1996

Developmental, Tissue, and Sub-Tissue Specific Expression of the Mammalian Apurinic/Apyrimidinic Endonuclease

Teresa Mari Wilson
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

DEVELOPMENTAL, TISSUE, AND SUB-TISSUE SPECIFIC EXPRESSION OF THE MAMMALIAN APURINIC/APYRIMIDINIC ENDONUCLEASE

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

GRADUATE PROGRAM IN MOLECULAR BIOLOGY

BY

TERESA MARI WILSON

CHICAGO, ILLINOIS

JANUARY, 1996

ACKNOWLEDGEMENTS

I would like to recognize the members of my committee for their helpful criticisms and assistance in developing a productive graduate school career: Dr. Nicholas Emanuele, Dr. Leonard Erickson, Dr. Hans-Martin Jäck, Dr. Russ Pieper and my advisor Dr. Mark Kelley. I would also like to thank Dr. Kelley for his guidance, support and friendship.

To my laboratory colleagues, I offer my appreciation for their help and friendship: Dr. James Carney, Dr. Margaret Halloran, Kent Hansen, Denise Scroggins, Dr. John Tentler, Dr. Shahab Uddin, Dr. David Wilson, III, Jonathan Yates, and Dr. Yi Xu.

I would like to extend my love and thanks to my husband and family.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF ABBREVIATIONS

A	adenine
aa	amino acid
AP	apurinic/apyrimidinic
APE	human apurinic/apyrimidinic endonuclease
APEX	mouse apurinic/apyrimidinic endonuclease
Apn1	*Saccharomyces cerevisiae* AP endonuclease
bp	base pair
BSA	bovine serum albumin
c	carboxyl
C	cysteine
cDNA	complementary deoxyribonucleic acid
Ci	Curie
CIAP	calf intestinal alkaline phosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
g	gram
G	guanine

GIT	guanidinium isothiocyanate
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
h	hour(s)
hAPE	human apurinic/apyrimidinic endonuclease
IPTG	isopropyl-β-D-thio-galactosidase
kb	kilobases
kDa	kilodalton
LB	Luria broth
M	molar
min	minute(s)
mg	milligram
ml	milliliter
mol	mole(s)
MOPS	3-[N-morpholino] propanesulfonic acid
MPG	methylpurine-DNA glycosylase
mRNA	messenger ribonucleic acid
N	amino
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
rAPEN	rat AP endonuclease
RNA	ribonucleic acid
Rrp1	recombination repair protein 1

s	second(s)
SDS	sodium dodecyl sulfate
T	thymine
TBE	tris boric acid EDTA
TBS	tris buffered saline
U	units
UDG	uracil-DNA glycosylase
µg	microgram
µl	microliter
µM	micromolar
UV	ultraviolet
X g	times gravity

ABSTRACT

The multifunctional mammalian apurinic/apyrimidinic (AP) endonuclease is responsible for the repair of AP sites in DNA. Apurinic and apyrimidinic sites are among the most common DNA lesions and can lead to polymerase halting, and, therefore, aberrant transcripts and even cell death. Consequentially, AP endonucleases have a critical role in the maintenance of DNA integrity and cell survival. This type of lesion results from the hydrolysis of the glycosylic bond between the base and deoxyribose and may occur through a variety of mechanisms including the following: First, AP sites can be produced by the effects of ionizing radiation and by oxidative agents that are the by-products of biological processes such as oxidative respiration of aerobic organisms, superoxide generation by activated macrophages and other immune cells, and by radiomimetic agents such as bleomycin. Second, these sites are the product of DNA *N*-glycosylases which recognize and catalyze the removal of damaged and incorrect bases from DNA. And third, spontaneous hydrolysis of the glycosylic bond occurs at a substantial rate: approximately 10,000 bases are lost per cell per day making this one of the most predominant forms of DNA damage.

Currently, little is known about the regulation and expression of mammalian AP endonucleases. Previous studies examining the tissue specific distribution of the AP endonuclease transcript and protein by Northern analysis and enzymatic assays, respectively, indicated that this gene is expressed in all tissues at relatively similar levels. The studies in this dissertation were designed to determine the developmental and cell specific pattern of expression of the rat and human AP endonuclease *in vivo*.

By utilizing direct techniques such as immunohistochemistry and *in situ* hybridization, the expression of mammalian AP endonucleases was demonstrated to be highly differential within various tissues and cell types and during the developmental process. These results allow us to begin to understand the regulatory and functional relationships of the AP gene products to the different subtissue cell types.

CHAPTER I

INTRODUCTION

The inherent chemical instability of DNA, the production of reactive oxygen species during normal cellular metabolism (Demple and Harrison 1994), and the continuous exposure to environmental mutagens represent a potential threat to the genetic information of cells (Friedberg, 1985; Larson *et al.*, 1985; Loeb and Preston, 1986; Saffhil *et al.*, 1985). If unrepaired, damaged DNA can have deleterious biological consequences for the organism and result in cell transformation or death (Friedberg, 1985). Thus, to protect against DNA damage, organisms have evolved an elaborate array of DNA repair mechanisms such as nucleotide excision repair (NER), mismatch/recombination repair and DNA base excision repair (BER) (Barnes *et al.*, 1993; Lindahl, 1992; Lindahl, 1994).

DNA base excision repair involves two major classes of repair enzymes, namely DNA glycosylases and apurinic/apyrimidinic (AP) endonucleases (Demple and Harrison, 1994; Doetsch and Cunningham, 1990; Lindahl, 1992; Sancar and Sancar, 1988). DNA glycosylases are enzymes that hydrolyze the N-glycosidic bond between the damaged base and the deoxyribose moiety, leaving behind an AP site on the DNA backbone (Demple and Harrison, 1994; Doetsch and Cunningham, 1990; Wallace,

1988). Cytotoxic lesions such as N^3-methyladenine, N^7-methylguanine, and N3-methylguanine, are all released by the N-methylpurine DNA glycosylase (MPG) enzyme (Mitra and Kaina, 1993; Sakumi and Sekiguchi, 1990; Sancar and Sancar, 1988). 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) (Demple and Harrison, 1994; Doetsch and Cunningham, 1990). Furthermore, tens of thousands of AP sites are produced per cell per day, primarily by spontaneous base hydrolysis, and cellular metabolites reacting with oxygen radicals (Lindahl, 1993). Unrepaired AP sites bypassed during DNA replication result in mutations and genetic instability (Loeb and Preston, 1986).

The major cellular enzymes initiating repair of AP sites ("class II" AP endonucleases) have been identified and characterized in bacteria, yeast, *Drosophila* and mammals (Demple and Harrison, 1994; Demple *et al.*, 1991; Doetsch and Cunningham, 1990). These repair proteins hydrolyze the phosphodiester backbone immediately 5′ to an AP site generating an abasic deoxyribose-5-phosphate which is released by a 5′-deoxyribophosphodiesterase (dRPase) or 5′-exonuclease followed by DNA synthesis and ligation. These enzymes have also been shown to contain repair activity for 3′-terminal oxidative lesions (Demple and Harrison 1994; Demple *et al.*, 1986; Henner *et al.*, 1983; Ramotar *et al.*, 1991). By hydrolyzing 3′-blocking

fragments from oxidized DNA, these enzymes can produce normal 3'-hydroxyl nucleotide termini, permitting DNA repair synthesis.

Recently, the human AP endonuclease (hAPE) repair enzyme has been found to display multiple functions. Besides AP site DNA repair activity, hAPE potentiates the binding of Jun-Jun homodimers or Fos-Jun heterodimers to DNA by alternating the redox state of these proteins (Walker *et al.*, 1993; Xanthoudakis and Curran, 1992; Xanthoudakis *et al.*, 1992; Xanthoudakis et al., 1994; Yao *et al.*, 1994). Hypoxic conditions in cell lines have been shown to increase AP endonuclease mRNA levels (Walker *et al.*, 1994; Yao *et al.*, 1994). AP endonuclease expression has recently been shown to be modulated in epithelium in a pig wound-healing model (Harrison *et al.*, 1995).

Currently our knowledge of the sites of AP endonuclease expression are limited. Northern analysis studies have shown that this gene is expressed in all tissues, albeit at relatively similar levels. Little, however, is known about whether this gene is differentially expressed in specific subpopulations of cells or differentially during development.

Bleomycin, a glycopeptide antibiotic, has been useful as a anti-tumor agent in the treatment of a number of neoplasms. The cytotoxic effect of this agent has been determined to be through DNA damage (Harrison and Lazo, 1987). A side effect of the clinical use of bleomycin is pulmonary fibrosis which is thought to be induced by the DNA damaging action of bleomycin (Lazo and Sebti, 1991; Harrison *et al.*, 1989). It has been demonstrated

that "activated" bleomycin reacts with the C-4′ position of the sugar residue generating a 4′-oxidized AP site along with strand breaks (Hecht, 1986). Additionally, 4′-oxidized AP sites have been shown to be an excellent substrate for endonuclease IV requiring 4-fold lower concentrations of the protein than for recognition of regular AP sites (Häring *et al.*, 1994). These results indicate that the base excision repair pathway, and AP endonuclease IV in particular, is involved in the repair of bleomycin induced 4′ oxidized AP sites.

The purpose of my dissertation research is to determine the developmental, tissue and sub-tissue specific expression of the major mammalian class II AP endonuclease. This will be accomplished utilizing in vivo methods of examination such as immunohistochemistry and *in situ* hybridization. Additionally, studies will be initiated on the ability of AP endonucleases to protect mammalian cells from the cell-killing effects of various chemotherapeutic agents including bleomycin.

The significance of these studies is two fold. First, these experiments allow us to begin to understand the regulatory and functional relationships of the AP gene products to the different subtissue cell types. And second, the protection of normal cells from the cell-killing effects of bleomycin will contribute significantly to future studies on the prevention/treatment of bleomycin induced fibrotic lung disease.

CHAPTER II

REVIEW OF RELATED LITERATURE

All organisms are continually subjected to DNA damaging agents, both endogenous and exogenous, which can lead to the disruption of RNA and DNA synthesis, cell killing, and, in the case of eukaryotes, aging and carcinogenesis. Of course, the ultimate effect of any DNA damage on an organism's viability depends upon the type and location of the damage within the DNA and the efficiency with which it is repaired (see Friedberg, 1985, for a comprehensive review of DNA damage and repair). In this chapter, the base excision DNA repair pathway will be discussed with an emphasis on mammalian AP endonucleases.

A. DNA Damage and Repair Mechanisms

DNA, being a highly reactive molecule, is the target of a wide variety of physical and chemical agents. Bases can be deaminated by heat and lost spontaneously or by the action of DNA-glycosylases; ionizing radiation can result in base fragmentation, ring opening, and single and double strand breaks; ultraviolet radiation produces pyrimidine dimers and 6-4 photoproducts (Sancar and Sancar, 1988). Additionally, DNA can be modified by the action of agents such as methyl methane

sulfonate (MMS) and the endogenous S-adenosyl methionine and by cross-linking agents such as cisplatin. However, one of the major sources of damage that DNA is exposed to on a daily basis are oxidative free radicals. These free radicals are the by products of biological processes such as oxidative respiration of aerobic organisms, superoxide generation by activated macrophages and other immune cells, and by radiomimetic agents such as bleomycin (Demple and Harrison, 1994). In fact, it has been determined that nearly 100 different types of free radical damage can occur (von Sonntag, 1987; Dizdaroglu, 1992).

To cope with the production of DNA damage, cells have evolved a complex array of DNA repair mechanisms. Direct methods of repair exist that simply reverse the covalent modifications of DNA. Examples include DNA photolyase (Sancar, 1994), an enzyme that catalyzes the breaking of the cyclobutane ring joining pyrimidines utilizing energy in light, and O^6-mGua DNA methyltransferase (MGMT) (Mitra and Kaina, 1993), a protein that stoichiometrically transfers alkyl groups from the O^6 position of guanine in DNA to a cysteine residue in its structure.

The excision repair pathways correct damaged DNA relying on the complementary strand for sequence information to repair the DNA duplex. In long patch repair, also referred to as nucleotide excision repair (NER), damaged bases are removed from DNA as an oligonucleotide of 27-29 bases (Huang *et al.*, 1992). This pathway is the principal mechanism for removing bulky

adducts from DNA that have been induced by UV and cross-linking agents. The human NER pathway has been extremely well characterized and has recently been reconstituted *in vitro* in a reaction that required approximately 30 polypeptides (Aboussekhra *et al.*, 1995). Short patch repair, or base excision repair (BER), is responsible for the removal of unusual or modified bases and the repair of apurinic/apyrimidinic (AP) sites. During the repair of modified bases, the base is removed by hydrolysis of the glycosylic bond resulting in a single nucleotide gap that is filled in without nick translation (Doetsch and Cunningham, 1990).

B. Apurinic/Apyrimidinic Sites

One of the most prominent types of DNA damage is apurinic/apyrimidinic (AP) sites. This type of lesion results from the hydrolysis of the glycosylic bond between the base and deoxyribose and may occur through a variety of mechanisms. First, spontaneous hydrolysis of the glycosylic bond occurs at a substantial rate: approximately 10,000 bases are lost per cell per day (Ames, 1989; Lindahl *et al.*, 1972). Second, AP sites can be produced by the effects of oxidative agents and ionizing radiation which lead to ring opening increasing the lability of the glycosylic bond (Friedberg, 1985). And third, these sites are the product of DNA *N*-glycosylases which recognize and catalyze the removal of damaged and incorrect bases from DNA (Sakumi *et al.*, 1990). While this list is not all inclusive,

these three mechanisms represent the major routes to the production of AP sites, the central intermediate in the base excision DNA repair pathway.

C. Base Excision DNA Repair

As previously mentioned, this repair pathway is responsible for the repair of damaged bases and AP sites. BER is the most highly conserved pathway from bacteria to humans denoting its importance in the maintenance of genetic integrity (Olsen *et al.*, 1989; Demple *et al.*, 1991). The general steps of this pathway include the recognition and release of the modified base by a specific *N*-glycoslyase followed by cleavage of the DNA phosphodiester backbone by an AP endonuclease or lyase. If necessary, blocking groups are removed by the action of either a deoxyribophosphodiesterase or a class II AP endonuclease. Next, the resulting gap is filled in by DNA polymerase and the nick sealed by DNA ligase (Figure 1).

1. DNA glycosylases

These are small enzymes (20-45 kDa) that act first within the BER pathway in the removal of modified or incorrect bases from DNA by hydrolyzing the N-glycosylic bond between the deoxyribose and the base. This action results in the production of an apurinic/apyrimidinic site. A variety of DNA glycosylases have been isolated from both prokaryotic and eukaryotic organisms. These include thymine glycol glycosylases (Demple and Linn, 1980),

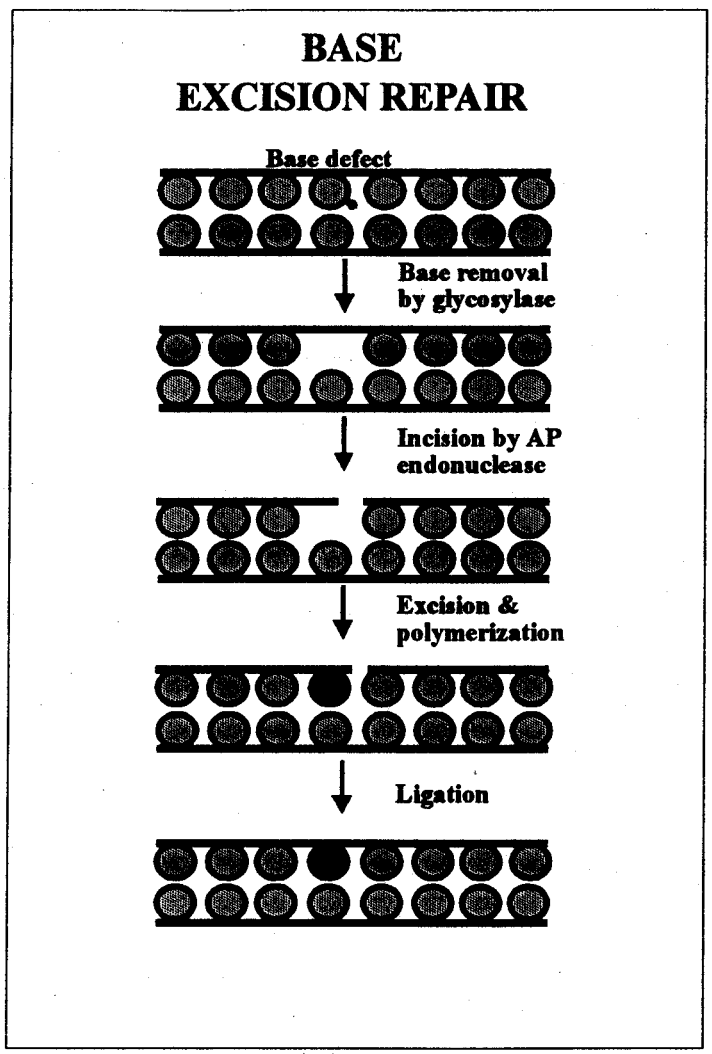


Figure 1. Schematic of base excision repair. This figure illustrates the general steps of base excision repair.

hypoxanthine-DNA glycosylase (Karran and Lindahl, 1980; Myrnes *et al.*, 1982), and formamidopyrimidine-DNA glycosylase (Lindahl, 1982; Bioteux *et al.*, 1987). The major and best characterized DNA glycosylases, 3-methyladenine-DNA glycosylase and uracil-DNA glycosylase, are describe below.

3-methyladenine (3-mAde), 7-methylguanine (7-mG), O^6-methylguanine (O^6-mG) and other alkylated bases are produced by exogenous alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU) and methylmethane sulfonate (MMS). Additionally, the endogenous S-adenosylmethionine methylates DNA at a slow but significant rate producing 3-mAde and 7-mG (Saffhill *et al.*, 1985). The generation of these alkyl adducts has been well characterized and shown to be highly mutagenic: especially 3-mAde, which, if not repaired, can block DNA replication (Lindahl *et al.*, 1988). *N*-glycosylases which remove 3-mAde have been isolated and cloned from a variety of organisms. The two methyl purine DNA-glycosylases (MPGs), Tag I and Tag II from *E. coli* have been studied in the greatest detail.

The Tag I enzyme, encoded by the *tag* gene, is composed of 187 amino acids with a molecular weight of 21 kDa (Sakumi *et al.*, 1986). This enzyme has been shown to excise 3-mAde and 3-ethylAde from methylated DNA and is inhibited by the reaction product 3-mAde; however, it is not active on other alkylated bases. Mutants lacking this enzyme are moderately sensitive to MMS and other alkylating agents (Friedberg, 1985).

Additionally, expression of this gene does not depend on treatments that lead to the SOS or adaptive response and is, therefore, considered to be constitutive (Sakumi *et al.*, 1990).

The Tag II enzyme is encoded by the *alkA* gene and consists of 282 amino acids molecular weight of 31.4 kDa. This enzyme removes 3-mAde and 3-mG from DNA with equal efficiency, and 7-mAde, 7-mG, and O^2-methylpyrimidines at slower rates (Thomas *et al.*, 1982). Unlike Tag I, this enzyme is not inhibited by free 3-mAde. Furthermore wild type *E. coli* treated (adapted) with alkylating agents show a dramatic increase in the number of Tag II molecules, and *alkA*$^-$ mutants are highly sensitive to MMS and MNNG (Sakumi *et al.*, 1990) indicating the importance of this enzyme in the repair of alkylated bases. The sequences of Tag I and Tag II reveal no significant homology reflecting differences in substrate specificity and, perhaps, mechanisms of action.

MPGs have been detected in, and cloned from, a wide variety of mammalian sources including rodents, bovine and human (O'Connor *et al.*, 1990; Engelward *et al.*, 1993; Male *et al.*, 1985; Chakravarti, *et al.*, 1991). These mammalian MPGs appear to be similar to the *E. coli* Tag II in that they have significant sequence similarity, are similar in molecular weight, and possess broad substrate specificity (O'Connor *et al.*, 1990). However, very little is currently known about the expression and regulation of this enzyme in mammals. Mitra and co-workers have published on the age-dependent modulation of

tissue-specific activity of 3-mAde-DNA glycosylase in female mice (Washington *et al.*, 1989). They noted that, in addition to differences in the enzyme levels between two stocks of mice for each organ examined, suckling animals (9 day old) have consistently lower levels of MPG than young adults (7-8 weeks old). Furthermore, MPG levels in adults (15-17 months) were significantly lower than those in young adults indicating an age-dependent modulation of MPG in female mice. Additionally, the level of MPG activity in the rat hepatoma cell line (H4) has been shown to increase after both single and repeated exposures to DNA damaging agents (O'Connor *et al.*, 1990). While this increase is not an adaptive response, it does suggest that MPG activity is inducible by DNA damage in rat liver cells. This response has not been observed in other mammals. Studies on the molecular mechanisms of MPG regulation are currently underway.

Uracil-DNA glycosylases, UDG, have been isolated from a number of organisms including *E. coli* (Varshney et al., 1988), herpes virus (Worrad and Caradonna, 1988) yeast (Percival et al., 1989) and humans (Olsen, et al., 1989; Wittwer et al., 1989; Muller and Caradonna, 1991). Uracil can arise in DNA by a variety of mechanisms: by mis-incorporation by a DNA polymerase, by spontaneous deamination of cytosine, by bisulfite and nitrous acid deamination of cytosine, and by DNA-(cytosine-5)-methyltransferases (Lindahl, 1994; Friedberg, 1985; Sancar and Sancar, 1988; Shen *et al.*, 1992). Additionally, nitric oxide, a constituent of cigarette smoke has been shown to

greatly accelerate the deamination of cytosine (Wink *et al.*, 1991). The incorporation of uracil or the deamination of cytosine results in a pre-mutagenic U:G mismatch that will cause a GC to AT transition mutation if left un-repaired. *E. coli* mutants for the *ung* gene display a 5 to 30 fold increase in the spontaneous mutation rate (Duncan and Weiss, 1982) while yeast *ung* mutants show a 20-fold increase in spontaneous mutations (Impellizzeri *et al.*, 1991).

All human tissues examined have been found to posses uracil-DNA glycosylase activity (Krokan *et al.*, 1983; Myrnes *et al.*, 1983). To date, three different uracil-DNA glycosylases have been characterized. A 26 kDa glycosylase was isolated from human placental cells that showed significant homology to the uracil-DNA glycosylases, *ung* genes, isolated from *E. coli*, yeast and herpes virus (Olsen et al., 1989). In fact, the human protein showed the highest similarity to the *E. coli* protein with 73% similarity when conservative amino acid substitutions were included. Cell cycle analysis of this gene's transcripts revealed an 8-12 fold increase in transcript levels during G1 phase with a 2-3 fold increase in total enzyme activity in the S-phase indicating that this gene is cell cycle regulated (Slupphaug *et al.*, 1991). The *ung* gene promoter was determined to posses an E2F element that is involved in the cell cycle regulation of a number of genes (Nevins, 1992).

A second uracil-DNA glycosylase of 36 kDa was isolated that shows significant homology to the cyclin protein family

(Muller and Caradonna, 1993). This gene was also demonstrated to be cell cycle regulated with 2-3 fold elevation of transcript levels at G1 phase with a subsequent rise in protein levels during S-phase. Kinetic analysis of the mRNA and protein induction suggested that expression of this gene is controlled by a repression mechanism (Muller and Caradonna, 1993).

A third uracil-DNA glycosylase was isolated from a human placental library utilizing monoclonal antibodies generated against the 36 kDa glycosylase (Meyer-Siegler *et al.*, 1992). Sequence analysis of the cDNA showed complete homology with glyceraldhyde-3-phosphate dehydrogenase. The transcription of this multifunctional gene was determined to be modulated by the proliferative state of the cell, and a correlation was found to exist between the transcription of the GAPDH/UDG gene and the level of uracil-DNA glycosylase activity (Meyer-Siegler *et al.*, 1992).

The developmental regulation of a 33 kDa uracil-DNA glycosylase was examined by *in vitro* biochemical assays in a rat model system (Weng and Sirover, 1993). In adult animals, liver, kidney and spleen displayed significant levels of activity while the brain, heart and lung had low activity. Glycosylase activity was minimal at early stages of life; however, maximal levels of activity were observed at 14-21 days after birth. These results led the authors to conclude that individual organs express this enzyme in a distinct and specific pattern during development.

2. DNA glycosylase-AP lyases

This type of DNA repair molecule releases modified nucleotides by a specific N-glycosylase activity and then hydrolyzes the phosphodiester bond 3′ to the abasic sugar producing 5′-hydroxyl and 3′-phosphate termini. To date, all AP lyases (class I AP endonucleases - Figure 1) have been found to possess associated DNA glycosylase activities.

Pyrimidine dimer-DNA glycosylase has been characterized in *M. luteus* and T4 phage-infected *E. coli*. The T4 phage gene (*denV*) has been cloned and found to encode a 16 kDa protein termed T4 endonuclease V (Radany *et al.*, 1984; Valerie *et al.*, 1985). Apparently, the two reactions are frequently uncoupled as the enzyme carries out the two cleavage reactions sequentially (Liuzzi *et al.*, 1987). Mutants deficient for the T4 endonuclease V display hypersensitivity to UV, indicating that this enzyme plays a vital role in phage survival (Lindahl, 1982). The *M. luteus* DNA-glycosylase is similar to T4 endonuclease V in that its glycosylase and AP endonuclease activities are largely uncoupled. However, mutants deficient in the DNA-glycosylase activity have wild-type UV resistance that has been atributed to the organism's possession of a nucleotide-excision repair system (Tao *et al.*, 1987). Endonuclease III, is a class I AP endonuclease and minor contributor to the total AP endonuclease activity in *E. coli*. This enzyme has been found to posses a glycosylase activity which is specific for hydrated pyrimidines induced by UV radiation (Bailly and Verly, 1987).

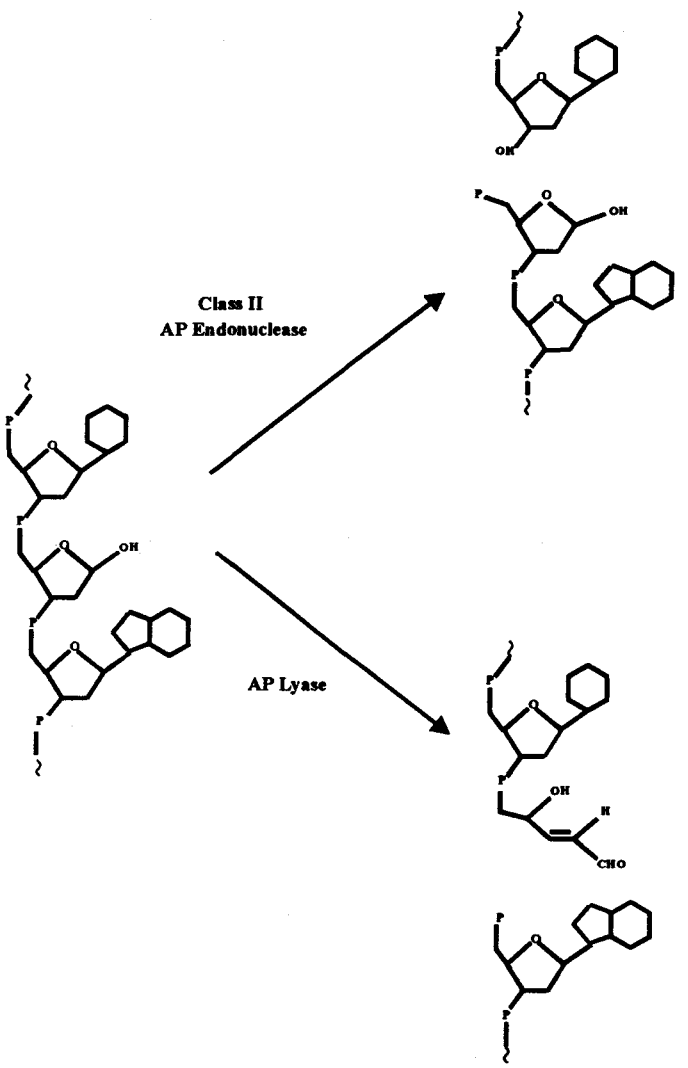


Figure 2. Schematic of the base excision repair pathways. Upper pathway: class II AP endonuclease. Lower pathway: class I AP endonuclease.

In addition to its role as a ribosomal protein, the multifunctional *Drosophila* S3 protein was recently determined to function as a class I AP endonuclease (Wilson *et al.*, 1994). The cDNA of this gene was cloned utilizing the cDNA of the rat S3 homologue and was found to encode a small protein of approximately 27 kDa. Even more recent was the discovery that, like all other AP lyases, S3 has an associated glycosylase activity that is specific for the recognition and repair of 8-oxoguanine residues, a major product of oxidative DNA damage (Kelley *et al.*, 1995).

3. AP endonucleases

Apurinic and apyrimidinic sites are among the most common DNA lesions and can lead to polymerase halting, and, therefore, aberrant transcripts and even cell death (Zhou and Doetsch, 1993). Consequentially, AP endonucleases have a critical role in the maintenance of DNA integrity and cell survival. The major cellular AP endonucleases belong to class II and can cleave the DNA backbone around an AP site 5′ leaving a 3′-hydroxyl and a 5′-phosphate termini (Doetsch and Cunningham, 1990)(Figure 2). The resulting 3′ hydroxyl group is an excellent substrate for DNA polymerase I and does not require further processing (Warner *et al.*, 1980). However, DNA polymerase I was found to be incapable of removing the abasic 5-deoxyribose molecule that is generated by this process (Mosbaugh and Linn, 1982). An enzyme, referred to as deoxyribose phosphatase (dRPase), that was first

characterized in *E. coli* appears to function soley in the removal of 5′ terminal deoxyriboses (Franklin and Lindahl, 1988). A functionally similar activity has been identified in human cells.

As with most other DNA repair enzymes, AP endonucleases have been most extensively studied in *E. coli*. This organism contains two major class II AP endonucleases: exonuclease III and endonuclease IV. Exonuclease III is a multifunctional class II AP endonuclease, and the major AP endonuclease activity in *E. coli* comprising approximately 90% of the cellular AP endonuclease activity (Rogers and Weiss, 1980). It was first detected as a combined 3′-phosphatase-exonuclease separated from DNA polymerase I during purification (Richardson *et al.*, 1964; Demple and Harrison, 1994). Exonuclease III is a single subunit enzyme of 28,000 kDa that requires divalent metal (Mg^{2+}) for it function (Sancar and Sancar, 1988). It has been shown to posses 3′→5′ exonuclease and 3′ phosphatase activity on duplex DNA and RNase H activity on RNA-DNA hybrids (Richardson *et al.*, 1964; Saporito and Cunningham., 1988; Weiss, 1981). Being a class II AP endonuclease, it incises on the 5′ side of an AP site, leaving a 3′ hydroxyl group and a 5′ phosphate (Kow, 1989). Exonuclease III mutants (*xth*) are modestly sensitive to killing by methyl methane sulfonate (MMS) (Friedberg, 1985; Weiss and Grossman, 1987), and near UV (Sammartano *et al.*, 1986), and are extremely sensitive to cell killing by H_2O_2 (Demple *et al.*,

1983). These agents all create fragmented bases and/or AP sites (Sancar and Sancar, 1988). The hypersensitivity to H_2O_2 is due to the lack of removal of 3′-terminal deoxyribose fragments from radical-induced DNA strand breaks (Demple *et al.*, 1986).

Endonuclease IV is a class II AP endonuclease which represents 8-10% of total AP endonuclease activity (Ljungquist *et al*, 1976; Saporito and Cunningham, 1986). It is a monomeric protein of 33 kDa and is fully active in the presence of EDTA (Sancar and Sancar, 1988). Endonuclease IV mutants (*nfo*) are sensitive to killing by the alkylating agents MMS and mitomycin C (Cunningham *et al.*, 1986). Genetic studies have demonstrated that endonuclease IV functions in the repair of oxidative DNA damage *in vivo*. *E. coli nfo* mutants are hypersensitive to killing by the oxidative agents bleomycin and *t*-butyl hydroperoxide. Additionally, *nfo* mutations exacerbate the sensitivity of *xth* mutants to H_2O_2 (Cunningham *et al.*, 1986). As will be discussed in detail in a later section, endonuclease IV was found to efficiently recognize 4′-oxidized AP sites generated by exposure of DNA to Fe(III)-bleomycin (Häring *et al.*, 1994).

The major AP endonuclease in *S. cerevisiae* is Apn1. This AP endonuclease is functionally similar to and structurally related to the *E. coli* endonuclease IV (Demple and Harrison, 1994). Apn1 is a class II AP endonuclease with 3′ PGA diesterase and 3′ phosphatase functions specific for duplex DNA

(Johnson and Demple, 1988), and its protein sequence of 367 residues has a 41% amino acid identity to *E. coli* AP endonuclease IV (Demple and Harrison, 1994). Additionally, like its *E. coli* homologue, Apn1 functions to repair both oxidative- and alkylation-induced DNA damage. Apn1 mutants are hypersensitive to killing by H_2O_2, *t*-butyl hydroperoxide, and alkylating agents (Popoff *et al*, 1990; Ramotar *et al.*, 1991). The hypersensitivity was determined to be the result of un-repaired oxidative strand breaks and apurinic sites in the yeast chromosomal DNA (Ramotar *et al.*, 1991).

Immunocytochemical analysis of Apn1 in *S. cerevisiae* demonstrated that it is localized in the nucleus (Ramotar *et al.*, 1993). The C-terminal segment of this protein, which is missing in its *E. coli* homologue, functions in the localization of Apn1 to the nucleus. Truncation of this region was shown to restrict >90% of the protein in the cytoplasm without affecting the stability or enzymatic activity of the enzyme as demonstrated by *in vitro* assays (Ramotar *et al.*, 1993).

Two distinct AP endonucleases have been partially purified from Drosophila embryos (Spiering and Deutsch, 1981). The molecular weights of these two proteins, 66 and 63 kDa, is significantly higher than AP endonucleases isolated from other organisms. Biochemical assays for AP endonuclease activity has indicated that the 63 kDa protein is a class I AP lyase (these cleave 3′ leaving a 3′-alpha, beta-unsaturated aldehyde) (Figure 2) and that the 66 kDa protein is a class III AP endonuclease

(these cleave 3′ to an AP site to yield an abasic sugar with a 3′-phosphoryl group)(Spiering and Deutsch, 1986). This was the first, and only, report of a class III AP endonuclease. The antibody generated against the major human HeLa AP endonuclease was found to cross react with both Drosophila proteins indicating that they possess antigenic regions of similarity. Utilizing this antibody, studies have shown that the 66 kDa protein localizes to the nucleus while the 63 kDa protein translocates to the mitochondria (Dr. James Carney, personal communication).

Drosophila possesses another AP endonuclease enzyme referred to as Rrp1 that was originally purified based on its strand transferase activity (Lowenhaupt *et al.*, 1989). The C-terminal 250 residues of this protein have clear homology to the exonuclease III family, while the remaining N-terminal residues are not related to any known sequences (Sander *et al.*, 1991a). Rrp1 is a class II AP endonuclease with a 3′5′ exonuclease activity against undamaged, double-stranded DNA (Sander *et al.*, 1991b; Nugent *et al.*, 1993).

AP endonuclease have been cloned from a variety of mammalians including mouse, calf thymus, human placenta and human (Seki *et al.*, 1991; Robson *et al.*, 1991; Robson and Hickson, 1991; Seki *et al*, 1991). All are class II AP endonucleases, are similar in their molecular weights, around 35.5 kDa and are closely related to one another with 91-93% identity. This striking level of homology extends to the

prokaryotic homologues and suggests a significant conservation of function during evolution from a common ancestor (Figure 3). The mammalian AP endonucleases will be discussed in detail in the next section.

D. Mammalian AP Endonucleases

While biochemical evidence suggests that multiple human AP endonucleases exist, the cDNA for only one has been isolated (Demple *et al.*, 1991). The purified protein of 37 kDa, termed APE, constitutes the main AP endonuclease activity of HeLa cells. This enzyme possesses all of the DNA repair functions of exonuclease III except for the 3′-exonuclease activity against undamaged DNA. Interestingly, the mouse homologue, APEX, was found to have exonuclease activity (Seki, 1991). While these homologues lack obvious amino acid changes, it has been suggested that the APE is lacking exonuclease activity due to sequence differences at an as yet undetermined conserved active site (Demple *et al.*, 1991). As previously mentioned, APE is a class II hydrolytic AP endonuclease that incises thephosphodiester bond just 5′ to the abasic site to generate a normal 3′-hydroxyl nucleotide terminus and a 5′-terminal deoxyribose-5-phosphate (Levin and Demple, 1990). Additionally, APE has 3′ repair activity enabling it to remove fragments of deoxyribose from the 3′ termini of DNA strand breaks produced by free-radical attack. However, this 3′-repair activity is weak equaling only about 1% of the AP-cleaving activity of

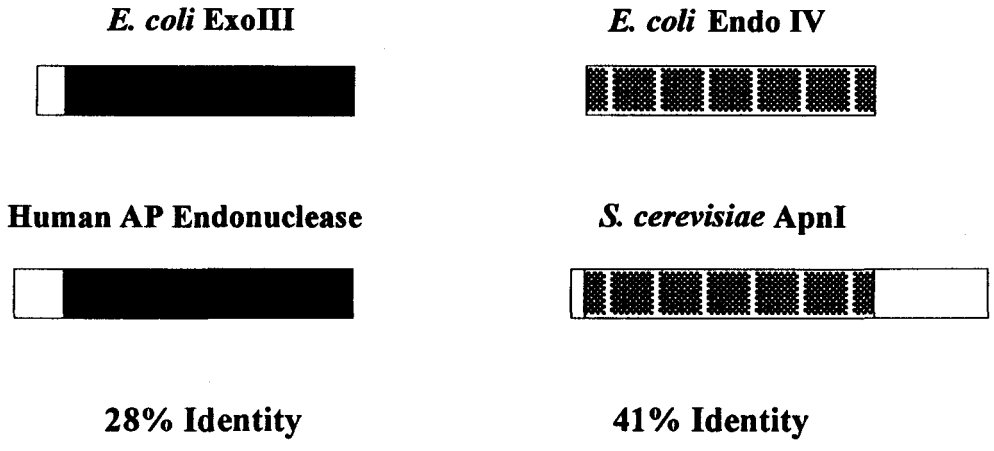


Figure 3. Comparison of the families of class II AP endonucleases.

exonuclease III in which the 3′-repair and AP-cleaving activities are about equal (Demple *et al.*, 1988; Levin *et al.*, 1988).

Complementation experiments indicate that hAPE can only partially replace its *E. coli* homologue, exonuclease III (Demple *et al.*, 1991). While APE conferred significant cellular resistance to the alkylating agent methyl methanesulfonate, it provided only slight resistance to the oxidative agent H_2O_2. As previously mentioned, exonuclease III-deficient *E. coli* are hypersensitive to the DNA damaging effects of hydrogen peroxide being unable to remove 3′-terminal deoxyribose fragments from free-radical induced DNA strand breaks (Demple *et al.*, 1983; Demple *et al.*, 1986). Therefore, considering the relatively weak 3′-repair activity of APE demonstrated *in vitro*, the human AP endonuclease appears to function poorly *in vivo* in the repair of 3′-terminal oxidative lesions and well in the repair of alkylation-induced AP sites (Demple *et al.*, 1991).

1. Multifunctional enzyme

Recently, it was discovered that APE is a multifunctional enzyme. While studying the redox factor termed Ref-1 which is responsible for the oxidation-reduction regulated DNA binding activity of Fos and Jun, Curran and co-workers discovered that this factor is identical to the previously cloned APE (Xanthoudakis, *et al.*, 1992a). The Ref-1/APE protein will for

the rest of the text be referred to as APE. *c-fos* and *c-jun* are members of a multigene family that is involved in signal transduction cascades associated with growth, differentiation, neuronal excitation, and cellular stress (Holbrook and Fornace, 1991; Morgan and Curran, 1991). Being early response genes, the expression of these two genes is induced rapidly and transiently in many cell types by a variety of extracellular stimuli, and are thought to function in the coupling of short-term signals initiated at the cell surface to changes in cellular phenotype by regulating the expression of specific target genes (Morgan and Curran, 1991). Fos and Jun proteins mediate their function as either Fos-Jun heterodimers or Jun-Jun homodimers; Fos does not form homodimers. In a reduced redox state, the homodimer/heterodimer is able to bind to activator protein-1 (AP-1) regulatory elements in the promoter region of target genes (Xanthoudakis *et al.*, 1992b; Nakabeppu *et al.*, 1988). The site of reduction-oxidation regulation of Jun DNA binding activity is a conserved cysteine residue (Abate *et al.*, 1990).

The redox activity of APE appears to be sensitive to the reduction-oxidation state of the protein; treatment of APE with thioredoxin was found to "activate" the redox activity of the protein (Xanthoudakis et al., 1992b). This finding suggested that a redox-sensitive group in APE is involved in the reductive activation of the FOS-JUN complex. To determine the region of the APE protein responsible for redox activity, Walker *et al*, 1993, performed deletion and site-directed mutagenesis analysis

of APE. All of the following results were derived from *in vitro* assays of recombinant APE protein. Truncation of the APE coding region indicated that the redox activity is mediated by sequences encoded within the 61 amino acid N-terminal domain. Additionally, it was found that this region is dispensable for the DNA repair functions of APE, but was necessary for reactivation of the DNA binding activity of oxidized Jun protein. As would be expected, this region is absent from exonuclease III which does not possess redox activity. By site-directed mutagenesis, cysteine 65 was identified as the redox active site and has been proposed to form a disulfide bridge with cysteine 93; the redox state of the two cysteines would mediate APEs ability to perform redox regulation of the Jun protein (Walker *et al.*, 1993).

A more complete study of the amino acid regions involved in either DNA repair or redox regulation was performed by Xanthoudakis *et al.*, 1994. N- and C-terminal truncated forms of histidine-APE fusion proteins were constructed and assayed for their redox and DNA repair activity. Gel retardation analysis utilizing purified FOS and JUN was used to determine the effect of the various deletions on redox activity. It was determined that deletion of the N-terminal 62 amino acids results in a 92% loss in redox activity while truncation to N-terminal amino acid 116 resulted in nearly a complete loss of function. However, the authors noted that the residual activity possessed by this deletion mutant may be attributed to carryover of DTT from a

preparative buffer, raising some concern about the accuracy of their results. C-terminal truncations indicated that amino acids critical for the redox activity of APE are contained within the first 127 amino acids of the protein. For DNA repair activity, the conversion of acid-depurinated supercoiled plasmid DNA to open circle plasmid DNA by the recombinant protein was monitored. The N-terminal 162 amino acids of APE were found to be non-essential to it's AP endonuclease activity. Together, these results indicate that the redox and AP endonuclease activities of APE are encoded by distinct amino acid regions within the protein and that these activities are functionally separate. It must be noted again that all of the studies on the redox activities of APE have been performed *in vitro* by biochemical analysis. The presence/significance of this activity *in vivo* awaits further study.

Recently, another activity was reported for APE. Okazaki, *et al.*, 1994, determined that APE is involved in negative regulation of the parathyroid hormone (PTH) gene by extracellular calcium. This repression of PTH expression is mediated, in part, by high calcium levels that increases the interaction of the nCaRE (negative calcium-responsive DNA element) in the upstream promoter region of the PTH gene with its nuclear binding protein nCaREB (nCaRE binding protein). This *in vitro* study using recombinant APE protein and nuclear extracts indicated that APE binds to nCaREBs potentiating their

binding to nCaREs. Again, the significance of this activity *in vivo* has not been demonstrated.

2. Gene regulation/expression

DNA repair genes have generally been considered to be "housekeeping" genes that are expressed at relatively the same basal level in all tissues. However, recent evidence has emerged which indicates that mammalian AP endonuclease gene expression is highly sensitive to various stimuli including the oxidative state of the cell along with differentiation and cell type.

First, the functional deletion of human AP endonuclease expression was performed in a human cell line. Hela cells were transfected with a construct that expresses antisense APE RNA (Walker *et al.*, 1994). While stable HeLa cell transfectants were normal in their growth rate, morphology and plating efficiency, they exhibited hypersensitivity to killing by a variety of alkylating and oxidative, agents but not to UV. This result was nearly identical to the study by Ono *et al.*, 1994, which demonstrated increased sensitivity of rat glioma cells expressing antisense AP endonuclease RNA to DNA damaging agents.

Of great interest in the HeLa cell study was the finding that the antisense transfectants were exceptionally sensitive to changes in oxygen tension. These cells displayed a high level of killing following exposure to both hypoxic (1% oxygen) and hyperoxic (100% oxygen) conditions. Examination of APE protein

levels indicated that the expression of this protein was induced following hypoxic stress. This observation, along with previous studies that failed to show induction of APE protein expression by DNA damaging agents, led the authors to suggest that the induced level of APE expression was necessary to counteract cytotoxic agents generated during hypoxic growth conditions, but not by DNA damaging agents (Walker *et al.*, 1994).

Second, the expression of the mouse AP endonuclease (APEX) gene during mouse brain development was investigated by *in situ* hybridization and northern blot analysis by Ono and co-workers (Ono *et al.*, 1995). Using *in situ* hybridization, APEX mRNA was found to be expressed at high levels in the telencephalon, brainstem, and cerebellum during the embryonic stages. During late embryonic and post-natal stages, APEX expression in the pyramidal and granular layers of the hippocampal formation, and in the cerebellum, was high. By northern analysis, the authors demonstrated that APEX mRNA levels decreased with development from embryonic to adult stages in the parietal cortex and cerebellum. The expression of c-fos and c-jun was also examined by northern analysis. c-fos mRNA expression in the parietal cortex and cerebellum was found to be undetectable at early developmental stages with a rapid rise of transcript levels at postnatal day 14. c-jun expression remained constant throughout development. From this study, the authors concluded that the expression of APEX is developmentally regulated with the highest levels of expression being in proliferative zones of the brain.

This pattern of expression was suggested to indicate that APEX plays a role in the proliferation and/or differentiation of the brain during development, perhaps through the redox modification of AP-1 binding proteins (Ono *et al.*, 1995).

E. Clinical Relevance of Apurinic/Apyrimidinic Sites

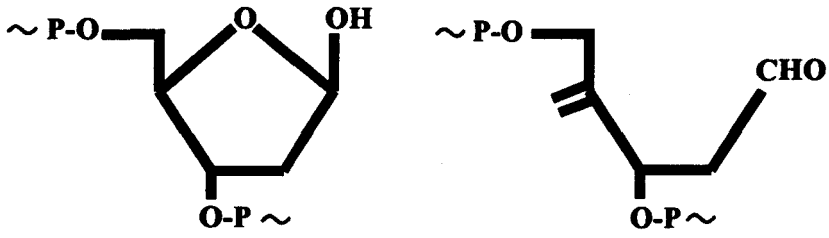
A wide variety of chemotherapeutic agents target DNA causing a variety of damage including adduct formation, cross-links, abasic sites, and single and double strand breaks. Bleomycin, a glycopeptide antibiotic, has been useful as a anti-tumor agent in the treatment of a number of neoplasms. The cytotoxic effect of this agent has been determined to be through DNA damage (Harrison and Lazo, 1987). Bleomycin forms a ternary complex with molecular oxygen and Fe(II)(bleomycin-Fe(II)-O_2) that undergoes a reduction reaction forming "activated" bleomycin capable of reacting with DNA forming AP sites, and single and double strand breaks (Lazo and Sebti, 1991). It has been suggested that the reduction of the bleomycin complex is mediated by endogenous reducing agents such as glutathione, NADH, ascorbate, and various electron transport proteins (Levy *et al.*, 1989).

A side effect of the clinical use of bleomycin is pulmonary fibrosis which tends to manifest itself after repeated administrations (Lazo and Sebti, 1991). A number of studies have indicated that the toxic effect of bleomycin toward lung tissue may be through DNA damage (Harrison *et al.*, 1989). Utilizing a

bleomycin sensitive strain of mice, Harrison *et al.*, 1989, demonstrated that the persistence of DNA damage, rather than the initial level of strand scission, is responsible for the development of fibrotic lung disease.

The study of DNA repair mechanisms involved in the repair of bleomycin-induced DNA damage has recently begun. Following repeated administrations of bleomycin to a sensitive mouse strain, one group discovered that, concomitant with initial inflammatory changes, there was a 2-fold increase in DNA polymerase β activity, a polymerase that is generally thought to be associated with base excision repair (Katsura *et al.*, 1992).

Häring *et al.*, 1994, recently demonstrated the involvement of AP endonucleases in the repair of bleomycin-induced DNA damage. Two forms of AP sites can occur: regular sites which result from the hydrolysis of the N-glycosylic bond, and oxidized sites that are the result of free radical attack at positions C- 1′, C-2′ or C-4′ of the sugar moiety (Figure 4). It has been demonstrated that "activated" bleomycin reacts with the C-4′ position of the sugar residue generating a 4′-oxidized AP site along with strand breaks (Hecht, 1986). In a study examining the recognition of oxidized AP sites by repair enzymes including endonuclease IV, exonuclease III, T4 endonuclease V, endonuclease III, and formamidopyrimidine-DNA glycosylase, it was found that 4′-oxidized AP sites are excellent substrates for endonuclease IV requiring 4-fold lower concentrations of the protein than for recognition of regular AP sites (Häring *et al.*,



"Regular" AP Site

4' - Oxidized AP Site

Figure 4. Coparison of the chemical structures of regular and oxidized sites of base loss (AP sites).

1994). Taken together, these results indicate that the base excision repair pathway, and AP endonuclease IV in particular, is involved in the repair of bleomycin induced 4′ oxidized AP sites. These findings have significant implications for future studies on the prevention/treatment of bleomycin induced fibrotic lung disease.

CHAPTER III

MATERIALS AND METHODS

A. Animals

Wistar-Harlan rats were supplied by Harlan Laboratories (Indianapolis, IN). For studies of adult animals, 400 gm male animals were used. For developmental studies, pregnant dams (gestation day 0 = day of sperm positive) were used. Animals were killed by decapitation while anesthetized. Specimens were rapidly dissected, immediately frozen in chilled 2-methylbutane (-20^0C) and stored at -80^0C.

B. Tissues

Human biopsy (duodenum, colon, liver and skin) and autopsy (brain) tissue sections were obtained from Dr. John Duguid of the Roudebush VA medical hospital, Indianapolis, IN and from Dr. Cunningham of the Tissue Procurement Service, University of Indiana University hospital, Indianapolis, IN.

C. Antibody

The polyclonal rabbit anti-human apurinic/apyrimidinic endonuclease antibody (hAPE) was previously produced in this

laboratory. The polyclonal rabbit anti-human MSH2 antibody was provided by Dr. Richard Fishel of the University of Vermont.

D. Oligonucleotides

Oligonucleotide primers were provided by the Wells Research Foundation Oligonucleotide Synthesis Facility.

E. Screening cDNA and Genomic Libraries

1. cDNA. A lambda gt11 rat testis library was screened with radioactive labeled rat AP endonuclease cDNA by standard protocol (Maniatis, *et al.*, 1982). Approximately 3.6 x 10^5 plaques were screened by plating 6 x 10^4 pfu/150 mm plate. The cell line XL1 Blue-MRA was incubated with the library phage for 20 minutes at 37^{0}C, mixed with prewarmed LB top agarose, and poured onto NZY agar plates. Plaques were nearly confluent and touching at the end of a six hour incubation at 37^{0}C. The phage DNA was transferred to 0.45 micron nytran membrane (Ambion, Austin, TX) by placing the membrane over the top agarose for 1 minute. This was followed by denaturation for 2 minutes in 1.5 M NaCl, 0.5 M NaOH and renaturation for 5 minutes in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0. After a brief rinse in 2X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), the filters were dried on whatman paper and the DNA crosslinked to the filter in a Stratalinker.

For hybridization, the filters were prehybridized for 1 hour at 42^{0}C with 50% formamide, 5X Denhardt's solution (0.5% polyvinly pyrolidone, 0.5% BSA, 0.5% ficol), 1% sodium dodecyl sulfate

(SDS), 1 M NaCl, 10 mM $NaPO_4$, pH 6.5, 0.1% pyrophosphate, and 250 mg/ml salmon sperm DNA (heat denatured by boiling 10 minutes). PCR generated rAPEN cDNA was radiolabeled utilizing the Decaprime kit from Ambion (Austin, TX) and added to the prehybridization solution and allowed to hybridize for 24 hours at 42°C. The filters were then washed in 0.2X SSC for 20 minutes at 65°C and wrapped in saran wrap. Autoradiographs were obtained by exposing film to the filters at -80°C overnight. Plaques with positive signals were picked and placed in 500 µl of SM (100 mM NaCl; 8 mM $MgSO_4$-H_2O; 50 mM Tris-HCl, pH 7.5; 0.01% gelatin) with 10 µl of chloroform and stored at 4°C. Three total rounds of plaque purification was completed until pure populations of a specific phage was isolated.

2. Genomic. A rat genomic DNA library (Stratagene, La Jolla, CA) was screened with the rAPEN cDNA fragment using the same procedure described above.

F. Purification of Lambda Phage DNA

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 Quiagen lambda purification procedure (Chatsworth, CA) and Promega Wizard columns (Madison, WI) were utilized. An inoculum of 6 x 10^5 plaque-forming units (pfu) was mixed with the appropriate bacterial host strain (XL1 Blue MRA cells for both the genomic and cDNA libraries) and allowed to incubate at 37°C for 20

minutes. Approximately seven ml of prewarmed LB soft agarose (42^{0}C) was added to the absorbed phage/bacteria mixture and spread onto a 150 mm NZY (86 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% casein hydrolysate, 1.5% Difco agar, pH 7.5) plate that was allowed to incubate at 37^{0}C for six hours. Ten ml of SM (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl pH 7.5, 0.1% gelatin) buffer was applied to the plate and an overnight incubation was performed at 4^{0}C on a nutator. The buffer was removed from the plate, mixed with 100 µl chloroform, and the debris pelleted by centrifugation at 8,000 x g for 10 minutes. The supernatant was then incubated with 10 mg/ml RNAse and 4 mg/ml DNAse at 37^{0}C for 30 minutes. Two ml of ice cold buffer L2 (30% polyethylene glycol 8000, 3 M NaCl) was added, gently mixed in, and the sample incubated on ice for 60 minutes. The sample was centrifuged at 12,000 X g for 10 minutes, the supernatant discarded, and the pellet resuspended in 1 ml of buffer L3 (100 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, pH 7.5) using a pipetter. One ml of buffer L4 (4% sodium dodecylsulfate) was added and mixed in followed by an incubation at 70^{0}C for 10 minutes. After a brief chilling on ice, 1 ml of buffer L5 (2.55 M potassium acetate, pH 4.8) was added and mixed and the sample centrifuged at 16,000 X g for 30 minutes, 4^{0}C. The supernatant was collected avoiding the potassium dodecylsulfate layer. The lambda DNA was purified and concentrated using the Promega Wizard DNA Clean-up System.

G. Subcloning rAPEN cDNA Into pBlueScript

The cDNA insert was released from the lambda arms by digestion with the restriction enzyme *Eco*RI (5U/µg DNA) at 37°C for 2 hours. The insert was isolated by electrophoresis of the digestion reaction on a 0.8% agarose gel followed by excision and elution from the agarose gel slice. The DNA fragment was purified by organic extraction and subsequently ligated into linearized pBSK KS(-) (Stratagene, La Jolla, CA) that had been digested with *Eco*RI and treated with calf intestinal alkaline phosphatase (CIAP)(BRL,Grand Island, NY). CIAP treatment was carried out by adding 2 µl of phosphatase enzyme (BRL; 24 U/ml) 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 3:1 ratio of insert:vector was mixed with 1X ligase buffer (10X ligase buffer = 300 mM Tris-HCl, pH 7.5; 100 $MgCl_2$; 100 mM DTT), ATP (final concentration of 1 mM), and 1 µl of T4 DNA ligase (Promega, Madison, WI; 1 Weiss U/ml) in a final volume of 20 µl. 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 *E. coli* HB101 bacteria cells.

H. Sequencing

One ml of an overnight culture containing the plasmid of interest was pelleted at 4,000 rpm for 1 minute in a 1.5 ml microfuge tube. The supernatant was aspirated and the pellet vortexed for 10 seconds. 0.5 ml of STET buffer (8% sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA and 5% Triton X-100) and 10 µl of lysozyme (10 mg/ml) was added to the pellet, mixed and incubated at room temperature for 2 minutes. The tube was transferred to a heat block at 100°C for 1 minute followed by centrifugation for 5 minutes at 14,000 rpm, 4°C. The pellet was removed and 10 µl of 10 N NaOH was added, the reaction mixed and incubated at 37°C for 10 minutes. 0.4 ml of ice-cold isopropanol was added and the tube placed at -20°C for 10 minutes. Following a 5 minute centrifugation, the supernatant was aspirated and the pellet washed with 70% ETOH. The pellet was then dried at 65°C, resuspended in 16 µl of dH_2O and placed on ice for the sequencing reaction.

Sequencing was performed utilizing the Sequenase Version DNA Sequencing Kit from USB. 7 µl of the plasmid sample was put into a clean 0.5 microfuge tube with 1 µl sequencing primer (2 pmol) and 2 µl 5X reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The primer was annealed to the DNA by heating for 2 minutes at 65°C and then cooling slowly to <35°C over a 30 minute period. After chilling the reaction on ice, it was mixed with 1 µl 0.1 M DTT, 2 µl labeling mix (1.5 µM each of dGTP, dCTP, and dTTP), 0.5 µl [^{35}S]dATP and 2 µl Sequenase Polymerase (3.25 U) and

allowed to incubate at room temperature for 5 minutes. Next, 3.5 µl of the labeling reaction was added to each termination mix (ddG, ddA, ddT, and ddC) and incubated an additional 5 minutes at 37°C. The reactions were stopped by the addition of 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and heated to 80°C for 2 minutes prior to loading onto a 6% polyacrylamide gel. The samples were electrophoresed at 60V for approximately 2.5 hours. The gel was dried under vacuum onto 3 mm Whatman chromatography paper (Whatman International Ltd., Maidstone, England) and exposed to film (Hyperfilm-MB, Amersham, Sweden) overnight.

I. Tissue Culture

All cultures were maintained at 37°C in an atmosphere of 95% air, 5% CO_2.

1. Nb2 T-cells. Nb2 T-cells (clone 11c), an immature rat T-cell line, were obtained from Dr. Li-Yuan Yu-Li of Baylor University, Houston, TX. The cells were cultured in maintenance medium consisting of Fisher's medium with 10% fetal bovine serum (FBS), 10% horse serum, 10^{-4} M 2-mercaptoethanol, 2 mM glutamine and penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively).

2. A1235 glioma cells. This is a human cell line that was obtained from Dr. Russ Pieper of Loyola University of Chicago, Maywood, IL. The cells were cultured in Dulbecco's

modified essential medium (DMEM) supplemented with 10% FBS, 2mM glutamine and penicillin-streptomycin (50 U/ml and 50 µg/ml).

3. KCNR neuroblastoma cells. This is a human cell that was provided by Dr. Kent Robertson of the University of Indiana School of Medicine, Indianapolis, IN. These cells were cultured in DMEM supplemented with 10% FBS, 2mM glutamine and penicillin/streptomycin (50 U/ml and 50 µg/ml).

4. HL-60 and HL-60R myeloid leukemia cells. These lines were provided by Dr. Kent Robertson of the University of Indiana School of Medicine, Indianapolis, IN. The cells were cultured in RPMI supplemented with 5% FBS, 2mM glutamine and penicillin/streptomycin (50 U/ml and 50 µg/ml). For differentiation experiments, the cells were diluted to 2 x 10^5 cells/ml in the above media that also contained 10^{-5} M retinoic acid dissolved in ethanol. Cell samples were collected every 48 hours over a six day period for Western analysis. Controls samples contained an equal amount of ethanol (1/200 vol ethanol).

5. 3T3 mouse fibroblast cells. This cell line was provided by Dr. David Williams of the University of Indiana School of Medicine, Indianapolis, IN. DMEM supplemented with 10% FBS and penicillin/streptomycin (50 U/ml and 50 µg/ml) was utilized as the culture medium.

J. Total RNA Extraction

Total cellular RNA was isolated using a modification of a procedure previously described (Chomcyznski and Sacchi, 1987). Approximately 100 mg of liver tissue or 20 x 10^6 Nb2 cells was homogenized in 500 µl of 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 by the addition 50 µl 2 M sodium acetate, pH 4.0, 500 µl phenol, and 200 µl chloroform:isoamyl alcohol 49:1, incubation on ice for 15 minutes, and centrifugation at 10,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube and the RNA precipitated by the addition of an equal volume of 100% isopropanol followed by a 15 minute incubation on dry ice. The RNA 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 50 µl of GIT (this step was omitted for tissue culture cells - the next step is the 70% ETOH wash). The pellet was fully dissolved by heating to 65°C and racking. The RNA was precipitated by the addition of 1/10 volume 3 M sodium acetate, pH 5.2 and an equal volume of 100% isopropanol followed by incubation on dry ice for 15 minutes and centrifugation. The pellet was then washed with 500 µl 70% ethanol, dried at 65°C for 2 minutes and resuspended in 50 µl of diethylpyrocarbonate (DEPC, 0.2%) treated water. Again, the pellet was dissolved by heating to 65°C. The final RNA sample was quantified for concentration and

purity using a Pharmacia GeneQuant (Piscataway, NJ) and stored at -80°C until needed.

K. Northern Analysis

1. Purified total RNA. 10 µg of total RNA was precipitated with 0.1 volume 3 M sodium acetate, pH 5.2 and 2.5 volumes of 100% ethanol on dry ice for 15 min 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 µl of RNA Loading Dye (50% formamide; 1X MOPS; 15% formaldehyde; 10% glycerol) and 1 µl of ethidium bromide (1 mg/ml) by heating at 65°C for 15 minutes with periodic mixing. After incubation at 65°C, the sample was spun down and fractionated on a formaldehyde-agarose gel (1X MOPS buffer (0.2 M MOPS, pH 7.0; 50 mM sodium acetate, 10 mM EDTA), 1.2% 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 by soaking it for 2 X 30 minute periods in 10X SSC at room temperature with gentle shaking. During the gel washing procedure, 0.22 micron nitrocellulose membrane (MSI, Westboro, Ma) was prewet in distilled water followed by a 15 minute soak in 10X SSC. The RNA was then transferred by capillary action overnight. RNA was

subsequently fixed to the membrane by crosslinking using a Stratagene UV Stratalinker 1800 (La Jolla, CA).

2. Premade Human Multiple Tissue Blot. Two premade Northern analysis blots were purchased from Clontech (Palo Alto, CA).

L. Reverse Transcription

Three µg of total RNA were added to a reaction tube containing 1 X reverse transcription buffer (50 mM Tris-HCl (pH 8.3) 75 mM KCl, 3 mM MgCl2), 10 mM dithiotreitol, 2 mM each dNTPs, 100 pmol oligo-(dT) primers (Bethesda Research Laboratories, Gaithersburg, MD), and RNase-free deionized distilled water to a final volume of 19 µl. This mixture was heated for 10 min at 65°C and quenched on ice, and 200 U Moloney murine leukemia virus RNase H^- reverse transcriptase (Superscript, Bethesda Research Laboratories) in 1 µl are added for a total reaction volume of 20 µl. This mixture was incubated at room temperature for 10 min and then at 42°C for 1 hour. The reaction was terminated by heating at 95°C for 5 min and quenching on ice. RNase-H (2U) was then added to the reaction and incubated for 20 min at 37°C.

M. Polymerase Chain Reaction

Five µl of the reverse transcription reaction from each sample or 1 µg of lambda or genomic DNA was diluted into a final volume of 100 µl in 1 X PCR buffer buffer (10 mM Tris-HCL, 1.5 mM

MgCl , 50 mM KCl, pH 8.3), 1U *Tfl* thermostable polymerase (Promega, Madison, WI) and 10 pmol of each 5′ and 3′ oligonucleotide primers. The oligonucleotides for multiple tissue RT-PCR analysis were as follows: 5′ oligonucleotide; 5′-CTGGACTTACATGATGAATGCCCG-3′ and 3′ oligonucleotide; 5′-GAAGAGATAACGCACTGGTCTCCT-3′. These oligonucleotides correspond to the region of the rat AP endonuclease gene at 748 bp 3′ of the ATG and 47 bp 3′ of the TGA, respectively. The oligonucleotide primers for genomic PCR analysis were:

A: 5′-ATGCCGAAGCGGGGGAAGAG-3′

B: 5′-CGAGCCAGAGACCAAGAAGAC-3′

C: 5′-GAGCTGACCAGTACTGATGG-3′

D: 5′-CATGAGCCACATTGAGATCC-3′

E: 5′-GAAGAGATAACGCACTGGTCTCCT-3′

The polymerase amplification was carried out using a MJ Research (Watertown, MA) programmable heating/cooling dry block for 25 cycles of amplification (94^{0}C, 30 sec; 60^{0}C, 1 min; 72^{0}C, 2 min), followed by 10 min at 72^{0}C. For RT-PCR analysis, a reaction was performed as described above, but without the addition of reverse transcriptase as a test for the presence of contaminating genomic DNA in the RNA samples. The oligonucleotide primers spanned at least one intron for genomic contamination controls.

Following amplification, 10 µl of each PCR product was electrophoresed on a 2% agarose gel containing 0.5 mg/ml ethidium bromide in Tris borate-EDTA buffer. After photography

of the gels using UV light, they were prepared for transfer and blotted onto nitrocellulose. The nitrocellulose was prehybridized in 5 x Denhardt's solution, 250 mg/ml denatured salmon sperm DNA, 1% (wt/vol.) sodium dodecyl sulfate (SDS), 0.1% tetrasodium pyrophosphate, and 50% deionized formamide for 12 h at 42°C. Hybridization was performed overnight under stringent conditions at 42°C in the same solution as that used for prehybridization, but included in the mixture was a [a-^{32}P]dCTP random primed AP endonuclease or histone H3.3 cDNA. The blots were washed under high stringency conditions in 1 x SSC and 0.5% SDS at room temperature for 15 min and then three times in 0.2 X SSC-0.5% SDS at 65°C for 20 min/wash. The washed filters were blotted dry and exposed to x-ray film at -70°C with enhancing screens (Dupont/New England Nuclear, Boston, MA).

N. 5'-End Mapping of rAPEN Transcript

The 5′ end of the rAPEN transcript was determined utilizing the 5′/3′ RACE Kit from Boehringer Mannheim (Indianapolis, IN). One µg of either the rat Nb2 T-cell line or rat testicular total RNA was mixed with a cDNA synthesis primer specific for the rAPEN transcript (5′-GAAGAGATAACGCACTGGTCTCCT-3′), deoxynucleotides, 5X cDNA synthesis buffer (250 mM Tris-HCL, 40 mM $MgCl_2$, 150 mM KCl, 5 mM DTT, pH 8.5), AMV reverse transcriptase (40 U) and the reaction brought up to 20 µl with dH_2O. This was incubated for 60 minutes at 55°C followed by a 10 minute incubation at 65°C. The cDNA was then purified using the Boehringer Mannheim High Pure PCR Product

Purification Kit and a homopolymeric tail appended using terminal transferase and dATP. 20 µl of the cDNA was mixed with 10X reaction buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM Kcl, pH 8.3) and 2 mM dATP. The reaction mixture was incubated for 3 minutes at 94°C and chilled on ice. One µl of terminal transferase (10 U) was added and the reaction incubated for 10 minutes at 37°C followed by 10 minutes at 70°C and chilling on ice. PCR amplification of the dA-tailed cDNA was accomplished utilizing an oligo dT-anchor primer with an attached known sequence and a nested rAPEN specific primer. The dA-tailed cDNA was mixed on ice with the oligo dT-anchor primer (5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTV-3'), the specific primer (5'-CATGAGCCACATTGAGATCC-3'), deoxynucleotides, Tfl DNA polymerase (1 U, Promega, Madison, WI), 10X reaction buffer (100 mM Tris-HCL, 15 mM MgCl2, 500 mM KCl, pH 8.3) and dH_2O up to 50 µl. This reaction mixture was subjected to the following PCR cycling conditions: 72°C for 5 minutes, 94°C for 3 minutes, 60°C for 1 minute, 72°C for 3 minutes followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 2 minutes with a final 72°C incubation for 10 minutes. A second round of PCR amplification was performed to obtain a visible band by agarose gel electrophoresis. The conditions were same as above except the 5 µl of the first PCR reaction was used with a second nested rAPEN specific primer (5'-GGCCGAATTCGAGCTGACCAGTACTGATGG-3'). The fragment was then subcloned into pBSK (Stratagene) utilizing the built in endonuclease restriction sites present in the oligo dT-

anchor primer (*Sal*I) and the second nested specific primer (*Eco*RI).

O. Genomic DNA Isolation

1. Nb2 DNA for Genomic PCR Analysis. Approximately 20 X 10^6 Nb2 cells were lysed in a 50 ml conical tube with 8 mls of basic lysis solution (17 mM $NaHCO_3$, 28 mM Na_2CO_3, 2mM EDTA pH 8.0, 0.5% N-lauroyl sarcosine, 300 µg/ml Proteinase K) by pipetting the lysate up and down two to three times with a 5 ml pipette. The lysate was incubated at 37°C for three hours with periodic mixing. The lysate was mixed with 0.5 volume phenol/0.5 volume chloroform by inverting the tube for 5-10 minutes until a phenol emulsion formed. The phases were separated by centrifugation at 2000 rpm for 5 minutes. The aqueous phase was removed to a new 50 ml conical tube and the organic extraction repeated once with a final chloroform extraction. The aqueous phase was transferred to a 25 ml Erlenmeyer flask containing 1.6 mls of 11 M ammonium acetate and mixed. 2.5 volumes of 95% ethanol was added, the flask tumbled until the DNA precipitated into threads, and the DNA spooled out and washed in 70% ethanol. The DNA was partially dried and dissolved in 400 µl dH_2O in a 1.5 ml microfuge tube by placing the tube at 37°C for 30 minutes with periodic tapping. Four µg of RNAse A was added to the DNA and the sample incubated at 37°C for 30 min. The organic extraction was repeated as described above. The DNA was precipitated out by the addition of 40 µl of 3 M sodium acetate and 1 ml of 95% ethanol. The DNA was

again spooled out, washed with 70% ethanol and dissolved in 400 µl dH_2O overnight at 4^0C.

2. 3T3 Transfectants. Genomic DNA was isolated from transfected 3T3 fibroblasts utilizing the Puregene DNA Isolation Kit from Gentra Systems, Inc. (Minneapolis, MN). Approximately 2 million cells were lysed on their growing substrate (tissue culture plate) with 600 µl cell lysis solution and RNase A treated (12 µg) for 30 minutes at 37^0C. Protein in the sample was removed by adding 200 µl protein precipitation solution, vortexing vigorously for 20 seconds, and centrifugation at 12,000 x g for 3 minutes forming a pellet. The supernatant was removed to a microfuge tube containing 600 µl 100% isopropanol and mixed by inverting the tube. The DNA was pelleted out by centrifugation for 1 minute, washed with 70% ethanol and dried. DNA hydration solution was added to the pellet and placed at 65^0C for 1 hour to rehydrate the DNA.

P. Biochemical Analysis of rAPEN Activity

PM2 DNA was partially depurinated by heating 70^0C at pH 5.2 for 15 minutes to produce roughly one AP site per DNA plasmid. Assay mixtures (50 µl) contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 170 fmol of PM2 [^{3}H]-DNA molecules (27 cpm/fmol molecules), and a sample of protein. Incubations were for 10 minutes at 37^0C. The number of nicks introduced into closed circular PM2 DNA was determined by a nitrocellulose filter binding assay.

Q. Purification of GST-rAPEN Protein From Inclusion Bodies

To construct a Glutathione S-Transferase (GST)/rat AP endonuclease fusion construct, the cDNA was ligated downstream of the GST coding region in the plasmid pGEX-3X. cDNA with appropriate restriction sites at each end (5'-BamHI; 3'-EcoRI) compatable with pGEX-3X vector restriction sites was generated by polymerase chain reaction utilizing the rat AP endonuclease pBSK plasmid as a template and the following primers:

5'- 5'-GGCCGGATCCATGCCGAAGCGGGGGAAGAG-3',

3'- 5'-GGCCAATTCTACAGTGCTAGGTAAAGGGT-3'.

The reaction mixture consisted of 1 µg of template, 10 pmols each primer, deoxynucleotides, Tfl DNA polymerase (1 U, Promega, Madison, WI), 1X reaction buffer (10 mM Tris-HCL, 1.5 mM MgCl2, 50 mM KCl, pH 8.3) and dH_2O up to 100 µl. The sample was subjected to the following PCR cycling conditions: 72°C for 5 minutes, 94°C for 3 minutes, 60°C for 1 minute, 72°C for 3 minutes followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 2 minutes with a final 72°C incubation for 10 minutes. The resulting PCR product was digested with *Bam*HI and *Eco*RI (5U each/µg DNA) at 37°C, gel purified, ligated into linearized pGEX-3X and transformed into competent cells. DH5α cells carrying the GST-rAPEN construct were inoculated into 50 ml of LB plus 100 µg/ml ampicillin and incubated overnight at 37°C with shaking. The culture was diluted 1:10 into prewarmed (37°C) LB plus AMP and allowed to incubate for 1 hour at 37°C with shaking. Expression of the recombinant protein was induced by the addition of isopropyl-*β*-*D*-thio-

galactoside (IPTG) to a final concentration of 0.1 mM followed by an additional 3 hours of incubation. The cells were then pelleted (2,000 rpm for 10 min.), washed once with PBS, repelleted and resuspended in 2 ml of PBS. To lyse the cells, they were sonicated at 45 watts 3 X 5 minutes with 30 seconds between bursts. Triton X-100 was added to a final concentration of 0.1% and the cell lysate was centrifuged at 8,500 x g for 10 minutes and the supernatant collected. The supernatant was then incubated in an elution column with 1 ml of glutathione-sepharose beads (Sigma, St. Louis, MO) overnight at 4°C on a nutator. The column was washed with 20 column volumes of PBS plus 0.1% Triton X-100. The protein was eluted with 50 mM Tris, pH 8.0, 10 mM glutathione and collected in 0.5 ml fractions. The fractions were analyzed by electrophoresis on a 12% SDS-polyacrylamide gel followed by coomassie blue staining.

R. Gel purification of GST-rAPEN for Antibody Production

To isolate the antigen for antibody production, the column purified GST-rAPEN protein samples were fractionated on a 12% SDS-polyacrylamide gel at 150 V for 1.5 hours. The gel was stained with comassie blue, destained, and the rAPEN band excised from the gel. The gel strip was then placed into dialysis tubing with 1 ml of 1X SDS electrophoresis buffer (0.125 M Tris-HCl; 0.96 M glycine; 0.5% SDS). The tubing was sealed and the antigen eluted in 1X SDS electrophoresis buffer at 150 V for 1 hour. The protein sample was dialyzed overnight

at 4°C against 1 L of dH_2O and quantified using the Bio-Rad protein assay. A total of 1.8 mg of antigen was provided to HTI bioproducts (La Jolla, CA) for antibody production.

s. Western Blot Analysis

Ten µg of each sample was fractionated on a 12% SDS-polyacrylamide gel at 150 V for 1 hour utilizing the BioRad Mini-Protean II system (BioRad, Hurcles, CA). The gel and 0.22 micron nitrocellulose membrane (MSI, Westboro, MA) were soaked in transfer solution (25 mM Tris-HCl, 192 mM Glycine, 20% Methanol) for 30 minutes. The protein was transferred to the filter (90 V for 1 hour). Following transfer, the gels were soaked in 1% blocking solution (Boehringer-Mannheim, Indianapolis, IN) for 1 hour and then incubated with primary antibody overnight at 4°C on a Robbins Scientific Western Incubation rotator (Sunnyvale, CA). Both rabbit anti-rAPEN and rabbit anti-hAPE antibodies were used at a dilution of 1:500 for Western blots. After four 10 minute washes with TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5), secondary antibody (peroxidase conjugated goat anti-rabbit, Boehringer-Mannhiem) was incubated with the blot for 30 minutes at room temperature. Cross-reacting proteins were detected using the Chemiluminescence Western Blotting Kit from Boehringer-Mannheim.

T. Purification of rAPEN Antibody

Following the transfer of GST-rAPEN to nitrocellulose membrane as described in the Western blot analysis protocol, the membrane was briefly stained with Ponceau S (0.2% Ponceau S, 3% trichloroacetic acid, 3% sulfosalicyclic acid) to visualize the recombinant protein. The strip of membrane containing the protein was cut out, destained with 20 mM Tris-HCL pH 7.5, and blocked in blotto (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5, 5% Carnation powdered milk) for 1 hour. The membrane strip was then incubated with three ml of immune sera overnight at 4^{0}C. The next day, the strip was washed three times for 10 minutes each in TBST. To remove the bound anti-GST-rAPEN antibody, 250 µl of ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL) was incubated with the strip in a humidified chamber for 20 minutes on a rotator and collected.

U. Immunocytochemical Staining of A1235 Cells for the Human AP Endonuclease

A1235 cells were grown on 60 mm plates to about 70% confluency. The cells were washed with PBS and fixed in a 2% paraformaldehyde/0.1% Triton X-100 solution for 10 minutes. The cells were then rinsed with PBS and the plate wiped dry leaving a small circle of buffer and cells (approximately 1.5 cm in diameter). Blocking of non-specific antibody binding sites was accomplished by incubating the cells with 1.5% ovalbumin and 1.5% bovine serum albumin in PBS for 30 minutes at room temperature.

The block was wicked off and the cells incubated with the rabbit anti-hAPE antibody at a 1:50 dilution in blocking solution for 3 hours at room temperature in a humidified chamber. The cells were washed 3 X 5 minutes with PBS followed by incubation with the secondary antibody (biotinylated goat anti-rabbit antibody) for 1 hour at room temperature. Following three more washes in PBS, the cells were incubated with Vectastain Elite ABC reagent (Vector Labs, Burlingame, CA) for 30 minutes and washed once with PBS. The signal was developed by incubating the cells with the chromagen diaminobenzidine (Vector Labs) for approximately 3 minutes followed by a brief rinsing in dH_2O. For counterstaining, the sections were incubated in 95% ETOH for 2 minutes, phyloxine eosin for 2 minutes, 95% ETOH for 2 minutes, rinsed with PBS and coverslipped utilizing Aqua-mount (Lerner Laboratories, Pittsburgh, PA).

V. Subcellular Fractionation

Approximately 20 x 10^6 tissue culture cells were dounce homogenized in 1 ml TES buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM sucrose) 15 times. PMSF was added to 0.5 mM and aprotinin to 0.25%. The homogenate was centrifuges at 1000 x g for 10 minutes at 4^0C to separate the crude nuclei from the post-nuclear supernatant (PNS). The PNS was centrifuged again to remove any remaining nuclei. The protein fractions were analyzed by Western blot analysis as described in Method S..

W. Immunohistochemistry

Human autopsy (brain) and biopsy (duodenum, liver, colon and skin) specimens were fixed in 4% paraformaldehyde, paraffin embedded and sectioned at 6 µm onto Superfrost/Plus slides (Fisher, Itasca, IL). The sections were then baked at 60°C for 30 minutes, deparaffinized in xylene, and hydrated through a series of graded alcohols (100%, 95%, 70%, 50%) to dH_2O. Non-specific antibody binding sites were blocked by incubation for 30 minutes with 10% goat serum in PBS (0.1 M, pH 7.4). Affinity purified primary anti-hAPE antibody (rabbit anti-human polyclonal) was then incubated with the sections overnight at 4°C at a concentration of 25 µg/ml in 10% goat serum/PBS. The sections were washed 3 x 5 minutes in PBS followed by incubation with the secondary antibody (goat anti-rabbit IgG, Vector Labs, Burlingame, CA) at a 1:100 dilution in 10% goat serum for 1 hour. Following two five minute PBS washes, the sections were incubated with an aviden/biotin solution (ABC elite kit, Vector Labs) for 45 minutes. The signal was then developed by incubation with DAB (Vector Labs). After development of sufficient signal, the sections were washed briefly in dH_2O and dehydrated through a graded alcohol series to xylene. The sections were coverslipped using Permount (Fisher, Itasca, IL) and photographed.

For histological staining, the coverslips were removed with xylene, the sections hydrated through a series of graded alcohols to dH_2O, and the sections stained with either hematoxylin/eosin (HE) or toluidine blue. For HE, the sections were soaked in

Carazzi's hematoxylin for 7.5 minutes, rinsed for 15 minutes in tap water and placed in 95% ETOH for 2 minutes. The sections were incubated in phyloxine eosin for 2 minutes, 95% ETOH for 2 minutes, 100% ETOH for 3 x 2 minutes, xylene for 3 x 2 minutes and coverslipped. For toluidine blue, the sections were incubated in a 25% solution of toluidine blue for 7 minutes followed by a brief rinse in dH_2O. The sections were dehydrated through graded ethanol to xylene and coverslipped.

X. Riboprobes for *In Situ* Hybridization

cRNA probes for *in situ* hybridization were generated by utilizing polymerase chain reaction (PCR) generated primers. The primers were constructed so that they contained either the SP6 (sense probe) or T7 (antisense probe) polymerase promoter recognition sequences. The primers used were as follows:

rAPEN/sense:

5'-AGGGATCCATTTAGGTGACACTATAGTAGTAAACACTGCTTCGGTG-3';

rAPEN/antisense:

5'GGATCCTAATACGACTCACTATAGGGAGGGTACTGATGGGTGAGTCCAGG-3'

Colony PCR reactions were performed to generate template from DH5α cells tranformed with plasmids containing the rAPEN cDNA. The PCR reactions consisted of 1 X PCR buffer (100 mM Tris-HCL, 15 mM MgCl , 500 mM KCl, pH 8.3), 1U *Tfl* thermostable polymerase (Promega) and 10 pmol of each 5' and 3' oligonucleotide primers, and a stab of bacterial cells. The polymerase amplification was carried out using a MJ Research programmable heating/cooling dry

block for 25 cycles of amplification (94°C, 30 sec; 60°C, 1 min; 72°C, 2 min), followed by 10 min at 72°C.

The riboprobe synthesis were labeled with using the Ambion Maxiscript SP6/T7 Kit (Austin, TX). The reactions consisted of 1 µg of PCR generated template, 2 µl 10 X buffer, 1 µl 200 mM DTT, 1 µl Rnasin, 1 µl dATP, dCTP and dGTP, 100 µCi ^{35}S-α-thio dUTP (New England Nuclear, Boston, MA), and 1 U SP6 or T7 polymerase. This mixture was incubated at 37°C for 1.5 hours. Percent incorporation and probe counts were determined by using a scinitllation counter.

Y. *In Situ* Hybridization

AP endonuclease gene expression was studied by *in situ* hybridization using cRNA probes. ^{35}S labeled anti-sense and sense probes were generated from the coding region of the rat AP endonuclease cDNA as described above. The tissue sections were incubated in 4% paraformaldehyde for 30 minutes, 0.2 N HCL for 30 minutes, acetylated in 0.1 M triethanolamine containing 0.2% acetic anhydride for 10 minutes, and dehydrated through a graded alcohol series. The sections were then covered with 50 µl of hybridization buffer (50% deionized formamide, 10% dextran sulfate, 5X SSPE, 0.1% SDS, 1X Denhardts, 2.5 mg/ml yeast tRNA, 10 mg/ml herring sperm DNA, 0.1% NaPPi, 10 mM rATP, rUTP, rCTP and rGTP) containing 5 x 10^6 cpm of probe per ml. Glass coverslips were applied, and the sections were incubated overnight at 55°C. The next day, coverslips were removed and the sections were washed in 2X SSC for 30 minutes at 20°C, RNAse A (5 mg/ml) for 60 minutes

at 37°C, 2X SSC for 30 minutes at 20°C, 0.1X SSC for 60 minutes at 60°C and 0.1X SSC for 30 minutes at 20°C. Sections were then dehydrated in alcohol containing 0.3 M ammonium acetate and dried.

Film autoradiographs were generated by exposing slides to Kodak SB5 film for 2 to 7 days. Emulsion autoradiographs were generated by dipping sections in prewarmed (42°C) Kodak NTB2 emulsion and stored in the dark with Dryrite for approximately 2 weeks. Sections were then developed by soaking in Kodak 19 developer for 4 minutes, rinsing quickly in dH_2O, soaking in Kodak fixer for 4 minutes and then stained with 0.5% toluidine blue to facilitate identification of anatomical structures.

Z. Construction of AP Endonuclease Vectors

A pUC18 plasmid containing the human SPC-A promoter region, the SV40 small T intron and poly A (approx. 0.4 kb) track was obtained from Dr. David Williams of the University of Indiana school of Medicine (Figure 5). The coding regions of the yeast AP endonuclease (ApnI), the human AP endonuclease (hAPE) and a truncated form of the human AP endonuclease (ΔhAPE) that is missing the first 450 bp but contains its normal nuclear localization signal KRGK (shown underlined in primer sequence) were amplified by polymerase chain reaction with primers that contained restriction sites allowing subcloning into the pUC18 plasmid multicloning site immediately downstream of the SPC-A promoter (Figure 5). The oligonucleotide primers were as follows:

ApnI -

5': 5'-GGCCGTCGACATGCCTTCGACACCTAGCTTTG-3'

3': 5'-GGCCGAATTCTTATTCTTTCTTAGTCTTCCTC-3'

hAPE -

5': 5'-GGCCGTCGACATGCCGAAGCGTGGGAAAAAGGG-3'

3': 5'-GGCCGAATTCTCACAGTGCTAGGTATAGGGTGA-3'

ΔhAPE -

5': 5'-GGCCGTCGACATGAAGCGTGGGAAACATGATCAGGAAGGCCGG-3'

3': 5'-GGCCGAATTCTCACAGTGCTAGGTATAGGGTGA-3'

The 100 µl PCR reaction consisted of 1U Tfl (Promega, Madison, WI), reaction buffer (10 mM Tris-HCL, 1.5 mM MgCl , 50 mM KCl, pH 8.3), 10 mM dNTPs and 10 pmol each oligonucleotide primer. The resulting PCR products were purified with the Promega Wizard PCR Cleanup System and digested with 10 U each *Sal*I and *Eco*RI at 37^{0}C for 2 hours. The restriction digests were then heat killed for 10 minutes at 72^{0}C and purified again. The plasmid was digested with *Sal*I and *Eco*RI, purified, ligated to the inserts and transformed into competent DH5a cells as described in Method D.. The pUC18-SPC-A/Apn1 construct was then digested with *Nde*I and *Sal*I to remove the 3.5 Kb SPC-A promoter region. The pUC18/Apn1 containing fragment was gel purified as previously described. A plasmid containing the human phosphoglycerate kinase(PGK) promoter region (provided by Dr. David Williams) was digested with *Nde*I and *Sal*I releasing the PGK promoter sequence that was then gel purified. The pUC18/Apn1 fragment was ligated to the PGK promoter

and the resulting ligation mixture transformed into competent DH5α cells. The pUC18-PGK/Apn1 construct was then digested with *Sal*I and *Hind*III to release the Apn1 fragment. The pUC18-PGK fragment was gel purified. The pUC18-SPC-A/hAPE and pUC18-SPC-A/ΔhAPE constructs were digested with *Sal*I and *Hind*III to release the AP endonuclease coding regions. Gel purified hAPE and ΔhAPE fragments were ligated to the pUC18-PGK fragment creating pUC18-PGK/hAPE and pUC18-PGK/ΔhAPE. These constructs were transformed into competent DH5α cells. For transfection into the mouse 3T3 fibroblast cell line, 100 μg of each of the six constructs was purified using the Quiagen tip 100 Plasmid Purification Kit (Chatsworth, CA). The plasmids were transfected into 3T3 fibroblasts utilizing DOTAP from Boehringer-Mannheim (Indianapolis, IN). Positive clones containing one of the six constructs were selected for using G418 (Gibco BRL, Grand Island, NY). After approximately ten days, G418 resistant colonies were visible and subcultured into separate wells of a 24 well plate. PCR analysis, using the primers made for plasmid construction, and Southern analysis were performed to detect cell lines carrying the transfected AP endonuclease sequences. One cell line for each of the six constructs was selected for further experimentation.

AA. Protection of Mouse 3T3 Fibroblasts from Bleomycin Induced DNA Damage by Transfected DNA Repair Vectors

Mouse 3T3 fibroblasts cell lines that had been transfected with and stably integrated by the AP endonuclease DNA repair constructs (SPC-Apn1, SPC-hAPE, SPC-ΔhAPE, PGK-Apn1, PGK-hAPE, PGK-ΔhAPE) were grown to confluency on 60 mm tissue culture plates. The normal growth media was replaced with media containing either 0, 10, 50 or 100 µg/ml bleomycin. The bleomycin was made up as a 4 mg/ml solution in 0.9% NaCl; the 0 µg/ml dose was 0.9% NaCl. After 1 hour of treatment, the media was removed, the cells washed twice with PBS and detached from the plates with 0.5 ml 0.25% trypsin-EDTA. 1.5 ml normal growth media was added to each plate and 5 X 10^4 cells were aliquoted to new 60 mm plates with 5 ml fresh growth media. A plate of each cell line at each bleomycin dose was collected and counted on a hemacytometer by trypan blue staining every 24 hours.

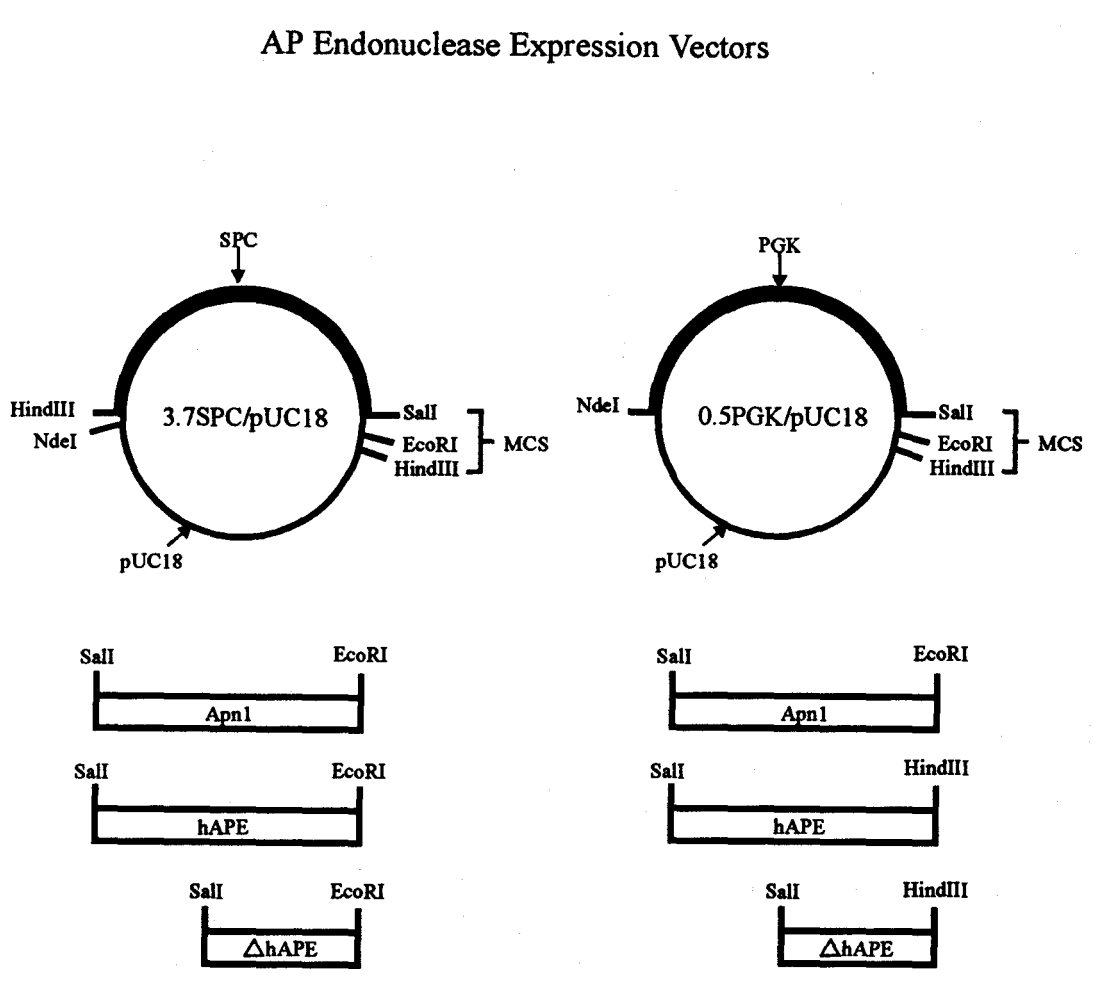


Figure 5. Schematic of AP endonuclease expression vectors

CHAPTER IV

RESULTS

A. Cloning and Sequencing of the rAPEN cDNA

To investigate the developmental, tissue and sub-tissue specific expression of a crucial base excision DNA repair enzyme, a cDNA was cloned that encoded the rat apurinic/apyrimidinic (AP) endonuclease. The cDNA was initially isolated by reverse transcription-polymerase chain reaction (RT-PCR) of total RNA isolated from the rat immature T cell line Nb2 using oligonucleotides specific to the mouse AP endonuclease translation initiation and termination codon regions (Seki *et al.*, 1991). Following the isolation, DNA sequencing of this clone was performed to verify that it was the rat AP endonuclease by nucleic acid homology to the mouse and human homologues (Demple *et al.*, 1991; Seki *et al.*, 1991). Due to the fact that the isolated cDNA clone may have posessed incorrect bases generated by PCR, the cDNA was used to screen a rat testis lambda gt11 cDNA library. Four positive phage clones were identified that were purified to homogeneity through a total of three successive screens. The cDNA inserts were isolated from the phage arms by restriction digests with *Eco*RI and all were

sequenced and determined to be the rat homologue of the *E. coli* exonuclease III family of conserved AP endonucleases. The other three inserts were later determined to encode the rat AP endonuclease by PCR analysis.

The original clone of 1198 bp was determined to contain an open reading frame that predicts a protein of 316 amino acids with a calculated molecular weight of 35.4 kDa (Wilson *et al.*, 1994). The rat AP endonuclease has 85% DNA identity to the human and 93% identity to the mouse AP endonuclease homologues. Amino acid identity between the rat and mouse is 97% while the rat and human have 93% identity. Alignment of the amino acid sequence of the rat protein with human and mouse homologues demonstrated the conserved nature of this DNA repair enzyme family (Figure 6)(Wilson, T. *et al.*, 1994). The highlighted cysteine 65 is conserved within all three proteins.

B. Genomic Organization of the rAPEN Gene

The human and mouse gene structures have previously been published (Demple *et al.*, 1991; Seki *et al.*, 1991). To determine the relative genomic structure of the rat AP endonuclease gene, PCR analysis was performed on Sprague-Dawley DNA derived from liver, and on a rAPEN genomic lambda gt11 clone (Figure 7). The results of the PCR reaction along with information gained from 5′ Rapid Amplification of cDNA Ends (5′RACE) analysis and the cDNA clone, indicated that three exons contain the rAPEN coding region. The structure of the rAPEN

```
hAPE    M P K R G K K G A V A E D G D E L R T E P E A K K S K T A A K K N D K E
rAPEN             R -   -       E   P K S       T         G         T E
APEX                -   A   D   E   P K S       T         G         T E

                                                                *
hAPE    A A G E G P A L Y E D P P D Q K T S P S A K P A T L K I C S W N V D G L
rAPEN             V                       A   G   S
APEX              V                           G   S

hAPE    R A W I K K K G L D W V K E E A P D I L C L Q E T K C S E N K L P A E L
rAPEN
APEX

hAPE    Q E L P G L S H Q Y W S A P S D K E G Y S G V G L L S R Q C P L K V S Y
rAPEN             T
APEX              T

hAPE    G I G D E E H D Q E G R V I V A E F D S F V L V T A Y V P N A G R G L V
rAPEN       E                       E     I
APEX    A   E                       E

hAPE    R L E Y R Q R W D E A F R K F L K G L A S R K P L V L C G D L N V A H E
rAPEN                                   D
APEX                                    D                               Y

hAPE    E I D L R N P K G N K K N A G F T P Q E R Q G F G E L L Q A V P L A D S
rAPEN                                       A         M
APEX

hAPE    F R H L Y P N T P Y A Y T F W T Y M M N A R S K N V G W R L D Y F L L S
rAPEN                 A
APEX                  A

hAPE    H S L L P A L C D S K I R S K A L A S D H C P I T L Y L A L -- 318 aa
rAPEN   Q                               G                         -- 316 aa
APEX                                    G                         -- 317 aa
```

Figure 6. Comparison of the amino acid sequence of rat AP endonuclease (rAPEN) with human (hAPE) and mouse (APEX) homologues. Differences in amino acids are noted below the hAPE sequence. The conserved cysteine residue is indicated by an asterisk (Wilson, T. *et al.*, 1994)

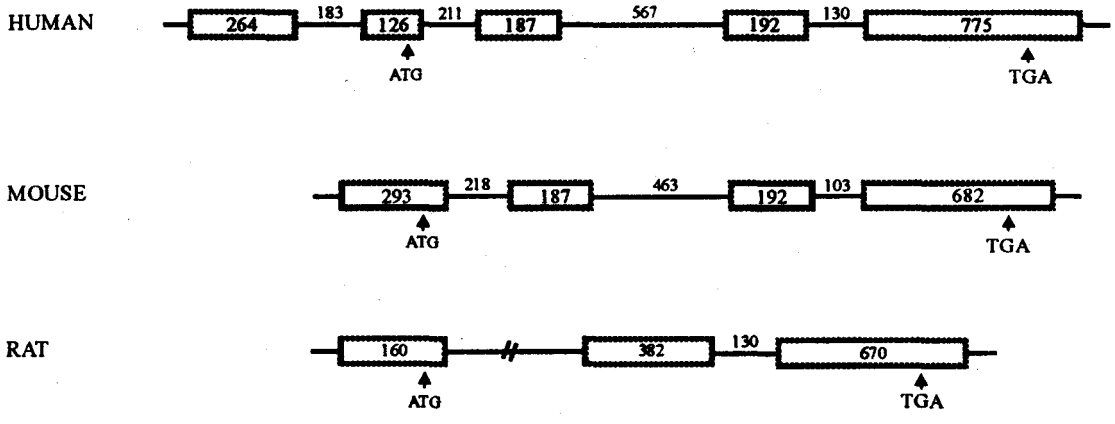


Figure 7. Comparison of the genomic structure of the rAPEN gene with human and mouse homologues. The relative structure of the rAPEN was determined by PCR analysis as described in the Materials and Methods section.

gene is different from its human and mouse counterparts which encode the AP endonuclease within four codons. The second to last intron present in the human, 567 base pairs, and mouse, 463 base pairs, genes is apparently absent from the rat AP endonuclease gene.

C. 5'RACE of rAPEN Nb2 and Testicular mRNA

cDNAs isolated from cDNA libraries are often missing 5'-flanking sequences, especially those that are generated using oligo dT primers. Therefore, 5'-RACE was performed on total RNA isolated from both the rat Nb2 T-cell line and 60-90 day old Sprague-Dawley testis to determine the 5'-end of the rAPEN transcript (Figure 8). In both Nb2 and testis, the transcriptional start site was found to be approximately 109 bases upstream of the translational start site. This is 26 bases longer than was isolated from the library cDNA. As is obvious from the alignment of the 5'RACE product and the cDNA clone, sequence differences exist between the two rAPEN transcripts. While the variance may be due to differences that may occur in isolated rat breeding colonies, the possibility also exists that alternative upstream codons are utilized.

D. Tissue Specific Expression of rAPEN mRNA

To examine the tissue specific expression of rAPEN mRNA, multiple tissue comparative RT-PCR analysis was performed on total RNA isolated from 60 - 90 day old male rats (Figure 9).

```
5′ RACE:  TTTCTACATACATACTCCATTCTTTGTGC--AGTGAGGGGCTCCCTGCCTCATT
cDNA:                                  CGGAGTGAGGGGCTCCCTGCCTCGTT

5′ RACE:    GGGAGG-----CAGCGTAGTAAACACTGCTTCGGTGCTCCAGACGCCTAAGGG
cDNA:       GGGAGGTCAGGCAGCGTAGTAAACACTGCTTCGGTGCTCCAGACGCCTAAGGG

5′ RACE:    CTTTCGTTACAGCGATG
cDNA:       CTTTCGTTACAGCGATG
```

Figure 8. Alignment of the 5′-end of rAPEN cDNAs isolated by 5′RACE and cDNA library screening.

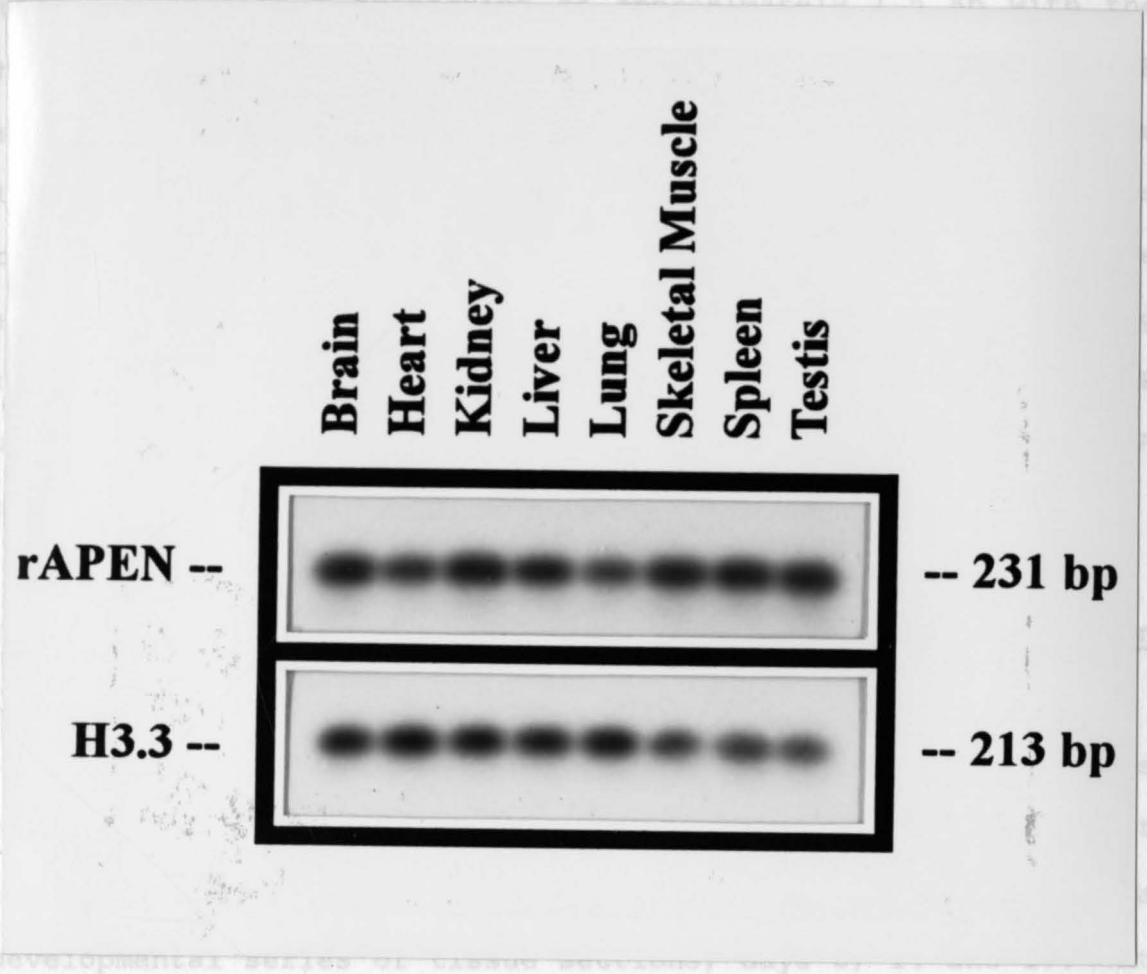


Figure 9. Comparative RT-PCR of the steady-state mRNA levels in various rat tissues for the AP endonuclease gene. Total RNA was isolated and subjected to RT-PCR (25 cycles) using the histone H3.3 transcript as an internal control for reverse transcription and amplification variance. The blot was transferred and probed with the rat cDNA for the AP endonuclease gene or a histone H3.3 specific DNA fragment.

After normalizing rAPEN mRNA levels to histone H3.3 levels, it was determined that the rat AP endonuclease gene was expressed in all tissues examined. Northern blot analysis of various tissues revealed a transcript of approximately 1.5 Kb with the highest signal present in testis and spleen (Figure 10). A similar pattern of expression has also been observed in human tissues as well (Figure 11). However, this type of analysis does not reveal the true nature of AP endonuclease tissue specific expression and can lead to erroneous and misleading conclusions concerning the lack of differential expression of AP endonuclease mRNA in various tissues, in particular, and DNA repair, in general.

E. *In situ* Hybridization Analysis of rAPEN Gene Expression During Fetal Development

To ascertain whether the AP endonuclease gene was differentially expressed in any tissues during fetal rat development, *in situ* hybridization was performed on a developmental series of tissue sections, days 8, 14 and 17. At all ages examined, which spanned periods of organogenesis and neurogenesis, a high level of AP endonuclease mRNA expression was present in all somatic sites examined (Figure 12). At gestation day (GD) 8, the fetus was examined within the uterus. A moderate hybridization signal was present over the entire embryo. At GD 14, the somatic pattern of labeling was widespread with a strong hybridization signal present over all

Figure 10. Multiple tissue Northern blot analysis rAPEN mRNA expression. In three separate studies, total RNA isolated from various tissues of a 60-90 day old male Sprague-Dawley rat was electrophoresed on a 1.25% agarose gel, blotted to Nytran and probed with labeled rAPEN cDNA. Total RNA loading was judged to be relatively similar by examination of RNA levels by ethidium bromide staining (data not shown).

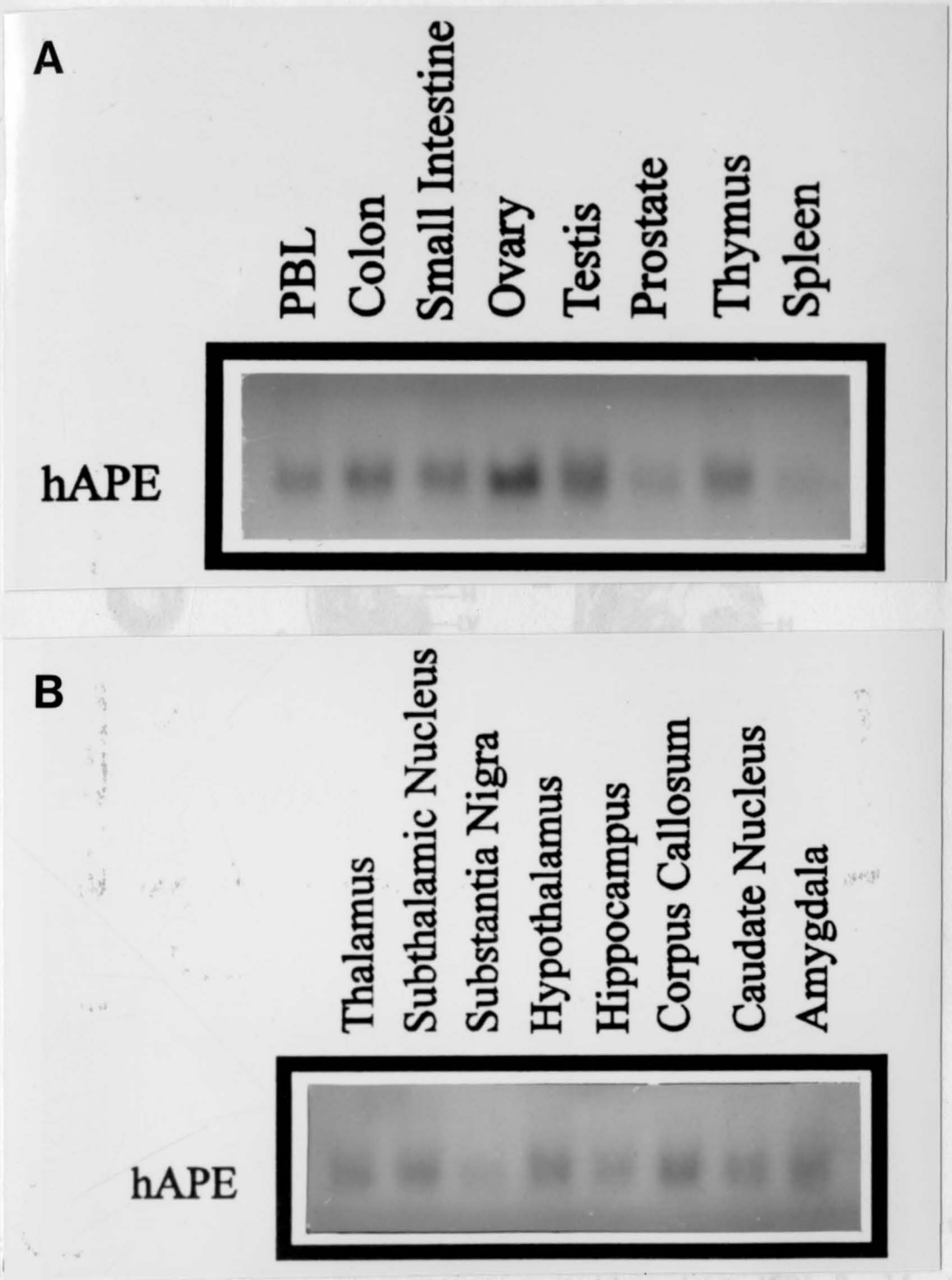


Figure 11. Multiple tissue Northern blot analysis of hAPE mRNA expression. Premade multiple tissue Northern blots, A and B, were obtained from Clontech that contained 2 µg poly A+ mRNA per lane. The blots were probed with the hAPE cDNA.

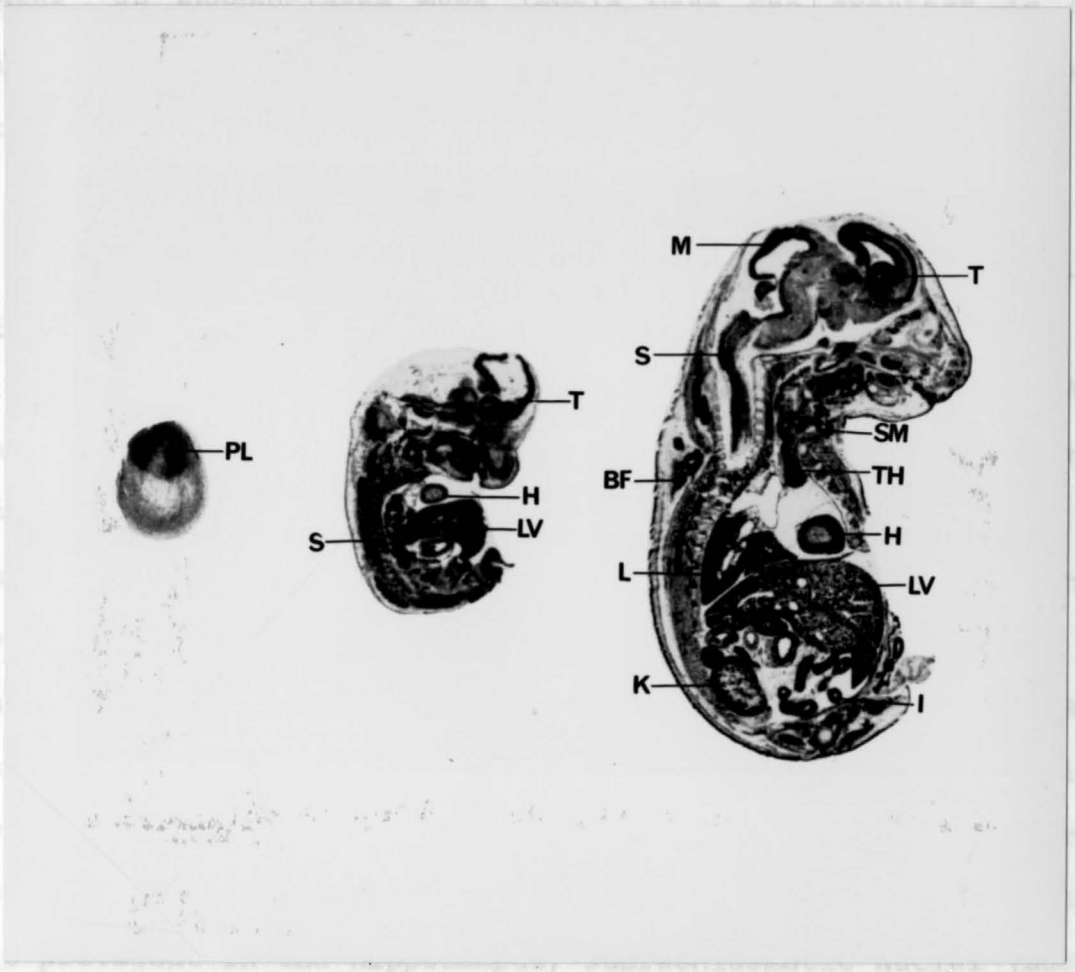


Figure 12. *In situ* hybridization of rAPEN mRNA during fetal development. The developmental expression pattern of AP endonuclease mRNA was examined at gestation days (GD) 8, 14, and 17, from left to right, respectively. PL = placenta, S = spinal cord, H = heart, LV = liver, T = telencephalon, M = mesencephalon, SM = submandibular gland, TH = thymus, I = intestine, K = kidney, L = lung, BF = brown fat. In all experiments, the sense RNA probe gave essentially no background.

organs that could be identified. While AP endonuclease mRNA was expressed at high levels in all structures examined, when relative hybridization signals were compared among different organs, AP endonuclease mRNA levels were the greatest in the liver, thymus and brain. At GD 17, the pattern was relatively unchanged to that observed at GD 14.

F. Distribution and Differential Expression of rAPEN mRNA in Adult Rat Brain

Following up on the striking observation that rAPEN mRNA was highly expressed in specific regions of the developing rat brain, the regional expression of the AP endonuclease gene in adult brain was examined by *in situ* hybridization. This investigation revealed a widespread but heterogeneous pattern of distribution of rAPEN transcript. The rat AP endonuclease mRNA was expressed at moderate levels throughout the entire brain; however, it was expressed at very high levels in several distinct brain regions (Figures 13 and 14). rAPEN mRNA was the most prevalent in the hippocampus, suprachiasmatic nuclei (SCN), the site of the circadian clock, and in the supraoptic nuclei (SON) and paraventricular nuclei (PVN), nuclei that regulate water level balance (Rivkees and Kelley, 1995). High levels of AP endonuclease expression were also found in the pyriform cortex (Figures 13 and 14), a region involved in olfactory signaling. Emulsion autoradiographs revealed that labeling in the cortex was heaviest over the pyramidal cells in layers III

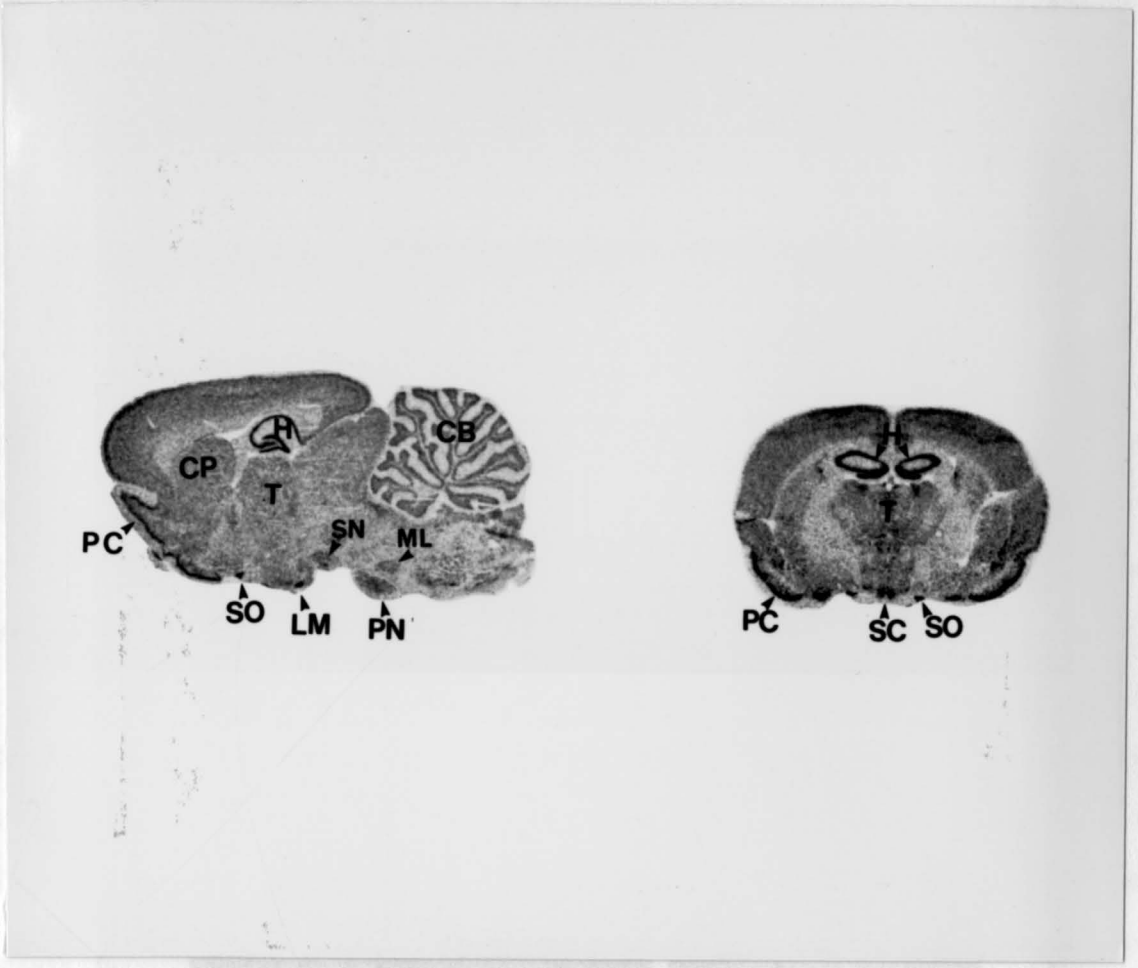


Figure 13. *In situ* hybridization of AP endonuclease mRNA on adult, male rat saggital and coronal brain tissue sections. AP endonuclease mRNA expression was examined using saggital or coronal tissue sections at 100 um intervals spanning the entire brains of rats (n = 3 or more per orientation, although only one section per orientation is shown). Areas of higher AP endonuclease expression are; pyriform cortex (PC), hippocampus (H), cerebellum (CB), suprachiasmatic nuclei (SC) and supraoptic nuclei (SO). Only the antisense probes are shown as the sense probes showed no background level of hybridization (see Figure 15). T = thalamus, LM = lateral mammilary body, SN= substantia nigra, PN= pontine nucleus.

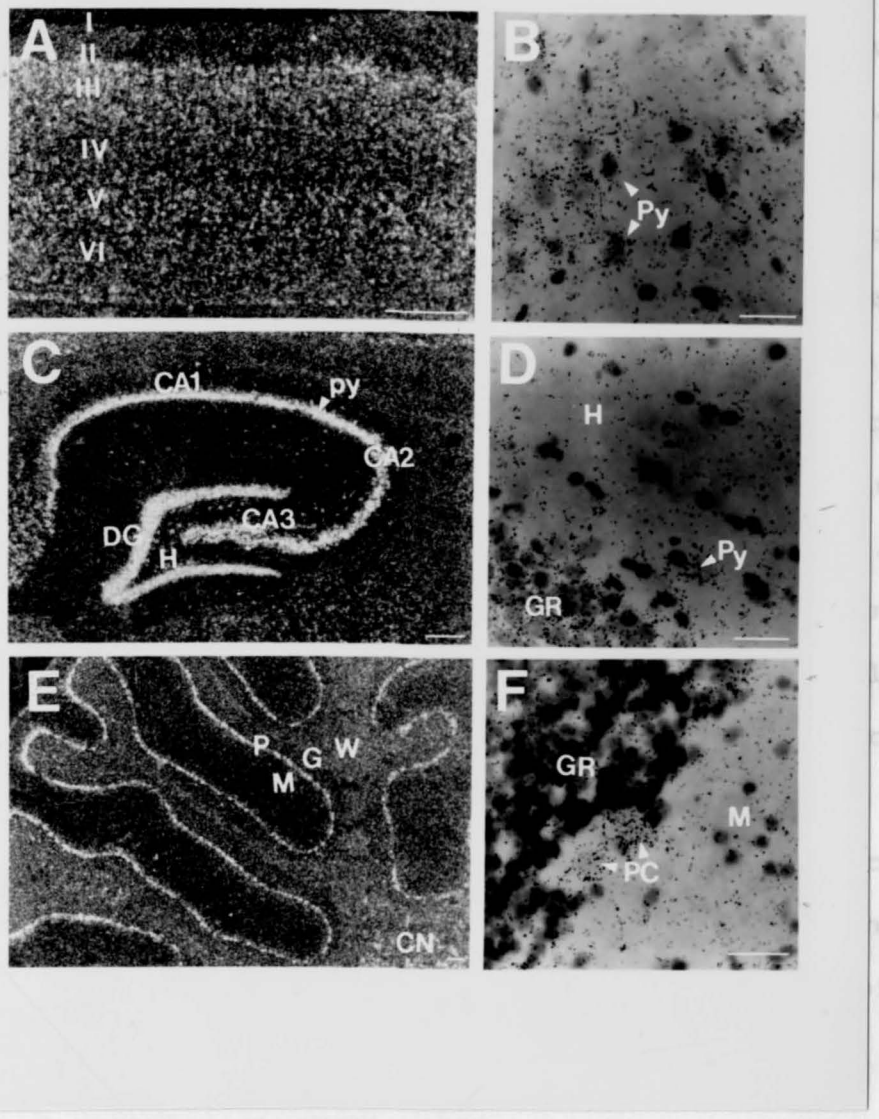


Figure 14. Emulsions of the *in situ* pattern of AP endonuclease gene expression in adult male rat brains. Labeling was seen in several cortical layers in emulsion autoradiographs (A & B). Detailed analysis of the hippocampus is presented in C & D and in the cerebellum in E & F. Py = pyramidal cells or neurons, H = dentate hilus, CA1, CA2 and CA3 are regions of Ammon's horn, Pc or P = Purkinje cells, M = molecular layer, G or GR = granule layer, W = white matter.

and V. Lower levels of labeling were also seen over cells in the other cortex layers (Figure 14).

rAPEN mRNA expression was also detected at high levels in the hippocampal formation (Figures 13 and 14), a region involved in short term memory and motor neuron control. Emulsion autoradiographs revealed heavy labeling over granule cells in the dentate gyrus (Figure 14). In the dentate hilus, labeling was present over pyramidal neurons and not over interneurons (Figure 14). In Ammon's horn, heavy labeling was seen over pyramidal cells in the CA1, CA2 and CA3 regions. Specific, but low level labeling of interneurons could be detected in the stratum oriens, lacunosum-moleculare, and radiatum (Figure 14).

In the cerebellum (Figures 13 and 14), Purkinje cells were the most heavily labeled cell type. In the molecular layer, there was low level labeling of cells, while in the granule layer, there was a moderate hybridization signal. The deep cerebellar nuclei also expressed high levels of AP endonuclease mRNA (Figure 14). In the basal ganglia, which contains different sub-populations of neurons, moderate labeling of all neurons was observed.

G. Differential Expression of rAPEN mRNA in Adult Rat Testis

With the finding of sub-tissue differential AP endonuclease gene expression in specific brain cells, another tissue of predicted importance for DNA BER activity, the testis, was examined. Because of the differential expression of AP

endonuclease gene expression in post-mitotic tissue, the expression pattern of these genes in a tissue undergoing vigorous cellular differentiation and cell-cycling was of interest. The testis are ideal for these *in vivo* studies since they fit this criteria and understanding DNA repair gene expression in developing germ cells has important ramifications for the production of mature sperm with intact and faithful genomic copies. Emulsion autoradiographs generated from *in situ* hybridization studies revealed that AP endonuclease mRNA expression varied with the stage of spermatogenesis (Figure 15). AP endonuclease mRNA was present at modest levels over spermatogonia and spermatocytes. However, the highest level of AP endonuclease mRNA expression was detected over round spermatids which are postmeiotic cells. Sense probes on these, and the previously shown developmental and brain sections, revealed no non-specific hybridization (Figure 15).

H. Overexpression of Recombinant GST-rAPEN Fusion Protein

To obtain rAPEN protein for DNA repair biochemical activity assays as well as for antibody production, recombinant glutathione S-transferase (GST)-rAPEN protein was overproduced and affinity and gel purified. The rAPEN coding region was amplified from the cloned cDNA by PCR using oligonucleotides specific to the nucleotide sequences of the translational start and stop codon regions. The resulting fragment of expected size was subcloned into the expression vector pGEX-3X for

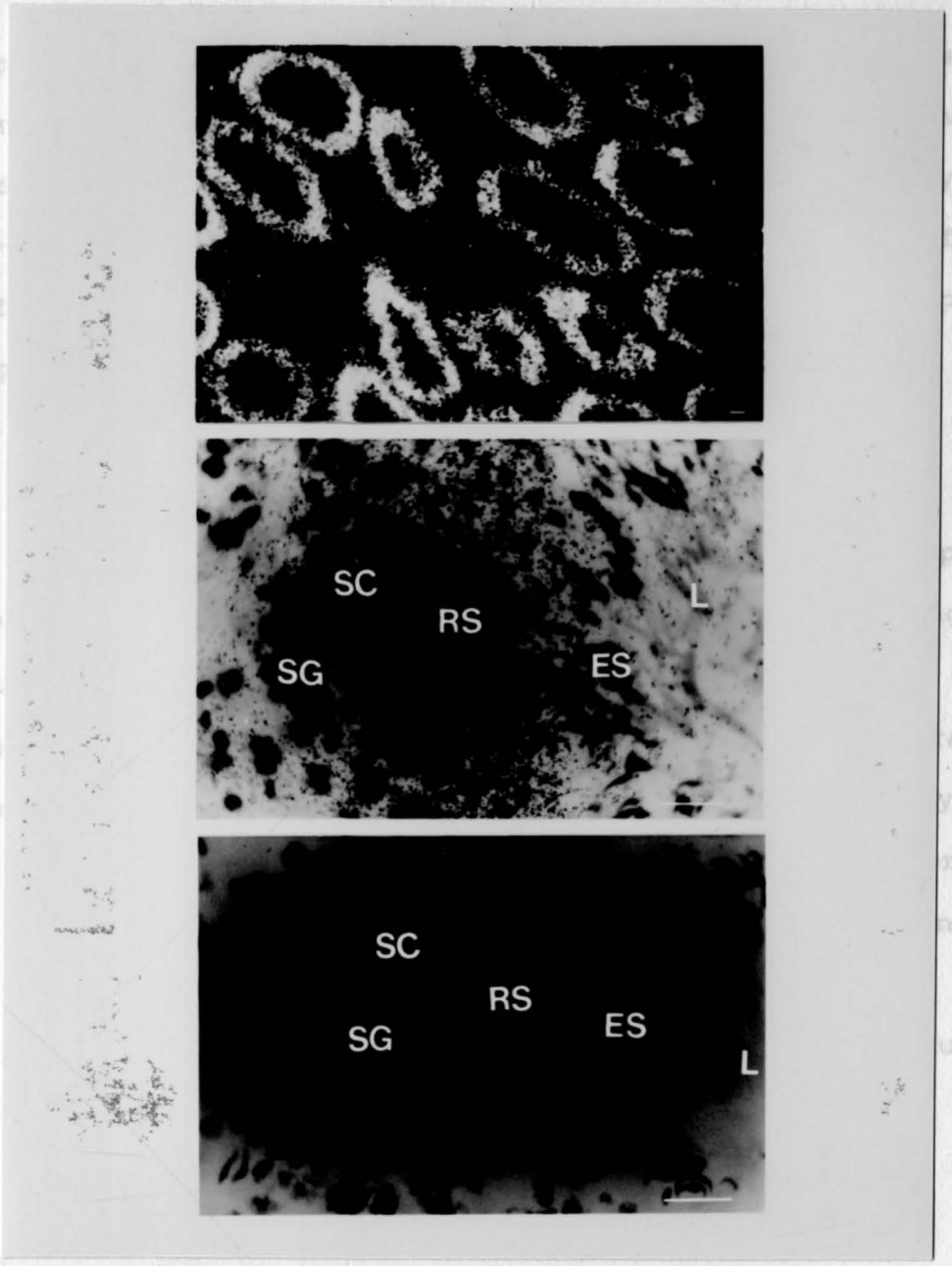


Figure 15. *In situ* hybridization for AP endonuclease mRNA on adult male testis. In three separate studies using testes from different animals, prominent hybridization signals were seen for AP endonuclease mRNA in developing round spermatids. SG = spermatogonia, SC = spermatocytes, RS = round spermatids, ES = elongating spermatids. The top panel is a dark field exposure of emulsions of a cross section through the testis tubules. The middle panel represents a magnified view of the intense pattern of AP endonuclease expression in the round spermatids. The bottom panel is an exposure of a sense RNA probe showing the lack of any background signal.

overproduction of a GST rAPEN fusion protein. The resulting recombinant protein was purified by affinity chromatography and was determined to have a molecular weight of approximately 63 kD (GST-26 kD, rAPEN-37 kD) s expected (Figure 16). The affinity purified GST-rAPEN was then further purified to apparent homogeneity by SDS-PAGE purification.

I. Biochemical Activity of rAPEN

The data presented in Table 2 demonstrate the specific activity for the rat AP endonuclease on AP containing DNA, verifying the AP endonuclease specific activity of the rat AP endonuclease. In direct comparisons of recombinant rat and human AP endonuclease activity, both had similar activity on AP DNA and no activity on non-AP containing DNA. These proteins were assayed as glutathione S-Transferase fusion proteins with the GST portion neither hindering the activity nor adding significantly to the specific activity of the rat and human AP endonucleases.

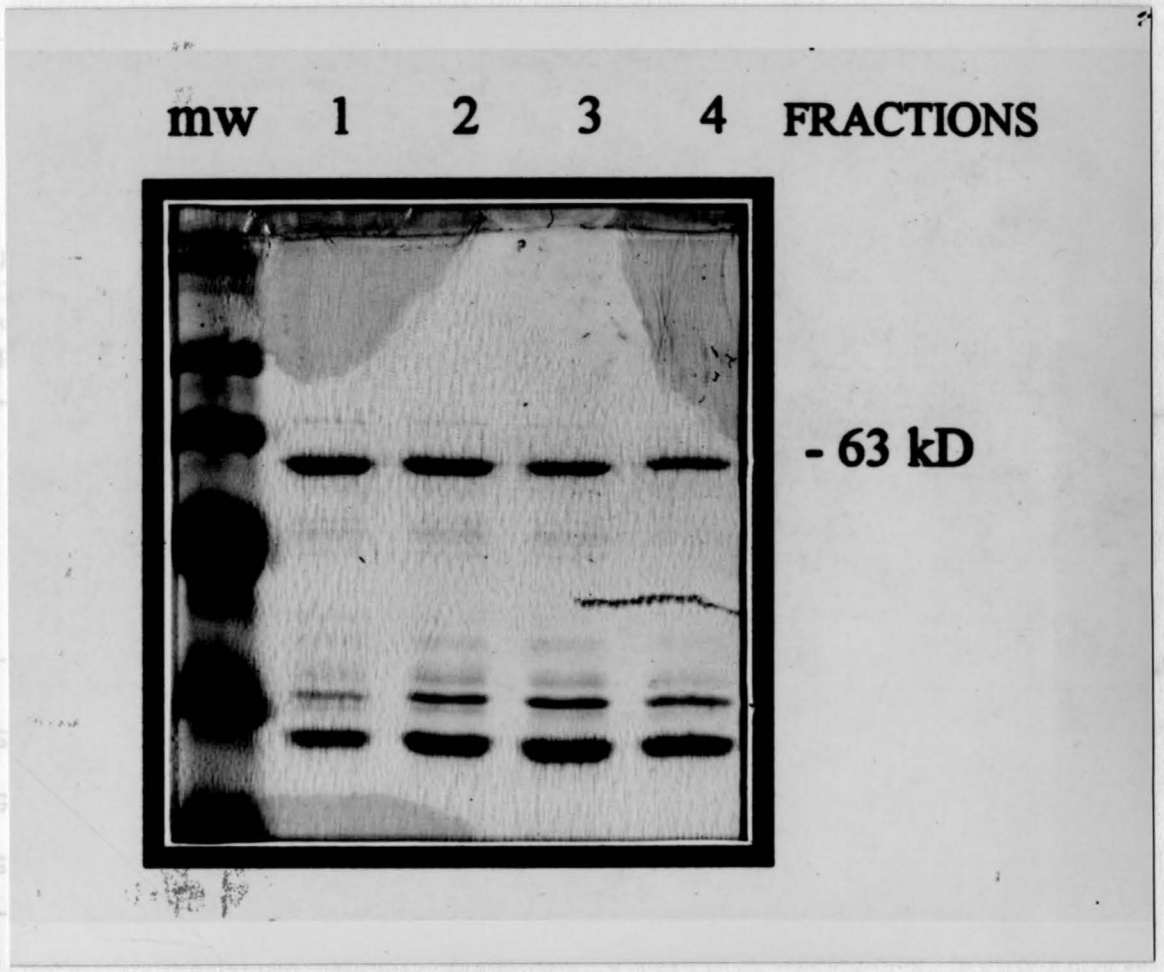


Figure 16. SDS-PAGE analysis of affinity purified recombinant GST-rAPEN protein. Supernatant containing GST-rAPEN fusion protein derived from whole cell sonicate was affinity purified on a glutathion-sepharose column. Fractions of 0.5 ml were eluted from the column and 10 µl of each was electrophoresed on a 12% polyacrylamide-SDS gel followed by coomassie blue staining. The arrow indicates the fusion protein of the predicted molecular weight.

TABLE 1

Rat AP endonuclease Repair Activity

Reactions (50 µl) contained 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 170 fmol of PM2 [^{3}H]-DNA in which the depurinated DNA substrate contained roughly one AP site per DNA circle and 50 ng of protein.

Addition	Nicks introduced/ DNA molecule	
	AP	NT
GST-rAP endonuclease	0.43	0.01
GST-hAP endonuclease	0.61	< 0.01
GST	0.11	0.09

Table I. GST-rAP endonuclease is the rat AP endonuclease (Huq *et al.*, 1995) and GST-hAP endonuclease is the human AP endonuclease (Demple *et al.*, 1991) produced as glutathione-S-transferase fusion proteins in *E. coli*.

J. Subcellular Expression of the rAPEN Protein

To examine the sub-cellular expression of the rat AP endonuclease protein in the rat Nb2 immature T-cell line, and the rat H19-7 hippocampal cell line, polyclonal rabbit anti-GST-rAPEN antibody was reacted with 10 µg each total cell extract, crude nuclei, and cytoplasm (post-nuclear supernatant), by Western analysis. The antibody specifically cross-reacted with a 37 kD protein in all lanes indicating that the rAPEN protein is located in both the nucleus and cytoplasm of both cell lines (Figure 17). While the bands appeared to be of relatively the same intensity, one must keep in mind that the amount of protein in nuclei only represent a small fraction of the total protein of the entire cell. Therefore, this procedure "enriches" the concentration of rAPEN in the nuclear lane as compared to the cytoplasmic. Additionally, the method of purification of the crude nuclei may have resulted in the presence of contaminating structures such as Golgi apparatus in this fraction that might have produced an incorrect result for the crude nuclei.

K. Specificity of the hAPE Antibody

The specificity of the polyclonal rabbit anti-hAPE antibody (Duguid *et al.*, submitted) was determined using total cellular extracts from the human cell lines A1235 (glioma), KCNR (neuroblastoma), and human liver (Figure 18). By Western blot analysis, the antibody cross-reacts with a single band at 37 kDa, the molecular weight of human APE.

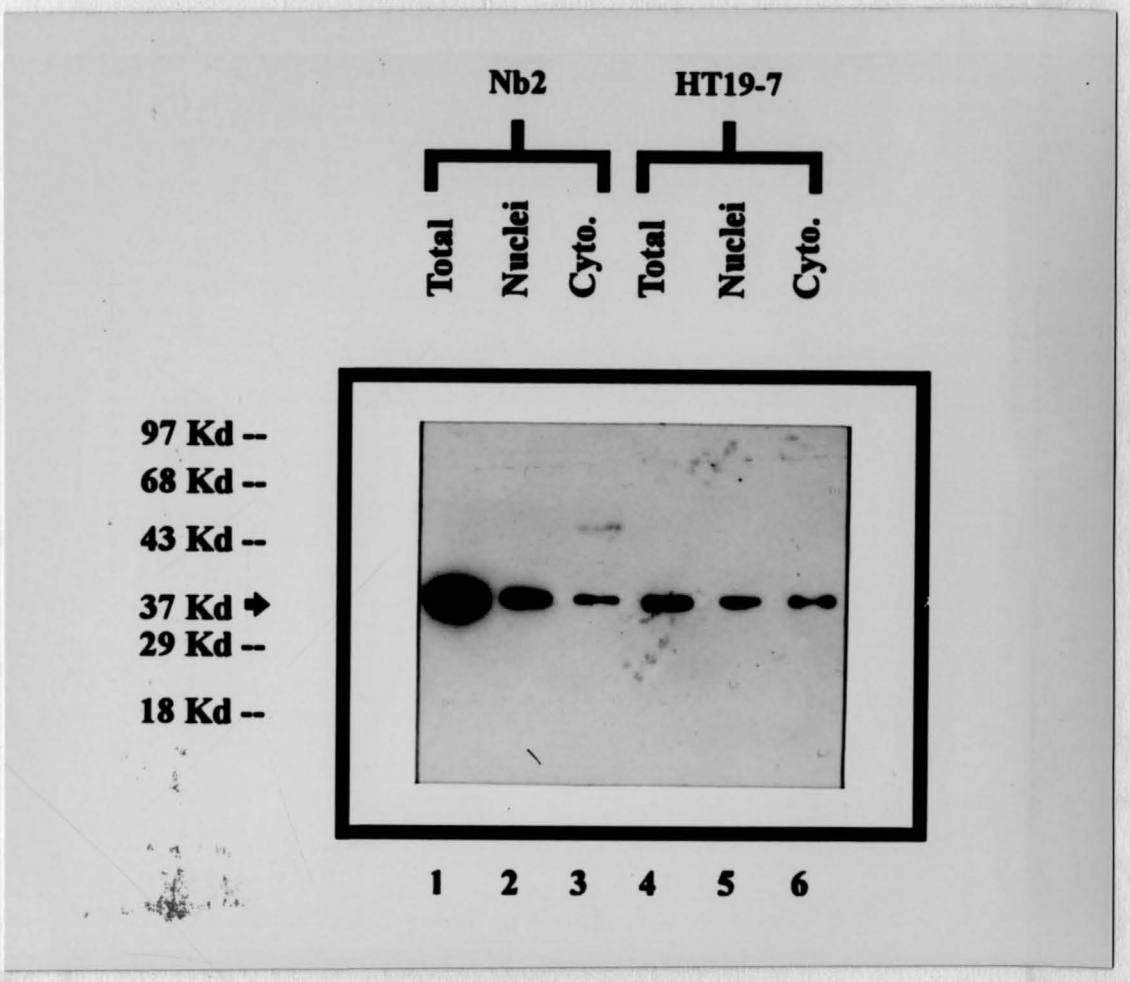


Figure 17. Western analysis of Nb2 and H19-7 subcellular fractions for rAPEN protein expression. Approximately 20 X 10^6 cells were dounce homogenized and separated into crude nuclei and cytoplasmic fractions by centrifugation. Ten µg of each of these fractions along with total cell extract was fractionated on a 12% polyacrylamide-SDS gel and transferred to nitrocellulose. The blots were incubated with anti-rAPEN antibody and the signal developed by chemiluminescence.

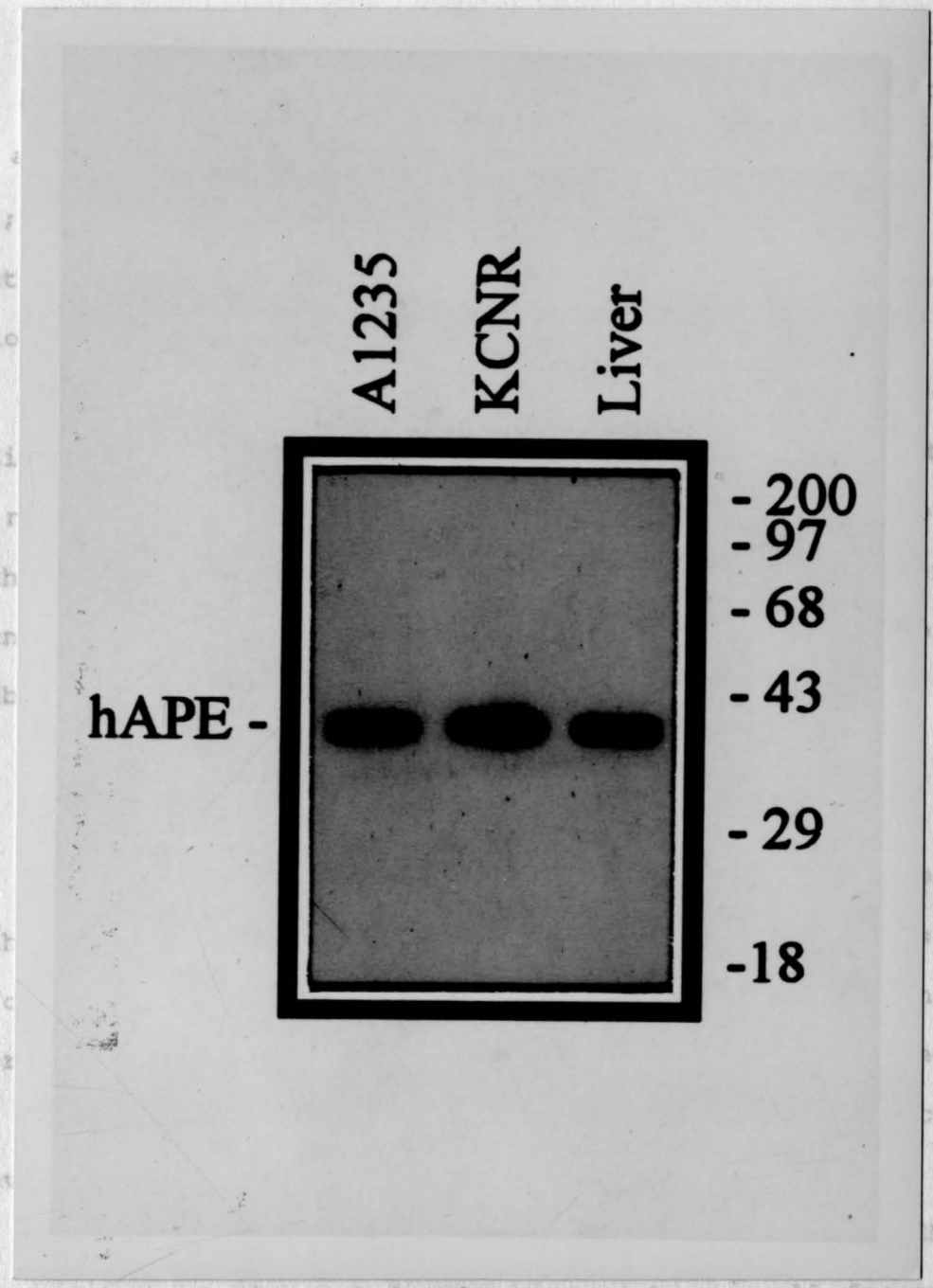


Figure 18. Demonstration of hAPE antibody specificity by Western analysis. Total cell extracts (20 ug) from the human glioma cell line A1235, neural blastoma KCNR and human liver were run on a 12% SDS-polyacrylamide gel, blotted and reacted with affinity purified antibody to the human AP endonuclease enzyme. Only a single protein band of 37 kDa was observed.

L. Subcellular Localization of hAPE in A1235 Glioma Cells

To determine whether our antibody performs similarly to the antibodies utilized in previous reports (Demple, *et al.*, 1991; Xanthoudakis, *et al.*, 1992), and to investigate the location of the protein in a brain cell line, the sub-cellular of location of hAPE protein in the human glioma cell line A1235 was determined by immunocytochemistry. As in the previous studies, the hAPE protein appeared to be primarily located in the nuclei of the cells with no readily visible signal present in the cytoplasm (Figure 19A). No signal was detected when pre-immune rabbit immunoglobulin was utilized as the primary antibody (Figure 19B).

M. Subcellular Fractionation of hAPE Protein

To determine the subcellular localization of hAPE protein in the human neuroblastoma cell line A1235, Western analysis was performed on extracts of crude nuclei and post-nuclear supernatant, (Figure 20). The antibody reacted with a single 37 kDa protein band (hAPE) that was present in all fractions; however, the total cell extract lane was overloaded.

As stated for the subcellular fractionation of rAPEN, the amount of protein in nuclei only represents a small fraction of the total protein of the entire cell. Therefore, this procedure "enriches" the concentration of hAPE in the nuclear lane as compared to the cytoplasmic.

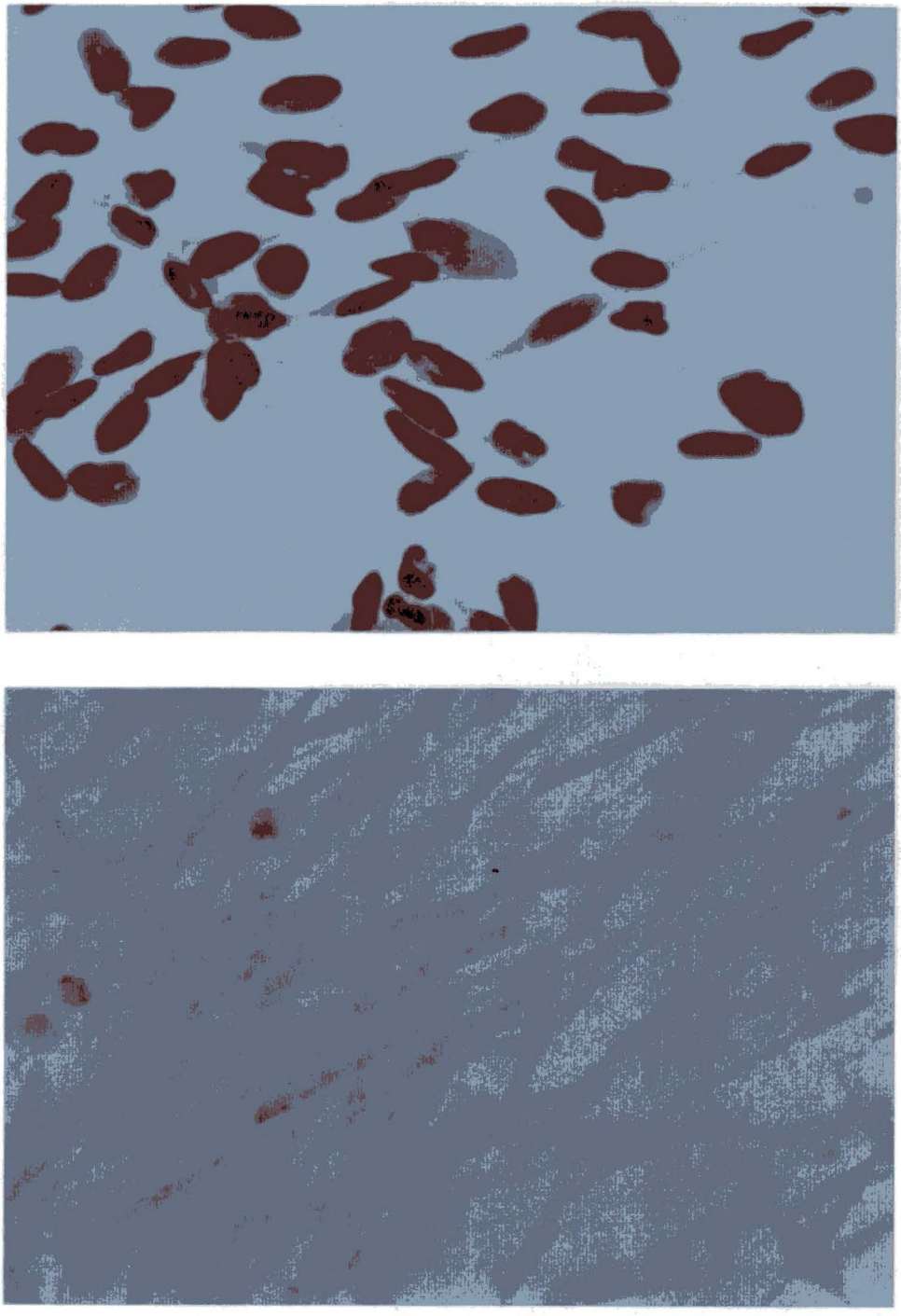


Figure 19. Subcellular localization of hAPE in A1235 glioma cells by immunocytochemistry. A1235 cells were incubated with anti-hAPE antibody and cross-reactivity detected using an aviden/biotin system. The cells were stained with phyloxine-eosin.

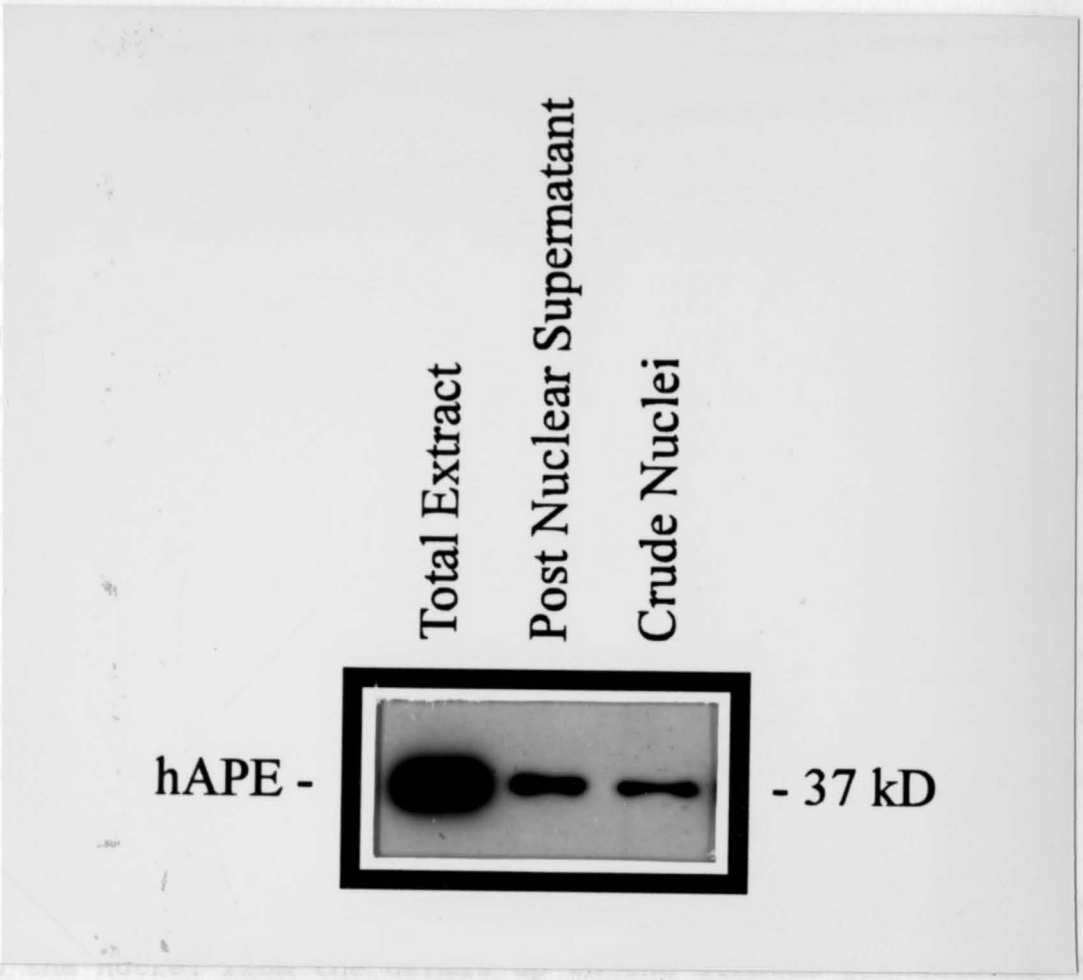


Figure 20. Western analysis of KCNR subcellular fractions for hAPE protein expression. Approximately 20 X 10^6 cells were dounce homogenized and separated into crude nuclei and cytoplasmic fractions by centrifugation. Ten µg of each of these fractions along with total cell extract was fractionated on a 12% polyacrylamide-SDS gel and transferred to nitrocellulose. The blots were incubated with anti-hAPE antibody and the signal developed by chemiluminescence.

N. Expression of hAPE in Duodenum, Hepatocytes and Epidermis

To characterize the expression of hAPE *in vivo*, immunohistochemistry of human biopsy specimens including duodenum, hepatocytes, and epidermis, was performed. The duodenum demonstrated a strong anti-hAPE antibody reaction with enterocyte nuclei of the proximal villus and the crypts of Lieberkuhn (Figure 21A). The nuclei of the distal villus showed little reaction. Hematoxylin and eosin staining of an adjacent section showed the continuity in appearance of the enterocytes across the region of discontinuity of hAPE staining (Figure 21B).

The distribution of hAPE staining in hepatocytes was dramatically different from that observed in the duodenum and A1235 cells. There was uniform staining of the cytoplasm and reticular staining of the nuclei that surrounds the nucleoli with clear staining of the nuclear rim (Figure 22A and 22B).

Anti-hAPE staining of the epidermis was uniform and strong in the nuclei from the dermis up to the stratum granulosum. The staining in the granulosum was somewhat less than that of the lower strata of the epidermis, and there was little or no staining of the strata lucidum and corneum; staining in the cytoplasm was present but considerably less strong (Figure 23A). An adjacent section showed that the hAPE staining was not restricted to the chromatin stained with hematoxylin, but was highly expressed throughout the entire nucleus (Figure 23B).

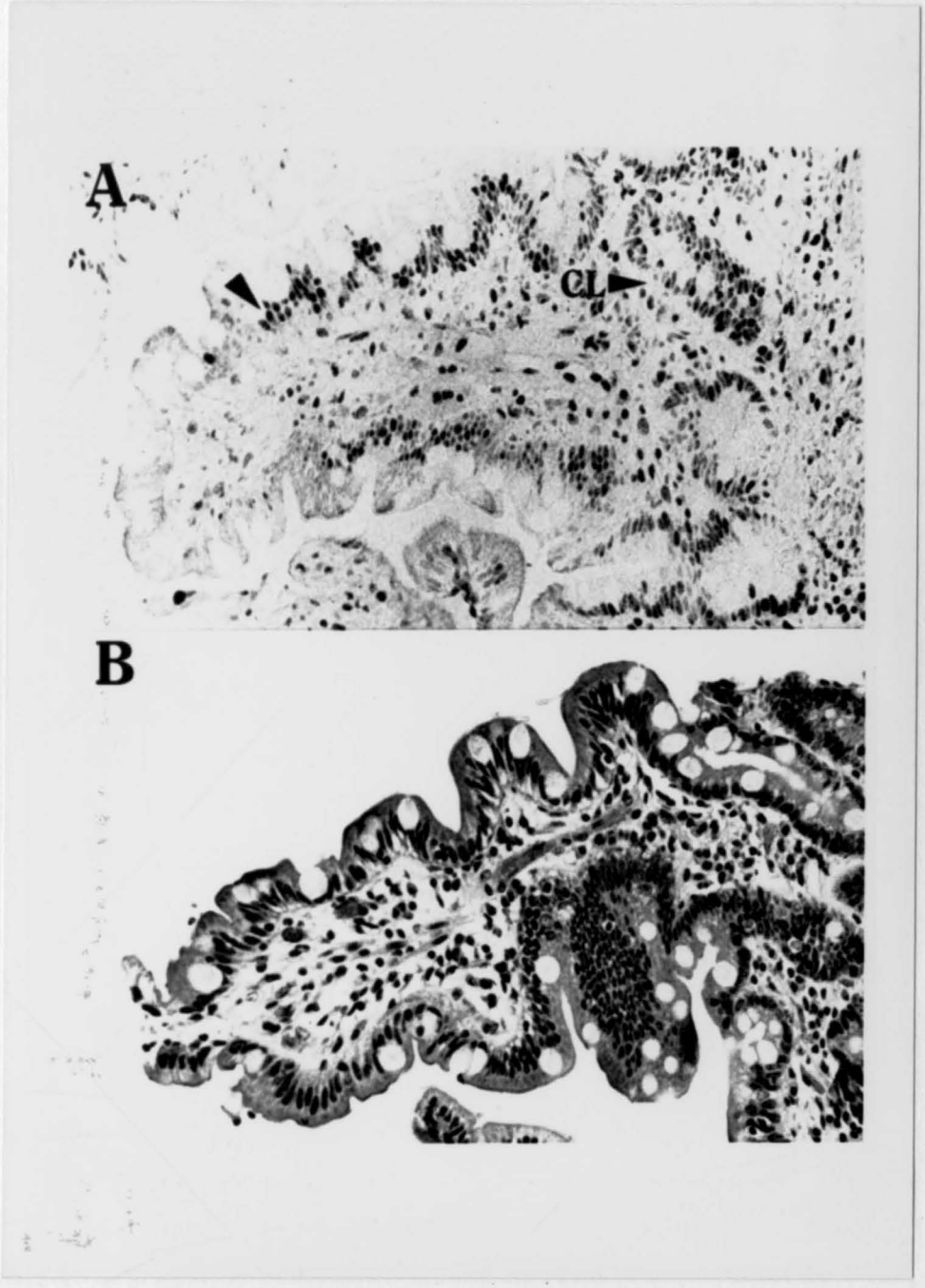


Figure 21. Immunohistochemistry analysis of hAPE expression in duodenum. A. hAPE immunohistochemistry demonstrates a strong reaction with enterocyte nuclei of the proximal villus and the crypts of Lieberkuhn (CL). The nuclei of the distal villus are not stained (see arrow). B. Hematoxylin and eosin staining of an adjacent section shows the enterocytes on the villus and in the crypts of Lieberkuhn (CL). Little difference is seen with the histochemical staining of the proximal and distal villus, compared to the immunohistochemical reaction with hAPE antibody.

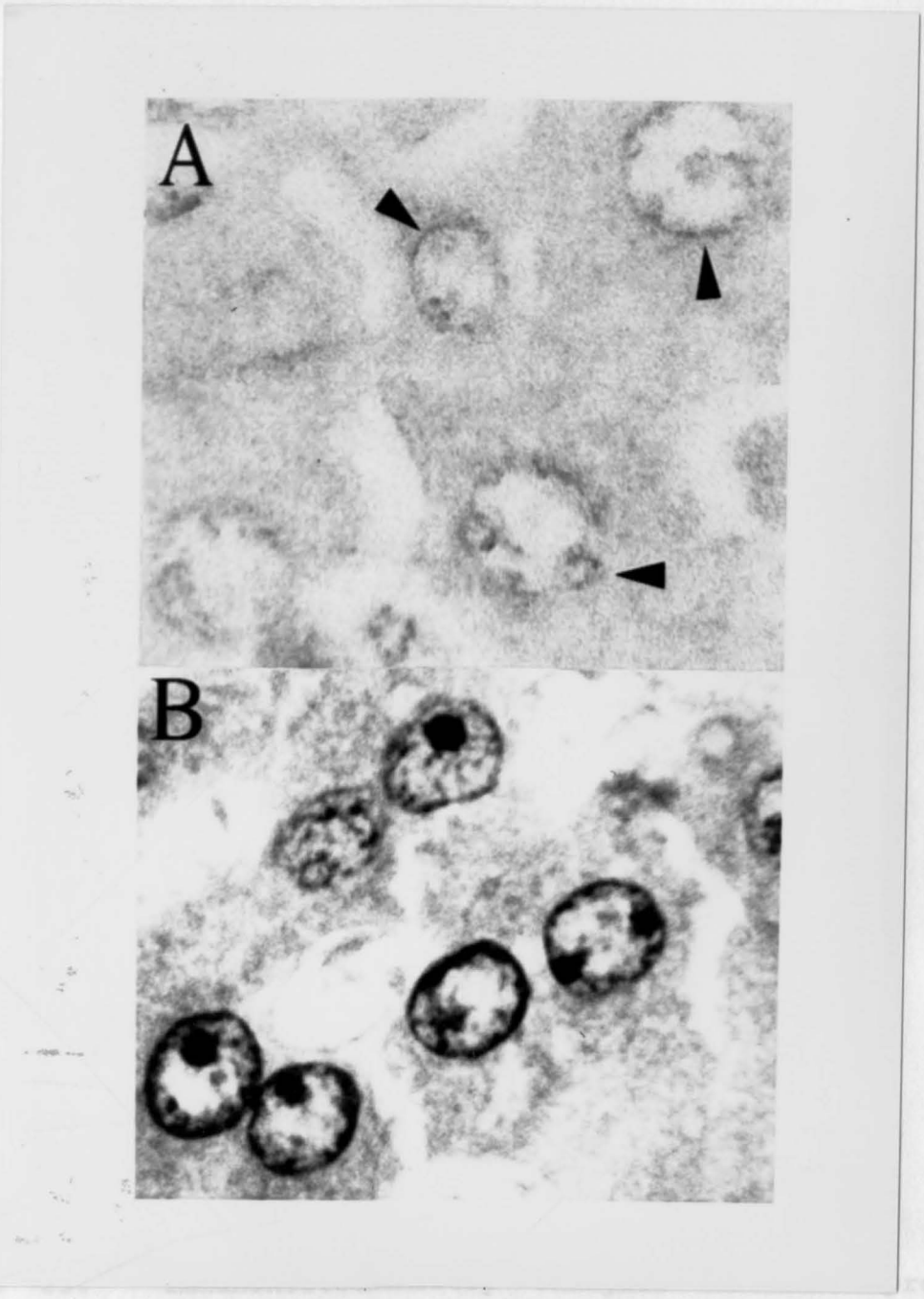


Figure 22. Immunohistochemistry analysis of hAPE protein expression in hepatocytes. A. hAPE antibody stains the cytoplasm and nucleus of hepatocytes; the nuclear rim is stained (vertical arrows) and the nucleoli are spared (horizontal arrow). B. Hematoxylin and eosin staining of an adjacent section.

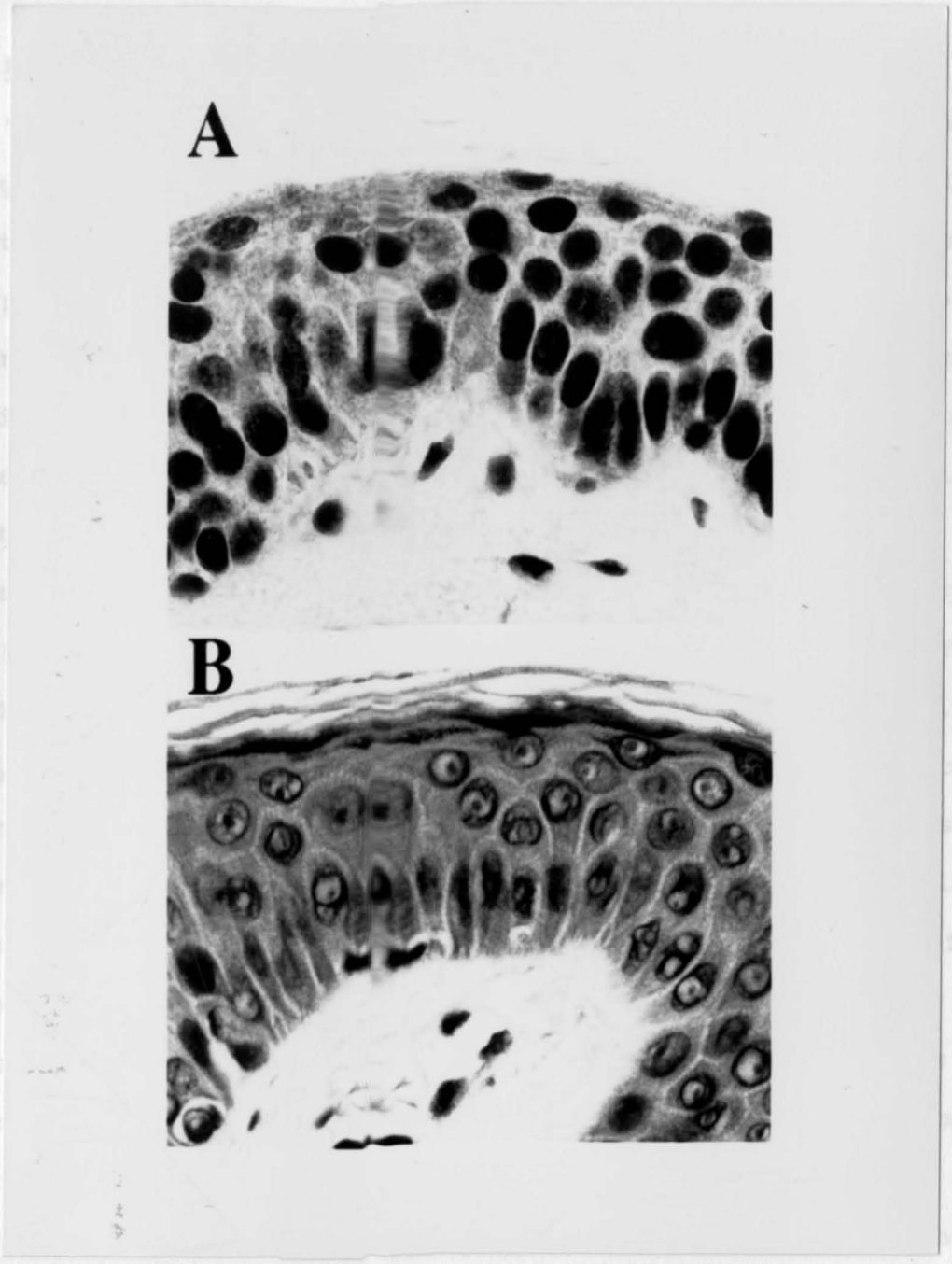


Figure 23. Immunohistochemistry analysis of hAPE protein expression in the epidermis. A. hAPE immunohistochemistry of the epidermis shows strong nuclear staining from the dermis up to the stratum granulosum. There the staining in the granulosum is somewhat less than the lower strata of the epidermis, and there is little staining of the strata lucidum and corneum. B. Hematoxylin and eosin staining of an adjacent section show that the strong, homogeneous hAPE staining of individual nuclei in the epidermis does not correspond to the hematoxylin staining of chromatin in these nuclei.

o. Distribution of hAPE Expression in Brain

The distribution of hAPE in the hippocampus is presented in Figure 24A, which shows antibody reactivity in neurons in the dentate gyrus and regions CA3 and CA4. No reaction was observed when pre-immune rabbit immunoglobulin was used in place of the primary antibody (Figure 24B). Further, this reaction was inhibited by preincubating the primary antibody with recombinant antigen (data not shown). At higher magnification, hAPE in the dentate gyrus (Figure 24C) and CA4 (Figure 24D) was seen to be primarily cytoplasmic in both sites, as observed in hepatocytes.

hAPE immunoreactivity in the cerebellum is shown in Figure 25A. The nuclei of some granule neurons were stained evenly and darkly, but others showed no reaction. In the Purkinje cells, the cytoplasm was stained uniformly while the nucleus was stained in a reticular manner around a circular structure in the center of the nucleus, and there was subtle staining of the nuclear rim. Subsequent staining of the same field with hematoxylin revealed that the circular structure was the nucleolus and the reticular hAPE staining material was chromatin (Figure 25B).

hAPE staining of the hypoglossal nucleus of the brainstem, where motor neurons that control tongue movement reside, demonstrated reactivity in discrete regions of the cytoplasm of these motor neurons (Figure 26A). Subsequent staining with cresyl violet and photography of the same field demonstrated

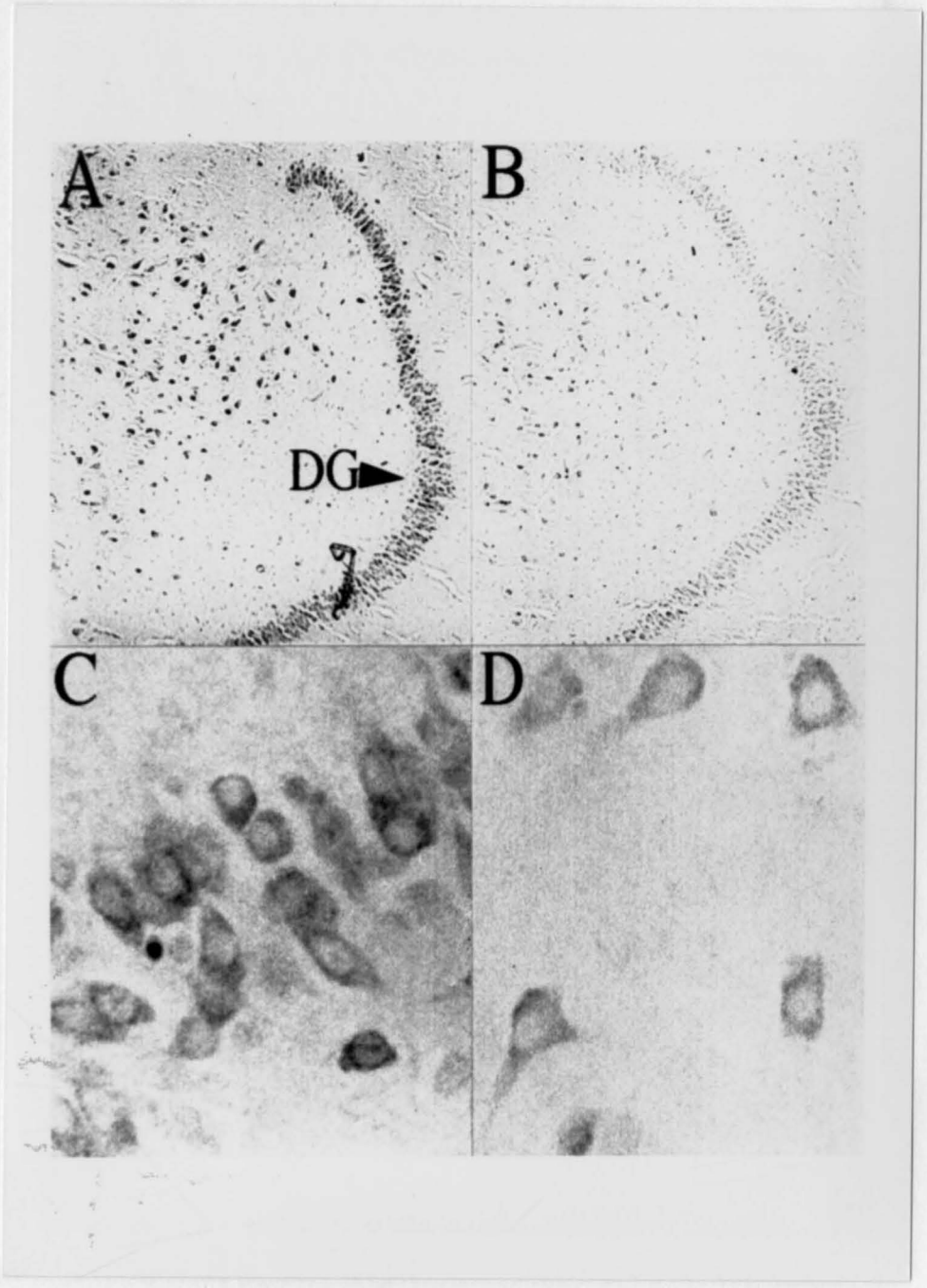


Figure 24. Immunohistochemistry analysis of hAPE protein expression in the hippocampus. A. Low magnification view shows staining of both the dentate gyrus and pyramidal cells of regions CA3 and CA4. B. Tissue stained with rabbit preimmune serum at a protein concentration three fold higher than the concentration used with hAPE indicates little, if any, background staining. C. and D. High magnification views of the dentate gyrus (C) and region CA4 (D).

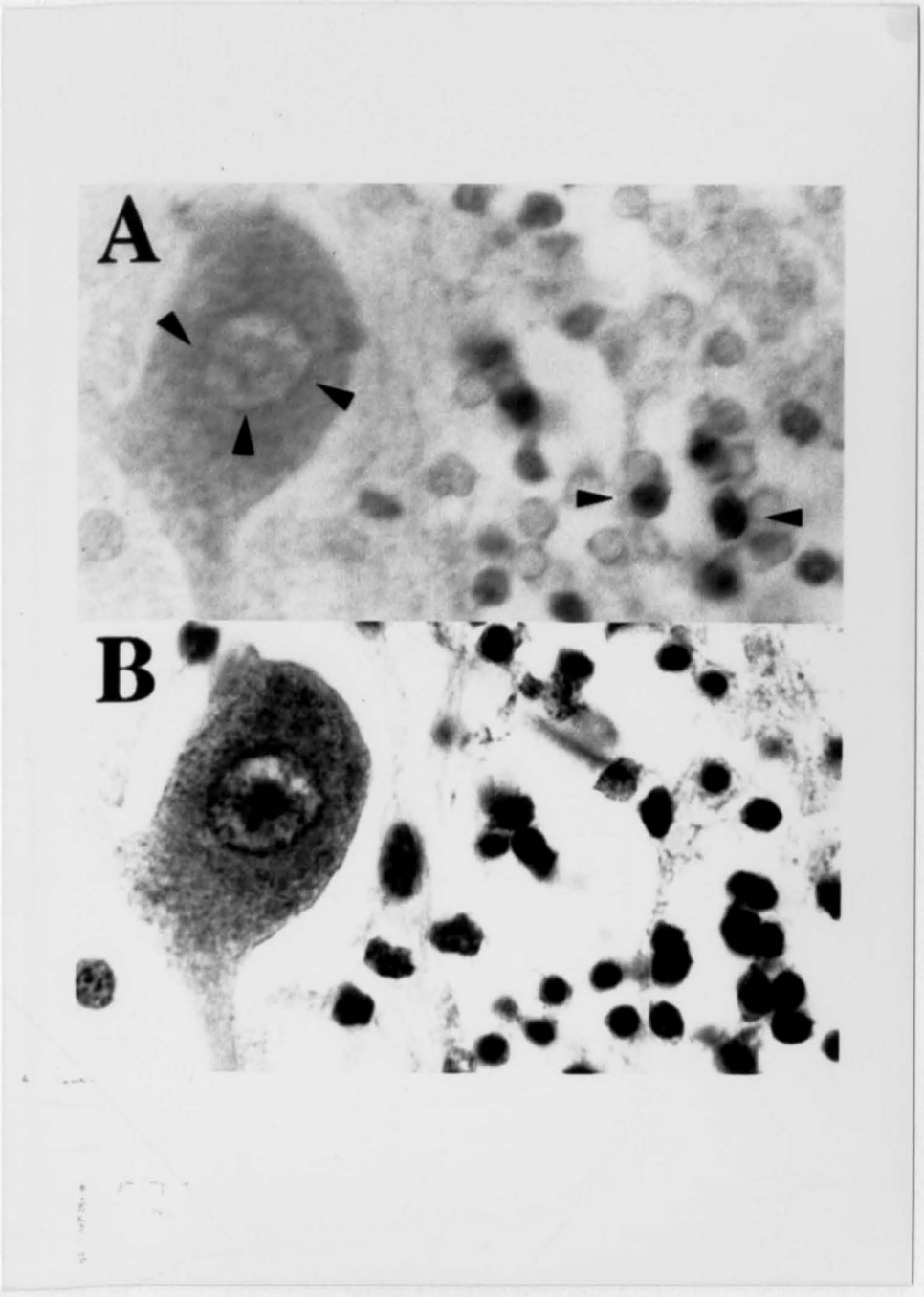


Figure 25. Immunohistochemistry analysis of hAPE protein expression in the cerebellum. A. hAPE immunohistochemistry demonstrates the strong staining of some granule neuron nuclei (horizontal arrows). The immunohistochemical reaction of the Purkinje cell nucleus suggests that the reaction spares the nucleolus and stains chromatin. This is confirmed by sequential staining of the same slide with hematoxylin, shown in B. Arrows indicate the Purkinje cell nuclear chromatin in panel A.

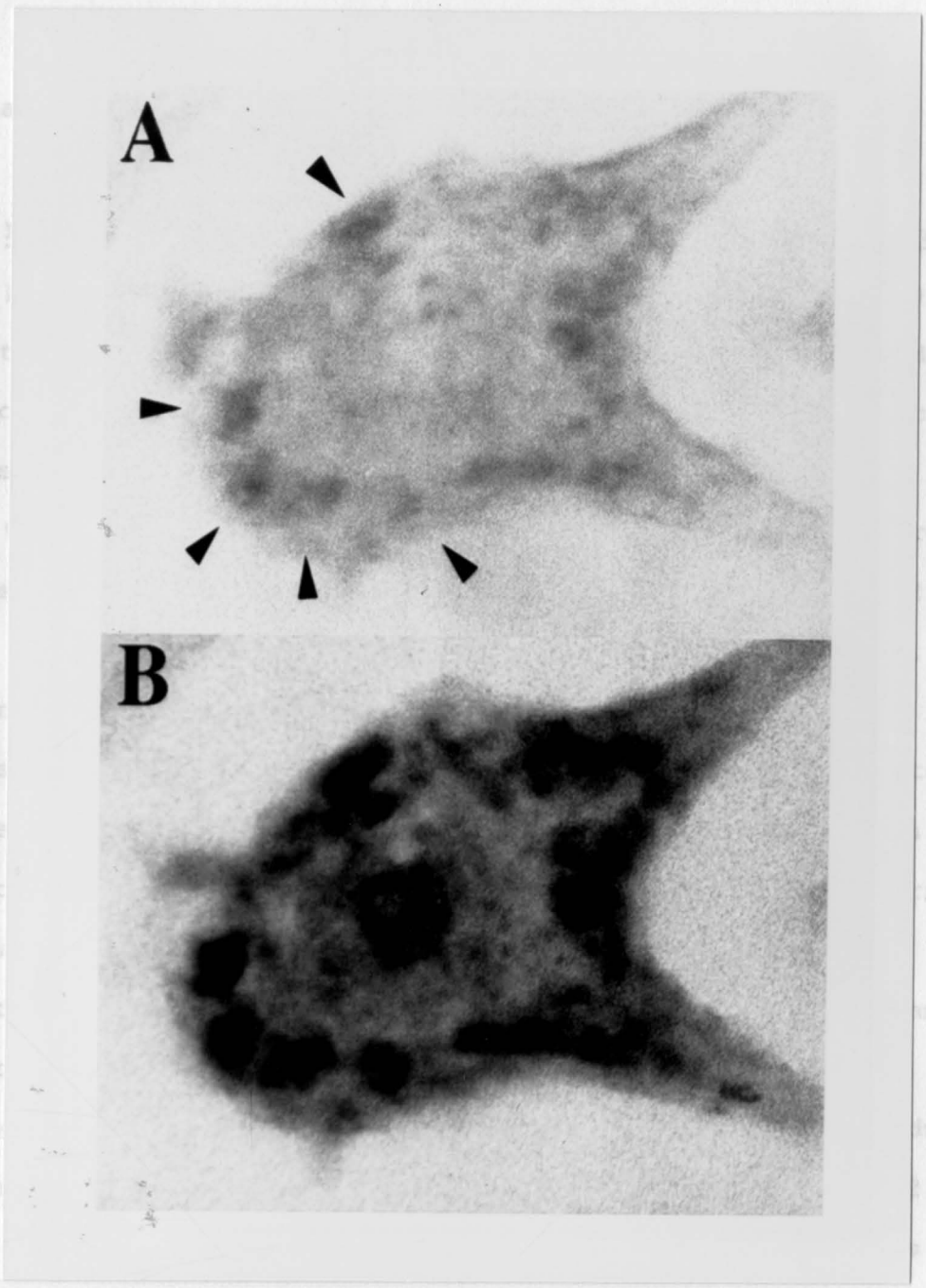


Figure 26. APE immunohistochemistry of hypoglossal nuclei motor neurons. A. The APE immunohistochemistry of hypoglossal motor neurons suggested that the reaction was with Nissl substance (aggregates of ribosomes that are prominent in motor neurons and are stained with cresyl violet). B. The coverslip was removed from the slide and the section was stained with cresyl violet. The same field that was photographed in panel A is shown in panel B, demonstrating that the APE reaction product does stain Nissl substance. Arrows indicate Nissl substance in panel A.

that the discrete hAPE staining corresponded to the Nissl substance (Figure 26B).

P. Effect of Differentiation/Apoptosis on hAPE Expression

Retinoic acid induces the myeloblastic leukemia HL-60 cell line to differentiate into granulocytes that subsequently die by apoptosis. To determine whether the human AP endonuclease expression is affected by or involved in apoptosis, HL-60 cells were treated with retinoic acid and examined for hAPE protein levels over a 6 day period. In addition, the HL-60R/BCl-2 cell line that undergoes differentiation but not apoptosis, was examined to elucidate whether any changes in hAPE protein expression were due to differentiation or apoptosis. Progression of the cells through the differentiation and apoptotic processes were monitored throughout the time course by observing changes in morphology and production of DNA degredation, respectively. Western analysis of cell samples collected every 2 days provided exciting results (Figure 27). In the HL-60 cell line, the level of hAPE protein gradually decreased over the 6 day period, while, in the HL-60R/BCl-2 cell line, hAPE levels remained constant. This result indicates that hAPE is affected by the process of apoptosis. The exact level (i.e. transcription, translation) of hAPE regulation during this process requires further study.

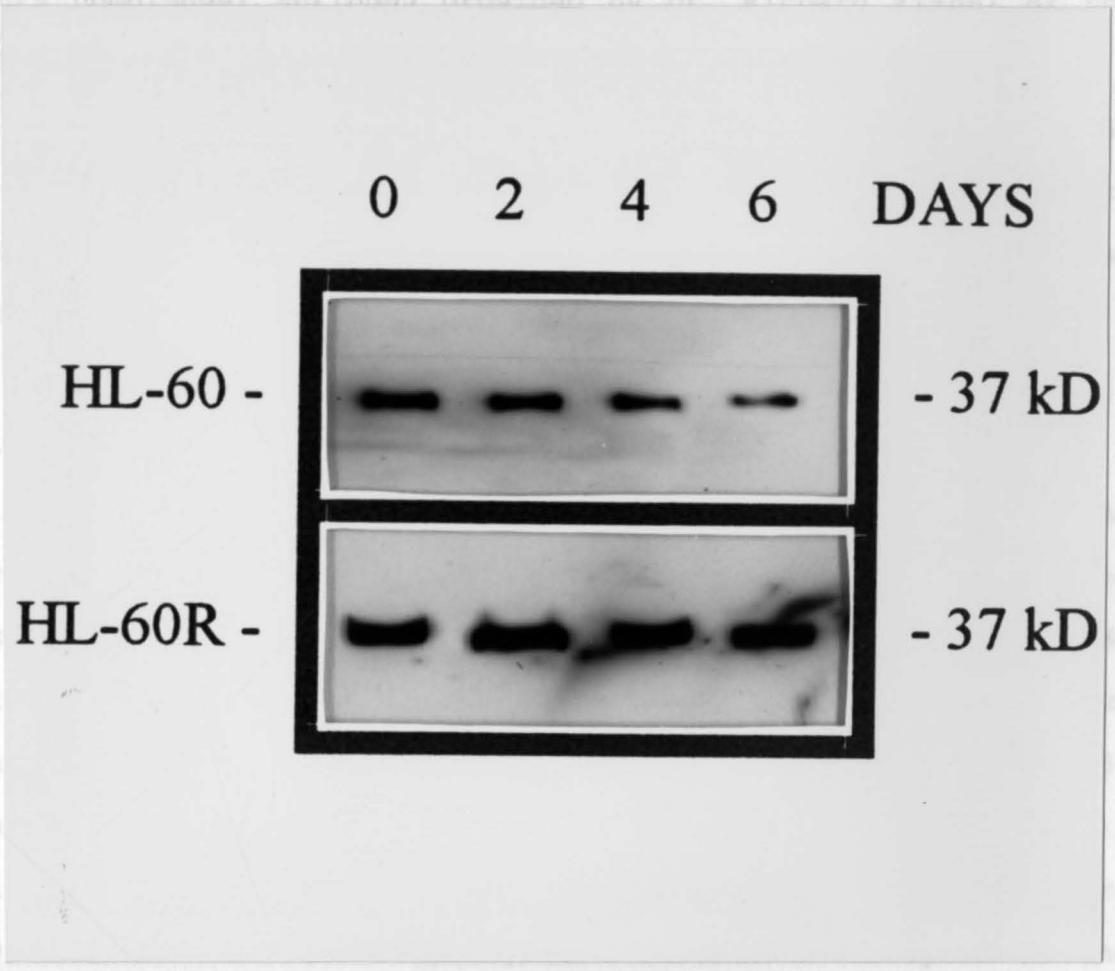


Figure 27. hAPE protein expression during the apoptotic process. The myeloid leukemia cell line HL-60 was induced to undergo apoptosis by treatment with retinoic acid. Cell samples were collected every two days over a six day course. Ten ug of total cell extract of each sample was fractionated on a 12% polyacrylamide gel and transferred to nitrocellulose. The blots were incubated with anti-hAPE antibody and cross-reactions detected by chemiluminescense.

Q. Specificity of the hMSH2 Antibody

The specificity of the polyclonal rabbit anti-hMSH2 (human *mutS* homologue) antibody provided by Dr. Richard Fishel of the University of Vermont was determined using total cellular extracts from the human cell lines A1235 (glioma) and HL-60 (myeloma). By Western blot analysis, the antibody cross-reacts with a single band at 105 kDa, the molecular weight of human MSH2 (Figure 28).

R. Expression of hMSH2 in Duodenum and Colon

To compare the localization of a DNA repair enzyme involved in a different repair pathway to that found for the human AP endonuclease, immunohistochemistry for the hMSH2 protein was performed. Immunohistochemistry of the duodenum demonstrated a strong reaction in the nucleus of striated-bordered cells in the crypts of Lieberkühn (DiFore, 1974), although the hMSH2 antibody did not recognize every cell (Figure 29). There was some staining of nuclei in enterocytes of the proximal villus and very sporadic, if any, staining of the distal villus nuclei occurred. Toluidine blue staining of the same section showed the continuity in appearance of the enterocytes across the region of discontinuity of MSH2 staining (Figure 29). Incubation with preimmune IgG as a primary antibody in place of the anti-hMSH2 antibody resulted in no staining of any cells (Figure 29).

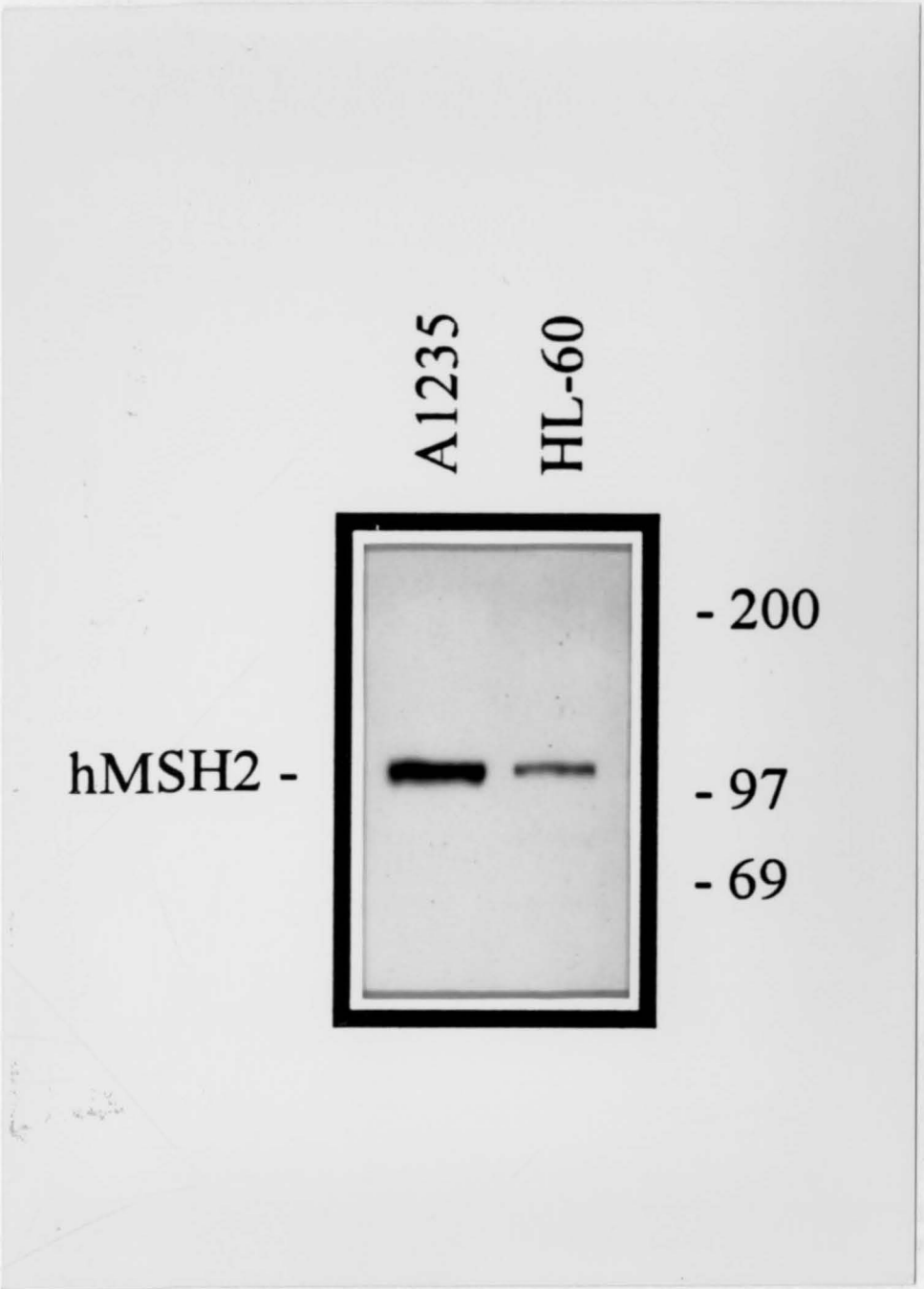


Figure 28. Specificity of the hMSH2 antibody. Total cell extracts (20 μg) from the human cell lines A1235 (astrocytoma), HL-60 (cell line of leukemic myeloid precursor cells), and human liver cell extract were run on a 7% SDS-polyacrylamide gel, blotted and reacted with purified antibody to the human MSH2 mismatch DNA repair enzyme. Only a single protein band of 105 kDa was observed.

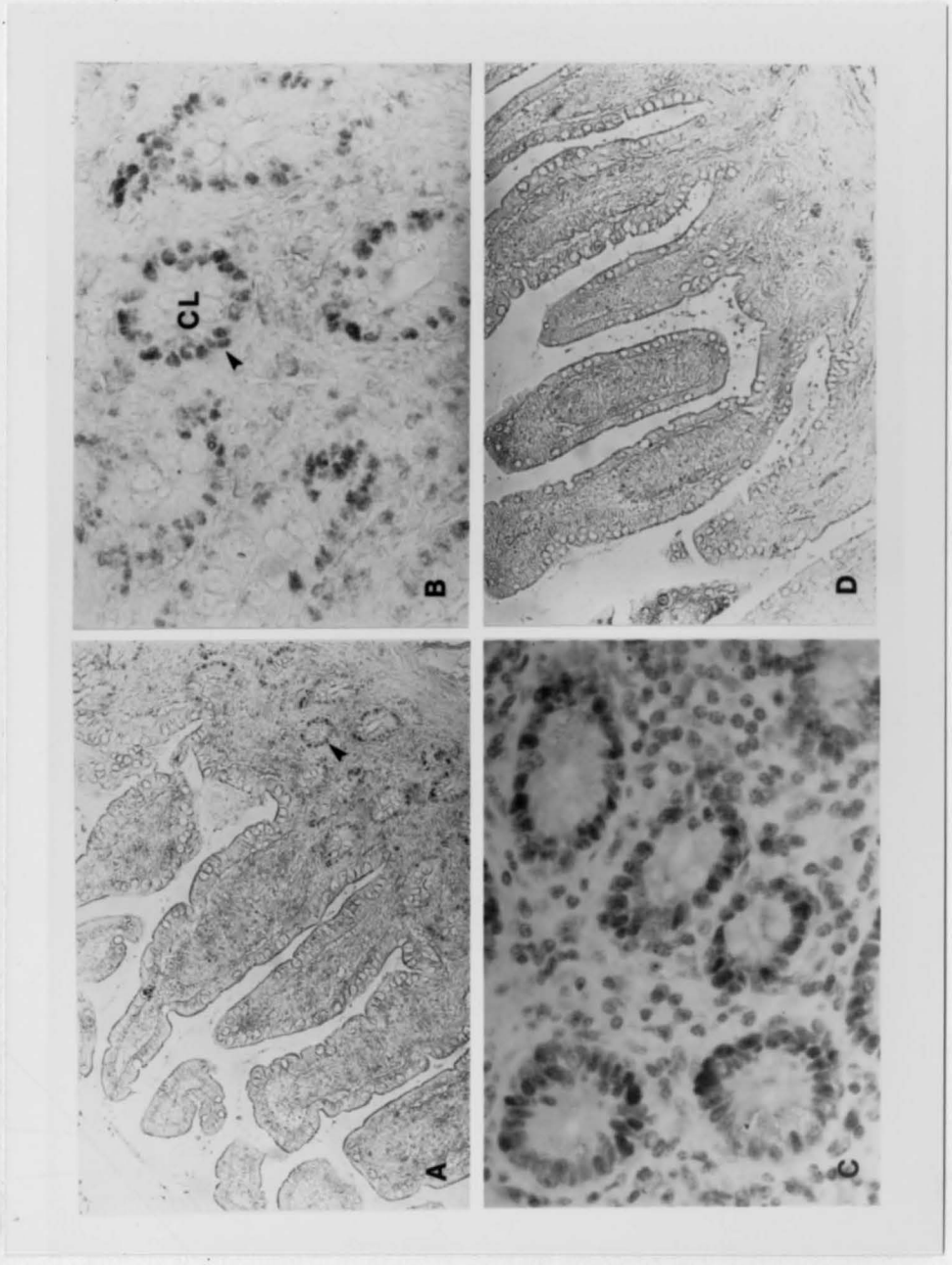


Figure 29. Immunohistochemistry analysis of hMSH2 expression in the duodenum. A-C: hMSH2 immunohistochemistry demonstrated a strong reaction with cells in the crypts of Lieberkühn (CL). The nuclei of the proximal or distal villus were not stained. Arrow points to crypt that is also shown at higher magnification in B. B: Higher magnification view of crypts of Lieberkühn shown in A (arrow). C: Crypts shown following counterstaining with toluidine blue. The antibody staining of hMSH2 appears as dark brown. Notice that not every cell in the crypts stained and that there was little staining of cells outside of the crypts. D: Preimmune IgG reaction with duodeum. No staining was observed for the preimmune IgG in the tissues.

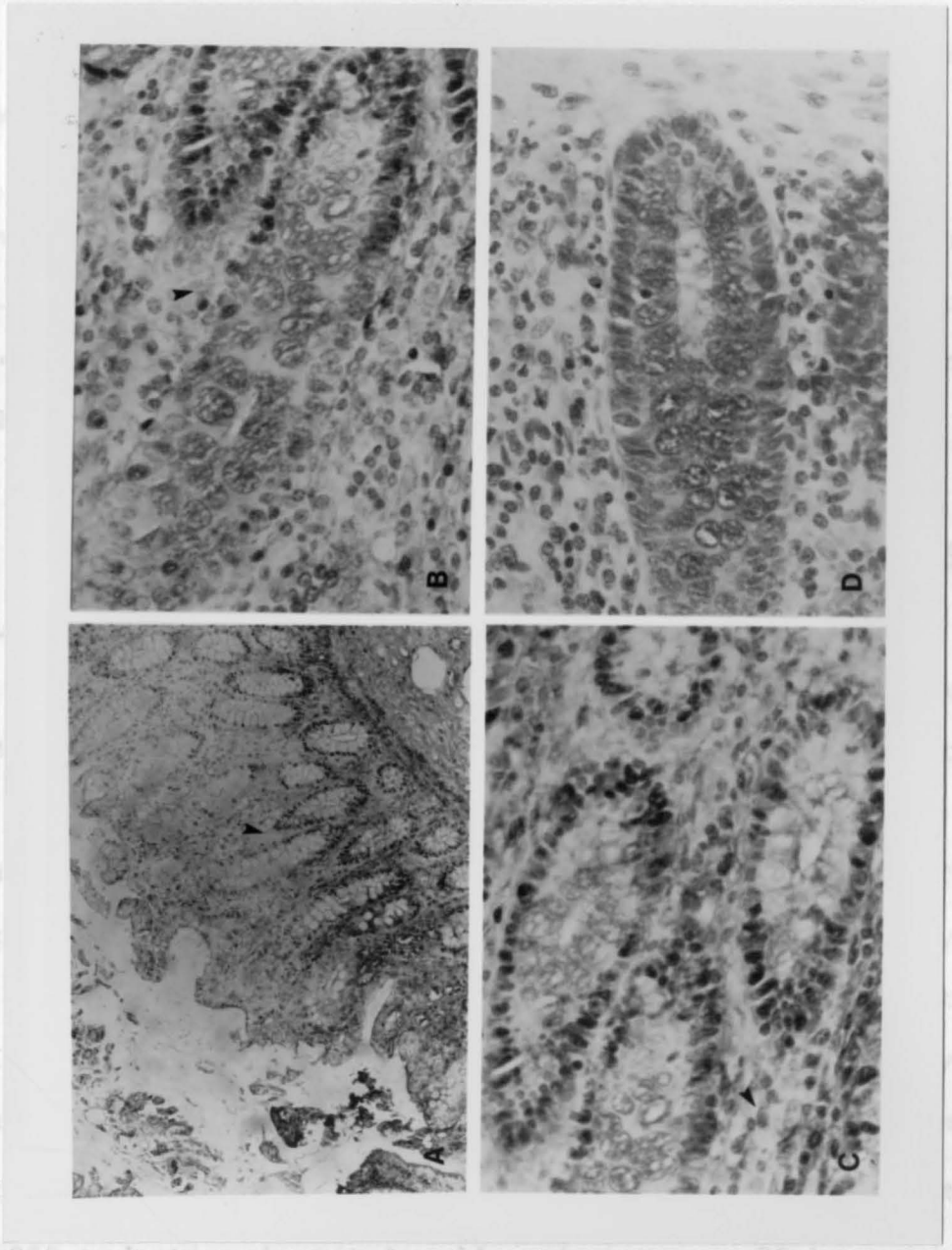


Figure 30. Immunohistochemistry analysis of hMSH2 in the colon. A-C. hMSH2 antibody staining of colon tissue. A: hMSH2 antibody staining without counterstain. B: Higher magnification view of crypts shown in A (arrow) and counterstained with toluidine blue. Notice the heavier staining in the lower third section of the crypt. Blue staining cells in the interior of the crypts are the goblet cells that did not react with hMSH2 antibody. C: In contrast to the results seen in duodenum, fibroblase cells (arrow) surrounding the crypts also react with hMSH2 antibody, although, as in the crypt cells, not every cell stained. D: Colon reacted with preimmune IgG showed no background antibody reaction.

In the large intestine (colon), the hMSH2 antibody predominantly stained the lower one to two thirds of the crypts and little, or no staining was observed in the outer third (Figure 30). Again, preimmune IgG did not result in non-specific background antibody staining of any cells (Figure 30). Goblet cells did not stain with the hMSH2 antibody (Figure 30), as observed in the duodenum (Figure 29).

S. Bleomycin Treatment of 3T3 Cells Transfected with the ApnI, hAPE and ΔhAPE Mammalian Expression Vectors

Bleomycin is a chemotherapeutic agent utilized for the treatment of a variety of carcinomas. The use of this drug has been limited due to the development of lung fibrosis in a relatively large percentage of patients. This side effect is thought to result from the DNA damaging effects of bleomycin in lung cells. Therefore, the development of a protective therapy for lung cells against the DNA damaging effects of bleomycin is desirable. An initial study of bleomycin treated mouse 3T3 fibroblasts indicated that a 50 μg/ml dose of bleomycin resulted in a 25% reduction in total cell number over a 72 hour period as compared to untreated 3T3 cells. To see if the yeast ApnI AP endonuclease or the human AP endonuclease could provide protection against the oxidative DNA damage induced by the radiomimetic agent bleomycin, 3T3 cells were transfected with one of six constructs: PGK-ApnI, PGK-hAPE, PGK-ΔhAPE, SPC-A-

ApnI, SPC-A-hAPE, or SPC-A-ΔhAPE. The PGK is the ubiquitously expressed human phosphoglycerate kinase promoter region that should direct the expression of the AP endonuclease coding region in the 3T3 cells. The SPC-A is a lung specific surfactant gene promoter sequence that should not direct transcription of the AP endonuclease coding region in 3T3 cells. The ΔPGK-hAPE and SPC-A-ΔhAPE constructs contain truncated hAPE coding regions that will produce DNA repair proficient proteins missing the N-terminal region purported to be involved in redox activities (Xanthoudakis *et al.*, 1994).

The transfected 3T3 cells were treated with either 0, 10, 50 or 100 μg/ml bleomycin for 1 hour, subcultured at 5 X 10^4 cells per plate and a sample counted every 24 hours. The preliminary results of the bleomycin treatment after 72 hours are depicted in Figure 31. PGK-ApnI transfected 3T3 cells exhibited an approximately 3-fold higher survival rate than the 3T3 cells alone. Additionally, PGK-hAPE displayed an approximately 2-fold increase in survival while the PGK-ΔhAPE construct did not confer any protection against bleomycin induced cell killing. The control vector, SPC-ApnI, did not provide any protection as expected.

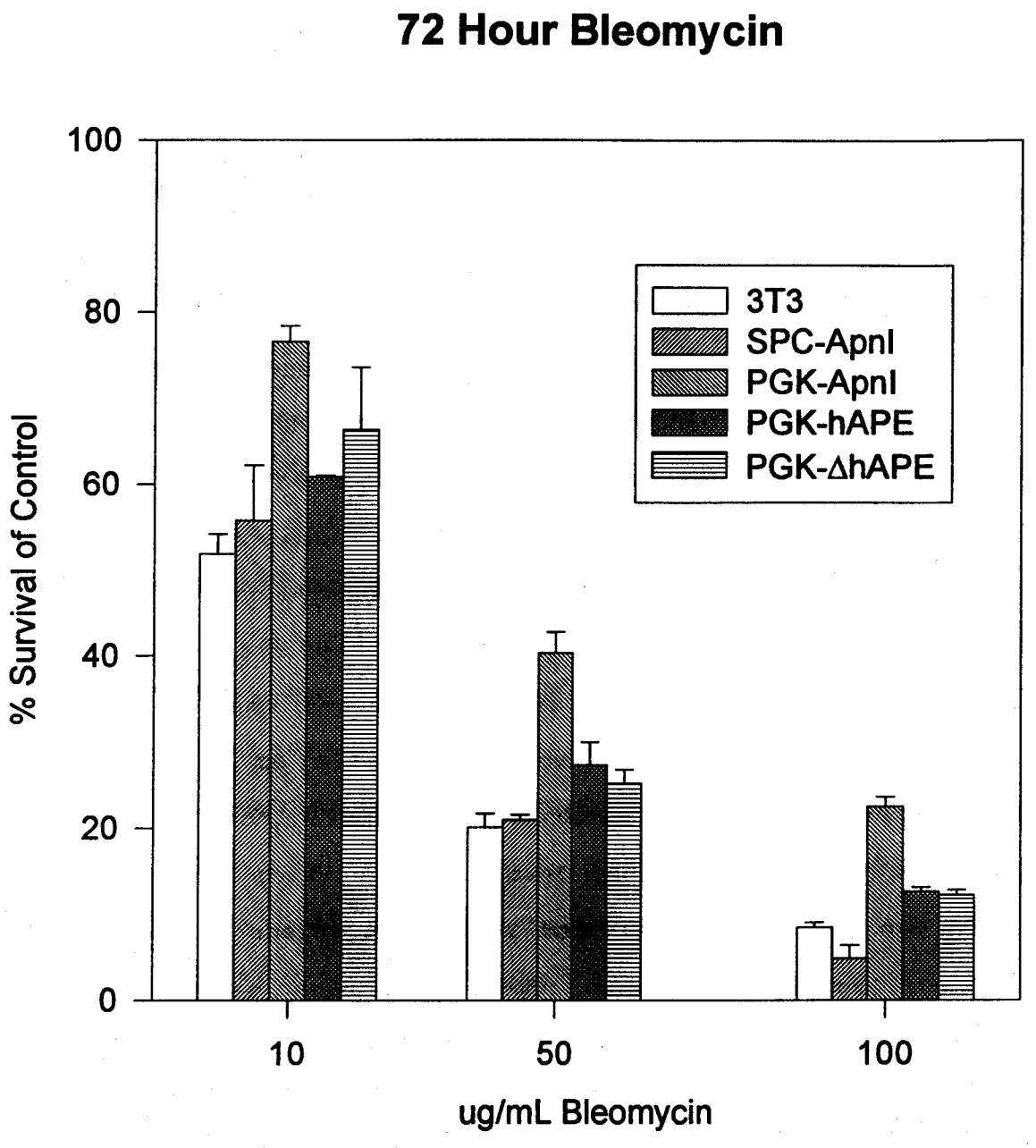


Figure 31. Enhanced survival of murine 3T3 fibroblasts transfected with AP endonuclease coding regions after treatment with bleomycin. 3T3 cells alone and transfected with either the PGK-ApnI, PGK-hAPE, PGK-ΔhAPE or SPC-ApnI expression vector were treated with 100 g/ml bleomycin for 1 hour and allowed to grow for an additional 72 hours. Total cell numbers were counted and the results presented as percentage survival relative to control.

CHAPTER V

DISCUSSION

Living organisms are continuosly exposed to DNA damaging agents, both exogenous and endogenous, that can form a wide array of DNA alterations and modifications. On a day to day basis, however, the formation of apurinic/apyrimidinic (AP) sites is one of the most common types of DNA damage, resulting from both spontaneous loss of bases and from oxidative free radicals that are byproducts of an oxidative respiratory system. Modified bases can lead to aberrant base pairing while AP sites in DNA have been shown to hinder transcription and, if not repaired, lead to altered transcripts and transcript levels (Zhou and Doetsch, 1993). The accumulation of DNA lesions has been postulated to be a significant and central cause of aging and neuronal degeneration (Warner and Price, 1989; Rao and Loeb, 1992; Boerrigter and Vijg, 1993; Weirich-Schwaiger *et al.*, 1994). Therefore, the role of the base excision repair pathway in the repair of damaged bases and AP sites in DNA is crucial.

While the *E. coli* homologue has been extensively characterized, very little is currently known about the regulation and expression of the mammalian AP endonuclease. The predominant theory proclaims that mammalian DNA repair genes in

general are "housekeeping" in nature and are not interesting from a differential expression/gene regulation point of view. Indeed, initial studies of this dissertation examining rAPEN mRNA expression by reverse transcription-polymerase chain reaction and nornthern analysis demonstrated relatively similar levels of transcript expression between the different tissues. However, as was demonstrated, this type of analysis does not reveal the true nature of AP endonuclease cell and tissue specific expression and can lead to erroneous and misleading conclusions concerning the lack of differential expression of AP endonuclease mRNA in various tissues, in particular, and DNA repair, in general. The purpose of this dissertation was to examine the developmental, tissue and sub-tissue specific expression of the mammalian class II AP endonuclease.

To begin to determine the expression of rAPEN *in vivo*, *in situ* hybridization was performed on adult rat brain sections and a series of fetal rat sections that spanned the developmental stages. Examination of the distribution of AP endonuclease mRNA in adult rat brain revealed a widespread, but heterogeneous pattern of distribution. Of these regions, most of which were either the more metabolically active brain cells or sites of excitatory amino acid (EAA) action, several sites were of particular interest. In agreement with previous observations, AP endonuclease mRNA expression was highly expressed in the suprachiasmatic nuclei, which is the site of a circadian pacemaker (Moore *et al.*, 1989; Rivkees and Kelley, 1994). AP

endonuclease mRNA levels were also very high in the supraoptic and paraventricular nuclei, which are involved in the regulation of water balance (Shen, 1992). Previous studies have shown that AP endonuclease mRNA in these structures appears to be constitutively expressed at a higher level than found in the majority of other cells and is not regulated by circadian, photic, or osmotic stimulation at the mRNA level (Rivkees and Kelley, 1994). Since these structures are important sites of c-Fos/Jun action (Aronin *et al.*, 1990; Kornhauser *et al.*, 1990; Rea *et al.*, 1993), high level AP endonuclease expression in these structures may be related to the c-Fos/Jun redox state regulation. However, AP endonuclease mRNA levels were found to remain unchanged with external perturbations known to induce c-Fos/Jun in these cells (Rivkees and Kelley, 1994). The high levels of AP endonuclease mRNA, and presumably AP endonuclease enzyme, may be present in these structures solely for DNA repair functions. The possibility remains that the AP endonuclease expression/activity is regulated at the translational and/or post-translational levels.

High levels of AP endonuclease mRNA expression were also detected in the hippocampal formation. This result was in agreement with the findings of Ono *et al*, 1995, on the expression of AP endonuclease in mouse brain. The hippocampus is a metabolically active tissue that is a major site of excitatory amino acid (EAA) action (Coltman and Monaghan, 1986). EAA are capable of inducing cell damage, which triggers oxidative damage

of DNA (Coltman and Monaghan, 1986). Because AP endonuclease is involved in oxidative DNA damage repair, the high level of AP endonuclease expression in these cells may be a protective mechanism evolved for this type of cellular damage. Furthermore, the results showed that high levels of AP endonuclease mRNA were present in granule cells of the dentate gyrus and pyramidal cells of Ammon's horn, which are also major sites of EAA action (Coltman and Monaghan, 1986). Thus, high level AP endonuclease gene expression may also protect neurons in these regions. Within this hypothesis, the observation that there was low level AP endonuclease mRNA expression in the dentate hilus is intriguing due to the fact that the hilus is a major site of hippocampal degeneration in seizure disorders and EAA toxicity models (Swanson, 1995). Studies to determine if low levels of AP endonuclease in this region contribute to the vulnerability of cells in the hilus to exocitotoxic damage may provide useful information.

In the cerebellum, high levels of AP endonuclease mRNA were observed in Purkinje cells. These cells are very metabolically active and are innervated by excitatory projections (Coltman and Monaghan, 1986). The deep cerebellar nuclei, which also receive excitatory projections (Coltman and Monaghan, 1986), also expressed high levels of AP endonuclease mRNA supporting the concept that the AP endonuclease is expressed at sites of EAA action or in metabolically active brain cells. Pyramidal cells of the cerebral cortex that

receive excitatory projections also expressed relatively high levels of AP endonuclease mRNA. Furthermore, DNA polymerase β, the enzyme responsible for BER DNA synthesis, has been localized in the adult rat brain to the cortex, cerebellum (particularly the Purkinje cells) and hippocampus, similar regions as demonstrated here for the AP endonuclease (Zucconi *et al.*, 1992).

During rat fetal development, AP endonuclease gene expression was very prominent. At the ages examined, which spanned periods of organogenesis and neurogenesis, high level AP endonuclease mRNA expression was present in all somatic sites examined. This pattern of expression during development may help protect the embryo and fetus against potential DNA replication errors that may occur during active periods of mitosis. Since pre- and post-natal AP endonuclease mRNA expression was not compared, it is not known if expression of this gene is greater during organogenesis than at later stages. At the gestational times studied, AP endonuclease mRNA levels were exceptionally high in the liver and thymus. During this period, the liver has a predominant role as an hematopoietic organ, as opposed to in adults when the liver is more involved in detoxification. The expression of the DNA BER pathway is currently being investigated in developing and purified populations of human hematopoietic cells in this laboratory. Individuals afflicted with a DNA-repair disorder display a wide range of clinical symptoms, including skeletal abnormalities and

altered growth. Further detailed analyses of the differential expression of the BER pathway during development may help to shed light on these observed clinical phenotypes.

When AP endonuclease mRNA expression was examined in the testis, rAPEN mRNA levels was found to vary with the stage of spermatogenesis. rAPEN mRNA expression was present at modest levels over spermatogonia and spermatocytes. However, the highest levels of rAPEN mRNA were detected over round spermatids. These are post-meiotic cells that mature into spermatozoa via a process that involves nuclear condensation (Clermont, 1972). Thus, although rAPEN mRNA was expressed throughout germ cell development, its expression was greatest after completion of meiosis during spermatozoa maturation. High level AP endonuclease expression at this stage would thus favor correction of DNA errors before mature sperm are formed.

The differential expression of the rat AP endonuclease gene in specific regions of rat brain, testis and particular tissues during development is in marked contrast to the expression of the NER gene ERCC3, which has been shown to be evenly and ubiquitously expressed in mouse brain during development (Hubank and Mayne, 1994). Therefore, the differential expression of the rAPEN transcript in the various tissue and subtissue regions is unique and not simply a general DNA repair gene mode of expression.

This type of analysis allows us to begin to understand the regulation and functional relationship of this gene product to

the various cell types while, at the same time, raising many new questions. For example, why is the level of expression of the AP endonuclease gene so strikingly high in the suprachiasmatic nuclei (SCN), paraventricular nuclei (PVN) and supraoptic nuclei (SON)? Why is AP endonuclease also highly expressed in pyriform cortex, hippocampus and cerebellum? What is its function during fetal development and what controls the level of expression? Why do round spermatids in developing germ cells express such a high level of the AP endonuclease mRNA compared to the other germ cell stages? While explanations have been suggested, many more studies, both *in vivo* and biochemical analyses, must be performed to arrive at answers to these questions.

Studies examining the correlation between rAPEN mRNA and protein expression in the various tissues would have been beneficial; however, this was not possible due to the ineffectiveness of the anti-rAPEN antibody in immunohistochemistry experiments. A new polyclonal antibody is currently being produced.

The striking results of the rAPEN *in situ* hybridization studies led to the analysis of human tissue samples. Fortunately, the anti-human AP endonuclease antibody previously generated in this laboratory was very effective for this method allowing analysis of hAPE protein expression in various human tissues (Duguid *et al.*, 1995).

To date, no studies have been performed on the sub-tissue distribution of hAPE in any human tissues. The only

immunohistochemical studies previously performed on human cells have used transformed cell lines. One such study demonstrated speckled staining of the nuclei of osteosarcoma cells in culture with little cytoplasmic staining (Demple, *et al.*, 1991), while the other showed nuclear rim staining in another cell line following the overexpression of a vector containing the hAPE cDNA (Xanthoudakis *et al.*, 1992a). Similar to these previous reports, the immunocytochemical staining of A1235 glioma cells presented in this dissertation demonstrated intense nuclear staining with little apparent cytoplasmic staining (Figure 19). Western analysis of sub-cellularly fractionated HL-60 cells demonstrated the presence of hAPE in all fractions examined, including crude nuclei, postnuclear supernatant, cytoplasm, ribosomes, and mitochondria (Figure 20). This result indicates that the hAPE protein is present in all the cell areas examined; however, due to the method of purification, the possibility exists that the mitochondrial fraction was contaminated with other cellular structures such as Golgi apparatus. The difference between the immunocytochemical and Western analyses results may be explaned by the fact that immunocytochemistry is a strictly qualitative form of analysis: hAPE may have been present in the cytoplasm of the examined cells but not at levels detectable by this method. As will be apparent by the discussion the follows, what has been observed in these *in vitro* studies is not necessarily the case *in vivo*.

Immunohistochemistry of hAPE *in vivo* using biopsy specimens demonstrated a diverse and drastically different picture of hAPE protein expression in the various organs and cell types. Two tissues have been identified in which the hAPE expression was markedly affected by the normal maturation of their cells. In the duodenum, enterocytes are generated in the crypts of Lieberkuhn and, over a period of approximately two to four days, they migrate out of the crypts and up the villi toward the tip where they are sloughed into the intestinal lumen (Wheater *et al.*, 1982). It was found that the enterocyte nuclei abruptly stopped staining with hAPE antibody well before they had reached the villus tip and at a time when they were morphologically normal. A similar pattern has been noted for alkaline phosphatase expression (Wheater *et al.*, 1982), which suggests that there is a marked change in gene expression that occurs in the maturation of enterocytes which is not reflected in their morphology. This pattern of staining was in contrast to what has been observed with the MGMT DNA repair protein. Immunohistochemical analysis of MGMT in rat duodenum showed a homogeneous staining pattern of enterocyte nuclei from the crypts all the way to the tips of the villi (Zaidi *et al.*, 1993). In addition, the antibody to the human MSH2 mismatch DNA repair protein demonstrates staining predominantly in the nuclei of the crypts with little staining in the cells of the villi (Wilson *et al.*, 1995), while MLH1, another human mismatch DNA repair protein is expressed in all enterocytes of the villi

similar to MGMT expression (unpublished data). Therefore, the various DNA repair proteins of several different pathways: direct reversal (MGMT), mismatch (MSH2, MLH1), and BER (hAPE) all show a different and distinct pattern of expression in the duodenum. The relationship of this differing pattern of expression for DNA repair proteins in one tissue, such as duodenum, is intriguing and requires further detailed investigation.

Epidermal cells are generated in the basal layer of the epidermis and over approximately 30 days migrate to the superficial layers where they keratinize and are sloughed (Wheater *et al.*, 1982). There was a clear morphological change that corresponded with the loss of hAPE antibody staining. As the cells keratinize and die, they appear to stop expressing hAPE. The staining of epidermal nuclei was far greater than that of any other tissue examined. This is not due exclusively to the fact that epidermal cells are continuously turning over as duodenal enterocytes turn over in much less time. Instead, it may reflect the exposure of the epidermis to ultraviolet light which can generate free radicals and, subsequently, produce abasic lesions. Furthermore, the uniform nuclear pattern of hAPE antibody staining is not present in enterocytes and epidermal cells solely because they are turning over as non-replicating cerebellar granular neurons also demonstrate this pattern.

Autopsy specimens were examined for hAPE expression in the brain. Large neurons in the cerebellum, hippocampus, and brainstem demonstrated prominent staining of the cytoplasm, and, when the nucleus was stained, the hAPE staining corresponded to chromatin staining demonstrated by hematoxylin, but sparing the nucleoli. Hepatocytes showed a similar staining pattern. Some cerebellar granule neuron nuclei and some enterocyte nuclei stained uniformly and darkly with both hAPE and hematoxylin while epidermal cells demonstrated a uniformly dark nuclear pattern with hAPE which did not correspond to the reticular staining of chromatin stained with hematoxylin. Another intriguing result of these studies was the cell to cell differential expression observed in the granule cells of the cerebellum. In contrast to the other tissues studied, granule cells displayed two strikingly different patterns of hAPE expression: numerous cells showed hAPE staining only in the cytoplasm while adjacent cells displayed an intense disperse nuclear staining. This result indicates a cell to cell difference in hAPE expression or half-life of the hAPE DNA repair mRNA or enzyme in these cells of the cerebellum. This was not seen in other brain cells; for example, hippocampal neurons all expressed hAPE staining protein primarily in the cytoplasm. This pattern of cell to cell variance, as well as the exclusive cytoplasmic staining of hippocampal neurons, warrants further investigation.

hAPE antibody recognized the Nissl substance of hypoglossal motor neurons. This was a specific reaction as there was not staining of Nissl substance when an adjacent section was stained with preimmune rabbit immunoglobulin (data not shown). The Nissl substance represents aggregates of ribosomes which are present in the cytoplasm of motor neurons. This staining pattern suggests that in these cells hAPE is associated with ribosomes and has a previously unrecognized role in ribosome function. It is possible that in the other cell types that demonstrate cytoplasmic hAPE staining, hAPE is associated with ribosomes as well, although this is not apparent due to the fact that these cells do not contain the large ribosomal aggregates present in motor neurons. These results are interesting in light of previously published data demonstrating that the ribosomal protein PO is an AP endonuclease (Kelley *et al.*, 1989; DeFelice, *et al.*, 1995) as is ribosomal protein S3 (Wilson *et al.*, 1994), with the former being a class II AP endonuclease and the latter a class I with associated glycosylase activity against 8-oxoguanine (Yacoub *et al.*, 1995). Both of these multifunctional ribosomal/DNA repair AP endonucleases have also been found to be associated with the nuclear matrix (DeFelice *et al.*, 1995; Wilson *et al.*, 1995).

The specificity of the immunohistochemical reactions employed was demonstrated by: a) the cross-reactivity of the hAPE antibody to only one protein upon Western blot analysis; b) reduced signal after preincubating the primary antibody with

purified antigen, and c) the absence of signal when pre-immune antibody was used.

The results of the immunohistochemical survey of human tissues also raises many questions. Why do granule cells of the cerebellum display such a diverse and cell-to-cell variability of hAPE protein expression? Why are hippocampal neurons of the dentate gyrus expressing hAPE protein predominantly in the cytoplasm? Why are there different nuclear staining patterns, from intense dispersed staining in skin enterocytes, epidermal cells, and duodenal cells versus the nuclear rim staining in Purkinje cells of the cerebellum or the punctate staining of liver nuclei? Why does hAPE protein completely disappear approximately 75% of the way out towards the distal tip of villi in the duodenum, while other DNA repair proteins are found throughout the villi, including the distal tip? Although there are various hypotheses for the differing patterns of expression, further studies are needed to determine the mechanistic principles of the findings presented in this dissertation.

A consideration that must be addressed when dealing with a purported multifunctional protein such as hAPE, concerns whether the observed pattern of expression has to do with the DNA repair capacity of the hAPE protein or the alleged redox factor function (Walker *et al.*, 1993; Xanthoudakis *et al.*, 1992a; Xanthoudakis *et al.*, 1992b; Xanthoudakis *et al.*, 1994). It has clearly been shown, *in vivo*, that the human AP endonuclease functions as a DNA repair protein (Demple *et al.*, 1991; Walker,

et al., 1994). Experiments using antisense constructs have shown clear cause and effect of lowered levels of hAPE protein and cell sensitivity to DNA damaging agents (Walker *et al.*, 1994). Furthermore, the human AP endonuclease has been shown to complement AP endonuclease deficient *E. coli* cells (Demple *et al.*, 1991). Low amounts of protein (picogram amounts) are highly active in acting on damaged DNA substrates (Demple *et al.*, 1991; Walker *et al.*, 1993; Huq *et al.*, 1995), while microgram amounts of AP endonuclease protein are needed for the other ascribed activities, and these activities have only been demonstrated in *in vitro* biochemical assays (Demple *et al.*, 1991; Xanthoudakis *et al.*, 1992b; Xanthoudakis *et al.*, 1994). Hypoxic and hyperoxic conditions have been shown to increase hAPE protein levels in tissue culture cells, and these results have been used to argue for the role of AP endonuclease in transcription factor redox activity (Yao *et al.*, 1994), but actual *in vivo* demonstration awaits further studies. Furthermore, these studies can as easily be interpreted as an indication of enhanced hAPE expression for the repair of oxidative lesions following oxygen level alterations (Walker *et al.*, 1994). In light of these considerations, it can be proposed that the pattern of expression of the hAPE protein described here reflects the DNA repair component of the hAPE protein.

This survey of *in vivo* cellular and subcellular expression of rAPEN mRNA and hAPE protein is the beginning of our understanding of the relationship of DNA BER and cell specificity of expression and has yielded several findings not predicted by previous studies using cell lines. These include the findings that: 1.) the expression of the mammalian AP endonuclease is spatially and temporally regulated; 2.) levels of mammalian AP endonuclease expression in brain is higher in metabolically active regions, perhaps in response to increased incidence of oxidative DNA damage; 3.) the finding that hAPE is associated with Nissl substance strongly suggests that hAPE either has functions that are not yet appreciated, or that its subcellular pattern of distribution is much more complex than initially appreciated using *in vitro* tissue culture cell lines; and 4.) the distinct levels and complex patterns of staining between tissues for the hAPE protein clearly suggests that this protein has diverse processes of regulation and trafficking in the numerous cell and tissue types studied that has not been observed during *in vitro* cell line studies.

One of the final experiments of this dissertation involved the examination of whether hAPE expression is modulated by the cellular process of apoptosis. The apoptotic process is important physiologically in that it controls the growth of both normal and neoplastic tissues. The myeloid leukemia HL-60 cell line has been well characterized and utilized for studies of both apoptosis and cellular differentiation. These cells can be

induced to undergo differentiation and apoptosis in response to treatment with retinoic acid (Nagy *et al.*, 1995). A varient of the HL-60 cell line that is mutant for the *bcl-2* gene, a gene involved in blocking the apoptotic signaling pathway, undergoes differentiation but not apoptosis (Robertson *et al.*, 1992). Therefore, with these two cell lines, HL-60 and HL-60R, it was possible to determine the effect of apoptosis and/or differentiation on hAPE expression. In the HL-60 cell line, hAPE protein levels decreased over a six day period. However, in the HL-60R cell line, hAPE levels remained constant. These results indicate that hAPE protein levels are reduced by the process of apoptosis but remain unaffected by differentiation. Interestingly, somewhat different results were obtained for the *myc* gene by Islas and Hanawalt, 1995. These authors observed a transcriptional down-regulation of c-myc mRNA levels during differentiation of HL-60 cells by TPA (12-*O*-tetradecanoyl-phorbol 13-acetate) which induces the cells to undergo differentiation but not apoptosis. This down-regulation is due to a block of transcriptional elongation and has also been observed with retinoic acid treatment (Bentley and Groudine, 1986). The decrease in hAPE protein levels during differentiation and apoptosis in HL-60 cells indicates that hAPE expression is downregulated by the apoptotic process. However, the mechanism of this downregulation has not yet been determined. hAPE mRNA levels need to be examined in this model system to provide more insight into the process.

The final study of this dissertation involved the attempt to protect mouse 3T3 fibroblasts from the DNA damaging effects of bleomycin. The preliminary results of the dose response experiments utilizing the various AP endonuclease expression vectors demonstrated that the yeast Apn1 AP endonuclease, under control of the human phosphoglycerate kinase promoter, is capable of providing a nearly three fold decrease in cell killing levels after treatment with 100 ug/ml dose of bleomycin. The cytotoxic effect of this agent in the induction of pulmonary fibrosis has been determined to be through DNA damage (Harrison and Lazo, 1987; Lazo and Sebti, 1991). It has been demonstrated that "activated" bleomycin reacts with the C-4′ position of the sugar residue generating a 4′-oxidized AP site along with strand breaks (Hecht, 1986). Additionally, 4′-oxidized AP sites have been shown to be an excellent substrate for endonuclease IV requiring 4-fold lower concentrations of the protein than for recognition of regular AP sites while exonuclease III has only minor repair activity on this lesion.(Häring *et al.*, 1994). Therefore, the ability of the yeast endonuclease IV homologue, ApnI, to provide an enhanced survival of 3T3 cells from bleomycin is not surprising. The protection of normal cells from the cell-killing effects of bleomycin will significantly contribute to the development of therapies for the prevention/treatment of bleomycin induced fibrotic lung disease.

LITERATURE CITED

Abate, C., Patel, L., Rauscher, F.J. III and Curran, T. (1990). Redox regulation of Fos and Jun DNA binding activity *in vitro*. Science **249**:1157-1161.

Aboussekhra, A., Biggerstaff, M., Lhivji, M.D.D., Vilpo, J.A., Moncollin, V., Podust, V.N., Protic, M., Hubscher, U., Egly, J.-M. Wood, R.D. (1995). Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell **80**:859-868.

Ames, B.N. (1989). Endogenous oxidative DNA damage, aging, and cancer. Free Radical Res. Comm. **7**:121-128.

Aronin, N., Sagar, S.M., Sharp, F.R. and Schwartz, W.J. (1990). Light regulates expression of a Fos-related protein in rat suprachiasmatic nuclei. Proc. Natl. Acad. Sci. **87**:19-26.

Bailly, V. and Verly, W.G. (1987). Escherichia coli endonuclease III is not an endonuclease but a β-elimination catalyst. Biochem. J. **242**:565-572.

Bailly, V. and Verly, W.G. (1989). AP endonucleases and AP lyases. NAR **17**:3617-3618.

Barnes, D.E., Lindahl, T. and Sedgwick, B. (1993). DNA repair (Review). Curr. Opinion Cell Biol., **5**:424-433.

Bentley, D.L. and Groudine, M. (1986). A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature **321**:702-706.

Boerrigter, M.E.T.I. and Vijg, J. (1993). Studies on DNA repair defects in degenerative brain disease. Age and Ageing. **22**:44-52.

Boiteux, S., O'Connor, T.R. and Laval, J. (1987). Formamidopyrimidine-DNA glycosylase of *Escherichia coli*: cloning and sequencing of the *fpg* structural gene and overproduction of the protein. EMBO J. **6**:3177-83.

Bonura, T., Schultz, r. and Friedberg, E.C. (1982). An enzyme activity from *Escherichia coli* that attacks single-stranded deoxyribopolymers and single-stranded

deoxyribonucleic acid containing apyrimidinic sites. Biochem. **21**:2548-2556.

Chakravarti, D., Ibeanu, G.C., Tano, K. and Mitra, S. (1991). Cloning and expression in *Escherichia coli* of a human cDNA encoding the DNA repair protein *N*-methylpuring-DNA glycosylase. J. Biol. Chem. **266**:15710-15715.

Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol. Rev. **52**:198-236.

Coltman, C.W. and Monaghan, D.T. (1986). Anatomical organization of excitatory amino acid receptors and their properties. Adv. Exp. Med. Biol. **203**:237-252.

Cunningham, R.P., Saporito, S., Spitzer, S.G. and Weiss, B. (1986). Endonuclease IV (*nfo*) mutant of *Escherichia coli*. J. Bacteriol. **168**:1120-1127.

DeFelice, S., Yacoub, A., Grabowski, D.T., Kelley, M.R. and Deutsch, W.A. (1995). *Drosophila* ribosomal protein PO contains AP endonuclease activity. J. Biol. Chem., in press.

Demple, B. and Linn, S. (1980). DNA N-glycosylases and UV repair. Nature **287**:203-208.

Demple, B., Halbrook, J. and Linn, S. (1983). *Escherichia coli* xth mutants are hypersensitive to hydrogen peroxide. J. Bacteriol. **153**:1079-1082.

Demple, B., Johnson, A.W. and Fung, D. (1986). Exonuclease III and endonuclease IV remove 3′ blocks from DNA synthesis primers in H_2O_2-damaged *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83**:7731-7735.

Demple, B., Greenberg, J.T., Johnson, A.W. and Levin, J.D. (1988). In: Mechanisms and Consequences of DNA Damage Processing, UCLA Symposia on Molecular and Cellular Biology. **83**:159-166.

Demple, B., Herman, T., and Chen, D. S. (1991). Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: Definition of a family of DNA repair enzymes. Proc. Natl. Acad. Sci. USA **88**:11450-11454.

Demple and Harrison, (1994). Repair of oxidative damage to DNA: Enzymology and biology. Ann. Rev. Biochem. **63**:915-948.

Doetsch, P.W. and Cunningham, R.P. (1990). The enzymology of apurinic/apyrimidinic endonucleases. Mut. Res. **236**:173-201.

Dizdaroglu, M. (1992). Oxidative damage to DNA in mammalian chromatin (Review). Mut. Res. **275**:331-342.

Duncan, B.K. and Weiss, B. (1982). Specific mutator effects of ung (uracil-DNA glycosylase) mutations in *Eshcerichia coli*. J. Bacteriol. **151**:750-755.

Engelward, B.P., Boosalis, M.S., Chen, B.J., Deng, Z., Siciliano, M.J. and Samson, L. (1993). Cloning and characterization of a mouse 3-methyladenine/7-methylguanine/3-methylguanine DNA glycosylase cDNA whose gene maps to chromosome 11. Carcinogenesis. **14**:175-181.

Franklin, W.A. and Lindahl, T. (1988). DNA deoxyribophosphodiesterase. EMBO J. **7**:3617-3622.

Friedberg, E.C. (1985). DNA Repair. Freeman, San Francisco, CA.

Gates, F.T. III and Linn, S. (1977). Endonuclease from *Escherichia coli* that acts specifically upon duplex DNA damaged by ultraviolet light, osmium tetroxide, acid, or X-rays. J. Biol. Chem. **252**:2802-2807.

Graves, R.J., Felzenszwalb, I., Laval, J. and O'Connor, T.R. (1992). Excision of 5'-terminal deoxyribose phosphate from damaged DNA is catalyzed by the fpg protein of *Escherichia coli*. J. Biol. Chem. **267**:14429-14435.

Häring, M., Rüdiger, H., Demple, B., Bioteux, S. and Epe, B. (1994). Recognition of oxidized a basic sites by repair endonucleases. NAR **22**:2010-2015.

Harrison, J.H. and Lazo, S. (1987). High dose continuous infusion of bleomycin in mice: a new model for drug-induced pulmonary fibrosis. J. Pharm. Exper. Therap. 243:1185-1194.

Harrison, J.H., Hoyt, D.G. and Lazo, S. (1989). Acute pulmonary toxicity of bleomycin: DNA scission and matrix protein mRNA levels in bleomycin-sensitive and resistant strains of mice.

Hecht, S.M. (1986). DNA strand scission by activated bleomycin group antibiotics (Review). Fed. Proc. **45**:2784-2789.

Henner, W.D., Grunberg, S.M. and Haseltine, W.A. (1983). Enzyme action at 3' termini of ionizing radiation-induced DNA strand breaks. J. Biol. Chem. **258**:15198-15205.

Huang, J.C., Svoboda, D.L., Reardon, J.T. and Sancar, A. (1992). Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the

6th phosphodiester bond 3′ to the photodimer. Proc. Natl. Acad. Sci. USA **89**:3664-3668.

Hubank, M. and Mayne, L. (1994). Expression of the excision repair gene, ERCC3 (excision repair cross-complementing) during mouse development. Brain Res Dev Brain Res. **81**:66-76.

Huq, I., Wilson, T.M., Kelley, M.R. and Deutsch, W.A. (1995). Expression in Escherichia coli of a rat cDNA encoding an apurinic/apyrimidinic endonuclease. Mutat. Res., in press.

Impellizzeri, K.J., Anderson, B. and Burgers, P.M. (1991). the spectrum of spontaneous mutations in a *Saccharomyces cerevisiae* uracil-DNA glycosylase mutant limits the function of this enzyme to cytosine deamination repair. J. Bacteriol. **173**:6807-6810.

Islas, A.L. and Hanawalt, P.C. (1995). DNA repair in the MYC and FMS proto-oncogenes in ultraviolet light-irradiated human HL-60 promyelocytic cells during differentiation. Cancer Res. **55**:336-341.

Johnson, A.W. and Demple, B. (1988). Yeast DNA 3′ repair diesterase is the major cellular apurinic/apyrimidinic endonuclease: substrate specificity and kinetics J. Biol. Chem. **263**:18017-18022.

Karran, P. and Lindahl, T. (1980). Hypoxanthine in deoxyribonucleic acid: Generation by heat-induced hydrolysis of adenine residues and release in free form by a deoxyribonucleic acid glycosylase from calf thymus. Biochem. **19**:6005-6011.

Katsura, H., Taguchi, T. and Kida, K. (1992). Alterations in DNA synthesis and cellular constituents in mouse lung following bleomycin injections. **6**:190-196.

Kelley, M.R., Venugopal, S., Harless, J. and Deutsch, W.A. (1989). Antibody to a human DNA repair protein allows for cloning of a *Drosophila* cDNA that encodes an apurinic endonuclease. Mol. Cell. Biol. **9**:965-973.

Kornhauser, J.M., Nelson, D.E., Mayo, K.E. and Takahashi, J.S. (1990). Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. Neuron **5**:127-134.

Kow, Y.W. (1989). Mechanism of action of *Escherichia coli* exonuclease III. biochem. **28**:3280-3287.

Krokan, H., Haugen, A., Myrnes, B. and Guddal, P.H. (1983). Repair of premutagenic DNA lesions in human fetal tissues:

evidence for low levels of o6-methylguanine-DNA methyltransferase and uracil-DNA glycosylase activity in some tissues. Carcinogenesis **4**:1559-1564.

Lazo, J.S. and Sebti, S.M. (1991). Bleomycins. In: Cancer Chemotherapy and Biological Response Modifiers Annual 12. Elsevier Science Pub.

Larson, K., Sahm, J., Shenkar, R. and Strauss, B. (1985). Methylation-induced blocks to *in vitro* DNA replication. Mut. Res. **236**:77-84.

Levin, J., Johnson, A.W. and Demple, B. (1988). Homogeneous *Escherichia coli* endonuclease IV. J. Biol. Chem. **263**:8066-8071.

Levin, J. and Demple, B. (1990). Analysis of class II (hydrolytic) and class I (beta-lyase) apurinic/apyrimidinic endonucleases with a synthetic DNA substrate. NAR **18**:5069-5075.

Levy, A., Manoharan, P.T., Fifkind, J.M., Walker, J.C., Haberle, F.C., Kumar, N.G., Glickson, J.D., Elgavish, G.A. and Mossbauer. (1989). NMR studies of the acid-induced reduction and changes in spin state of ferric bleomycin. Biochem. Biophys. Acta. **991**:97-108.

Lindahl, T. and Nyberg, B. (1972). Rate of depurination of native DNA. Biochem. **11**:3610-3618.

Lindahl, T. (1982). DNA repair enzymes (Review). Ann. Rev. Biochem. **51**:61-87.

Lindahl, T., Sedgwick, B., Sekiguchi, M. and Nakabeppu, Y. (1988). Regulation and expression of the adaptive response to alkylating agents (Review). Ann. Rev. Biochem. **57**:133-157.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA (Review). Nature **362**:709-715.

Lindahl, T. (1994). Instability and decay of the primary structure of DNA. Nature **362**:709-715.

Loeb, L.A. and Preston, B.D. (1986). Mutagenesis by apurinic/apyrimidinic sites (Review). Ann. Rev. Genet. **20**:201-230.

Lowenhaupt, K., Sander, M., Hauser, C. and Rich, A. (1989). *Drosophila melanogaster* strand transferase. J. Biol. Chem. **264**:20568-20575.

Lungquist, S., Lindahl, T. and Howard-Flanders, P. (1976). Methyl methane sulfonate-sensitive mutant of Escherichia coli deficient in an endonuclease specific for apuriic sites in deoxyribonucleic acid. J. Bacteriol. **126**:646-653.

Liuzzi, M., Weinfeld, M. and Paterson, M.C. (1987). Selective inhibition by methoxyamine of the apurinic/apyrimidinic endonuclease activity associated with pyrimidine dimer-DNA glycosylases from *Micrococcus luteus* and bacteriophage T4. Biochem. **26**:3315-3321.

Male, R., Helland, D.E. and Kleppe, K. (1985). Purification and characterization of 3-methyladenine-DNA glycosylase from calf thymus. J. Biol. Chem. **260**:1623-1629.

Meyer-Siegler, K., Rahman-Mansur, N., Wurzer, J.C. and Sirover, M.A. (1992). Proliferative dependent regulation of the glyceraldehyde-3-phosphate dehydrogenase/uracil DNA glycosylase gene in human cells. Carcinogenesis **13**:2127-2132.

Mitra, S. and Kaina, B. (1993). Regulation of repair of alkylation damage in mammalian genomes. Prog. Nuc. Acid Res. **44**:109-140.

Moore, R.Y., Shibata, S. and Bernstein, M. (1989). In Development of Circadian Rhythmicity and Photoperiodism in Mammals, Perinatology Press, pp. 1-24.

Morgan and Curran. (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun (Review). Ann. Rev. Neurosci. **14**:421-451.

Mosbaugh, D.W. and Linn, S. (1982). Characterization of the action of *Escherichia coli* DNA polymerase I at incisions produced by repair endodeoxyribonucleases. J. Biol. Chem. 257:575-583.

Muller, S.J. and Caradonna, S. (1993). Cell cycle regulation of a human cyclin-like gene encoding uracil-DNA glycosylase. J. Biol. Chem. **268**:1310-1319.

Myrnes, B., Guddal, P.H. and Krokan, H. (1982). Metabolism of dITP in HeLa cell extracts, incorporation into DNA by isolated nuclei and release of hypoxanthine from DNA by a hypoxanthine-DNA glycosylase activity. NAR **10**:3693-3701.

Myrnes, B., Giercksky, K.E. and Krokan, H. (1983). Interindividual variation in the activity of o6-methyl guanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. Carcinogenesis **4**:1565-1568.

Nagy, L., Thomazy, V.A., shipley, G.L., Fesus, L., Lamph, W., Heyman, R.A., Chandraratna, R.A.S. and Davies, P.J.A. (1995). Activation of retinoid x receptors induces apoptosis in HL-60 cell lines. Mol. Cell. Biol. **15**:3540-3551.

Nakabeppu, Y., Ryder, K. and Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell **55**:907-915.

Nevins, J.R. (1992). Transcriptional regulation: a closer look at E2F. Nature **358**:375-376.

Nugent, M., Huang, S.M. and Sander, M. (1993). Characterization of the apurinic endonuclease activity of *Drosophila* RrpI. Biochemistry **32**:11445-11452.

O'Connor, T.R. and Laval, F. (1990). Isolation and structure of a cDNA expressing a mammalian 3-methyladenine-DNA glycosylase. EMBO J. **9**:3337-33342.

Okazaki, T., Chung, U., Nishishita, T., Ebisu, S., Usuda, S., Mishiro, S., Xanthoudakis, S., Igarashi, T. and Ogata, E. (1994). A redox factor protein, ref1, is involved in negative gene regulation by extracellular calcium. J. Biol. Chem. **269**:27855-27862.

Olsen, L.C., Aasland, R., Witter, C.U., Krokan, H.E. and Helland, D.E. (1989). Molecular cloning of human uracil-DNA glycosylase, a highly conserved DNA repair enzyme. EMBO J. **8**:3121-3125.

Ono, Y., Furuta, T., Ohmoto, T., Akiyama, K. and Seki, S. (1994). Stable expression in rat glioma cells of sense and antisense nucleic acids to a human multifunctional DNA repair enzyme, APEX nuclease. Mut. Res. **315**:55-63.

Ono, Y., Watanabe, M., Inoue, Y., Ohmoto, T., Akiyama, K., Tsutsui, K. and Seki, S. (1995). Developmental expression of APEX nuclease, a multifunctional DNA repair enzyme, in mouse brains. Dev. Brain Res. **86**:1-6.

Percival, K.J., Klein, M.B. and Bergers, P.M.J. (1989). Molecular cloning and primary structure of the uracil-DNA glycosylase gene from *Saccharomyces cerevisiae*. J. Biol. Chem. **264**:2593-2598.

Popoff, S.C., Spira, A.I., Johnson, A.W. and Demple, B. (1990). Yeast structural gene (*APN1*) for the major apurinic endonuclease: Homology to *Escherichia coli* endonuclease IV. Proc. Natl. Acad. Sci. USA **87**:4193-4197.

Radany, E.H., Naumovski, L., Love, J.D., Gutekunst, K.A., Hall, D.H. and Friedberg, E.C. (1984). Physical mapping and complete nucleotide sequence of *denV* gene of bacteriophage T4. J. Virol. **52**:846-856.

Ramotar, C., Popoff, S.C., Gralla, E.B. and Demple, B. (1991). Cellular role of yeast Apn1 apurinic endonuclease/3' diesterase: Repair of oxidative and alkylation DNA damage and control of spontaneous mutation. Mol. Cell. Biol. **11**:4537-4544.

Ramotar, C., Kim, C., Lillis, R. and Demple, B. (1993). Intracellular localization of the Apn1 DNA repair enzyme of *Saccharomyces cerevisiae*. J. Biol. Chem. **268**:20533-20539.

Rao, K.S. and Loeb, L.A. (1992). DNA damage and repair in brain: relationship to aging. Mut. Res. **275**:317-329.

Rea, M.A., Michel, A.M. and Lutton, L.M. (1993). Is Fos expression necessary and sufficient to mediate light-induced phase advances of the suprachiasmatic circadian oscillator? J. Biol. Rhyth. **8**:559-564.

Richardson, C.C., Lehman, I.R. and Kornberg, A. (1964). A deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli*: Characterization of the exonuclease activity. J. Biol. Chem. **239**:251-258.

Rivkees, S.A. and Kelley, M.R. (1994). Expression of a multifunctional DNA repair enzyme, apurinic/apyrimidinic endonuclease (APE;REF-1) in the suprachiasmatic, supraoptic and paraventricular nuclei. Brain Research **666**:137-142.

Robertson, K.a., Emami, B. and Collins, S.J. (1992). Retinoic acid-resistant LH-60R cells harbor a point mutation in the retinoic acid receptor ligand-binding domain that confers dominant negative activity. Blood **80**:1885-1889.

Robson, C.N., Milne, A.M., Pappin, D.J. and Hickson, I.D. (1991). Ilsonation of cDNA clones encoding an enzyme from bovine cells that repairs oxidative DNA damage in vitro: homology with bacterial repair enzymes. NAR **19**:1087-1092.

Robson, C.N. and Hickson, I.D. (1991). Isolation of cDNA clones encoding a human apurinic/appyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in *E. coli xth* (exonuclease III) mutants. NAR **19**:5519-5523.

Rogers, S.G. and Weiss, B. (1980). Exonuclease III of *Escherichia coli* K-12, an AP endonuclease. Meth. Enz. **65**:201-211.

Saffhill, R., Margison, G.P. and O'Connor, P.J. (1985). Mechanisms of carcinogenesis induced by alkylating agents. Biochem. Biophys. Acta **823**:111-145.

Sakumi, K., Nakabeppu, Y., Yamamoto, Y., Kawabata, S., Iwanaga, S. and Sekiguchi, M. (1986). Purification and structure of 3-methyladenine-DNA glycosylase I of *Escherichia coli*. J. Biol. Chem. **261**:15716-15766.

Sakumi, K. and Sekiguchi, M. (1990). Structures and functions of DNA glycosylases. Mut. Res. **236**:161-172.

Sammartano, L.J., Tuveson, R.W. and Davenport, R. (1986). Control of sensitivity to inactivation by H_2O_2 and broad-spectrum near-UV radiation by the *Escherichia coli* katF locus. J. Bacteriol. **168**:13-21.

Sancar, A. (1994). Structure and function of DNA photolyase. Biochem. **33**:2-9.

Sancar, A. and Sancar, G.B. (1988). DNA repair enzymes. Ann. Rev. Biochem. **57**:29-67.

Sander, M., Lowenhaupt, K., Lane W.S. and Rich, A. (1991a). Cloning and characterization of Rrp1, the gene encoding *Drosophila* strand transferase: Carboxy-terminal homology to DNA repair endo/exonucleases. NAR **19**:4523-4525.

Sander, M., Lowenhaupt, K. and Rich, A. (1991b). Drosophila Rrp1 protein: An apurinic endonuclease with homologous recombination activities. Proc. Natl. Acad. Sci. USA **88**:6780-6784.

Saporito, S.M. and Cunningham, R.P. (1988). Nucleotide sequence of the *nfo* gene of *Escherichia coli* K-12. J. Bacteriol. **170**:5141-5145.

Seki, S., Akiyama, K., Watanabe, S., Matsuhika, M., Ideda, S. and Tsutsui, K. (1991). cDNA and deduced amino acid sequence of a mouse DNA repair enzyme (APEX nuclease) with significant homology to *Escherichia coli* exonuclease III. J. Biol. Chem. **266**:20797-20802.

Shen, E., Dun, S.L., Ren, C. and Dun, N.J. (1992). Hypovolemia induces Fos-like immunoreactivity in neurons of the rat supraoptic and paraventricular nuclei. J. Auton. Nerv. Sys. **37**:227-230.

Shen, J.-C., Rideout, W.M., III and Jones, P.A. (1992). High frequency mutagenesis by a DNA methyltransferase. Cell **71**:1073-1080.

Slupphaug, G., Olsen, L.C., Helland, D., Aasland, R. and Krokan, H.E. (1991). Cell cycle regulation and in vitro hybrid arrest analysis of the major human uracil-DNA glycosylase. NAR **19**: 5131-5137.

Spiering, A.L. and Deutsch, W.A. (1986). *Drosophila* apurinic/apyrimidinic DNA endonucleases. Characterization of mechanism of action and demonstration of a novel type of enzyme activity. J. Biol. Chem. **261**:3222-3228.

Swanson, T.H. (1995). The pathophysiology of human mesial temporal lobe epilepsy. J. Clin. Neurophys. **12**:2-22.

Tao, K., Noda, A. and Yonei, S. (1987). The roles of different excision-repair mechanisms in the resistance of *Micrococcus luteus* to UV and chemical mutagens. Mut. Res. **183**:231-239.

Thomas, L., Yang, C.-H. and Goldthwait, D.A. (1982). Two DNA glycosylases in *Escherichia coli* which release primarily 3-methyladenine. Biochem. **21**:1162-1169.

Valerie, K., Henderson, E.E. and de Riel, J.K. (1985). Identification, physical map location and sequence of the *denV* gene from bacteriophage T4. NAR **12**:8085-8096.

Varshney, U., Hutcheon, T. and van de Sande, J.H. (1988). Sequence analysis, expression, and conservation of *Escherichia coli* uracil-DNA glycosylase and its gene (*ung*). **263**:7776-7784.

von Sonntag, C. (1987). In: The chemical basis of radiation biology. London: Taylor and Francis.

Walker, L.J., Robson, C.N., Black, E., Gillespie D. and Hickson, I.D. (1993). Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. Mol. Cell. Biol. **13**:5370-5376.

Walker, L.J., Craig, R.B., Harris, A.L. and Hickson, I.D. (1994). A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress. Nucl. Acids Res. **22**:4884-4889.

Wallace, S.S. (1988). AP endonucleases and DNA glycosylases that recognize oxidative DNA damage (Review). Envir. Mol. Mut. **12**:431-477.

Warner, H.R. and Price, A.R. (1989). Involvement of DNA repair in cancer and aging. J. Geron.: Biol. Sci. **44**:45-54.

Washington, W.J., Foote, R.S., Dunn, W.C., Generoso, W.M. and Mitra, S. (1989). Age-dependent modulation of tissue-specific repair activity for 3-methyladenine and O6-methylguanine in DNA in inbred mice. Mech. Age. Develop. **48**:43-52.

Weirich-Schwaiger, H., Weirich, H.G., Gruber, B., Schweiger, M. and Hirsch-Kauffmann, M. (1994). Correlation between senescence and DNA repair in cells from young and old individuals and in premature aging syndromes. Mut. Res. **316**:37-48.

Weiss, B. (1981). Exodeoxyribonucleases of *Escherichia coli*, in: The Enzymes, P.D. Boyer (ed), Academic Press, New York, pp. 203-231.

Weiss, B. and Grossman, L. (1987). Phosphodiesterases involved in DNA repair. Adv. Enzymol. **60**:1-34.

Weng, Y. and Sirover, M.A. (1993). Developmental regulation of the base excision repair enzyme uracil DNA glycosylase in the rat. Mut. Res. **293**:133-141.

Wheater, P.R., Burkitt, H.G. and Daniels, V.G. Functional Histology. 2nd ed. New York:Churchill Livingstone. 1987.

Wilson, T.M., Rivkees, S.A., Deutsch, W.A. and Kelley, M.R. (1995). Differential expression of the apurinic/apyrimidinic (APE/ref-1) muntifunctional DNA base excision repair gene during fetal development and in adult rat brain and testis. Mut. Res. In press.

Wilson, T.M., Ewel, A., Duguid, J.R., Eble, J.N., Lescoe, M.K., Fishel, R. and Kelley, M.R. (1995). Differential ecllular expression of the human MSH2 repair enzyme in small and large intestine. Cancer Res. In press.

Wilson, T.M., Carney, J.P. and Kelley, M.R. (1994). Cloning of the multifunctional rat apurinic/apyrimidinic endonuclease (rAPEN)/redox factor from an immature T cell line. NAR **22**:530-531.

Wilson III, D.M., Deutsch, W.A. and Kelley, M.R. (1994). *Drosophila* ribosomal protein S3 contains an activity that cleaves DNA at apurinic/apyrimidinic sites. J. Biol. Chem. **269**:25359-25364.

Wink, D.A., Kasprzak, D.S., Maragos, C.M., Elespuru, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrews, A.W., Allen, J.S. and Keefer, L.K. (1991). Science **254**:1001-1003.

Wittwer, C.U., Bauw, G. and Krokan, H.E. (1989). Purification and determination of the NH_2-terminal amino acid sequence of uracil-DNA glycosylase from human placenta. Biochem. **28**:780-784.

Worrad, D.M. and Caradonna, S. (1988). Identification of the coding sequence for herpes simplex virus uracil-DNA glycosylase. J. Virol. **12**:4774-4777.

Xanthoudakis, S. and Curran, T. (1992a). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J. **11**:653-665.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y.C. and Curran, T. (1992b). Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. EMBO J. **11**:3323-3335.

Xanthoudakis, S., Miao, G.G. and Curran, T. (1994). The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. Proc. Natl. Acad. Sci. **91**:23-27.

Yacoub, A., Kelley, M.R., Doetsch. P. W., Augeri, L. and Deutsch, W.A. (1995) *Drosophila* ribosomal protein S3 contains 8-oxoquanine and AP lyase DNA repair activities. Submitted.

Yao, K.S., Xanthoudakis, S., Curran, T. and O'Dweyer, P.J. (1994). Activation of AP-1 and nuclear redox factor, Ref-1, in response of HT29 colon cancer cells to hypoxia. Mol. Cell. Biol. **14**:5997-6003.

Zaidi, N.H., Potten, C.S., Marqison, G.P., Cooper, D.P. and O'Connor, P.J. (1993). Tissue and cell specific methylation, repair and synthesis of DNA in the upper gastrointestinal tract of Wistar rats treated with single doses of N-methyl-N^1-nitro-N-nitrosoquanidine. Carcinogenesis **14**:1981-1990.

Zhou, W. and Doetsch, P.W. (1993). Transcription bypass or blockage at single-strand breaks on the DNA template strand: effect of different 3′ and 5′ flanking groups on the T7 RNA polymerase elongation complex. Biochem. **33**:14926-14934.

Zucconi, G.G., Carcereri de Prati, A., Menegazzi, M., Cosi, C. and Suzuki, H. (1992). DNA repair enzymes in the brain. DNA polymerase beta and poly (ADP-ribose) polymerase. Annals NY Acad. Sci. **663**:432-435.

VITA

The author, Teresa M. Wilson, was born in Peoria, Illinois on December 2, 1966 to Marion and Yoko Wilson.

In December, 1990, Mrs. Wilson received a Bachelor of Arts in Microbiology from Southern Illinois University in Carbondale, Illinois. The following year, she enrolled in the Program in Molecular Biology at Loyola University Chicago, Maywood, Illinois and joined the laboratory of Mark R. Kelley, Ph.D., in the Spring of 1992. In 1993 she moved with her advisor, Dr. Mark R. Kelley, to Indiana University Medical Center, where she performed all her studies on the expression of a mammalian DNA repair protein, apurinic/apyrimidinic endonuclease.

Mrs. Wilson has accepted a position as a post-doctoral fellow in the laboratory of Richard A. Fishel, Ph.D, at the Thomas Jefferson Cancer Institute, Philadelphia, Pennsylvania.

The dissertation submitted by Teresa M. Wilson has been read and approved by the following committee:

Mark R. Kelley, Ph.D., Director
Associate Director, Wells Center for Pediatric Research
Associate Professor, Departments of Pediatrics and Biochemistry and Molecular Biology
Indiana University School of Medicine

Nicholas Emanuele, M.D.
Director, Division of Endocrinology and Metabolism
Professor, Department of Medicine
Loyola University Chicago

Leonard C. Erickson, Ph.D.
Assistant Director, Loyola University Cancer Research Center
Director, Program in Molecular Biology
Professor, Departments of Medicine and Pharmacology
Division of Hematology/Oncology
Loyola University Chicago

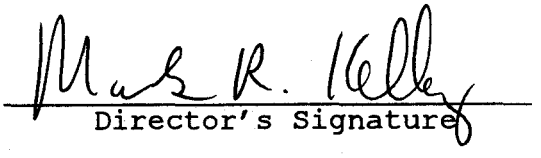
Hans-Martin Jäck, Ph.D.
Assistant Professor, Department of Microbiology and Immunology
Loyola University Chicago

Russell Pieper, Ph.D.
Assistant Professor, Department of Medicine
Division of Hematology/Oncology
Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Nov. 17, 1995
Date


Director's Signature