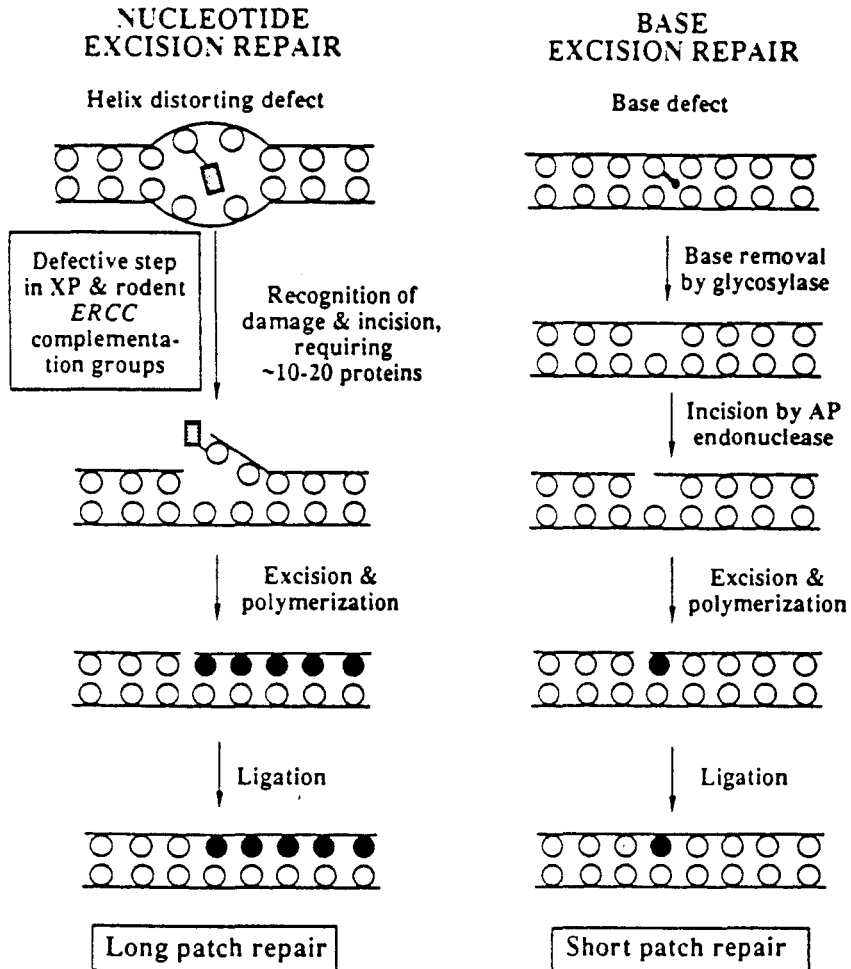


Figure 3: Schematic of excision repair processes illustrating the steps of nucleotide excision repair and base excision repair [Reprinted with permission from Wiley-Liss, a division of John Wiley and Sons, Inc. (Thompson, 1989)].

glycosidic bond between the damaged base and the deoxyribose moiety, leaving behind an AP site on the DNA backbone. AP sites produced by the action of N-glycosylases are acted upon by AP endonucleases, which can make an incision either 3' to the AP site (class I AP lyase) or 5' to the AP site (class II AP endonuclease). All those enzymes shown to contain class I AP lyase activity possess an associated DNA glycosylase activity; however, not all glycosylases are AP lyases. Class II AP endonucleases are the major enzymes responsible for the repair of AP sites in DNA.

i. DNA Glycosylases

DNA glycosylases can be defined as enzymes which recognize specific DNA base modifications and catalyze the hydrolysis of the N-glycosylic bond that links a base to the deoxyribose-phosphate backbone of DNA (Figure 4; for review see Sancar and Sancar, 1988; Wallace, 1988; Sakumi and Sekiguchi, 1990). This enzymatic activity results in the generation of an AP site. To date, several DNA glycosylases have been identified and are classified into two major families: 1) enzymes that possess only DNA glycosylase activity and 2) enzymes that contain both a DNA glycosylase activity and an associated class I AP lyase activity; that is, enzymes that catalyze a beta-elimination cleavage of the phosphodiester bond 3' to an AP site.

Enzymes which carry out only N-glycosylase activity are

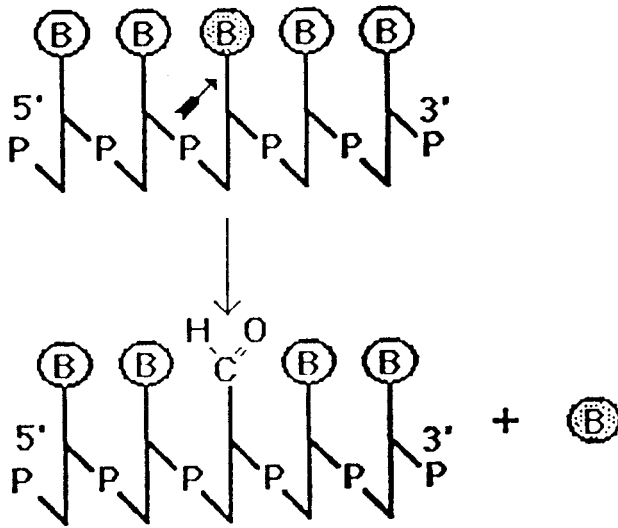


Figure 4: Processing of base damage by a DNA-glycosylase. A modified base (shaded "B") is removed from the DNA strand by an N-glycosylase activity to produce an apurinic/aprimidinic (AP) site (modified from Haukenes *et al.*, 1990).

uracil-DNA glycosylase, 3-methyladenine-DNA glycosylase, hypoxanthine-DNA glycosylase, and hydroxymethyluracil-DNA glycosylase. Each of these proteins received its name based on the type of DNA adduct it recognizes and releases. Enzymes that contain both N-glycosylase activity and AP lyase activity are T4 endonuclease V, endonuclease III, and formamidopyrimidine-DNA glycosylase. The latter of these proteins will be discussed later.

Uracil-DNA Glycosylase

Uracil in DNA may result from deamination of cytosine, which may give rise to transition mutations, or from misincorporation of dUMP residues during DNA synthesis (Lindahl, 1993). Repair of uracil via DNA glycosylase activity was first detected in *E. coli* (Lindahl, 1974). Uracil-DNA glycosylase activity has since been demonstrated in various other organisms, including yeast and mammals (Crosby et al., 1981; Sekiguchi et al., 1976; Wist et al., 1978; Krokan and Wittwer, 1981). It was subsequently discovered that mammalian cells possess both a nuclear and mitochondrial form of uracil-DNA glycosylase, which will be discussed in detail later (Anderson and Friedberg, 1980; Wittwer and Krokan, 1985; Domena and Mosbaugh, 1985; Domena et al., 1988).

The genes encoding the *E. coli* (*ung*) and yeast (*UNG1*) proteins have been cloned (Duncan and Chambers, 1984; Varshney et al., 1988; Percival et al., 1989). As expected, *E. coli*

ung mutants exhibit increased spontaneous mutations of the GC to AT transition type (Duncan and Miller, 1980; Duncan and Weiss, 1982). In addition, the genes for the uracil-DNA glycosylase enzymes of herpes simplex virus types 1 and 2, as well as for varicella-zoster, Shope fibroma, and Epstein-Barr virus, have been isolated (Baer et al., 1984; Davidson and Scott, 1986; Caradonna et al., 1987, McGoech et al., 1988; Worrad and Carradonna, 1988; Upton et al., 1993). A comparison of the predicted amino acid sequences of the proteins from *E. coli*, yeast and these viruses reveals that uracil-DNA glycosylase is a highly conserved protein. These enzymes are relatively small (20-45 kDa) and show no requirement for a cofactor. Recently, experiments have demonstrated that cell extracts of *E. coli* and of human cell lines carry out repair of uracil residues in DNA mainly by replacing a single nucleotide (Dianov et al., 1992). This process occurs as follows: 1) release of the uracil residue by uracil-DNA glycosylase, 2) incision of the resulting AP site by a class II AP endonuclease, 3) excision, not by an exonuclease, but by a DNA deoxyribophosphodiesterase, 4) gap-filling by DNA polymerase beta (Sweasy and Loeb, 1992), and 5) sealing of the nick by DNA ligase. DNA deoxyribophosphodiesterase acts during the excision repair pathway to remove the sugar-phosphate residue from an endonucleolytically incised AP site, prior to gap filling and ligation (Franklin and Lindahl, 1988).

Interestingly, three distinct human uracil-DNA glycosylases have been identified (Olsen *et al.*, 1989; Wittwer *et al.*, 1989; Vollberg *et al.*, 1989; Muller and Caradonna, 1991). One of the human uracil-DNA glycosylases (27 kDa) displays homology to the enzymes from yeast, animal viruses, and prokaryotes (Olsen *et al.*, 1989). Transcript levels of this human gene were found to be increased 8 to 12 fold during the G1 phase and total protein activity elevated 2 to 3 fold during the S phase (Slupphaug *et al.*, 1991), suggesting a cell-cycle regulation of this gene. In addition, this protein demonstrates a faster repair rate at sites of U:A base pairs as compared to U:G base pairs, which may indicate that sequence specific repair may be a determinant to be considered in mutagenesis (Eftedal *et al.*, 1993). The second human uracil-DNA glycosylase (36 kDa), which displays no homology to the other glycosylases, shows significant similarity to the cyclin protein family. This protein is also a G1 phase-regulated protein; that is, its protein levels are elevated during the G1 phase (Muller and Caradonna, 1993). This cell-cycle regulation of repair is believed to serve as a defense mechanism to reduce cytotoxicity, mutagenesis, and carcinogenesis by prescreening DNA prior to replication to remove miscoding lesions and thus to ensure the fidelity of DNA replication. Both groups of investigators believe that their protein (the 36 kDa and 27 kDa) is the major nuclear version and have presented compelling evidence to support

their claims. However, it is generally accepted that the 36 kDa species is the nuclear protein and the 27 kDa species is the mitochondrial protein (Seal *et al.*, 1987; Domena *et al.*, 1988). Interestingly, a third human uracil-DNA glycosylase was identified as the 37 kDa subunit of glyceraldehyde-3-phosphate dehydrogenase (Meyer-Siegler *et al.*, 1991).

Uracil-DNA glycosylase activity was recently found to be differentially regulated in various tissues during development, where specific activity was at its highest around the time of birth (Weng and Sirover, 1993). In addition, a uracil-DNA glycosylase enzyme deficiency has been connected to the human genetic disease, Bloom's syndrome (Yamamoto and Fujiwara, 1986; Vollberg *et al.*, 1987; Seal *et al.*, 1991).

Methylpurine-DNA Glycosylase

Lesions, such as O6-methylguanine and O4-methylthymine are repaired by DNA methyltransferases and in some situations by nucleotide excision repair (Samson *et al.*, 1988). Conversely, cytotoxic lesions such as N3-methylpurines and O2-methylpyrimidines which block DNA replication, are corrected by two distinct DNA glycosylases in *E. coli*, encoded by the *alkA* and *tag* genes (Thomas *et al.*, 1982). Both AlkA (TagII) and Tag (TagI) release N3-methyladenine and N3-methylguanine, but Alk A also removes O2-methylthymine and O2-methylcytosine (Yamamoto *et al.*, 1983; Evensen and Seeberg, 1982; Karran *et al.*, 1982; Bjelland *et al.*, 1993). Little homology exists

between the products of *tag* and *alkA* genes, reflecting their differences in substrate specificity as well as reaction mechanisms. Tag is a 22 kDa protein and is constitutively expressed. AlkA expression, on the other hand, is enhanced upon exposure of cells to low concentrations of alkylating agents, and for this induction presence of the intact *ada* gene is essential (Nakabeppu *et al.*, 1984b). Thus, *alkA* apparently belongs to the *ada* regulon and is part of the adaptive response. AlkA has a molecular weight of 32 kDa (Nakabeppu *et al.*, 1984a). Mutants in either gene or in both genes display higher sensitivity to alkylating agents than do their wild-type counterparts (Nakabeppu *et al.*, 1984a; Clarke *et al.*, 1984).

The gene encoding the *S. cerevisiae* homolog (MAG) to AlkA glycosylase has been isolated (Chen *et al.*, 1989; Chen *et al.*, 1990; Berdal *et al.*, 1990). Similar to the *alkA* gene, the *MAG* gene is induced upon exposure to relatively non-lethal levels of alkylating agents and specifically protects yeast cells against alkylation induced cell death. In addition, the cDNAs for mouse (AAG), rat, and human (ANG, MPG or MAG) have also been cloned (Engelward *et al.*, 1993; O'Connor and Laval, 1990, 1991; Chakravarti *et al.*, 1991; Samson *et al.*, 1991). The mammalian proteins, which appear to release N3-methyladenine, N7-methylguanine, and N3-methylguanine, are all around 30-35 kDa and share extensive amino acid identity. Thus far, although there is no evidence that the mammalian

genes are inducible, their expression is regulated in a tissue-specific and age-dependent fashion (Washington *et al.*, 1988, 1989).

Other DNA Glycosylases

Two other enzymes which possess only N-glycosylase activity are hypoxanthine-DNA glycosylase and hydroxymethyluracil-DNA glycosylase. Proteins that remove hypoxanthine, a premutagenic product resulting from deamination of adenine, by glycosylic cleavage have been purified from *E. coli*, calf thymus and HeLa cells (Harosh and Sperling, 1988; Dianov and Lindahl; Myrnes *et al.*, 1982). *E. coli* has since been found to have 2 distinct enzymes responsible for the repair of hypoxanthine in DNA. These enzymes are 55 kDa and 30 kDa in size, and the former requires Mg^{2+} for its activity. The mammalian proteins have a molecular weight in the 30-40 kDa range and apparently have no cofactor requirement (Karran and Lindahl, 1980).

Hydroxymethyluracil is produced in DNA by ionizing radiation or by oxidative damage to thymine residues (Hutchinson, 1985; Halliwell and Aruoma, 1991). Activity, which is insensitive to the presence of EDTA and distinct from uracil-DNA glycosylase activity, specific for hydroxymethyluracil has been identified in calf thymus and mouse cells (Cannon-Carlson *et al.*, 1989; Hollstein *et al.*, 1984). Interestingly, this activity has not been detected in

bacteria or yeast (Boorstein et al., 1987). To date, neither of the genes encoding hypoxanthine- or hydroxymethyluracil-DNA glycosylase have been cloned.

There has also been a report of a protein, purified from human tissues and cells, that binds double-stranded oligonucleotides containing a single 1,N6-ethenoadenine, an adduct produced by a variety of environmental chemicals (Rydberg et al., 1991). This enzyme demonstrates glycosylase activity both to this DNA lesion and to 3-methyladenine (Singer et al., 1992). Surprisingly, 3-methyladenine-DNA glycosylase behaves in an identical manner on the same adducts. Whether these repair enzymes are actually distinct activities requires cloning of the 1,N6-ethenoadenine-DNA glycosylase gene.

ii. Glycosylases/AP Lyases

In prokaryotes, *Micrococcus luteus* and T4 phage-infected *E. coli*, enzymes have been characterized that remove cyclobutane pyrimidine dimers (Gordon and Haseltine, 1980). These enzymes contain a specific N-glycosylase activity (Figure 4) as well as an AP lyase activity (Figure 5) that, in combination, catalyze a two-step DNA incision process at sites of dimers which initiates the base excision repair pathway. The T4 phage gene (*denV*) has been cloned and sequenced (Radany et al., 1984; Valerie et al., 1985); however, the gene encoding the *M. luteus* protein has not been identified.

Endonuclease V, the *denV* gene product, has a molecular weight of 16 kDa, and like the *M. luteus* enzyme (Grafstrom et al., 1982), has no cofactor requirement. Both enzymes are extremely specific for pyrimidine dimers, but neither shows homology to photolyase. The two reactions performed by the enzyme, hydrolysis of the glycosylic bond and cleavage of the phosphodiester bond, are frequently uncoupled (Liuzzi et al., 1987). In addition, it was found that a critical factor for T4 endonuclease V activity is the relative distance between the active site alpha-NH₂ terminus and those domains responsible for DNA binding and pyrimidine dimer recognition (Schrock and Lloyd, 1993). The X-ray structure of this protein has been solved and the structure-function relationship is close to being elucidated (Morikawa et al., 1992).

Recently, the first eukaryotic enzyme of this type was identified from *S. cerevisiae* (Hamilton et al., 1992). This yeast protein appears to function in a similar fashion when compared to its prokaryotic counterparts, endonuclease V and the *M. luteus* enzyme. However, definitive characterization of the enzyme awaits further investigation.

One of the primary N-glycosylases for the repair of oxidative base damages in *E. coli* is endonuclease III (Cunningham and Weiss, 1985). Endonuclease III, which is encoded for by the *nth* gene, recognizes a wide spectrum of radiolysis products including thymine glycol, dihydrothymine,

urea, cytosine hydrates, and other base modifications (Breimer and Lindahl, 1984; Asahara *et al.*, 1989). In addition to its N-glycosylase activity, this enzyme possesses an associated class I AP lyase activity (Bailly and Verly, 1987). Studies have shown that the N-glycosylase activity precedes the AP lyase activity and that these activities appear to be able to act independently. The 25 kDa endonuclease III enzyme has no cofactor requirement but is inhibited by the presence of Mg²⁺ (Kow and Wallace, 1987). Unlike any other known DNA repair enzyme, endonuclease III has an iron-sulfur cluster embedded in the protein (Cunningham *et al.*, 1989). Endonuclease III has been recently crystallized and its three-dimensional structure elucidated (Kuo *et al.*, 1992).

Eukaryotes possess a number of enzymes that recognize and remove a wide variety of base modifications produced by ionizing radiation and oxidizing agents (Wallace, 1988). One of these enzymes has been designated redoxyendonuclease to emphasize the common action mechanism to that of endonuclease III. In fact, these eukaryotic proteins appear to be the functionally conserved analogs to *E. coli* endonuclease III. To date, redoxyendonuclease activity has been detected in yeast, bovine, and human cells (Doetsch *et al.*, 1987; Gossett *et al.*, 1988).

The debate as to whether or not *Drosophila* contain DNA glycosylase activity continues. Recently, researchers were able to detect uracil-DNA glycosylase activity in *Drosophila*

eggs as well as third instar larvae (Morgan and Chlebek, 1989). However, several studies have been unable to identify repair of uracil-containing DNA in extracts from *Drosophila* (Friedberg et al., 1978; Deutsch and Spiering, 1982; Breimer, 1986). Instead, *Drosophila* were found to possess a nuclease activity (not a glycosylase activity) specific for uracil-DNA, but only in third instar larvae. Thus, it has been proposed that uracil-containing DNA acts as a target for the nucleolytic breakdown of DNA during histolysis in insects, raising into question the need for uracil-DNA glycosylase activity (Deutsch, 1987). In fact, a recent report suggests that insect populations which undergo pupation do not contain detectable levels of this repair activity. This would permit cellular destruction during development by utilizing uracil-containing DNA as a target (Dudley et al., 1992).

Tests, thus far, to detect DNA glycosylase activity for the repair of N7-methylguanine and N3-methyladenine have been unsuccessful in *Drosophila*, although removal of these bases was observed (Green and Deutsch, 1983). The inability to detect N-methylpurine glycosylase activity suggests that *Drosophila* may not rely on base excision repair for the removal of modified bases in DNA. Thus, it has been proposed that the DNA methyltransferase of *Drosophila* may function to repair a broader spectrum of DNA damage (Guzder et al, 1991). There exists, however, a report of DNA glycosylase activity which excises fragmented thymine residues from DNA,

contradicting the idea that *Drosophila* do not contain DNA-glycosylase activity (Breimer, 1986). Certainly, more investigation is required.

iii. AP Endonucleases

The major cellular enzymes initiating the repair process for AP sites, the so-called "class II" AP endonucleases, have been identified and characterized in bacteria, yeast and mammalian systems, including humans cells (for review see Doetsch and Cunningham, 1990). These repair proteins hydrolyze the phosphodiester backbone immediately 5' to an AP site generating a normal 3'-hydroxyl nucleotide which can prime DNA repair synthesis (Figure 5). Moreover, these enzymes have also been shown to contain repair activity for 3'-terminal oxidative lesions (Henner *et al.*, 1983; Demple *et al.*, 1986; Johnson and Demple, 1988a and b; Ramotar *et al.*, 1991b). By hydrolyzing 3'-blocking fragments from oxidized DNA, these enzymes can produce normal 3'-hydroxyl nucleotide termini, permitting DNA repair synthesis. To date, only the AP endonuclease enzymes of microbial systems have been well characterized both biochemically and molecularly. However, large steps have been made recently in cloning eukaryotic AP endonucleases and identifying their cellular function(s).

In *E. coli*, the major AP endonuclease enzymes are exonuclease III and endonuclease IV. Exonuclease III comprises approximately 90% of the cellular AP endonuclease

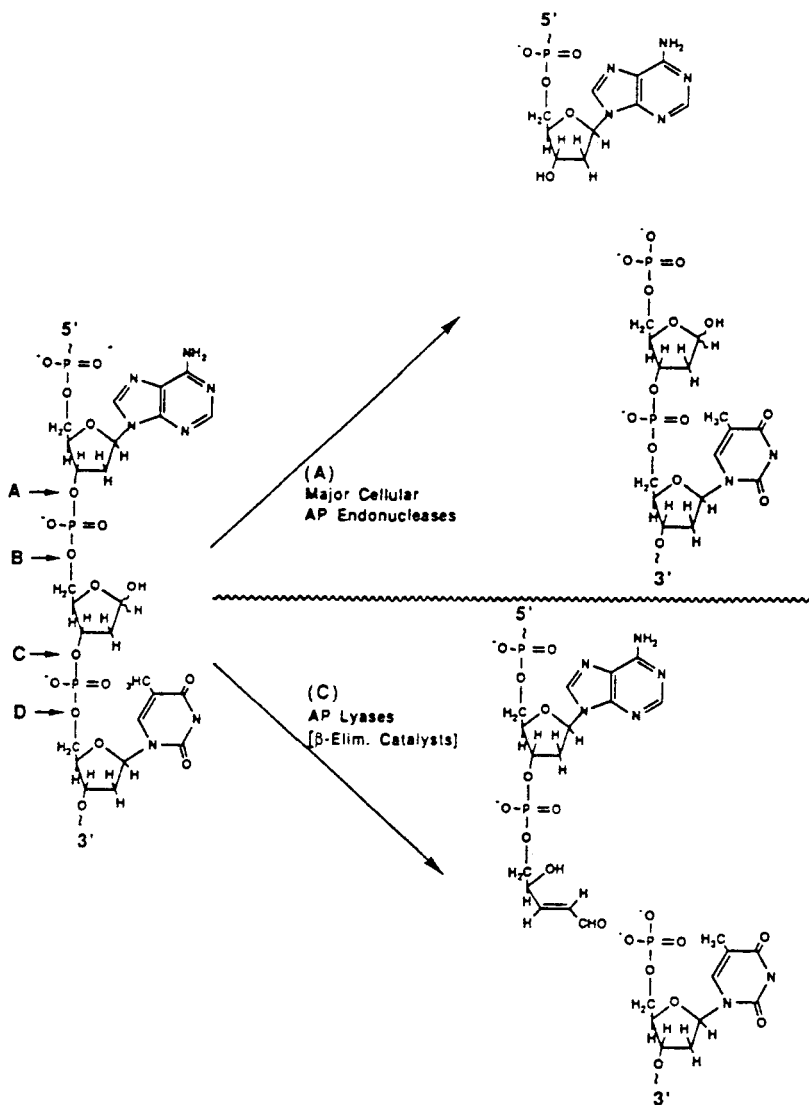


Figure 5: Potential sites of phosphodiester bond cleavage adjacent to AP sites. 4 possible sites of cleavage exist, but only 2 are used by the vast majority of enzymes. AP endonucleases (class I) cleave hydrolytically at site A to yield a 5'-terminal residue of deoxyribose 5-phosphate and a 3'-terminal residue of deoxyadenosine. AP lyases (class II) cleave by a β -elimination mechanism at site C to yield a 3'-terminus which is an α,β -unsaturated aldehyde (the 3'-ester of deoxyadenosine 3'-phosphate with the 5'-hydroxyl group of (4R)-4-hydroxy-trans-2-pental) and a 5'-terminal residue of deoxythymidine 5'-phosphate. The bases flanking the AP site were selected arbitrarily. The base ring hydrogens have been omitted for simplicity [Reprinted with permission from Elsevier Science Publishers B V (Doetsch and Cunningham, 1990)].

activity (Rogers and Weiss, 1980) and greater than 95% of the total activity for removal of blocked 3' ends (Demple *et al.*, 1986), while endonuclease IV accounts for much of the residual activity (Ljungquist *et al.*, 1976).

Exonuclease III, encoded by the *xth* gene (Saporito *et al.*, 1988), was also identified as endonuclease II and endonuclease VI because of its multiple enzymatic activities (Friedberg and Goldthwait, 1969; Kirtikar *et al.*, 1975a, 1975b; Gossard and Verly, 1978). In fact, this protein was originally purified as a byproduct of DNA polymerase I (Richardson and Kornberg, 1964). As mentioned, exonuclease III is the major class II AP endonuclease in *E. coli* and incises on the 5' side of an AP site, leaving a 3' hydroxyl and a 5' phosphate (Kow, 1989). This 3' hydroxyl group is a substrate for DNA polymerase I (Warner *et al.*, 1980) and does not require further processing. In addition to its AP endonuclease activity, exonuclease III demonstrates phosphodiesterase, exonuclease, phosphatase and RNase H activities (Weiss, 1981). Recently, exonuclease III was shown to have 5' endonuclease activity against urea residues (Kow and Wallace, 1985). Exonuclease III is a 28 kDa protein and has an absolute requirement for magnesium. *E. coli xth* mutants are slightly sensitive to killing by MMS, which produces AP sites, and near UV, and are extremely sensitive to hydrogen peroxide (Cunningham *et al.*, 1986). Conversely, this mutant strain displays no sensitivity to gamma rays or

bleomycin.

Endonuclease IV, encoded by the *nfo* gene, is the other main class II AP endonuclease of *E. coli* (Saporito and Cunningham, 1988). Like exonuclease III, endonuclease IV exhibits many activities such as phosphatase, phosphodiesterase, as well as endonuclease activity against DNA containing urea residues (Kow and Wallace, 1987). Atomic absorption analysis reveals that this protein contains several zinc atoms as well as manganese, which appear to be involved in the mechanism of action (Levin *et al.*, 1988, 1991). *Nfo* mutants have increased sensitivity to the alkylating agents MMS and mitomycin C, as well as the oxidant tert-butyl hydroperoxide (Cunningham *et al.*, 1986). Moreover, these mutants are hypersensitive to bleomycin exposure, but are not sensitive to gamma rays or hydrogen peroxide which implies that endonuclease III and endonuclease IV have different biological roles. Two other minor apurinic activities have also been detected in *E. coli*, these being endonuclease V (Gates and Linn, 1977; Demple and Linn, 1982b) and endonuclease VII (Bonura *et al.*, 1982).

S. cerevisiae contains a single major AP endonuclease/3'-repair diesterase encoded by the *APN1* gene (Popoff *et al.*, 1990). *Apn1* protein has many biochemical properties in common with endonuclease IV (Johnson and Demple, 1988a, b) and comparison of the predicted amino acid sequences of these two enzymes indicated that they are remarkably homologous. *Apn1*

accounts for greater than 97% of yeast AP endonuclease and 3'-repair diesterase activities. Mutants in *apn1* are hypersensitive to oxidative DNA damage and alkylating agents, reaffirming the importance of this protein in carrying out DNA repair (Ramotar *et al.*, 1991b). Interestingly, when compared to wild-type strains, *Apn1*-deficient yeast strains were also found to display higher spontaneous mutation rates when grown in either aerobic or anaerobic conditions. This result suggests that there exist several endogenous mutagens, not just reactive oxygen species, capable of generating DNA damage that requires the repair functions of *Apn1*. In addition, the 41 kDa *Apn1* protein can function in bacteria lacking exonuclease III and endonuclease IV in a manner similar to its proposed functions in yeast (Ramotar *et al.*, 1991a). Earlier studies identified at least five chromatographically distinct apurinic activities in yeast, however, the cloning of these genes and the determination of their relationship to *Apn1* awaits further investigation (Armel and Wallace, 1978, 1984; Chang *et al.*, 1987).

Overall, *E. coli* lacking exonuclease III or yeast deficient for *Apn1* demonstrate hypersensitivity to both oxidative and alkylating agents supporting the idea that these enzymes participate in two distinct pathways of DNA repair: removal of 3'-blocking fragments that result from oxidative DNA damage and restoration of alkylation-induced AP sites (Johnson *et al.*, 1988a, b; Popoff, *et al.*, 1990; Ramotar *et*

al., 1991b). These findings clearly indicate that these enzymes play a critical role in protecting cells against agents which cause alterations in DNA composition.

Two AP endonucleases have been partially purified from *Drosophila* embryos and are resolved by phosphocellulose chromatography (Spiering and Deutsch, 1981). The estimated molecular weights of these two proteins are 66 kDa and 63 kDa, much larger than those enzymes characterized from other organisms. Whether these proteins are encoded by different genes or the same gene and modified post-translationally awaits further investigation. Interestingly, antibody generated to the major HeLa AP endonuclease (Ape) cross-reacts with both these *Drosophila* proteins (Kane and Linn, 1981), suggesting they are at least antigenically similar. In addition, studies have shown that the 66 kDa protein resides in the nucleus, whereas the 63 kDa protein translocates to the mitochondria (James P. Carney, personal communication). Initial assays of AP activity using partially purified fractions of the 63 kDa and 66 kDa proteins indicate that these enzymes incise AP DNA via class I AP lyase and class III AP endonuclease mechanisms respectively (Spiering and Deutsch, 1986). Class III AP endonucleases cleave on the 3'-side of an AP site generating a deoxyribose 3'-phosphate and 5'-OH termini. This finding is the first report of a class III type enzyme and needs to be confirmed by additional studies.

Another *Drosophila* enzyme able to initiate the repair of

abasic sites in DNA has been cloned and is termed Rrp1 (Recombination Repair Protein 1, Sander *et al.*, 1991b). This enzyme was originally purified based on its strand transferase activity, indicating a possible involvement in recombination (Lowenhaupt *et al.*, 1989). The carboxy terminus of this protein displays significant homology to exonuclease III (Sander *et al.*, 1991b). Rrp1 has Mg^{2+} dependent AP endonuclease, 3' exonuclease, and strand transfer activities, in addition to a single stranded DNA renaturation activity (Sander *et al.*, 1991a, b). Recent results indicate that the carboxy terminus, and therefore, presumably the activity involving exonucleolytic processing of double-stranded DNA, is necessary for the initiation of the strand transfer reaction (Sander *et al.*, 1993). Recent reports of a multifunctional ribosomal/class II AP endonuclease (AP3) will be discussed later.

AP endonucleases have been purified to apparent homogeneity from a variety of mammalian sources including mouse, calf thymus, human placenta, and HeLa cells (Seki *et al.*, 1991a; Haukanes *et al.*, 1989; Ivanov *et al.*, 1988; Henner *et al.*, 1987; Cesar and Verly, 1983; Shaper *et al.*, 1982; Kane and Linn, 1981). The activities have similar molecular weights around 37 kDa and require magnesium. Each of these enzymes appears to be a class II AP endonuclease.

Using a synthetic DNA substrate that contains 3'-PGA esters (3'-O-PO₃-CH₂-CHO) as the predominant damages, two

major diesterases were identified in HeLa cells (Chen et al., 1991). Interestingly, these activities were found to correspond with the AP endonuclease activities already reported. In fact, one of the enzymes was found to be identical to a previously studied HeLa AP endonuclease (Kane and Linn, 1981) and was cloned (*APE*, Demple et al., 1991; *HAP1*, Robson and Hickson, 1991; *HAP1h*, Cheng et al., 1992b). *Ape* shows significant homology to the AP endonucleases of *E. coli* (exonuclease III), *Drosophila* (*Rrp1*), mouse, and bovine (Seki et al., 1991b; Robson et al., 1991). The *APE* cDNA maps to position 11.2-.4 of chromosome 14 in the human genome, a locus not previously identified with any known human disease thought to involve DNA repair (Harrison et al., 1992; Robson et al., 1992). However, in contrast to exonuclease III and endonuclease IV of *E. coli* and *Apn 1* of *S. cerevisiae*, which display approximately equal 3'-repair activity and AP-cleaving activity (Levin et al., 1988; Johnson et al., 1988a, b), the HeLa enzyme, *Ape*, shows low 3'-repair activity (approximately 1% of the AP-cleaving activity; Demple et al., 1988). Furthermore, the *Ape* protein only partially complements repair-deficient *E. coli*, conferring significant cellular resistance to MMS, an alkylating agent, but little resistance to hydrogen peroxide, an oxidative agent (Demple et al., 1991). This information, in agreement with the biochemical evidence, suggests that the *Ape* protein may function well *in vivo* in repairing alkylation-induced AP sites but poorly in

removing oxidative 3'-terminal deoxyribose fragments. Instead HeLa cells possess a second distinct enzyme which displays 3'-repair and AP-cleaving activity at more similar levels (Chen *et al.*, 1991). This second human enzyme most likely is responsible, *in vivo*, for the removal of 3'-blocking fragments generated by reactive oxygen species. Molecular genetic analysis of this second AP endonuclease/3'-repair diesterase from HeLa cells, and thus cloning the gene, would assist in efforts to delineate the biological role(s) of this human DNA repair protein.

Regulation of DNA repair enzymes is an important part in how cells cope with environmental stress and maintain genetic integrity. *E. coli*, yeast and HeLa cells express high constitutive levels of exonuclease III, Apn1 and Ape, respectively (Dempfle *et al.*, 1991). This finding appears to indicate that the repair functions of these enzymes are constantly required in the face of continual cellular production of endogenous mutagens. In addition, expression of these enzymes in high levels would permit rapid repair of substantial amounts of DNA damage resulting from abrupt assaults by DNA-damaging agents, without requiring the synthesis of new proteins. This would explain why the levels of exonuclease III and Apn1 appear unaffected following exposure to various oxidizing agents (Dempfle and Halbrook, 1983; Chan and Weiss, 1987; Johnson and Dempfle, 1988a). *E. coli* endonuclease IV, however, which is normally expressed at

relatively low levels, is inducible to levels comparable to exonuclease III upon exposure to superoxide generators, such as paraquat (Chan and Weiss, 1987). To date, human genes encoding DNA repair enzymes appear non-inducible and systems such as the adaptive response and SOS response of *E. coli* have not been found.

b. Nucleotide Excision Repair

It has been widely accepted that the nucleotide excision repair pathway is responsible for the removal of DNA lesions which produce a significant distortion in the DNA backbone (Van Houten, 1990). In fact, it has been shown that specific types of damage, such as bulky chemical adducts and pyrimidine dimers, are excised directly by the operation of complex endonucleases. This process can be broken down into five major steps: damage recognition, incision, excision, repair DNA synthesis, and ligation to restore the structural and biological integrity to the damaged DNA.

In *E. coli* the UvrABC nuclease complex initiates the repair of over twenty different DNA lesions (for review see Grossman and Yeung, 1990; Selby and Sancar, 1990; Van Houten, 1990). Most of these lesions induce significant distortion to the DNA helix. However, recent findings have revealed that DNA lesions such as O⁶-methylguanine, thymine glycol and AP sites, which produce minor structural alterations, are also substrates for UvrABC. Both thymine glycols and AP sites were

normally thought to be repaired exclusively via the base excision pathway. Thus, it would appear that nucleotide excision repair may be an alternate route for the removal of some DNA lesions repaired by base excision repair or by direct reversal.

The UvrABC complex consists of three gene products, UvrA, UvrB, and UvrC and is an ATP-dependent nuclease (Husain *et al.*, 1986; Arikan *et al.*, 1986; Sancar *et al.*, 1984b). The UvrA protein appears to be the damage recognition subunit of the UvrABC excinuclease, primarily because this protein is the only component of the UvrABC complex that is capable of interacting with DNA (Yeung *et al.*, 1986a, b). The DNA-binding activity of UvrA has been attributed to putative "zinc finger" sites (Doolittle *et al.*, 1986; Navaratnam *et al.*, 1989). This protein binds as a dimer, presumably by interacting via a helix-loop-helix motif found in its structure. Recently, mutations in this motif were shown to eliminate (UvrA)₂ dimer specificity for UV-damaged DNA, likely by preventing protein dimerization (Wang and Grossman, 1993). Once bound to DNA, the UvrA-DNA complex delivers UvrB to damaged sites (Orren and Sancar, 1989). UvrC does not associate with either UvrA or UvrB directly. Instead, once UvrA has been displaced, the UvrB-DNA complex becomes the natural substrate for UvrC-induced incision (Visse *et al.*, 1992). The UvrABC excinuclease incises the lesion-containing DNA twice on the same DNA strand at the 8th phosphodiester

bond 5' and at the 4th or 5th phosphodiester bond 3' from the damaged site. This incision event results in the displacement of a 13 bp oligonucleotide from the DNA helix (Sancar and Rupp, 1983; Yeung et al., 1983). Lastly, both *uvrA* and *uvrB* genes are inducible by the *recA* gene product as part of the SOS response (Little and Mount, 1982), a mechanism used by *E. coli* to cope with bulky lesions in its DNA. *UvrC*, on the other hand, is expressed at low constitutive levels and is non-inducible.

In eukaryotes, yeast and mammals, a total of at least 10 excision repair genes have been identified (Friedberg, 1991; Bootsma and Hoeijmakers, 1991). However, little is known about the molecular mechanism of this repair reaction. Amino acid sequence comparison suggests that at least three DNA helicases operate in eukaryotic nucleotide excision. In addition, a striking sequence conservation is noted between human and yeast repair proteins. However, no eukaryotic homologs of the *UvrABC* proteins have been characterized. Nonetheless, the isolation and detailed phenotypic analysis of yeast and human mutants that are abnormally sensitive to killing by ultraviolet radiation has led to the elaboration of a detailed genetic framework with which to approach the molecular biology and biochemistry of nucleotide excision repair (NER) in eukaryotes (Thompson, 1989).

Five yeast genes which have been associated with NER (*RAD1*, *RAD2*, *RAD3*, *RAD4* and *RAD10*) are believed to be involved

in the primary biochemical events of damage-specific recognition of bulky base damage and damage-specific incision of DNA at or near such sites (Madura and Prakash, 1986; Miller et al., 1982a and b). A mutation in any one of these genes renders the cell highly sensitive to UV irradiation. Five additional genes (*RAD7*, *RAD14*, *RAD16*, *RAD23*, and *RAD24*) have been identified that confer a phenotype of partially defective NER in yeast mutants. At present, the roles of these ten proteins are unknown.

The *RAD1* gene has been cloned and sequenced (Higgins et al., 1983). Unfortunately, this 126 kDa protein shows no homology to any other protein, thus, offering no indication as to its biological function. The gene is expressed at low constitutive levels and is non-inducible by treatments with UV or other DNA-damaging agents. The gene encoding Rad2 has also been cloned and sequenced (Madura and Prakash, 1986). Rad2 is a protein of 117 kDa which, like Rad1, demonstrates no obvious amino acid sequence homologies with other known proteins, making it difficult to determine a potential cellular function. *RAD2* gene expression is inducible in response to UV irradiation and during meiosis (Madura and Prakash, 1986, 1990a; Siede et al., 1989). The 87 kDa Rad3 protein has been characterized most extensively. This protein has been found to contain a DNA dependent ATPase activity as well as a helicase activity (Sung et al., 1988; Harosh et al., 1989; Bailly et al., 1991; Naegli et al., 1992a, b, 1993). It is

believed that the Rad3 helicase activity allows this enzyme to unwind damaged DNA or to displace RNA transcripts allowing for repair. Alternatively, following the incision of the DNA, this activity may be involved in removing the damaged strand. Interestingly, deletion mutants of *RAD3* are lethal indicating that this gene product is required for cell viability. The *RAD4* gene has been isolated, but as is the case with many of the others, there is no clearly defined biological function for the protein (Couto and Friedberg, 1989). This enzyme has a predicted molecular weight of 87 kDa and shows significant homology to yeast photolyase. It has been proposed that this region of homology serves as a pyrimidine dimer binding domain. The *RAD7* gene encodes a 63 kDa protein of unknown biological function (Perozzi and Prakash, 1986). *RAD7* gene expression is inducible by UV irradiation and rises 15 fold upon entering meiosis as seen with *RAD2*, *RAD6*, *RAD18*, and *RAD23* (Jones et al., 1990). With Rad7 deficient yeast strains, there is evidence that Rad23 may be able to carry out some of the functions normally performed by Rad7. *RAD23* gene encodes a protein of unknown purpose (Madura and Prakash, 1990). The *RAD10* gene has been isolated and found to encode a 24 kDa protein which displays a region of similarity to the consensus sequence for a helix-loop-helix DNA binding domain (Bardwell et al., 1990). Rad10 protein binds single-stranded DNA and activates the renaturation of complimentary single-stranded DNA (Sung et al., 1992). In addition, both Rad1 and

Rad10 appear to be involved in mitotic recombination and have recently been shown to physically associate *in vitro* (Schiestl and Prakash, 1988, 1990; Bardell et al., 1992). The RAD1/RAD10 recombination pathway is distinct from the conventional RAD52 pathway.

As with yeast, many components of the excision repair system in higher eukaryotes have been revealed indirectly through the analysis of mutant cell lines with aberrant DNA repair (for review see Thompson, 1989). These mutants have been derived from two sources, human syndromes associated with repair defects and damage-sensitive rodent cell lines isolated in the laboratory. In particular, cells from individuals with the sunlight-sensitive disorders of xeroderma pigmentosum (XP) and Cockayne's syndrome exhibit reduced DNA excision repair. These inherited conditions display considerable genetic diversity, with seven complementation groups and a variant form in XP (A to G, and V) and three or more complementation groups in Cockayne's syndrome. In addition, hundreds of repair mutants have been isolated from rodent cell lines, mainly from Chinese hamster cells (Hickson and Harris, 1988). These mutant lines have been assigned to 10 different complementation groups by analysis of cell hybrids. Some of the groups appear to correspond to human XP groups, while others do not. The human genes that correct repair defects in rodent cells have been designated ERCC (Excision Repair Cross-Complementing) genes. By transfection of repair mutants with

DNA from normal cells, sequences can be selected that confer resistance to mutagenic agents. Genes cloned this way include *XPAC*, *ERCC-1*, *ERCC-2*, *ERCC-6*, and two *ERCC* genes that are apparently equivalent to XP genes, *ERCC-3/XPBC* and *ERCC-2/XPDC* (for review see Thompson, 1991). The predicted protein sequences of these genes have features that suggest that their products interact with DNA. However, despite hints of activities deduced from DNA sequences, few biochemical functions have been firmly established, and proteins have yet to be isolated in sufficient quantities for further study. It has been theorized that some of these proteins are involved in recognition of DNA damage, in endonucleolytic incision at damaged sites, and in disassembly of chromatin to allow access for repair enzymes. In addition, some proteins may be accessory factors that link transcription to DNA repair or regulatory factors that control the expression or activity of other repair genes.

The human and murine DNA repair genes that complement the defect of excision repair in group A XP cells have been cloned and named *XPAC* genes (Tanaka *et al.*, 1989, 1990). These gene products have a predicted molecular weight of 31 kDa and contain a zinc-finger motif, a feature of many DNA-binding proteins. Recently, homologs to the murine and human *XPAC* genes have been isolated from chicken, *Xenopus laevis*, and *Drosophila melanogaster* (Shimamoto *et al.*, 1991). In addition, it was found that the yeast *RAD14* gene, which

encodes a highly hydrophilic protein of 247 amino acids, is similar to *XPAC* (Bankmann et al., 1992). The XP-C complementing clone *XPCC* has been identified and shares limited homology to the yeast *RAD4* gene. The predicted molecular weight of this protein is 93 kDa and no definitive biological role has been determined (Legerski and Peterson, 1992). The human excision repair gene *ERCC-1* has been isolated and sequenced (van Duin et al., 1986). This gene encodes a protein of 33 kDa that shows significant amino acid sequence homology to the yeast protein Rad10. Human *ERCC-2* clones have also been identified and this 87 kDa gene product displays high homology to the yeast Rad3 protein (Weber et al., 1990). The similarity of *ERCC2* and Rad3 provides insight into their biochemical function and suggests a role for *ERCC2* in both cell viability and DNA repair. The human *ERCC-2* DNA repair gene is also able to correct the defect of XP complementation group D (Flejter et al., 1992). The human excision repair gene *ERCC-3* has been cloned and will be discussed in detail later (Weeda et al., 1990). Briefly, the yeast homolog *SSL2* (*RAD25*) to *ERCC-3* was identified because of its role in translation initiation (Gulyas and Donahue, 1992). Complementation of the DNA repair defect in XP group G cells permitted the cloning of a human cDNA (*XPGC*), which is related to yeast *RAD2* (Scherly et al., 1993). In addition, studies revealed that the XP group G and group 5 correcting (*ERCC-5*) proteins are identical (O'Donovan and Wood, 1993). *ERCC-6*, a

gene involved in preferential repair in eukaryotes and which corrects the repair defect of Cockayne's syndrome group B, has been identified (Troelstra *et al.*, 1990, 1992). The 168 kDa ERCC6 protein contains seven consecutive domains conserved between DNA and RNA helicases. These regions bear striking homology to segments in recently discovered proteins involved in transcription regulation (SNF2, MOT1, and brm), chromosome stability (Iodestar), and DNA repair (RAD16, RAD54, RAD5, ERCC2, ERCC3) (Troelstra *et al.*, 1992). Thus far, several of the human genes involved in nucleotide excision repair have been identified; however, to understand this repair pathway, the biological roles of these genes needs to be determined.

Using a cell-free system capable of nucleotide excision repair, some of the biochemical properties and components of the human nucleotide excision repair pathway have been characterized. For instance, the human excision nuclease was found to remove thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer (Huang *et al.*, 1992). In addition, it was reported that human DNA excision repair required the replication protein, single-stranded DNA-binding protein (Coverley *et al.*, 1991)

E. 8-Oxoquanine and Its Repair

N7-methylguanine, the major product after treatment of DNA with simple alkylating agents and a minor product of the

necessary methyl transfer function of SAM, has a very alkali-labile imidazole ring which is rapidly cleaved in mild alkaline solution to form a ring-opened base, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (FaPy). Using a DNA substrate which contains primarily radioactively labeled FaPy residues, DNA-glycosylase activity that specifically removed this base modification from DNA was first discovered in *E. coli* extracts (Chetsanga and Lindahl, 1979). It was later shown that the primary physiological substrate for this glycosylase was 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG; Figure 6; Tchou *et al.*, 1991).

Experiments analyzing 8-oxodG formation revealed that this adduct is generated in cellular DNA during normal metabolic processes or by various oxygen radical-producing agents such as ionizing radiation and hydrogen peroxide (Kasai *et al.*, 1986). In fact, the presence of oxygen dramatically increases the yields of 8-hydroxypurines, while not affecting the yields of formamidopyrimidines (Gajewski *et al.*, 1990). Recently, it has been demonstrated that 8-oxodG is formed in DNA upon exposure to ultraviolet radiation, UVB (Beehler *et al.*, 1992). This adduct has clearly been shown to cause mutations both *in vivo* (Wood *et al.*, 1990; Moriya *et al.*, 1991; Cheng *et al.*, 1992a; Moriya, 1993) and *in vitro* (Kuchino *et al.*, 1987; Shibutani *et al.*, 1991) and has been demonstrated to induce G to T and A to C transversions. Thus, the formation of 8-oxodG in DNA could be a significant source

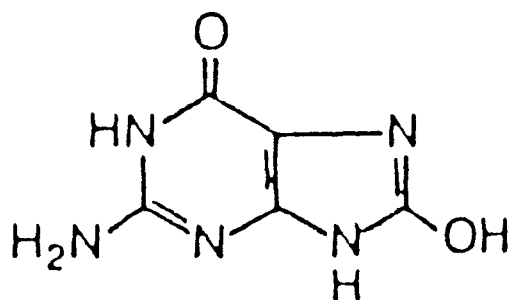


Figure 6: The chemical structure of 8-hydroxyguanine.

of mutations resulting in the increased likelihood of tumor promotion and carcinogenesis shown to be associated with this lesion (Floyd, 1990). Considering the mutagenic potential of this adduct and the fact that it is produced by endogenous reactive oxygen species generated under normal physiological conditions (cellular respiration), it is critical that organisms have a mechanism(s) for its removal.

A cell's initial defense against oxidizing agents is scavenging enzymes such as superoxide dismutase and catalase which catalytically remove reactive oxygen species from the intracellular environment. In addition, the removal of 8-oxodG from the cellular environment is carried out in *E. coli* (MutT), as well as humans, by an activity that functions at the nucleotide level (Bhatnagar *et al.*, 1991; Mo *et al.*, 1992). This activity, in an attempt to prevent the incorporation of 8-oxodG formed in the cellular nucleotide pool, hydrolyzes 8-oxoguanine to its corresponding nucleoside monophosphate.

If 8-oxodG should be present in DNA, an enzyme responsible for repair of this adduct has been identified and cloned in *E. coli*, formamidopyrimidine-DNA glycosylase (*fpg*) (Boiteux *et al.* 1987). *Fpg* is a 30 kDa protein which demonstrates N-glycosylase activity for FaPyAde, FaPyGua, 8-oxodG, and to a minor extent 8-oxodA (Chung *et al.*, 1991a; Boiteux *et al.*, 1992). *Fpg* also demonstrates glycosylase activity for other minor lesions induced by many chemical

carcinogens (Chetsanga and Frenette, 1983; Boiteux *et al.*, 1989). Homogeneous preparations of *E. coli* Fpg protein contain, not only glycosylase activity, but an EDTA-resistant AP endonuclease activity (Bailly *et al.*, 1989; O'Connor and Laval, 1989; Boiteux *et al.*, 1990; Chung *et al.*, 1991a). Fpg catalyzes the nicking of both the phosphodiester bonds 3' and 5' of AP sites in DNA leaving 3'-phosphate and 5'-phosphate ends. This 3' terminus is not a substrate for DNA polymerase I. These results suggest that Fpg cleaves in a class I beta-elimination reaction which is immediately followed by a delta-elimination reaction. Thus, Fpg is an AP lyase. Interestingly, studies have found that Fpg can also excise 5'-terminal deoxyribose phosphate (dRp) from damaged DNA (Graves *et al.*, 1992). dRp is the product of incision of DNA by class II AP endonucleases. This 5-terminus is not efficiently removed by the 5' to 3' exonuclease activity of DNA polymerase I and requires enzymatic removal, apparently, in some cases, by the *fpg* gene product. Atomic absorption spectrophotometric analysis shows that there is one zinc per Fpg protein molecule.

The bacterial gene encoding the Fpg protein has been found to be associated with an *E. coli* mutator phenotype, *mutM* (Michaels *et al.*, 1991). As one may predict, the *mutM* strain was previously characterized as a mutator strain that results specifically in GC to TA transversions (Cabrera *et al.*, 1988). As an additional line of defense against the detrimental

effects of 8-oxodG, *E. coli* contain a glycosylase activity, *mutY*, that specifically recognizes AG mismatches and removes misincorporated adenines opposite guanine residues (Lu and Chang, 1988; Au et al., 1989; Micheals et al., 1992). This activity presumably will allow the cell another opportunity to repair guanine residues, particularly 8-oxodG derivatives.

An endonuclease activity in human polymorphonuclear neutrophils that repairs 8-oxodG residues from DNA has been identified (Chung et al., 1991b). However, until now, no gene encoding a eukaryotic protein possessing 8-oxodG glycosylase activity had been isolated.

F. DNA Repair and Human Disease

The involvement of DNA repair systems in preventing carcinogenesis is clearly demonstrated by the high incidence of cancer found associated with several well-recognized DNA-repair-defective human diseases [i.e. xeroderma pigmentosum (XP), ataxia telangiectasia (AT), Cockayne's syndrome (CS), Bloom's syndrome (BS), and Fanconi's anemia (FA)]. In addition to these diseases, there exist several other syndromes that are DNA damage sensitive and are suspect for a DNA repair deficiency (for review see Hoeijmakers and Bootsma, 1992; Bohr et al., 1989). Only those diseases that have been well characterized will be discussed.

Clinical features of XP include extreme hypersensitivity to ultraviolet (UV) irradiation associated with neurological

abnormalities and mental impairment. It appears that the repair deficiency of XP is at an early step in the excision repair pathway such as damage recognition or incision (Cleaver, 1990; Bootsma and Hoeijmakers, 1991). There are seven complementation groups of XP and a variant group, indicating at least eight genes in the repair process--some of which have been identified.

CS is characterized by dwarfism, neurological abnormalities, mental retardation, and hypersensitivity to UV-induced DNA damage. This human disease appears to be a deficiency in the preferential DNA repair of actively transcribed genes. Recently, a gene involved in preferential repair of transcribed sequences in eukaryotes, *ERCC-6*, was isolated and characterized (Troelstra *et al.*, 1992)

Patients suffering from BS display a photosensitivity and are at higher risk for developing hemopoietic cancer and/or immunodeficiency. In addition, BS patients demonstrate a 15-fold increase in the rate of spontaneous sister chromatid exchanges. Evidence has been obtained for altered biochemical properties of partially purified DNA ligase I in several BS cell lines (Willis and Lindahl, 1987). However, no coding mutations have been encountered in the ligase I gene of a number of BS patients (Strathdee *et al.*, 1992; Petrini *et al.*, 1991). Instead two missense mutations occurring in both alleles of the DNA ligase I gene were detected in a human fibroblast strain, 46BR (Barnes *et al.*, 1992). This strain

was derived from a patient who displayed symptoms of immunodeficiency, stunted growth, and sun sensitivity. Other laboratories have found Bloom's syndrome cells to have altered uracil-DNA glycosylase activity. Nevertheless, the disorder for Bloom's syndrome has not been convincingly identified.

FA is a disease characterized by skeletal abnormalities, bone marrow hypofunction, mental deficiency, and leukemia. This disease displays a high incidence in spontaneous chromosome damage such as gaps, breaks, and chromosomal translocations. FA is thought to be caused by a defect in the ability to repair DNA interstrand cross-links. A gene conferring wild-type resistance to DNA cross-linking agents upon transfection into cells of FA complementation group C recently has been identified (Strathdee et al., 1992).

Clinical symptoms of AT include telangiectasia, cerebellar ataxia, immunodeficiency, and neurological abnormalities. AT is characterized by hypersensitivity to x-irradiation as well as several other DNA-damaging agents and shows an increase in spontaneous chromosome rearrangements. AT cells have recently been found to be defective in their ability to inhibit DNA synthesis after exposure to exogenous mutagens. In fact, three participants (AT genes, p53, and *GADD45*) were identified which are involved in the signal transduction pathway that controls cell cycle arrest following DNA damage (Kastan et al., 1992). Abnormalities in this pathway have the potential to adversely affect cell survival

and genomic integrity following certain types of DNA damage.

G. Ribosomal Protein S3

Ribosomes are ribonucleoprotein cellular organelles required for the synthesis of proteins (for review see Wool, 1991). The ribosomal proteins and ribosomal nucleic acids make up the specific binding sites for mRNA, tRNA, as well as the initiation, elongation, and termination factors. In addition, these protein and nucleic acid components catalyze peptide bond formation during protein synthesis. The focus of much of the research on ribosomes has been to determine the relationship between ribosomal structure and function. Thus, studies to elucidate the chemistry of ribosomal constituents and to isolate the various components have been ongoing.

Eukaryotic ribosomes are composed of two subunits which are designated by their sedimentation coefficients: the smaller is 40S and the larger 60S. The 40S subunit has a single molecule of RNA, designated 18S RNA, and 30 to 35 proteins. The 60S subunit has 3 molecules of RNA, 5S, 5.8S, and 28S, and 40 to 50 proteins. The subunits associate, forming the functional 80S ribosome, and are held together by noncovalent bridges. While a great deal is known about prokaryotic ribosomes (principally *E. coli*), far less is known of eukaryotic ribosomes. However, progress is being made especially with ribosomes from yeast and rat. Although comparatively little has been done with ribosomes from humans, it is believed that they closely resemble in both structure

and function the particles from other mammals.

The nucleotide sequences of the four RNA species--5S, 5.8S, 18S, and 28S--have been determined for human ribosomes. This sequence information has helped in the determination of the secondary structure of these RNA molecules. In contrast, the amino acid sequences of only about half the ribosomal proteins have been elucidated. Thus, efforts to isolate and identify all the protein components of a single mammalian (the rat) ribosome are continuing.

It has been estimated that there exist approximately 70 to 80 unique ribosomal proteins; about 30 in the small subparticle and 40 in the large (Wool, 1991). Steps have been taken to obtain appreciable amounts of pure ribosomal proteins in hopes to carry out sequence studies, to raise antibodies, and to determine binding to rRNA. During these efforts, specific nomenclature has been adopted to identify each of the ribosomal proteins. All protein components of the small 40S subparticle are indicated with an S (S followed by a number, i.e. S3), whereas all compounds of the large 60S subunit are symbolized with an L. Protein components from either the small or large subunit were further characterized by their two-dimensional electrophoretic patterns and given a specific number to identify them (McConkey *et al.*, 1979). S3 was originally classified as a core constituent of the 40S ribosomal subunit and migrates to a specific location during two-dimensional gel electrophoresis (Collatz *et al.*, 1976).

Thus, in future studies, the S3 ribosomal protein was identified by its two-dimensional electrophoretic mobility.

Experiments designed to determine the location of proteins within the 40S ribosomal subunit found that S3 was crosslinked to 18S rRNA (Svoboda and McConkey, 1978). In addition, studies uncovered that S3 can be crosslinked [using a variety of agents such as UV irradiation, 2-iminothiolane, sodium periodate, and diepoxybutane] to initiation factors eIF-2 (Westermann *et al.*, 1979) and eIF-3 (Tolan *et al.*, 1983), Met-tRNA_f (Westermann *et al.*, 1981), as well as mRNA (Takahashi and Ogata, 1981). In separate experiments, S3 was also determined to be located, at least with part of its polypeptide chain, in close proximity to the mRNA binding region (Stahl and Kobets, 1981). Furthermore, antibodies raised against pure preparations of ribosomal protein S3 strongly inhibited the binding of the ternary complex (Met-tRNA_f X eIF-2 X GTP) to the 40S subunit (Bommer *et al.*, 1980). Taken together, these results suggest that a domain of S3 is involved directly in binding the ternary complex and that S3 contributes to the formation of the P-site on the small subunit of eukaryotic ribosomes. Furthermore, these findings clearly indicate an involvement of S3 in the initiation of translation.

The cDNA encoding rat ribosomal protein S3 was isolated by screening a rat cDNA library with radioactively labeled *Xenopus leavis* ribosomal protein S1 DNA, the prokaryotic

homolog to S3 (Chan *et al.*, 1990). Preliminary identification of the protein specified by the reading frame of the isolated rat cDNA was made when comparing the deduced amino acid composition with peptide sequence derived from purified S3. The amino acid sequence obtained from purified ribosomal protein S3 matched exactly with residues (158-172) predicted from the nucleotide sequence of the cDNA. In addition, *in vitro* transcription followed by *in vitro* translation yielded a protein that migrated on two-dimensional gel electrophoresis to a position that had the coordinates of S3.

The cDNA encoding the human S3 ribosomal protein was isolated from a human colon cDNA library (Pogue-Geile *et al.*, 1991). Interestingly, this clone was identified as one of six genes whose level of expression was increased in adenocarcinoma of the colon (Pogue-Geile *et al.*, 1991). To detect genes that were overexpressed in colorectal tumors relative to normal colonic mucosa, differential screening of cDNA libraries was performed. In addition to S3, several other mRNAs encoding ribosomal proteins, including S6, S8, S12, L5 and P0, were found to be elevated in colorectal tumors and polyps.

H. Multifunctional Repair Enzymes

Recently there has been a surge of evidence implicating several proteins as having more than one cellular function. For example, a glycolytic enzyme, lactate dehydrogenase-5, has

been reported to be identical to a helix destabilizing protein (Williams et al., 1985). The epidermal growth factor receptor exhibits partial DNA topoisomerase II activity (Mroczkowski et al., 1984). Furthermore, a human DNA repair enzyme, uracil-DNA glycosylase (one of three identified), was found to be the 37 kDa subunit of glyceraldehyde-3-phosphate dehydrogenase (Meyer-Seigler et al., 1991). These are a few cases where proteins display two very distinct biochemical activities.

Interestingly several DNA repair proteins demonstrate sequence similarities to other protein families. For instance, the predicted amino acid sequence of a cDNA encoding one of the human uracil-DNA glycosylases shows striking similarity to members of the cyclin protein family (Muller and Caradonna, 1993). In addition, a human cDNA encoding a structure-specific recognition protein that binds specifically to DNA modified with cisplatin contains a region of identity to a portion of the high mobility group protein HMG1 (Bruhn et al., 1992).

More specifically, a class of proteins is beginning to emerge as transcriptional or translational regulators that are encoded by genes involved in DNA repair. A *Drosophila* gene, isolated by immunoscreens using antibody to a human AP endonuclease, is homologous to human ribosomal associated protein P0 (Kelley et al., 1989; Grabowski et al., 1991a). Interestingly, the *Drosophila* protein, AP3, contains AP endonuclease activity and cuts AP DNA in a class II hydrolytic

mechanism. In addition, this protein associates both with ribosomes and with the nuclear matrix, suggesting a role in ribosomal function as well as nucleic acid metabolism. SSL2, a factor which is involved in promoting ribosomal binding and scanning of mRNA, was found to be the yeast homolog to ERCC3, a component of the human excision repair machinery (Gulyas and Donahue, 1992). An *SSL2* (*RAD25*) allele devised to resemble the defective *ERCC-3* gene associated with XP/CS renders these yeast hypersensitive to ultraviolet irradiation (Weeda *et al.*, 1990; Gulyas and Donahue, 1992; Park *et al.*, 1992). These results suggest that SSL2 as well as ERCC3 may have two functions, one defined by a UV repair defect, and a second essential function that is related to translation initiation. Moreover, both SSL2 and ERCC3 contain nuclear targeting sequences, indicating that they may be translocated to the nucleus to carry out their DNA repair function (Friedberg, 1992). *Haywire* mutants, the *Drosophila* homolog to the human *ERCC-3* gene, display several of the phenotypic traits affiliated with XP and CS, such as ultraviolet sensitivity, motor defects, and reduced life span (Mounkes *et al.*, 1992). Thus, these proteins appear to associate with ribosomes to regulate translation as well as within the nucleus to carry out DNA repair. Another multifunctional protein with a role in DNA repair is the major human AP endonuclease which is also a regulator of the DNA-binding activity of several transcription factors via reduction-oxidation mechanism

(Xanthoudakis *et al.*, 1992). Finally, eukaryotic DNA binding factors, YB-1 and dbpA, have been isolated by screening expression libraries with DNA probes containing either specific promoter sequence elements or AP sites (Hasegawa *et al.*, 1991). The human counterparts to these two proteins have also been identified based on their ability to selectively bind AP DNA (Lenz *et al.*, 1990). Thus, it appears that these proteins may be involved in regulating transcription as well as repairing abasic sites in DNA.

Recently, ribosomal proteins S27 and S29 have been shown to contain zinc finger-like motifs, domains commonly found in DNA- and/or RNA-binding proteins (Chan *et al.*, 1993). Thus, these proteins might have functioned, and still may, as transcriptional regulators. Since ribosomes were at one time composed of solely RNA, it is likely that ribosomal proteins were recruited based on their ability to increase ribosomal and translational efficiencies (Dr. Ira Wool, University of Chicago, personal communication). Therefore, it seems logical to predict that, prior to recruitment of these proteins, they were performing some other function involving nucleic acid interaction and/or RNA-DNA metabolism (*i.e.* DNA repair).

CHAPTER III

MATERIALS AND METHODS

A. Materials

Enzymes and chemicals were purchased from Amersham (Arlington Heights, IL), BRL (Gaithersburg, MD), New England BioLabs (Beverly, MA), Pharmacia (Piscataway, NJ), Promega (Madison, WI), Sigma (St. Louis, MO), and Stratagene (La Jolla, CA). Radioisotopic [α - 32 P]dCTP (3000 Ci/mmol) and [γ - 32 P]ATP (3000 Ci/mmol) were purchased from NEN (Wilmington, DE), [3 H]UTP (15 Ci/mmol) was purchased from Amersham. Oligonucleotides were obtained from National Biosciences, NBI (Plymouth, MN). Nytran membranes were purchased from Amersham.

B. Care of *Drosophila*

Drosophila melanogaster (Oregon R) stocks were maintained at 25°C and transferred to fresh media about every two or three weeks. When growing flies at 18°C, which slows their development, flies were transferred every four weeks. Food was prepared by mixing 100 ml of molasses and 14.8 g of agar in one liter of water. This mixture was boiled for 10 minutes and 400 ml of water, 100 ml of cornmeal and 41.2 g of brewer's yeast were added. The media was again boiled for 10 minutes

and allowed to cool to the touch before adding 22.5 ml of tegosept (10% methyl p-hydrobenzoate (w/v) in 95% ethanol) and 8.0 ml of propionic acid. The media was subsequently poured into bottles or vials and allowed to harden overnight.

C. Transformation of Competent Bacteria Cells with Supercoiled Plasmid DNA

A plasmid containing the rat S3 ribosomal protein cDNA was graciously provided by Dr. Ira Wool of the University of Chicago, Illinois (Chan *et al.*, 1990). Upon receipt of the recombinant DNA molecule, the plasmid (ampicillin resistance gene) was used to transform competent bacterial HB101 F⁺ cells (kanamycin resistance gene present on the episome).

Competent HB101 cells were prepared according to the protocol designed by Chung *et al.* (1989). A single colony of bacteria was aseptically inoculated into LB (1% tryptone, 0.5% NaCl, 0.5% yeast extract, 1.5% bactoagar) media containing the appropriate antibiotic (kanamycin (50 µg/ml) for HB101 F⁺ cells). Cultures were grown overnight in an environmental shaker (225-275 rpm) at 37°C. In the morning, the fresh overnight culture was diluted 1:100 into prewarmed (37°C) LB broth containing the appropriate antibiotic (kanamycin) and grown as before to an OD600 of 0.3 to 0.4. For long term storage, the cultures were cooled on ice for 10 minutes and an equal volume of cold (4°C) 2X TSS (LB containing 10% PEG av. mol. wt. 8000, 5% DMSO, 20-50 mM Mg⁺² [MgSO₄ or MgCl₂], pH

6.5) was added. The cells were then frozen in dry ice and stored at -80°C . For immediate use, 1 ml of bacterial culture was aliquoted into a microfuge tube and the cells pelleted by centrifugation at $1000 \times g$ for 5 minutes. The supernatant was aspirated and the cells were resuspended in $100 \mu\text{l}$ of 1X TSS and used immediately.

Transformations were carried out by adding 1 ng of supercoiled plasmid DNA to $100 \mu\text{l}$ of either previously frozen or freshly prepared competent cell culture (Chung *et al.*, 1989). Frozen cells were thawed on ice just prior to use. The cell/DNA solution was gently mixed and allowed to incubate on ice for 5 to 60 minutes. After incubation, $500 \mu\text{l}$ of TSS plus 20 mM glucose was added and the sample placed in the 37°C water bath for 1 hour to permit expression of the antibiotic-resistance gene. This cell/DNA mixture was then aseptically spread onto a prewarmed (37°C) LB plate containing the appropriate antibiotic(s) [ampicillin ($100 \mu\text{g/ml}$) for the S3 plasmid]. The plate was placed at 37°C overnight and checked in the morning for transformant colonies. These transformants were subsequently analyzed for the presence of recombinant S3 plasmid DNA.

D. Preparation of Frozen Sterile Bacterial Cultures

For long-term storage of bacteria, 20% glycerol stocks were prepared. A single colony of cells was aseptically transferred to a tube containing 2 ml of LB medium

supplemented with the appropriate antibiotic. This culture was grown overnight at 37°C in an environmental shaker. Of the 2 ml, 800 µl of cells were placed in a sterile tube and mixed with 200 µl of sterile glycerol. The cells were frozen at -70°C and stored indefinitely. Bacteria were recovered by streaking a sample of the frozen stock onto the appropriate LB agar-antibiotic plate.

E. Plasmid DNA Purification

Plasmid DNA was isolated using the alkaline lysis technique as described by Maniatis *et al.*, 1989. A single bacterial colony was inoculated into the appropriate antibiotic containing LB media (ampicillin for S3). For quick analysis of plasmid, 2 ml cultures were used (minipreps)--larger volumes for larger preparations. The culture was grown overnight in an environmental shaker at 37°C. The overnight culture was transferred to a microcentrifuge tube and the bacteria pelleted by centrifugation at 10,000 x g for 1 minute. The media was aspirated off and the bacteria resuspended in 250 µl of ice-cold P1 solution (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 400 µg/ml RNase A; 250 µl of P1 for each 1.5 ml of bacteria culture). Once the pellet was fully resuspended, 250 µl of P2 solution (200 mM NaOH, 1% SDS) was added, the tube inverted several times, and the mixture allowed to incubate at room temperature for 5 minutes. After incubation at room temperature, 250 µl of P3 (2.55 M potassium

acetate, pH 4.8) was added and the tube placed on ice for 15 minutes. The bacterial lysis solution (bacterial cell lysate) was mixed thoroughly and centrifuged at 10,000 x g for 15 minutes at 4°C. The resulting supernatant was transferred to a new tube without disrupting the precipitant formed. Columns were utilized to isolate highly purified plasmid DNA for methods such as sequencing. Otherwise, DNA was precipitated from the supernatant by the addition of 0.6 volumes of isopropanol. The mixture was incubated at -20°C for at least 30 minutes, but the incubation can be extended indefinitely. Plasmid DNA was pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C. The supernatant was aspirated off and the pellet washed with 70% ethanol. After centrifugation at 10,000 x g for 2 minutes at room temperature and removal of the supernatant, the nucleic acid was dried at 65°C until all remaining ethanol evaporated. The DNA was dissolved in a minimal volume of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) by vortexing and heating to 65°C. The plasmid DNA solution was stored at 4°C for short-term storage or frozen at -20°C for long-term storage. The plasmid DNA was analyzed by restriction endonuclease digestion for the presence of the S3 cDNA insert by cutting with *Hind*III and *Bam*HI under the conditions specified by the manufacturer. After digestion, the sample was fractionated in a 0.85% agarose gel containing 0.5 µg/ml ethidium bromide and 1X TBE buffer (20X TBE = 1.78 M Tris-HCl; 1.78 M boric acid; 4 mM EDTA, pH 8.0). Results

were analyzed by viewing the nucleic acid on a UV transilluminator.

F. Release and Gel Purification of DNA Fragments

Plasmid containing the rat S3 ribosomal protein cDNA was purified. To isolate the S3 cDNA fragment, approximately 10 μ g of plasmid DNA was digested with 20 U of *Hind*III and 20 U of *Bam*H1 in appropriate enzyme buffer as described by the manufacturer (BRL) at 37°C for 4 hours. To stop the reaction, the digestion mixture was heated to 65°C for 10 minutes and 0.1 volume of 10X loading dye (25% ficoll, 0.25% orange G) added. The sample was loaded onto a 0.85% agarose gel prepared in 1X TBE. Samples were run at a constant voltage of 5V/cm length of gel in 1X TBE buffer until clear separation of vector and insert was obtained. The band corresponding to the S3 cDNA insert was detected with a UV handheld transilluminator (302 nm) and excised. This gel slice was blotted dry on Whatman 3MM filter paper and transferred to a punctured sterile 0.8 ml microcentrifuge tube that had been plugged with about 2-3 mm of sterile glass wool. The 0.8 ml tube was placed into a 1.5 ml microcentrifuge tube and centrifuged at 10,000 x g for 10 minutes. The eluant containing the DNA fragment was then analyzed by agarose gel electrophoresis and an approximate concentration determined. This fragment was used in random primer labeling reactions, for subcloning, and to screen *Drosophila* cDNA and genomic

libraries.

G. Labeling Double-Stranded DNA Fragments with Random Hexamers

Double stranded DNA molecules, which were used as hybridization probes, were labeled to high specific activity with [α - 32 P]dCTP (NEN) by the random hexanucleotide primer method (Feinberg and Vogelstein, 1983). Approximately 25 ng of DNA template was brought to a final volume of 30 μ l with sterile water in a microcentrifuge tube and denatured by heating at 100°C for 3 minutes. The tube was rapidly chilled on ice for 3 minutes. The labeling reaction was prepared by adding 10 μ l of 5X labeling buffer (250 mM Tris, pH 8.0, 25 mM MgCl₂, 10 mM DTT, 1 mM HEPES, pH 6.6, 27 A260 U/ml of hexanucleotide primer), 2 μ l 1.5 mM dNTP's (dATP, dGTP, dTTP, final concentration of 20 μ M each), 2 μ l nuclease-free acetylated BSA (1mg/ml, final concentration of 400 μ g/ml), 5 μ l α - 32 P dCTP (50 μ Ci, 3000 Ci/mmol), and 1 μ l of Klenow enzyme (final concentration 100 U/ml) in a final volume of 50 μ l to the denatured template (500 ng/ml). The labeling reaction was gently mixed and allowed to incubate at room temperature for 1 hour. To terminate the reaction, 2 μ l of 0.5 M EDTA, pH 8.0 (final concentration of 20 mM) and 48 μ l of sterile water was added and the tube was heated to 100°C for 3 minutes. The tube was subsequently chilled on ice for 3 minutes and can be stored at -20°C or used immediately.

Removal of unincorporated labeled dCTP was carried out by gel filtration using Sephadex G-50 columns. Sephadex G-50 was prepared in a 20 mM NaOH; 1 mM EDTA solution, autoclaved, and stored at room temperature. To make the spun column, a 1 ml syringe was plugged with sterile glass wool and G-50 Sephadex added to the column. The Sephadex was packed by centrifugation at 1000 x g for 5 minutes. The denatured, labeled probe was added to the column, spun at 1000 x g for 5 minutes, and collected.

Percent incorporation was determined by comparing the amount of radioactivity left in the column to the radioactivity collected. Specific activity in dpm/ μ g was calculated by the following computation: (starting label, 50 μ Ci)x(fraction of label incorporated, 0.5 for 50%)x(2.2×10^6 dpm/ μ Ci)x(40, if 25 ng of DNA is being labeled).

H. Screening a Lambda gt10 cDNA Library with Radioactively labeled DNA Probes

A 5.5 to 7.5 day, early pupal lambda gt10 library supplied by Dr. Thomas Kornberg (University of California, San Francisco) was screened with radioactively labeled rat S3 cDNA using standard procedures (Maniatis et al., 1982). Approximately 1.2×10^5 plaques were screened by plating 3×10^4 pfu/150 mm plate.

Plating bacteria were prepared by inoculating a single C600 bacterial colony into 50 ml of LB medium, supplemented

with 0.2% maltose, in a 250 ml flask and grown overnight in an environmental shaker at 37°C. Cells were pelleted from the overnight culture by centrifugation at 1000 x g for 10 minutes. The supernatant was discarded and the cell pellet resuspended in sterile 0.01 M MgSO₄, pH 7.5 (0.4 x the volume of the original culture). These cells were stored at 4°C for up to three weeks.

For infection, 100 µl of the plating bacteria (10⁸ cells) was mixed with 30,000 pfu (as determined by titer experiments) of the pupal phage library and incubated for 20 minutes at 37°C. Quickly, 9 ml (for 150 mm plates, 3 ml for 82 mm plates) of prewarmed (42°C) LB soft agarose (LB media + 0.72% agarose) was added to the infection sample and poured onto prewarmed (42°C) LB plate (LB media + 1.5% bactoagar). The plate was swirled while pouring to ensure even spreading of the agarose over the plate. Plates were allowed to cool at room temperature for 5 minutes before being placed at 42°C. The plates were incubated until plaques were just beginning to make contact with one another. Following incubation at 42°C, plates were cooled at 4°C for 20 minutes prior to lifts. Meanwhile, nitrocellulose filters were labeled.

For lifting, the filter membrane was placed onto the top agarose, avoiding all air bubbles. The alignment of the filter was marked by asymmetrically stabbing through the filter into the agar with a syringe needle. After 1 minute, the filter was carefully removed using forceps and placed into

the DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH) plaque side up for 1 minute. The filter was partially dried and transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 minutes. The filter was then rinsed in 3X SSC (20X SSC = 3 M NaCl; 0.3 M sodium citrate, pH 7.0) for 3 minutes and subsequently placed on paper towels to dry. DNA was fixed to the membranes by drying in a vacuum oven for 1 hour.

Hybridization conditions with the rat S3 cDNA on *Drosophila* RNA and DNA were determined previously by Northern and Southern blot experiments, respectively. Identical conditions were used when screening the library. Basically, filters were prehybridized for at least 1 hour at 42°C with 30% formamide, 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 10 mM NaPO₄, pH 6.5, 0.1% pyrophosphate, and 250 µg/ml salmon sperm DNA (heat denatured by boiling 10 minutes). Rat S3 cDNA, radioactively labeled using the random primer technique, was added to the prehybridization solution and allowed to hybridize 16 to 24 hours at 42°C. Filters were washed once in 1X SSC at room temperature for 15 minutes and twice with 1X SSC at 65°C for 20 minutes. These filters were wrapped in saran wrap and autoradiographs obtained by exposure to XAR-5 film using intensifying screens at -80°C. Plaques representing positive signals were picked and placed in 500 µl of SM (100 mM NaCl; 8 mM MgSO₄-H₂O; 50 mM Tris-HCl, pH 7.5; 0.01% gelatin)/0.3%

chloroform and stored at 4°C until subsequent screening experiments were performed. Clones were purified until all plaques were positive before further analysis was carried out.

I. Screening a Genomic Library with DNA Probes

A *Drosophila* genomic DNA library, a gift from Dr. Tom Maniatis, was screened with the rat S3 cDNA fragment using the same procedure as described for screening a lambda cDNA library and using the C600 bacterial stock.

J. Purification of Lambda Phage DNA and Isolation of Candidate Inserts

After positive phage clones were purified to homogeneity through successive screens, phage DNA was isolated to permit further analysis of the insert DNA. To isolate lambda phage DNA, the procedure described by the Lambda Quick! Kit (Bio101, Inc., La Jolla, CA) was utilized. An inoculum of 10^5 plaque-forming units (pfu) was mixed with the appropriate bacterial host strain (C600 cells for both the genomic and cDNA libraries) and allowed to incubate at 37°C for 20 minutes. Approximately 5 ml of prewarmed LB soft agarose (42°C) was added to the absorbed phage/bacteria mixture and aseptically spread onto an 85 mm LB plate containing no antibiotics. The soft agarose was permitted to harden and the plates were incubated at 37°C overnight. Following the overnight incubation, the plates were cooled at 4°C for fifteen minutes

and 3 ml of Phage Lift Buffer was added. The plates were incubated for an additional six hours at 4°C while rotating. The phage containing liquid was removed from the plates with a pasteur pipet and the debris pelleted from the liquid by centrifugation at 10,000 x g for 10 minutes. The resulting supernatant was filtered through a 0.45 micron filter to remove residual bacteria. To the filtered phage, the provided Nuclease Mixx (10 mg/ml of RNase and DNase) was added to a final concentration of 5 µg/ml. Lambda Phage Drop Buffer, which precipitates the phage, was added to a final concentration of 25% and the new mixture incubated at 37°C for 20 minutes and then at 4°C overnight. The phage were pelleted by centrifugation at 12,000 x g for 10 minutes and the supernatant discarded. The phage pellet was resuspended in 150 µl of TE and transferred to a microcentrifuge tube. An equal volume of Lambda Phage Lysis/Binding Buffer was added to the phage solution. The sample was vortexed and heated to 70°C for 10 minutes. To the 300 µl of liberated phage DNA, 5 µl of Lambda DNA Glassmilk (binding capacity of 1 µg phage DNA/µl) was gently mixed into solution. The binding reaction was carried out for 10 minutes at room temperature with intermittent mixing to keep the matrix in suspension. The Lambda DNA Glassmilk was spun down by centrifugation at 10,000 x g for 5 minutes. The pellet was washed once with 150 µl of Lambda Phage Lysis/Binding Buffer and twice with 200 µl of Lambda DNA Wash Buffer. The final pellet was permitted to dry

at room temperature for 3 minutes, resuspended in 50 μ l TE, and incubated at 65°C for 5 minutes. The Glassmilk was removed by centrifugation at 10,000 x g for 1 minute and the supernatant transferred to a new tube. This supernatant contained the purified lambda DNA.

Inserts from the purified lambda phage DNA were isolated by digesting the entire 50 μ l sample with *EcoRI* as described by the manufacturer and eluting the DNA fragments from agarose gel slices. These inserts were subsequently subcloned into the appropriate vector.

K. Subcloning of Isolated DNA Fragments

DNA fragments were excised and eluted from agarose gels. These DNA fragments were subsequently ligated into linearized plasmids that had been cut with the appropriate endonuclease(s) and treated with calf intestinal alkaline phosphatase (CIAP). CIAP treatment was carried out by adding 1 μ l of phosphatase enzyme (BRL; 24 U/ μ l) to the plasmid restriction digest and allowing the reaction to proceed for an additional hour at 37°C. The enzyme was killed by incubating the reaction at 65°C for 15 minutes and extracting with phenol/chloroform twice. Digested/CIAP treated plasmid was gel purified and used in ligation reactions. For ligations, a 10:1 ratio of insert:vector was mixed with 1X ligase buffer (10X ligase buffer = 300 mM Tris-HCl, pH 7.5; 100 MgCl₂; 100 mM DTT), ATP (final concentration of 1 mM), and 1 μ l of T4 DNA

ligase (New England BioLabs; 1 Weiss U/ μ l) in a final volume no greater than 20 μ l. Ligations were performed at 14°C overnight. T4 DNA ligase was inactivated by heating the reaction to 65°C for 10 minutes. The tube was cooled on ice for 2 minutes and the entire reaction transformed into competent bacteria cells.

L. Sequence Analysis of Isolated Clones

Sequence analysis of purified recombinant plasmid DNA was performed using the *fmol* DNA Sequencing System (Promega). Purified plasmid DNA was isolated using either the Magic Minipreps DNA Purification System (Promega) or the Qiagen column system (Chatsworth, CA). For either system, 2 to 10 ml of an overnight bacterial culture, which contained the recombinant plasmid of interest, was grown at 37°C in the environmental shaker. Cells were pelleted by centrifugation at 1000 x g for 5 minutes and cell lysates prepared.

For the Magic Minipreps DNA Purification System, plasmid DNA was isolated in the following manner. One ml of the Magic Minipreps DNA Purification resin was added to the bacterial cell lysate and mixed by inverting the tube. This resin/DNA mix was then pipeted into a 3 ml syringe barrel and the slurry pushed into the Magic Minicolumn which had been attached to the end of the syringe. The resin/DNA in the Magic Minicolumn was washed with 2 ml of Column Wash Solution by gently pushing the wash solution through the syringe barrel with the syringe

plunger. The Minicolumn was further dried by placing it into a microcentrifuge tube and spinning at 10,000 x g for 20 seconds. The Minicolumn was transferred to a new microcentrifuge tube and the nucleic acid eluted with 50 μ l of 65°C TE. The preheated TE was applied to the Minicolumn, allowed to incubate 1 minute at room temperature, and centrifuged at 10,000 x g for 20 seconds. The Minicolumn was discarded and the plasmid DNA stored appropriately.

As an alternative method, Qiagen columns were used. For this procedure, plasmid DNA was isolated in the following manner. A Qiagen-tip 20 was equilibrated with 1 ml of QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% ethanol, 0.15% Triton X-100). The bacterial cell lysate was applied to the equilibrated column and allowed to empty by gravity flow. The column was washed with 2 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% ethanol). The DNA was then eluted with 800 μ l of QF (1.25 M NaCl, 50 mM MOPS, pH 8.2, 15% ethanol) and collected in a microcentrifuge tube. The DNA was precipitated with 0.5 volumes isopropanol at -20°C, pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C, washed with 70% ethanol, dried at 65°C and resuspended in TE for proper storage. Concentrations of plasmid DNA solutions were determined by absorbance at A_{260} .

The nucleotide sequences of genomic and cDNA inserts were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). For detection of extended/terminated

polymerase chain reaction (PCR) products, generated during the sequencing reactions, a ^{32}P end-labeled oligonucleotide primer was utilized. Oligonucleotide primers were labeled by mixing 10 pmol primer, 10 pmol gamma-labeled ATP (3.0 μl of 3,000 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{l}$), 1 μl of T4 polynucleotide kinase 10X Buffer (0.5 M Tris-HCl, pH 7.6; 0.1 M MgCl_2 ; 50 mM DTT; 1 mM spermidine-HCl), 5 units of T4 Polynucleotide Kinase in a final volume of 10 μl and allowing the reaction to incubate at 37°C for 30 minutes. The kinase was inactivated by heating the tube to 90°C for 2 minutes and the end-labeled primers were stored at -20°C. For each set of four sequencing reactions, 130 fmol of template plasmid DNA, 5 μl of fmol Sequencing 5X Buffer (250 mM Tris-HCl, pH 9.0; 10 mM MgCl_2), 1.5 pmol of labeled primer and water were mixed in a final volume of 16 μl . In addition, four 0.5 ml microcentrifuge tubes were labeled (A, C, G, T) and 2 μl of the appropriate d/ddNTP mix was added. These tubes were stored at 4°C until needed. To the primer/template mix, 1.0 μl of Sequencing Grade *Taq* DNA polymerase (5 u/ μl) was added. To each of the four tubes containing d/ddNTP mix, 4 μl of the primer/template/enzyme mix was added. One drop (20 μl) of mineral oil was layered onto each reaction mixture, the tubes spun briefly and placed into the thermal cycler which had been preheated to 95°C. The PCR cycling profile was as follows: 95°C for 2 minutes; 95°C for 30 seconds (denaturation), 42°C for 30 seconds (annealing) and 70°C for 1 minute (extension)

repeated for 30 cycles total; hold at 10°C. At the completion of the thermocycling program, 3 μ l of *fmol* Sequencing Stop Solution (10 mM NaOH; 95% formamide; 0.05% bromophenol blue; 0.05% xylene cyanole) was added to each tube. The reaction tubes were centrifuged briefly and stored at -20°C until fractionated. Prior to loading on a sequencing gel, the reactions were heated to 90°C for 2 minutes.

To prepare the sequencing gel, the sequencing plates were washed and rinsed with 100% ethanol. The large plate was treated on occasion with a 5% dichloromethyl silane diluted with chloroform. Once the plates were cleaned and dried, the spacers were put into place, the plates were aligned and taped accordingly. To pour the gel, 90 to 95 ml of sequencing gel matrix [6% polyacrylamide (19:1, acrylamide:bis)] and 7.55 M Urea in 1X Sequencing TBE (0.1 M Tris-HCl, 83 mM boric acid, 1 mM EDTA, pH 8.3) was filtered and transferred to a clean beaker. 210 μ l of 25% APS and 75 μ l of TEMED were added to catalyze polymerization and stirred into solution. A 60 ml syringe was used to pour the gel, the gel was poured, the comb placed, and the plates clamped. The gel was allowed to polymerize overnight covered with Saranwrap. Electrophoresis of the sequencing reactions was carried out at 55 mA, 2200 volts, and 110 watts in 1X sequencing TBE. At the conclusion of electrophoresis, the sequencing gel was fixed in 10% methanol, 10% acetic acid for 20 minutes, transferred to Whatman and dried down in a BioRad gel dryer at the

appropriate setting for 1.5 hours.

M. Polytene Chromosome Preparations and In Situ Hybridization

Subbed slides were prepared as described by Ashburner (1989). Basically, a mixture of 1% gelatin and 0.1% chromium K sulfate was prepared at 60°C, allowed to cool, and used to treat clean microscope slides. The slides were allowed to dry and stored in a dust-free environment.

Salivary glands from mature third-instar larvae were dissected in 45% acetic acid and polytene chromosomes squashed onto subbed slides (Pardue, 1986). The salivary glands were removed using a pair of forceps by pinching off the anterior end of the larva and pulling the glands through the opening. Much of the fat body was then removed without disrupting the glands. One or two salivary glands were transferred to a subbed slide containing a drop of 45% acetic acid and covered with a coverslip. The coverslip was lightly tapped with the erasure end of a pencil to force the chromosomes out of the cells and to spread the chromosome arms. This procedure was monitored by checking periodically the condition of the chromosomes with a phase contrast microscope. When the chromosomes were well spread, a paper towel was placed over the coverslip and, using a thumb, the coverslip was pressed down very hard without sliding. The slide was maintained at 42°C for 15 minutes while keeping it damp with 45% acetic

acid. It was then transferred onto a flat piece of dry ice with the coverslip side up and left until the preparation was frozen (at least 5 minutes). The slide was removed from the dry ice, the coverslip quickly lifted off with a razor blade, and the slide plunged into 95% ethanol for 10 minutes. This wash step was repeated two more times before the slide was allowed to air-dry. Squashes were stored at 4°C for several months.

Chromosomes were pretreated prior to hybridization. Each slide was washed once in 2X SSC at 65°C for 30 minutes and once in 2X SSC at room temperature for 2 minutes. To reduce the nonspecific sticking of radioactively labeled probes, the slide was acetylated by suspending it in a large volume of 100 mM TEA, pH 8.0, and, while vigorously stirring, acetic anhydride was added to a final concentration of 5 ml/L. After the acetic anhydride had been added, the stirring was stopped and the slide allowed to sit for 10 minutes. The slide was washed twice in 2X SSC at room temperature for 5 minutes. Dehydration was then carried out by washing twice for 5 minutes in 70% ethanol and twice for 5 minutes in 95% ethanol. The slide was allowed to air dry before hybridization.

Chromosomes were denatured by incubating the slide in freshly prepared 70 mM NaOH for 3 minutes. The slide was washed in 2X SSC for 5 minutes and then dehydrated. *Drosophila* S3 cDNA was labeled with biotinylated dUTP using the random-primer technique. Approximately 50 ng of DNA was

denatured by boiling 3 minutes in a final volume of 27 μ l and placed on ice for 3 minutes. After incubation on ice, 10 μ l of 5X reaction buffer, 5 μ l dNTP's (dATP, dGTP, dCTP), 2 μ l BSA, 2.5 μ l ^3H -dTTP (Amersham), 2.5 μ l bio-16-dUTP (Amersham), and 1.0 μ l Klenow was added and the mixture allowed to incubate at room temperature for 1 hour (when available Sequenase should be used instead of Klenow). Unincorporated nucleotides were removed by fractionation through a G50 spun column. The labeled probe was precipitated with 0.1 volume 3 M sodium acetate, pH 5.2 and 2.5 volumes 100% ethanol in the presence of 2.5 μ l yeast tRNA (10 mg/ml). The nucleic acid was pelleted by centrifugation at 10,000 g for 15 minutes at 4°C, washed with 70% ethanol, dried at 65°C for 5 minutes, and resuspended in 5 μ l. Of this 5 μ l, 1 μ l was counted for ^3H in 5 ml scintillation fluid to which 100 μ l sterile water was added. The remaining 4 μ l was used for hybridization by adding 4 μ l formamide (final concentration 40%) and 2 μ l of 20 X SSC (final concentration 4 X). The 10 μ l of probe was denatured and cooled and placed onto a prepared slide. A coverslip was placed on top of the solution and the sample sealed with 2 coats of rubber cement. Hybridization was carried out for at least 18 hours at 40°C.

Following the incubation, the slide was subjected to an intense wash procedure. The coverslip was removed and the slide was rinsed in 2 X SSC at room temperature for 15 minutes, in 2 X SSC three times at 35°C for 10 minutes, and

once in 2 X SSC at room temperature for 5 minutes. The slide was then placed in Buffer 1 (0.1 M Tris, pH 7.6, 0.1 M NaCl, 2 mM MgCl₂, 0.05% Triton X-100) plus 3% BSA for 10 minutes at room temperature. Most of this solution was drained onto a paper towel and the slide was transferred to a moisture chamber where it was incubated with 200 μ l of Buffer 1 containing BRL streptavidin (final concentration 2 μ g/ml) for 10 minutes at room temperature. The solution was drained as before and the slide was washed three times in Buffer 1 at room temperature for 3 minutes. The slide was drained of all solution and 200 μ l of Buffer 1 containing BRL biotin (alkaline phosphatase, final concentration of 1 μ g/ml) was added. The slide was incubated at room temperature for 10 minutes in a moisture chamber. The slide was washed twice in Buffer 1 for 3 minutes at room temperature and then washed twice in Buffer 2 (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂) for 5 minutes at room temperature. The slide was transferred to the moisture chamber and incubated in the dark for 2 hours at room temperature with NBT (100 μ g/ml) and BCIP (50 μ g/ml) solution made up in Buffer 2. Color development was monitored using the microscope. To stop the reaction the slide was rinsed in 20 mM Tris, pH 7.6, 5 mM EDTA for 5 minutes at room temperature. The slide was mounted in glycerol and stored at 4°C and/or photographed. For banding patterns, chromosomes were stained with orcein.

N. Drosophila Chromosomal DNA Isolation

Genomic DNA was isolated according to the procedure of Kidd et al. (1983). Approximately 120 adult flies were collected and stored in microcentrifuge tubes at -70°C . The flies were removed from -70°C and 300 μl of solution A (10 mM Tris-HCl, pH 7.5, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine, 5% sucrose) added. The flies were homogenized on ice and to the homogenized sample 300 μl of solution B (1.25% SDS, 0.3 M Tris-HCl, pH 9.0, 0.1 M EDTA, 5% sucrose) was added. This mixture was incubated at 65°C for 45 minutes. After incubation, 90 μl of 8 M potassium acetate was added and the sample was placed on ice for 60 minutes. Debris was pelleted by centrifugation at 10,000 x g for 1 minute in a microcentrifuge. The resulting supernatant was removed and transferred to a new tube and the pellet discarded. To the supernatant two volumes of chloroform was added. The sample was vortexed and centrifuged for three minutes at 10,000 x g. The top, aqueous layer was removed and saved. Two volumes of 100% ethanol was added to the aqueous phase and the nucleic acid pelleted immediately by centrifugation at 10,000 x g for three minutes. The supernatant was aspirated and the pellet dissolved in 200 μl TE2 (10 mM Tris-HCl, pH 8.0, 2 mM EDTA). The nucleic acid was again precipitated by the addition of two volumes of 100% ethanol and pelleted as before. The final pellet was washed with 70% ethanol, dried at 65°C , and resuspended in 100 μl of TE2.

O. Restriction Endonuclease Digestion and Southern Blot Hybridization

Restriction endonuclease digestions were carried out as recommended by the manufacturer. Basically, ten micrograms of high molecular weight chromosomal DNA was digested with 40 units of restriction enzyme, with the appropriate reaction buffer, for 8 to 16 hours at 37°C in the presence of 10 µg of DNase-free RNase. To stop the reactions, samples were heated at 65°C for 5 minutes and 10X DNA loading dye was added to a final concentration of 1X. Samples were subsequently loaded onto an agarose gel and fractionated at a constant voltage of 5 V/cm of gel length. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for visualization and photographed. Southern blotting was performed as described by Southern (1975). For preparation of DNA, the gel was soaked for 8 to 10 minutes at room temperature with gently agitation in 0.25 N HCl. The gel was then rinsed in distilled water and twice immersed in 0.5 N NaOH, 1.5 M NaCl for 20 minutes to permit denaturation of the DNA. The gel was neutralized by soaking it twice in 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl for 20 minutes. While the gel was soaking, the nytran membrane was prepared and the blot apparatus set up. Transfer of the DNA to the membrane was mediated by capillary movement of the solvent 10 X SSC through the gel and membrane and was performed for 16 to 24 hours. The DNA was immobilized to the filter by crosslinking with a Stratagene Stratalinker and

baking in a vacuum oven for one hour at 80°C. This membrane was stored at -20°C for hybridizations at another time. Hybridizations were performed as described with 50% formamide.

P. Polymerase Chain Reaction (PCR) Amplification of the S3 Coding Region from Genomic DNA

To isolate and clone genomic S3 fragments which contain the entire coding region, PCR amplification was employed (Davies et al., 1989). PCRs were performed on 1-2 µg of *Drosophila* chromosomal DNA. The reaction mixture contained 1-2 µg of genomic DNA, 10 µl of 10X PCR Reaction Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), 200 µM each of dATP, dCTP, dGTP, and dTTP, 100 pmoles of each oligonucleotide (dS3-met and dS3-term, see section on thermal cycle amplification S3 cDNA), and 2.5 U of Taq DNA polymerase in a final volume of 100 µl. Each sample was layered with 100 µl of sterile mineral oil, briefly spun, and placed in a programmable thermo-cycler. The cycle program consisted of 1 cycle of 3 minutes at 95°C (denaturation); 1 minute at 60°C (annealing); 3 minutes at 72°C (elongation), 30 cycles of 2 minutes at 95°C; 2 minutes at 60°C; 9 minutes 54 seconds at 72°C, an incubation at 72 C for 10 minutes, and a holding temperature of 18°C. At the completion of the program, 100 µl of chloroform was added to remove the mineral oil from the sample. The tube was mixed and centrifuged at 10,000 x g for 2 minutes and the aqueous phase transferred to another tube.

PCR products were analyzed by agarose gel electrophoresis on a 1.5% gel and visualized with UV light.

Q. Purification of PCR Products and UDG Cloning

Contaminating amplification primers and unincorporated nucleotides were removed using Promega's Magic PCR Preps DNA Purification System. The aqueous phase from the completed PCR reaction (30-300 μ l) was transferred to a new tube and 100 μ l of Direct Purification Buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8 at 25 C), 1.5 mM MgCl₂, 0.1% Triton X-100) was added. This mixture was vortexed briefly and 1 ml of Magic PCR Preps Resin was added. The resin/DNA solution was vortexed three times over a 1 minute period. One disposable 3 ml luer-lock syringe was used with each PCR product. The resin/DNA mix was pipeted into the syringe barrel and the slurry gently pushed into the attached Minicolumn with the syringe plunger. The Minicolumn was then washed with 2 ml of 80% isopropanol. To remove any residual isopropanol, the Minicolumn was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 12,000 x g for 20 seconds. The Minicolumn was transferred to a new microcentrifuge tube and the DNA eluted with 50 μ l of 65°C TE buffer. The heated TE buffer was applied to the resin, allowed to incubate for 1 minute at room temperature, and the Minicolumn centrifuged as above. The collected DNA sample was stored at 4°C or -20°C. This purified DNA fragment was subsequently used for subcloning into the appropriate

vector.

For uracil DNA glycosylase (UDG) cloning, 10-50 ng of the PCR product, 25 ng of the pAMP 1 vector DNA (25 ng/ μ l), and 1 U of UDG (1 U/ μ l) were mixed in a final volume of 20 μ l. These components were subsequently incubated at 37°C for 30 minutes. After annealing, one-tenth of the reaction was used for transformation. Transformants were analyzed for recombinant plasmid DNA and inserts sequenced.

R. RNA Extraction and Northern Analysis

Total cellular RNA was isolated using a modification of a procedure previously described (Chomczynski and Sacchi, 1987). *Drosophila* from various stages of development were collected and stored at -80°C until needed. These flies (approximately 120 flies) were homogenized in 500 μ l of 4 M guanidinium thiocyanate (GIT) buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarcosyl and 0.1 M 2-mercaptoethanol). The homogenate was extracted with 50 μ l 2 M sodium acetate, pH 4.0, 500 μ l phenol, and 100 μ l chloroform:isoamyl alcohol 49:1, mixed, and incubated on ice for 15 minutes. The sample was centrifuged at 10,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and the nucleic acid precipitated by the addition of an equal volume of isopropanol and incubated on dry ice for 30 minutes. The nucleic acid was pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C. The isopropanol was

aspirated and the resulting pellet resuspended in 100 μ l (dependent on size of pellet) of GIT. The pellet was fully dissolved by heating to 65°C and occasionally mixing. Nucleic acid was precipitated by the addition of 0.1 volume 3 M sodium acetate, pH 5.2 and an equal volume of isopropanol, and by incubating on dry ice for 15 minutes. The nucleic acid was pelleted. This pellet was washed with 500 μ l 70% ethanol, dried at 65°C for 2 minutes, and resuspended in 200 μ l of diethylpyrocarbonate (DEPC, 0.2%) treated water. Again the pellet was dissolved by heating to 65°C. The final RNA sample was stored at -80°C until needed.

To determine the concentration of RNA in each sample, A_{260} absorbance was performed using 5 μ l. For electrophoresis, 10 μ g of total RNA was precipitated with 0.1 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol on dry ice for 15 minutes. RNA was pelleted by centrifugation at 10,000 x g at 4°C for 15 minutes, the supernatant aspirated and the nucleic acid washed with 70% ethanol. The resulting pellet was dried at 65°C until all residual ethanol was evaporated. The RNA was resuspended in 20 μ l of RNA Loading Dye (50% formamide; 1X MOPS; 15% formaldehyde; 10% glycerol) and 1 μ l of ethidium bromide (1mg/ml) by heating at 65°C for 15 minutes with intermittent mixing. After incubation at 65°C, the sample was quickly spun down and fractionated on a formaldehyde-agarose gel. Gels were prepared with 1X MOPS buffer (0.2 M MOPS, pH 7.0; 50 mM sodium acetate, 10 mM EDTA),

1.2% ultra pure agarose, and 5% formaldehyde. The formaldehyde was added just prior to pouring the gel. RNA electrophoresis was performed at 80 V in 1 X MOPS buffer. At the completion of electrophoresis, the gel was photographed.

The gel was prepared for transferring (Fourney *et al.*, 1988) by soaking it for two 20 minute periods in 10X SSC at room temperature with gentle shaking. During the gel washing procedure, the membrane was prewet in distilled water for 5 minutes followed by a 5 minute soak in 10X SSC. The RNA was then transferred by capillary action overnight. RNA was subsequently fixed to the membrane by crosslinking using the UV stratalinker and by baking for 1 hour in the vacuum oven at 80°C. The membrane was placed in a sealable bag and stored at -20°C until needed.

8. Mapping the Transcriptional Start Site by 5'RACE

Total RNA was isolated and used for first strand cDNA synthesis with the 5'RACE system (BRL). Total RNA (<1 µg) and MAP oligonucleotides (2 pmoles, S3-MAP, 5' TCAGCTCCCGAATGCGAC 3') were mixed in a final volume of 13 µl (RNase free DEPC-treated water). The tube was placed at 70°C and allowed to incubate for 10 minutes to denature the RNA. The mixture was placed on ice for 1 minute and the contents collected by brief centrifugation. To the tube, 2 µl of 10X synthesis buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl, 25 mM MgCl₂, 1 mg/ml BSA), 1 µl of 10 mM dNTP mix, 2 µl of 0.1 M DTT, and 1 µl of

RNasin were added. The reaction mixture was mixed and the tube incubated at 42°C for 2 minutes before adding 1 μ l of SUPERSCRIPT RT. First strand synthesis was carried out for 30 minutes at 42°C. To inactivate the RT, the components were placed at 55°C for 5 minutes. The contents were collected by brief centrifugation, 1 μ l of RNase H added, and the reaction incubated at 55°C for an additional 10 minutes. At the completion of this step, the tube was either placed on ice to continue or stored at -20°C indefinitely.

Purification of cDNAs was performed using the GlassMAX DNA Isolation Spin Cartridge System (BRL). To begin, 100 μ l/sample of distilled water was equilibrated to 65°C and the binding solution (6 M NaI) was equilibrated to room temperature. 4.5 volumes (95 μ l) of binding solution was added to the first strand reaction. The cDNA/NaI solution was transferred to a GlassMAX Spin Cartridge, and the cartridge capped and centrifuged at 13,000 x g for 20 seconds. The flowthrough was saved until recovery of the cDNA confirmed. To the cartridge, 0.4 ml of cold (4°C) 1X wash buffer was added. The cartridge was centrifuged at 13,000 x g for 20 seconds, the flowthrough discarded, and this wash step repeated two more times. The cartridge was then washed with 400 μ l of cold (4°C) 70% ethanol. To remove all residual ethanol, the cartridge was centrifuged again at 13,000 x g for 1 minute. The spin cartridge was inserted into a fresh recovery tube, 32 μ l of 65°C water added, and the unit

centrifuged at 13,000 x g for 30 seconds to elute cDNA.

Half of the above cDNA sample (16 μ l of the 32 μ l) was transferred to an eppendorf tube and used for the TdT tailing. The cDNA was incubated at 70°C for 10 minutes and then chilled on ice for 1 minute. To the 16 μ l of cDNA, 1 μ l of 10X synthesis buffer, 2 μ l of 2 mM dCTP, and 1 μ l of TdT (10 U/ μ l) were added. The components were mixed and incubated at 37°C for 10 minutes. The TdT was heat inactivated by placing the tube at 70°C for 10 minutes. The contents were collected by brief centrifugation and the tube placed on ice.

Tailed cDNA was amplified directly by PCR without prior purification. The following components were mixed in a 0.5 ml microcentrifuge tube: 34 μ l of sterile water, 5 μ l of 10X synthesis buffer, 1 μ l of 10 mM dNTP mix, 2 μ l of 5'-S3 oligonucleotide (10 μ M; 5'-(CAU)₄CGCCCAGCACCTGCTGGG-3'), 2 μ l of anchor primer (10 μ M), 5 μ l of dC-tailed cDNA, and 1 μ l of Taq DNA polymerase in a final volume of 50 μ l. The contents were mixed and overlaid with 100 μ l of sterile mineral oil. The components were collected by brief centrifugation and subjected to thermal amplification. The polymerase chain reaction (PCR) profile was identical to that one used for amplification of the S3 cDNA (see next section). PCR products were gel purified and subcloned into the pAMP vector using the UDG cloning system.

T. Thermal Cycle Amplification of S3 cDNA

Plasmid DNA containing the S3 cDNA insert was purified using the Qiagen technique. The purified plasmid was linearized by digesting with *ScaI* as detailed by the manufacturer. The digested DNA was subsequently gel purified and the resulting nucleic acid precipitated with 0.1 volume 3 M sodium acetate, 2.5 volumes 100% ethanol at -20°C overnight. This purified, linearized plasmid DNA was used for amplification of the S3 coding region.

To overexpress the protein encoded by S3, a cDNA was prepared for subcloning by PCR. This cDNA was amplified using oligonucleotides designed to the amino-terminus (5'-CGTGGGATCCCCATGAATGCGAACCTTCCG-3'; dS3-met, *Bam*H1 site underlined) and the carboxy-terminus of the S3 protein (5'-CGATGAATTCTTACAAAACCTTTCGCCTCGG-3'; dS3-term, *Eco*R1 site underlined). In addition, these oligonucleotides were synthesized with the appropriate restriction endonuclease sites to enable the directional cloning of the amplified PCR product into the expression vector pGEX-3X (Pharmacia). PCR reactions were carried out with 100 ng of template DNA (linearized plasmid), 100 pmol of each oligonucleotide (dS3-met, dS3-term), 200 μ M of each deoxynucleotide triphosphate, 1 X PCR Reaction Buffer, and 2 U of Taq DNA polymerase in a final volume of 100 μ l. The reaction mixture was layered with 100 μ l sterile mineral oil and subjected to one cycle of 95°C for 3 minutes; 60°C for 1 minute; 72°C for 3 minutes, forty

cycles of 95°C for 30 seconds; 60°C for 1 minute; 72°C for 2 minutes, and a 72°C elongation period for 10 minutes. PCR products were extracted once with phenol/chloroform (1:1), once with chloroform, and precipitated by the addition of sodium acetate and ethanol as above. Nucleic acid was pelleted by centrifugation at 10,000 x g for 15 minutes at 4°C. The resulting DNA pellet was resuspended in sterile water and digested with *Eco*R1 and *Bam*H1. Organic extractions of phenol/chloroform and chloroform were performed, and the nucleic acid precipitated in the presence of 10 µg of yeast tRNA (10 mg/ml). The final pellet was dissolved in 10 µl of T.E. This PCR product was subcloned into the pGEX-3X vector (which had been digested with the same restriction enzymes as the insert, treated with calf intestinal alkaline phosphatase, and gel purified). The ligation mixture was transformed into competent bacteria cells (HB101 for antigen production; RPC503 and MutM for biochemical analysis) and the transformants analyzed for the existence of recombinant DNA molecules.

U. Overexpression of Glutathione S-Transferase:S3 Fusion Protein for Antigen Production and Biochemical Analysis

Colonies containing pGEX-3X:S3 recombinant plasmid DNA molecules were streaked out and used for overexpression experiments. For overexpression, bacterial cultures (50 ml) containing the GST-S3 fusion construct were grown overnight at 37°C in the appropriate media [100 ng/µl ampicillin (pGEX), 50

ng/ μ l kanamycin (HB101 or RPC503)]. Overnight cultures were diluted 1 to 10 in fresh, pre-warmed (37°C) LB medium supplemented with the appropriate antibiotics and grown for 1 hour at 37°C. Expression of the S3 fusion protein was induced by adding isopropyl-*B-D*-thio-galactoside (IPTG) to a final concentration of 0.1 mM and allowing the cells to grow for an additional 3 hours at 37°C. Cells were harvested by centrifugation at 1000 x g for 10 minutes, washed with PBS, pH 7.4, and collected as before. Packed cells were resuspended in 2 ml of PBS, lysed by mild sonication (three 15 second bursts) on ice, and the insoluble proteins pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C. The S3 fusion protein was insoluble. Induction experiments performed at 30°C permitted increased solubility of the S3 fusion protein, but not to a great extent. Therefore, it was necessary to solubilize the S3 fusion prior to purification. The insoluble recombinant proteins (inclusion bodies) were resuspended in 2 ml 0.1 M glycine-NaOH (pH 9.0) containing 8 M urea (Frorath et al., 1992). Proteins were subsequently fractionated from the Urea using spun-column chromatography with Sephadex G-25 equilibrated in 0.1 M glycine-NaOH (pH 9.0). Basically, a 3 CC syringe with glass wool was layered with Sephadex G-25 and the Sephadex packed by centrifugation at 1000 x g for 5 minutes. The sample was applied to the column and spun as above. The passthrough fraction contained the fusion protein and was stored overnight at 4°C to permit

refolding of the protein. The fusion-containing fraction was diluted to 15 ml with PBS containing 1% Triton X-100 and loaded onto a glutathione Sepharose 4B column, pre-equilibrated with PBS. Binding of the GST-S3 protein was carried out within the column for 20 minutes on a neutator at 4°C. The sample was allowed to flow through the column. The column was subsequently washed with 20 column volumes of PBS containing 1% Triton X-100. The fusion protein was eluted with 50 mM Tris, pH 7.5 containing 10 mM glutathione by incubation on the neutator at 4°C for 5 minutes. The sample was then collected from the column and analyzed by SDS-polyacrylamide gel electrophoresis. This protein was used as antigen for antibody production (native).

Protein used for biochemical activity assays was purified from RPC503 or *mutM* bacteria. The above methodology was used for purification of GST-S3 from RPC503 or *mutM*, except that induction with IPTG was carried out for 5 hours. [RPC503 *Escherichia coli* (from Dr. Richard P. Cunningham, SUNY, Albany) mutants lack apurinic/aprimidinic (AP) endonuclease activity and are defective in exonuclease III (*xth*), endonuclease IV (*nfo*), and endonuclease III (*nth*). *mutM E. coli* (from Dr. Jeffery Miller, University of California, Los Angeles) are defective in FaPy-DNA glycosylase activity, the enzyme activity responsible for repair of 8-oxoguanine.]

The factor Xa cutting site, which allows for the removal of the glutathione S-transferase portion of the polypeptide,

was not utilized because it was found to be unnecessary for detecting activity or for antibody production. However, when performed, 1 ml of GST-S3 protein was cut with 2 μ l of factor Xa in the presence of 1 mM CaCl₂ overnight at 4°C.

V. SDS-Polyacrylamide Gel Electrophoresis

Protein samples were solubilized by boiling in 1X SDS sample buffer (2X = 0.125 M Tris-HCl, pH 6.8; 20% glycerol; 2% SDS; 2% B-mercaptoethanol; 0.001% bromophenol blue) for 5 minutes. Debris was pelleted prior to loading onto the gel by centrifuging at 10,000 x g for 2 minutes. The gel unit was cast and the gel poured according to the Bio Rad specifications. For the separating gel, appropriate volumes of 30% acrylamide/0.8% bisacrylamide, 4X Tris-HCl/SDS (1.5 M Tris-HCl containing 0.4% SDS), pH 8.8, and water were mixed. To catalyze polymerization, 50 μ l of 10% ammonium persulfate and 10 μ l of TEMED were added and swirled into solution. Using a pasteur pipet, the separating gel solution was transferred to the glass plate sandwich and layered with isobutyl alcohol. Following polymerization, the isobutyl alcohol was removed, the gel rinsed with distilled water and the stacking gel poured. The stacking gel consists of 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4X Tris-HCl/SDS (0.5 M Tris-HCl containing 0.4% SDS), pH 6.8, and 3.05 ml of water. To this mixture, 25 μ l of 10% ammonium persulfate and 5 μ l of TEMED were added. The stacking gel

solution was slowly added to the top of the separating gel and the comb placed, without introducing air bubbles. The stacking gel was allowed to polymerize, the wells rinsed with distilled water and the electrophoresis unit set up according to specifications. Prepared samples were loaded into the wells and electrophoresed in 1X SDS electrophoresis buffer (0.125 M Tris-HCl; 0.96 M glycine; 0.5% SDS) at a constant current of 15 mA (125 volts) until the Bromophenol Blue tracking dye ran off the gel. The gel was processed accordingly.

W. Staining of SDS-Polyacrylamide Gels

After electrophoresis, gels were transferred to a container and stained with 45% methanol, 10% acetic acid, 0.1% coomassie Blue R-250 for 30 minutes. To destain, gels were rinsed with several washes of 20% methanol, 10% acetic acid. Prior to drying, gels were soaked in 20% methanol, 3% glycerol for at least 30 minutes, preferably overnight. Alternatively, gels were silver stained to further analyze purity of protein fractions.

When silver staining, gels were soaked in 50% methanol (reagent grade)/10% acetic acid for at least 1 hour with 2 to 3 changes of the solution. The gel was rinsed with water for 30 minutes with 3 changes. While the gel was rinsing in water, the following solutions were prepared: solution A, 0.8 g of silver nitrate in 4 ml of water; solution B, mix 21 ml of 0.36% NaOH with 1.4 ml of 14.8 M (30%) ammonium hydroxide; and

solution C, A was added to B dropwise with constant vigorous stirring, allowing brown precipitate to clear. Solution C was brought to a final volume of 100 ml with water. The final water rinse was poured off and the gel stained in solution C for 15 minutes with gentle agitation. The gel was washed twice in deionized water and soaked for 2 minutes as above. Solution D was prepared by mixing 0.5 ml 1% citric acid with 50 μ l 38% formaldehyde in a final volume of 100 ml. The gel was then washed with solution D. Silver stained bands appeared within 10 minutes or the developing solution was changed. The reaction was stopped by rinsing the gel in 1% acetic acid. The gel was washed overnight with several changes of water and dried down.

X. Electroelution of Protein from SDS-Polyacrylamide Gels

For isolation of gel purified (denatured) antigen, column purified GST-S3 fusion protein was fractionated in a 12% SDS-polyacrylamide gel. The fusion protein was excised from the gel and this gel strip placed into dialysis tubing with 2 ml of 1X SDS electrophoresis buffer. The tubing was sealed on both sides and the antigen eluted in 1X SDS electrophoresis buffer at 80 V for 1 hour. The protein was stained prior to electroelution for visualization if needed.

Y. Western blotting

Gels, Whatman, and membranes were soaked in

electroblotting buffer (25 mM Tris-HCl; 193 mM glycine; 20% methanol) for 15 minutes prior to transferring. Proteins separated on SDS-polyacrylamide gels, were transferred to nitrocellulose sheets by electroblotting in a Transblot BioRad transfer apparatus in 25 mM Tris, 192 mM Glycine, 20% Methanol at 150 mA (70 V). The transfer was carried out for 1 hour at 4°C. Following protein transfer, the filter was blocked with Blotto [1X TBST (10X TBST = 1.5 M NaCl; 100 mM Tris-HCl, pH 8.0; 0.5% Tween 20; 2% NP-40; 0.2% SDS); 5% Carnation dried milk; 0.02% sodium azide] for 1 hour at room temperature on a rotator. The primary antibody was diluted accordingly in Blotto and incubated with the filter at 4°C overnight. The filter was washed 3 times in 1X TBST for 10 minutes at 4°C. The ¹²⁵I-labelled secondary antibody (Amersham) was diluted (3 μCi/blot) in blocking buffer and incubated with the filter for 2 hours at 4°C. Visualization of the proteins was performed based on the type of secondary antibody utilized.

Z. Production and Purification of Antibodies

An anti-*Drosophila* S3 antibody, anti-dS3, was obtained by injection of rabbits with about 15 μg of each (native and denatured) purified GST-S3 fusion protein preparation (Harlow and Lane, 1988). Animals were initially injected with the antigen in an emulsion containing equal volume of Hunter's TiterMax. Preimmune serum was collected at the time of the first injection using the ear bleed method. Red blood cells

were removed from the serum by incubation at 4°C overnight and centrifugation at 3000 x g for 10 minutes. Serum (the supernatant) was stored in aliquots at -20°C. Rabbits were anesthetized prior to injections and bleeds with ketamine (35mg/kg) and rompun (5 mg/kg). Animals were boosted every 3 to 5 weeks with an emulsion containing an equal volume of antigen and incomplete Freund's adjuvant. At this time, anti-dS3 serum was harvested (5 ml of blood/lb).

For affinity purification of anti-dS3, approximately 5 µg of purified GST-S3 fusion was electrophoresed on a 12% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The region of nitrocellulose which corresponded to the location of the fusion protein was cut into a strip and used to affinity purify the antibody. The filter strip was blocked with Blotto for 1 hour at room temperature and incubated with 3 ml of anti-dS3 serum on a rotator at 4°C overnight. The filter was washed 3 times with 1X TBST for 10 minutes each. To elute the antibody, the strip was covered in a minimal volume of Elution Buffer (Pierce), placed in a damp chamber and incubated for 20 minutes on the rotator at room temperature. The Elution Buffer was passed over the strip several times and transferred to a labeled tube for storage at 4°C. The sample was subsequently dialyzed against PBS, pH 7.4 for 18 hours and termed affinity purified anti-dS3.

AA. Purification of Post-Nuclear and Nuclear Fractions

Following the procedure of Fisher *et al.* (1982), flies were fractionated into post-nuclear (cytoplasm) and nuclear components. All steps were performed at 4°C. Flies collected from any stage were suspended in 10 volumes of Extraction Buffer (50 mM Tris-Cl, pH7.5, 50 mM NaCl, 5 mM MgCl₂, 250 mM sucrose, 1 mM PMSF, 0.5% Aprotinin), homogenized in a dounce homogenizer, and filtered through nylon mesh to produce the filtered crude homogenate (FCH). This homogenate was centrifuged at 1000 x g for 10 minutes and the resulting supernatant saved and termed the post-nuclear supernatant (PNS). The crude nuclear (CN) pellet was resuspended in 5 volumes of the Extraction Buffer by gentle vortexing and the centrifugation repeated. This wash step was repeated and the final nuclear pellet resuspended in 1 volume of 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂. These fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. For the developmental Western, only the PNS and the purified nuclei (N) were fractionated.

BB. Subfractionation of *Drosophila* Nuclei

Embryos of 4 to 24 hours were collected, washed with distilled water, and dechorionated by rinsing with 0.4% NaCl, 0.03% Triton X-100 for 1 minute and with 50% Chlorox for 3 minutes. Embryos were thoroughly washed with distilled water, blotted dry, and weighed. Samples were used immediately or

stored at -70°C for several months. Purified nuclei were used for subnuclear fractionation (Fisher *et al.*, 1982). All steps were performed at 4°C unless otherwise indicated. Purified nuclei were nuclease treated by the addition of DNase I and RNase A to a final concentration of $10\ \mu\text{g/ml}$ and $8\ \mu\text{g/ml}$, respectively, and incubated at 37°C for 15 minutes. The digested nuclei were centrifuged for 10 minutes at $1000\ \times\ g$, the nuclease supernatant removed, and the pellet resuspended in 0.9 volumes of $290\ \text{mM}$ sucrose, $10\ \text{mM}$ Tris-HCl, pH 7.5, and $0.1\ \text{mM}$ MgCl_2 . After resuspension, 0.1 volumes of 20% Triton X-100 was added and the mixture allowed to incubate on ice for 10 minutes. The sample was centrifuged at $1000\ \times\ g$ for 10 minutes and the supernatant saved (TXS). The pellet was resuspended in 0.5 volumes of $100\ \text{mM}$ Tris-Cl, pH 7.5, $290\ \text{mM}$ sucrose, $0.1\ \text{mM}$ MgCl_2 followed by the addition of $2\ \text{M}$ NaCl. After a 10 minute incubation on ice, the sample was centrifuged for 10 minutes at $10,000\ \times\ g$. The supernatant (SS-1) was saved and the pellet extracted again with $1\ \text{M}$ NaCl as above. The centrifugation was repeated and the final pellet was designated the *Drosophila* subnuclear fraction (DSNF). These fractions were analyzed by immunoblotting techniques for the presence of the S3 protein.

CC. Preparation of *Drosophila* Chromatin

Embryos of 4 to 24 hours were collected as detailed previously and purified chromatin isolated (Wu *et al.*, 1979;

Kelly and Hart, 1989). Embryos were plunged into ice-cold buffer A/1 M sucrose [buffer A contains 60 mM KCl, 15 mM NaCl, 1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. All operations were performed at 4°C. Embryos were Dounce-homogenized and the homogenate filtered through one layer of nylon mesh. The filtrate was centrifuged at 480 x g for 9 minutes. The pellet of unbroken cells and chorion debris was discarded. The supernatant was made 0.2% Nonidet P-40, vortexed vigorously and centrifuged for 10 minutes at 4300 x g. The pellet of crude nuclei (CN) was resuspended in buffer A*/1 M sucrose (buffer A* is buffer A without EDTA and EGTA) and centrifuged through a crude gradient prepared from buffer A*/1.5 M sucrose and buffer A*/1.8 M sucrose at 19,600 x g for 20 minutes in a swinging bucket rotor (Beckman SW28). The supernatant was aspirated off, the inner wall wiped of debris, and the purified nuclear pellet resuspended in 10 mM Tris-HCl, pH 7.5, 0.29 M sucrose, 0.1 mM MgCl₂, and 2% Triton X-100. The nuclei solution was incubated on ice for 10 minutes and centrifuged for 10 minutes at 1000 x g. The supernatant was saved and the pellet washed twice with 75 mM NaCl, 24 mM EDTA, pH 8.0. All centrifugations, unless otherwise stated, were performed at 1000 x g for 10 minutes. The second supernatant from the above washes was saved and the pellet of disrupted nuclei washed twice with 10 mM Tris-HCl, pH 8.0 and then in 5 mM

Tris-HCl, pH 8.0. Chromatin was prepared from this pellet by overlaying the sample onto 5 mM Tris-HCl, pH 8.0, 1.7 M sucrose solution and centrifuging at 50,000 x g for 3 hours. The resulting chromatin pellet was washed twice with 10 mM Tris-HCl, pH 8.0 by centrifugation at 12,000 x g for 10 minutes. The final purified chromatin (C) pellet was resuspended in 10 mM Tris-HCl, pH 8.0, quantitated using the Bio-Rad protein assay, and analyzed by SDS-polyacrylamide gel electrophoresis and Western blot studies.

DD. Preparation of Ribosomes and Mitochondria from *Drosophila* Embryos

Purified ribosomal and mitochondrial fractions were isolated according to the standard procedures established by Elkou et al. (1986) and Tomkinson and Linn (1986), respectively. All steps were performed at 4°C. In general, embryos were dounce homogenized in 10 mM Tris-HCl (pH 7.5), 1.0 mM EDTA and 0.25 M sucrose (TE sucrose). The nuclei and cell debris were pelleted by centrifugation at 1000 x g for 10 minutes. The post-nuclear supernatant was centrifuged again as above and the mitochondria were removed from this supernatant by centrifugation at 20,000 x g for 20 minutes. The post-mitochondrial supernatant was retained and is the soluble fraction from which ribosomes are purified. The mitochondrial pellet was washed and resuspended in TE sucrose, loaded onto a 1.0/1.5 M sucrose step gradient in TE, and

centrifuged at 82,000 x g for 60 minutes. Mitochondria were removed from the interface between the sucrose solutions, diluted by the addition of 5 volumes of TE sucrose, and pelleted by centrifugation at 20,000 x g for 20 minutes. These mitochondria were resuspended in TE sucrose and used for Western blot analysis. To isolate ribosomes, the post-mitochondrial supernatant was centrifuged at 100,000 x g for 60 minutes. The supernatant was removed and the pelleted ribosomes were suspended in 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5M KCl and recovered after centrifugation at 100,000 x g for 60 minutes. Ribosomes were resuspended in TE sucrose and used for Western blot analysis.

EE. Determination of Protein Concentration

Protein concentrations for all fractions were determined by the Bio-Rad protein assay as specified by the manufacturer. Equal amounts of protein from each fraction were separated on an SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with affinity purified *Drosophila* S3 antibody. Detection of cross-reacting protein species was performed with ¹²⁵I-anti-rabbit-IgG (Amersham).

CHAPTER IV

RESULTS

A. Cloning and Sequencing of the *Drosophila* S3 Ribosomal Protein cDNA

I have isolated a cDNA which encodes the *Drosophila* ribosomal protein S3 by screening a 5.5 to 7.5 day pupal lambda gt10 library with radioactively labeled rat S3 cDNA. The rat S3 cDNA clone was provided by Dr. Ira Wool, University of Chicago (Chan et al., 1990). Fourteen positive signals were originally identified of which four phage clones containing S3 coding sequences were purified to homogeneity through successive screens. Inserts were subsequently isolated from these four lambda DNA constructs and subcloned into pBluescript SK⁺ (Stratagene) for further analysis. Restriction digestion of these recombinant plasmids with *Eco*R1 (designed to release the S3-containing fragment) was performed to distinguish the largest clone. The complete nucleotide sequence of the largest cDNA insert (approximately 1.0 kb) was determined (Figure 7). Both strands of this cDNA were sequenced in their entirety at least once and various regions were sequenced multiple times, both during initial sequencing studies and during the mutant strain analysis (data not shown). The *Drosophila* clone includes a single open reading

		M N A N L	
1	CCCCCCCCCTTTTCTGCGCACCACGTTTCCGATTTCGACGTCAAAATGAATGCGAACCTT		5 60
6	P I S <u>K K R K</u> F V S D G I F K A E L N E		25
61	CCGATTTCCAAGAAACGCAAGTTCGTTTCCGATGGCATCTTCAAGGCTGAATTGAACGAG		120
		*	
26	F L T R E L A E D G Y S G V E V R V T P		45
121	TTCTGACTCGCGAACTCGCCGAGGATGGCTACTCCGGCGTGGAGGTCCGTGTGACCCC		180
46	S R T E I I I M A T K T Q Q V L G E K G		65
181	TCCCGCACTGAGATCATCATCATGGCCACCAAGACCCAGCAGGTGCTGGGCGAGAAGGGC		240
		*	
66	R R I R E L T A M V Q K R F N F E T G R		85
241	CGTCGCATTCCGGGAGCTGACCGCCATGGTGCAGAAGCGTTTCAACTTCGAGACCGGACGC		300
86	I E L Y A E K V A A R G L C A I A Q A E		105
301	ATTGAGTTGTACGCCGAGAAGGTGGCCGCTCGTGGCCTGTGCGCCATTGCCAGGCTGAG		360
106	S L R Y K L T G G L A V R R A C Y G V L		125
361	TCGCTGAGGTACAAGCTCACCGGAGGACTGGCCGTCGCTCGTGCTGTGCTATGGTGTGCTC		420
126	R Y I M E S G A K G C E V V V S G K L R		145
421	CGCTACATCATGGAGTCCGGAGCCAAGGGCTGCGAGGTGCTCGTGTCCGGCAAACCTGCGT		480
		*	
146	G Q R A K S M K F V D G L M I H S G D P		165
481	GGTCAGCGTGCCAAGTCGATGAAATTCGTCGATGGCCTGATGATCCACTCGGGAGATCCG		540
166	C N D Y V E T A T R H V L L R Q G V L G		185
541	TGCAACGACTATGTCGAGACCGCCACCCGTCATGTGCTCCTGCGCCAGGGAGTGCTTGGT		600
186	I K V K V M L P Y D P K N K I G P K K P		205
601	ATCAAGGTCAAGGTCAATGTTGCCCTACGACCCCAAGAACAAGATCGGCCCAAGAAGCCG		660
206	L P D <u>N V S</u> V V E P K E E K I Y E T P E		225
661	CTGCCCGACAATGTGTCGTTGTTGGAGCCCAAGGAGGAGAAGATCTACGAGACGCCCGAG		720
226	T E Y K I P P P S K P L D D L S E A K V		245
721	ACCGAGTACAAGATCCCCCGCCAGCAAGCCACTGGACGATCTGTCCGAGGCGAAAGTT		780
246	L		
781	TTGTAAACCTAGTTTTACTGTTTCATCCAATGAGCGACTAGCTGAAAGAGTTGCCCTCAA		840
841	TACAACAAAACAACAGA _n		

Figure 7: Nucleotide and deduced amino acid sequence of *Drosophila* ribosomal protein S3 cDNA. Candidate cDNA fragments were sequenced by the dideoxy-chain termination method using the fmol polymerase chain reaction kit as sold by Promega. The *Drosophila* clone (869 nucleotides) includes a single 738 nucleotide open reading frame. A putative nuclear localization signal (KKRK) is overlined. A potential Asn glycosylation site is marked by an underline. Three potential phosphorylation sites are indicated by **BOLD** and an asterisk. Two potential poly(A) addition signals (AAUACA AND AACAAA) are indicated by double underlines.

frame which predicts a protein of 246 amino acids with an estimated molecular weight of 27,470 D. The initiation codon occurs in the context CAAAUGA which conforms to an optimal translation start consensus sequence (A/CAAA/CAUG) (Cavener, 1987; Kozak, 1991). In addition, two potential polyadenylation sequences, AAUACA and AACAAA, exist at the 3' end of the cDNA insert (Birnstiel *et al.*, 1985). Analysis of the primary amino acid sequence indicates that S3 is rich in basic amino acids (pI>10). This feature may explain why the protein migrates with an apparent molecular weight of approximately 32,000 D on SDS-polyacrylamide gels. Moreover, at the carboxy-terminus, 12 of the last 55 residues are proline, a residue found to deter the formation of an alpha helix. The protein contains well defined calcium/calmodulin-dependent protein kinase (R-X-X-S/T) and cAMP-dependent protein kinase (R-X-S/T) phosphorylation sites (Kennelly and Krebs, 1991), a potential Asn glycosylation site (Wold, 1981), as well as a putative targeting sequence for nuclear translocation (KKRK) (Garcia-Bustos *et al.*, 1991; Silver, 1991).

B. Similarity of S3 Ribosomal Proteins from Various Species

Alignment of the amino acid sequences of the S3 ribosomal proteins from human (Zhang *et al.*, 1990; Poque-Geile *et al.*, 1991), rat (Chan *et al.*, 1990) and *Drosophila* reveals the conserved nature of this polypeptide (Figure 8). The

Drosophila encoded S3 protein is approximately 80% identical to both the rat and human ribosomal proteins. Although the overall amino acid sequence of the various S3 molecules is very similar, the *Drosophila* protein is slightly larger in size (246 amino acids as compared to 243 amino acids for human and rat) and is quite divergent at the carboxy-terminus and, to a lesser extent, the amino-terminus. In addition, the *Drosophila* gene product is the only protein species to contain a potential domain for Asn glycosylation. Interestingly, while the *Drosophila* and the human protein species have a consensus nuclear localization signal (KKRK), the rat S3 protein possesses an altered sequence (KNRK). The three putative phosphorylation sites depicted are a feature shared by the S3 gene products from each organism.

C. Sequence Similarities to the Yeast NUC2 Gene Product

Recently, it was discovered that ribosomal protein S3 of each organism possesses a region of homology to a yeast nuclease, Nuc2 (Dr. Burbee, personal communication at the Nuclease meetings Tamaroon, CO). These two proteins display 56% similarity across a nine amino acid stretch and 50% similarity over a 24 amino acid domain (Figure 9).

D. Southern Blot Analysis of *Drosophila* Chromosomal DNA

To determine the copy number of the S3 gene, Southern blot analysis, using the full length S3 cDNA as a probe, was

Human	1	MAVQISKKRKFVADGIFKAELNEFLTRELAEDGYSGVEVRVTPTRTEIII	50
Rat		<u>N</u>	
Dros	1	MNANLP S S	
Human	51	LATRTQNVLGEKGRRIRELTAVVQKRFGFPEGSVELYAEKVATRGLCAIA	100
Rat			
Dros		M K Q M N ET RI A	
Human	101	QAELRYKLLGGLAVRRACYGVLRFIMESGAKGCEVVVSGKLRGQRAKSM	150
Rat			
Dros		S T Y	
Human	151	KFVDGLMIHSGDPVNYVDTAVRHVLLRQGVLGIVKIMLPWDPTGKIGP	200
Rat			S
Dros		C D E T V Y KN	
Human	201	KKPLPDHVSIVEPKDEILPTTPISEQGGKPELPAMPQPVPTA	243
Rat			P
Dros		N V E KIYE ET Y IPP SK LDDLSEAKVL	246

Figure 8: Comparison of the predicted amino acid sequences of the human, rat, and *Drosophila* S3 proteins (Wilson et al., 1993). This comparison was derived using the GCG Sequence Analysis Software Package and the compare program. Amino acid differences in the rat and *Drosophila* proteins are indicated below the human S3 sequence. A putative nuclear localization signal for the human and *Drosophila* protein, which is missing for rat, is overlined. The potential Asn glycosylation site for the *Drosophila* S3 protein, which is absent from rat and human, is marked by an underline. The three potential phosphorylation sites, which are present in each of the proteins, are indicated by **BOLD** and an asterisk. The *Drosophila* S3 protein is 246 amino acids while the rat and human protein species are 243 amino acids.

RAT S3	ATR <u>TQ</u> <u>N</u> <u>V</u> <u>L</u> G	-- X8	--	L <u>T</u> AVVQ <u>K</u> RFGFPEGSVRLY <u>A</u> EKVA
HUMAN	ATR <u>TQ</u> <u>N</u> <u>V</u> <u>L</u> G	-- X8	--	L <u>T</u> AVVQ <u>K</u> RFGFPEGSVRLY <u>A</u> EKVA
DROS	AT <u>K</u> T <u>Q</u> <u>Q</u> <u>V</u> <u>L</u> G	-- X8	--	L <u>T</u> AVVQ <u>K</u> RFGFPEGS <u>V</u> ELY <u>A</u> EKVA
YEAST	ATR <u>TQ</u> <u>D</u> <u>V</u> <u>L</u> G	-- X8	--	L <u>T</u> LLVQ <u>K</u> RFKYAPGTIVLY <u>A</u> ERVQ
NUC2	<u>S</u> <u>L</u> <u>Y</u> <u>S</u> <u>I</u> <u>D</u> - <u>L</u> <u>G</u>	-- X11	--	<u>V</u> <u>T</u> <u>L</u> <u>P</u> <u>L</u> <u>L</u> <u>K</u> <u>R</u> <u>P</u> <u>N</u> - <u>S</u> <u>R</u> <u>S</u> <u>T</u> <u>D</u> <u>V</u> <u>V</u> <u>F</u> <u>A</u> <u>S</u> <u>T</u> <u>S</u> <u>T</u>
YEAST S3	ATR <u>TQ</u> <u>D</u> <u>V</u> <u>L</u> G	-- X8	--	L <u>T</u> LLVQ <u>K</u> RFKYAPGTIVLY <u>A</u> ERVQ
NUC2	<u>S</u> <u>L</u> <u>Y</u> <u>S</u> <u>I</u> <u>D</u> - <u>L</u> <u>G</u>	-- X11	--	<u>V</u> <u>T</u> <u>L</u> <u>P</u> <u>L</u> <u>L</u> <u>K</u> <u>R</u> <u>P</u> <u>N</u> - <u>S</u> <u>R</u> <u>S</u> <u>T</u> <u>D</u> <u>V</u> <u>V</u> <u>F</u> <u>A</u> <u>S</u> <u>T</u> <u>S</u> <u>T</u>

Figure 9: Sequence similarities of *Drosophila* S3 protein with a yeast nuclease, Nuc2. A region of homology shared by the S3 protein from several organisms and Nuc 2 is depicted. Similar amino acids are indicated with an underline and identical residues signified with an overline.

performed under stringent conditions on high molecular weight chromosomal DNA isolated from *Drosophila* and digested with various restriction enzymes. *Bam*H1, *Eco*R1, and *Pst*1, none of which cleaves the S3 cDNA, generated S3-containing DNA fragments ranging from 4.0 to 4.7 kb, whereas *Hind*III, which cleaves the S3 cDNA once, generated two fragments of 3.3 kb and 0.7 kb (Figure 10). In addition, the combination of either *Bam*H1/*Pst*1 or *Eco*R1/*Pst*1 produced a single S3-containing DNA fragment of slightly greater than 2.0 kb. The results obtained from the various other restriction digestion experiments were consistent with the above findings and permitted the construction of a preliminary restriction map (Figure 13). This information suggests that the S3 transcriptional unit is contained within a region of no more than 2.0 to 3.0 kb and that *Drosophila* S3 is probably encoded by a single gene. Moreover, since S3 cDNA sequences hybridized to only a single cytological locus, it was hypothesized that the S3 gene is a single-copy gene. Supportive evidence regarding the S3 copy number was obtained from experiments executed in another laboratory (Andersson et al., personal communication). Here, researchers found that a P-element insertion into the S3 loci at position 94F/95A, as determined by sequence analysis, resulted in a *Minute* phenotype. Mobilization of this single P-element reverted the flies back to a wild-type phenotype. Thus, the P-element mutagenesis studies, which were found to produce a *Minute*

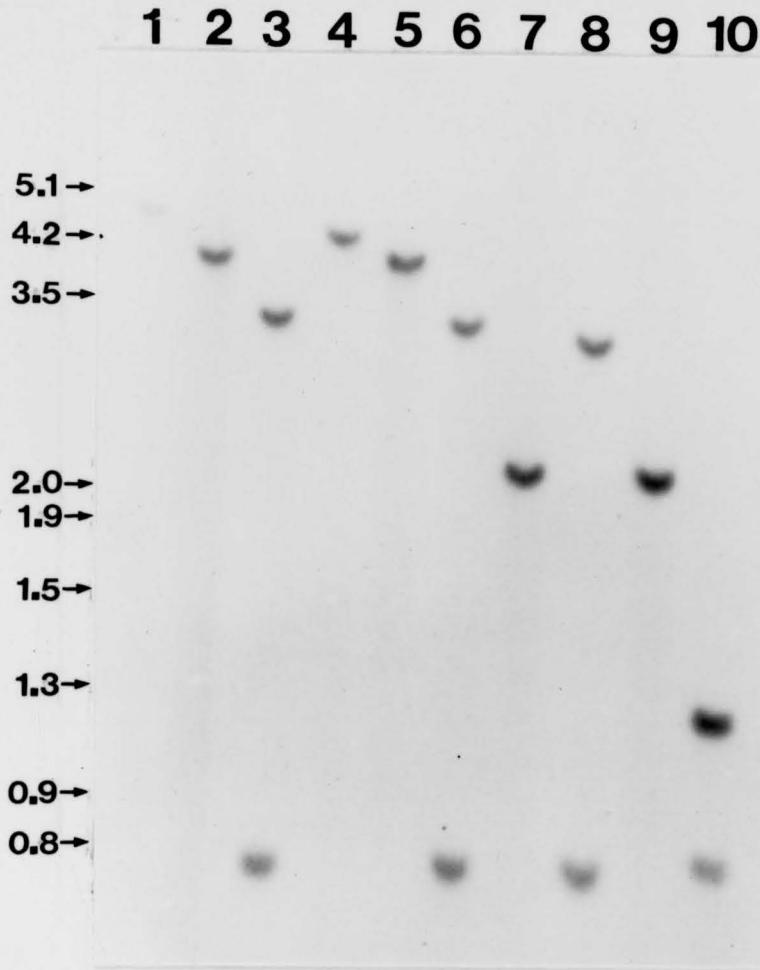


Figure 10: Restriction endonuclease digestion of *Drosophila* high molecular weight chromosomal DNA and Southern blot analysis with full length *Drosophila* S3 cDNA. Genomic DNA (10 ug) isolated from adult *Drosophila* flies was digested with *Bam*H1 (lane 1), *Eco*R1 (lane 2), *Hind*III (lane 3), *Pst*I (lane 4), *Bam*H1 and *Eco*R1 (lane 5), *Bam*H1 and *Hind*III (lane 6), *Bam*H1 and *Pst*I (lane 7), *Eco*R1 and *Hind*III (lane 8), *Eco*R1 and *Pst*I (lane 9), or *Hind*III and *Pst*I (lane 10), electrophoresed on a 0.85% agarose gel, blotted to Nytran and probed with labeled *Drosophila* S3 cDNA.

phenotype by disruption of the ribosomal protein gene S3, suggest that this gene is, in fact, a single-copy gene. However, the possibility of a tandem duplication event cannot be eliminated.

E. Chromosomal Localization of the S3 Gene

In situ hybridization of S3 cDNA sequence to *Drosophila* polytene chromosomes was able to detect only a single location, 94F/95A, for the S3 gene (Figure 11B). To verify the location of S3 on chromosome 3R, *in situ* hybridization was performed simultaneously with a known marker, the Hsp70 gene, which maps to regions 87A and 87C (Figure 11A; Lis *et al.*, 1978; Livak *et al.*, 1978; Schedl *et al.*, 1978). Recently, the mapping of S3 to region 94F has been confirmed by studies using P-element induced mutation (Andersson *et al.*, personal communication). Interestingly, this region had previously been identified as a strong *Minute* (Ferrus, 1975). Minutes are a class of genes that, when present in the hemizygous condition, produce a characteristic phenotype consisting of short slender bristles and delayed development. At least one other *Minute* locus has been shown to encode a ribosomal protein (Kongsuwan *et al.*, 1985). Presumably, most if not all of this group of over 50 phenotypically similar *Drosophila* mutations affect ribosomal protein genes.

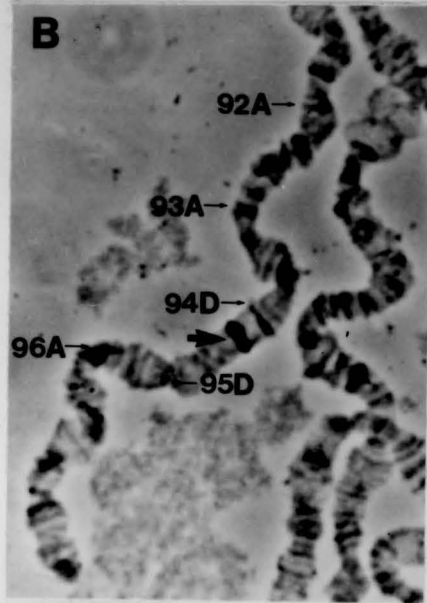
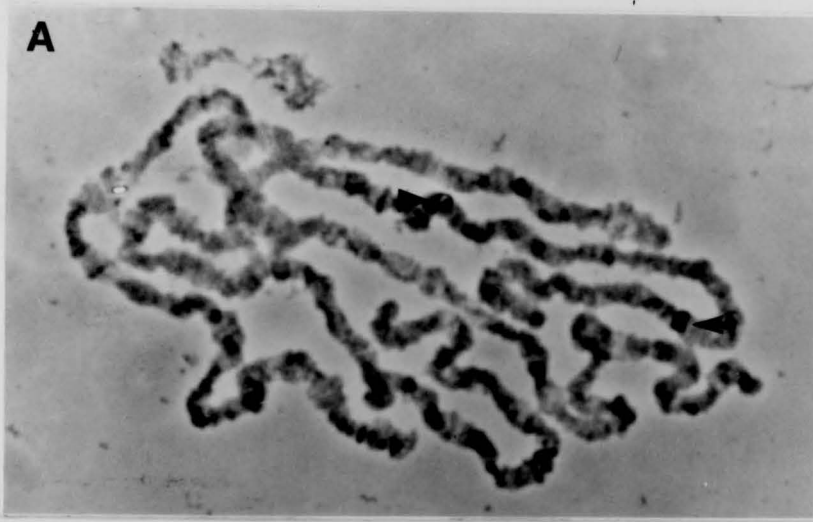


Figure 11: *In situ* hybridization to polytene chromosome squashes from third instar larvae with biotinylated *Drosophila* S3 cDNA. Isolated S3 fragment was biotinylated using the random-priming method and hybridized to a single region, 95A, on chromosome 3R as shown by the arrows with tails (panels A and B). The Hsp70 clone, which maps to positions 87A and 87C on chromosome 3, was used as a localization marker and is indicated by the arrowhead (panel A). Also depicted are other cytological positions (panel B).

F. Genomic Organization of the *Drosophila* S3 Gene

Genomic fragments encompassing the *Drosophila* S3 translational open reading frame were isolated by PCR amplification of chromosomal DNA using specific oligonucleotides. These oligonucleotides were generated to the C-terminal and N-terminal amino acids of the S3 protein predicted from the open reading frame of the previously characterized cDNA. PCR products were subsequently subcloned into pAMP 1 (BRL) and sequenced (Figure 12). The genomic organization (Figure 13) was determined based upon comparison analysis with sequence data obtained from the aforementioned cDNA (Figure 7). The S3 gene consists of two exons and a single intron of 263 nucleotides in length. This intron is located between nucleotides 36 and 37 of the coding region and contains consensus splice site junctions (donor site, gt; acceptor site, ag). Thus, exon1 encodes the first 12 amino acids of the *Drosophila* S3 ribosomal protein and exon2 encodes the remaining 234 residues.

In humans, a small nucleolar RNA (U15A) gene resides within the first intron of the human gene encoding ribosomal protein S3 (Tycowski et al., in press). However, the *Drosophila* intron shows no sequence homology to U15A or to any other known RNA molecule.

The transcriptional start site was determined using the 5' Rapid Amplification cDNA Ends (RACE) system (BRL)..

5' Untranslated CTTTTCTGCGCACCACGTTTCCGATTCGACGTCAA

Exon1

1 ATGAATGCGAACCTTCCGATTCCAAGAAACGCAAG 36

Intron1

gttaattggggaaaaatgagacaggattacctccggcatgtaatgcagtgccgtacggttcggttgt
ttcatcggagttgtgtgaaaaacccggccggaaagcgcgaaaatccatcgggttcggagttaacc
tcaaagcagatggcgctcccaaaaactttggaactcaacaacgcgtggtgaaacaacaacaaca
gaaggaggattgctaggtgtgcaatagatgtggaatgcggattataatggtttctttttgctccc
cag (263)

Exon2

37 TTCGTTTCCGATGGCATCTTCAAGGCTGAATTGAACGAGTTCCTGACTCGCGAACTCGCCC
AGGATGGCTACTCCGGCGTGGAGGTCGGTGTGACCCCTCCCGCACTGAGATCATCATG GCC
ACCAAGACCCAGCAGGTGCTGGGCGAGAAGGCCGTCGCATTCGGGAGCTGACCGCCATGGTGCA
GAAGCGTTTCAACTTCGAGACCGGACGCATTGAGTTGTACGCCGAGAGGTGGCCGTGGCC
TGTGCGCCATTGCCAGGCTGAGTCGCTGAGGTACAGCTCACCGGAGACTGGCCTCCGTCGT
GCTTGCTATGGTGTGCTCCGCTACATCATGGAGTCGGGAGCCAAGGGCTGCGAGGTCGTGCTG
CGGCAAACTGCGTGGTCAGCGTGCCAAGTCGATGAAATTCGTGATGGCCTGATGATCCACTCGG
GAGATCCGTGCAACGACTATGTCGAGACCGCCACCGTCATGTGCTCCTGCGCCAGGGAGTGCTTG
GTATCAAGGTCAAGGTATGTTGCCCTACGACCCCAAGAACAAGATCGGCCCAAGAAGCCGCTG
CCGACAATGTGTCCGTTGTGGAGCCCAAGGAGGAGAAGATCTACGAGACGCCCGAGACCGAGTA
CAGATCCCCCGCCAGCAAGCCACTGGACGATCTGTCCGAGGCGAAAGTTTTGTAA 738

ACCTAGTTTTACTGTTTCATCCAATGAGCGACTAGCTGAAAGAGTTGCCCTCAAATACAACAAAAC
AACAGAAACAAAAAATAAAAAA 3' Untranslated

Figure 12: The genomic sequence was determined using the fmol polymerase chain reaction kit from Promega. Exon nucleotide sequence (translated and non-translated) is in capital letters whereas the intron sequence is in lowercase type. Numbers 1-738 represent the translational coding region. Consensus splice site junctions are indicated with an underline. A potential (polymorphic) *Hind*III restriction site is boxed.

Drosophila melanogaster S3 gene

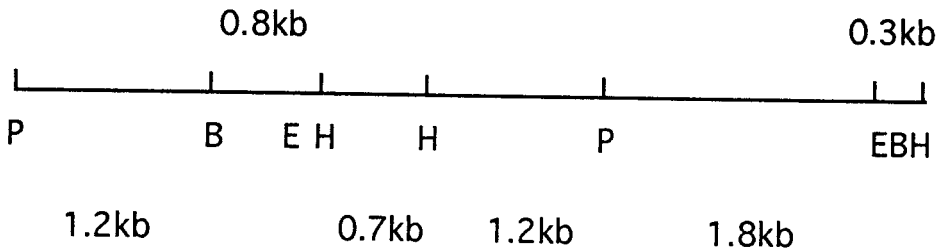
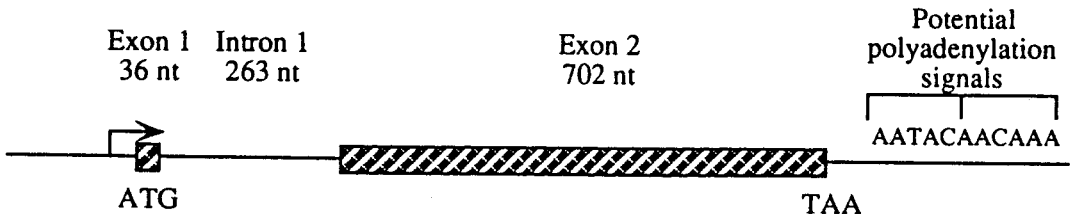


Figure 13: Schematic representation of the genomic organization of the *Drosophila* S3 gene as determined by sequence analysis. The transcriptional start site, two exons (hatched box), the one intron, and the nucleotide lengths of each are indicated. The translational start and stop condons as well as the potential polyadenylation signals are also displayed. In addition, a preliminary restriction map was devised based on Southern blot analysis (B=*Bam*H1; E=*Eco*R1; H=*Hind*III; P=*Pst*1).

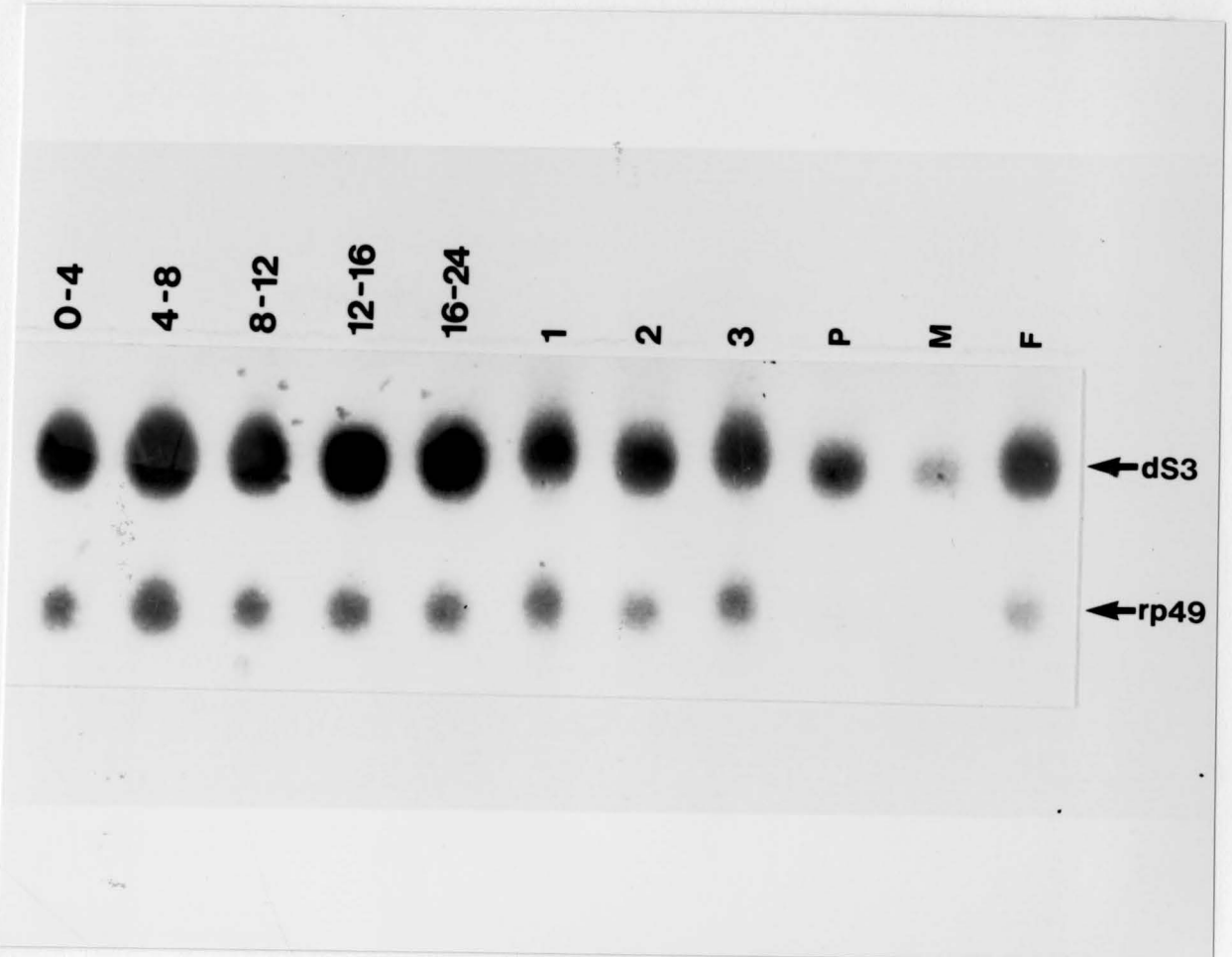
Oligonucleotides were designed to known internal sequences of the S3 mRNA in order to ultimately clone the 5' region of this transcript. PCR amplification was performed on prepared *Drosophila* cDNA and the resulting product subcloned into pAMP 1 (BRL) and sequenced. The 5' start site was found to initiate at a cytosine (Figure 12).

Southern blot analysis indicates that *Hind*III cleaves within the S3 coding region (Figure 10). However, sequence analysis of the S3 gene did not uncover a *Hind*III restriction site, indicating that this site may be polymorphic.

G. Developmental Expression Pattern of the S3 Transcript

Using the full length S3 cDNA as a probe, a single mRNA species of approximately 1.0 kb was detected by Northern blot analysis of total RNA prepared from each stage of *Drosophila* development (Figure 24). The presence of the S3 transcript in the 0-4 hour embryo RNA indicates that this mRNA is maternally inherited. Moreover, S3 gene expression appears to be constitutive during the *Drosophila* life cycle as no obvious change in the level of mRNA expression was observed. The reduced levels of S3 mRNA in pupae and adult male are a result of a loading artifact which was evident when using rp49 gene sequences as a control for normalization (O'Connell and Rosbash, 1984). In addition, experiments comparing adult male and female S3 transcript levels were repeated and confirm that

there is no significant difference in the quantities of S3



antibody production in rabbits (Figure 15).

The integrity and specificity of anti-ds3 antibody was determined by Western blot analysis (data not shown). Pre-immune serum was compared to serum isolated after initial

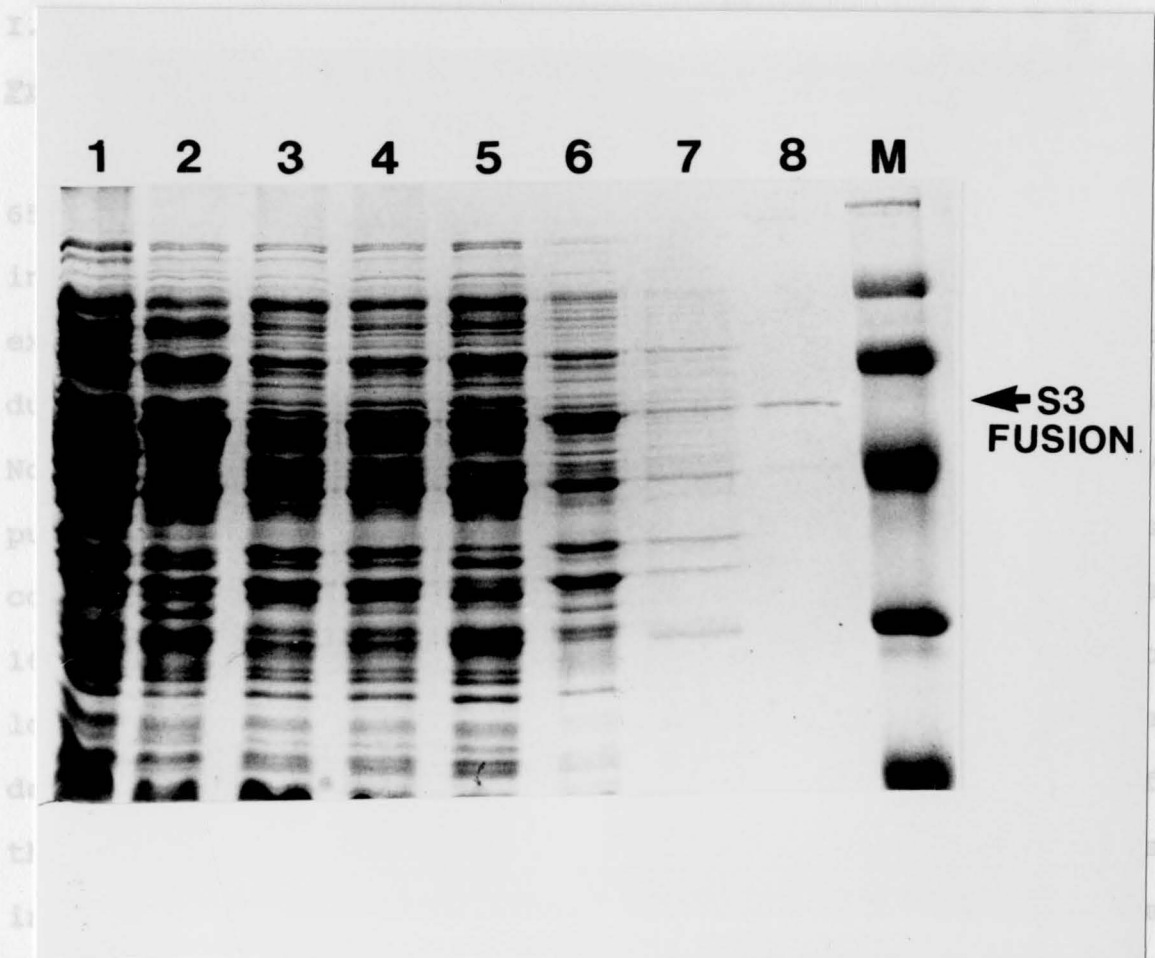
Figure 14: Developmental Northern blot analysis for the *Drosophila* S3 transcript. Total RNA (10 ug) was extracted from 0-4 hr embryos, 4-8 embryos, 8-12 hr embryos, 12-16 hr embryos, 16-24 hr embryos, first instar larvae, second instar larvae, third instar larvae, pupae (mixed population), adult male and adult female using the Guanidinium-thiocyanate procedure and fractionated on an 1.2% agarose-formaldehyde gel. RNA was subsequently transferred to Nytran and probed with radiolabeled *Drosophila* S3 cDNA fragments. The *rp49* clone was used for normalization.

there is no significant difference in the quantities of S3 mRNA (data not shown). Furthermore, Northern blot analysis of total RNA prepared from head, thorax, and abdomen of mixed populations detected no difference in S3 mRNA levels or specificity in S3 gene expression (data not shown).

H. Purification of S3 Fusion Protein and Antibody Production

Fragments containing the S3 open reading frame were obtained by PCR amplification of full length S3 cDNA with specific oligonucleotides generated to the nucleotide sequences of the amino and carboxy terminal 4 amino acids of S3. These fragments, which contained the appropriate restriction sites, were subsequently subcloned into the expression vector pGEX-3X for overproduction of a glutathione S-transferase S3 fusion protein. Affinity purified S3 fusion protein was used for DNA repair activity assays as well as for antibody production in rabbits (Figure 15).

The integrity and specificity of anti-dS3 antibody was determined by Western blot analysis (data not shown). Pre-immune serum was compared to serum isolated after initial injections of S3 fusion protein preparations and indicated that specific polyclonal antibodies were raised to this protein (data not shown). These antibodies were subsequently affinity purified and used to neutralize S3 repair activity and to determine S3 protein localization by Western analysis.



was approximately a 70% (post-nuclear) to 30% (nuclear) distribution ratio of the S3 protein (Figure 17). However,

Figure 15: Coomassie blue stained SDS-polyacrylamide gel of purification steps designed to obtain purified *Drosophila* S3 fusion protein. *Drosophila* S3 protein was overexpressed and purified as a glutathione S-transferase (GST) fusion using the pGEX-S3 system. Crude extracts (25 ug) from HB101, GST-S3 containing cells, IPTG induced (lane 1) and non-induced (lane 2), and from RPC503, GST-S3 containing cells, IPTG induced (lane 3) and non-induced (lane 4) were obtained. Fractions were isolated from RPC503 procedure to monitor the purification scheme. These fractions include, soluble protein following sonication of cells (lane 5, 25 ug), insoluble protein following sonication (lane 6, 10 ug), glutathione column passthrough (lane 7, 1 ug), and column purified GST-S3 (lane 8, 100 ng). Similar results were obtained after silver staining gel.

I. S3 Protein Expression in Post-Nuclear and Nuclear Fractions During Development

Extracts from *Drosophila* embryos (4-24 hour), larvae (48-65 hour), pupae (mixed population), and adults were separated into post-nuclear and nuclear fractions to analyze the expression pattern and localization of the S3 gene product during development. Consistent with the developmental Northern blot analysis, Western blot studies using affinity purified anti-dS3 revealed that the S3 protein was constitutively expressed throughout the life cycle (Figure 16). Interestingly, a portion of the S3 protein was found to localize to the nucleus, the amount being dependent on the developmental stage of *Drosophila*. Densitometric scanning of the autoradiographs from the developmental immunoblots indicated that during embryonic, larval and adult stages there was approximately a 70% (post-nuclear) to 30% (nuclear) distribution ratio of the S3 protein (Figure 17). However, during the pupal stage there was approximately a 1:1 ratio. For normalization, extracts containing equal amounts of protein, as determined by the Bio-Rad Protein Assay, were fractionated and transferred. Moreover, the comparable SDS-polyacrylamide gel showed similar levels of protein staining for each sample (data not shown). These procedures for normalization of protein loading were employed for all subsequent immunoblot studies.

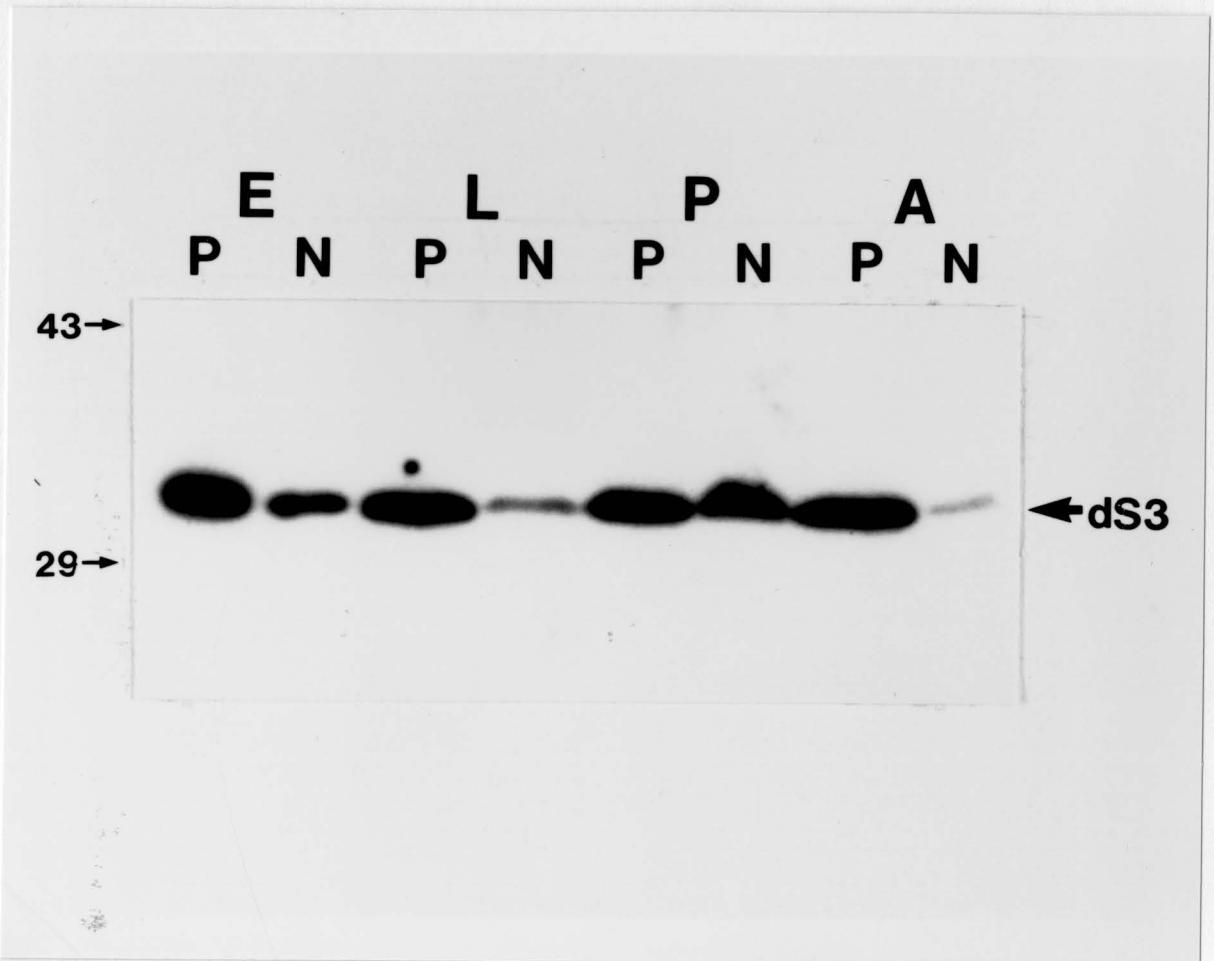


Figure 16: Western blot analysis of *Drosophila* S3 protein in post-nuclear and nuclear fractions from various developmental stages. Post-nuclear (PNS) and nuclear (N) protein (100 ug) from the various *Drosophila* developmental stages [E=embryo (4-24 hour), L=larvae (48-65 hour), P=pupae (mixed population), and A=adult] was fractionated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. The level of S3 protein expression was determined using affinity purified polyclonal antibody generated to purified glutathione-S-transferase:S3 fusion protein. S3 protein was detected with ^{125}I -labeled anti-rabbit IgG secondary antibody.

S3 Protein Expression

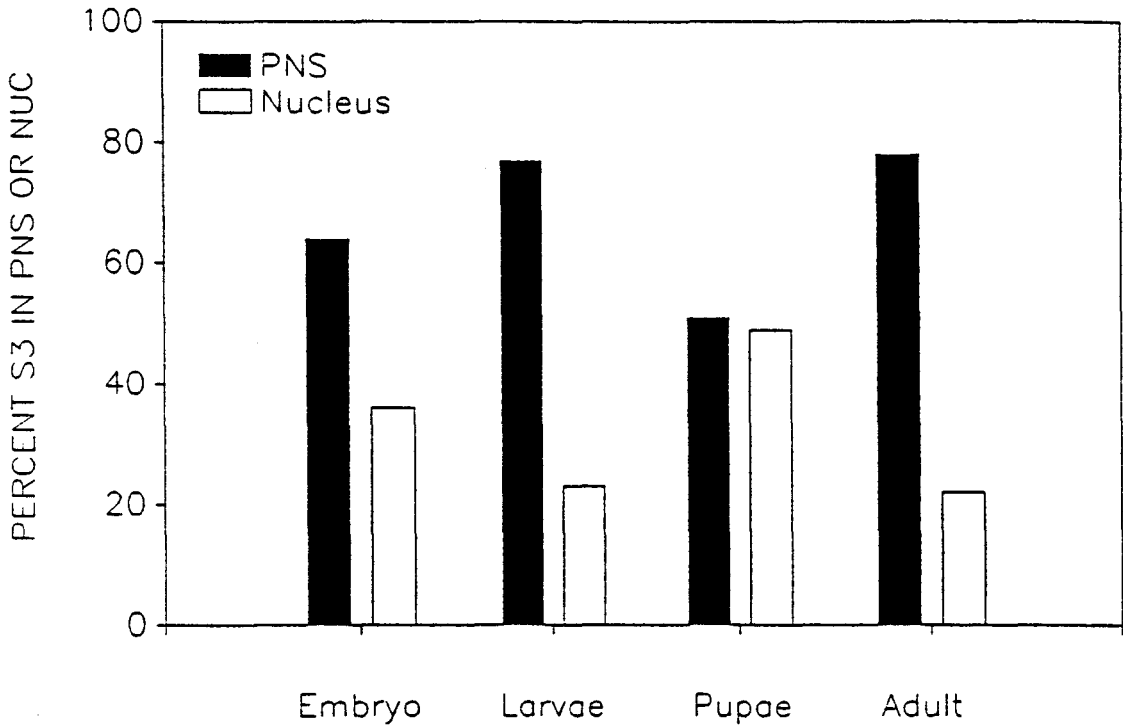


Figure 17: Graphic presentation of the relative abundance of post-nuclear and nuclear S3 protein during the indicated developmental stages. The autoradiograph of the above western blot was densitometrically scanned and the percentage of S3 protein in the post-nuclear and nuclear fractions of each stage was determined.

J. Sub-Cellular Localization of the S3 Gene Product

Drosophila 4-24 hr embryos were fractionated into subcellular components to verify the location of the S3 protein in ribosomes. Subcellular extracts were analyzed by Western blot studies using affinity purified antibody generated against S3 fusion proteins. These experiments, as expected, clearly demonstrated that the *Drosophila* S3 gene product is found in the cytoplasm and in association with ribosomes (Figure 18). S3 protein was not found in purified mitochondrial preparations.

K. Sub-Nuclear Localization of the S3 Gene Product

To address the possibility that ribosomal protein S3 functions in DNA metabolism, sub-nuclear fractions of *Drosophila* 4-24 hour embryos were obtained. Using affinity purified *Drosophila* S3 antibody, antibody cross-reacting material was detected in a nuclear matrix preparation via Western blot analysis (Figure 19). Moreover, topoisomerase II was also serologically identified in this nuclear matrix preparation (Berrios et al., 1985). Unfortunately, I was unable to obtain an antibody which could be used as a negative control; that is, antibody which recognizes a protein known not to associate with the nuclear matrix.

To further characterize the presence of the *Drosophila* S3 protein in the nucleus, 4-24 hr embryos were fractionated into

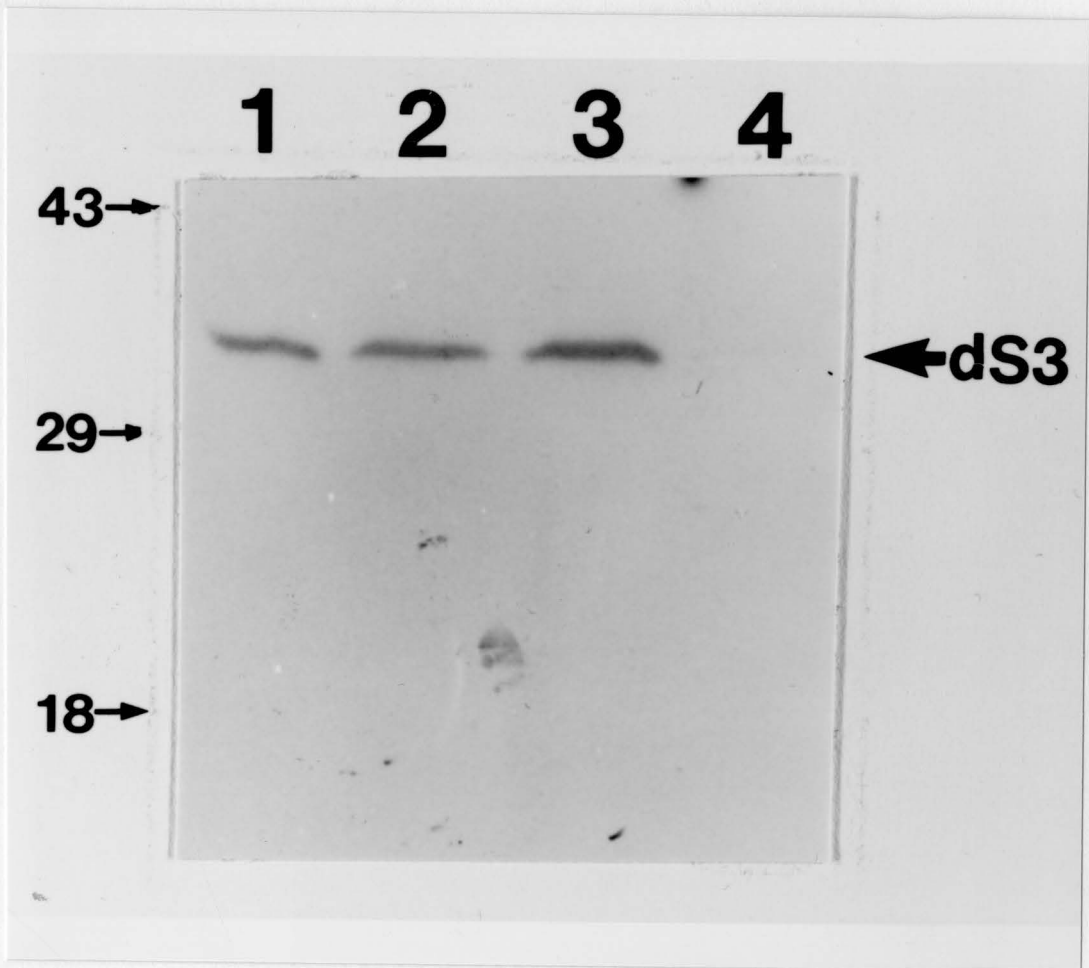


Figure 19: Western blot analysis of a nuclear matrix preparation to determine sub-nuclear localization of the *Drosophila* S3 gene product. Embryos (4-24 hour) extracts were separated into filtered crude lysosomate (PCM, 10 ug), post-

Figure 18: Western blot analysis to determine sub-cellular localization of the *Drosophila* S3 gene product. *Drosophila* embryos were fractionated into post-nuclear supernatant (lane 1), cytosol (lane 2), purified ribosomes (lane 3), and purified mitochondria (lane 4). Equal concentrations of the above protein samples (50 ug) were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Anti-dS3 cross-reacting material was visualized by probing with ^{125}I anti-rabbit IgG secondary antibody and autoradiography. on the same blot following anti-dS3 probing.

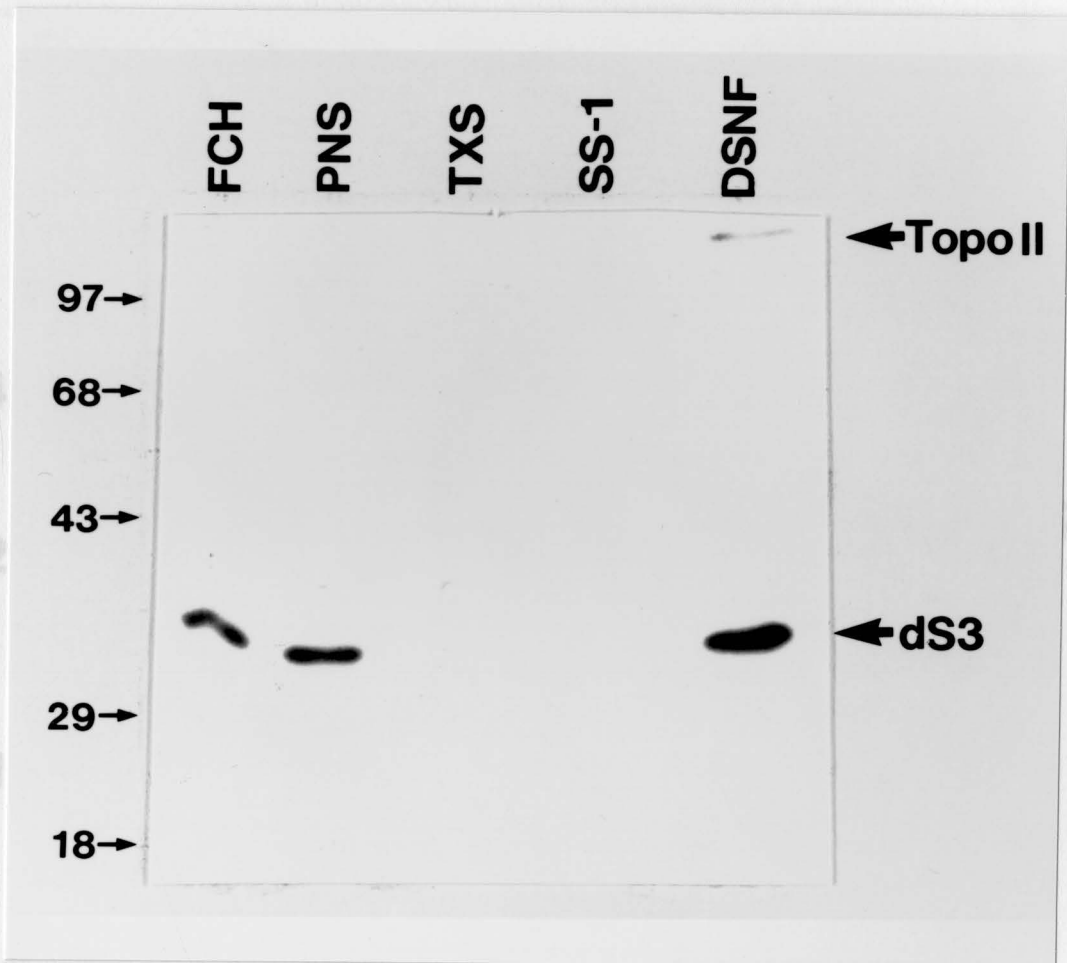
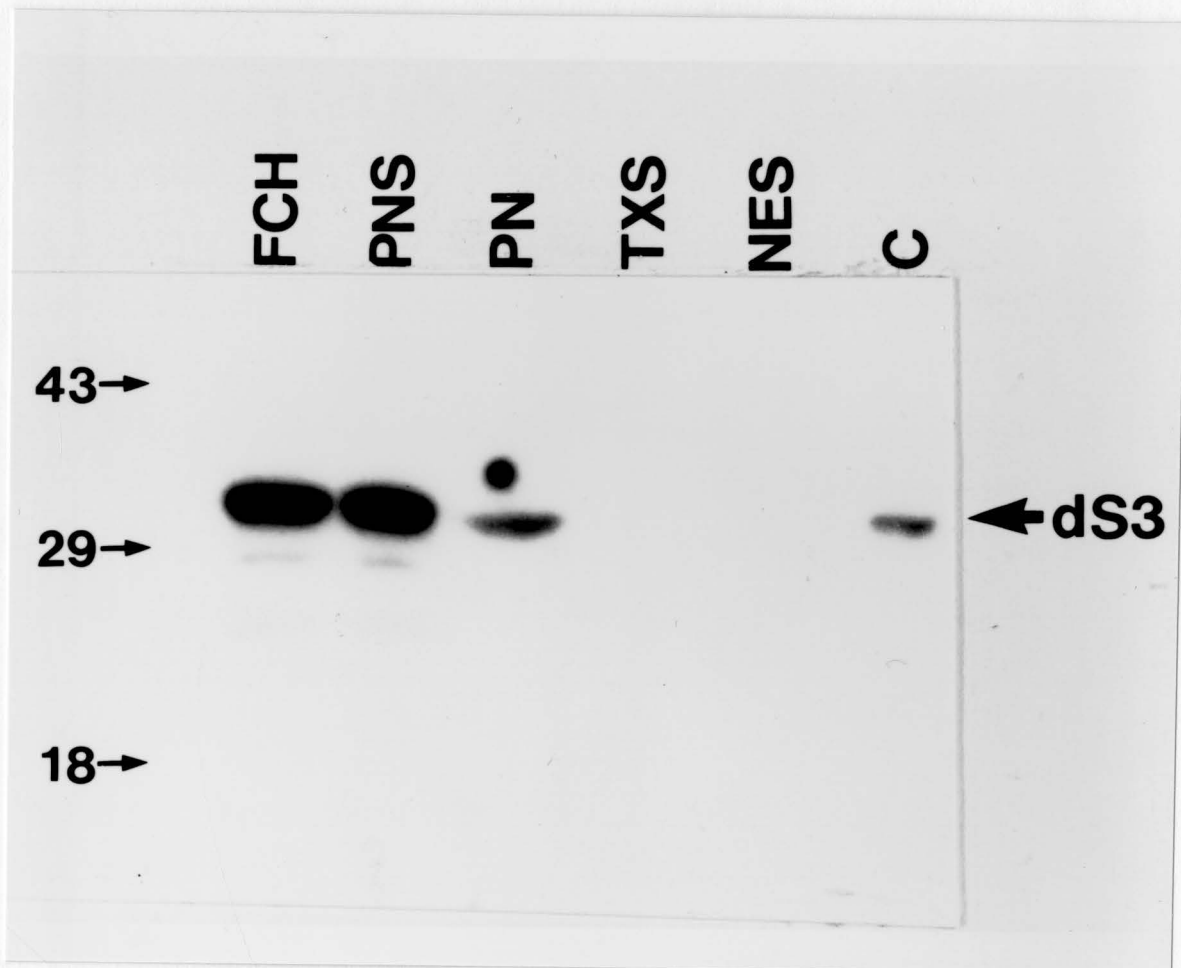


Figure 19: Western blot analysis of a nuclear matrix preparation to determine sub-nuclear localization of the *Drosophila* S3 gene product. Embryo (4-24 hour) extracts were separated into filtered crude homogenate (FCH, 10 ug), post-nuclear supernatant (PNS, 10ug), supernatant after extraction with Triton X-100 (TXS, 10 ug), supernatant after extraction with 1 M NaCl (SS-1, 10 ug), and the *Drosophila* subnuclear fraction (DSNF, 10 ug) and analyzed. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. The S3 protein was detected with affinity purified antibody to the *Drosophila* S3 polypeptide and ¹²⁵I anti-rabbit IgG secondary antibody. Antibody generated against topoisomerase II, a nuclear matrix protein, was used on the same blot following anti-dS3 probing.

sub-nuclear components and the chromatin purified. Western



E. coli mutants defective for exonuclease III (*xth*) and endonuclease IV (*nfo*), or triple mutants also lacking endonuclease III (*nth*), or the *mutM* strain. Recombinant S3 protein was purified from bacterial lysates by affinity

Figure 20: Western blot analysis of a chromatin preparation to determine sub-nuclear localization of the *Drosophila* S3 gene product. Embryos (4-24 hours) were fractionated into filtered crude homogenate (FCH, 50ug), post-nuclear supernatant (PNS, 50 ug), purified nuclei (PN, 10 ug), supernatant after extraction with Triton X-100 (TXS, 10 ug), supernatant after washing with NaCl/EDTA (NES, 1 ug), and chromatin (C, 100 ng). Samples were electrophoresed and evaluated as in Figure 19.

sub-nuclear components and the chromatin purified. Western blot analysis of these protein extracts indicates a tight association of the S3 protein within the chromatin complex (Figure 20). Both these findings are consistent with the presence of a nuclear localization sequence in the N-terminal domain of the S3 protein.

L. DNA Repair Activity of *Drosophila* Ribosomal Protein S3

In collaboration with Dr. Walter A. Deutsch (Louisiana State University) and Dr. Paul W. Doetsch (Emory University), purified S3 fusion protein was assayed for its ability to cleave at sites of base loss or base damage. The 246 amino acid open reading frame of S3 was overproduced in DNA repair deficient *Escherichia coli* as a glutathione S-transferase fusion protein, using the pGEX-3X expression system as described earlier. The bacteria used for overexpression were *E. coli* mutants defective for exonuclease III (*xth*) and endonuclease IV (*nfo*), or triple mutants also lacking endonuclease III (*nth*), or the *mutM* strain. Recombinant S3 protein was purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B columns. This purification resulted in a major silverstaining protein band at a molecular weight predicted for the fusion (59 kDa), and occasionally two very minor bands at 45 kDa and 21 kDa (Figure 15).

Dr. Walter A. Deutsch and colleagues performed experiments which tested the ability of S3 to cleave the phosphodiester backbone adjacent to an apyrimidinic (AP) site in DNA. Using a poly(dA:dT) substrate containing AP sites bordered by a labeled phosphate 5' to the abasic sugar, the position of enzymatic cleavage, relative to the AP site, was determined for S3 fusion protein (Figure 21). These tests revealed that the release of labeled product from the AP DNA substrate was most substantial when primary incubations with S3 were followed by the addition of *E. coli* exonuclease III, a known class II hydrolytic AP endonuclease that cleaves DNA 5' to an AP site. This result suggests that S3 acts as a class I beta-elimination catalyst which cuts AP DNA so as to leave a 3'-terminal base-free sugar or modified sugar (Figure 22). In other words, the combination of a class I lyase and class II endonuclease is required for release of the radioactively labeled AP site. The labeled products generated by S3 and exonuclease III treatments were analyzed by descending paper chromatography and found to be consistent with the idea that S3 is a class I B-elimination catalyst.

As an alternative method to investigate S3 activity on DNA containing AP sites, Dr. Paul W. Doetsch and colleagues incubated either S3 or *E. coli* endonuclease III in the presence of a 139 bp 5' 32p end labeled DNA fragment containing a single AP site. The DNA scission products were

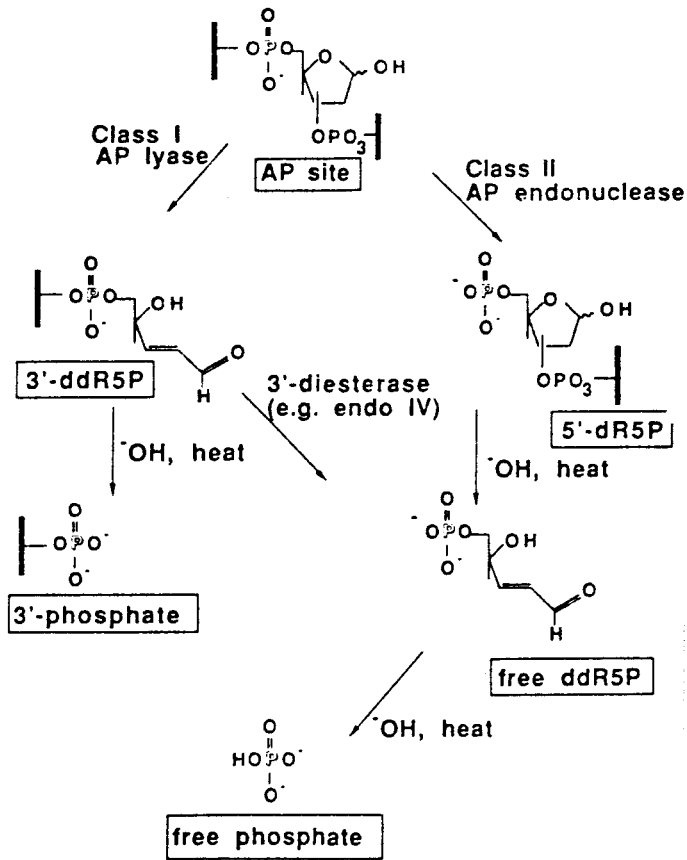


Figure 21: Principle of class I and class II AP endonuclease assays. The AP site is drawn attached to DNA at both ends (thick vertical bars). The ³²p-labelled phosphate (outlined and shadowed) is on the 5'-side of the AP site. Cleavage of AP sites by class II AP endonucleases follows the right-hand pathway, the action of class I AP lyases the left-hand pathway. The products expected from secondary reactions are shown [Reprinted with permission from Oxford University Press (Levin and Demple, 1990)]. Endo: endonuclease.

subsequently electrophoresed on a DNA sequencing gel. Both S3 and endonuclease III, which cut at AP sites via a beta-elimination reaction, generated DNA cleavage products that comigrated, indicating similar 3'-termini. This finding was consistent with the notion that S3 is an class I AP lyase.

Further biochemical characterization of S3 (work performed by Dr. Walter A. Deutsch) showed that its AP lyase activity is not dependent upon $MgCl_2$ and is insensitive to the presence of EDTA. Furthermore, the addition of tRNA to the reaction inhibits the ability of S3 to cleave at sites of base loss, which does not appear to be the case for the *E. coli* FaPy-DNA glycosylase. Most importantly, the activity of S3 towards AP sites is specifically inhibited by affinity purified antibody raised against the S3 fusion protein. This antibody did not alter the AP endonuclease activity of exonuclease III, suggesting that only the activity of S3 is immunoprecipitated by this antibody.

To date, all class I AP lyase enzymes contain an associated DNA glycosylase activity that recognizes and releases specific DNA base modifications. These enzymes process a particular DNA adduct via a combined DNA N-glycosylase/AP lyase mechanism. Thus, to address whether S3 recognizes and cleaves at specific DNA base modifications (work performed by Dr. Paul W. Doetsch), a 3' end-labeled DNA fragment containing either thymine glycols or ultraviolet (UV)

photoproducts was incubated with purified S3 fusion. The scission products resulting from S3-mediated cleavage were subsequently analyzed on DNA sequencing gels. S3 was found to incise UV-damaged DNA at particular guanine residues, a subset of damages recognized by *E. coli* endonuclease III, an N-glycosylase/AP lyase that acts on various forms of oxidative (ie thymine glycols) and UV-induced DNA damage. On the other hand, S3 did not display nicking activity on DNA substrates containing thymine glycols.

The guanine photoproduct recognized by S3 was suspected to be 8-oxoguanine and therefore the ability of S3 to cleave at this DNA adduct was analyzed (work performed by Dr. Paul W. Doetsch). S3 protein was incubated with a 5' end-labeled DNA fragment containing a single 8-oxoguanine residue and the products of the reaction evaluated. It was determined that S3 specifically incised at this residue by catalyzing a class I beta, delta-elimination reaction.

CHAPTER V

DISCUSSION

Genetic material is continuously being assaulted by a variety of agents, both intracellular and extracellular, that react with DNA to cause DNA damage (Friedberg, 1985). In addition, DNA composition is altered by spontaneous errors which arise due to the intrinsic instability of the nucleic acid structure and the infidelity of DNA synthesis. Probably the most common forms of DNA damage are those introduced by reactive oxygen species, which are generated during normal cellular metabolism or from exposure to mutagenic agents such as ionizing radiation (Ames, 1987; Hutchinson, 1985). One of the most dangerous base modifications produced as a result of free radical attack on DNA is the premutagenic lesion 8-oxoguanine (Floyd, 1990; Cheng et al., 1992a). This lesion has been shown to cause GC to AT transitions and is likely one of the primary causes of tumor promotion and carcinogenesis associated with reactive oxygen species. Thus, to avert the deleterious effects of 8-oxoguanine, organisms have evolved systems to prevent the accumulation of free radicals and to repair this adduct when present in DNA (Grollman and Moriya, 1993).

A variety of scavenging molecules such as superoxide

dismutase, glutathione peroxidase, and catalase exist in living cells to compete with DNA for reactive oxygen species (Halliwell, 1991). These enzymes catalytically remove radicals from the intracellular environment, protecting cells against the formation of oxidative DNA damage. In addition, to prevent the incorporation of 8-oxoguanine during DNA synthesis, both *E. coli* and human cells possess an activity that removes 8-oxo-dGTP from the cellular nucleotide pool by converting 8-oxo-dGTP to its monophosphate form (Bhatnagar et al., 1991; Mo et al., 1992). Should reactive oxygen species or 8-oxo-dGTP escape the detection of these front line defenses, additional mechanisms exist that repair 8-oxoguanine when present in DNA. One such mechanism, characterized in *E. coli*, involves a DNA glycosylase activity which specifically removes misincorporated adenines opposite guanine residues, particularly 8-oxoguanine derivatives (Au et al., 1989; Michaels et al., 1991, 1992). This process of mismatch repair would presumably allow the organism a second opportunity to correct 8-oxoguanine lesions in DNA. In addition, an enzyme has been identified in *E. coli* which displays N-glycosylase and AP lyase activities towards both formamidopyrimidine (FaPy) and 8-oxoguanine residues (Chetsanga and Lindahl, 1979; Bailly et al., 1989; Tchou et al., 1991). This protein (FaPy-DNA glycosylase) was originally detected based on its ability to repair FaPy adducts (Chetsanga and Lindahl, 1979). However, in view of the widespread distribution of 8-

oxoguanine in cellular DNA and the demonstrated miscoding and mutagenic properties of this lesion, it is assumed that 8-oxoguanine is the primary physiological substrate for this glycosylase. The gene (*fpg*) encoding this bacterial protein has since been isolated (Boiteux et al., 1987). Taken together, it appears that organisms have developed several activities that are specifically directed towards protecting against the mutagenic consequences of 8-oxoguanine.

In eukaryotic cells, the enzymatic repair of 8-oxoguanine is not nearly as well understood. Recently, an activity has been identified from human polymorphonuclear neutrophils that cleaves both 5' and 3' to 8-oxoguanine residues in DNA (Chung et al., 1991b). Surprisingly, further characterization of this activity revealed that, unlike the bacterial protein, FaPy is not a substrate for the human enzyme, suggesting that more advanced organisms may possess two distinct activities for the repair of 8-oxoguanine and FaPy DNA adducts. Until now, no gene encoding a eukaryotic protein possessing N-glycosylase activity for 8-oxoguanine has been cloned. I report here the isolation of a gene from *Drosophila melanogaster* which encodes a protein that demonstrates repair activity for this DNA lesion. Interestingly, this protein is 80% identical at the amino acid level to the rat and human ribosomal protein, S3 (Wilson III et al., 1993).

My interest in ribosomal protein S3 as having a possible role in DNA repair developed for several reasons. First of

all, our laboratory has been studying another ribosomal protein which displays DNA repair activity. A *Drosophila* cDNA (AP3) was isolated by screening an expression library with antibody raised against a human apurinic/apyrimidinic (AP) endonuclease (Kelley et al., 1989). Computer searches revealed that AP3 was the *Drosophila* homolog to the human ribosomal associated protein P0 (Grabowski et al., 1991a). Subsequent experiments have shown that AP3 demonstrates class II AP endonuclease activity on AP DNA (Deutsch et al., personal communication). In addition, AP3 was found to associate both with ribosomes and within the nuclear matrix, reaffirming the idea that this protein has a role in ribosomal function as well as DNA metabolism, similar to what will be described here for *Drosophila* S3.

In light of the recent discoveries which have identified several dual functional proteins with roles in DNA repair, it now appears quite possible that proteins have the capacity to carry out more than one cellular function. For instance, the yeast protein SSL2, the homolog to the human excision repair gene *ERCC-3* which is associated with xeroderma pigmentosum (XP) and Cockayne's syndrome (CS; Weeda et al., 1990), is involved in ribosomal binding and scanning of mRNA (Gulyas and Donahue, 1992). A mutant *SSL2* allele constructed to resemble the defective *ERCC-3* gene found in XP/CS patients confers ultraviolet light sensitivity to these yeast cells, but does not effect the function of this protein in the process of

translation initiation. Mutations in the *Drosophila* homolog to *ERCC-3*, *haywire*, result in UV sensitivity, CNS defects, ataxia, and lethality, phenotypic traits commonly affiliated with individuals suffering from XP/CS (Mounkes et al., 1992). Thus, *ERCC3*, *SSL2*, and *haywire* represent proteins that appear to have two functions, one defined by a UV repair defect, and a second essential function related to gene expression at the level of translation initiation. Another unexpected finding with regard to multifunctional proteins with roles in DNA repair is the observation that the major human AP endonuclease is also a regulator of AP-1 DNA binding (Xanthoudakis et al., 1992). Other examples of multifunctional proteins exist, but will not be discussed here (Table 1). Nonetheless, it appears that a class of proteins is beginning to emerge as transcriptional and translational regulators that are encoded by genes involved in DNA repair (Friedberg, 1992a).

Perhaps one of the most interesting properties associated with S3 is the observation that in humans it is among 6 ribosomal genes whose transcript levels are elevated in colorectal tumors (Pogue-Geile et al., 1991). Also in this class is S6, a tumor suppressor gene. S6 has been suggested to somehow be involved in the selective translation of particular mRNAs and in controlling cell growth, proliferation, and the immune response system. Many of these conclusions were drawn from studies on the *Drosophila* homolog of human S6, *air8* (aberrant immune response; Watson et al.,

YEAST	DROSOPHILA	HUMAN	FUNCTION	HUMAN DISEASE
SSL2 (Rad25)	<i>haywire</i>	ERCC3	Translation Initiation/ CNS mutant in <i>Drosophila</i>	XP - B/CS
---	66/63 APE	hAPE	AP Endo. / Jun-Fos Redox	
A0	AP3	P0	Ribosomal / AP Endo.	
*	S3	S3	Ribosomal / AP lyase 8-oxoguanine recognition	XP - D
---	Rrp1	---	Strand transferase / AP Endo./ homologue of ExoIII	
---	---	GAPDH	Uracil Glycosylase / tRNA binding GAPDH	
---	---	UDG	Uracil Glycosylase / cyclin-A	
---	---	ERCC6	Helicase / trans. reg. / chromo. stab. preferential repair of act. genes	CS - B
---	---	YB1	Binds AP DNA / transcription factor	

Table 1: Multifunctional DNA repair proteins.

1992). Moreover, mammalian S6 ribosomal protein shows developmentally regulated phosphorylation that is often associated with cell growth and tumorigenesis. Another ribosomal protein found to be overexpressed in colorectal tumors is P0 or AP3. As discussed earlier, this protein displays AP DNA repair activity. Thus, three of the six proteins (S3 being the other) within this class appear to have biological functions involved in tumorigenesis.

Human ribosomal protein S3 appears to be identical to mammalian endonuclease III characterized in the laboratory of Dr. Stuart Linn, U.C. Berkeley (personal communication). Endonuclease III, in studies conducted so far, seems to act on heavily UV-irradiated DNA and appears to be defective in XP complementation group D (XPD; Kuhnlein et al., 1978). This finding combined with the fact that several DNA repair proteins have recently been identified as being multifunctional and that S3 was one of six mRNAs overexpressed in colorectal cancers stimulated my interest in pursuing studies of ribosomal protein S3, using *Drosophila* as a model eukaryotic system. The utilization of *Drosophila* permits detailed genetic analysis of S3 as a means towards understanding the structure-function relationship of this multifunctional protein. In fact, as has been discussed, experiments performed in *Drosophila* have already produced some highly insightful results related to multifunctional proteins encoded by ribosomal and DNA repair genes.

The conserved nature of S3 permitted isolation of both cDNA and genomic clones encoding the *Drosophila* homolog (Figure 8). Thus, by screening *Drosophila* libraries with radioactively labeled rat S3 cDNA fragments (Chan et al., 1990), several clones containing S3 sequences were ultimately purified. These clones were sequenced, the putative protein domains identified (Figure 7), and the genomic organization elucidated (Figures 12 and 13). PCR amplification of chromosomal DNA was also used to isolate S3 genomic fragments for sequencing and elucidating the S3 gene structure.

Predicted amino acid sequence from the cDNA open reading frame revealed a consensus nuclear localization signal within the amino terminus of the *Drosophila* ribosomal protein S3, suggesting a nuclear function for this protein (Figure 7). Interestingly, this targeting signal was also present in the human S3 protein, but absent from the rat protein. To determine the localization of the S3 protein, Western blot analysis was performed on cytoplasmic and nuclear fractions prepared from a variety of organisms. Based on the conserved nature of the S3 protein, one might expect polyclonal antibodies generated to the *Drosophila* protein (anti-dS3) to cross-react with the S3 protein from yeast, rat, and human extracts. However, this was not the case. The inability of anti-dS3 to recognize these proteins may ultimately explain why differences (if any are detected) are seen in DNA repair activity and/or localization of the various S3 gene products.

In any case, whether the rat protein, which lacks a nuclear localization signal, translocates into the nucleus was unable to be determined using anti-dS3 antibody (data not shown). On the other hand, sub-cellular/sub-nuclear localization experiments in *Drosophila* found S3 protein to be in tight association with the nuclear matrix and chromatin (Figures 19 and 20), consistent with the presence of the nuclear targeting sequence in the amino-terminal domain of the S3 protein. Likewise, immunohistochemistry studies using antibody to the human S3 protein detected S3 in the nucleus of human cells (Dr. Stuart Linn, personal communication). These findings further suggest that S3 may be involved in DNA metabolism.

As expected, *Drosophila* S3 protein was also found in purified ribosome preparations (Figure 18). Previous studies carried out in rat have indicated that ribosomal protein S3 participates in controlling the interaction of the pre-initiation complex with the ribosome (Bommer et al., 1980; Westerman et al., 1979, 1981; Stahl and Kobets, 1981; Takahashi and Ogata, 1981; Tolan et al., 1983), implying a role in protein synthesis. Thus, much like ERCC3, these data suggest that S3 is involved in translation initiation, and therefore, regulation of gene expression, as well as DNA repair processes.

Analysis of the predicted amino acid sequence of the *Drosophila* S3 protein identified three potential sites for phosphorylation, which are present in the human and rat

proteins, and one putative site for glycosylation, which is absent from these mammalian proteins (Figure 8). The inability to detect alternative protein forms in *Drosophila* extracts by Western blot analysis implies that the S3 protein may not undergo post-translational modification involving glycosylation. However, direct studies to assess whether or not the S3 protein is glycosylated were not implemented. On the other hand, experiments utilizing HeLa cells have shown that the S3 protein is a substrate for cyclic AMP-dependent protein kinase enzymes (Issinger and Beier, 1980). Thus, the sites of phosphorylation may be involved in regulating 1) the distribution of S3 between the ribosome and the nucleus and/or 2) the DNA repair activity of the protein. Although initial experiments were performed to address this question in *Drosophila* Schneider cells, results proved inconclusive. Work done by Dr. Stuart Linn's laboratory indicates that in XPD cells the S3 protein is unable to be phosphorylated, which may result in a decreased nuclear localization and therefore a reduced repair capacity (personal communication). This finding implies that XPD cells contain a defective kinase or that S3 contains an altered phosphorylation site. However, to date, sequencing of either the human S3 protein or the cDNA from XPD cells has not been performed. Certainly, more studies in this area need to be carried out.

Recent findings have uncovered that near the amino-terminus of the S3 gene product exists a region which exhibits

similarity to a domain of the yeast Nuc2 nuclease (Figure 9; Dr. Burbee, personal communication). The function of this domain at present is unknown but may be responsible for the incision activity displayed by the S3 enzyme. Thus, plans to delete this portion of S3 and to subsequently assay for both N-glycosylase and AP lyase activities have been made. In addition, random mutagenesis studies of the S3 cDNA would provide further insight as to the structure-function relationship of this protein.

Whereas mammalian ribosomal protein genes are present in multiple copies with only one of the genes being functional, the *Drosophila* S3 gene was found to exist in a single copy. This conclusion was reached based on results obtained from Southern blot analysis of genomic DNA digested with various combinations of restriction endonucleases (Figure 10) and *in situ* hybridization of polytene chromosomes (Figure 11). In addition, P-element mutagenesis revealed that only one locus contains a functional S3 gene (Andersson et al., personal communication). *In situ* chromosomal localization experiments mapped the S3 gene to position 94F/95A, a region previously identified as containing a *Minute* gene. As mentioned, this locus was confirmed to contain the S3 gene by the isolation of a P element insertion into this region.

Minutes are a class of phenotypically identical organisms that are thought to be defective in genes encoding ribosomal proteins. Homozygotes are lethal at the late embryonic, early

larval stage, while heterozygotes are slow developers, small in size, and females typically have a reduced fertility rate. These characteristics imply that the S3 gene product is necessary for cell viability, a feature expected for a protein involved in translation initiation. To date, five mutant alleles exist at the locus 94F/95A, but they have not been characterized. Studies to identify the mutation present in the heterozygotes for each of these five alleles have been unsuccessful, but extremely informative. Southern blot analysis indicates that the mutation is not the result of a gross chromosomal aberration (data not shown). Moreover, Northern and Western blot experiments reveal that these mutants display no significant change in the levels of mRNA or protein, respectively, when compared to their wild-type counterparts (data not shown). Thus, the alteration is suspected to be a point mutation within the essential ribosomal domain of S3, resulting in the production of a non-functional ribosomal protein. In all likelihood, this mutation would not effect the DNA repair capacity of S3. Based on all the available data, I hypothesize that the *Drosophila Minutes* contain a mutation(s) which affects the ribosomal domain of S3 and that XPD cells possess a mutation which alters the DNA repair domain of S3.

Sequence analysis revealed that the *Drosophila* S3 gene consists of two major exons interrupted by a single 263 bp intervening sequence, which contains consensus splice site

junctions (Figures 12 and 13). This genomic simplicity is in marked contrast to the complex organization of the functional human S3 gene, which is composed of several exons and introns (Tycowski et al., submitted). Interestingly, one of these introns in the human gene was found to encode a small nucleolar RNA species (Tycowski et al., submitted). These studies established that U15 snRNA is transcribed as part of the human S3 pre-mRNA and then excised from the intron to yield mature U15 snRNA molecules. On the other hand, the sequence of the *Drosophila* S3 intron displays no homology to U15 RNA, implying, from an evolutionary standpoint, that introns are mobile elements of relatively recent origin.

Expression analysis of both mRNA (Figure 14) and protein (Figure 16) uncovered that *Drosophila* S3 is constitutively expressed throughout development. The fact that the S3 transcript is present in total RNA isolated from 0-4 hour embryos indicates that this message is maternally derived. In addition, there does not appear to be much change in the overall level of either S3 message or gene product during the life cycle. This result would be expected for a ribosomal protein required for translation initiation. Moreover, it would explain why mutations in both S3 alleles would result in a lethal phenotype. Preliminary studies performed with Schneider cells as well as 2nd instar larvae indicate that S3 gene expression (protein and transcript) is non-inducible and that protein localization is unaffected by exposure to either

low or high doses of ultraviolet light (data not shown). This finding is consistent with other experiments conducted in eukaryotic systems where genes encoding DNA repair enzymes were non-inducible following treatments with DNA-damaging agents. Nonetheless, knowing that S3 is a repair enzyme for 8-oxoguanine, the S3 gene may respond to conditions of oxidative stress, which produce high levels of this DNA adduct. To date, induction experiments with hydrogen peroxide or other free radical-producing agents have not been performed. Finally, while AP3/P0, another ribosomal/DNA repair protein, was found to be constitutively overexpressed in human Mer- cell lines (MGMT deficient) as compared to Mer+ cell lines (MGMT proficient), S3 expression was found to be unaltered (data not shown; Grabowski et al., 1991). Thus, *Drosophila* S3 gene expression appears to be unaffected by these various situations as well as others not discussed. However, the fact that S3 transcript levels are elevated in colorectal cancers suggests that transcriptional regulation of the S3 gene does exist. Therefore, studies to analyze, in detail, the promoter elements of the S3 gene are being developed.

One potentially exciting feature of S3 is its localization pattern seen during development. During all the stages, except pupae, S3 protein is present primarily in the cytoplasm at approximately 70%, with the remaining 30% found in the nucleus (Figure 17). The pupal stage, however,

displays equal distribution of the S3 gene product between these two cellular compartments. It should be noted that these results were obtained by densitometric scans of autoradiographs which can be unreliable if outside the linear range. However, these experiments were repeated several times with similar conclusions. Why S3 is dispersed differently is unknown. One may predict that during pupal development, the repair function of S3 is required at a greater extent to prevent the formation of mutations during histolysis. Permanent mutations are more likely to occur during times of rapid cell division, when DNA repair systems have less opportunity to scan for DNA damage and DNA synthesis is progressing at a frequent pace. Thus, increased levels of DNA repair enzymes may prevent the accumulation of DNA damage and subsequent erroneous replication of these adducts.

Much of the data discussed thus far is suggestive of S3 having a role in DNA repair. However, firm biochemical evidence was required before making such a conclusion. In this regard, S3 was found to cleave at sites of 8-oxoguanine in DNA, implying that S3 contains N-glycosylase as well as AP lyase activities (Kelley et al., submitted). However, firm resolutions regarding N-glycosylase activity await the construction of substrates containing 8-oxoguanine so that the release of this adduct may be monitored. There has been some concern that the 8-oxoguanine containing oligonucleotide undergoes spontaneous loss of this damaged base, resulting in

an AP substrate, and that S3 is, therefore, cleaving as an AP lyase and not a glycosylase/lyase. Based upon the reported stability of the 8-oxoguanine lesion, I conclude that S3 possesses DNA glycosylase activity for this adduct and therefore represents the first eukaryotic gene cloned with this activity. It is clear, however, that S3 possesses AP lyase activity.

The enzymatic characteristics of S3, when presented with an AP site in DNA, are more similar to the *E. coli* FaPy-DNA glycosylase than to the human activity identified. Specifically, the *Drosophila* S3 activity, like the bacterial enzyme, but unlike the human enzyme, lacks any cofactor requirement, and is insensitive to the presence of EDTA. However, the *Drosophila* enzyme, unlike FaPy-DNA glycosylase of *E. coli*, is sensitive to tRNA. Further biochemical characterization of the *Drosophila* S3 protein is presently being carried out by the laboratory of Dr. Walter A. Deutsch at Louisiana State University.

The concern arises as to whether S3 protein fractions are contaminated. *Drosophila* S3 protein was overexpressed as a fusion with glutathione S-transferase (GST) using the pGEX-3X system. This fusion protein was subsequently purified on GST affinity columns which resulted in a major coomassie blue or silver staining protein band at the predicted molecular weight of 60 kDa (Figure 15). To reduce the possibility of contaminating enzymes, overexpression experiments were

performed in DNA-repair deficient *E. coli*. Bacterial strains used for these experiments included double mutants defective for exonuclease III (*xth*) and endonuclease IV (*nfo*), triple mutants also defective for endonuclease III (*nth*), or *mutM* mutants lacking FaPy-DNA glycosylase. Since, 1) results did not vary regardless of the *E. coli* strain used, 2) internal controls such as GST, which was purified in an identical manner as the fusion, did not display DNA repair activity, and 3) the AP lyase activity detected was inhibited by affinity purified antibody to S3, it was concluded that the activities identified are those of S3 and not some contaminant. Clearly, these findings place S3 in the aforementioned class of multifunctional proteins with roles in DNA repair.

Initial studies have been directed towards understanding the biochemistry of S3 as a repair enzyme for DNA damage. However, it would be interesting if S3 had a "repair" role while associated with ribosomes. Since S3 can be crosslinked to mRNA, it obviously has the capability of interacting with transcripts while present in the ribosomal complex. Thus, experiments are being designed to examine whether S3 can bind RNA and if S3 acts on damaged RNA, in particular AP RNA. It could be hypothesized that, should S3 encounter messenger RNA that contains an AP site, S3 would incise adjacent to this site, aborting translation, and preventing potentially aberrant protein translation. Along these lines, the repair capacities of both endogenous ribosomal S3 protein and nuclear

S3 protein should be assessed.

The fact that ribosomal proteins contain multiple activities is an interesting finding. Since it has been suggested that ribosomes at one time were composed of solely RNA, during evolution, ribosomal subunits must have recruited protein molecules to assist in carrying out functions such as protein synthesis. Thus, it would be logical to hypothesize that proteins most likely recruited are those capable of interacting with nucleic acid. These proteins may have originally been transcriptional regulatory factors, DNA repair enzymes, or other DNA-binding proteins, and presently are functioning as ribosomal components, while also performing their original roles. S3 may represent a DNA repair enzyme which developed the ability to expedite the process of translation initiation. Alternatively, S3 may have originally been a protein involved in RNA binding and/or metabolism and has evolved the capacity to carry out DNA repair.

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VITA

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In September, 1985, Mr. Wilson entered Bucknell University in Lewisburg, Pennsylvania, and received the degree of Bachelor of Arts in both Biology and Political Science in June, 1989. That year, he enrolled in the Program in Molecular Biology at Loyola University Chicago, Maywood, Illinois. Soon after, he joined the laboratory of Mark R. Kelley, Ph.D., where he initiated his studies on DNA repair processes in *Drosophila melanogaster*. In 1992, Mr. Wilson was awarded a fellowship by the Arthur J. Schmitt Foundation.

Mr. Wilson has accepted a position as a post-doctoral fellow in the laboratory of Bruce Demple, Ph.D., at Harvard University, School of Public Health, Boston, Massachusetts. Here, he will continue his research on DNA repair mechanisms using human systems.

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