The Toxicity and Mutagenicity of the Aflatoxin B_1 Formamidopyrimidine DNA Adduct

by

Maryann E. Smela

B.S. Biology, Minor in Chemistry
Massachusetts Institute of Technology, 1996

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Signature of Author:_____
Maryann E. Smela
Division of Bioengineering and Environmental Health
February 22, 2002

Certified by:____
John M. Essigmann
Professor of Chemistry and Toxicology
Thesis Supervisor

Accepted by:____
Ram Sasisekharan
Associate Professor of Bioengineering and Environmental Health
Chairman Graduate Student Committee

Thesis Committee

Gerald N. Wogan_____
Professor of Chemistry and Toxicology
Chairman, Thesis Committee

Steven R. Tannenbaum_____
Professor of Chemistry and Toxicology
Committee Member

Bevin P. Engelward_____
Assistant Professor of Toxicology
Committee Member

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ABSTRACT

Aflatoxin B_1 (AFB_1) is a fungal metabolite that contaminates the food supply in certain areas of the world. It is produced by *Aspergillus flavus* and related fungi that grow on improperly stored foods such as corn, rice, and peanuts. Epidemiological studies have shown a correlation between exposure to AFB_1 and incidence of hepatocellular carcinoma (HCC). Mutations in p53 are observed in over 50% of the HCC samples studied, and a unique mutational hotspot occurs at the third position of codon 249 in this gene, yielding almost exclusively GC to TA transversions. It is of interest to evaluate the mutagenic properties of specific chemical structures of AFB_1 adducts in order to determine which of these may be responsible for the mutations that may play a role in the formation of HCC. The primary DNA adduct formed by the epoxide of AFB_1 is the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua) adduct, which can lead to two secondary lesions, an apurinic site or a ring opened formamidopyrimidine (FAPY) adduct, which itself has two rotameric forms.

This study focuses on of the determination of how well cells tolerate each of the AFB_1-FAPY rotamers and of the type and frequency of mutations caused by the persistent AFB_1-FAPY adduct in a site specifically modified M13 viral vector transfected into *E. coli*. Four major results were concluded from this work. First, one of the rotamers of AFB_1-FAPY is a strong block to DNA replication, even when bypass polymerases are employed by the cell. Second, the G to T mutation frequency of the AFB_1-FAPY adduct is at least six fold greater than that observed for the AFB_1-N7-Gua adduct. Third, a spectrum of mutations that is unique to the AFB_1-FAPY adduct was observed. Fourth, cell strains expressing different bypass polymerases responded differently when challenged with the AFB_1-FAPY and AFB_1-N7-Gua DNA adducts. These results show that AFB_1-FAPY is the most toxic and mutagenic species of aflatoxin adduct studied to date.

Thesis Supervisor: John M. Essigmann
Title: Professor of Chemistry and Toxicology

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AAF	2-acetylaminofluorene
AF	2-aminofluorene
AFB_1	aflatoxin B_1
AFB_1-FAPY	aflatoxin B_1-formamidopyrimidine
AFB_1-N7-Gua	8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1
AP	apurinic
Arg	arginine
ATP	adenosine triphosphate
B(a)P	benzo(a)pyrene
BER	base excision repair
CYP	cytochrome P_{450}
dAMP	deoxyadenosine monophosphate
DHVB	duck hepadnavirus B
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dRpase	deoxyribose phosphatase
E. coli	*Escherischia coli*
Exo III	exonuclease III
Gln	glutamine
HBV	hepatitis B virus
HBsAg	hepatitis B virus surface antigen
HBx	hepatitis B x protein
HCC	hepatocellular carcinoma
HPLC	high pressure liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight

NER	nucleotide excision repair
NMR	nuclear magnetic resonance
Oltipraz	4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PNA	peptide nucleic acid
PNK	polynucleotide kinase
Pol	polymerase
PROBE	primer-oligonucleotide base extension
RNA	ribonucleic acid
RR	relative reactivity
RRF	restriction resistant fraction
S. typhimurium	*Salmonella typhimurium*
Ser	serine
T_m	melting temperature
tRNA	transfer RNA
Trp	tryptophan
UDG	uracil DNA glycosylase
UV	ultraviolet
WHVB	woodchuck hepadnavirus B
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XP	xeroderma pigmentosum

Chapter 1

Literature Survey

Aflatoxins

Many fungal species are capable of producing mycotoxins, apparently as a self-defense mechanism. The species *Aspergillus flavus* produces a class of toxins called aflatoxins (Stoloff et al., 1977). These consist of aflatoxins B_1, B_2, G_1, and G_2, which differ slightly in chemical structure (Asao et al., 1963). They can be metabolized *in vivo* to form aflatoxins B_{2a}, M_1, M_2, P_1 and Q_1, as well as aflatoxicol and aflatoxicols M_1 and H_1, and aflatoxin B_1 dihydrodiol (Busby, Jr. and Wogan, 1984).

Aflatoxin B_1 Epidemiology

Aflatoxin B_1 (AFB_1) (Figure 1.1) first attracted attention when it was discovered to be the agent responsible for a multitude of livestock deaths in 1960 (Goldblatt, 1969). It was found to be a contaminant in animal feed, causing severe problems for agriculture. Concern arose over this contaminant in human food supplies, since supplements given to undernourished children were found to be tainted with this toxin (Stoloff et al., 1977). It was soon discovered that not only was this chemical toxic, but it was also carcinogenic (Busby, Jr. and Wogan, 1984) and mutagenic (Rogers and Newberne, 1967; Ong, 1975). Early epidemiological evidence demonstrated a correlation between exposure to AFB_1 and incidence of liver cancer (Campbell and Salamat, 1971; Peers et al., 1976; Peers and Linsell, 1973; Shank et al., 1972a; Shank et al., 1972b; Van Rensburg et al., 1974). Hepatitis B virus (HBV) is also common in areas of the world where there is high exposure to AFB_1, and it was discovered that these two factors have a synergistic effect upon the formation of hepatocellular carcinoma (HCC). In over 50% of HCC cases studied in these areas, a characteristic G to T mutation is observed at the third position of

codon 249 of the p53 tumor suppressor gene (Hsu et al., 1991; Bressac et al., 1991). It remains to be determined whether this specific sequence is an exceptional target for mutations caused by AFB_1, or if the mutation is selected for expansion once it occurs. However, each of these scenarios shares the fundamental first step of generating a G to T mutation.

To date, over 2000 HCC samples from all over the world have been examined for the target mutation described above (Shen and Ong, 1996; Katiyar et al., 2000; Kirk et al., 2000; Yang et al., 1997; Rashid et al., 1999; Shimizu et al., 1999; Vautier et al., 1999; Boix-Ferrero et al., 1999; Lunn et al., 1997). In regions where exposure to aflatoxin is high - namely Quidong and Tongan (China), India, Southern Africa, The Gambia, Mozambique, and Senegal - 116 out of 262 (44%) of the total HCC cases examined show a predominance of G to T mutations at the third position of codon 249 of the p53 gene (Katiyar et al., 2000; Kirk et al., 2000; Vautier et al., 1999; Yang et al., 1997; Rashid et al., 1999; Shimizu et al., 1999). In contrast, in regions of low exposure to aflatoxin, namely, Australia, Europe, Japan, and the USA, only 17 out of 1273 (1%) of the HCC samples examined had mutations at this site (Katiyar et al., 2000; Vautier et al., 1999; Boix-Ferrero et al., 1999; Shimizu et al., 1999). In regions where exposure to aflatoxin is moderate - namely, Beijing, Shanghai, Xian, Hong Kong, Singapore, South Korea, Taiwan, Thailand, Vietnam, Southern Asia, South Africa and Egypt, 40 out of 568 (7%) HCC cases examined had mutations at the third position of codon 249 of the p53 gene (Shen and Ong, 1996; Rashid et al., 1999; Lunn et al., 1997; Lunn et al., 1999). This p53 mutation appears to be unique to AFB_1-induced liver tumors, as tumors presumably induced by other factors fail to consistently show this genetic change.

The correlation between exposure to aflatoxin and the hotspot at codon 249 suggests two possibilities. First, aflatoxin may be particularly reactive with this nucleotide owing to its surrounding sequence. Data obtained *in culture* suggest that this may not be the case, since adducts form at other positions in the p53 gene as well (Denissenko et al., 1998). Second, it is possible that this mutation is somehow due to the combined effects of AFB_1 exposure and HBV infection, either directly or through separate pathways, since this mutation is observed much more often in areas of the world where exposure to both agents is very common. In both cases, if mutations in codon 249 significantly debilitate the function of p53, the loss of function of this protein should give cells a selective growth advantage, since p53 is an important tumor suppressor gene. The p53 protein product is a transcriptional activator that has been shown to be involved in cell cycle regulation (el Deiry et al., 1993), play a role in the apoptosis pathway (Miyashita and Reed, 1995; Graeber et al., 1994; Linke et al., 1996; Schwartz and Rotter, 1998; Choisy-Rossi et al., 1999), and be involved with DNA repair (Schwartz and Rotter, 1998; Janus et al., 1999; Oren, 1999; Amundson et al., 1998). AFB_1 and HBV may act in concert, or they may induce completely different pathways, the combined effects of which lead to the selection of the hallmark p53 mutation and the ultimate endpoint of HCC.

p53 and HBV

HBV infection has been demonstrated to be associated with increased incidence of HCC. In general, a person with HBV is seven times more likely to develop liver cancer, regardless of their level of exposure to AFB_1 (Shen and Ong, 1996). Thus, the association of HBV infection

and HCC formation has been the focus of many studies. Several groups have shown that one of the gene products of HBV, the hepatitis B x protein (HBx), binds to and inactivates the p53 protein (Truant et al., 1995; Wang et al., 1994). Although there is some controversy over this issue, experiments suggest that the binding of HBx to the C-terminal region of p53 inhibits p53 sequence specific DNA binding and thereby inhibits its role as a transcriptional activator (Wang et al., 1994). The same group has also demonstrated that expression of HBx may inhibit p53-induced apoptosis (Buchhop et al., 1997). In contrast, another group has shown that p53, in cells exposed to HBV, can still moderately activate some genes *in vivo* (Puisieux et al., 1995). Other work, while failing to observe a direct interaction between p53 and HBx (Terradillos et al., 1998; Su et al., 2000; Schaefer et al., 1998; Lee and Rho, 2000) has determined an alteration in either the localization, the phosphorylation status, or the transcription of the wild type p53 during HBx expression. Yet others have probed the total cellular RNA in liver cell lines expressing HBx, searching for genes that are up- or down-regulated, and no effect was observed on p53 RNA (Han et al., 2000). An increase in the number of hallmark codon 249 mutations has been observed when a cell line that expresses the HBx protein is exposed to increasing levels of AFB_1 (Sohn et al., 2000). Perhaps it is not the direct association of HBx with p53, but some other effect that HBx has on the cell, such as an inhibition of apoptosis in p53 mutant cells, that promotes mutant selection.

Experiments have examined the ability of different p53 mutants to modulate *in trans* the transcriptional activity of wild-type p53 (p53 acts functionally as a tetramer) on a reporter construct in HCC cell lines (HEP-3B) possessing an integrated HBx gene (Forrester et al., 1995).

It has not been confirmed, however, that these cells actually do express the HBx protein. The p53 mutants tested included Arg to Trp at codon 248 and Arg to Ser at codon 249. The codon 248 mutant enhanced the level of transcription to 132% of wild-type, while the codon 249 mutant, which can result from a G to T mutation at the third position of this codon, drastically reduced the level of transcription to 18% of wild-type. Control experiments were carried out in tumor cell lines from lung, prostate, mesothelial, and mammary epithelial tissues, all of which do not express HBx. The codon 249 mutant exhibited a similar effect in lung (20% of wild-type), and a milder effect in prostate (80% of wild-type), mesothelial (52% of wild-type), and mammary (75% of wild-type) tissues. When any of the mutants are expressed in a cell line that does not contain wild-type p53, no transcriptional activation of the reporter construct is seen. In another study the presence of HBx has been demonstrated to promote apoptosis following DNA damage in a cell line containing wild type p53, while it promotes a growth advantage in a cell line containing AFB_1-induced mutant p53 (Sohn et al., 2000).

AFB_1, p53, and HBV

Although exposure to HBV increases the risk of HCC, the risk is even higher in an individual who is both positive for HBV and is exposed to aflatoxin (Shen and Ong, 1996; Chen et al., 1996; Ross et al., 1992). One study indicates that patients with positive urinary AFB_1 antigen are three times more likely to develop HCC, patients with positive HBV surface antigen (HBsAg) are about seven times more likely to develop HCC, and when both AFB_1 and HBsAg are present, patients are sixty times more likely to develop this disease (Ross et al., 1992). Thus,

there seems to be a synergism between HBV and AFB_1 exposure for the development of HCC. Interestingly, a relationship has been observed between the presence of HBsAg and the mutational hotspot at codon 249 of p53 (Katiyar et al., 2000; Kirk et al., 2000; Shen and Ong, 1996). Twelve percent of the 2019 HCC cases examined for the mutational hot spot at codon 249 were examined for the presence of HBsAg. In regions of high aflatoxin exposure, 47% (82 out of 175 total) of HBV positive HCC samples examined had a G to T transversion at position three of codon 249 of p53. About 42% (18 out of 43) of the HBV negative HCC samples from these regions had the same mutation. For the samples taken from regions of moderate aflatoxin exposure, only 35% (15 out of 43 total) of the HBsAg positive samples had the G to T transversion. None of the 11 HBsAg negative samples in these regions exhibited the mutation. Other work (Su et al., 2000) has demonstrated that in areas of low aflatoxin exposure, HCC samples from people who are positive for HBsAg also have p53 mutations, although they have not yet determined what these mutations are. These studies help establish the relationship between this hotspot mutation in tumors and HBV infection.

AFB_1 Metabolism

The types and positions of the mutations induced by AFB_1 may differ among species due to a difference in AFB_1 activation (Mace et al., 1997). Human SV40 immortalized cell lines expressing different human cytochrome P_{450} (CYP) enzymes (CYP1A2, CYP2A6, CYP2B6, or CYP3A4) gave rise to different mutational spectra, regarding the proportions of transitions and transversions at each position that was mutated in codons 249 and 250 of a globally modified p53 gene. Indeed, AFB_1 exposure itself alters expression of some genes, including those for

certain CYPs, which are involved in activation, and glutathione S-transferase, which is involved in detoxification, of AFB_1 (Harris et al., 1998). HBV infection is also thought to influence the regulation of such genes (Chomarat et al., 1998). From species to species, and even within the human population itself (Chi et al., 1998), the branch between the activities of the enzymes of AFB_1 activation and detoxification may explain the differences observed in response to the toxin (McGlynn et al., 1995).

Dietary factors can influence the activation or inhibition of different metabolic enzymes. Foods such as green tea and carotenoids and phytochemicals such as coumarin and indole-3-carbinol can activate enzymes that play a role in AFB_1 detoxification (Kelly et al., 2000; Gradelet et al., 1998; Qin et al., 2000). Phenolic compounds are also observed to have antimutagenic activity toward AFB_1. Additionally, several drugs have been shown to increase AFB_1 detoxifying enzymes, and these include butylated hydroxyanisole, diethyl maleate, ethoxyquin, β-naphthoflavone, phenobarbitol, and oltipraz (Kelly et al., 2000).

Chemoprevention of HCC

Oltipraz is a synthetic dithiolthione (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione) similar to the natural products found naturally in cruciferous vegetables (Figure 1.2). It was originally used as a treatment for schistosomiasis, and it was discovered to increase the detoxification potential of the host (Bueding et al., 1982). It was these data that led to the study of oltipraz as a chemopreventive agent. Studies *in vivo* and *in culture* have demonstrated an increase in the levels of Phase II detoxification enzymes, such as glutathione-S-transferases A1,

A2, and M1, and a decrease in Phase I activation enzymes, such as cytochrome P_{450}s 1A2 and 3A4 (Langouet et al., 1995a; Langouet et al., 1995b). Oltipraz has also been observed to directly inhibit CYP1A2 (Sofowora et al., 2001). It has been demonstrated as a possible chemopreventive drug in cancers such as those of the lung, breast, trachea, colon, bladder, pancreas, skin, and liver (Clapper, 1998). It is hypothesized to be particularly effective against AFB_1-induced liver cancer, since the enzymes it regulates are those that are involved with the activation and detoxification of AFB_1. Currently, clinical trials with oltipraz are underway in Qidong (China) that have shown a 51% decrease in AFB_1 metabolites in treated patients. Further studies will elucidate whether this drug plays a role in reducing HCC incidence (Wang et al., 1999). In addition oltipraz has been shown to inhibit HBV transcription and to up-regulate the expression of wild type p53 in cell culture (Chi et al., 1998).

Animal Models for HCC

p53 is at least 92% homologous across the species listed in Table 1 (Duflot et al., 1994). Codons 248 and 249 in the animal models discussed here are analogous to human codons 248 and 249, and they are believed to be within the DNA binding regions of these proteins. The G to T hotspot mutation at the codon analogous to the human codon 249 in the p53 genes of other species has not been identified. To date, all attempts to recreate this hotspot by exposing animals to aflatoxin have not been successful. However, it is possible that this failure to generate the hotspot is due solely to the differences among the p53 sequences of different species (Table 1). A different sequence context may alter the reactivity of AFB_1 toward the analogous codon 249 sequence. Table 1 illustrates, from a compilation of data, that there is no mutation analogous to

the human p53 codon 249 mutation (Arg to Ser) in primates, rats, mice, ducks, woodchucks, tree shrews, or ground squirrels when these animals are exposed to aflatoxin. A possible reason that no analogous mutation has been found may be that, at least in woodchucks, tree shrews (opposite strand mutation), and ground squirrels a G to T transversion in the third position of the codon results in a silent mutation. Additionally, some of the species tested do not have a species-specific form of hepatitis B. The HBV status of several species studied is also illustrated in Table 1.

If HBV and AFB_1 are both required for the selection of a p53-inactivating mutation at the third position of codon 249, it is important for an animal model to be able both to contract HBV mediated disease and to have a similar p53 codon sequence in the DNA binding region. Similar to the woodchuck and ground squirrel models noted above, ducks contract a form of hepatitis B, duck hepadnavirus B (DHVB), but the predominant mutation in ducks at codon 249 is Arg to Leu, which may not be as detrimental as Arg to Ser for p53 activity (Duflot et al., 1994). In the case of primates and rats, even though the mutation at codon 249 would give rise to an amino acid change of Arg to Ser, studies were carried out in the absence of HBV (Fujimoto et al., 1992; Hulla et al., 1993). Currently, rats transgenic for HBV are being developed (Gong and Almasan, 1999). Mice naturally have an Arg to Leu amino acid change in codon 249; we note, however, that mice have been engineered to have an Arg to Ser mutation at codon 246, which is analogous to the human Arg to Ser mutation at codon 249 (**AG<u>G</u>C** to **AG<u>T</u>C**) (Ghebranious and Sell, 1998). One study has been conducted where mice either with or without the codon 246 mutation were simultaneously exposed to AFB_1 and were positive for the HBsAg. This mutation causes

an increase in high-grade liver tumor formation in the presence of AFB_1 (from 0% to 14% when homozygous for wild type p53 and 14% to 71% when heterozygous for wild type p53), and even more so in the presence of both AFB_1 and HBsAg (from 62% to 100% when homozygous for wild type p53). Again, in these studies, it has not been proven that HBx is expressed in these animals.

In animal models it is important for the species-specific form of hepadnavirus to have all the characteristics of human HBV, including the HBx protein, and this protein should be at least similar to that found in human HBV. It is known that DHVB does not have the HBx protein. Different species differ in their ability to metabolize and repair AFB_1 (Monroe and Eaton, 1988; Ramsdell and Eaton, 1990; Borroz et al., 1991; Buetler et al., 1992; Yanagimoto et al., 1997; Rivkina et al., 1994; Tokusashi et al., 1994; Toshkov et al., 1990; Transy et al., 1994; Gemechu-Hatewu et al., 1997; Sotomayor et al., 1999; Fields et al., 1999). For example, mice have different proportions of metabolizing and protective enzymes, so most of the aflatoxin they ingest is inactivated and excreted before it can form DNA adducts (Monroe and Eaton, 1988; Ramsdell and Eaton, 1990; Borroz et al., 1991; Buetler et al., 1992). On the other hand, woodchucks show an increase in conversion of AFB_1 to the epoxide when they are exposed to woodchuck hepadnavirus B (WHVB) (Gemechu-Hatewu et al., 1997). Recently, the tree shrew has been proposed a useful animal model that can be infected with human HBV (Yan et al., 1996; Park et al., 2000). This animal model shows the same synergism that humans do when they are infected with HBV and exposed to AFB_1. Of four tumors from animals that were positive for both AFB_1 and HBV exposure, no codon 249 mutations have been observed. Three

of the tumors have mutations elsewhere in p53, and only one of these is in the DNA binding domain of the protein (Park et al., 2000). This result implies there may be some other factor, such as a co-carcinogen or phenotypic trait, that may play a role specifically in the development of human HCC and may somehow lead to the selection of the codon 249 mutation. Further studies should help elucidate the mechanisms of known contributors to HCC and suggest other possible factors in the etiology of this disease.

Sequence Specificity of AFB_1 Binding and Mutational Hotspots

The reactivity of aflatoxin toward different DNA sequences has been studied by a number of groups (Yu et al., 1990; Refolo et al., 1985; D'Andrea and Haseltine, 1978; Denissenko et al., 1998; Denissenko et al., 1999). One systematic study (Benasutti et al., 1988) illustrates that the reactivity of AFB_1 toward a guanine residue is highly dependent on the bases immediately 5' and 3' to the central guanine. A run of 3 guanines is the most reactive sequence overall. In the 5' position the reactivity profile is G (1.0) > C (0.8) > A (0.4) > T (0.3), and in the 3' position it is G (1.0) > T (0.8) > C (0.3) > A (0.2). (A numerical value for relative reactivity is given in parentheses. These values were calculated such that the 3' value can be multiplied by the 5' value to yield a number that can be used to compare the reactivities of the different bases flanking a central G. Herein, these values will be denoted 'relative reactivity', RR). The sequence context-specific reactivity for AFB_1 holds true for DNA globally modified by AFB_1 (Benasutti et al., 1988; Misra et al., 1983; Marien et al., 1987; D'Andrea and Haseltine, 1978). The sequence-specificity for AFB_1 reactivity has been divided into three classes, and these are

referred to in the literature as "weak (RR ~0.23)", "intermediate (RR ~0.44)", or "strong (RR ~0.5)" sites for AFB_1 reactivity. Not only can the sequence context affect the reactivity of AFB_1, but it has also been suggested that a pre-existing modification at a neighboring base can do so as well. Several reports in the literature demonstrate that AFB_1 adduct formation, as well as several other types of DNA damage, is modulated by the presence of 5-methyl cytosine at CpG sites (Chen et al., 1998; Denissenko et al., 1997; Mathison et al., 1993; Tommasi et al., 1997; Tang et al., 1999). One contradictory report, however, states that cytosine methylation has no influence over aflatoxin reactivity (Ross et al., 1999).

As indicated earlier, the predominant mutational hotspot found in human HCC is a G to T transversion in the third position of codon 249 of the p53 gene (**AGG**C: codon 249 in bold, targeted G underlined, 3' base of target G in normal type). The RR value for this context is 0.3, which falls somewhere between weak and intermediate reactivity toward AFB_1. This mutation results in an Arg to Ser alteration in the p53 protein. Evidence is provided that indicates that AFB_1 reacts with the third position of codon 249 of p53 *in vitro* (Puisieux et al., 1991). Position two of codon 249 (RR = 0.4) was not reactive towards AFB_1, even though Benasutti's rules predict it to be 1.3 times more reactive than the third base. This study also demonstrates that AFB_1 reacts with 20% of the bases in exons 5-8 of the p53 gene, ~85% of which are guanines that are in several different sequence contexts. Another group has also observed that significant adduct formation occurred in several codons in exons 7 and 8 of the p53 gene, as well as at the third position of codon 249 (Denissenko et al., 1999). In yet another study (Aguilar et al., 1993), G to T and C to A transversions were observed at positions adjacent to the hallmark mutation site

at codon 249 of p53 (AGG to ATG and **AGGC** to **AGGA**), demonstrating that in different systems, reactivity toward AFB_1 and the correlation between reactivity and mutation may be different. The most relevant model to study would be *in vivo* mammalian systems.

Providing further support for the view that AFB_1 binds to and causes mutations in certain sequence contexts, data illustrate that four out of seven of the mutational hotspots from human *xeroderma pigmentosum* (XP) cells, observed in the *supF* gene of the pS189 shuttle vector, are at AGG sequences (Levy et al., 1992). Three of these hotspots are located at the third G [the 3' base was either a G (RR = 1.0), a T (RR = 0.8), or an A (RR = 0.2)]. One AGG sequence contained hotspots at both the second (RR = 0.4) and third G. It is not clear whether mutations at both of the guanine positions are due to aflatoxin binding at one site or at two different sites. Using a polymerase arrest assay, this study showed that there was more blockage at the third G than at the second G of the AGG sequence. This indicates that although the sites that are hotspots for predominantly G to T mutations are intermediate or strongly reactive towards AFB_1, not all of these sites incurred the level of mutations consistent with their reactivity, and some sequences that should only be weakly reactive are actually hotspots for mutation. These data are consistent with other studies, whereby different systems were utilized for the analysis of AFB_1-induced mutations (Denissenko et al., 1999; Trottier et al., 1992; Prieto-Alamo et al., 1996; Sambamurti et al., 1988). This implies that another factor, such as DNA secondary structure, may play a role in AFB_1 modification and/or mutagenesis. As mentioned above, it is possible that an aflatoxin-modified guanine residue at the third position of the AGG sequence may give rise to mutations at the second position as well. This separation of mutation site from the site of

covalent modification may be due to distortion of the base 5' to the modified guanine as a consequence of intercalation of the AFB_1 moiety on the 5' face of that guanine (Bailey et al., 1996) (Figure 1.5).

In Vivo Models of Mutational Hotspots Induced by AFB_1

There is substantial evidence that the activation of cellular *ras* (c-*ras*) genes by specific single-base mutations may be an important step involved in the transformation of normal cells to malignant cells (Bos et al., 1987; Vogelstein et al., 1988; McMahon et al., 1986). This activation event is manifested as mutations in codon 12 of the c-Ki-*ras* gene in DNA in rat liver tumors (McMahon et al., 1990; Soman and Wogan, 1993). Table 2 illustrates the sequences of codons 11, 12, and 13 of rat, trout, and humans and the relative reactivity of each guanine to that in the third position of codon 249. The first and second positions of codon 12 incur mutations in rat, and the second positions of both codon 12 and 13 incur mutations in trout (Chang et al., 1991). In humans mutations have been observed at the first and second positions of codon 12 in the Ha-*ras* proto-oncogene (Riley et al., 1997). Interestingly, the c-Ki-*ras* sequence context (**AGG**A) for both mutations in rainbow trout is analogous to that of codon 249 of p53 (**AGG**C), except that the bases 3' to the mutated G are different (an A in the case of codon 12 and a T in the case of codon 13). These data and others suggest that the third position of the sequence AGG is a hotspot for AFB_1 mutation regardless of the 3' base (Levy et al., 1992). Rats and humans do not incur mutations in codon 13, possibly due to a base other than adenine 5' to the GG sequences. It is also possible that the different sequences form different DNA secondary structures, which may be more or less accessible to the DNA repair machinery.

In vivo studies conducted in the *lacI* gene in transgenic rats and mice examined sequences surrounding mutated guanines to determine if the mutations observed were sequence context dependent (Dycaico et al., 1996). Mutations were observed in six different sequence contexts in mice. In half of these, the targeted guanine is in the third position of a CGG context, with either a C (RR = 0.4), an A (RR = 0.3), or a T (RR = 0.8) in the 3' position. With regard to the empirical rules for aflatoxin reactivity in specific contexts, one out of the six sequences would be considered strong, two out of the six would be considered intermediate, and three out of the six contexts would be considered weak. Mutations were also observed in 25 different sequence contexts in rats, where 8 are predicted to have weak reactivity and the rest of which have intermediate to strong reactivity toward AFB_1. Of all the types of mutations observed, ~ 48% of those in mice and ~ 66% of those in rats were found at CpG sites. Only 17% of the sequences examined in mice and 4% of the sequences examined in rats did not have at least one G or C immediately adjacent to the mutated G. Sequences that have at least one G or C adjacent to a central G have an average RR of 0.44, while sequences without an adjacent G or C have an average RR of 0.18. In a separate study rats treated with AFB_1 in the presence or absence of the liver regeneration process were evaluated for p53 mutations (Lee et al., 1998). Only one mutation (20%) occurs at a CpG site. One out of five (20%) of the sequences examined does not have a G or a C adjacent to the mutated base (on two occasions, the mutated base was a C; these mutations could have been due the Gs in the opposite strand). In a third study, carried out in the human *HPRT* gene (Cariello et al., 1994), a hotspot for both base substitution and frameshift mutations occurs in a run of six guanines. These data once again show that although certain

sequence contexts exhibit weak reactivity towards AFB_1, they may still incur mutations. We note, however, that they also support Benasutti's rules for preferential AFB_1 binding to GC-rich regions.

In summary of the aforementioned studies, some of the mutational hotspots occurred in sequences that, according to Benasutti's empirical rules, are intermediate or strong spots for AFB_1 reactivity. We note that the atomic environment of some of the sequences may render certain sites more favorable for AFB_1 intercalation (Johnson and Guengerich, 1997). Most of these hotspots for reactivity and mutation are within sequences where a G is located 5' to the base involved in the mutation. The base 3' to the modified G appears to be associated with mutation less consistently and may not be as crucial as the 5' base. Taking a minimalist approach, one could say that all that is required for a mutational hotspot for AFB_1 is a GG sequence with the modification/mutation at the second G. In fact, some have described hotspots in just this way (Levy et al., 1992). It is noted, however, that the AFB_1-induced mutations in the aforementioned studies are not observed at every site, within the particular gene analyzed, where a strong or intermediate sequence for AFB_1 reactivity occurs. Further investigation of potential differences in the mechanism of AFB_1 reactivity toward different sequences, potential differences in repair of various AFB_1 modified sequences, and the influence of different DNA secondary structures on AFB_1 reactivity should shed further light on the correlation between hotspots for AFB_1 modification and AFB_1 mutations.

Repair of AFB_1

The characteristics of the mutational spectrum of aflatoxin are influenced by at least three factors: (i) the preference of one G over another for reaction, (ii) the preference for a base to be erroneously replicated more frequently in one context compared with another, and (iii) the preference for repair of an adduct in one context over others. One would expect that DNA repair deficient cells present an opportunity to examine the contributions of lesion formation and misreplication [points (i) and (ii)] separated from the complications of preferential repair [point (iii)]. Several mechanisms of DNA repair exist that may play a role in the repair of AFB_1 adducts. These repair systems include nucleotide excision repair (NER), base excision repair (BER), and recombination.

Nucleotide Excision Repair

NER systems exist in both prokaryotes and eukaryotes. Figure 1.3 illustates NER in bacteria, which involves the UvrABC endonuclease. The damaged DNA is recognized and nicked by the UvrABC complex several bases up- and downstream of the damage, and the oligonucleotide segment containing the damage is removed. DNA polymerase I (polI) is then recruited to fill in the gap (Friedberg et al., 2001). This mechanism is particularly suited for the repair of bulky adducts, since it recognizes the distortion in DNA caused by these adducts (Machius et al., 1999; Routledge et al., 1997). Early studies with AFB_1 indicated that cells deficient in one of the components of NER (*uvrA*) were sensitive to treatment with AFB_1 (Sarasin et al., 1977). Subsequently, DNA repair assays have demonstrated that AFB_1 adducts are repaired by NER *in vitro* (Oleykowski et al., 1993).

Humans have a similar mechanism for NER (Friedberg et al., 2001), and this system has been shown to play a role in repair of AFB_1-DNA adducts. Mutational spectra were studied in cells with the NER deficiency disorder, XP (Levy et al., 1992). All sequence contexts that should be strongly reactive toward AFB_1 may not incur mutations, since preferential repair of some sequences may be taking place. Surprisingly, most of the contexts that were hotspots in repair proficient cells were predicted to have only weak to intermediate reactivity toward AFB_1, whereas in XP cells, most of the hotspots occurred in intermediate to strong contexts. This result suggests that sequences that are more reactive to AFB_1 may also be more prone to undergo DNA repair, implying a role for secondary structure of the DNA or of the chromatin itself in these processes. It has also been observed that HBx can inhibit NER, either by binding directly to repair proteins or to the damaged DNA itself, making it possible that AFB_1 adducts persist preferentially in patients who have been exposed to both HBV and AFB_1 (Terradillos et al., 1998; Becker et al., 1998; Jia et al., 1999).

Base Excision Repair

BER is also a process that is conserved from bacteria to humans (Figure 1.4). This mechanism of DNA repair involves recognition and removal of only the damaged base by a DNA glycosylase, nicking of the DNA by an AP endonuclease, repair of the DNA backbone by dRpase, and filling in the resulting gap by DNA polymerase and ligase (Friedberg et al., 2001). BER assays (Chetsanga and Frenette, 1983) have been performed on AFB_1 adducts *in vitro*, demonstrating that this may also be a possible mechanism of repair for these types of adducts

(Chetsanga and Frenette, 1983). Additionally, XRCC1, an enzyme involved in base excision repair in humans, has been shown to play a role in AFB_1 adduct repair. This enzyme has several polymorphisms, and *in vivo* studies have been conducted that demonstrate that individuals with the *399 Gln* allele have increased levels of AFB_1 adducts (Lunn et al., 1999). This result implies a role for BER in removal of these adducts in humans as well.

Recombination

Homologous recombination (Figure 1.5) is a mechanism used by bacterial and mammalian cells not only as a mechanism of DNA repair, but also in normal cellular functions such as allelic conversion (Lodish et al., 1995) and antibody diversification (Leder, 1982). Early studies in which cells deficient in RecA were treated with AFB_1 demonstrate that these cells were sensitive to this toxin (Ueno and Kubota, 1976; Ichinotsubo et al., 1977). Not much was known about recombination at that time, however, and the sensitivity was attributed to one of the other known functions of RecA, particularly its involvement in regulation of NER gene expression. Recently, recombination has been proposed as a mechanism by which bacteria repair DNA damage such as that caused by cisplatin (Zdraveski et al., 2000). The Essigmann lab has carried out preliminary studies to test this hypothesis for AFB_1 as well. Additionally, studies have been conducted in yeast that demonstrate that AFB_1 may be repaired by a recombination-mediated mechanism (Sengstag et al., 1996; Sengstag, 1997).

Lesion Bypass of AFB_1

If cells are unable to repair AFB_1 adducts soon after they form, these adducts will be encountered by the replicative DNA polymerase, polymerase III (polIII). If polIII cannot bypass the lesion, other bypass polymerases may take over DNA replication temporarily. Once the lesion is bypassed, polIII resumes normal replication (Sutton et al., 2000). There are several bypass polymerases belonging to the Y-class of DNA polymerases that are present in all organisms from bacteria to man (Ohmori et al., 2001). In bacteria these include polymerase V (UmuDC and MucAB) and polymerase IV (DinB). DinB is analogous to human polκ (Masutani et al., 1999), and UmuDC (and MucAB) is analogous to human polη (Gerlach et al., 1999).

Escherischia coli (*E. coli*) can be induced for the SOS response upon exposure to UV light, which activates the error-prone UmuDC bypass polymerase (Figure 1.6) (Witkin, 1976; Friedberg et al., 2001; Sommer et al., 1993; Tang et al., 1999). MucAB, an analogous bypass polymerase isolated from *Salmonella typhimurium* (*S. typhimurium*), is regulated in a similar manner and has been demonstrated to induce higher mutation frequencies when challenged with ABF_1 and other forms of DNA damage (Blanco et al., 1982; Blanco et al., 1986; Kulaeva et al., 1995; Hauser et al., 1992; Perry et al., 1985; Woodgate and Sedgwick, 1992; Watanabe et al., 1994; Foster and Sullivan, 1988; Bennett et al., 1988; Lawrence et al., 1996; O'Grady et al., 2000).

Site Specific Analysis of Different Chemical Structures of AFB_1

Many studies have been conducted that evaluate the mutational spectrum observed in DNA and cells that have been globally modified with the reactive metabolite of AFB_1, the *exo*-8,9-epoxide or other electrophilic derivatives. As noted above the G to T mutation is predominantly observed in all of these studies, although they do not elucidate which specific chemical structure of AFB_1-DNA adduct may be responsible for causing these mutations (Foster et al., 1983; Basu and Essigmann, 1990; Hsu et al., 1991; Bressac et al., 1991; Benasutti et al., 1988; Denissenko et al., 1999; Kamiya et al., 1992; Levy et al., 1992; Puisieux et al., 1991; Bailey et al., 1996; Foster et al., 1988; Muench et al., 1983; Misra et al., 1983). The epoxide can react with DNA to form the primary AFB_1-DNA adduct, 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N7-Gua) (Essigmann et al., 1977; Martin and Garner, 1977; Lin et al., 1977; Groopman et al., 1981; Croy et al., 1978; Croy and Wogan, 1981a; Croy and Wogan, 1981b), which can then break down into two secondary lesions, the apurinic (AP) site or the AFB_1-formamidopyrimidine (FAPY) adduct (Busby, Jr. and Wogan, 1984) (Figure 1.7).

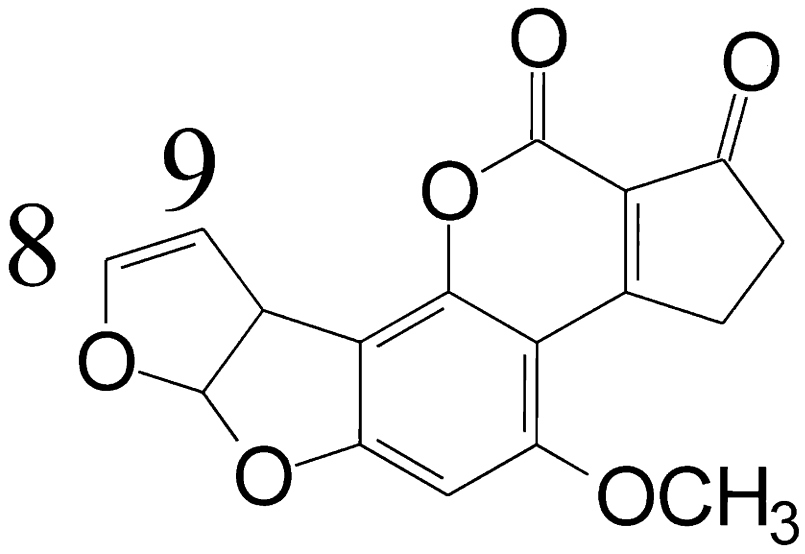
The AFB_1-N7-Gua adduct has been studied in a site-specific M13 viral system in *E. coli* (Bailey et al., 1996). This adduct primarily causes G to T mutations, although at a very low frequency (4%). An interesting property of this adduct is that a significant number of the total mutations observed (13%) occurred 5' to the site at which the adduct forms. In contrast, the AP site-containing genome gave rise only to mutations at the site of damage. The mutational asymmetry observed for AFB_1-N7-Gua is consistent with structural models indicating that the aflatoxin moiety of the adduct is intercalated on the 5' face of the guanine residue (Figure 1.8A)

(Gopalakrishnan et al., 1990). These results suggest a molecular mechanism that could explain an important step in the carcinogenicity of AFB_1. The observation that mutations occur at the 5' base suggests that mutational spectra, obtained in the studies discussed above, may harbor mutations at bases 5' to potentially modified guanines. Based on NMR data it is hypothesized that the bulkiness of the adduct interferes with the base being inserted opposite the base 5' of the lesion upon replication (Johnston and Stone, 1995) (Figure 1.8B). The AFB_1-FAPY adduct is similar in structure to the AFB_1-N7-Gua adduct. NMR data show that when adducted to DNA, both structures stabilize the duplex, AFB_1-N7-Gua by 3-5°C and AFB_1-FAPY by 15°C. While the AFB_1-N7-Gua adduct kinks the DNA slightly, the AFB_1-FAPY adduct unwinds the duplex without a significant effect on secondary structure (Figure 1.8C) (Mao et al., 1998; Johnston and Stone, 1995). These differences in DNA secondary structure may lead to differences in lethality, mutagenicity, and repair of the two chemical forms of the adduct.

AFB_1-FAPY

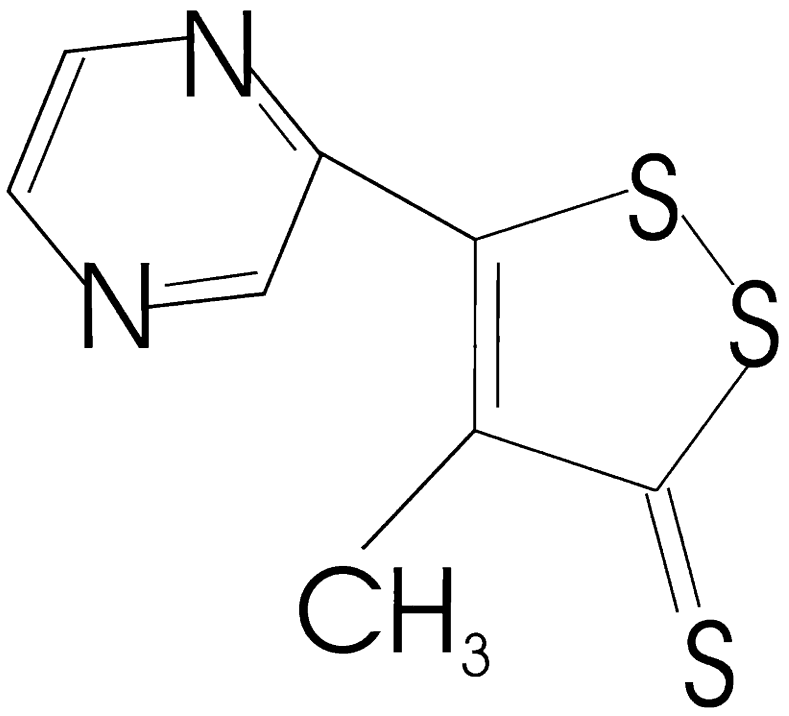
Formamidopyrimidines can be formed in DNA by any type of damage that occurs at the N7 position of guanine, such as methylation or electrophilic attack by agents like AFB_1 and mitomycin C. The FAPY residues formed by these damaging agents characteristically exhibit two or more species, in single nucleotides as well as in oligonucleotides, when evaluated by methods such as high pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) (Boiteux et al., 1984; Tomasz et al., 1987; Chetsanga and Makaroff, 1982; Chetsanga et al., 1982). The AFB_1-FAPY adduct is an equilibrium of two forms (FAPY mix) separable by HPLC, dubbed FAPY major and FAPY minor (FAPY major was formerly referred to as AFB_1-

FAPY, and minor was formerly referred to as an unidentified adduct, "Peak F" (Irvin and Wogan, 1985), or an unknown product (Groopman et al., 1981); additionally, the major and minor adducts could possibly be assigned to peaks IV and I, respectively, in (Lin et al., 1977)). It was later shown that the two species of AFB_1-FAPY were, in fact, isomers (Hertzog et al., 1982), and they were denoted as major and minor based on the predominance of one form over the other. NMR studies have revealed the structure of the FAPY major adduct in a DNA duplex (Mao et al., 1998). While the exact chemical structure of the FAPY minor form of the adduct remains elusive, several possible structures have been proposed, and these will be discussed throughout this work.



Aflatoxin B_1 (AFB_1)

Figure 1.1: Structure of aflatoxin B_1.



Oltipraz

Figure 1.2: Structure of Oltipraz. Oltipraz, (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione), has chemoprotective properties against AFB_1.

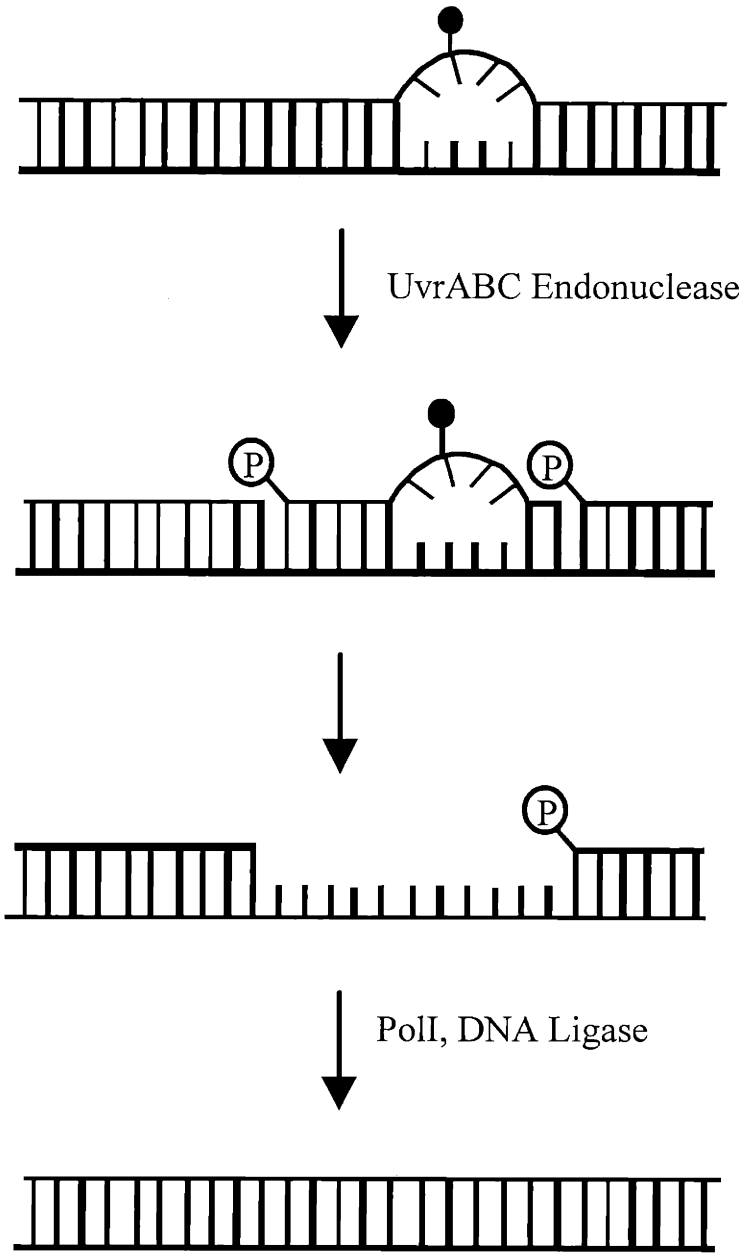


Figure 1.3: Nucleotide excision repair. DNA damage forms a structure that is recognized by the UvrABC endonuclease. Nicks are made several bases 5' and 3' to the lesion, and an oligonucleotide containing the damage is removed. PolI fills in the gap, and DNA ligase seals the nick.

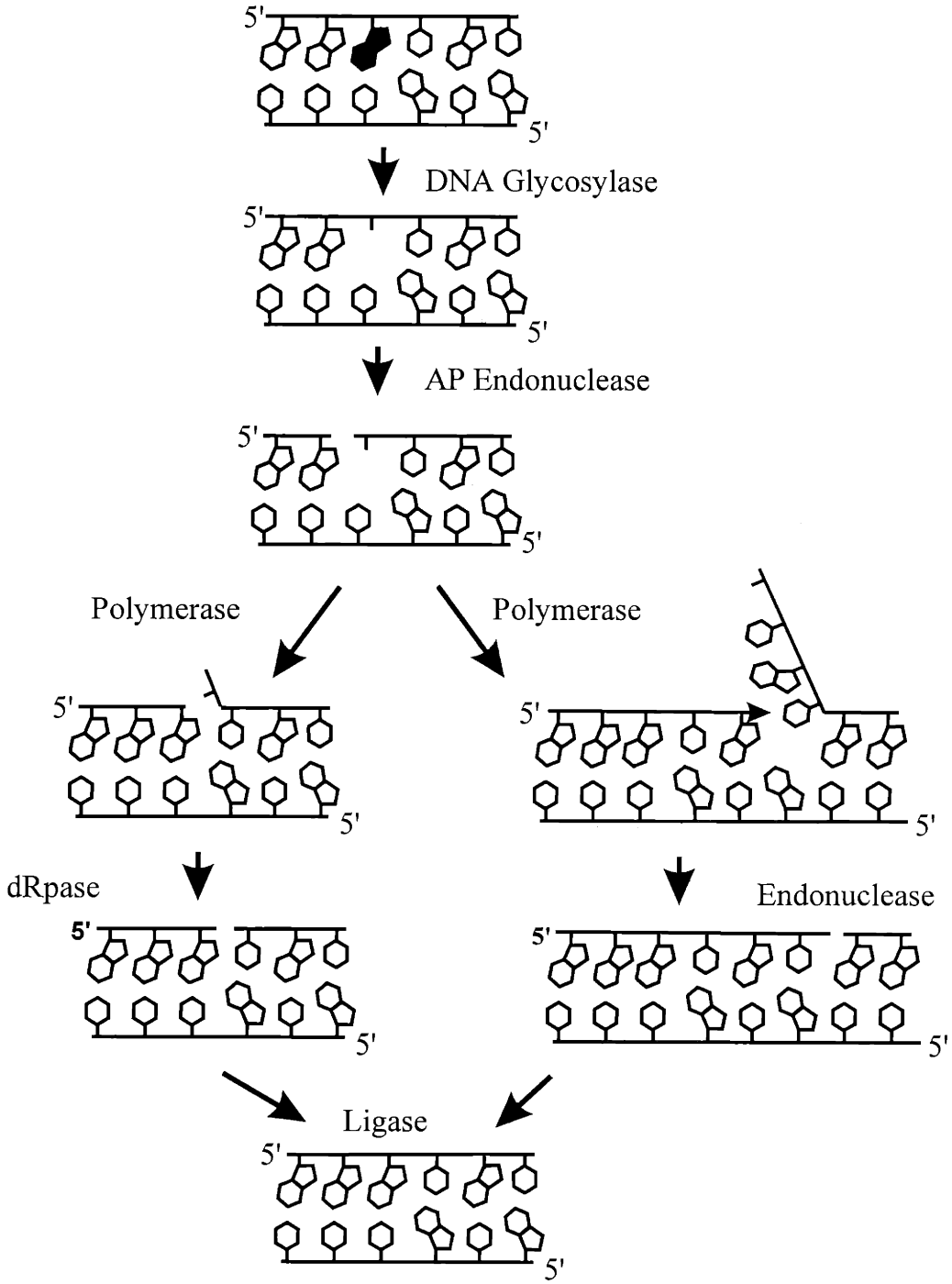


Figure 1.4: Base excision repair. A damaged base is removed from DNA by a glycosylase. AP endonuclease creates a nick in the phosphate backbone. Formation of a single stranded flap may or may not occur, and a polymerase fills in the resulting gap in DNA. An endonuclease or dRpase removes any extraneous bases, followed by ligation of the remaining nick. (Used with permission from Engelward, B.P.)

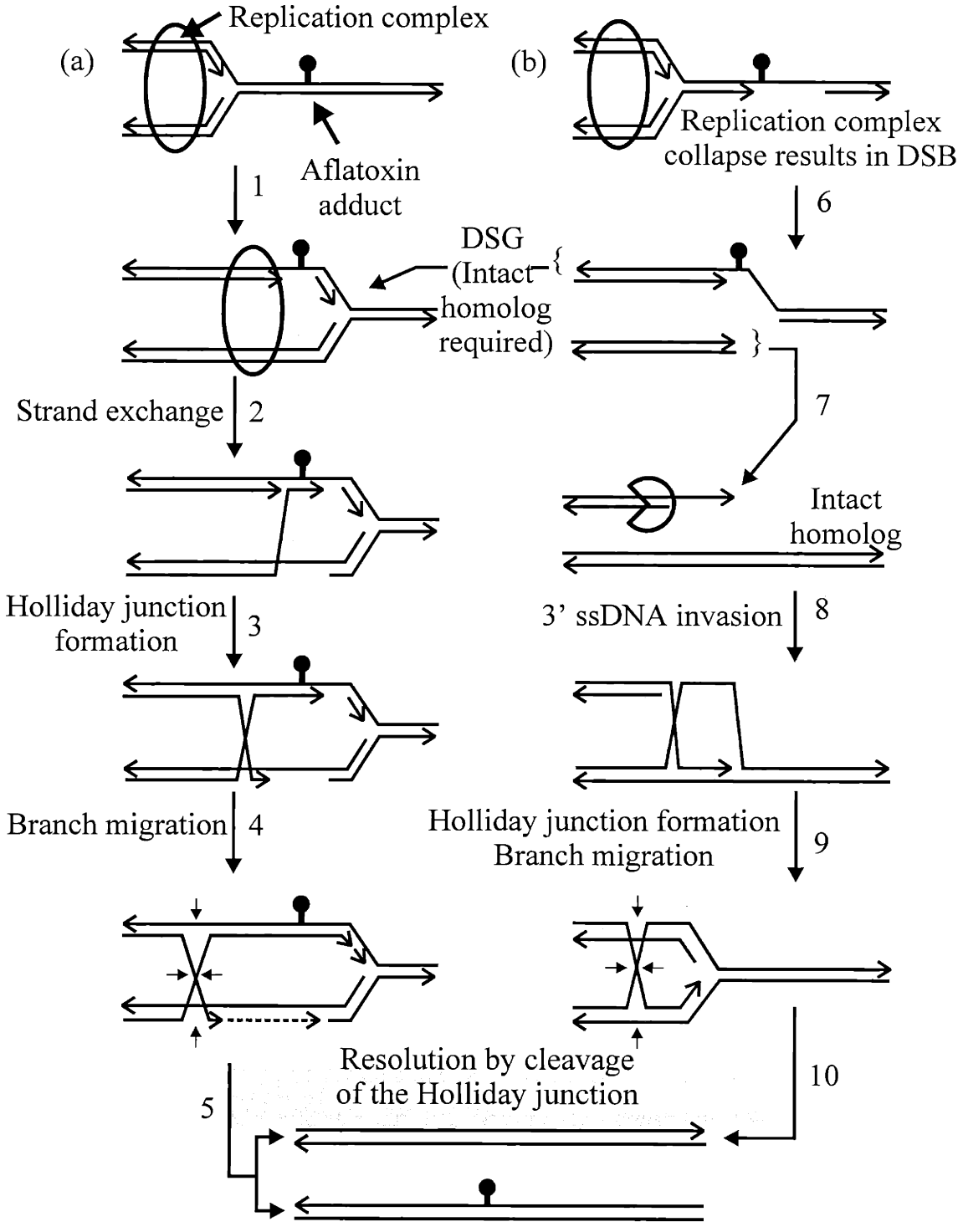


Figure 1.5: Recombination. AFB_1-DNA adducts can either stall the replication complex or possibly cause a double stranded break. Several steps in two branches of the recombination pathway can then take place, resulting in error free bypass of AFB_1 adduct. (Adapted from Zdraveski, Dissertation, MIT, 2001.)

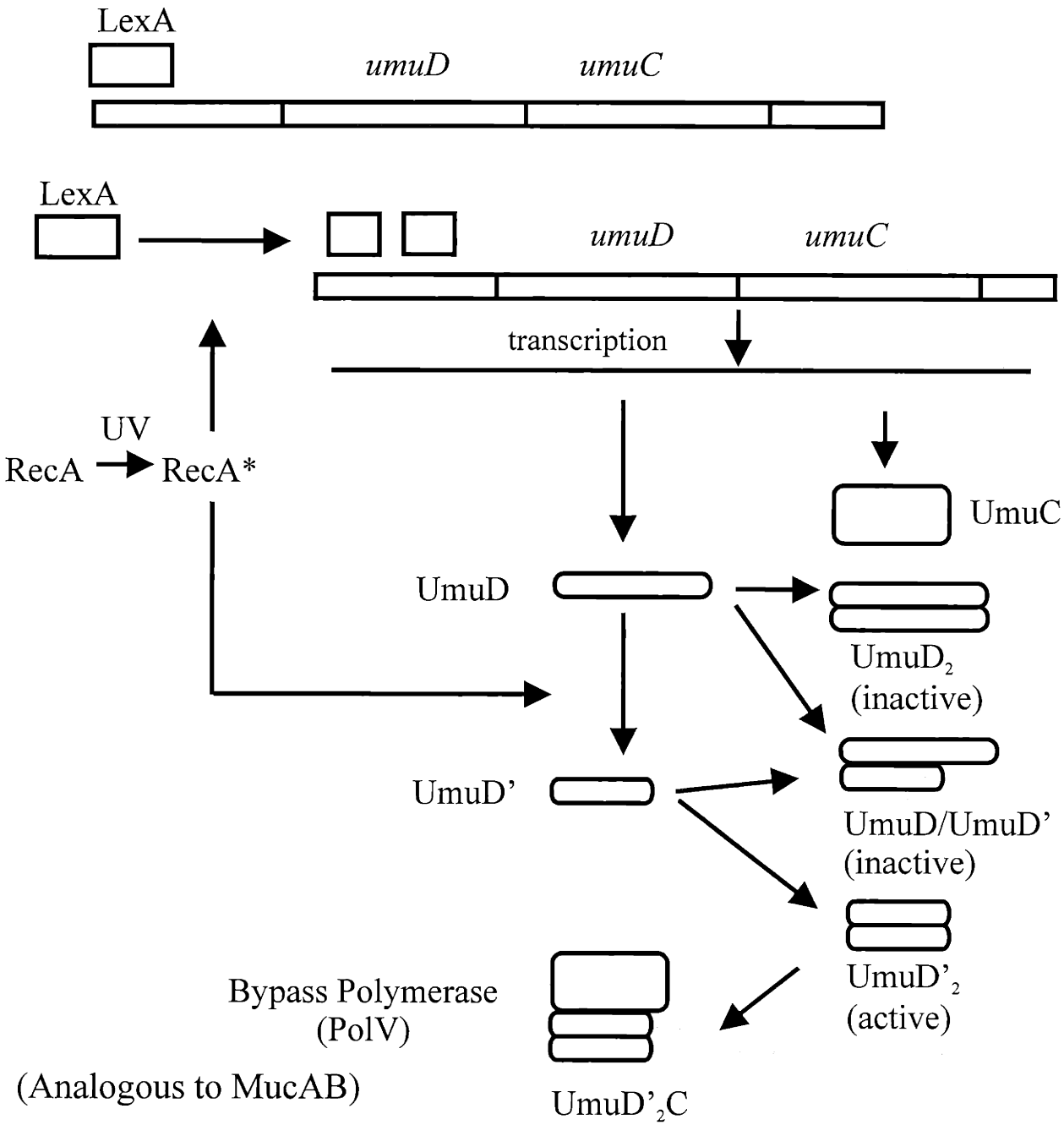


Figure 1.6: Induction of the bypass polymerase UmuDC. *E. coli* is irradiated with UV light, which converts RecA to its active form, RecA*. RecA* can then cleave LexA, the natural repressor of the umuDC operon. Transcription and translation can then occur, producing the UmuD and UmuC proteins. UmuD is not active as transcribed. It must be further cleaved to its active form either via RecA* or its own autocatalytic activity, yielding UmuD'. UmuD' then dimerizes and forms a complex with UmuC, which, in total, make up the bypass polymerase, polV. (Adapted from Friedberg, *et al.*, DNA Damage and Repair, 1995.)

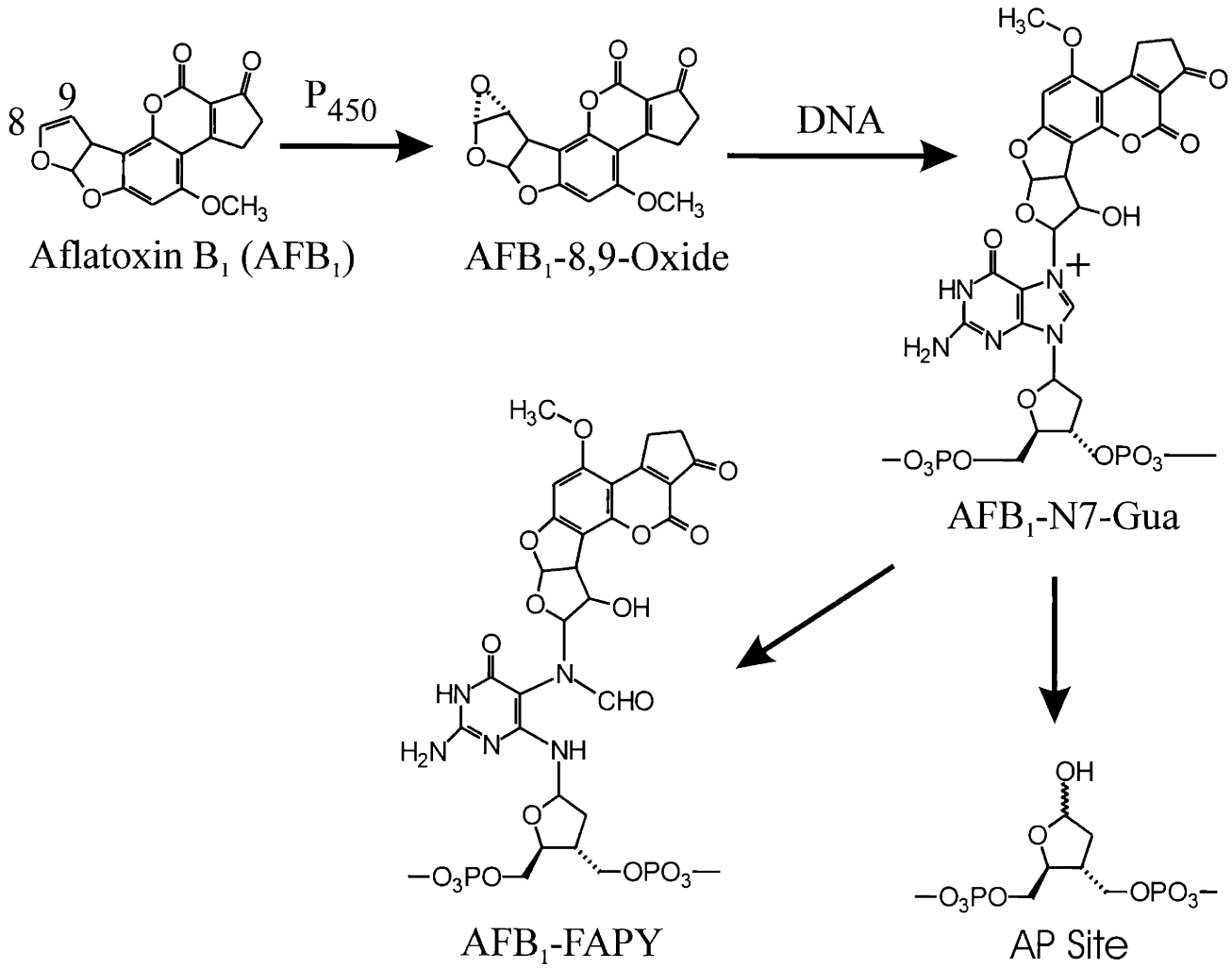


Figure 1.7: Structures of aflatoxin adducts. Aflatoxin B_1 is acted upon in the liver by cytochrome P_{450} (isozymes 1A2 and 3A4) to form the 8,9-exo-epoxide. This can react at the N7 position of guanine, generating the AFB_1-N7-Gua DNA adduct, which can subsequently break down into two secondary lesions, the AP site and the AFB_1-FAPY lesion.

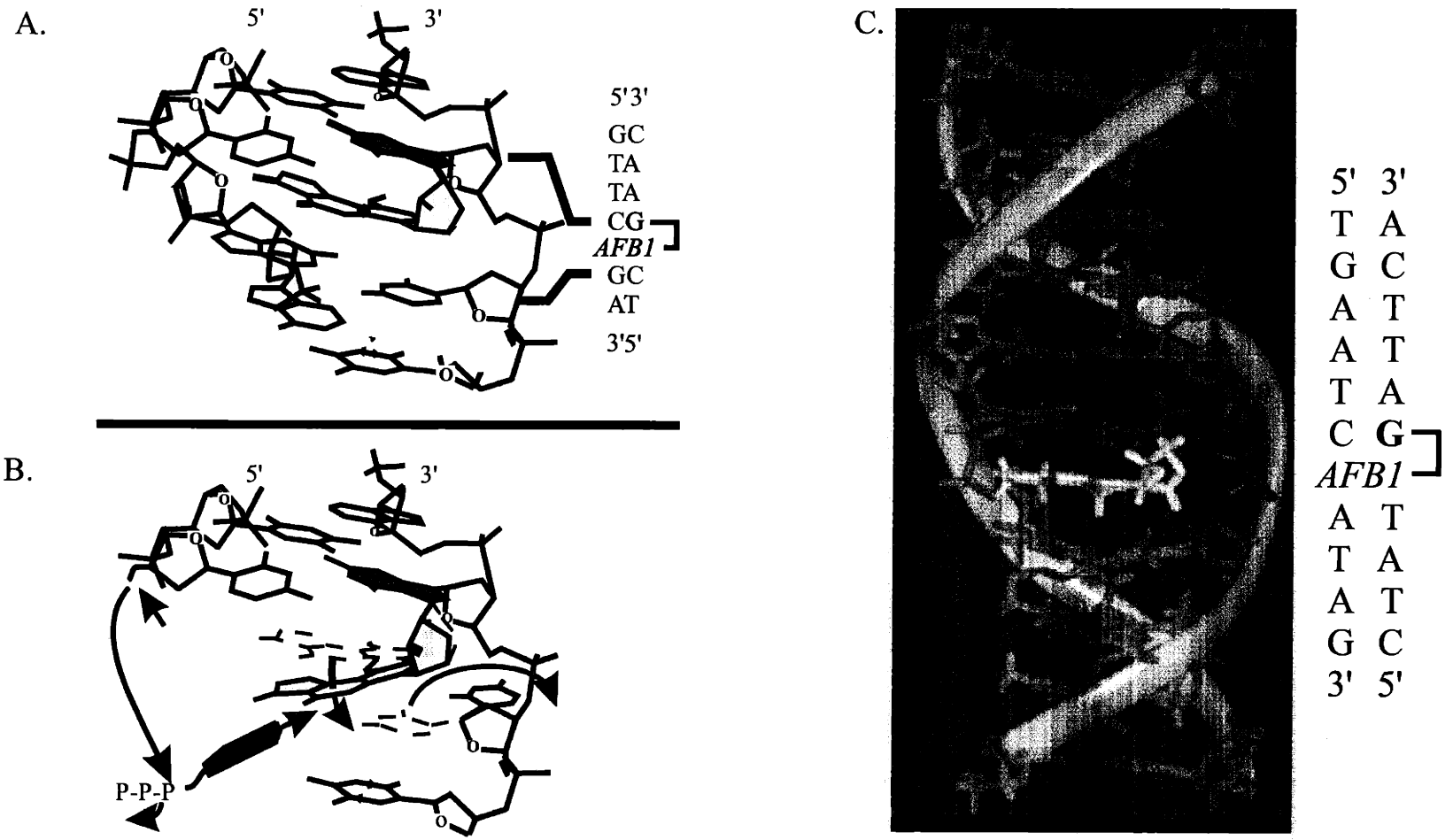


Figure 1.8: NMR solution structures of AFB_1-DNA complexes. A. AFB_1 (yellow) intercalates 5' to the guanine (red) to which it is covalently attached. The result is a slightly kinked DNA duplex. B. Upon replication it is hypothesized that the AFB_1 moiety of the AFB_1-N7-Gua adduct can interfere with the incoming base not only at the site of modification, but also at the 5' base (black). C. The AFB_1 moiety of AFB_1-FAPY also intercalates 5' to the guanine to which it is covalently attached, unwinding the duplex by 15° and increasing the T_m of the duplex by about 15°C. (A. and B. adapted from Bailey, Dissertation, MIT, 1996. C. adapted from Mao, *et al.*, *Biochemistry*, 1998).

Species	Human		Primate		Rat		Mouse[b]		Woodchuck		Duck		Tree Shrew		Ground Squirrel	
Codon[c,d] Sequence[e] Amino acid	248 CGG Arg	249 AGG Arg	248 CGG Arg	249 AGG Arg	248 CGC Arg	249 CGG Arg	248 CGC Arg	249 CGA Arg	248 CGA Arg	249 CGG Arg	248 CGT Arg	249 CGC Arg	248 CGG Arg	249 CGC Arg	248 CGC Arg	249 CGG Arg
Base change Amino acid	CGT Arg	AGT Ser	CGT Arg	AGT Ser	CTC Leu	CGT Ser	CTC Leu	CTA Leu	CTA Leu	CGT Arg	CTT Leu	CTC Leu	CGT Arg	CTC Leu	CTC Leu	CGT Arg
Base change Amino acid	CTG Leu	ATG Met	CTG Leu	ATG Met		CTG Leu			CTG Leu			CTG Leu			CTG Leu	
Viral status	HBV		None		Transgenic for HbsAg		Transgenic for HBV envelope		WHVB		DHVB		HBV		GSHV	

[a]Actual mutations observed only in humans. Mutations shown in other species are proposed AFB_1-induced G to T transversions.
[b]Studies in mice transgenic for the HBV envelope protein did not evaluate the third position of codon 249 of p53, and those that did evaluate codon 249 did not involve aflatoxin.
[c]Codons noted are functional equivalent of p53 codons 248 and 249 in humans.
[d]The first base of codon 250 in each species above is a C.
[e]Sequence context relative reactivity: CGG = 0.8; CGC = 0.3; GGA = 0.3; CGA = 0.2; AGG = 0.3; CGT = 0.6; GGC = 0.3.

Table 1: Expected amino acid changes arising from G to T mutations at p53 codons 248 and 249 in different Species[a].

Species	Rat			Trout			Human		
Codon	11	12	13	11	12	13	11	12	13
Sequence	GCT	GGT (Gly)	GGC	GCA	GGA (Gly)	GGT (Gly)	GCC	GGC (Gly)	GGT
RR G1[a] RR G2[b]	0.2 0.8	0.2 0.4			0.3 0.3	0.3 0.8		0.8 0.4	0.8 0.8
Mutation		GAT (Asp) TGT (Cys) GTT (Val)			GTA (Val)	GTT (Val)		TGC (Trp) GTC (Val)	

[a]Relative reactivity of the first G in the codon
[b]Relative reactivity of the second G in the codon

Table 2: Species specific sequence differences among ras codons - correlation of predicted reactivity with reactive forms of aflatoxin B_1.

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Chapter 2

Structure and Integrity of the Aflatoxin B_1-Formamidopyrimidine Adduct

Introduction

This chapter will describe the present state of knowledge on the chemical nature of the AFB_1-FAPY adduct. As mentioned in the literature survey, AFB_1-N7-Gua can break down into two secondary structures, an AP site and the AFB_1-FAPY adduct (Essigmann et al., 1977; Martin and Garner, 1977; Lin et al., 1977; Groopman et al., 1981; Croy et al., 1978; Croy and Wogan, 1981a; Croy and Wogan, 1981b; Busby, Jr. and Wogan, 1984) (Figure 1.7). The FAPY adduct itself has two forms, FAPY major and FAPY minor. Several methods were employed to probe the structures and stabilities of these two species.

AFB_1-FAPY adducts have been observed *in vitro* and *in vivo* (D'Andrea and Haseltine, 1978; Croy and Wogan, 1981b; Busby, Jr. and Wogan, 1984). It was previously thought that the two chemical species observed by HPLC when the AFB_1-FAPY adduct was present were due to two chemically different DNA adducts. FAPY major was formerly referred to as AFB_1-FAPY, while the minor form was referred to as an unidentified adduct ("Peak F" (Irvin and Wogan, 1985)), or an unknown product (Groopman et al., 1981). Additionally, the major and minor forms of the adduct could possibly be assigned to peaks IV and I, respectively, in another early work (Lin et al., 1977). There was some debate over whether the ring opening reaction that occurs to form FAPY adducts is a result of cleavage only of the 8-9 bond of guanine or is a mixture of cleavage products at the 7-8 bond and the 8-9 bond. Two cleavage products could account for two isomers. It was later demonstrated that the two species of AFB_1-FAPY observed were, in fact, isomers (Hertzog et al., 1982), and they were denoted as "major" and "minor" based on the predominance of one form over the other. These data were in agreement with those

observed for 7-methylguanine and other FAPY derivatives (Chetsanga and Makaroff, 1982; Chetsanga et al., 1982), which are also present as two or more isomers in single nucleotides as well as in oligonucleotides (Boiteux et al., 1984; Tomasz et al., 1987). Rotational isomers are similarly observed for other DNA adducts, such as 2-aminofluorene (AF) (Belguise-Valladier and Fuchs, 1995), 2-acetylaminofluorene (AAF) (Harsch and Vouros, 1998), benzo(a)pyrene (B(a)P) (Kozack et al., 2000), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Brown et al., 2001).

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight) mass spectrometry (Karas et al., 2000; Hillenkamp et al., 1991) is a very sensitive technique used widely to analyze the mass of proteins (Roepstorff, 2000; Fenselau, 1997; Kussmann and Roepstorff, 2000), small molecules (Wang and Sporns, 2000; Harvey et al., 1998; Frison-Norrie and Sporns, 2001), DNA (Griffin et al., 1999; Taranenko et al., 1996; Bray et al., 2001; Braun et al., 1997; Higgins et al., 1997; Jurinke et al., 1996; Bentzley et al., 1996; Smirnov et al., 1996; Chiu and Cantor, 1999; Crain and McCloskey, 1998) and DNA adducts (Tretyakova et al., 2001; Bartolini and Johnston, 2000; Siegert et al., 1996; Kozekov et al., 2001; Brown et al., 2001; Hwang et al., 1999; Harsch and Vouros, 1998; Ni et al., 1998; Wang and Sporns, 1999; Wang et al., 1999). This technique can be used to detect products that differ in as little as 2 mass units, which makes it ideal for such procedures as DNA sequencing (Pieles et al., 1993), detection of single nucleotide polymorphisms (Griffin et al., 1999; Taranenko et al., 1996; Bray et al., 2001; Braun et al., 1997; Higgins et al., 1997), and determining what types of DNA adducts are present in an oligonucleotide (Tretyakova et al., 2001; Harsch and Vouros, 1998; Ni et al., 1998; Wang

and Sporns, 1999; Wang et al., 1999; Bartolini and Johnston, 2000; Siegert et al., 1996; Kozekov et al., 2001; Brown et al., 2001). MALDI-TOF mass spectrometry was performed on each of the forms of the AFB_1-FAPY adduct in order to determine if their chemical makeup is the same.

Materials and Methods

Enzymes and Chemicals

*Eco*RI (20,000 U/ml), *Hae*III (10,000 U/ml), *Hinf*I (10,000 U/ml), T4 polynucleotide kinase (PNK) (10,000 U/ml), T4 DNA ligase (400,000 U/ml), exonuclease III (Exo III) (100,000 U/ml), and uracil DNA glycolsylase (UDG) (1000 U/ml), were from New England Biolabs. γ-^{32}P-dATP was from Perkin Elmer. Tetraethylammonium acetate (TEAAc) was purchased from Applied BioSystems. G-50 and G-25 Sephadex columns were from Roche. Centricon-3 concentrators and ZipTip C18 pipet tips were from Millipore.

Oligonucleotides

A 13 base oligonucleotide d(CCTCTTCGAACTC), a 53 base uracilated oligonucleotide scaffold d(AAAACGACGGCCAGUGAAUUGAGUUCGAAGAGGCACUGAAUCAUGGUCA-UAGC), a 12 base oligonucleotide d(ATTGGTAAGTGC), and a 14 base oligonucleotide d(ATTGGTAAGTCCGA) were obtained from Gibco BRL. A 24 base oligonucleotide d(CACTACCTCTTCGAACTCAATTCA) was purchased from Integrated DNA Technologies for analytical purposes. All oligonucleotides were purified using PAGE. The bold G indicates the modified base and the underlined sequence within the 13 base oligonucleotide indicates a unique *Sful* restriction site. The underlined sequence in the scaffold is complementary to the 13 base insert, and 20 bases on either side of this sequence are complementary to the flanking regions of the M13mp7L2 genome. Dr. Constance M. Harris (Department of Chemistry, Vanderbilt University, Nashville, TN) or Dr. Michelle L. Hamm (Essigmann lab, MIT) made AFB_1-N7-Gua- and AFB_1-FAPY-modified 13 and 24 base oligonucleotides.

Adduct Formation

Oligonucleotides (13 or 24 bases) containing single AFB_1-N7-Gua and AFB_1-FAPY residues were synthesized based on previously published procedures (Bailey et al., 1996). First, the oligonucleotides were gel purified and 100-250 nanomoles were resuspended in 90 ml of buffer (10 mM $NaHPO_4$, pH 7, 100 mM NaCl, 50 mM EDTA). One mg of AFB_1 epoxide (Gopalakrishnan et al., 1989) in 50 ml methylene chloride was then added and the mixture was shaken for 10 minutes at 4°C. The same amount of AFB_1 epoxide was added and shaken at 4°C for 15 minutes. The solution was then extracted twice with 100 ml of methylene chloride and desalted on a G-25 Sephadex column. Oligonucleotides containing AFB_1-N7-Gua adducts were separated from unreacted DNA by HPLC using a Beckman Ultrasphere C18 reverse phase column (part # 235329). Buffer A was 0.1 M TEAAC, pH 7.5, and buffer B was acetonitrile. The gradient was 8-12% B from 0-40 min, and from 12-25% B from 40-60 min. Fractions were collected and desalted using Centricon-3 concentrators at 4°C. A portion of the AFB_1-N7-Gua lesions was converted to AFB_1-FAPY lesions through incubation in 0.1 M $NaHPO_4$, pH 10, at 37°C for 4 hours. Oligonucleotides containing AFB_1-FAPY lesions were separated from those containing AFB_1-N7-Gua lesions using the HPLC conditions above (Figure 2.1A). FAPY minor formed first, and this peak was collected and rerun through the HPLC column (Figure 2.1B). The same HPLC conditions were used to separate the FAPY major from FAPY minor.

Characterization of Oligonucleotides

The AFB_1-FAPY-modified 13 or 24 base oligonucleotides (FAPY mix) were analyzed on HPLC using the conditions above (Figure 2.2). Fractions containing either FAPY major or FAPY minor were collected, and these, as well as FAPY mix, were further characterized. As soon as each fraction was collected, it was immediately desalted using Centricon-3s at 4°C (approximately 6 hours). The integrity of each adduct was checked via HPLC after this step. The 13 base oligonucleotides were slightly too short to give a good yield after desalting, so there was not enough product to do the following HPLC analyses adequately. Therefore, the 24 base oligonucleotide was used for this purpose (Figure 2.3). Next, each of the 3 adduct-containing oligonucleotides were phosphorylated using 10U T4 PNK per 20 picomoles of DNA. Kinase reactions were carried out for 15 min in 1 mM ATP, 20 mM DTT, 50 mM MOPS pH 6.6, 10 mM $MgCl_2$, and 50 μg/ml BSA at 16°C. Adduct integrity was checked via HPLC (Figure 2.4). The oligonucleotides were then kept an additional hour at 16°C to mimic the ligation reaction, although no ligase was added, as it would interfere with HPLC analysis (Figure 2.5). Lastly, the oligonucleotides were subjected to uracil glycosylase by adding 2U UDG per 0.4 picomoles of DNA and incubating at 16°C for an additional 1.5 hours, once again leaving out the exonuclease III, as that would digest the DNA and interfere with HPLC analysis (Figure 2.6).

Additionally, to determine if any AFB_1-N7-Gua adducts, AP sites, or unmodified oligonucleotides contaminated the AFB_1-FAPY-containing oligonucleotides, treatment with heat and base was employed. This would cleave oligonucleotides containing AFB_1-N7-Gua adducts or AP sites, leaving AFB_1- FAPY or unmodified oligonucleotides intact. The oligonucleotides were heated

to 80°C for 15 minutes in MOPS buffer, pH 6.6, followed by boiling at 100°C for 10 minutes in 1 M NaOH and neutralization with 0.12 M HCl. Half of the reaction was saved at this point and stored at -80°C. The other half was subjected to 1 M piperidine treatment at 90°C for 1 hour, which would cleave either AFB_1-N7-Gua- or AFB_1-FAPY-containing oligonucleotides. The piperidine was evaporated off, and the DNA was resuspended in water. The products were then analyzed on a 20% polyacrylamide gel (Figure 2.7).

MALDI-TOF Mass Spectrometry

Thirteen base oligonucleotides containing either FAPY major, FAPY minor, FAPY mix were analyzed on a PerSeptive Biosystems Voyager Elite DE MALDI-TOF instrument by Dr. Paul T. Henderson (Essigmann lab, MIT). Unmodified oligonucleotides (12 base and 14 base) were used as internal calibration standards. Ten picomoles of the sample and each standard were mixed together in a 10 μl volume and desalted with ZipTips. They were then resuspended in the MALDI matrix (3% anthranilic acid, 1% nicotinic acid, 46% acetonitrile, 27% 100 mM ammonium citrate) and spotted onto the sample plate. Spectra were obtained in the reflector mode with a laser energy of 2400, an accelerating voltage of 25,000 V, a grid voltage of 95%, a guide wire voltage of 0.28%, and a delay time of 120 ns. Each spectrum was an average of 128-256 laser shots (Figure 2.8).

Genome Construction

Genomes were made (Figure 2.9) by first digesting bacteriophage M13mp7L2 DNA at a unique restriction site with 10U *Eco*RI per μg DNA for 4 hours at 23°C. The digest was carried out in 50 mM NaCl, 100 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, and 100 μg/ml BSA. The linearized

genome was then annealed to a 53-mer uracilated scaffold, in a 2-fold molar excess, by heating at 80°C for 5 minutes and then cooling to room temperature overnight to form a "gapped" structure. Four separate ligation reactions were done with each of the 13-base inserts: AFB_1-N7-Gua, FAPY major, FAPY mix, and unmodified control. The inserts were first 5'-phosphorylated using 10U T4 PNK per 20 picomoles of DNA. Kinase reactions were carried out in 1 mM ATP, 20 mM DTT, 50 mM MOPS pH 6.6, 10 mM $MgCl_2$, and 50 μg/ml BSA at 16°C in order to ensure the integrity of the adduct. Ligations were done by adding an equimolar amount of phosphorylated 13 base insert to the gapped genome, using the same buffers as for the kinase reaction and 160U of T4 DNA ligase per 0.48 picomoles of DNA. The reaction was incubated at 16°C for 1 hour. The uracilated scaffold was then removed by adding 10U ExoIII and 2U UDG per 0.4 picomoles of DNA and incubating at 16°C for an additional 1.5 hours. Genomes were then desalted on G-50 Sephadex columns, which were pre-equilibrated with water, by centrifuging at 2000x*g* at 4°C. Products were analyzed via agarose gel (Figure 2.10).

Genome Characterization

In order to check the ligation efficiencies of modified and unmodified inserts and the integrity of the adducts, genomes were characterized after they were constructed. Genomes were constructed as above, except that 13 base inserts were 5'-phosphorylated with ^{32}P-γ-dATP. Completed genomes were then heated in the presence of acid-base and/or treated with piperidine as described for ***Characterization of Oligonucleotides*** in order to determine the purity of each adduct. The single stranded genome was then annealed to the 53-mer uracilated scaffold and subjected to

digestion with restriction enzymes *Hae*III and *Hinf*I (40U per 0.11 picomoles DNA) (Figure 2.11). The digests were run on a 20% polyacrylamide gel for analysis (Figure 2.12).

Results

Adduct Formation

The oligonucleotides were made by Dr. Constance M. Harris or Dr. Michelle L. Hamm. AFB_1-8,9-exo-epoxide was adducted to DNA to form primarily AFB_1-N7-Gua adducts. A certain percentage of these adducts depurinate to form AP sites (Figure 1.7). The conditions used for HPLC were able to resolve the peaks containing the adducts and the AP sites. The oligonucleotides containing the AFB_1-N7-Gua adducts were separated and subjected to heat and mild base treatment to form AFB_1-FAPY adducts, although this process does not go to completion (Figure 2.1A). The primary adduct that formed was the FAPY minor adduct. This adduct was reinjected into the HPLC, and it can be seen as two peaks, FAPY major and FAPY minor (Figure 2.1B). These two peaks themselves were separated and subjected to the conditions used for constructing genomes (Figures 2.3-2.6).

Characterization of Oligonucleotides

After the FAPY major and FAPY minor peaks were HPLC purified and desalted, they were analyzed via HPLC to determine if adduct integrity survived the desalting procedure. The 13 base oligonucleotides did not yield enough material after desalting in order to fully characterize them. The limited amount of material present implied that FAPY major retained its integrity after desalting, whereas a certain percentage of FAPY minor converted to FAPY major. It was at this point that the decision was made to carry out lethality and mutagenicity experiments (see **Chapters 3** and **4**) only with FAPY major or FAPY mix.

It was determined that the 13 base oligonucleotide was too close to the exclusion limit of the Centricon-3s to give a high enough yield in order to fully characterize adduct stability via HPLC. In order to determine conclusively the integrity of the adducts during the genome construction procedure, a 24 base oligonucleotide of similar sequence was constructed. It yielded enough material to carry out the various genome construction steps. After desalting on Centricon-3s, each adduct was tested for conversion to the other (Figure 2.3). Approximately 11-15% of the FAPY major adduct was converted to FAPY minor, and *vise versa*. For FAPY mix the ratio of major to minor remained roughly the same. This implies that 6 hours at 4°C is enough to cause a shift toward equilibrium in each of the pure FAPY adducts, but when equilibrium is already achieved (FAPY mix), it is not significantly affected by these conditions.

The first step in the genome construction procedure involved 5' phosphorylation of the oligonucleotides. After phosphorylation of a 24 base oligonucleotide containing either FAPY major, FAPY minor, or FAPY mix, HPLC was used to determine adduct integrity. FAPY mix maintained a similar ratio of the major to minor forms (Figure 2.4A). For the oligonucleotides containing either FAPY minor or FAPY major, the amount of other species present did not change significantly from that after the desalting step (Figure 2.4 B and C). The ligation step was tested next. Oligonucleotides were subjected to 1 hour at 16°C with all the buffers used in the ligation reaction, sans ligase. The results looked similar to those after the phosphorylation reaction (Figure 2.5). Lastly, the oligonucleotides were incubated with Exo III and UDG at 16°C

for 90 minutes with all the buffers, sans ExoIII. Once again the results were similar to both of the above reactions (Figure 2.6). Taken together, these results imply that most of the equilibrium shift occurs directly after the desalting procedure.

Modified and control 13 base oligonucleotides were subjected to acid/base/piperidine treatment and PAGE analysis to determine whether there was contamination with unmodified or AP site DNA. Intact, unmodified oligonucleotides are resistant to this method of cleavage. Modified oligonucleotides run slightly higher on the gel, and cleavage products are 6 bases long. Heating AFB_1-N7-Gua oligonucleotides produces an AP site, and subsequent treatment with base yields cleavage of the DNA. AFB_1-FAPY oligonucleotides remain intact throughout this treatment; however, they are subject to cleavage by piperidine. AFB_1-N7-Gua oligonucleotides were contaminated with about 30% AFB_1-FAPY adducts (Figure 2.7). This conclusion was based operationally on the fact that these oligonucleotides were resistant to the heat and acid-base treatment but were cleaved by piperidine. New AFB_1-N7-Gua oligonucleotides were synthesized and found to be pure by the HPLC methods described above. It was demonstrated that both the FAPY major- and FAPY minor-containing oligonucleotides had only about 1% contamination with either the AFB_1-N7-Gua adduct or the AP site, and after piperidine treatment, approximately 95% of the product was cleaved, and the remaining 4-5% was due to unmodified DNA.

MALDI-TOF Mass Spectrometry

In order to determine whether the peaks observed in the HPLC traces (Figures 2.2-2.6) represented chemically unique structures, MALDI-TOF mass spectrometry was performed on 13 base oligonucleotides containing either FAPY major, FAPY minor, or FAPY mix. Twelve base and 14 base oligonucleotides were used as internal standards, and in each case, only one peak was observed for the FAPY-containing oligonucleotide (Figure 2.8). This peak had a *m/z* ratio of 4191.73, 4191.97, and 4191.99, respectively, for each of the three samples. This indicates that only one chemical composition makes up each of the two adducts, implying that they are either rotational isomers (Figure 2.13A) or structural isomers (Figure 2.13B).

Genome Construction

Single stranded M13mp7L2 viral vectors were digested with *Eco*RI, which cleaves at a hairpin structure in the genome (Messing et al., 1977). Agarose gel analysis was used to determine the completion of the reaction by comparing the circular versus linear forms of the genome (1% agarose gel for 5 hours at 120 volts in 1xTBE). Gels were post stained in ethidium bromide. This procedure was done anew for each set of transformations. In all experiments 100% cleavage was observed (Figure 2.10, compare control, Lane 1, to digest, Lane 2). Next, a 53-mer uracilated scaffold was annealed to the cleaved genome in order to hold it together for ligation of the adduct-containing insert. The product of the annealing step (gapped genome) could also be visualized by agarose gel, appearing as a smear between the linear and circular markers (Figure 2.10, Lane 3). After the ligation of the insert (either modified or unmodified, as a control) into the gapped DNA and subsequent digestion of the scaffold, a full circular product

can be observed on the agarose gel, signifying an intact genome containing a single AFB_1 adduct (Figure 2.10, Lanes 4-7). It should be noted that the yield of intact modified genomes was lower than the unmodified control (Figure 2.10, compare Lanes 5-7 to Lane 4), indicating that the bulkiness of the AFB_1 adducts may inhibit proper annealing and ligation of the insert. Additionally, the formation of genomes containing the AFB_1-N7-Gua adduct (Figure 2.10, Lane 5) was better than that of those containing FAPY mix (Figure 2.10, Lane 6), which, in turn, was better than that of those containing FAPY major (Figure 2.10, Lane 7). The amount of DNA used in each transformation (see **Chapter 3**) was adjusted according to how much intact circular product appeared on the gel.

Genome Characterization

In order to determine the ligation efficiencies of oligonucleotides containing AFB_1 adducts to control oligonucleotides, the genomes were subjected to the characterization procedures described above. The oligonucleotide inserts were 5'-end labelled with ^{32}P, and genomes were constructed. Once the construction procedure was complete, genomes were desalted and once again annealed to the 53 base scaffold to form a double stranded region of DNA. A 31 base stretch of the region containing the insert was excised by *Hae*III/*Hinf*I digestion (Figure 2.11). An intact 31 base oligonucleotide signified a complete ligation, whereas a 21 base or 23 base oligonucleotide were the result of either a 5' only or 3' only ligation. This experiment demonstrated similar ligation efficiencies for each of the adduct-containing inserts (~30%-40%), and both of these were about half that for the unmodified insert (~60%) (Figure 2.12). If the excised regions were subsequently treated with acid/base and piperidine, it was possible to

determine if there was an adduct present. If an adduct were present, there would be cleavage at the adducted site, yielding a 15 base or 6 base oligonucleotide. Control oligonucleotides or genomes without adducts would be resistant to such treatment. Oligonucleotides containing AFB_1-FAPY adducts were completely digested to 15 bases or 6 bases when treated with piperidine. Those containing AFB_1-N7-Gua adducts exhibited a 30% contamination with AFB_1-FAPY, as demonstrated by their resistance to acid/base treatment and subsequent digestion with piperidine. These results are consistent with those of the oligonucleotide characterization procedure prior to genome construction.

Discussion

Adduct Formation

When the methods described above are used to convert the AFB_1-N7-Gua adduct to AFB_1-FAPY, it is the FAPY minor form of the adduct that forms first. Both the FAPY major and FAPY minor adducts have been observed in a single adducted base (Croy and Wogan, 1981a; Croy and Wogan, 1981b; Hertzog et al., 1982) and in single [(Bailey et al., 1996) and this work] and double stranded DNA (Mao et al., 1998). The kinetics of conversion of FAPY minor to FAPY major has not yet been determined in detail. It has been observed in the 13 base oligonucleotide that there is some conversion of FAPY minor to FAPY major, and *vise versa,* in a matter of hours at 4°C, until both species are present in roughly a 2:1 ratio of major:minor. This result implies that temperature is only a factor in conversion of one form of the adduct to another up to a certain point. When the Stone group attempted to study the FAPY adduct by NMR spectroscopy, there were no clear results until the DNA containing the adduct was stored at 4°C for weeks. The resulting structure was interpreted to be that of FAPY major (Figure 1.5C) (Mao et al., 1998). This form of the adduct stabilizes the DNA duplex in two ways. First, it increases the T_m of the duplex by 15°C. Second, it unwinds the duplex by approximately 15°, increasing stacking interactions between the intercalated aflatoxin moiety and the neighboring bases. Moreover, it is possible that there is a hydrogen bond between the formyl proton of the FAPY major adduct and the exocyclic amino group on a neighboring adenine. The NMR solution structure of the AFB_1-N7-Gua adduct, in contrast, reveals that the adduct kinks slightly the DNA helix (Gopalakrishnan et al., 1990) (Figure 1.5A). The structure of the FAPY minor adduct remains elusive, but some possible conformations are discussed below.

Characterization of Oligonucleotides

After individual FAPY major and FAPY minor peaks were collected and desalted at 4°C for 6 hours, it was noted that about 15% of each had converted to the other species. However, in subsequent steps no further conversion was observed after nearly 3 hours at 16°C, even though the 2:1 ratio of major:minor had not yet been achieved. Additionally, the FAPY mix sample retained its major:minor ratio throughout the procedure as well. These results have several implications. It is possible that the equilibrium observed in FAPY mix cannot be perturbed significantly under the conditions used. It might require a higher temperature, longer incubation period, or different buffer conditions in order for a shift to take place. Also, in order to reach the 2:1 equilibrium, several conformational steps may be required. Six hours at 4°C followed by nearly 3 hours at 16°C only allow for roughly an 8:1 ratio to be observed, either major:minor or minor:major. Again, another set of conditions may be required to reach the 2:1 equilibrium.

MALDI-TOF Mass Spectrometry

As a result of the mass spectrometry performed in this work, it can be concluded that the major and minor forms of the FAPY adduct are of the same mass. These data and others support the hypothesis that two or more rotational isomers of the FAPY adduct exist (Hertzog et al., 1982; Tomasz et al., 1987). There are several bonds around which AFB_1-FAPY can rotate, suggesting several possible confirmations for these rotational isomers (Figure 2.13A). A ball-and-stick model kit was used to observe several possible conformations of FAPY minor in both a single adducted nucleotide and a trinucleotide sequence of double stranded DNA. It appears that both the single nucleotide and the trinucleotide sequence would allow for both 5' and 3'

intercalation of FAPY, where there is the possibility of hydrogen bonds holding the molecule in place. This possibility, however, may not afford enough of a structural difference to be observed by HPLC. A more likely scenario is that the two FAPY rotamers can be attributed to an intercalated and an extrahelical form of the adduct (Figure2.13C). These types of adduct conformations have been proposed for AFB_1-N7-Gua (Loechler et al., 1988) and B(a)P (Kozack et al., 2000). FAPY minor may also be a conglomerate of several conformations before settling on the FAPY major structure (Figure 2.13B). A flexible extrahelical form of the adduct would interfere significantly with the phosphate backbone of DNA, and this point will be discussed further in subsequent chapters.

The sequences used in the NMR studies of AFB_1-N7-Gua and AFB_1-FAPY major were not identical, which raises the question of whether sequence context is the cause of the difference in the secondary structure of DNA or whether the structure of the adduct itself is causing this difference. The resulting secondary structure of DNA in turn may affect whether the adducts could block replication, as has been observed for both the AFB_1-N7-Gua and AFB_1-FAPY adducts in many *in vitro* systems (Levy et al., 1992; Yu, 1983; Refolo et al., 1985; Yu et al., 1990; Marien et al., 1987; Johnston and Stone, 2000). Conversely, the preexisting secondary structure of the DNA may also affect aflatoxin binding, in turn affecting the types and frequencies of mutations these adducts form.

It is possible that AFB_1 itself induces a DNA secondary structure observed for some other DNA adducts, such as 2-aminofluorene (AF) (Belguise-Valladier and Fuchs, 1995). It has been

observed that AF can cause –2 frameshift mutations, and these are believed to result from slippage of the DNA strand containing the AF adduct. The "looped out" structure is thought to be stabilized by the AF adduct. In much the same way the AFB_1 adducts used in this study may cause a "looped out" region of partial double stranded nature (Figure 2.14A), although no frameshift mutations have been observed for these adducts. It is known that intercalators prefer the stacked environment of a double stranded structure, and since the adducts in this study are initially in single stranded DNA, it may be energetically more favorable for this secondary structure to form. This phenomenon may also preferentially occur in some sequences over others.

Genome Construction and Characterization

It is noteworthy to mention that it is more difficult to construct genomes using AFB_1-modified single stranded oligonucleotides than it is to construct genomes that contain the unmodified control. The ligation efficiency for the modified DNA is about half that of the control. Two possible explanations are as follows. First, it is possible that in the single stranded oligonucleotide, the "looped out" model described above (Figure 2.14A) applies. Therefore, the oligonucleotide would be only partially complementary to the scaffold (Figure 2.14B), and may be more likely to ligate at either the 5' or the 3' end, but not both. Second, it is possible that the bulkiness of the AFB_1 adduct itself does not allow for simple annealing of the 13 base oligonucleotide to the scaffold. AFB_1 may intercalate more easily into a DNA duplex that is already formed, rather than be restricted to the limits of forced annealing in our system.

Conclusions

AFB_1-FAPY is a mixture of two species, having the same *m/z* ratio, which are rotational isomers. These two species undergo a complex equilibrium that may depend on temperature, time, and buffer conditions. The molecular environment of the DNA duplex may also play a role in this equilibrium.

Future Work

Several experiments can be done to take the results of this work one step further. First, the kinetics of conversion of FAPY minor to FAPY major should be examined in detail. Experiments should be performed under different temperature, time, and buffer conditions to determine the dynamics of how equilibrium is reached and how it can be perturbed. Ideally, it would be of interest to mimic *in vivo* conditions and utilize the possible factors that can affect them. These experiments can be attempted *in vitro*, first using chemical buffers, then cell extracts.

Second, molecular modeling should be used to further analyze the possibilities of sequence dependent AFB_1-FAPY binding. This has been done quite successfully for AFB_1-N7-Gua (Loechler et al., 1988) and for B(a)P (Kozack et al., 2000) in order to help elucidate the possibilities of adduct conformation. All immediate 5' and 3'sequence contexts should be applied for both the AFB_1-FAPY adduct. This analysis could help determine if hydrogen bonding can play a role in stabilizing certain DNA structures, and if each of the AFB_1 adducts has the same effect on DNA (i.e., unwinding by 15° or kinking the helix) regardless of the sequence context. Ideally, NMR structures should be solved to confirm each of these possibilities, but this may prove to be a daunting task. However, in the long run, this information could possibly help to elucidate the reason for p53 codon 249 hotspot mutation by determining what particular characteristics of this sequence make it favorable for AFB_1 binding and mutagenesis.

Third, the structure of FAPY minor should be solved. A few simple experiments can be carried out to test the possibility that the FAPY minor adduct is extrahelical in nature. First, a footprinting experimenting can be done using ethylnitrosourea, which binds to the phosphate backbone of DNA. This compound will not bind to any region obstructed by the adduct. Second, a fluorescence experiment can be performed, utilizing linear dichroism (Eriksson and Norden, 2001). AFB_1 fluoresces at 360 nm while intercalated in DNA. If it is not intercalated, the fluorescence may be quenched. Third, it is possible that FAPY minor is a 3' intercalated structure. By using a site-directed intercalator, such as psoralen, the site 3' to the modified G can be blocked (Kobertz et al., 1997). If only one peak is observed by HPLC after this block is in place, than 3' intercalation may be possible for FAPY minor. Fourth, if FAPY minor is rotating through several conformations, it may be possible to observe this phenomenon using EPR spectroscopy, or perhaps to isolate one of the conformations. Lastly, analysis of single adducted nucleotides using multiple spectroscopic techniques can be performed to evaluate the different rotational isomers (Tomasz et al., 1987).

Fourth, the "looped out" structure should be analyzed to determine if it can occur in single stranded oligonucleotides that contain an AFB_1 adduct. One way to probe this question is to use non-denaturing PAGE to determine if hairpin-like structures can form in oligonucleotides of varying length, as well as in a single stranded plasmid. These experiments will not only resolve whether such a phenomenon is preventing certain DNA manipulations from occurring efficiently, but they will also clarify a possible mechanism for blocking replication, signaling for DNA repair, or bypassing the lesion. However, it has been noted, that these structures, at least with

respect to AF-DNA adducts (Belguise-Valladier and Fuchs, 1995), most likely will result in frameshifts, which have not been observed for AFB_1 in this work. This point will be discussed further in **Chapter 4**.

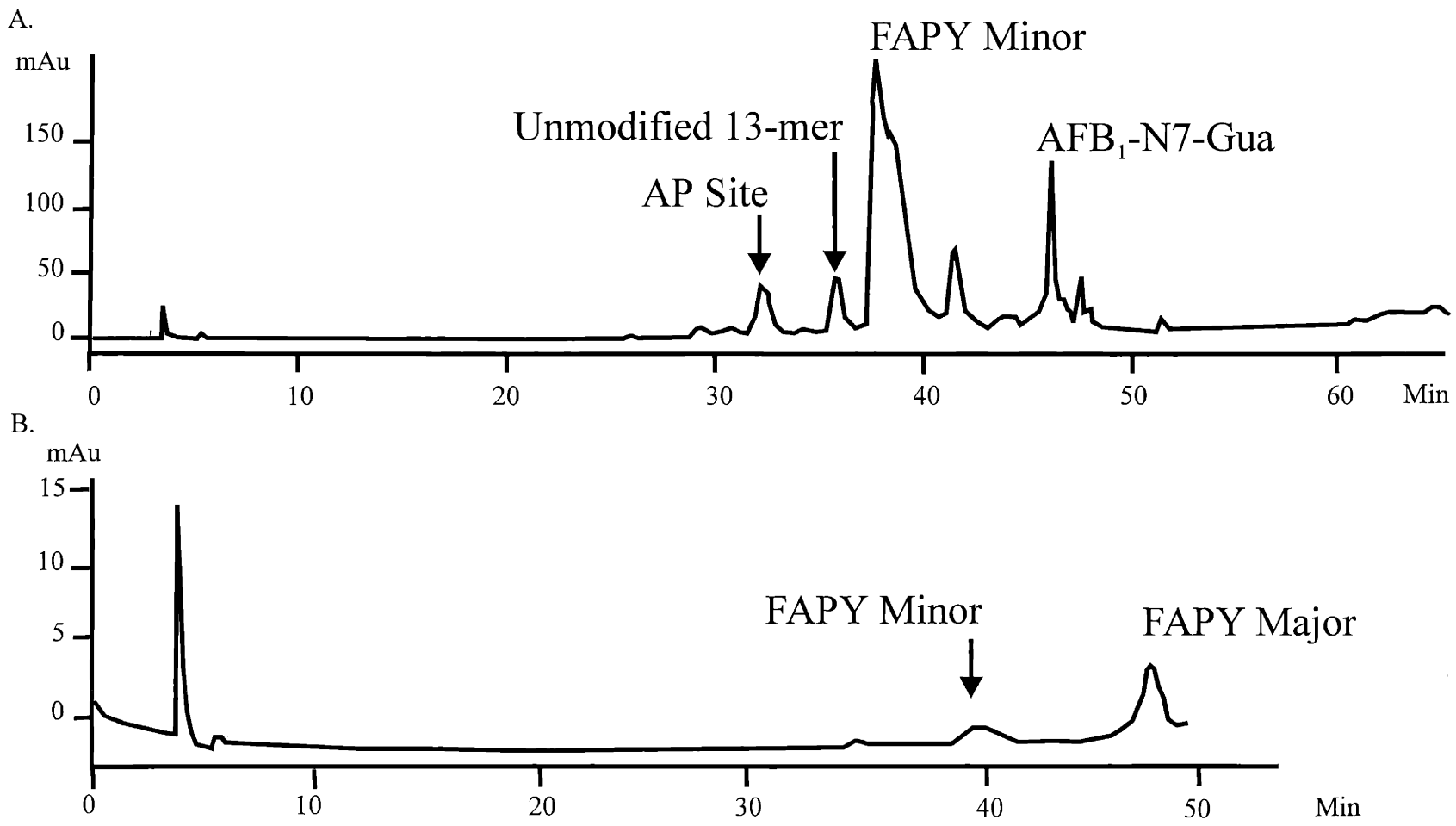

Figure 2.1: HPLC trace of 13 base oligonucleotide after incubation with AFB_1-epoxide. A. Aflatoxin epoxide was incubated with ssDNA. The minor rotamer of the adduct forms first. This reaction does not go to completion, so HPLC must be employed to separate out the desired product. This trace shows that all products can be successfully separated under the following conditions. B. The peak labeled FAPY minor in part A was isolated and a portion reinjected to ensure purity.

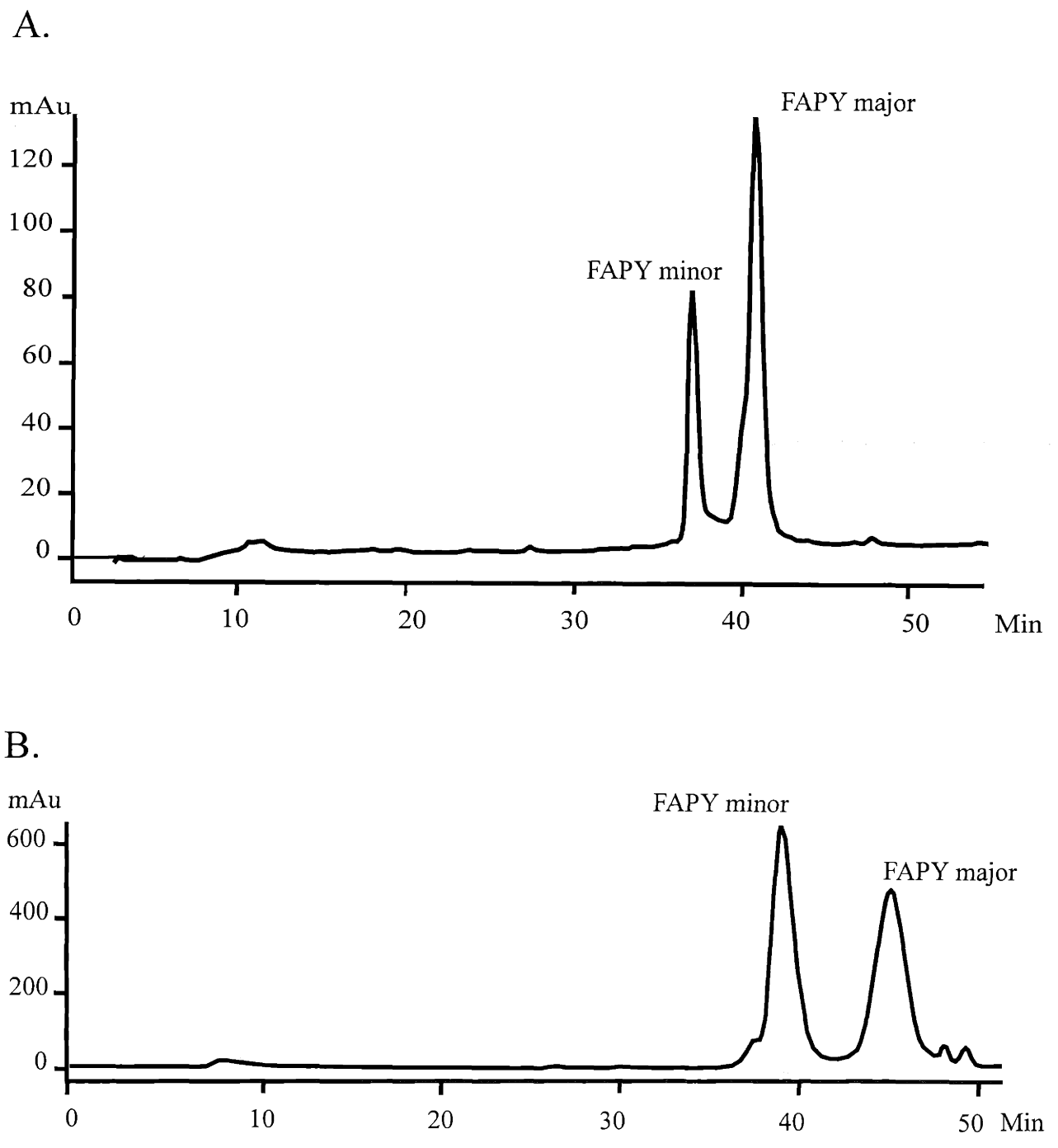

Figure 2.2: AFB_1-FAPY adducted 13 base and 24 base oligonucleotides. Aflatoxin epoxide was allowed to react with either a 13 base (A) or 24 base (B) oligonucleotide, as described in the text. Both the FAPY major and FAPY minor forms can be separated as observed in each case.

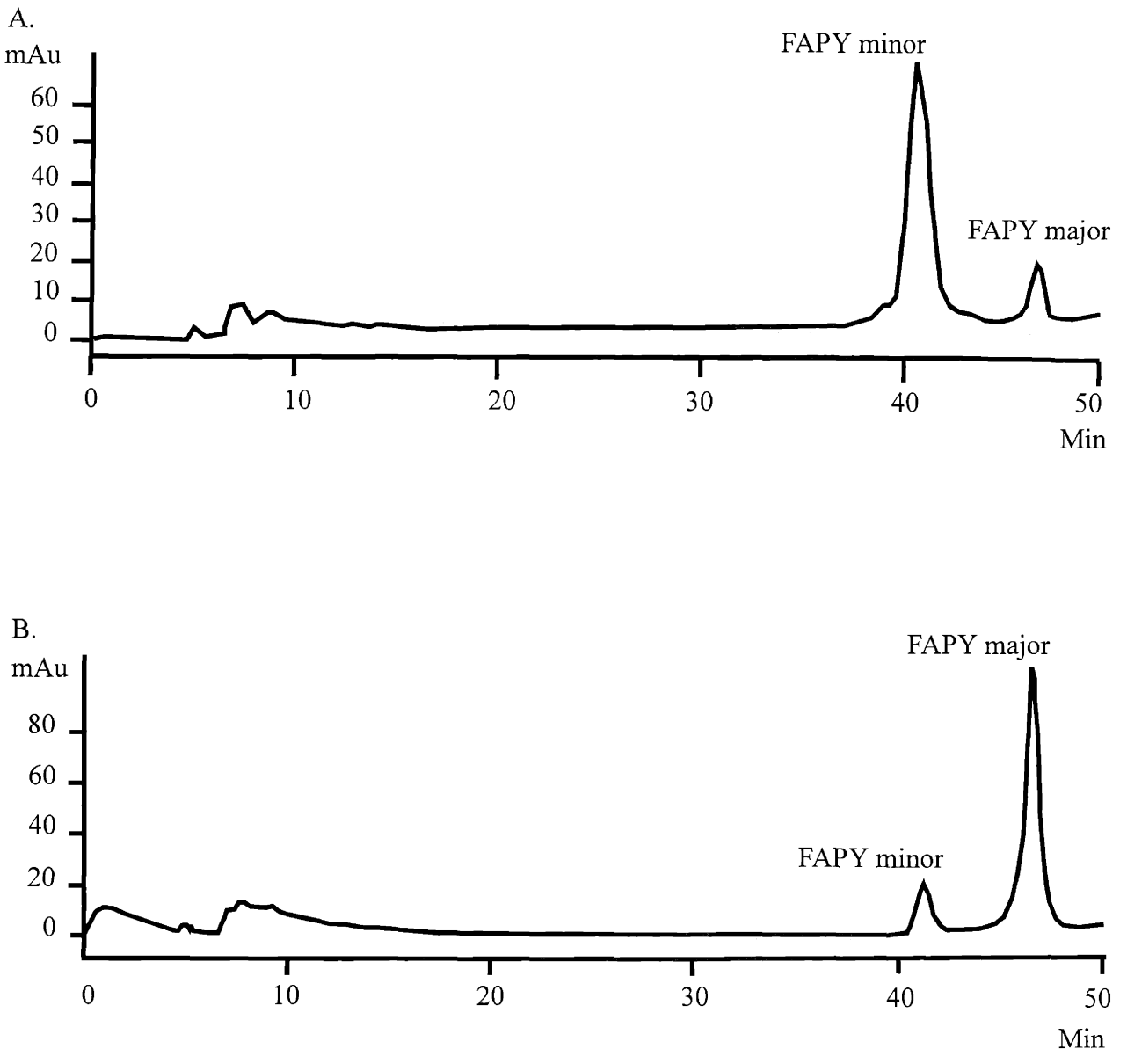

Figure 2.3: Integrity of FAPY adducts after HPLC separation and Centricon-3 desalting. Twenty-four base oligonucleotides containing AFB_1-FAPY adducts were sparated on HPLC. Fractions were collected that corresponded to the major and minor peaks. Each of these fractions was desalted using Centricon-3 concentrators. Their stability was monitored by injection in to the HPLC once again. A. FAPY minor fraction. B. FAPY major fraction.

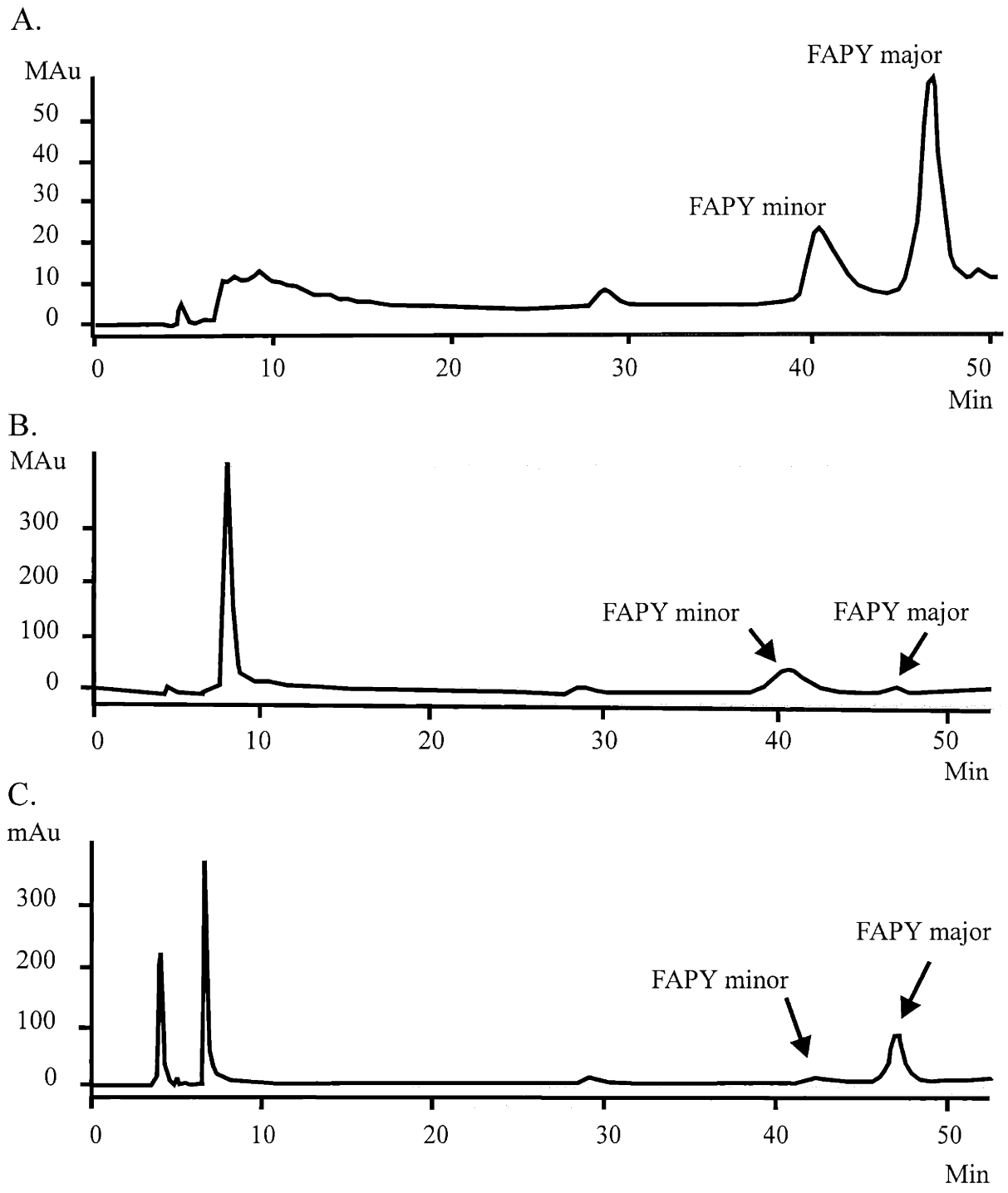

Figure 2.4: Integrity of FAPY adducts after phosphorylation. Twenty-four base oligonucleotides containing AFB_1-FAPY adducts that were desalted on Centricon-3 concentrators were subjected to phosphorylation reactions using T4 polynucleotide kinase. The integrity of the adduct was analyzed via HPLC. A. FAPY mix. B. FAPY minor. C. FAPY major.

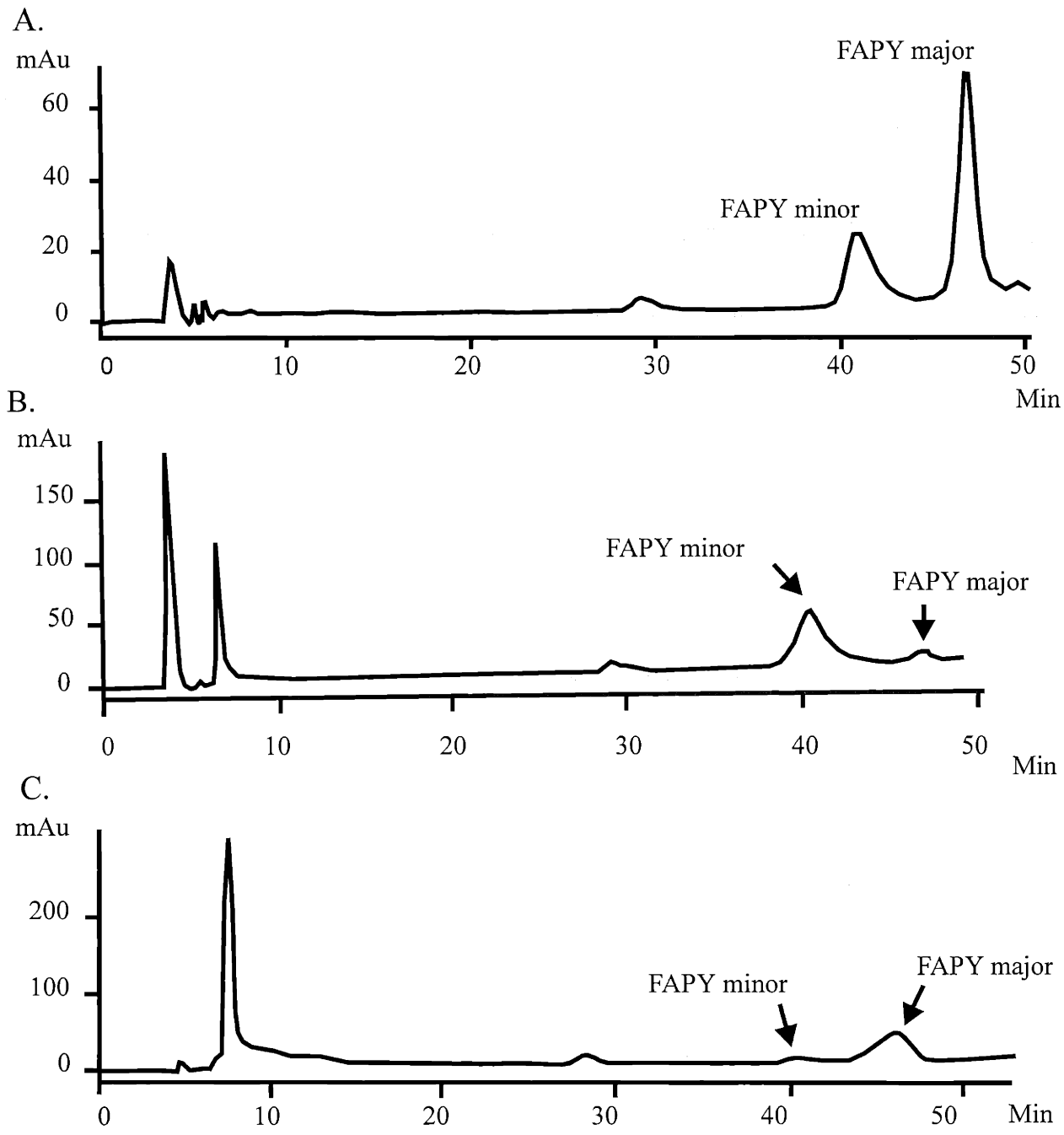

Figure 2.5: Integrity of FAPY adducts after mock ligase treatment. Twenty-four base oligonucleotides containing AFB1-FAPY adducts that were both desalted on Centricon-3 concentrators and put through the phosphorylation reactions were then subjected to conditions used for ligation reactions with T4 DNA ligase. However, samples were mock treated (buffers only, no ligase) due to the fact that adding ligase to the reactions would produce multimers that would skew the HPLC analysis. A. FAPY mix. B. FAPY minor. C. FAPY major.

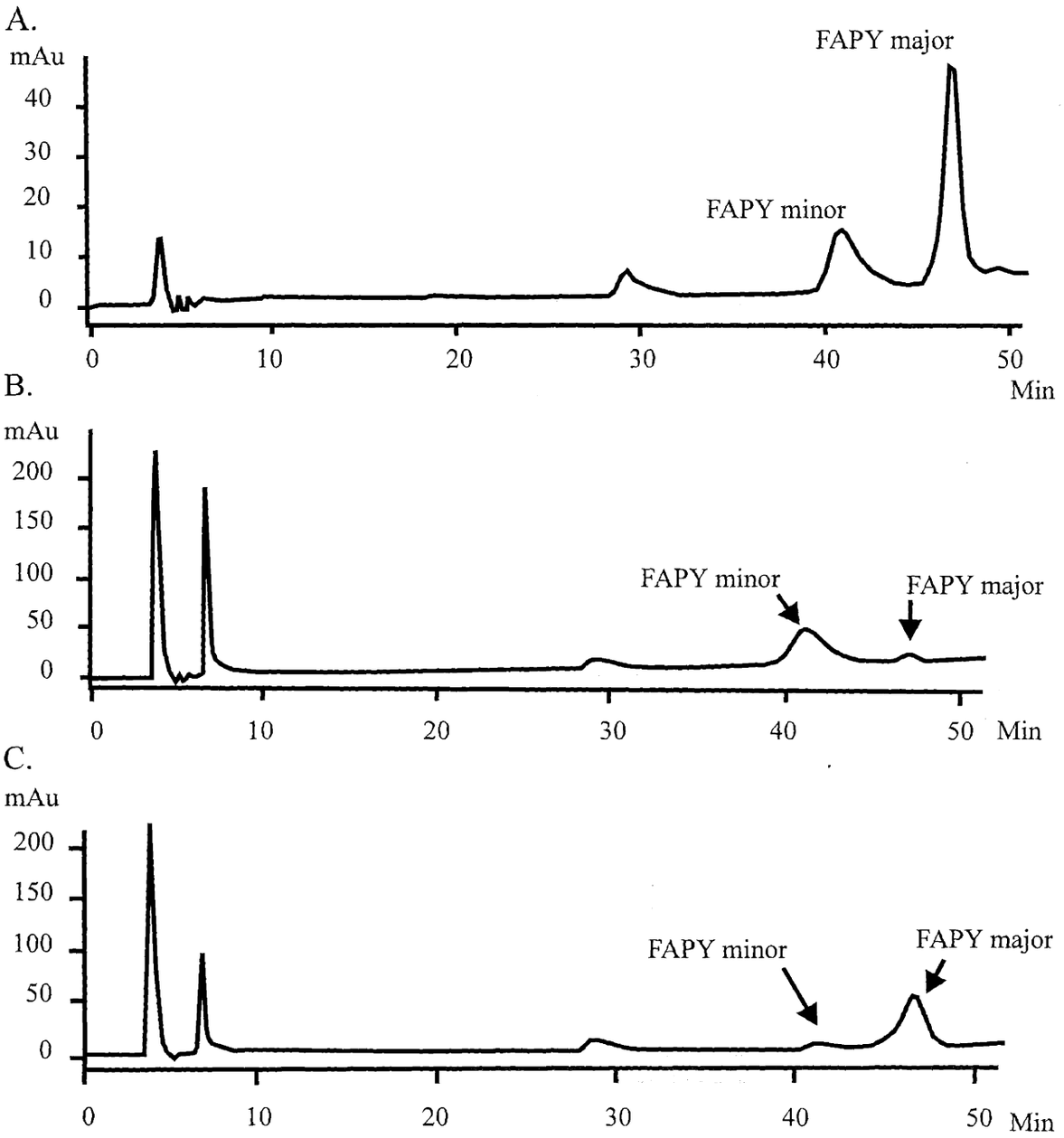

Figure 2.6: Integrity of FAPY adducts after UDG and mock Exo III treatment. Twenty-four base oligonucleotides containing AFB_1-FAPY adducts that were desalted on Centricon-3 concentrators, phosphorylated, and put through the mock ligase reaction were then subjected to conditions for treatment with UDG and Exo III. However, samples were only mock treated with Exo III due to the fact that this enzyme will degrade the oligonucleotides. A. FAPY mix. B. FAPY minor. C. FAPY major.

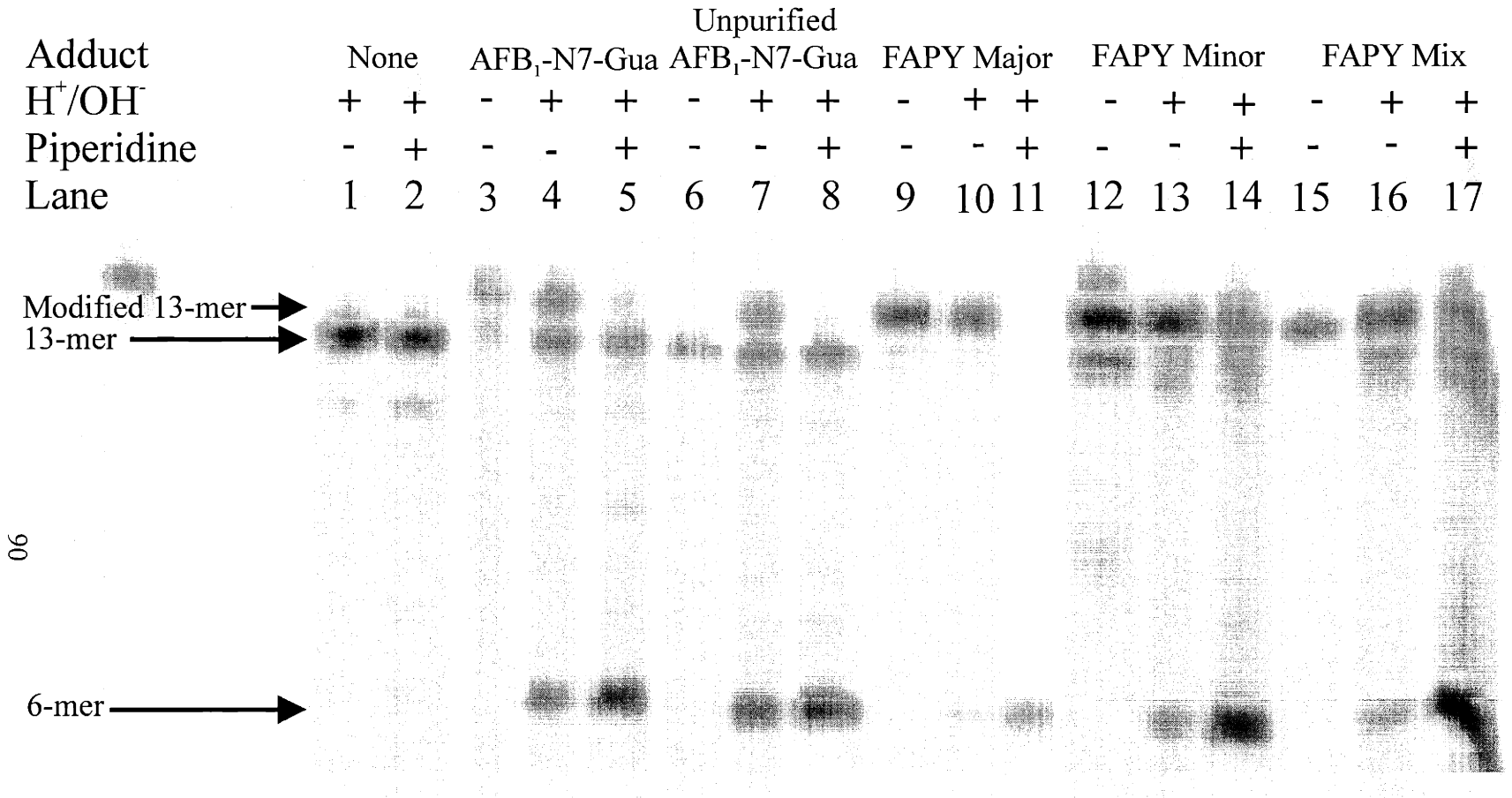

Figure 2.7: Characterization of oligonucleotides. Thirteen base oligonucleotides that were either unmodified or contain one form of an aflatoxin were analyzed to confirm the purity of the adduct present. Samples were either mock treated, heated with acid and base, and/or treated with piperidine. Lanes 1-2, unmodified 13-mer; lanes 3-5, AFB_1-N7-Gua; lanes 6-8, unpurified AFB_1-N7-Gua; lanes 9-11, AFB_1-FAPY major; lanes 12-14, AFB_1-FAPY minor; lanes 15-17, AFB_1-FAPY mix. Lanes 3, 6, 9, 12, and 15 are untreated controls. Lanes 1, 4, 7,10, 13, and 16 are samples heated in the presence of acid and base. Lanes 2, 5, 8, 11, 14, and 17 are samples treated with acid, base, and piperidine. Arrows indicate different sized fragments.

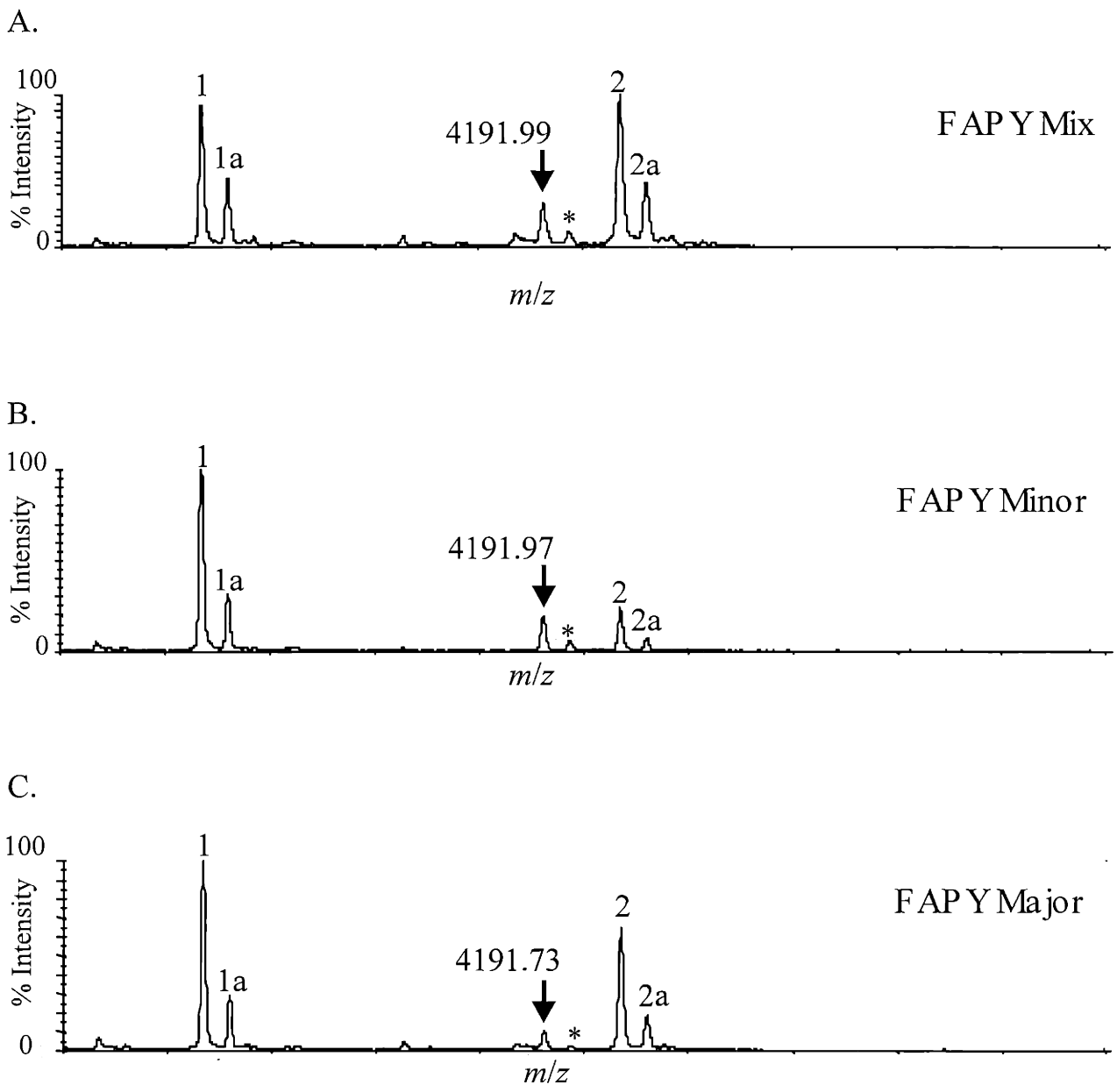

Figure 2.8: Mass spectrometry of AFB_1-FAPY. Thirteen base oligonucleotides containing either A. FAPY mix, B. FAPY minor, or C. FAPY major were mixed with internal standards, 12 base (1) and 14 base (2) oligonucleotides. Peaks labelled 1a, *, and 2a are artifacts corresponding to each of the oligonucleotides in the sample. The *m/z* ratios for the FAPY containing 13 base oligonucleotides are indicated by the arrows.

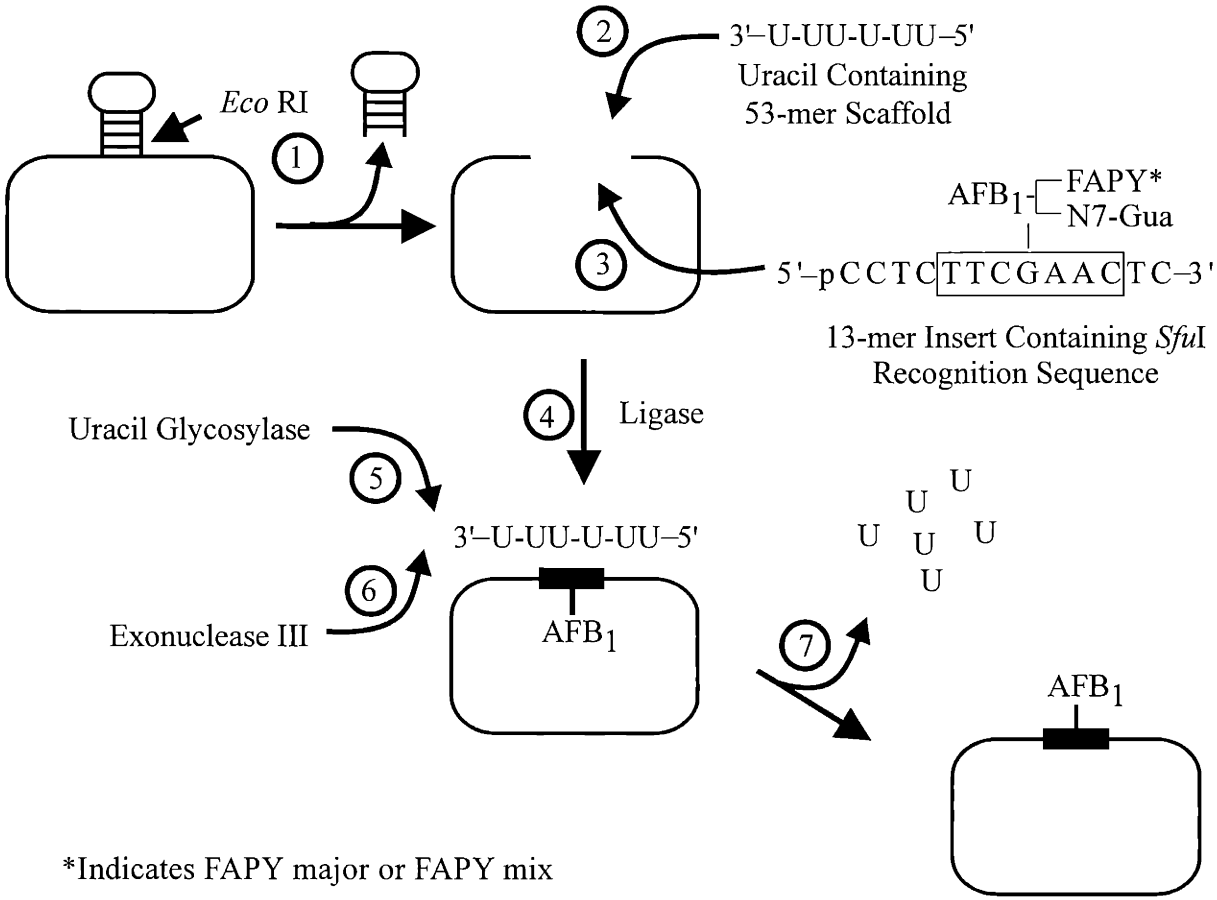

Figure 2.9: Genome construction. Single stranded M13mp7L2 viral genomes were cleaved with the *EcoR*I restriction enzyme (1) to yield a linear piece of DNA. This linear DNA was annealed to a 53 base uracil-containing oligonucleotide scaffold (2), which is complementary to 20 bases on either side of the excised region, forming a "gapped" structure. Either an unmodified or aflatoxin-containing 13 base oligonucleotide that was complementary to the unannealed 13 base region of the scaffold was then inserted into the gap (3). The oligonucleotide was then ligated (4). The scaffold was then removed using uracil glycosylase (5) and Exonuclease III (6), yielding a degraded scaffold (7) and an intact viral genome with a single modified guanine.

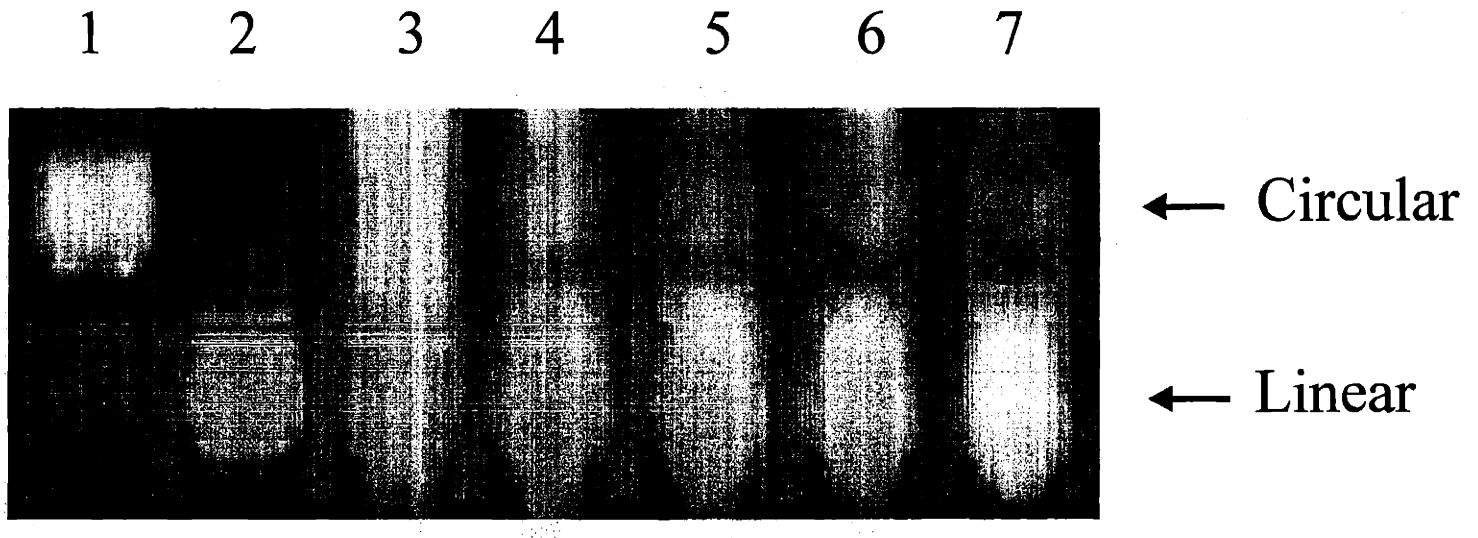

Figure 2.10: Agarose gel of genome construction products. Genomes were constructed and run on a 1% agarose gel in 1x TBE buffer at 120 V for 5.5 hours. Gel was poststained in 5 μg/ml ethidium bromide. Lane 1, M13mp7L2; lane 2, *EcoR*I digest; lane 3, "gapped" structure; lane 4, unmodified insert ligation; lane 5, AFB_1-N7-Gua ligation; lane 6, AFB_1-FAPY mix ligation; lane 7, AFB_1-FAPY major ligation.

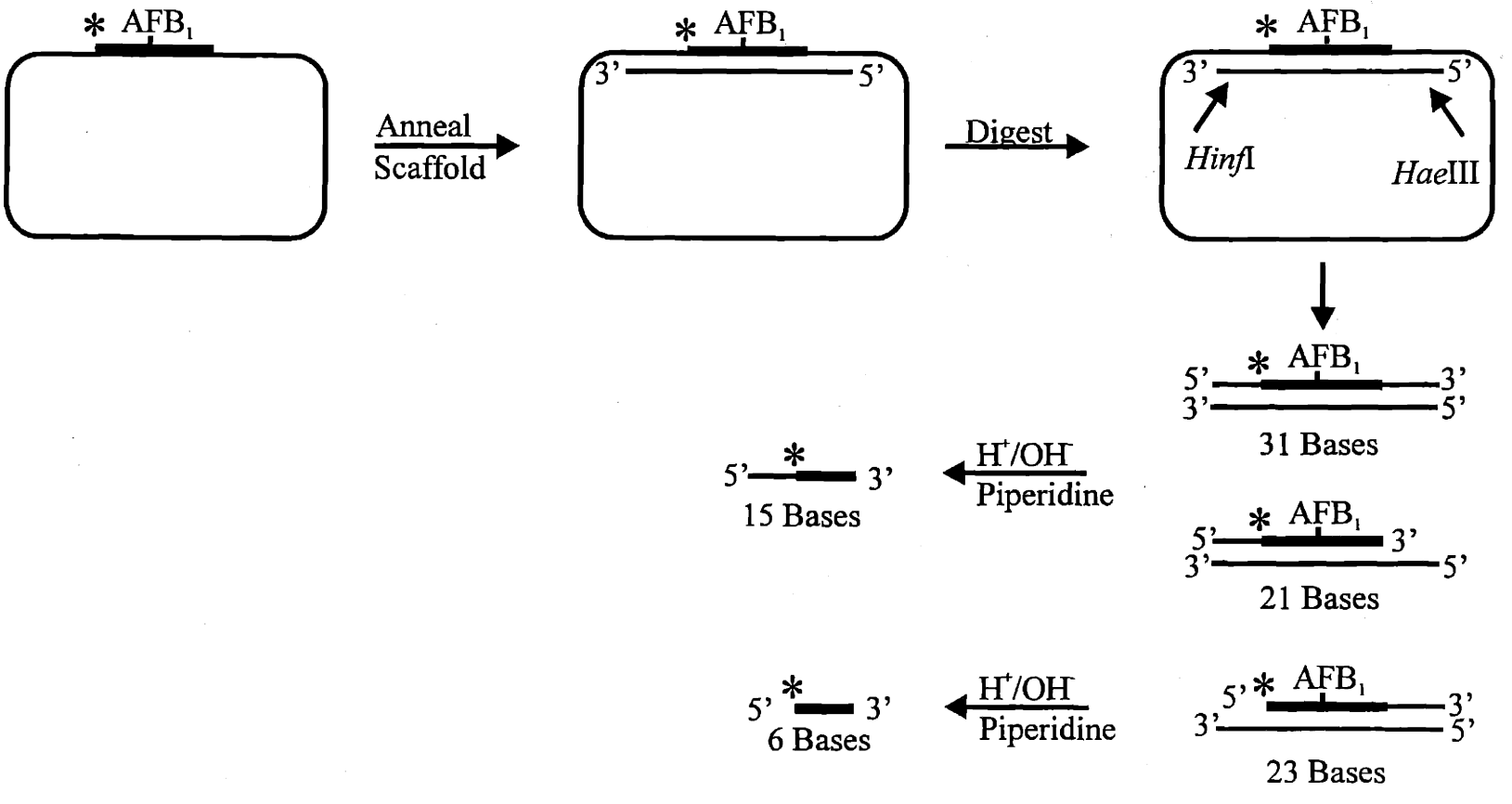

Figure 2.11: Schematic of characterization of AFB_1-containing genomes. After genomes were constructed, characterization experiments were carried out to determine the integrity of the adducts after the construction procedure. First, the 53 base scaffold was once again annealed to the genome, yielding double stranded DNA regions that contained the *Hae*III and *Hinf*I restriction sites. Upon digestion with these enzymes, a 31 base region containing the insert is excised from the genome. Twenty-one or 23 base products correspond to incomplete ligations, giving an indication of the ligation efficiency of each insert. The products were then treated with acid and base and/or piperidine. Unmodified products will remain intact while modified products will be cleaved,yielding either 6- or 15-base products.

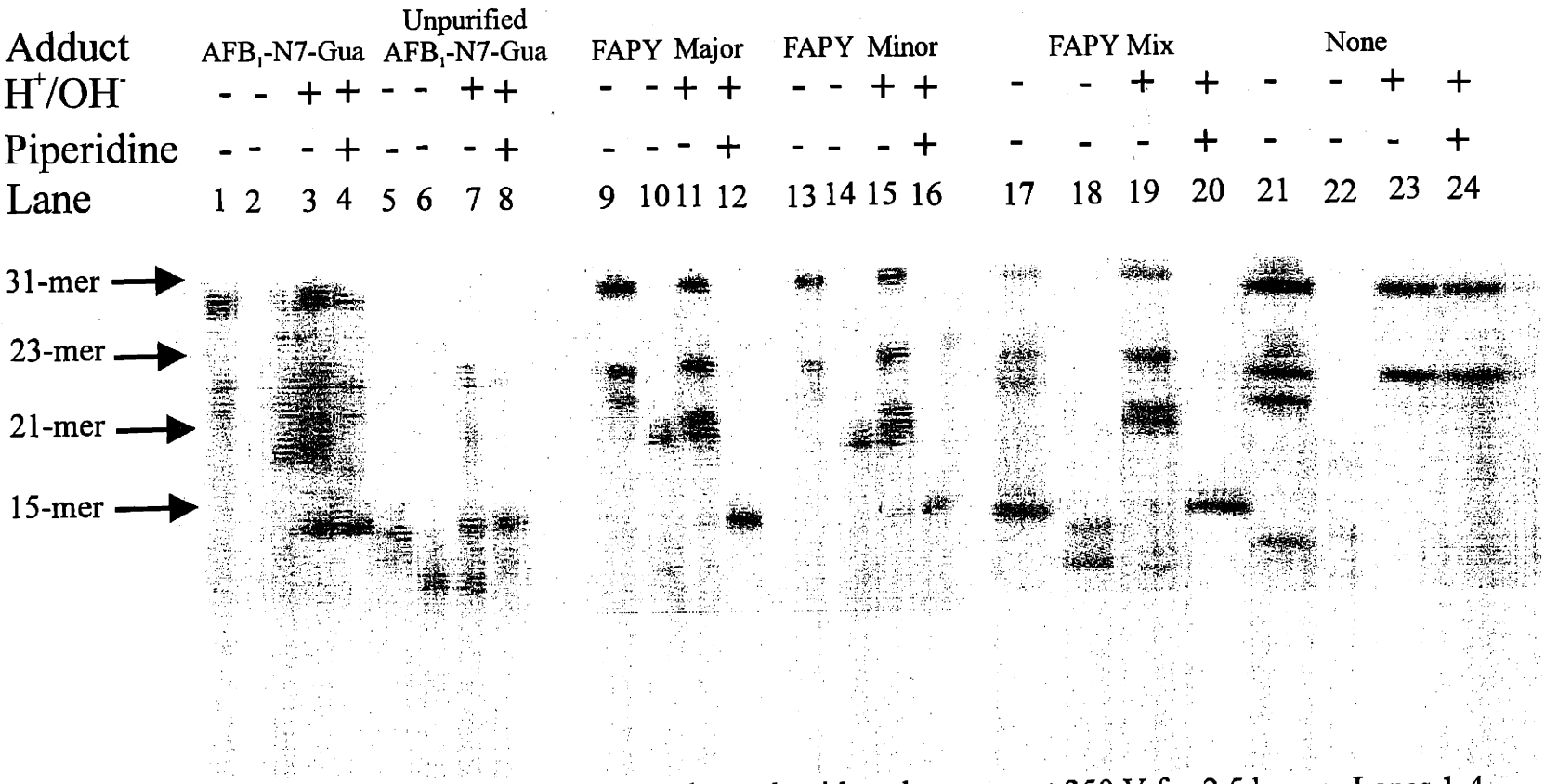

Figure 2.12: Genome characterization. A 20% polyacrylamide gel was run at 350 V for 2.5 hours. Lanes 1-4, AFB_1-N7-Gua; lanes 5-8, unpurified AFB_1-N7-Gua; lanes 9-12, AFB_1-FAPY major; lanes 13-16, AFB_1-FAPY minor; lanes 17-20, AFB_1-FAPY mix; lanes 21-24, unmodified control. Lanes 1, 5, 9, 13, 17, and 21 are untreated controls. Lanes 2, 6, 10,14, 18, and 22 are samples treated with ExoIII and UDG. Lanes 3, 7, 11, 15, 19, and 23 are samples heated in the presence of acid and base. Lanes 4, 8, 12, 16, 20, and 24 are samples treated with acid, base, and piperidine. Arrows indicate different sized fragments.

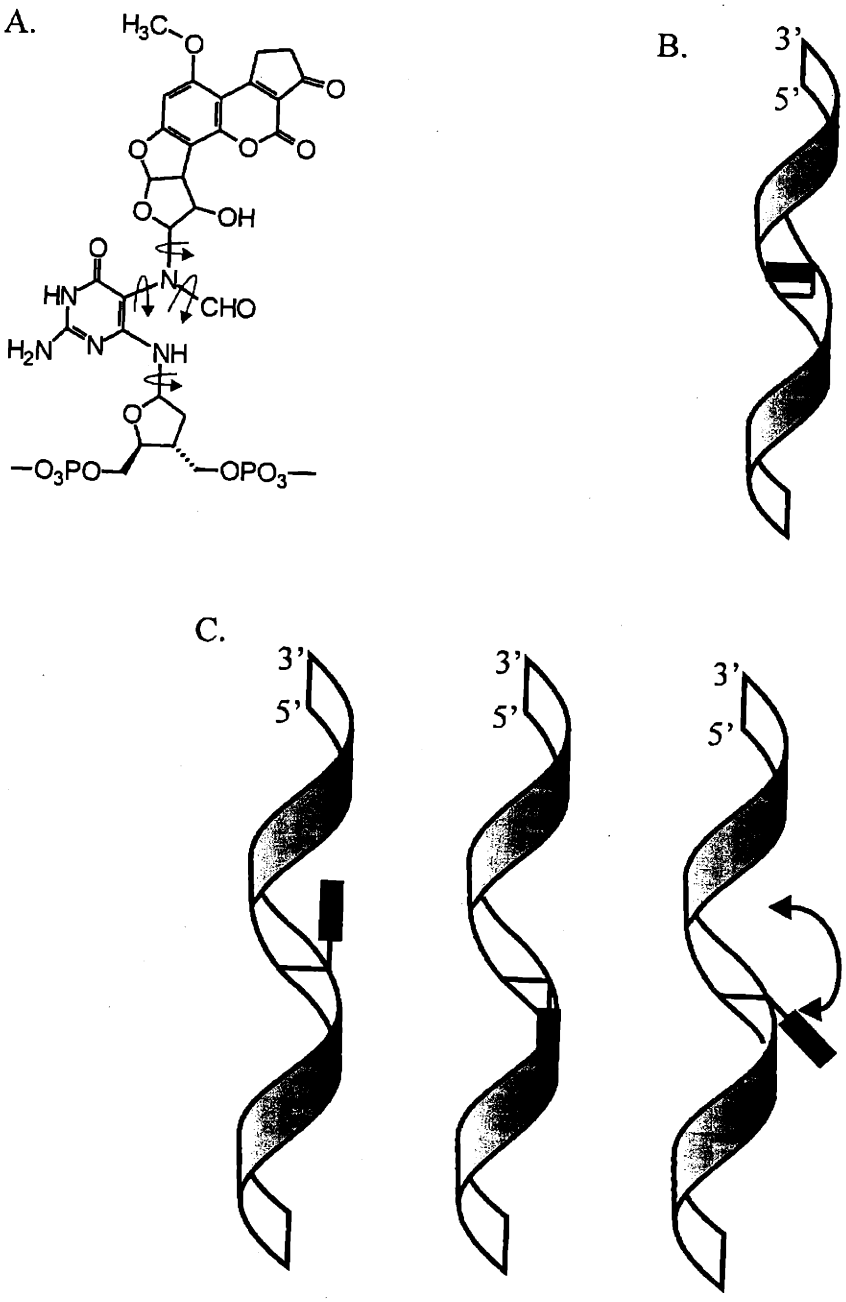

Figure 2.13: Possible structures of FAPY minor. A. There are several bonds around which the AFB_1-FAPY molecule can rotate. B. Orientation of FAPY major in DNA (5' intercalation). C. Possible structures of FAPY minor rotamers.

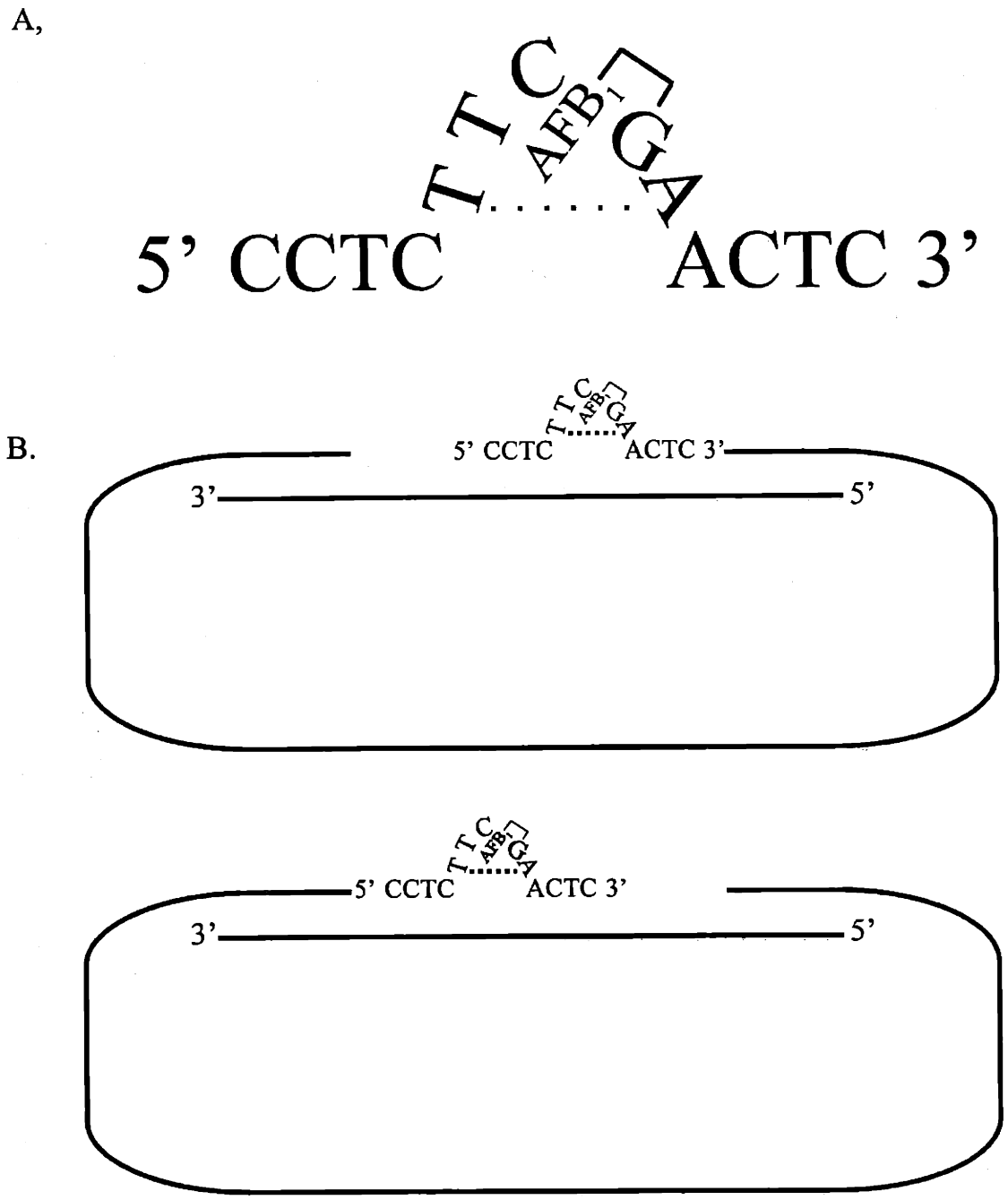

Figure 2.14: Possible "looped out" structure of AFB_1 DNA. A. AFB_1 adducts in single stranded DNA may stabilize a "looped out" structure by intercalation into the region with a partial double stranded nature. B. "Looping out" in the 13 base inserts may afford for partial ligation, either 5' only or 3' only.

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Chapter 3

Tolerance of the Aflatoxin B_1 Fomamidopyrimidine Adduct by Cells Expressing Different Bypass Polymerases

Introduction

This chapter focuses on how cells that express different bypass polymerases tolerate different AFB_1-DNA adducts. A system was developed that 1) employed the use of DNA modified with one specific chemical form of the AFB_1 adduct, 2) allowed for host cells with different gene expression patterns, 3) screened for wild type and mutant DNA sequences, and 4) the facile isolation of DNA for further analysis.

The M13 Bacteriophage

As discussed in the previous chapter, the first point above can be addressed by using a M13 viral genome that is site-specifically modified to contain an AFB_1-DNA adduct of choice. This single stranded genome contains a stem-loop region that can be manipulated by genetic engineering techniques (Messing et al., 1977; Bailey et al., 1996a; Basu et al., 1993; Mackay et al., 1992; Hines and Ray, 1980; Kramer et al., 1984). This stem-loop region is located within the gene for the α-subunit of β-galactosidase (Van Den Hondel et al., 1975; Van Den Hondel and Schoenmakers, 1975; Van Den Hondel and Schoenmakers, 1976; Messing et al., 1977). M13mp7L2 is specifically constructed such that a frameshift is incorporated; that is, a nonsense polypeptide is produced upon transcription and translation. It is within this region that we inserted the 13 base oligonucleotide described in **Chapter 2**, which will shift the gene back into frame, producing a functional protein.

M13 is a lytic filamentous bacteriophage containing a single stranded genome (Kessler-Liebscher et al., 1975). It infects bacteria by injecting its DNA, which the host cell machinery then replicates. A temporary double stranded form of the genome is produced, designated the "replicative

form". This DNA is then transcribed and translated, producing the phage coat proteins, and the assembly of progeny phages takes place. The progeny phages are packaged with only single stranded DNA [the (–) strand] and proceed to lyse the host cell. This releases the progeny phages, which in turn infect and lyse the surrounding bacteria, forming a plaque. In the system used here M13 DNA that contains a single AFB_1 adduct is electroporated into a specific host cell. If the host cell expresses a polymerase that can efficiently bypass the adduct, the genome will be replicated successfully, with or without a mutation at the site of DNA damage. The M13 life cycle can be carried out, and the cells will be lysed, forming a plaque. If the adduct is a block to replication, or if the host cells are knocked out for the polymerase necessary to bypass the adduct, the genome will not be replicated, there will be no lysis, and no plaque will form (Hines and Ray, 1980). It is in this manner that we can tell how well a cell is able to tolerate each specific type of DNA adduct.

It should be noted that the cell strains into which the M13 genomes are electroporated are termed "transfection strains" and those that produce the lawn of bacteria are termed "plating bacteria". The transfection strains and the plating bacteria are each unique. It is only necessary for the transfection strain to posses the desired genetic background, since it is in this strain that the M13 genomes will undergo the first round of replication. Once this first encounter takes place, any mutations will be fixed and propagated by subsequent rounds of replication. Therefore, the plating bacteria serve solely as a medium for infection and lysis by progeny phage. However, the plating bacteria do play a role in the LacZ assay, as described below.

The LacZ Assay

The *lacZ* gene produces β-galactosidase, an enzyme involved in the break down of lactose to glucose and galactose (Beckwith and Zipser, 1970). The β-galactosidase α-subunit is an integral part of this enzyme, which will only cleave its substrate if this subunit is present. The cell strains used in this study have a deletion in the α-subunit of their genomic copy of the *lacZ* gene. Therefore, upon electroporation of the M13 genomes described above, only those genomes that produce a functional copy of the α-subunit will be able to complement this deletion. Any cell strain that has this genomic deletion can be used in this experimental system, which addresses the second point above.

The complementation assay can be visualized using a mock substrate for β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). When this substrate is cleaved, it is dark blue in color. In order to carry out this reaction successfully, isopropyl-β-D-thiogalactopyranoside (IPTG) is used to stimulate transcription (Beckwith and Zipser, 1970). In this system, the successful insertion of the 13 base oligonucleotide will allow for transcription of a wild type copy of the α-subunit, producing a fully functional protein, and cleavage of the mock substrate will occur. Since the M13 phage lyses the cells, a dark blue plaque is observed on a lawn of plating bacteria.

The above system was engineered to screen for a specific type of mutation, a G to T transversion, at the site of AFB_1 modification (Bailey et al., 1996b). The sequence of the 13 base oligonucleotide was chosen such that this mutation would produce the *ochre* stop codon, TAA.

In a wild type cell strain, this mutation would lead to a clear plaque, since an incomplete α-subunit would result. However, this event would make the mutation indistinguishable from frameshifts, large deletions, or uncut M13 genomes. To make it possible to observe the ochre mutants specifically, the plating bacteria used here contain an *ochre* suppressor tRNA, which partially reads through the stop codon, resulting in a light blue plaque. Other point mutations and small in-frame deletions yield dark blue plaques. This screening method allows for the third point above to be addressed. The mutational aspect of this system will be discussed in detail in **Chapter 4**.

After the transfection strain is electroporated with M13 DNA and a portion is plated, the remaining cells are diluted and grown in liquid culture. This process allows cells to replicate and become infected with phages, lyse, and expel phages into the media to infect other cells. After this culture is grown, the cells and debris can be removed by pelleting, leaving behind media that contain progeny phages. These progeny phages accurately represent the proportion of wild type and mutant DNA, and they in turn can be scored as such when plated. This procedure affords a more than adequate supply of samples to be processed via DNA sequencing, addressing the fourth point above.

Materials and Methods

Enzymes and Chemicals

The *Sfu*I restriction enzyme (10,000 U/ml) and G-50 and G-25 Sephadex columns were obtained from Roche. IPTG and X-gal were purchased from GOLD Biothechnology. Dimethylformamide (DMF) was purchased from Fischer Scientific. Maxiprep kits were from Qiagen. M13mp7L2 was from C.W. Lawrence.

Cell Strains

E. coli strains used were the following: DL7 (AB1157; lacΔU169) and DL7/pGW16 (DL7; pGW16 *mucAB* Amp^r) (Essigmann lab, MIT); GW8023 (AB1157; *sul*A11; ilv^{TS}; lacΔU169; pro^+; Δ*umuDC* 595::cat) and a P1 lysate (*DinB*::kan) (Graham Walker, MIT); and NR9050 (F' *prolacIZΔM15*, Δ*prolac*, *suB*) (R. Schaaper, NIH).

Construction of a UmuDC Knockout Strain that Contains MucAB

Plasmid DNA (pGW16) containing the gene for the MucAB bypass polymerase was isolated from the DL7/pGW16 cell strain. An overnight culture of DL7/pGW16 was grown in LB media containing 50 μg/ml ampicillin, diluted 1:1000, and grown for 8 hours at 37°C. Another 1:1000 dilution was made into a final volume of 500 ml and was incubated at 37°C for 15 hours. A Qiagen maxiprep kit was used in order to isolate the plasmid DNA. A Beckman-Coulter DU-65 UV spectrophotometer was used to quantitate the DNA at $\lambda = 260$ nm. An overnight culture of GW8023, a UmuDC knockout strain, was grown and diluted 1:50. The subculture grew for 1.75 hours, yielding an OD_{600} of 0.461. GW8023 was prepared for

electroporation as described in the following section. Cells were electroporated with 4 ng pGW16 plasmid DNA and were immediately plated on LB plates containing 50 μg/ml of ampicillin. One colony was picked and grown overnight in ampicillin. An 850 μl aliquot was added to 150 μl of 80% glycerol and stored at –80°C. This cell strain was designated MES1 (AB1157; Δ*umuDC*; pGW16 *mucAB* Amp^r).

Construction of dinB *Knockout Strains*

Overnight cultures of DL7 (WT) and GW8023 (*umuDC* knockout) were grown in LB media. Five ml of overnight culture were mixed with 5 ml of MC buffer (0.1 M $MgSO_4$, 0.005 M $CaCl_2$) and incubated at 37°C for 15 minutes. Three 100 μl aliquots of each cell strain were added to Eppendorf tubes containing: 1) no P1 lysate, 2) 5 μl P1 lysate, and 3) 10 μl P1 lysate. These mixtures were then incubated at 37°C for 20 minutes. Next, 200 μl of 1 M sodium citrate was added to each tube, and the entire volume from each tube was plated on LB plates containing 40 μg/ml of kanamycin. The plates were incubated overnight, and colonies were selected, grown using 40 μg/ml of kanamycin in liquid culture, and frozen down as above. The cell strains were designated MES2 (AB1157; lacΔU169; Δ*dinB*::kan) and MES3 (AB1157; Δ*umuDC*; Δ*dinB*::kan).

Transformation of AFB_1-Modified Genomes into Cell Strains

Once genomes were constructed and desalted, as in ***Genome Construction***, **Chapter 2**, they were kept on ice and used immediately to transform either DL7, DL7/pGW16, GW8023, MES1, MES2, or MES3. All of these cell strains were used without UV irradiation; however, DL7 and DL7/pGW16 additionally underwent UV irradiation. Cells were prepared for transformations

according to whether or not they would undergo UV irradiation to induce the SOS response. First, a single colony was inoculated into 10 ml of LB medium (strains containing pGW16 had 50 μg/ml ampicillin added to the media) and grown at 37°C overnight (10-15 hours). These cultures were then diluted 1:50 and grown for 1.5-2 hours at 37°C (OD_{600} between 0.5 and 0.6). The cells were kept on ice for 10-15 minutes prior to centrifugation. Cells were transferred to 250 ml centrifuge bottles and centrifuged 5 minutes at 4000x*g* (5000 rpm in a Sorvall GSA rotor); the supernatant was decanted, and the cells were thoroughly resuspended in 50 ml of ice cold, sterile water. The cells that were to be UV irradiated were resuspended in ice cold, sterile 10 mM $MgSO_4$ instead. Cells were then transferred to petri dishes (25 ml/dish) and irradiated in a Stratalinker UV crosslinker at 45 J/m^2. The cells were then immediately transferred to an equal volume of pre-warmed 2xLB medium and incubated at 37°C for 40 minutes. All cells were centrifuged again for 10 minutes at 8000x*g* (7000 rpm in a Sorvall GSA rotor), regardless of UV irradiation. The supernatant was decanted, the cells were resuspended in 25 ml of sterile, ice-cold water, and they were transferred to a smaller centrifuge tube. Cells were then centrifuged for 10 minutes at 13,000x*g* (9500 rpm in a Sorvall SA-600 rotor), the supernatant was decanted, and the cells were resuspended in the volume appropriate for transformations (200 μl/transformation).

Plating bacteria (NR9050) were grown as above, except the 1:50 dilution was allowed to grow for approximately 3 hours. These cells were removed from the incubator prior to transformations and allowed to cool slowly at room temperature until ready for use (10-15 minutes). They were then mixed with IPTG and X-Gal [300 μl cells, 40 μl X-gal (40 mg/ml in DMF), and 10 μl IPTG (20 mg/ml in water) per transformation] and kept on ice while being used. Approximately 0.1 picomoles of genome in a volume of 10 μl were mixed with 190 μl of the transfection strain in 2

mm ice cold BTX cuvettes and electroporated with a BTX Electro Cell Manipulator 600 system. Electroporations were conducted at 2.5 kV/resistance, 129 Ω resistance timing, and 800 μF capacitance timing. Cells were immediately given 1 ml of SOC medium and diluted as appropriate. Appropriate volumes of electroporated cells were then added to 350 μl of the plating bacteria/X-gal/IPTG mixture, added to 2.5 ml of soft agar, and plated on B-broth plates. Plates were placed in a 37°C incubator overnight, and plaques were counted the following day. The remaining electroporated cells were added to 2 ml of SOC medium and incubated in a roller drum at 37°C for 1.5 hours. They were then aliquotted into Eppendorf tubes and microcentrifuged at 15,000x*g* (13,200 rpm) for 5 minutes. The supernatant was decanted into a fresh tube and the centrifugation was repeated. The supernatant, which contained the progeny phage, was then stored at 4°C until it was needed for further use.

Pooling Progeny Phages

Progeny phages were plated at appropriate dilutions to obtain titer and to confirm the results of the electroporations. Cultures of NR9050 cells were grown and prepared as above with IPTG and X-gal. Dilutions of progeny phages were made in SOC media and were mixed with the plating bacteria/IPTG/X-gal mixture in 2.5 ml of soft agar. They were plated on B-broth plates and incubated overnight at 37°C. Plaques were counted the following day.

Results

Construction of Cell Strains

Cell strains MES1 (*umuDC* knockout expressing MucAB), MES2 (*dinB* knockout), and MES3 (*umuDC* and *dinB* double knockout) were successfully constructed. MES1 was constructed by electroporating a MucAB-expressing plasmid into a cell strain that had *umuDC* knocked out. MucAB is constitutively expressed due to a mutation in the promoter binding site for the repressor LexA (McNally et al., 1990) (Figure 1.6). MES2 and MES3 were constructed using a knock in procedure. This was achieved by using a P1 lysate to transduce the *dinB*::*kan* allele into the genome via homologous recombination into the open reading frame of *dinB* (Figure 3.1). The bacterial strains (MES2 and MES3) containing the newly integrated allele were selected for their resistance to kanamycin. This procedure was carried out with both the wild type (DL7) and *umuDC* knockout (GW8023) strains. It should be noted that the open reading frame of *dinB* also controls three other genes of unknown function, *yafN*, *yafO*, and *yafP* (www.ncbi.nlm.nih.gov:80/PMGifs/Genomes /micr.html). As studied so far, these genes do not appear to have an effect on mutagenesis (V. Godoy, Walker lab, MIT, personal communication). It is also of note that GW8023 and DL7 are not isogenic, but they do not differ significantly, to our knowledge, in ways that would affect these studies.

Transformation of AFB_1-Modified Genomes

Genomes containing an unmodified insert or an insert containing either the FAPY major, FAPY mix, FAPY minor, or AFB_1-N7-Gua adduct were electroporated into *E. coli*. Cells expressing different bypass polymerases were used in these experiments to probe how well they tolerated AFB_1 adducts. These polymerases were either expressed, due to exposure to UV light (Figure 1.6) or to constitutive expression from plasmid DNA in the case of MucAB, or knocked

out, as in the UmuDC and DinB controls. Cells not induced for the SOS response (DL7) or cells that expressed the bypass polymerases UmuDC (UV irradiated DL7), MucAB (DL7/pGW16), or both UmuDC and MucAB (UV irradiated DL7/pGW16) were used in the electroporations. Two cell strains were used as controls to determine if UmuDC is a "leaky" gene: a UmuDC knockout (GW8023) and a UmuDC knockout that expresses MucAB (MES1). Additionally, two more cell strains were used to determine if DinB (MES2 and MES3) plays a role in tolerating AFB_1.

After electroporation the cells were mixed with a plating bacterium (NR9050) that contains an *ochre* suppressor. The oligonucleotides were engineered such that a wild type insert allows proper in frame reading of the *lacZ* sequence, generating a dark blue plaque. A G to T mutation at the site of modification (GAA to TAA) forms a stop codon that can be partially read through by the *ochre* suppressor tRNA, leading to a light blue plaque. Other point mutations and small in-frame deletions also yield dark blue plaques. Clear plaques would result from frameshifts, large deletions, or uncut M13 genomes (Figure 3.2).

Each adduct-containing genome was electroporated into each cell strain 5-30 separate times to yield one set of data. Every graph shown represents four sets of data, each set generated from a separate genome construction event (**Appendix A**). Upon transformation the total number of blue plaques was scored as the number of infective centers. Each infective center represents a single transformation event by one M13 genome that was successfully replicated in the host cell, yielding a wild type or mutant outcome. Thus, we can determine how effectively the replication machinery of the cell can handle the challenge of a single AFB_1 adduct.

Figure 3.3 compares the number of infective centers per ml of transformed cells for genomes containing an unmodified, AFB_1-N7-Gua, FAPY major, or FAPY mix oligonucleotide insert in each of the genetic backgrounds described above. The numbers above each bar represent the total number of transformations carried out for that strain. Unmodified inserts were tolerated an average of 1-3 orders of magnitude better than those containing a modified insert. When cells were challenged with the FAPY major adduct alone, the M13 viral genome was replicated less efficiently, yielding fewer blue plaques (infective centers), than when a comparable genome containing FAPY mix was present. Therefore, FAPY mix is not as lethal a block to replication as FAPY major alone, so it is possible that cells that survive the challenge with AFB_1-FAPY contain mostly the FAPY minor component of this mixture. Indeed, a preliminary set of transformation data was obtained for genomes that contained predominantly FAPY minor, and they exhibit similar properties to those containing FAPY mix (data not shown). Cells tolerated the AFB_1-N7-Gua adduct one to two orders of magnitude better than the FAPY mix adduct. This difference indicates that the AFB_1-N7-Gua adduct is far less effective as a replication block when compared to the AFB_1-FAPY adduct.

The cell strains employed in these studies responded uniquely to different forms of AFB_1 adduct. Several observations can be made about this point. First, the reverse pattern of lesion tolerance is observed for the AFB_1-N7-Gua adduct and the AFB_1-FAPY mix adduct. When challenged with FAPY mix, cells expressing MucAB survive the best, whereas when cells are challenged with AFB_1-N7-Gua, those expressing UmuDC survive the best. Second, cells expressing MucAB exhibit the most survival when challenged with AFB_1-FAPY mix and the unmodified control. Third, cells expressing UmuDC exhibit the most survival when challenged

with AFB_1-N7-Gua or the unmodified control. Fourth, when both UmuDC and MucAB are simultaneously expressed by the cell, the UmuDC phenotype is observed. Fifth, UmuDC bypasses AFB_1-N7-Gua 1000-fold better than AFB_1-FAPY. Sixth, there is 1000-fold more lesion bypass, independent of the adduct present, when UmuDC is expressed as opposed to when it is knocked out. Finally, UmuDC is leaky; i.e., it is active at a constitutive level when not intentionally induced.

Preliminary results for the **dinB** *Knockout*

One set of experiments has been performed using MES2 and MES3. These cell strains have been electroporated with genomes containing AFB_1-FAPY mix 8 times each. The results obtained for the UmuDC knockout and the DinB knockout are similar, but when both genes are knocked out, there is no survival at all. The suggestion to initiate studies on DinB came late in this work. The studies are less far advanced than those on UmuDC and MucAB.

Plating Progeny Phage

When progeny phages are grown, the number of blue plaques increases due to the expansion of the phage population. Unlike the infective centers obtained immediately after the electroporations, these plaques are probably not an indication of survival. Instead, the ratio of light blue to dark blue plaques is used to calculate mutation frequency and to obtain the titer of the progeny phages in order to carry out the experiments described in the following chapter. The calculation of percent light blue plaques from progeny phage data can be found in the **Appendix A.**

Discussion

AFB_1-FAPY Major

In vivo (Levy et al., 1992) and *in vitro* (using polI) (Refolo et al., 1985) polymerase arrest experiments have demonstrated that both AFB_1-N7-Gua and AFB_1-FAPY adducts block DNA synthesis on single stranded DNA templates, particularly those sequences capable of double stranded configuration. However, these earlier experiments do not take into account the two different species of the FAPY adduct. This study has established that the AFB_1-FAPY major adduct is the strongest *in vivo* block to replication of all the aflatoxin adducts studied here, regardless of which bypass polymerase is induced to help the cell overcome the DNA damage. One reason for this observation may be that the FAPY major form of the adduct is intercalated into the DNA helix (Figure 1.8C) (Mao et al., 1998) and stabilizes the helix in two ways: 1) by unwinding it by 15° and increasing the stacking interactions, and 2) by increasing the T_m of the duplex by 15°C. The adduct may also be involved in hydrogen bonding with neighboring bases. This stabilization may make it more difficult the polymerases to bypass the lesion (Figure 3.4A). However, there is one confounding factor to be considered. These studies initially involve single stranded DNA, so the first round of replication would not involve the properties of the DNA duplex.

It is possible that the structure of the single stranded DNA used here resembles that of double stranded DNA enough so that the hypothesis stated above may still be applied to these results (Figure 3.5B). A second possibility is that the AFB_1-FAPY adduct itself induces a structural change in the single stranded DNA, forcing a more favorable environment for itself. A hypothetical model for such a structure can be found in Figure 2.14A. Alternatively, AFB_1-

FAPY major may be able to evade replication blockage on first pass, allowing synthesis of the opposite strand. The complementary strands can then bind together to form double stranded DNA, and this can, in turn, lead to the more stable DNA duplex (Figure 3.5C). One last hypothesis is that DNA repair plays a role in cell survival. If DNA containing FAPY minor lesions is recognized and repaired early on, the cells will survive. The repair process is sometimes mutagenic (Figures 1.3 and 1.4), due to the fact that the polymerase (polI) that fills in the gaps formed during the repair process has a low fidelity (Friedberg et al., 2001). This property could explain the mutations observed. The structure of FAPY major may not signal repair, and therefore, it could subsequently encounter the DNA polymerases that become blocked and cause cell death. Excision repair assays (Oleykowski et al., 1993; Chetsanga and Frenette, 1983) have been performed on AFB_1-FAPY adducts, but there has not been a distinction between the FAPY major and FAPY minor isomers in these studies.

The AFB_1-FAPY major lesion undoubtedly contributes to the toxicity of AFB_1, which could be extrapolated to humans, since mammalian cells have similar lesion bypass mechanisms [DinB is analogous to human polκ (Masutani et al., 1999), and UmuDC is analogous to human polη (Gerlach et al., 1999)]. In fact, studies have shown that similar mutations result when either bacterial UmuDC or human polη respond to DNA lesions such as *cis-syn* thymine dimmers (Masutani et al., 1999; Napolitano, et al. 2000) or B(a)P adducts (Lenne-Samuel, et al. 2000; Chiapperino, et al. 2002). Additionally, similar DNA repair systems, such as nucleotide excision repair (Figure 1.3), base excision repair (Figure 1.4), and recombination (Figure 1.5), are conserved from bacteria to humans (Friedberg et al., 2001).

AFB_1-FAPY Mix

In general cells challenged with AFB_1-FAPY mix survive an order of magnitude better than those challenged with AFB_1-FAPY major. Since the FAPY major adduct is lethal to cells, it can be concluded that the surviving cells contain primarily the FAPY minor component of the adduct. FAPY minor may be less lethal to cells due to its structure, as proposed in **Chapter 2**, specifically Figure 2.13. The fact that cells show a different survival pattern depending on which lesion of AFB_1-FAPY is present is consistent with work in which similar bulky DNA adducts such as PhIP (Brown et al., 2001), AAF (Harsch and Vouros, 1998), and AF (Belguise-Valladier and Fuchs, 1995) exhibit the characteristics of structural duality. These studies and others propose that a DNA adduct in one conformation may be easily bypassed and may or may not be mutagenic, whereas in another conformation it may be a complete block to replication and be detrimental to the cells (Neidle et al., 1984).

AFB_1-N7-Gua

Phages containing the AFB_1-N7-Gua adduct generate about two orders of magnitude more infective centers than those containing the AFB_1-FAPY adducts, indicating that the AFB_1-N7-Gua adduct is a less formidable threat to the replication machinery of the cell. In some cases the cells even survive as well as unmodified genomes. This, too, is most likely based on structure. Since this adduct kinks the DNA helix, it may be more easily bypassed by the replicative polymerase and UmuDC, subsequently increasing survival. Additionaly, the kinked DNA structure may be a better substrate for DNA repair enzymes. It should be noted that Bailey *et al.* carried out preliminary experiments examining the comparative tolerance of cell strains expressing UmuDC and/or MucAB for the AFB_1-N7-Gua adduct (Bailey et al., 1996b). The

focus of that work was on the mutagenicity of this adduct, and it did not attempt to investigate quantitatively cell survival. There is a difference between those results and the ones presented here, but this work performed more quantitative experiments.

Studies have demonstrated AFB_1-N7-Gua to be a replication block *in vitro* (Refolo et al., 1985). These studies were not carried out in cell extracts; they were conducted only in the presence of polI, which is not the functional replicative polymerase that encounters these adducts *in vivo*. DNA repair assays have also been performed to show that this adduct is repaired by NER (Oleykowski et al., 1993), although the AFB_1-FAPY adduct is equally repaired in this system. Consequently, AFB_1-N7-Gua may not be as strong a replication block as AFB_1-FAPY, and it may be repaired more efficiently. Taken together, these results support the notion that AFB_1-N7-Gua is better tolerated than AFB_1-FAPY.

Tolerance of Cell Strains for AFB_1 Adducts

It is evident that cells expressing different bypass polymerases respond differently to the various AFB_1-DNA adducts. There are two main conclusions that are supported by the data shown here. The first is that the structures of the different adducts elicit unique responses from the different bypass polymerases expressed by the cells. In support of this statement, it is observed that when cells are challenged with AFB_1-FAPY mix, those expressing MucAB exhibit the highest survival, whereas when cells are challenged with AFB_1-N7-Gua, those expressing UmuDC survive the best. A plausible explanation may be that these two adducts form unique secondary structures with DNA, and UmuDC may be better at recognizing and bypassing AFB_1-N7-Gua, while MucAB may be better at recognizing and bypassing AFB_1-FAPY mix.

Additionally, the AFB_1-N7-Gua adduct is tolerated as well as unmodified DNA when UmuDC is expressed. This observation implies that UmuDC efficiently recognizes and easily bypasses this form of the adduct. This is not the case for AFB_1-FAPY mix, since it is best tolerated by MucAB. It is possible that UmuDC cannot efficiently recognize the structure formed by AFB_1-FAPY mix, so MucAB has time to replace the former polymerase and take over replication (Figure 3.5C). Indeed, UmuDC bypasses AFB_1-N7-Gua about 1000-fold better than AFB_1-FAPY.

Additionally, the polymerase that is indigenous to the cell strain used will be the dominant factor in conducting bypass of the adduct. UmuDC is the natural SOS-induced bypass polymerase of *E. coli* (Witkin, 1976; Friedberg et al., 2001; Gerlach et al., 1999; Woodgate and Sedgwick, 1992; Sommer et al., 1993; Tang et al., 1999). MucAB is native to *S. typhimurium*, and it is generally thought to be more mutagenic than UmuDC (Blanco et al., 1982; Blanco et al., 1986; Kulaeva et al., 1995; Hauser et al., 1992; Woodgate et al., 1991; Foster et al., 1988; O'Grady et al., 2000; Watanabe et al., 1994; Bennett et al., 1988; Szekeres, Jr. et al., 1996; Perry et al., 1985). There may be some factors that promote the use of *E. coli* proteins over their foreign counterparts. This hypothesis is supported by the observation that when both UmuDC and MucAB are simultaneously expressed, the UmuDC phenotype is observed, regardless of which adduct is present. Additionally, when UmuDC is expressed, there is a 1000-fold increase in survival compared to cell strains where UmuDC had been knocked out.

These data also support evidence that suggests the UmuDC bypass polymerase may interact with polIII, the *E. coli* replicative DNA polymerase (Sutton et al., 2000; Sutton et al.,

1999; Jonczyk and Nowicka, 1996; O'Grady et al., 2000). It is thought that polIII is normally replicating the DNA, and when it encounters damage, it switches over to UmuDC in order to bypass that damage (Figure 3.5A). Once the lesion is bypassed, polIII resumes normal replication. It is unclear whether UmuDC is physically associated with polIII or if it is in the vicinity and is actively recruited upon encountering DNA damage (Figure 3.5B). If UmuDC is unable to bypass a DNA adduct successfully, the complex may stall, allowing for MucAB to resume (Figure 3.5C). MucAB may be the most efficient bypass polymerase, but it may not have an opportunity to carry out its function unless UmuDC defers. It is possible that UmuDC does not associate with polIII at all, and the difference in survival may solely be due to a direct competition between the two polymerases. In this case UmuDC may bind more tightly to the DNA damage, but it may not be able to repair all types of damage. Therefore, it eventually gets competed away by MucAB.

Finally, it has been demonstrated that the UmuDC operon is "leaky". This implies that even when the SOS response is not induced, UmuDC is expressed at low levels, regardless of whether or not DNA damage is present. The reason for this could solely be due the stress of electroporation or the presence of viral DNA. This result also supports the model in Figure 3.5B, in that UmuDC would have to be constitutively present at low levels and may subsequently be upregulated upon the encounter of DNA damage.

Knockouts and the Role of DinB

It has been discussed above that the UmuDC knockout shows 1000-fold less survival than cell strains that are not induced for the SOS response, implying "leakiness". In cell strains

that have been knocked out for UmuDC but express MucAB, the survival should resemble that of cell strains expressing MucAB alone where UmuDC is not knocked out. They do so in the case of the FAPY mix adduct; in the unmodified control, they survive less well than MucAB alone, and in the case of AFB_1-N7-Gua, they survive better than MucAB alone. One explanation of this result is that in the case of AFB_1-FAPY mix, MucAB is carrying out its nascent function. In cell strains challenged with AFB_1-N7-Gua, MucAB takes over, since UmuDC is no longer available to compete it away or inihibit its function. This theory is supported by the fact that UmuDC has a greater opportunity to recognize AFB_1-N7-Gua, and it is not directly competitive because MucAB works just as well in the UmuDC knockout. This observation supports the model for interaction with polIII over the direct competition model.

DinB is another polymerase (polIV) that may play a role in bypassing AFB_1 adducts. It was hypothesized that DinB could be redundant for lesion bypass in cells that are knocked out for UmuDC. Preliminary results obtained for the DinB knockout resemble those of the UmuDC knockout, implying that one or the other polymerase must be present in order for some survival to occur when challenged with AFB_1-FAPY mix. The DinB/UmuDC double knockout exhibits no survival at all, which implies that without either of these polymerases present, there is no hope of surviving the damage.

Conclusions

Several conclusions can be drawn from the studies discussed in this chapter. First, the AFB_1-FAPY major adduct is the most lethal to cells. It either strongly blocks DNA polymerase or evades detection by DNA repair enzymes. Second, the survival observed when cells are challenged with AFB_1-FAPY mix may be solely due to the FAPY minor form of the adduct. It can be concluded that structure plays a role in this difference. Third, the AFB_1-N7-Gua adduct is tolerated better than AFB_1-FAPY, perhaps due to structural differences as well. Fourth, adduct structure may also play a role in how they are recognized and processed by the UmuDC and MucAB bypass polymerases. Fifth, the phenotype observed when UmuDC and MucAB are simultaneously expressed corresponds to that of UmuDC alone, illustrating that the indigenous polymerase exhibits a dominant effect over an artificially introduced one. Sixth, UmuDC is a "leaky" operon, showing a level of increased survival when compared to the UmuDC knockout.

Future Work

AFB_1-FAPY

Since AFB_1-FAPY major is lethal to cells, experiments should be conducted to determine why this is so. Polymerase arrest experiments can be performed in single stranded DNA containing only AFB_1-FAPY major adducts to determine if they block replication. These experiments should be carried out using several DNA polymerases, including the replicative bacterial polymerase, polIII, and its mammalian analog, pol α (Tsurimoto et al., 1990). Additionally, the bypass polymerases UmuDC, MucAB, and DinB, as well as their analogs in mammalian cells, polκ (Masutani et al., 1999) and polη (Gerlach et al., 1999), should be tested. Similarly, these can be carried out using AFB_1-FAPY mix to observe any differences.

DNA repair assays should be carried out using FAPY major alone as well as FAPY mix. These should involve bacterial as well as mammalian NER and BER systems. The Essigmann lab has begun this course of experimentation by transfecting plasmids that contain predominantly either the AFB_1-N7-Gua adduct or the AFB_1-FAPY adduct into *E. coli* strains that are knocked out for NER, BER, or both. Survival of these plasmids is scored by transcription of a reporter gene on the plasmid. If the plasmid gets repaired, transcription can occur. Preliminary results show that both adducts are preferentially repaired by NER, but BER does not appear to contribute. FAPY glycosylase, bacterial Mut M, and its human homolog, hOGG, have also been studied *in vitro* to determine if these BER enzymes can remove AFB_1-FAPY adducts from DNA. Preliminary results show that this is not the case. In each of these studies, there is no distinction between FAPY major and FAPY minor (M. Hamm and Y. Alekseyev, personal communication).

AFB_1-N7-Gua

Studies should be carried out as above to determine if the AFB_1-N7-Gua is a block to replication using the relevant polymerases in both bacterial and mammalian systems. This form of AFB_1 adduct must also be tested more specifically in DNA repair assays, using BER, NER, and recombination. These experiments would have to be carried out in reconstituted systems in order to ensure the integrity of this DNA adduct.

UmuDC and MucAB

Structures of the DNA binding sites of the UmuDC and MucAB bypass polymerases should be solved and compared. Structural information will help in determining if the structural differences of the AFB_1-DNA adducts determines which polymerase is better at recognizing and bypassing them. Additionally, experiments should be carried out to determine conclusively if there is a physical interaction between polIII and UmuDC and if an interaction exists between MucAB and polIII as well. Preliminary studies show an interaction between the UmuD and UmuD' subunits with certain subunits of polIII, but the entire complex of proteins has not yet been shown to associate (Sutton et al., 2000; Sutton et al., 1999). To demonstrate this interaction, one can first perform protein crosslinking studies. Additionally, time lapse microscopy can be employed to detect fluorescently-tagged proteins while analyzing their response to DNA damage. Studies should also be carried out to determine if there is a difference in binding constants of MucAB and UmuDC for both modified and unmodified DNA.

Studies with DinB

More experiments must be carried out involving MES2 and MES3 to determine whether DinB actually plays a role in bypassing AFB_1 adducts. DinB should also be included in all the tests proposed for UmuDC and MucAB.

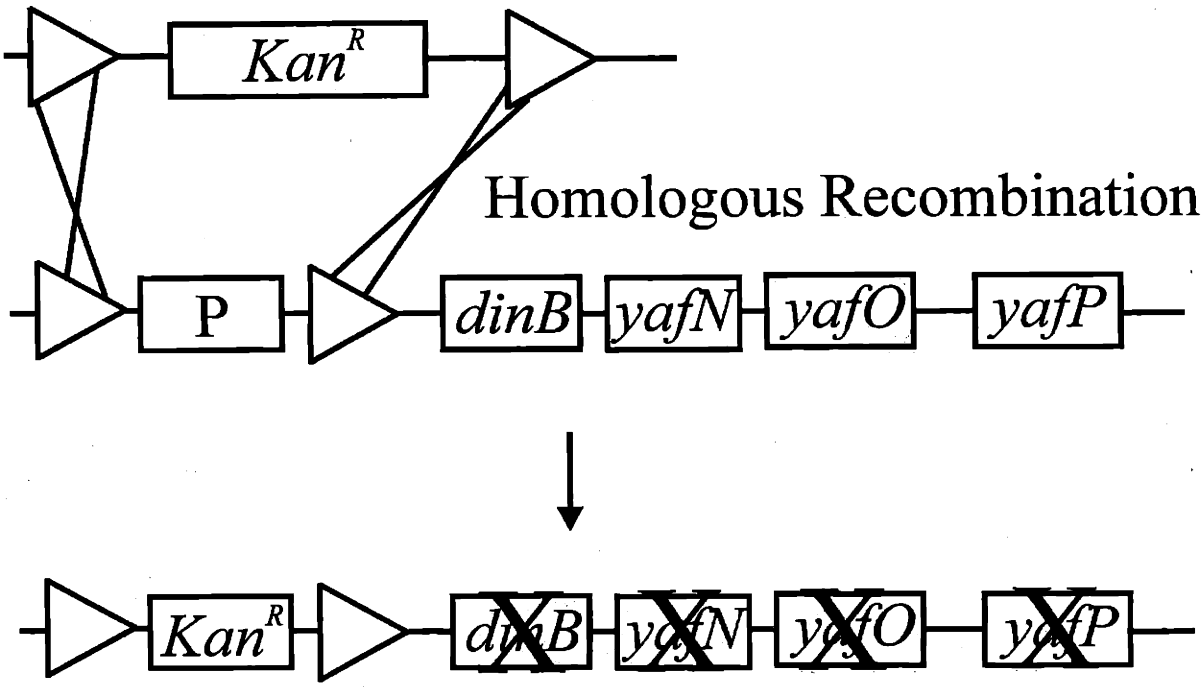

Figure 3.1: Construction of MES2 and MES3. A bacteriophage P1 lysate, grown in a host containing the allele *dinB*::*Kan*, is a deletion of the *dinB* gene open reading frame (P) and a replacement with the kanamycin marker. The P1 lysate was used to transduce the *dinB*::*kan* allele into DL7 and GW8023 genomes via homologous recombination. The genes for *yafN*, *yafO* and *yafP* are also under the control of this promoter..

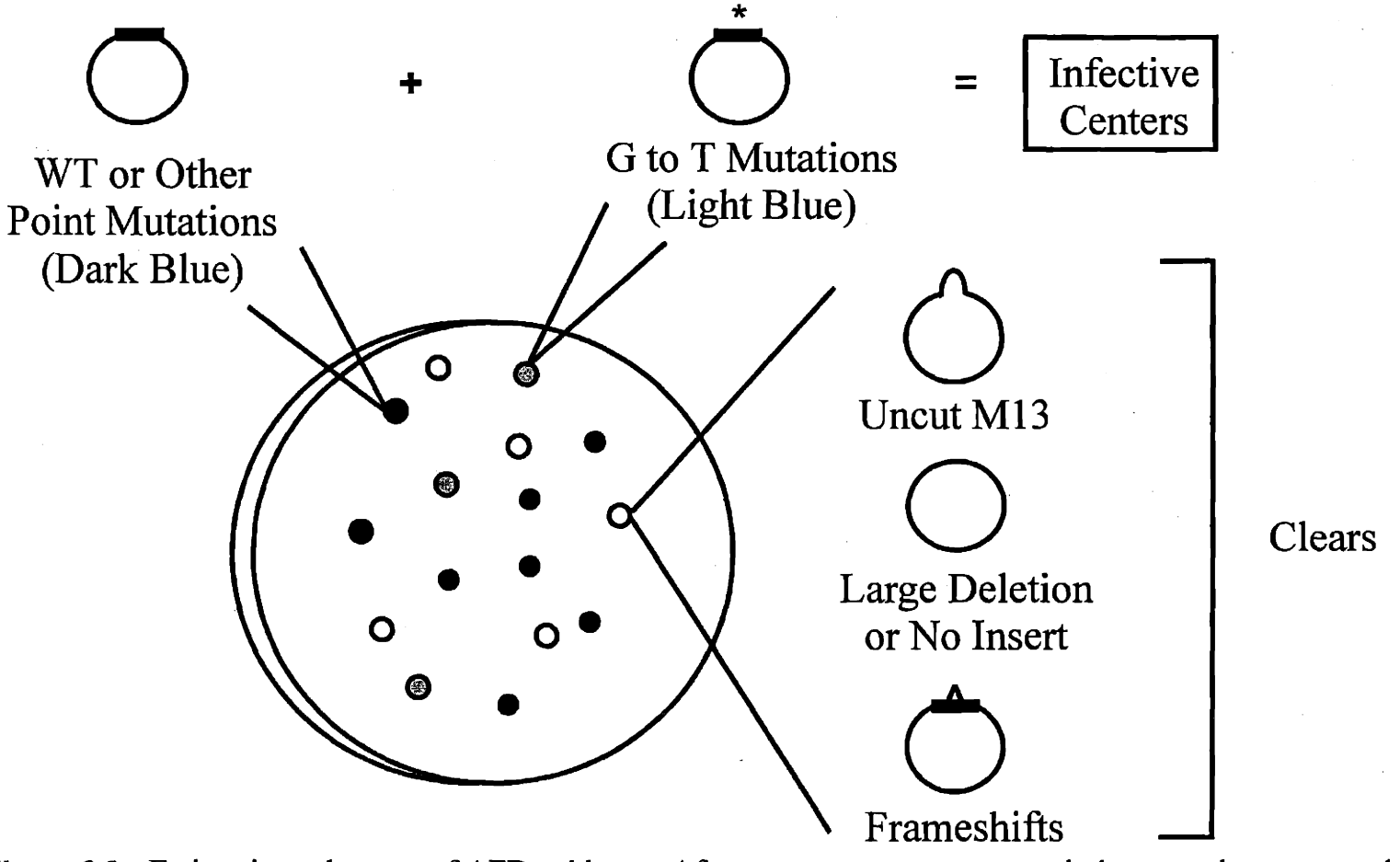

Figure 3.2: Estimating tolerance of AFB_1 adducts. After genomes are constructed, they are electroporated into one of the eight cell strains described in the text. The plating bacteria contain an ochre suppressor. If DNA is replicated successfully and does not contain a G to T mutation, a dark blue plaque will arise on the plate. If a G to T mutation occurs, a stop codon, TAA, will result and will be partially read through by the *ochre* suppressor tRNA, yielding a light blue plaque. The total number of blue plaques is indicative of the tolerance of the cell for the DNA adduct. Clear plaques are the result of either very large deletions, genomes without inserts, or frameshift mutations.

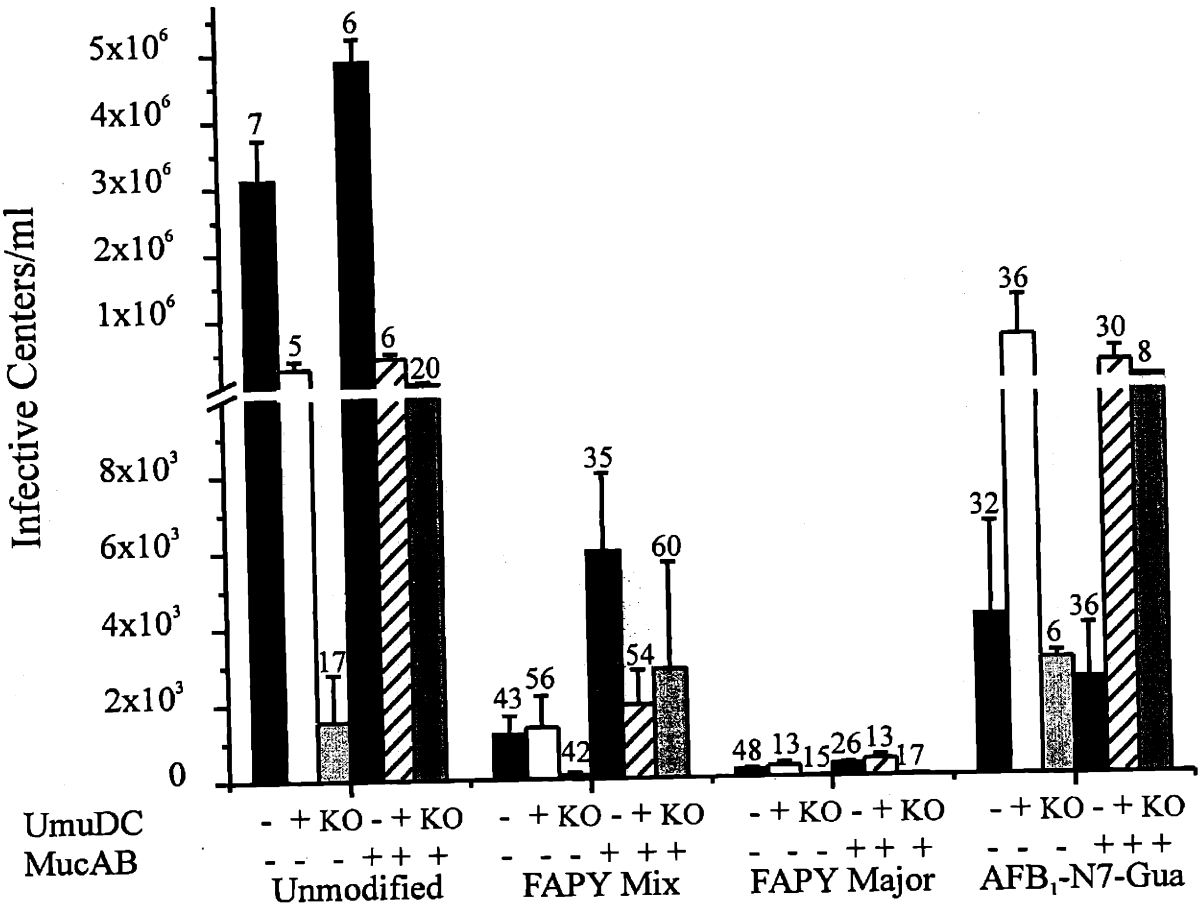

Figure 3.3: Tolerance of cell strains for the AFB_1-DNA adducts. Modified genomes containing either AFB_1-FAPY mix, AFB_1-FAPY major, AFB_1-N7-Gua, or an unmodified site were electroporated into cell strains that were either not induced for the SOS response (black bars), cell strains expressing UmuDC (white bars), MucAB (dark gray bars), or both UmuDC and MucAB (hatched bars), or cell strains that were knocked out for UmuDC and either did (medium gray bars) or did not (light gray bars) express MucAB. The graph represents the number of infective centers per ml of transfected cells, and the numbers over the bars indicate the number of times each electroporation was repeated. The x-axis indicates which bypass polymerase is expressed by the cell. Error bars reflect the standard error of the mean.

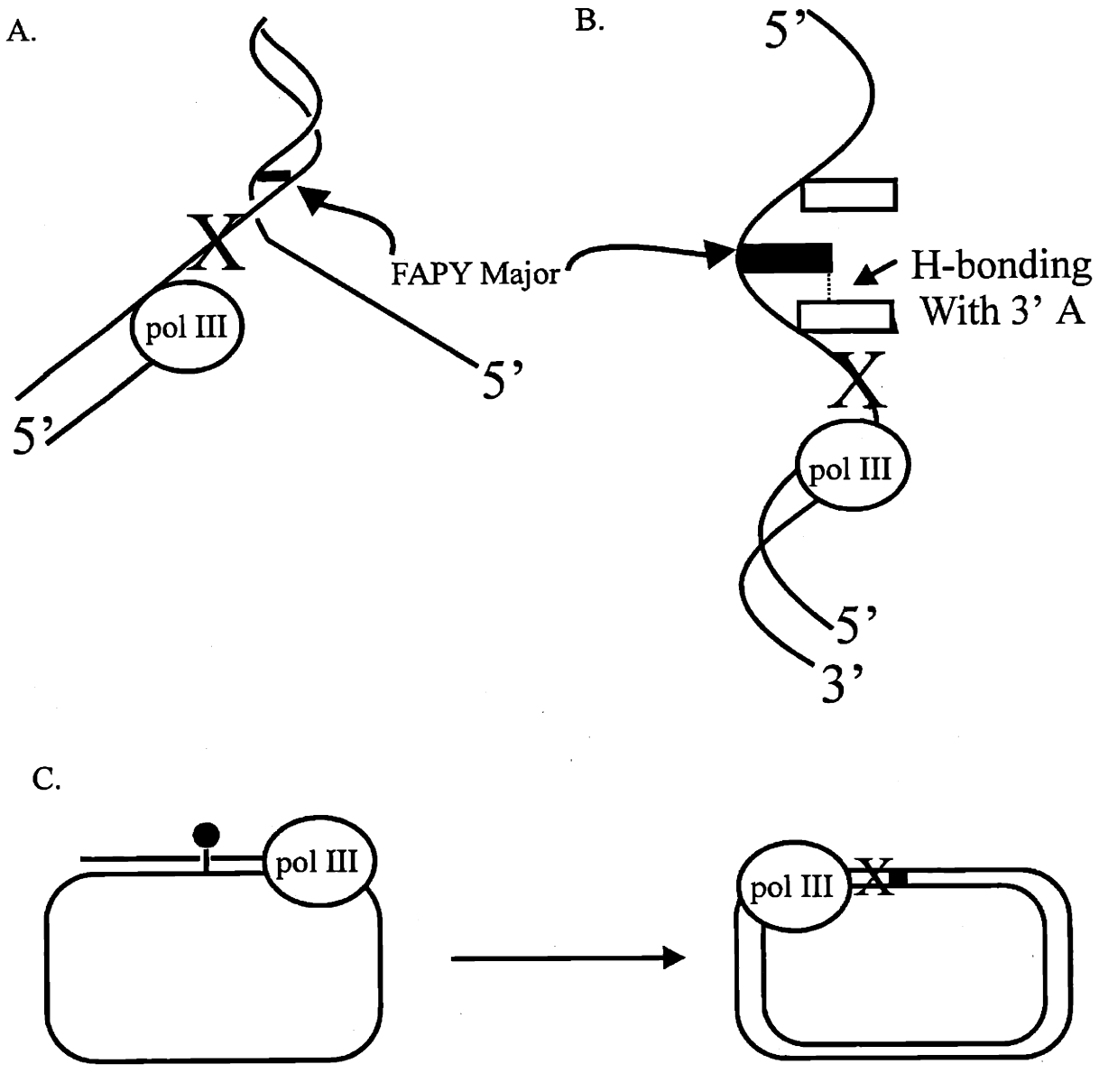

Figure 3.4: Models for lethality of FAPY major. A. AFB_1-FAPY major stabilizes double stranded DNA, making it impossible for topoisomerases to unwind it or polymerases to bypass it. B. Single stranded DNA may mimic the properties of duplex DNA when FAPY major is present. C. Single stranded DNA may undergo one round of replication past the FAPY major adduct. The resulting opposite strand can then anneal to the original strand containing the adduct, and a strong replication block can result in cell death.

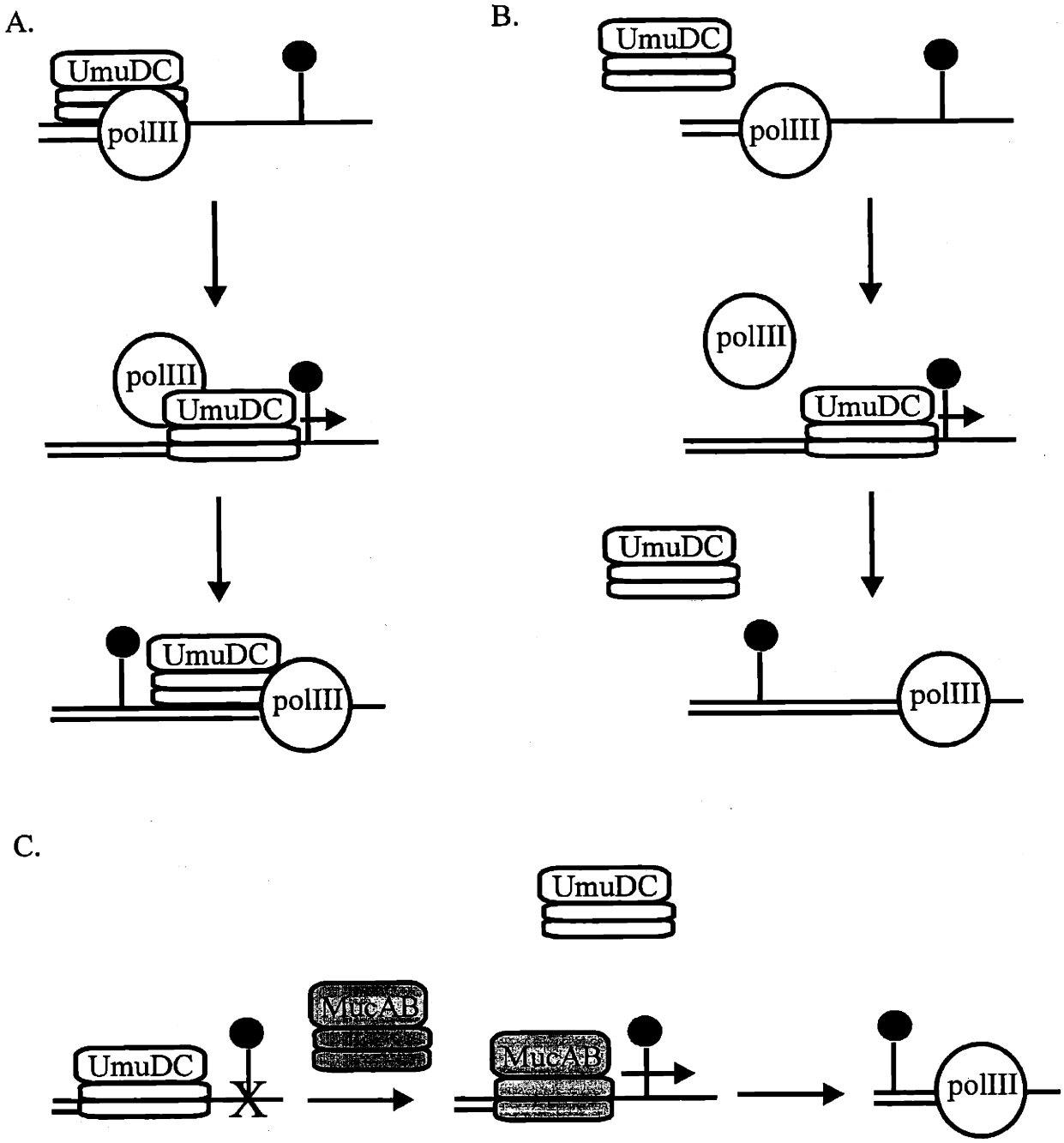

Figure 3.5: Model for polIII interaction with UmuDC. UmuDC may be covalently associated with (A) or recruited by (B) polIII upon its encounter with AFB_1-DNA adducts. UmuDC takes over replication past the adduct, and then polIII resumes replication. In the case that UmuDC cannot replicate past the DNA adduct (C), MucAB is able to compete away the complex and take over lesion bypass.

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Chapter 4

Mutational Properties of the AFB_1-Formamidopyrimidine Adduct

Introduction

The purpose of work described in this chapter was to demonstrate the mutagenic potential of AFB_1-FAPY. This issue has been addressed in two ways: 1) the assay was designed to score G to T mutations using color selection, and 2) enrichment of the phage population was carried out to test for other types of mutants. The mutagenicity of AFB_1-FAPY was also compared directly to that of AFB_1-N7-Gua.

Mutagenesis Studies

Over the years, experiments have been conducted to determine if certain chemicals or biological molecules are able to cause mutations in DNA. Some of these chemicals and the types of mutations they induce include: 1) alkylating agents such as *N*-methyl-*N*-nitrosourea, *N*-ethyl-*N*-nitrosourea, and *N*-methyl-*N*-nitrosoguanidine (G to A transitions) (Mehta and Ludlum, 1978; Abbott and Saffhill, 1979; Gemignani et al., 2001); 2) bulky DNA lesions such as B(a)P (G to T transversions, G to A transitions, and frameshifts) (Loechler, 1995), PhIP (G to T and G to A transversions and G to C transitions) (Brown et al., 2001), AF and AAF and 4-aminobiphenyl (G to T and G to A transversions and G to C transitions) (Belguise-Valladier and Fuchs, 1995; Fuchs et al., 1981; Fuchs et al., 1983; Bichara and Fuchs, 1985; Verghis et al., 1997), and AFB_1 (G to A transitions and G to T transversions) (Foster et al., 1983; Basu and Essigmann, 1990; Hsu et al., 1991; Benasutti et al., 1988; Bressac et al., 1991; Denissenko et al., 1999; Kamiya et al., 1992; Levy et al., 1992; Puisieux et al., 1991; Lawrence et al., 1990; Bailey et al., 1996; Foster et al., 1988; Muench et al., 1983; Misra et al., 1983); 3) nucleotide cyclizers such as methyl glyoxal, malonaldehyde, acrolein, crotonaldehyde, vinyl halides, carbamate

esters, and (haloalkyl)nitrosoureas (Basu et al., 1993); 4) ionizing radiation (T to A transversions and T to C transitions) (Basu et al., 1989) and UV light (G to A and A to G transitions) (Lebkowski et al., 1985; Hauser et al., 1986; Protic-Sabljic et al., 1986; Bredberg et al., 1986; Brash et al., 1987); 5) the antitumor agent Cisplatin (G to T and A to T transversions and G to A transitions) (Brouwer et al., 1981). These experiments have been carried out either *in vitro*, *in culture*, or *in vivo*.

Many studies that evaluate the mutational spectrum observed in DNA and cells that have been treated with these chemicals have been conducted. In the case of AFB_1, the most frequently observed mutation induced by chemically reactive forms of the adduct (e.g., the AFB_1 epoxide or other electrophilic derivatives) is the G to T transversion. This is the principal mutation found in several experimental systems: in the endogenous *lacI* gene in an SOS-induced *E. coli* strain that contains the *mucAB* mutagenesis enhancing operon (Foster et al., 1983); in human cells replicating an AFB_1-modified pS189 shuttle vector (Trottier et al., 1992; Levy et al., 1992); in the *ras* gene of rainbow trout liver (Chang et al., 1991); in the human Ha-*ras* proto-oncogene (Riley et al., 1997); in the transgenic C57BL/6N (BigBlue) mouse treated with phorone (a glutathione depleting agent) (Autrup et al., 1996); in the *lacI* gene in transgenic C57BL/6 mice and F344 rats given a single dose of AFB_1 (2.5 mg/kg in mice; 0.125 mg/kg in rats) (Dycaico et al., 1996); in an intrasanguinous host-mediated assay (Prieto-Alamo et al., 1996); in human hepatocytes grown in culture (Denissenko et al., 1999); in human liver tumors (Hsu et al., 1991; Bressac et al., 1991); in the human HPRT gene (Cariello et al., 1994); and in AFB_1-exposed rats that either did or did not undergo partial hepatectomy (Lee et al., 1998). Both G to T and G to A

mutations are induced with equal efficiency in other systems by metabolically activated AFB_1 (Mace et al., 1997) and the AFB_1 8,9-dichloride model for the AFB_1 epoxide (Sambamurti et al., 1988).

The mutations that result from different types of DNA damage indicate the mechanism of action of these chemicals, whether it is to modify DNA bases (alkylating agents, cyclizers, radiation) or to bind covalently to DNA (bulky adducts, antitumor agents). It is possible that each of these agents have more than one mechanism by which they damage DNA, but these global treatment studies cannot distinguish among these mechanisms. To address the issue of how different forms of DNA damage can result from one damaging agent, Weissmann and coworkers (Weissmann, 1974) first developed the concept of site-specific mutagenesis. This type of assay consists of modifying a single stranded or double stranded plasmid or viral DNA with a specific type of DNA damage, ensuring its stability in the system, and determining its mutagenicity (described in detail in **Chapter 3**). This system was used to study the AFB_1-N7-Gua adduct, which was determined to primarily cause G to T mutations, although at a very low frequency (4%) (Bailey et al., 1996). The AFB_1-FAPY adduct is similar in structure to the AFB_1-N7-Gua adduct, but it is a distinct chemical entity that may contribute differently to the mutational spectrum observed in global modification studies. The assay described above has been employed in this work to determine the types of mutations caused by the structurally unique AFB_1-FAPY adduct.

Causes of G to T Mutations

The studies described above discuss how different types of DNA damage result in different types of mutations. DNA damage by an alkylating agent can result, for example, in the altered nucleotide O^6-methyl-G, which can mispair with T, leading to a G to A mutation (Figure 4.1). Bulky DNA adducts work by a different mechanism. They do not simply add a functional group to an existing nucleotide, but they themselves intercalate into and/or covalently bind to DNA in some manner, affording multiple possibilities for mechanisms of mutation. AFB_1-N7-Gua has been studied in a site-specific system (Bailey et al., 1996) to determine its mutagenecity, and it was found to cause G to T mutations. Additionally, an NMR solution structure indicates that when intercalated into DNA, AFB_1-N7-Gua distorts the helical structure and causes neighboring base pairs to stack differently (Mao et al., 1998) (Figure 1.8A). Upon DNA replication the incoming base has the opportunity to mispair with this adduct, and the preferred pairing is ^{AFB}G:A (Figure 4.2A). T and G mispair somewhat less with the adduct (Figure 4.2B and C), resulting in fewer G to A and G to C mutations. Since this DNA adduct is large, it has the ability to interfere with the incoming nucleotide opposite the base 5' to the modified site as well (Mao et al., 1998) (Figure 1.8B). This result supports the notion that not all mutations are the result of a DNA adduct at one location; adducts can have an effect over a range of bases.

Structure-Induced Mutations

A single DNA adduct can exhibit several different conformations in DNA that may ultimately lead to various types of mutations (Kozack et al., 2000; Kozack et al., 1999; Shukla et al., 1997a; Shukla et al., 1997b; Jelinsky et al., 1995; Belguise-Valladier and Fuchs, 1995;

Brown et al., 2001). Molecular modeling has been conducted as a proof of principle that these structures can exist, and biological data demonstrate that one form of DNA damage can lead to several mutation types. In some of the cases, there is evidence that there are actually two or more forms of the adduct present, although their structural differences cannot be determined until further experiments are conducted. In other cases the structural difference is known, and that difference corresponds to the biological data. This work evaluates the effect different AFB_1-FAPY can have on mutational patterns.

Materials and Methods

Enzymes, Chemicals, Oligonucleotides, and Cell Strains

The *Sfu*I restriction enzyme was obtained from Roche. Sequenase kits were from Amersham Life Sciences. α-^{35}S-dATP and γ-^{32}P-dATP were from New England Nuclear Life Sciences. Oligonucleotides d(CTCTTC**G**AACTCAATTCA), d(CTCTTCA**A**ACTCAATTCA), d(CTCTTC**C**AACTCAATTCA), and d(CTCTTC**T**AACTCAATTCA) and complementary strands (scaffolds) d(ATGAATTGAGTT**T**GAAGAGC), d(ATGAATTGAGTT**G**GAAGAGC), d(ATGAATTGAGTT**A**GAAGAGC), and d(ATGAATTGAGTT**C**GAAGAGC) were from Gibco. (Underlined sequence = *Sfu*I restriction site, bold = target base changed for assay.) GW5100 (JM103, P1$^-$) was from Graham Walker, MIT. Midi prep kits were from Qiagen.

Pooling Progeny Phages

Transformation experiments were carried out 5-30 times per sample per cell line in order to generate enough progeny phages to pool for enrichment of mutant DNA. A total of 20,800 infective centers from each sample were required in the phage pool in order to isolate enough DNA for further experiments. Twenty-six hundred infective centers from each of the 8 most successful independent transformations were pooled together. The pooled phages were incubated with a pregrown culture of GW5100 cells (single colony inoculation into 10 ml 2xYT medium overnight, diluted 1:50 and grown for 30 minutes at 37°C) for 5 hours at 37°C on a roller drum. Replicative form DNA was then isolated using a Qiagen Midi double stranded plasmid preparation kit.

SfuI Specificity and Efficiency

*Sfu*I specificity was tested by using an oligonucleotide cleavage assay. Oligonucleotides (18 bases) containing either a G, C, A, or T at the potential AFB_1-modification site were labeled with ^{32}P and were annealed to complementary strands (20 bases) by heating to 80°C for 5 minutes and cooling down to room temperature. Once annealed, 0.5 picomoles of DNA duplexes were incubated for 2 hours at 37°C with or without 30U of the *Sfu*I restriction enzyme. The products were run on a 20% polyacrylamide gel for 1.5 hours at 400V. The gel was exposed on a phosphorimager plate for 1.5 hours and imaged using a STORM 840 system from Molecular Dynamics. Results are shown in Figure 4.3.

Progeny phages were isolated from phage originally containing unmodified DNA that was transfected into each of 4 cell strains (uninduced for SOS, expressing UmuDC, MucAB, or both UmuDA and MucAB). Progeny phages were also isolated from phages originally modified with AFB_1-FAPY mix and transfected into cell strains expressing both UmuDC and MucAB. They were pooled as above, and the DNA was digested according to the enrichment procedure below.

Enrichment of Mutant Genomes

Approximately 0.5 picomoles of DNA isolated from pooled phage was subject to cleavage by 30U *Sfu*I, a restriction enzyme that cleaves at a unique site within the 13 base region of the inserted oligonucleotide (see "**Oligonucleotides**" in Chapter 1). Mutant genomes were refractory to cleavage, while wild type genomes were linearized with greater than 90% efficiency (Table 3). Digested products, as well as mock-digested controls, were analyzed on agarose gels to determine

linearization. Approximately 0.1 picomoles of digested or control DNA was desalted on a G-25 Sephadex column and electroporated as in **Chapter 3**, using DL7 as the transfection strain and NR9050 as the plating bacteria. Dark blue plaques were scored either as wild type that was undigested or mutants other than G to T transversions. Clear plaques were scored as either transformations of undigested M13, M13 genomes without inserts, or large deletion mutants. Light blue plaques were scored as G to T transversion mutants at the site of modification (Figure 4.4).

DNA Sequencing

Plaques were picked from plates containing transformants that were the result of the enrichment procedure. Sequencing was performed on DNA that had been originally modified with AFB_1-FAPY mix and was isolated from the cell strain that expressed both UmuDC and MucAB. A sampling of light blue plaques, 256 dark blue plaques, and 100 clear plaques were sequenced. DNA was prepared in the following manner.

Plaques were soaked in 100 µl 1xTE buffer, a 90 µl aliquot was removed and mixed with 100 µl of GW5100 overnight culture, then this was added to 5 ml of 2xYT medium. These cultures were allowed to grow for 6 hours in a roller drum at 37°C. Approximately 1.5 ml were aliquotted into eppendorf tubes and microcentrifuged at 15,000x*g* for 5 minutes. The supernatant was decanted into a tube containing 200 µl 20% PEG/2.5 M NaCl, vortexed, and kept overnight at 4°C. The tubes were centrifuged again and the supernatant was decanted and discarded. The tubes were centrifuged once more in order to ensure no residual cells remained in the tube, and the rest of the supernatant was aspirated. The pellet was then resuspended in 20 µl of 1xTE and boiled 2 minutes prior to

annealing the sequencing primers. The DNA was then sequenced according to instructions in the Sequenase kit. ^{35}S-α-dATP was used in the reactions. Thin 8% polyacrylamide gels were run at 2000 volts for 1.5 hours, and gels were fixed in 10% methanol, 10% acetic acid. Gels were then dried on a BioRad model 1125B gel drier at 80°C for 1 hour. They were exposed to Kodak x-ray film for approximately 48 hours before they were developed and analyzed.

Results

G to T Mutations

The experimental system allowed the facile scoring of G to T mutations using the convenient light blue/dark blue color selection described above. Figure 4.5A shows the G to T mutation frequency of the FAPY mix adduct in each of the four genetic backgrounds used in this study. Wild type cells not induced for SOS showed the lowest number of mutations, roughly 15%. Those expressing UmuDC showed a 17% mutation frequency, while those knocked out for UmuDC only had a 3% mutation frequency. The MucAB expressing cells showed a 27% mutation frequency, while those knocked out for UmuDC that express MucAB similarly showed a 21% mutation frequency. Finally, cells expressing both UmuDC and MucAB showed a 32% mutation frequency. The only significant difference observed here is that cells expressing MucAB or both MucAB and UmuDC have a slightly higher G to T mutation frequency than the other cell strains. This difference is most likely due to the presence of the MucAB bypass polymerase.

The G to T mutation frequency observed for the AFB_1-N7-Gua adduct was 4% in the cell strain expressing MucAB (Bailey et al., 1996). Upon repeating this experiment for all four cell strains used in these studies, the mutation frequency was found to be between 2-6% (Figure 4.5B), which agrees well with prior results and allows for a direct comparison of the two studies. This result emphasizes the striking difference in mutagenic properties between the AFB_1-N7-Gua and the AFB_1-FAPY adducts, establishing the fact that FAPY adducts are at least six times more likely to cause G to T mutations as their molecular predecessor.

Efficiency and Specificity of the Sfu*I Restriction Enzyme*

In order to determine the specificity of the *Sfu*I restriction enzyme, a cleavage assay was conducted using oligonucleotides that had each of the 4 bases at the position to be potentially mutated by AFB_1. The wild type (G-containing) oligonucleotide contains the *Sfu*I restriction site, whereas this site is abolished in the other three oligonucleotides. Bands that correspond to 20 bases in length represent uncut DNA, while those that correspond to 12 bases represent the enzyme cleavage product (Figure 4.3). Lanes 2, 4, 6, and 8 are the samples incubated with the enzyme, while lanes 1, 3, 5, and 7 represent the controls. Lanes 1 and 2 contain the wild type (G) sequence. Lanes 3 and 4 contain a G to A substitution, lanes 5 and 6 contain a G to C substitution, and lanes 7 and 8 contain a G to T substitution. The results demonstrate that there is no cleavage of any sequence other than that of the wild type restriction site.

Control experiments were performed with unmodified inserts to determine the efficiency of the *Sfu*I enzyme. A pool of DNA was subjected to *Sfu*I digestion, while a control was mock treated with buffer. A comparison of the number of infective centers obtained from the digested versus the control DNA showed that the efficiency of the enzyme was greater than 96% in phage pooled from each of the four genetic backgrounds (Table 3). This means that the restriction digest should eliminate at least 96% of the wild type DNA from the pool, increasing the number of mutants observed in each transformation.

Enrichment for Non-G to T Mutations

In order to simplify the analysis, the number of non-G to T mutations were only evaluated in the cell strain expressing both UmuDC and MucAB. These mutations were expected to be infrequent, so a method to enrich the phage pool for mutants by eliminating the majority of wild type DNA was used. Progeny phages obtained from the eight transformations with the FAPY mix adduct that gave the highest number of transformants in the cell strain expressing both UmuDC and MucAB were pooled. The population was expanded in cell culture, and the double stranded (replicative form) M13 DNA was isolated. The oligonucleotide inserts were engineered such that they contained a unique *Sfu*I restriction site that encompassed the target base for evaluation of mutagenesis (Bailey et al., 1996). If this and the surrounding positions retained their wild type sequence, the restriction enzyme would successfully cut this DNA, rendering it unable to transform *E. coli*. However, if a mutation occurred within the restriction site, the DNA would be refractory to digestion and therefore able to transform *E. coli* successfully (Figure 4.4). Results are shown in Table 3, and this cell strain exhibited an upper limit of 14% non-G to T mutations.

Non-G to T Mutations

Progeny phages DNA resulting from AFB_1-FAPY modified genomes that were transfected into cells expressing both UmuDC and MucAB was subjected to treatment with *Sfu*I or was mock treated. The number of dark blue plaques in the digested sample divided by the number of dark blue plaques in the mock treated sample is termed the Restriction Resistant Fraction (RRF) and is a first approximation of the mutation frequency. Sequencing the dark blue

plaques in the digested samples and multiplying the percent of mutants by the RRF (14%) yields the true frequency of each type of mutation present (Figure 4.6A). It should be noted that all the dark blue plaques did not consist of wild type or mutant sequences; some were the result of the genetic engineering procedures employed in the experiment. For example, some sequences were M13 DNA that had been ligated back together without an insert, containing only a few extra random bases to put the gene back in frame. Others were small deletions that result from "chew back" of the DNA after enzyme digestion and ligation of the resulting product. Since these deletions would be present in both the digested and mock treated samples, they are included in the total used to calculate the fraction of each type of mutation observed. The following overall mutation frequencies were observed: G to A = 1%, total 5' mutations (C to G and C to T) = 1%, a specific quadruple mutation = 1%, and other multiple mutations = 3%. There were several other types of mutations that occurred (Figure 4.6B and Table 4) totaling another 3% overall. For comparison, these genetic conditions induced a G to T mutation frequency of 32%, yielding a total overall mutation frequency of nearly 40%.

When considering only the dark blue plaques that were mutants, it was determined how each specific type of mutation contributed to the total (Figure 4.6B). The following percentages were observed: G to A mutations = 18%, total 5' mutations (C to T and C to G) = 16%, a specific quadruple mutation = 16%, and other multiple mutations = 49%. Figure 4.6B and Table 4 illustrate the different multiple mutations and their contribution to the total. One surprising result was the presence of a specific quadruple mutation. This sequence was as follows: CCTCT**AA<u>A</u>G**ACTC. The bold bases are mutations, and the underlined base is the initial site of

modification. The mutation at the target site was a G to A, and these were not included in the calculation of the total number of G to A mutations. The two 5' transversions are T to A and C to A, and the 3' transition is A to G, none of which were included in the calculation of the total number of 5' or 3' mutations. Several small deletions consisting of the codon containing the modified G (GAA) were also observed. They made up about 10% of the non-G to T mutations. One other rare occurrence was the point mutation of one or two bases located five or six positions upstream of the modified G, most often a C to T or C to G.

Discussion

G to T Mutations

A G to T mutation was predominantly observed for the AFB_1-N7-Gua adduct in the mutation analysis system used in this study. This form of the adduct, however, was only weakly mutagenic, with a mutation frequency of 2-6% (Figure 4.5B). By contrast, we have established that the AFB_1-FAPY adduct produced the same mutation with a frequency of up to 32% (Figure 4.5A). Because this adduct is highly persistent in the genome, it has great potential to play a role in hepatocarcinogenesis. The G to T mutation frequency in the cell strains that express both UmuDC and MucAB is less than additive with respect to those cell strains that express each polymerase individually, implying that both polymerases may compete to bypass the same adduct.

The same trend observed for survival is not observed for G to T mutation frequency. For the AFB_1-FAPY mix adduct, cells expressing MucAB have the most tolerance for the adduct, and, therefore, it is expected that this cell strain would exhibit the highest G to T mutation frequency (Figure 3.3). Although it is not significantly different from the cell strain expressing both UmuDC and MucAB, the mutation frequency is slightly lower (27% as opposed to 32%). Cells expressing both UmuDC and MucAB exhibit the survival phenotype of those expressing UmuDC alone; however, this is not the case for the G to T mutation frequency. One reason for this could be that the different bypass polymerases have different fidelities and efficiencies. MucAB has been observed to be more efficient at lesion bypass than UmuDC, bypassing several types of DNA lesions with ease and exhibiting low fidelity (Foster et al., 1988; Perry et al., 1985;

Kulaeva et al., 1995; Hauser et al., 1992; Woodgate and Sedgwick, 1992; Bennett et al., 1988; Szekeres, Jr. et al., 1996). When both polymerases are expressed, UmuDC may be predominantly involved in lesion bypass, inhibiting MucAB from taking over, but when MucAB eventually replaces UmuDC, the lesion is highly mutagenic. However, UmuDC remains relatively efficient at mutagenic lesion bypass, due to the fact that when it is knocked out, G to T mutations are reduced 5-fold.

One study (O'Grady et al., 2000) has suggested that the types of mutations observed when bypass polymerases are employed may be a direct function of the polymerase, not a result of the DNA adduct. This observation may be applied to mammalian bypass polymerases as well, although the specific type of mutation induced may differ. If prokaryotic and eukaryotic bypass polymerases cause the same types of mutations, than the system used here can be extrapolated to mammalian systems. Studies have demonstrated that bacterial polIV and its human counterpart, polκ, differ in how they bypass DNA adducts such as AAF (Ohashi et al., 2000; Napolitano et al., 2000)and B(a)P (Lenne-Samuel et al., 2000). Bacterial polV and its human counterpart, polη, exhibit some similarities and some differences when challenged with different DNA lesions. *Cis-syn* thymine dimers (Napolitano et al., 2000; Masutani et al., 1999) and B(a)P adducts (Lenne-Samuel et al., 2000; Chiapperino et al., 2002) yield similar results when bypassed by these polymerases. AAF adducts, in contrast, produce unique events when confronted with polV and polη (Συζυκι ετ αλ., 2001).

Model for the Mutagenic Contribution of AFB_1-FAPY

It is possible to construct a scenario in which both AFB_1-N7-Gua and AFB_1-FAPY contribute to the genetic changes observed in HCC. Shortly after aflatoxin exposure, AFB_1-N7-Gua is abundant and may generate a low frequency of G to T mutations. Over time the majority of adducts are either removed from DNA or converted to AFB_1-FAPY, which can persist for weeks to months as the dominant progenitor to genetic changes. For most of the period that an animal is undergoing carcinogenic transformation, AFB_1-FAPY is principally the highly mutagenic lesion in DNA. Early data were used to show quantitavely how each form of AFB_1 adduct could contribute to observed G to T mutations (Croy and Wogan, 1981). Values were obtained for the number of adducts per base pair, which were easily converted to the number of adducts per cell for each time point (Table 5). One factor to consider is that in rat liver DNA, a certain population of the cells is undergoing replication at any given time, so even though an adduct may be present at a particular time point, it does not mean that it will come in contact with a replicative polymerase at that time. Since it is not known what percentage of rat liver cells is replicating at any given time, it was assumed that all the adducts observed at one time point would undergo replication. This number is an upper limit for the number of mutations that can be formed.

The number of adducts per cell in rat liver was multiplied by the observed aflatoxin mutation frequency in bacteria for each type of adduct (4% in the case of AFB_1-N7-Gua and 32% in the case of AFB_1-FAPY) in order to get the maximum possible number of G to T mutations formed per cell at each time point (Table 5). This calculation also assumes that the mutation frequencies generated for bacterial polymerases are comparable to those of mammalian

polymerases. With these assumptions in mind, the results establish that the AFB_1-N7-Gua adduct is never the predominant cause of G to T mutations. This point is even more strongly emphasized if we look at a more realistic scenario in which the cells undergo replication once every 24 hours. At the 24 hour time point most of the AFB_1-N7-Gua adducts have either converted to AFB_1-FAPY or have been removed from DNA. Therefore, the AFB_1-FAPY adduct is the major contributor of these mutations at every time point, most strikingly at the 72 hour time point, when it is more than 600 times as effective as its molecular precursor. The ratio of mutations formed by each type of adduct holds true, even though the numbers used are upper limits, due to the fact that the same percentage of cells should be replicating at any given time.

One factor to note in the above calculations is that neither the quantity nor the mutagenic potential of AP sites was taken into account. This absence is due to the fact that they were not able to be calculated in the original experimental procedure (Croy and Wogan, 1981). It has been reasonably suggested that the premutagenic lesion responsible for the observed G to T transversions in bacterial systems would be the AFB_1-induced AP site, since dAMP is the most common base inserted opposite AP sites in *E. coli* induced for the SOS response (Lawrence et al., 1990; Loeb et al., 1986). We note, however, that the mutational specificity of the AP site in mammalian cells is different; in fact, studies conducted in different mammalian systems have demonstrated different preferences for the base inserted opposite an AP site (Kamiya et al., 1992; Gentil et al., 1992; Gentil et al., 1990; Gentil et al., 1984; Klinedinst and Drinkwater, 1992). AP sites are also generated in DNA by endogenous mechanisms (Lindahl and Nyberg, 1972), so cells are able to process these lesions quite efficiently. In *E. coli* the mutational properties of the

AFB_1-N7-Gua lesion and the AP site (Bailey et al., 1996) were compared when each lesion was situated at a specific site within the bacteriophage M13 genome. The predominant mutation for both lesions is a G or AP to T transversion. The data on the AP site are in accord with previous studies on the mutational specificity of this lesion in *E. coli* (Lawrence et al., 1990). These data indicate that AP sites may play a role in the causation of G to T mutations observed in human HCC. However, mammalian cells have evolved sophisticated mechanisms to process this type of DNA damage, so AP sites may be efficiently repaired before they have a chance to become mutgenic.

Adduct Structure and Mutagenicity

It would be of interest to know how AFB_1 adduct structure influences its biological properties. In principle, repair enzymes may recognize AFB_1-N7-Gua better than AFB_1-FAPY owing to the more profound effect of AFB_1-N7-Gua on DNA structure. In support of this view, excision repair systems preferentially remove AFB_1-N7-Gua from the DNA of human fibroblasts (Oleykowski et al., 1993; Chetsanga and Frenette, 1983). The lower mutation rate observed for this form of the adduct can be explained by this fact as well. Since the FAPY adduct has a more subtle effect on DNA architecture and increasing the local melting temperature of the DNA duplex, may evade repair, which is in line with its persistence *in vivo*. Additionally, its structure in DNA implies that it has the potential to pair predominantly with A (Figure 4.2A), causing G to T mutations. The longevity of the FAPY adduct, combined with its high mutagenic potential, make it a dangerous lesion.

Enrichment for Non-G to T Mutations

Previous work (Bailey et al., 1996) shows that non-G to T mutations caused by AFB_1-N7-Gua are extremely rare (a fraction of a percent). Although these mutations may not be biologically relevant, they can detail important information about the structure of DNA adducts. In order to investigate these mutations, however, a procedure to enrich the population for them must be carried out. The alternative involves sequencing thousands of dark blue plaques to get an adequate representation of each type of mutation present.

The procedure employed in this work utilizes a restriction enzyme recognition site. This restriction enzyme shows a high fidelity towards its substrate (Figure 4.3). It will not cleave DNA that contains a mutated restriction site. It also cleaves its substrate greater than 96% of the time, which makes the enrichment procedure extremely efficient. One drawback to this enrichment procedure is that it can only be applied to one DNA sequence. In order for the effects of sequence context on adduct formation, tolerance, and mutagenicity to be probed, a new enrichment procedure would have to be developed (see **Appendix B**).

The mutant enrichment procedure was carried out for cells expressing both UmuDC and MucAB. The RRF was determined to be 14%. This set of enriched DNA was further analyzed, using DNA sequencing, to obtain the true mutation frequency. The percent of sequenced plaques that actually were mutants were multiplied by the RRF to get the true non-G to T mutation frequency, which was roughly 8%. This brings the total G to T plus non-G to T mutation

frequency up to nearly 40%. The true mutation frequency in other genetic backgrounds may be similar to these results.

Non-G to T Mutations

Several types of non-G to T mutations were observed for the AFB_1-FAPY mix adduct (Figure 4.6B and Table 4). These mutations are attributed primarily to the FAPY minor form of the adduct, since FAPY major is usually a lethal replication block. The mutagenic properties of FAPY minor are similar to those of AFB_1-N7-Gua in that they both induce G to A mutations, 5' mutations, and tandem mutations (a mutation at the site of modification and the 5' site occurring simultaneously) approximately 1% of the time. This indicates that FAPY minor may have some structural similarities to AFB_1-N7-Gua at least part of the time. Most of the time, however, mutations that are unique to FAPY minor are observed.

These unique mutations consist of small deletions, quadruple mutations, and combinations of target mutations and upstream point mutations. It is hypothesized that the FAPY minor adduct may either consist of an extrahelical conformation in which the adduct interferes with several positions in the DNA via interactions with the phosphate backbone, or it may alternate among several intermediary structures before reaching the low energy FAPY major structure (Figure 2.13). This conformational flexibility of the adduct may interfere with the replication of several bases that are adjacent to the modified site, resulting in the multiple mutations observed. This hypothesis is supported by the reproducibility of the quadruple mutants. Alternatively, these mutations may be a result of the DNA repair process. If NER is

taking place, the excised region may be filled in by the error-prone polI resulting in mutations distal to the damaged site (Figure 1.3). Such mutations would taper off once the normal replicative DNA polymerase resumed its function. This result may change the way that mutational hotspots are evaluated. Mutational hotspots do not always coincide with sites of adduct formation (Smela et al., 2001), and one DNA adduct can be responsible for more than one mutation (Courtemanche and Anderson, 1999).

Conclusions

Several conclusions can be drawn from this work. First, the G to T mutation frequency for the AFB_1-FAPY adduct is at least six times that of the AFB_1-N7-Gua adduct. Second, the pattern of mutations observed for AFB_1-FAPY mix in each cell strain is not consistent with how each of the cell strains tolerates this DNA lesion. This inconsistency may be due to the fact that MucAB is a more efficient error-prone bypass polymerase than UmuDC. Third, FAPY minor can cause some mutations that are similar to those observed for AFB_1-N7-Gua and some that are unique to its own structure. This spectrum of mutations implies that FAPY minor has either an extrahelical conformation or exhibits conformational flexibility that results in multiple mutations. Alternatively, it may also be repaired by NER, and the error prone polI may be responsible for multiple mutations. Fourth, these data demonstrate that DNA damage at one site can lead to mutations at other sites as well, explaining why mutational hotspots do not always coincide with sites of adduct formation.

Future Work

Mutagenesis in Different Sequence Contexts

One of the challenges of evaluating mutational spectra site-specifically is to do so in all possible sequence contexts. Global modification studies have evaluated where adducts form and where mutational hotspots occur in DNA. These two pieces of information do not always coincide, possibly due to the fact that one DNA adduct can be responsible for mutations observed at sites other than the target site (Smela et al., 2001). All chemical structures of AFB_1 should be evaluated for their mutagenecity in each of the 16 possible immediate sequence contexts. This is a difficult challenge if the techniques described in this work are used to evaluate each sequence context individually. First, a color selection assay for G to T mutations (or at least for one type of mutation) would have to be engineered, since formation of the GAA to TAA *ochre* suppressor would only work successfully for those sequence contexts having A as the 3' base. Second, a separate mutant enrichment procedure would have to be developed for each sequence context, since the *SfuI* restriction site would no longer exist. Alternatively, a unifying mutant enrichment procedure could be developed.

Mutant Identification or Enrichment Procedures

Better techniques should be developed to either identify mutations that occur at very low frequencies or to enrich for mutations universally. There are several characteristics that describe a good mutant enrichment procedure. First, it should be able to enrich for mutations in any given sequence context. Second, it should be able to enrich for mutations at more than one target site, including multiple mutations as well. Third, it should be easy to use and easily adaptable to

different systems. Preliminary experiments have been performed to test a number of ideas for new mutant identification or enrichment procedures. These ideas include plaque hybridization, ligation mediated cleavage, hairpin hybridization, addition of fluorescent dideoxynucleotides, the PROBE assay, and a PNA enrichment procedure. They are described in **Appendix B**.

Determining the Structure-Mutagenicity Relationship

Site specific mutagenesis experiments should be carried out for the chemicals that have been observed to exhibit different conformations in DNA (B(a)P, PhIP, and AF). These experiments could help to elucidate how different known structures of DNA adducts can lead to specific types of mutations. It could also help to elucidate what the possible structure of a DNA adduct could be based on its mutagenic properties.

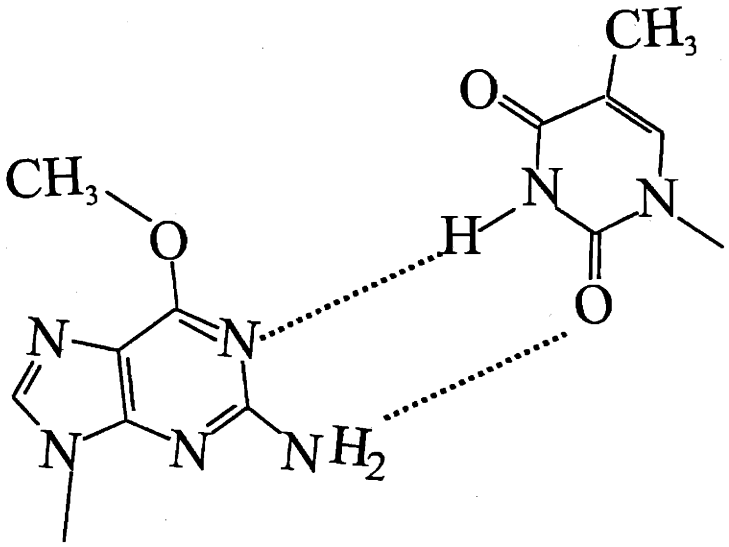
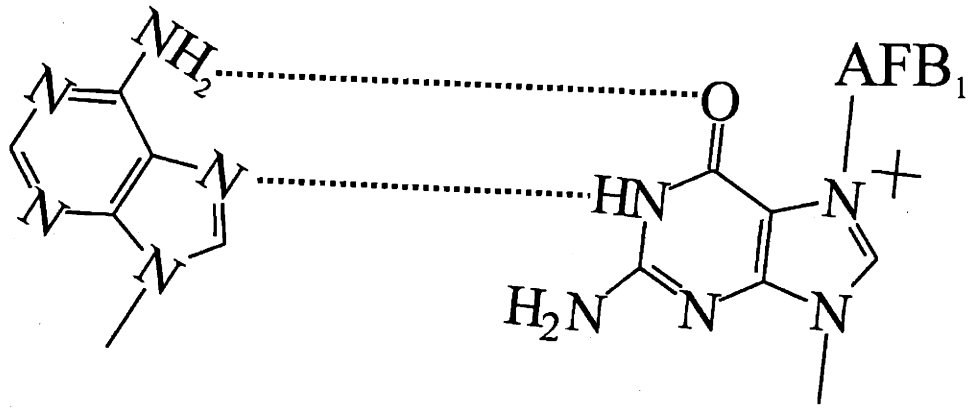
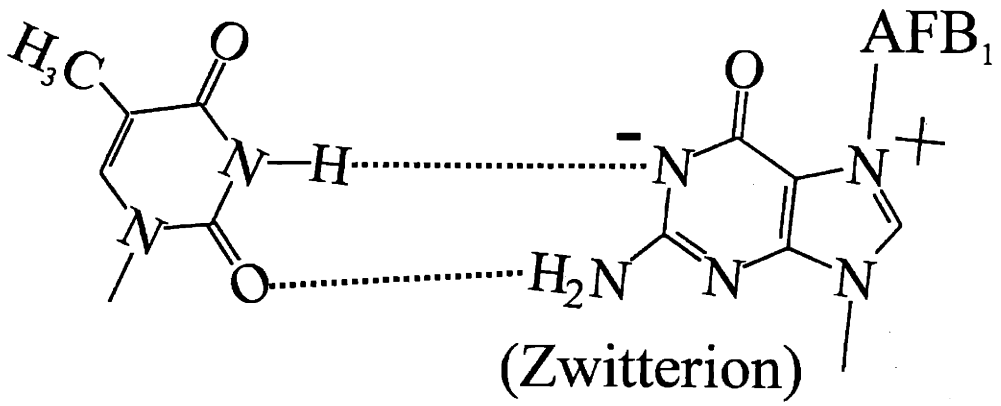


Figure 4.1: Mispair of O^6-methyl-G with T. Upon DNA replication T can mispair with O^6-methyl-G. During the next round of replication, the T correctly pairs with an A, leading to a G to A mutation.

A.



B.



C.

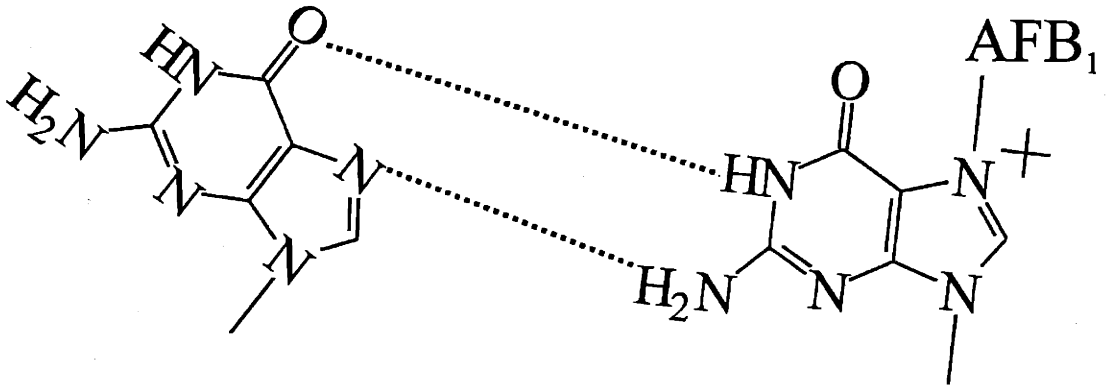


Figure 4.2: AFB_1 mispairs. A. $^{AFB1\text{-}FAPY}G$ mispairs with A, leading to G to T mutations. B. $^{AFB1\text{-}FAPY}G$ mispairs with T, leading to G to A mutations. C. $^{AFB1\text{-}FAPY}G$ mispairs with G leading to G to C mutations.

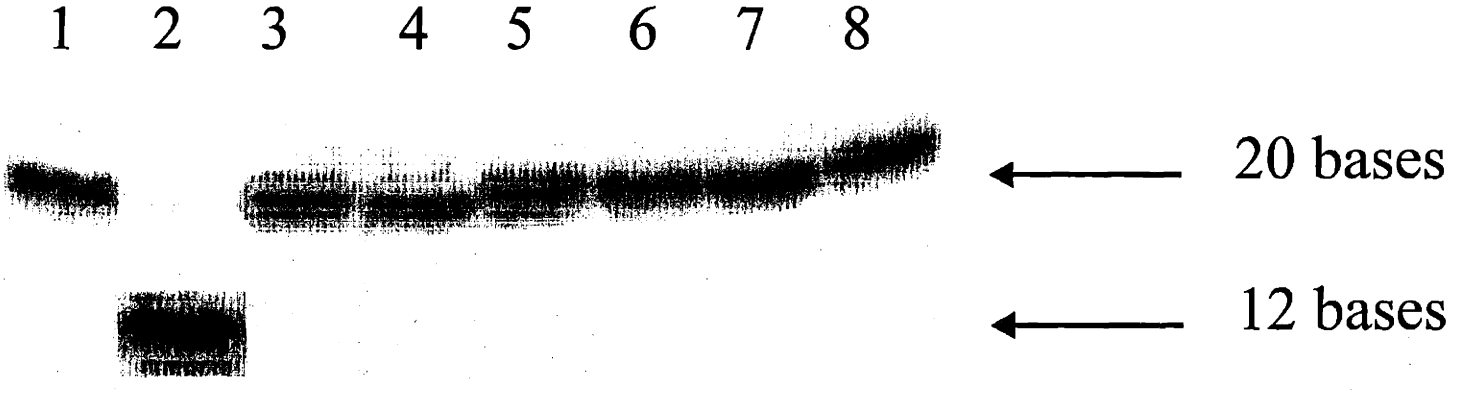


Figure 4.3: Efficiency of *Sfu*I. The *Sfu*I restriction enzyme was incubated with 4 oligonucleotide duplexes (Lanes 2, 4, 6, and 8). Control reactions without the enzyme (Lanes 1, 3, 5, and 7) were done in parallel. Twenty base oligonucleotides were labeled with ^{32}P and cleavage products resulted in a 12 base labelled fragment. The enzyme recognition sequence was only present in lanes 1 and 2. Lanes 3 and 4 contain a G to A substitution, lanes 5 and 6 contain a G to C substitution, and lanes 7 and 8 contain a G to T substitution in the restriction site.

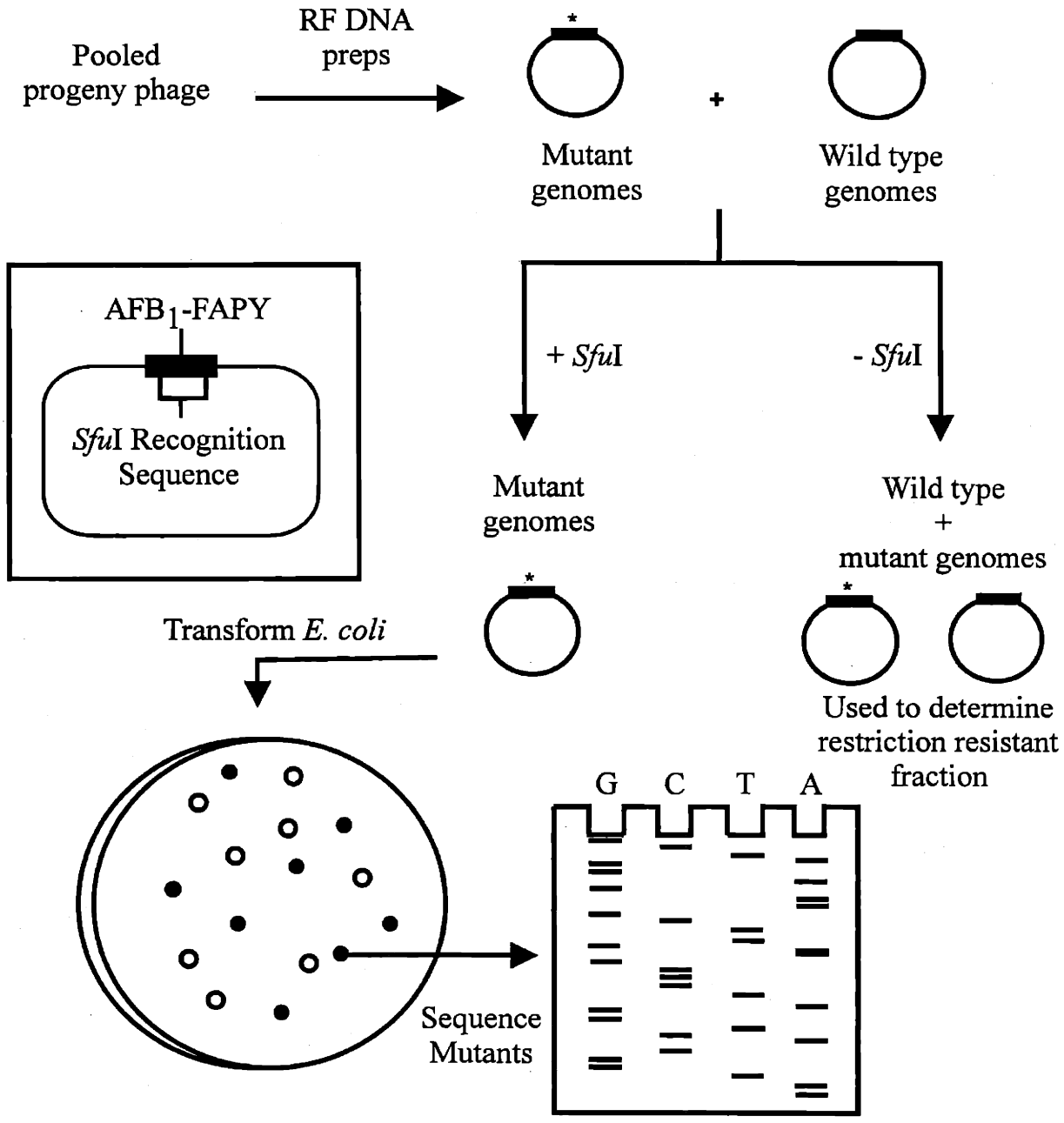


Figure 4.4: *Sfu*I enrichment procedure. After cells are electroporated with modified DNA, progeny phages are produced and pooled. Progeny contain both mutant and wild type sequences. In order to enrich the pool for mutants, the DNA is subjected to digestion with the *Sfu*I restriction enzyme, which cleaves wild type DNA and allows mutant DNA to remain intact. After digestion the DNA is once again electroporated into *E. coli*, and the dark blue plaques are picked and Sequenced.

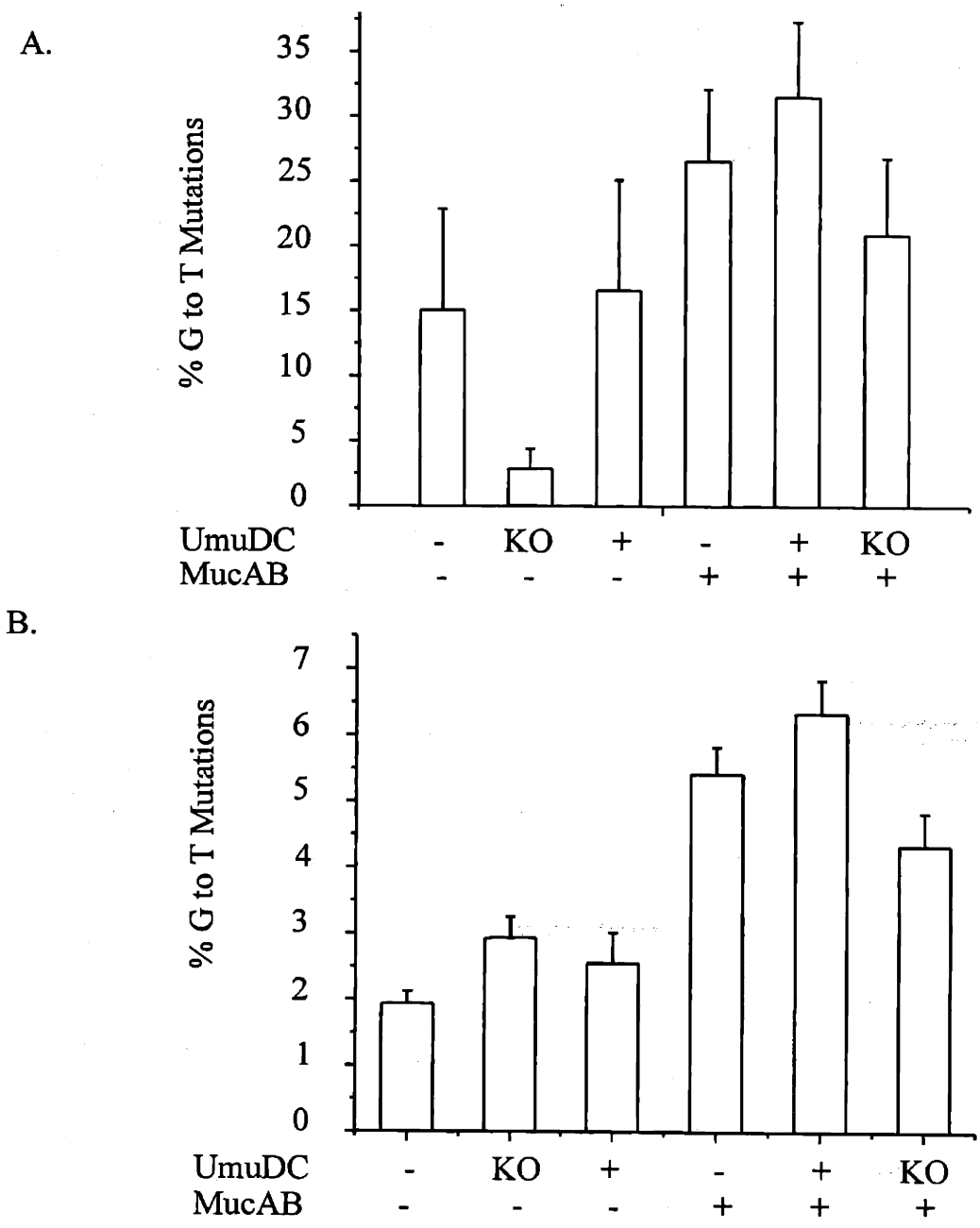
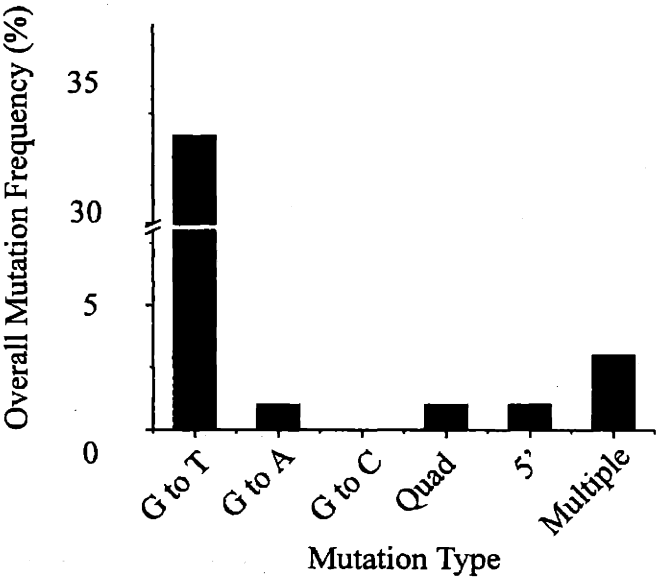


Figure 4.5: G to T mutation frequencies for AFB_1-FAPY mix and AFB_1-N7-Gua. G to T mutation frequencies were determined by dark blue/light blue color selection. The number of light blue plaques was divided by the total number of dark blue plus light blue plaques to get the percent light blue, or percent G to T mutants. The data represent an average of four experiments. Error bars represent standard error of the mean. A. G to T mutation frequency for AFB_1-FAPY mix. B. G to T mutation frequency for AFB_1-N7-Gua.

A.



B.

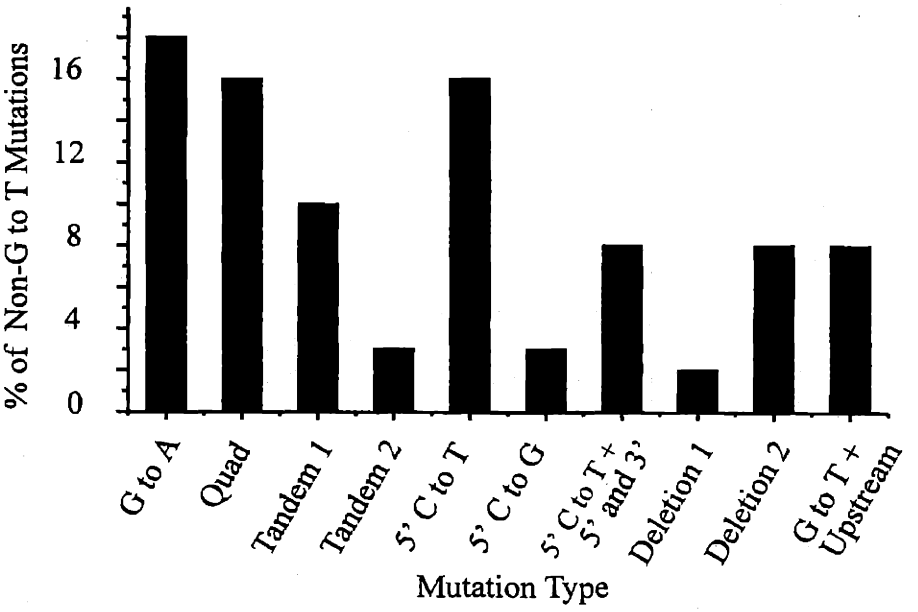


Figure 4.6: Mutation frequencies for non-G to T mutants. A. Overall mutation frequency of major non-G to T mutants as compared to the G to T frequency. B. Breakdown of all non-G to T mutations. Percentage was calculated by dividing the number of each specific mutation by the total number of non-G to T mutations observed.

Dark Blue Infective Centers/ml (Digest/Control)			
SOS Status		Modification	
UmuDC	MucAB	None	AFB_1-FAPY Mix
-	-	0.02	ND
+	-	0.01	ND
-	+	<0.03	ND
+	+	0.038	0.14

Table 3: *Sfu*I efficiency. Phages resulting from transformations with unmodified or AFB_1-FAPY mix containing M13 genomes were pooled from cells expressing different bypass polymerases. The efficiency of the enzyme was greater than 96%, and the RRF for the modified genome was 14%. (ND = Not Determined)

Mutation Type	Sequence (5'-3')	Occurrence (%)
Wild Type	CCTCTTCGAACTC	
Quadruple	CCTCT**AAAG**ACTC	16%
Tandem 1	**CTGCTTGA**AACTC	10%
Tandem 2	CCTCTT**GA**AACTC	3%
5' C to T	CCTCTTTGAACTC	10%
5' C to G	CCTCTT**GG**AACTC	3%
5' C to T + 5' and 3'	CTCCATTGAAGTC	8%
Deletion 1	C**GA**CTTC(ΔGAA)CTC	2%
Deletion 2	C**G**TCTTC(ΔGAA)CTC	8%
G to T + Upstream	CATCTTCTAACTC	8%

Table 4: Sequences of non-G to T mutations. Mutations and percentage of occurrence are given.

Time	Adducts/Liver Cell[a]		G to T Mutations/Liver Cell[a]		Mutagenecity Ratio
	AFB_1-N7-Gua[b]	AFB_1-FAPY[c]	AFB_1-N7-Gua[b]	AFB_1-FAPY[c]	AFB_1-FAPY : AFB_1-N7-Gua
2h	188,000	24,000	7500	7600	1.0
4h	153,000	29,000	6140	9200	1.5
12h	64,000	35,000	2600	11,000	4.2
24h	27,000	43,000	1100	14,000	13
48h	2200	20,000	89	6300	71
72h	220	18,000	9	5600	600

[a] Calculations based on data from Croy, et al.
[b] Assuming a 4% G to T mutation frequency.
[c] Assuming a 32% G to T mutation frequency.

Table 5: Possible contribution of different AFB_1 DNA adducts to G to T mutations in human cells. Data are based on experiments conducted by Croy, *et al* (1981). Assumptions are made that extrapolate bacterial data from experiments performed here to mammalian polymerases. The mutagenicity ratio compares the number of G to T mutations for AFB_1-FAPY to those of AFB_1-N7-Gua.

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Appendix A:

Data for Lesion Tolerance and G to T Mutation Frequency

Introduction

The data tables presented in this appendix illustrate the number of infective centers per milliliter for each transformation (IC/ml) and the percent of G to T mutations for each transformation (% Light Blue). Each IC/ml value consists of the total number of light blue and dark blue plaques observed for one transformation event, and each value shown in the tables is the average of two or more replicate platings. The percentage of G to T mutations for each transformation was calculated by plating progeny phages and dividing the number of light blue plaques by the total number of blue plaques. The number of % Light Blue values does not necessarily coincide with the number of IC/ml values due to the fact that progeny phages can be plated multiple times in order to obtain sufficient data. Additionally, the number of IC/ml values may be greater in some cases if values for % Light Blue were unable to be calculated. The percent G to T mutations for the unmodified control and FAPY major were roughly zero, so these data were not calculated.

Data for Unmodified Control

Set Number	Induced Polymerase	IC/ml
1	None	1745000
		2390000
		1590000
		2195000
		3470000
		4340000
		6065000
	UmuDC	740000
		281000
		91000
		158000
		110500
	MucAB	6425000
		4745000
		4980000
		4260000
		4820000
		4030000
	UmuDC and MucAB	650000
		581500
		421000
		394000
		236000
		256000

Data for Unmodified Control

Set Number	Induced Polymerase	IC/ml
1	UmuDC Knockout	5935
		5340
		3605
		2400
		1530
		1445
		815
		1200
		115
		395
		220
		205
		215
		300
		230
		100
		1000

Data for Unmodified Control

Set Number	Induced Polymerase	IC/ml
1	UmuDC Knockout and MucAB	1730
		2770
		3170
		2830
		950
		1780
		1400
		1345
		60800
		40500
		57100
		61050
		78000
		86000
		72000
		28700
		97000
		59000
		71000
		83000

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	None	900	3.5
		2900	23.1
		2200	9.7
		1300	5.3
		3100	60
		2200	23.1
		2000	40
		3600	68.6
		200	78.6
		2100	28.6
		1300	
		1000	
		1000	
		1250	
		4330	
		2400	
		1485	
	UmuDC	1500	93.3
		1200	25
		500	20
		700	21.1
		300	28.6
		800	41.7
		500	100
		300	14.6
		0	62.5
		1100	2.5
		1900	14
		900	2.6
		1600	1.6
		200	
		500	
		1400	
		1200	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	MucAB	7300	15.1
		15700	15.3
		10400	23.1
		10100	21
		9900	24.2
		9800	26.5
		9300	14
		4300	23.3
		6030	56.5
		4990	50
		4430	65.2
		4000	50
		4870	
		15150	
		11400	
		15100	
	UmuDC and MucAB	3100	19.4
		2600	19.2
		1700	23.5
		2200	72.7
		2400	87.5
		900	17
		800	44.4
		4000	16.3
		4600	12.5
		3100	37.5
		6010	5.9
		5780	23.9
		5100	22.5
		4470	42.3
		4000	33.3

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	UmuDC Knockout	660	8.5
		430	4
		380	8.3
		460	0
		100	
		70	
		250	
	UmuDC Knockout and MucAB	700	0
		1150	11.8
		820	25
		670	
		795	
		810	
		360	
	DinB Knockout	60	
		0	
		0	
		40	
		110	
		5	
		55	
		20	
	DinB and UmuDC Double Knockout	0	
		0	
		0	
		0	
		0	
		0	
		0	
		0	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
2	None	435	70.3
		290	16.7
		375	4.2
		315	4.6
		645	9.1
		740	33.3
		330	12.3
		40	8.8
		125	6.1
		220	53.3
		150	18.2
		10	20
		10	
		1850	
		905	
		560	
		425	
	UmuDC	865	18.2
		335	21.3
		430	13
		395	18
		325	22.2
		30	33.3
		60	62.5
		40	50
		320	
		90	
		10	
		10	
		70	
		2340	
		5300	
		4320	
		3410	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
2	MucAB	4740	28.1
		3000	30.9
		1325	28.1
		3535	24.1
		3720	23.5
		485	33.3
		1175	34.6
		1860	36.8
		3150	29.7
			23.7
			31.6
			28.2
			10
			19.2
			51.4
	UmuDC and MucAB	65	25
		30	66.7
		20	66.7
		40	26.7
		30	52.9
		30	60
		275	30
		185	33.3
		75	50
		100	57.1
		145	10
		70	35.3
		100	66.7
		235	25.9
		535	50
		265	43.5
		80	33.3
		30	75
		10	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
2	UmuDC Knockout	110	
		140	
		120	
		70	
		70	
		0	
		0	
		110	
		30	
		10	
		10	
		10	
	UmuDC Knockout and MucAB	24400	0
		21300	0
		26800	0
		19500	0
		16300	
		10420	
		8655	
		6880	
		7640	
		4235	
		12500	
		21600	
		20300	
		14100	
		21100	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
3	None	2400	0
		1497	
		1000	
		1193	
		2445	
		900	
		633	
		825	
	UmuDC	6200	2
		2100	0.8
		1985	0.8
		3425	4
		1000	3
		600	
		690	
		3205	
		1760	
	MucAB	3564	23
		1517	27
		6000	59
		3390	36
		3583	30
		2767	
		1170	
		2710	
	UmuDC and MucAB	5365	28
		4560	28
		5205	21
		4060	27
		2400	20
		3100	35
		1400	32

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
3	UmuDC Knockout	10	
		10	
		10	
		10	
		20	
		30	
		0	
		0	
		30	
	UmuDC Knockout and MucAB	0	20
		90	25.8
		80	16.3
		40	4.6
		95	19
		90	35.7
		0	17.8
		75	11.1
			8.1
			9.5
			15.8
			14.9
			19.5
			15.4
			11.1
			12.5
			9.2
			12.8
			8.3
			3.1
			21.6
			31.3
			21.4
			15.5
			7.7

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
4	None	210	0
		200	
		60	
		90	
		80	
		60	
		10	
		10	
	UmuDC	2806	0.5
		4000	1.3
		2663	
		3478	
		2000	
		1625	
		1270	
		1300	
		0	
		1400	
		3840	
		3690	
		3410	
	MucAB	3000	2.9
		6400	10.6
		572	6.9
		1555	14.1
		3379	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
4	UmuDC and MucAB	1800	19.1
		3700	11.3
		964	8.6
		899	6.1
		928	21.1
		587	6.7
		1408	9.8
		5040	22.2
		6220	71.4
		4100	33.3
		590	50
		3655	39
	UmuDC Knockout	175	
		160	
		115	
		80	
		65	
		50	
		95	
		80	
		50	
		20	
		50	
		165	
		40	
		30	

Data for FAPY Mix

Set Number	Induced Polymerase	IC/ml	% Light Blue
4	UmuDC Knockout and MucAB	10	
		21	
		10	
		10	
		825	
		2700	
		725	
		510	
		690	
		620	
		510	
		1030	
		750	
		595	
		865	
		715	
		540	
		725	
		520	
		285	
		275	
		350	
		60	
		310	
		185	
		60	
		80	
		110	
		60	
		170	

Data for FAPY Major

Set Number	Induced Polymerase	IC/ml
1	None	55
		60
		200
		60
		110
		775
		210
		577
		220
		50
		55
		30
	UmuDC	0
		30
		705
		1000
		430
		245
		420
		320
	MucAB	490
		400
		20
		70
		290
		335
		750
		640
		485
		510

Data for FAPY Major

Set Number	Induced Polymerase	IC/ml
1	UmuDC and MucAB	10
		530
		265
		845
		975
		1275
		520
		470
	UmuDC Knockout	50
		75
		30
		0
		10
		10
		0
		0
	UmuDC Knockout and MucAB	0
		50
		80
		0
		55
		30
		30
		20

Data for FAPY Major

Set Number	Induced Polymerase	IC/ml
2	None	110
		0
		0
		10
		0
		0
		905
		640
		595
		725
		565
		310
	UmuDC	150
		105
		100
		185
		75
	MucAB	350
		340
		605
		250
		185
		160
		260
		250
	UmuDC and MucAB	160
		240
		175
		105
		160

Data for FAPY Major

Set Number	Induced Polymerase	IC/ml
2	UmuDC Knockout	10
		0
		20
		0
		10
		0
		0
	UmuDC Knockout and MucAB	20
		30
		0
		40
		30
		0
		20
		20
		40

Data for FAPY Major

Set Number	Induced Polymerase	IC/ml
3	None	420
		485
		130
		115
		170
		95
		155
		90
		140
		10
		20
		55
	MucAB	1210
		400
		350
		320
		60
		205
		165
		215

Data for FAPY Major

Set Number	Induced Polymerase	IC/ml
4	None	0
		0
		0
		0
		0
		70
		500
		210
		165
		90
		230
		295
	MucAB	200
		220
		80
		145
		175
		215

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	None	559000	1.8
		701000	1.1
		390000	2.3
		452000	1.6
		486000	2.1
		397000	1.5
		323000	0.62
		347000	1.2
			1.2
			0.91
			3.5
			2.2
			1.3
			1.2
			3.8
			2.2
	UmuDC	132750	1.2
		140300	1.1
		102450	1.1
		101850	1.3
		89900	7.6
		72100	1.6
		87750	1.5
		63800	1.7
		181500	0
		60300	1.4
		65450	3.5
		45150	2.9
		50550	3
		42800	1.9
		49200	0
			1.8

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	MucAB	39050	7.5
		19200	4.4
		15950	5.7
		28850	7.1
		21200	6.2
		29350	15.6
		20800	10.5
		18150	6.5
			5.8
			6.7
			7.3
			11.8
			5.4
			13
			6.1
			15
			4.2
	UmuDC and MucAB	53850	10.5
		52400	8.3
		67000	12.5
		52250	0
		57700	14.1
		58250	8.6
		33100	11.9
		53250	9.2
			8.6
			6.6
			10
			6.8
			4.7
			5.2
			7.5
			8.4

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	UmuDC Knockout	2850	0
		3515	5.6
		2730	2.2
		2815	2.6
		3660	5.8
		2635	2.2
			3.2
			1.8
			0
			1.2
			5.4
			1.1
			4.9
			1.4
			5.1
			2.3
			6.5
			0.89
			0
			1.6
			4.2
			1.4
			2.6
			2.1
			2.9
			1.5
			2.9
			1.5
			5.1
			2.2
			6.8
			2.7
			4.8
			3
			5.3

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
1	UmuDC Knockout and MucAB	76100	4.1
		72900	2.9
		52800	2.2
		44900	6.5
		44300	5.9
		48500	4.8
		48500	4.7
		32800	1.8
			5.9
			4.5
			0
			7.6
			10.3
			8.5
			20
			2.5

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
2	None	5055	2.8
		7015	1.6
		6700	2.9
		4630	1.7
		7500	4
		5175	2.9
		5295	0
		5750	2.1
		17100	3.1
		10700	0.85
		5585	0.96
		3445	0
		3050	1.6
		2120	2.1
		4350	2.5
			1.8
			1.7
			2.5
			1.8
			2.7
			1
			1.6
			2.3
			0.45
	UmuDC	98000	2.5
		117500	1.3
		140400	1.9
		162300	1.2
		99600	2.9
		133700	0.88
		85000	2.9
			1.2
			4
			2

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
2	MucAB	24600	8.5
		17800	5.3
		21300	7.4
		26400	0
		11400	5.4
		12000	15.2
		16100	6.7
		20900	5.3
			4.6
			23.3
			6.6
			3.7
			8.2
			14.3
			4.6
			5.6
			9.8
			6.3
	UmuDC and MucAB	70600	8.3
		48700	7.1
		84500	8.7
		74400	7.7
		66500	12.5
		83200	9.2
		60200	2.1
		66000	6
			15.6
			10.1
			6.5
			8.5
			6.7
			8.6
			4.4
			7.3

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
3	None	880	0.89
			3.1
			1.9
			3.3
	UmuDC	80100	3.8
		71000	3.3
		113000	3
		62000	4.1
		7250	2.9
		21000	0.74
			0.72
			1.9
			1.4
			1.6
			1.7

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
3	MucAB	3160	6.8
		480	8.7
		3000	12.5
		250	5.6
		1815	10.3
		1985	1.2
		955	1.8
		1035	4.4
		765	8.2
		1200	4.4
		515	0
		530	6.3
			6.5
			4.6
			6
			6.8
			5
			9.9
			6
			1.8
			3.8
			5.2
			4.7
			8.1
			4.8
			7
			4.3
			5.6
			4.3
			5.9
			5.9
			0
			5.3
			2.4
			7.4

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
3	UmuDC and MucAB	40250	6.5
		55000	5.5
		38000	3.7
		49000	2.7
		44000	6.9
		37000	5.2
			6.8
			6.3
			3.3
			8.1
			4.7
			13.2
			6
			4.4
			2.2
			3
			6.4
			6.1
			7.6
			8.9
			7.1
			0
			7.7
			5.4
			6.6
			7.9
			7.1
			6.3
			6.7
			6.9
			8.6
			6.3
			3
			9.8
			6.1

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
4	None	261000	2.8
		221000	1.9
		172000	1.9
		185000	5.1
		149000	3.4
		169000	0
		209000	2.4
		137000	0
			2.7
			4.2
			1.7
			0
	UmuDC	71250	10.8
		87550	0
		62400	6.8
		61200	0
		71250	9.9
		67100	12.5
		62950	10.2
		56350	0
			6.8
			0
			6.7
			0
			7
			16.7
			0

Data for AFB_1-N7-Gua

Set Number	Induced Polymerase	IC/ml	% Light Blue
4	MucAB	18350	0
		27250	1.4
		20600	2.2
		25450	1.6
		24800	1.9
		22650	2.3
		20700	0
		13350	2.8
			8.6
			2.3
			5.7
			2.2
			4.4
			0
			2.5
			10
	UmuDC and MucAB	47500	6
		39900	7.7
		45850	5.2
		40600	4.6
		36900	5.8
		60750	5.4
		38700	6.8
		40250	6.1
			5.2
			14.9
			5.5
			0
			5.4
			1.9
			10.1
			0
			4.6
			2.7

Appendix B:

Mutant Identification or Enrichment Procedures

Introduction

Better techniques should be developed to either identify mutations that occur at very low frequencies or to enrich for mutations universally. There are several characteristics that describe a good mutant enrichment procedure. First, it should be able to enrich for mutations in any given sequence context. Second, it should be able to enrich for mutations at more than one target site, including multiple mutations as well. Third, it should be easy to use and easily adaptable to different systems. Preliminary experiments have been performed to test a number of ideas for a new mutant identification and enrichment procedures. These ideas include plaque hybridization, ligation mediated cleavage, hairpin hybridization, addition of fluorescent dideoxynucleotides, the PROBE assay, and a PNA enrichment procedure. They are described below.

Mutation Identification Procedures

Plaque Hybridization

This idea was based on the property of DNA sequences to hybridize differently to perfectly matched and mismatched sequences. In theory 21 base oligonucleotides that are complementary to each possible mutant type would be used as probes. Two radiolabelled oligonucleotide probes were tested here: one complementary to the inserted DNA sequence and one complementary to a G to T base substitution of this sequence (a single mismatch). Phages known to contain either a wild type or G to T substitution in the region of interest were plated. Plaque lifts were performed in duplicate for each plate, and the duplicate plaque lifts were incubated with either the wild type or G to T complement. The result obtained demonstrated cross hybridization between the wild type and mismatch probes, indicating that this method was not sensitive enough to use as an enrichment procedure. Several different buffering conditions were tested.

One Step Addition of Fluorescent Oligonucleotides

This idea is similar to DNA sequencing, but it has yet to be tested. The premise is to obtain DNA from progeny phages and anneal a primer that is complementary and terminates 1 base 5' to the target site. The length of this primer can be modified to evaluate mutations at any site. Once annealed to the template, the primer would be incubated with sequenase version 2.0 and all 4 dideoxynucleotide bases. The 4 bases would be fluorescently labeled with different dyes, as they are for automated DNA sequencing. The chain will terminate at the site of interest,

containing a fluorescent dideoxynucleotide of a certain color. The percentage of each color present would quantitate the types of mutations present.

The PROBE Assay

The PRimer-Oligonucleotide Base Extension (PROBE) assay was developed by others (Jalanko et al., 1992; Paunio et al., 1996; Pastinen et al., 1996) (Figure B.3). It involves the use of an oligonucleotide probe that hybridizes a few bases upstream of the target sequence. The probe will be incubated with the template phages DNA in four separate reaction tubes, each containing one of the four dideoxynucleotides and the other 3 deoxynucleotides. Each tube will then be incubated with sequenase version 2.0 and an extension reaction will occur. The chain will terminate differently depending on which base is present at the target site, and the results could be analyzed using PAGE. This assay was tested for sensitivity by mixing oligonucleotides of different lengths at different ratios and running them out on the gel. It was determined that this procedure is

successful for detecting mutations above a threshold of 5%. It was not sensitive enough to detect very low frequency mutations.

Mutation Enrichment Procedures

Ligation-Mediated Cleavage

This method employs the same principles of differential hybridization as well as the discriminatory ability of *Taq* ligase and cleavage of a type IIs restriction site. Two 19 base oligonucleotides could be annealed to the phage DNA as shown in Figure B.1, one having its 3' base complementary to the target base and one having its 5' base complementary to the base 5' to the target base. If these two oligonucleotides are perfectly complementary to the sequences to which they are annealed, they can be ligated together efficiently. This procedure relies on the fidelity of *Taq* ligase to only ligate oligonucleotides that are paired with perfectly matched sequences. If a mismatch occurs between the oligonucleotide and the template, the ligase would not join the two oligonucleotides together. If the oligonucleotides are ligated successfully, a substrate for the type IIs restriction enzyme, *Bbs*I, will be formed. *Bbs*I will recognize a site upstream from the target site and will cleave between the target and 5' base. Using different oligonucleotides complementary to each wild type sequence to be tested would allow for universal use of this technique. The downfall of this enrichment procedure was due to the fact that *Taq* ligase was not able to be completely inactivated or removed from the reaction. The ligase continued to contaminate the reaction even after *Bbs*I cleavage, ligating cleaved products back together.

Hairpin Hybridization

This technique also employed the use of differential hybridization with that of a type IIs restriction enzyme. A 54 base oligonucleotide containing a self complementary hairpin region

was engineered such that the region involved in the hairpin would form the *Fok*I recognition sequence, whereas the region not involved in the hairpin would be complementary to the target DNA. The region complementary to the target DNA contained a site that could be variable, and this site was at the posititon 5' to the targeted base. The *Fok*I restriction enzyme would have a recognition sequence but would only cleave the product if the region complementary to the target sequence was annealed properly (Figure B.2). The reason this method could work better than the previous differential hybridization technique would be that this technique employs the fidelity for *Fok*I to cleave a product that has its 5' end hybridized securely rather than be impeded by a mismatched base. This technique proved successful to an extent, but not to the degree of sensitivity required for enrichment of low frequency mutations. Different mismatched bases were cleaved with different efficiencies, introducing bias into the system. This method was also attempted with an engineered destabilizing mismatch, a nitropyrrole derivative, at a single position. The purpose of this would be to make different mismatches equally unstable. It proved to better the experimental results; however, the technique still proved to be somewhat biased.

PNA Enrichment Procedure

This technique once again employs the use of differential hybridization. However, PNA:DNA hybrids are used instead of DNA:DNA hybrids. PNA is much more selective in its descrimination between perfectly matched and mismatched sequences. One mismatch can lead to destabilization of the PNA:DNA duplex by 20°C. PNA cannot be analyzed by PAGE, however, since it is neutral and will not be pulled into the gel matrix. Therefore, an experimental procedure was designed involving biotinylated PNA and streptavidin coated magnetic beads.

PNA was hybridized to a pool of DNA containing a known percentage of mismatched and perfectly matched sequences. It was incubated at a temperature at which the perfect complements would remain hybridized while the mismatches would melt away. The mixture was then exposed to a magnet, which would pull all the PNA:DNA hybrids into a pellet, leaving behind a supernatant containing mutant genomes. The supernatant could be pipetted out and used to transform *E. coli*. This experiment showed that there was irreproducible cross reaction between wild type and mutant sequences. The cross reaction may be due to mutant DNA physically being pulled over along with wild type PNA:DNA hybrids, even though the mutants are not hybridized to the PNA.

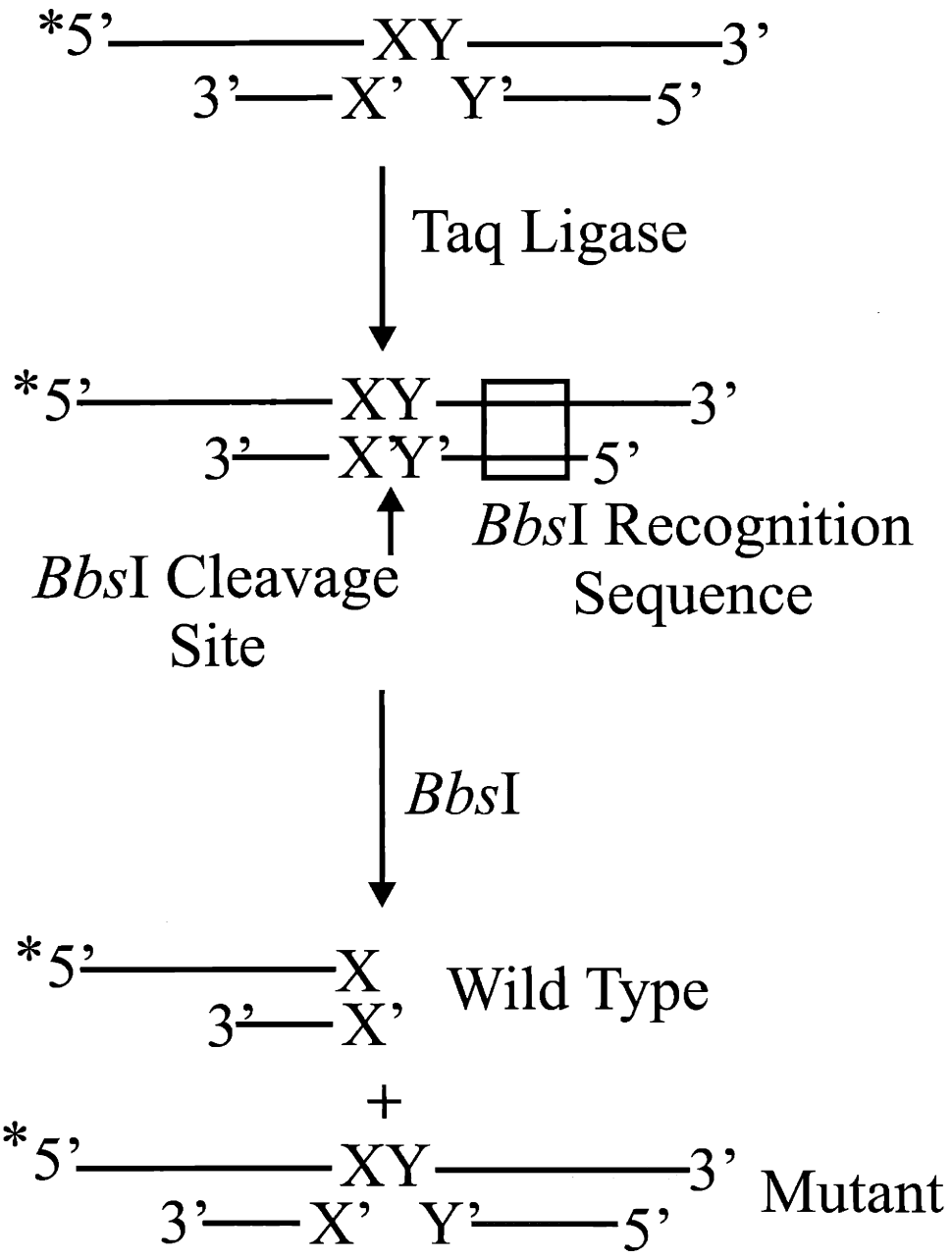


Figure B.1: Ligation-mediated cleavage enrichment procedure. Two 19 base oligonucleotides are annealed to a target. These two oligonucleotides are ligated by *Taq* ligase if they are perfectly complementary at the indicated positions. After ligation, the double stranded DNA is subjected to type IIs restriction enzyme digestion with *Bbs*I. Cleavage of the radiolabelled target will only occur if the DNA is double stranded (*ie*, ligated together) at the cleavage site.

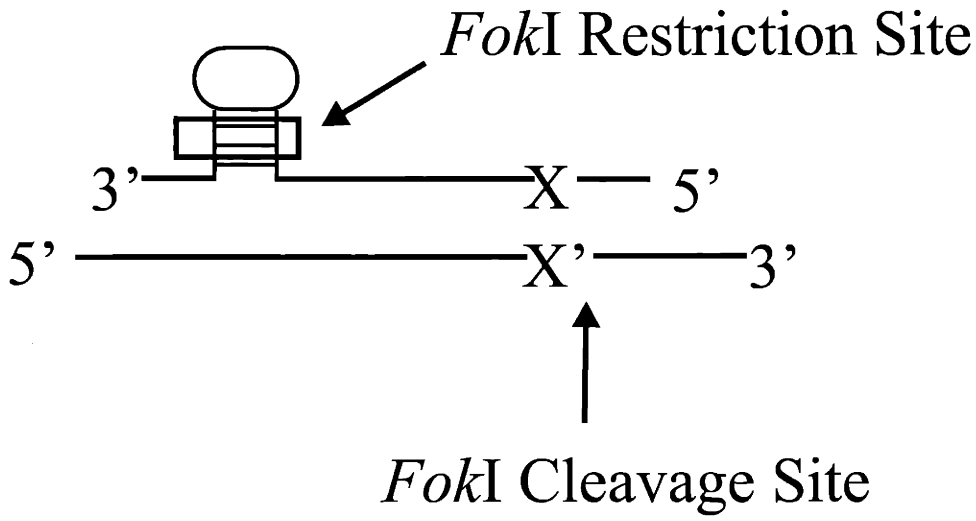
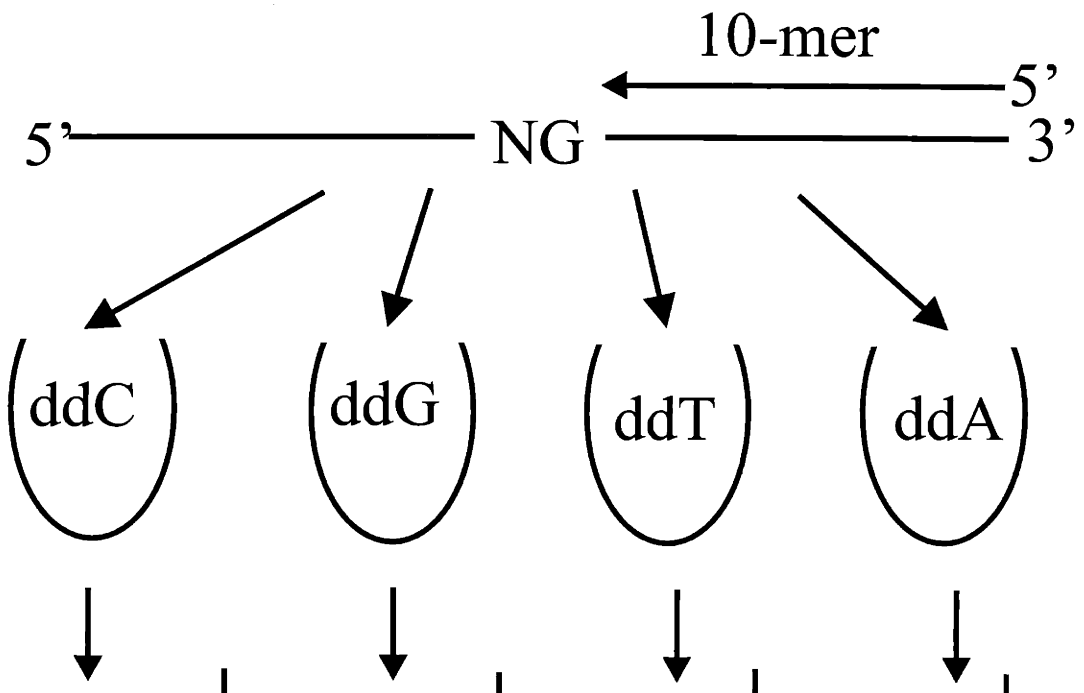


Figure B.2: *Fok*I enrichment procedure. An oligonucleotide with a self complementary region is annealed to the target sequence. Cleavage will only occur if the complementary region is perfectly matched. A mismatch will prevent cleavage.



100% 11	100% 15	100% 12	100% 13	WT (N=A)
96% 11 4% 19	4% 11 96% 15	100% 12	100% 13	G→C
100% 11	100% 15	0.5%20 99.5%12	99.5%13 0.5% 12	A→T
96% 11 4% 19	4% 11 96% 15	0.5% 20 99.5% 12	99.5% 13 0.5% 12	G→C A→T
96% 11 4% 19	4% 11 96% 15	99.5% 12 0.5% 20	100% 13	G→C A→C

Figure B.3: PROBE assay. A 10 base primer is annealed to the target and extended by sequenase version 2.0. Four separate extension reactions are carried out for each sample, each one containing a different dideoxynucleotide. The chart shows what percent of each length oligonucleotide would be produced in each tube for each mutation shown at the right. Examples are only shown for G to C mutations at the target site at a rate of 4%, A to T or A to C mutations at the 5' site at a rate of 0.5%, or a combination of these mutations.

Reference List

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Paunio,T., Reima,I., and Syvanen,A.C. (1996). Preimplantation diagnosis by whole-genome amplification, PCR amplification, and solid-phase minisequencing of blastomere DNA. Clin. Chem. *42*, 1382-1390.

Biographical Note

Maryann Smela was born and raised in the Ironbound section of Newark, NJ by her parents, Edward J. and Anna M. Smela (nee Sidor), and her grandmothers, Katherine Smela and Victoria Sidor. She has two older sisters, Jean A. Stroud and Teresa M. Barrett, and 5 nieces and nephews. Maryann attended grammar school at St. Casimir's parish, spending nine years with a close group of friends who remain dear to this day. She played Ironbound Little League and Senior League softball since the age of ten years old and was a member of the Polish Falcons of America bowling, gymnastics, and cultural dance club since the age of five. She also attended Polish language classes for three years.

Maryann attended high school at Science High in Newark, a magnet school and former brewery located downtown in the business district. She was a member of the varsity softball, bowling, volleyball, and track teams, and she was active in the math club, school newspaper, and independent science research project team. Maryann also attended a special program to learn advanced biology at Essex County College during her senior year.

Maryann was accepted to MIT in 1992, and attended for four years of undergraduate education, receiving her bachelor's degree in biology with a minor in chemistry in 1996. She was a member of the varsity softball team and the junior varsity volleyball team. She was also a teacher for the SAT preparatory program, intramural sports coordinator for her undergraduate dormitory, and a member of several intramural sports teams. Maryann was also a student manager of the dormitory dining facility and, later, an undergraduate researcher for Lisa A. Bailey and John M. Essigmann.

Although Maryann was accepted to graduate programs at Cornell, Stanford, Princeton, Harvard, and Johns-Hopkins, she remained at MIT under the advisement of John M. Essigmann. During her years in graduate school, she became an umpire, player, team captain, and co-commissioner for the MIT Community Summer Softball league. She also organized departmental teams in summer volleyball and coordinated other activities for her laboratory group. She also became a member of the MIT Women's Ice Hockey Club in 1998, taking over the captain's chair in 2001. In her third year of graduate school, Maryann and her fiancé Alfred M. Timinski (her high school sweetheart) became graduate resident tutors at the Third West hall of the East Campus undergraduate dormitory, where she played "big sister" to forty undergraduate students. Maryann has also supervised five undergraduate researchers and one technician in her time at the lab. She was also the teaching assistant for one of the courses taught by Professors John M. Essigmann and Robert Langer. Maryann will receive her PhD from the Division of Boengineering and Environmental Health (formerly the Division of Toxicology) in June 2002.