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Perspective

Mechanisms of Frameshift Mutations: Insight from Aromatic Amines

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Introduction

This perspective explores mechanisms of frameshift mutagenesis as revealed by studies of selected aromatic amines and amides. The data are drawn primarily from studies of three compounds: *N*-2-(acetylamino)fluorene (AAF),[1] its deacetylated counterpart *N*-2-aminofluorene (AF), and its halogenated derivative *N*-2-(acetylamino)-7-iodofluorene (AAIF). The structures of these compounds and that of *N*-acetoxy-*N*-2-(acetylamino)fluorene (N-Aco-AAF), an electrophilic compound commonly used to introduce AAF adducts into DNA, are shown in Figure 1.

AF and AAF have generated much interest because they are excellent model chemicals on several levels. First, as aromatic amines, they have yielded critical information on the roles of metabolic activation in the mutagenicity and carcinogenicity of this important group of compounds (reviewed in refs 1–3). Second, they are the most thoroughly studied of the many carcinogens that form adducts on the C8 position of guanine in DNA; as such, they provide insight into the biological consequences of an important class of DNA damage. Among the compounds that bind to the C8 position are mutagens formed in cooked foods (see ref 4 for examples) and nitroaromatic compounds (*e.g.*, nitrofluorene, nitropyrene), a major group of environmental carcinogens formed by the incomplete combustion of organic materials (*5*). Third, the effects of AAF and AF adducts on DNA conformation have been characterized biophysically and reveal linkages between macromolecular structure and toxicological effects, especially mutagenesis. More than 50 years ago AAF and AF were being developed as potential pesticides, but the agricultural use of both compounds was terminated when AAF was found to be carcinogenic (reviewed in ref 1). Though they do not have significant industrial use today, these compounds continue to yield important insight into mechanisms of

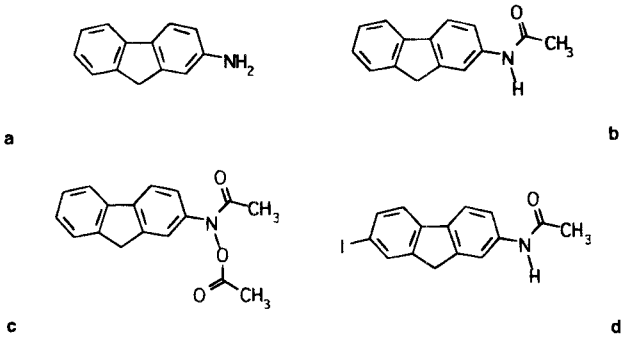

Figure 1. Structures of aromatic amines and amides. (a) *N*-2-Aminofluorene. (b) *N*-2-(Acetylamino)fluorene. (c) *N*-Acetoxy-*N*-2-(acetylamino)fluorene. (d) *N*-2-(Acetylamino)-7-iodofluorene.

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[1]Abbreviations: 9AA, 9-aminoacridine; AAF, *N*-2-(acetylamino)-fluorene; AAIF, *N*-2-(acetylamino)-7-iodofluorene; AF, *N*-2-aminofluorene; BAA, bromoacetaldehyde; DEPC, diethyl pyrocarbonate; HA, hydroxylamine; N-Aco-AAF, *N*-acetoxy-*N*-2-(acetylamino)fluorene; N-Aco-AAFF, *N*-acetoxy-*N*-2-(acetylamino)-7-fluorofluorene; N-OH-AAF, *N*-hydroxy-*N*-2-(acetylamino)fluorene; N-OH-AF, *N*-hydroxy-*N*-2-aminofluorene; TLS, translesion synthesis.

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mutagenesis and carcinogenesis.

A comprehensive review by Heflich and Neft (*1*) provides an excellent overview of the chemistry, metabolism, carcinogenicity, and genetic toxicology of AAF, AF, and related compounds. In this perspective, we focus on the insight that these compounds have yielded into mechanisms by which chemicals induce frameshift mutations. We begin our perspective by introducing frameshift mutations and models of frameshift mutagenesis. We then summarize data on the mutagenicity of AAF and related compounds and discuss current research on mechanisms of frameshift mutagenesis. We conclude by tying these observations together in models that explain the frameshift mutagenicity of aromatic amines. The models entail the hindrance of replication, the formation and stabilization of mispaired premutational intermediates in DNA, and the generation of mutations from these intermediates.

Background on Frameshift Mutations and Mutagens

Changes in DNA sequence at a localized site within a gene are called gene mutations or point mutations. The two major kinds of point mutations are base-pair substitutions and frameshift mutations (reviewed in ref 6). In the former, one base pair in DNA (*e.g.*, G:C) is replaced by another (*e.g.*, A:T), but the number of base pairs is not altered. Unlike base-pair substitutions, frameshift mutations have gained or lost base pairs relative to the original sequence. The simplest classification of frameshifts is by the number of base pairs involved. For example, the gain of a single G:C base pair (*e.g.*, 5′GGGG3′ → 5′GGGGG3′) can be described as a +1 frameshift, and the deletion of two base pairs (*e.g.*, loss of adjacent G:C and C:G base pairs in the mutation 5′GGCGCC3′ → 5′GGCC3′) can be described as a −2 frameshift. Most commonly, frameshifts involve the gain or loss of one or two base pairs, thereby altering the reading frame of the genetic code. Because every codon after the point of the mutation is changed, the gene product is grossly altered, and it is apt to be incomplete because of the generation of a nonsense codon in the new reading frame. Frameshift mutations therefore lead to nonfunctional gene products, and their phenotypic effects depend on how the absence of the gene function affects viability and metabolism.

A mutation spectrum, revealed by DNA sequence analysis, is comprised of the kinds of mutations that occur and their distribution within a gene. Spectra of spontaneously occurring and induced mutations show that the distribution of mutations within a gene is often highly nonrandom. Some sites, called hotspots, are much more mutable than others. A clear example of a hotspot is found in studies of the *lacI* gene of *Escherichia coli*, in which 71% of 729 spontaneous mutations were in a small region of the gene (positions 620−632) whose sequence is $[5'TGGC3']_3$ (*7*). Induced mutations are also clustered in hotspots, as evidenced by 98% of *lacI* mutations induced by the acridine mustard ICR-191 being +1 and −1 frameshifts in runs of repetitive G:C base pairs (*8*).

Chemical mutagenesis entails chemical or physical alteration of the structure of DNA. Frameshift mutagens may stimulate the induction of mutations by reacting with DNA covalently or by noncovalent interactions. Acridine compounds, perhaps the most familiar frameshift mutagens, intercalate between DNA base pairs and, in some cases, also bind to DNA covalently (*9*, *10*). Intercalation is sufficient for mutagenesis, in that the growth of bacteria in the presence of a simple acridine such as 9-aminoacridine (9AA) leads to base pairs being gained or lost when the DNA containing the intercalated planar ring system is replicated (*9*, *11*). 9AA primarily induces additions and deletions of single base pairs in monotonous runs of a single base (*e.g.*, GGGGG). In contrast to simple intercalating agents, most mutagens form covalent adducts in DNA. Even among the acridines, compounds that both intercalate and form covalent adducts, such as the acridine mustards or nitroacridines, are more potent mutagens than the simple intercalators. Besides being more potent, the reactive acridines induce a more diverse array of mutations, including −2 frameshifts at alternating G:C base pairs in addition to −1 and +1 frameshifts in monotonous runs of bases (*10*, *11*).

While some mutagens are electrophiles that form covalent adducts in DNA directly, many are not themselves electrophilic but are converted into electrophiles in mammalian metabolism (reviewed in refs 2 and 6). For example, polycyclic aromatic hydrocarbons can be activated by several mammalian tissues into diol epoxides that react with DNA to form adducts. Studies of the biological effects of such compounds must make provision for their metabolic activation or use reactive derivatives that are electrophilic without metabolic activation. For this reason, the reactive N-Aco-AAF (Figure 1) has been used as a substitute for AAF, and *N*-hydroxy-*N*-2-aminofluorene (N-OH-AF) has substituted for AF in many of the studies that we discuss.

AAF and AF have been used extensively to explore mechanisms of mutagenesis. As experimental compounds, they offer the advantage that the chemical lesions that they form in DNA are well-characterized. Not only are the adducts known, but there is substantial information on how they alter the conformation of DNA. AAF and related compounds therefore offer an opportunity to relate specific genetic effects to defined chemical adducts and physical alterations of DNA.

Models of Frameshift Mutagenesis

Streisinger Slippage Model. A well-known model for frameshift mutagenesis was proposed by Streisinger and colleagues several decades ago (*12*, *13*). The Streisinger model, which continues to have much support (*9*), explains frameshift mutagenesis as a consequence of slipped mispairing. According to the model, frameshift mutations are generated by slippage, or localized pairing out of register, during the replication of DNA. Slippage, which occurs at sites of repetitive bases (*e.g.*, GGGGG), leaves an unpaired base or a few bases bulged out of the helix. During DNA replication, such a bulge in the template strand can lead to deletion, while a bulge in the primer strand can lead to insertion of an extra base (or bases) in the new strand.

Misalignment during replication occurs readily in runs of repetitive bases because proper pairing can occur adjacent to the bulge. Repetitive bases are therefore more prone to spontaneous +1 and −1 frameshifts than are nonrepetitive sequences. The gain or loss of a single base from a monotonous run of bases is the most obvious case of frameshift mutagenesis by slippage. The model, however, is not restricted to +1 and −1 frameshifts or to monotonous runs, in that it can be extended to frameshifts arising in other repetitive sequences, such

as the loss of two base pairs (*e.g.*, G:C and C:G) from a region of repetitive GpC dinucleotides (*e.g.*, GCGCGCGC).

The unusual case of a hotspot in the *E. coli lacI* gene illustrates how repetitive sequences are prone to the gain or loss of one unit of the repetition. The sequence $[5'TGGC3']_3$ is a strong hotspot for spontaneous mutations in *lacI*. Most mutations at this site are +4 frameshifts with an extra TGGC and −4 frameshifts that have lost a TGGC sequence (*7*). Less frequent mutations at this site can also be attributed to slippage, such as the +8 insertion (*i.e.*, +TGGCTGGC). While mutagenesis at the *lacI* hotspot is explainable by slippage, it appears to be modulated by other factors, in that the ratio of +4 additions to −4 deletions varies among strains that are altered in DNA repair (*7*), SOS induction (*14*), or sequence upstream from the repeat (*15*).

Nick-Processing Model. The slipped mispairing model does not explain all observations on the induction of frameshift mutations, and a nick-processing model has been proposed by Ripley and colleagues (*16*, *17*) to explain acridine-induced mutagenesis in bacteriophage T4. Nick processing depends on the DNA-nicking activity of T4 topoisomerase and the polymerase and exonuclease activities of T4 DNA polymerase (*16*, *17*). The nick-processing model does not require a repetitive sequence. The applicability of the nick-processing model beyond frameshifts at nonrepetitive sequences in phage T4 requires further exploration, but it seems likely that frameshift mutations may arise by several mechanisms. In this respect, slipped mispairing should not be regarded as explaining all frameshift mutagenesis, but it explains the induction of + and − frameshift mutations in repetitive sequences; these mutations comprise a prominent part of the mutation spectrum of many frameshift mutagens (*9*, *10*).

Applicability of Slippage Models to Induced Frameshift Mutagenesis. The slippage model nicely explains the generation of spontaneous frameshift mutations in repetitive sequences. Its applicability to induced mutagenesis depends on the slippage process being favored by adducts in DNA or by molecules that associate with DNA noncovalently. Frameshift mutagens may increase the opportunity for mutations to arise by enhancing the formation of slipped mutagenic intermediates or by stabilizing them (*9*). For example, there is evidence that acridines preferentially bind to the DNA bulges associated with slipped mispairing and may stabilize these mispaired structures (*9*). In the case of covalent adducts in DNA, an understanding of mutagenesis requires the elucidation of mechanisms by which specific adducts favor the formation of premutational intermediates or the processing of these intermediates into mutations. In this perspective, we explore these issues focusing on the AAF adduct as a model frameshift mutagen.

Effects of Aromatic Amines on DNA Structure

Chemistry and Metabolism of AAF and AF. AAF and AF require metabolic activation into electrophilic forms in order to be mutagenic and carcinogenic (reviewed in ref 1). The metabolism of AAF, AF, and related compounds is summarized in Figure 2. The first step in the metabolic activation of AAF and AF is N-oxidation catalyzed by a cytochrome P450 monooxygenase, giving rise to *N*-hydroxy-*N*-2-(acetylamino)fluorene (N-OH-AAF) and N-OH-AF, respectively. N-OH-AF may react with DNA via an arylnitrenium ion or be converted into an ester in a sulfotransferase or *O*-acetylase reaction; the esters react with DNA even more effectively than N-OH-AF. N-OH-AAF does not react with DNA directly but, like AF, may be converted into a reactive ester in a sulfotransferase reaction. The esters of AF and AAF readily give rise to electrophilic arylnitrenium ions that attack DNA. In order to circumvent the need for metabolic activation, many studies of AAF and AF adducts are conducted with the direct-acting carcinogens N-Aco-AAF (*18*) and N-OH-AF (*19*), respectively.

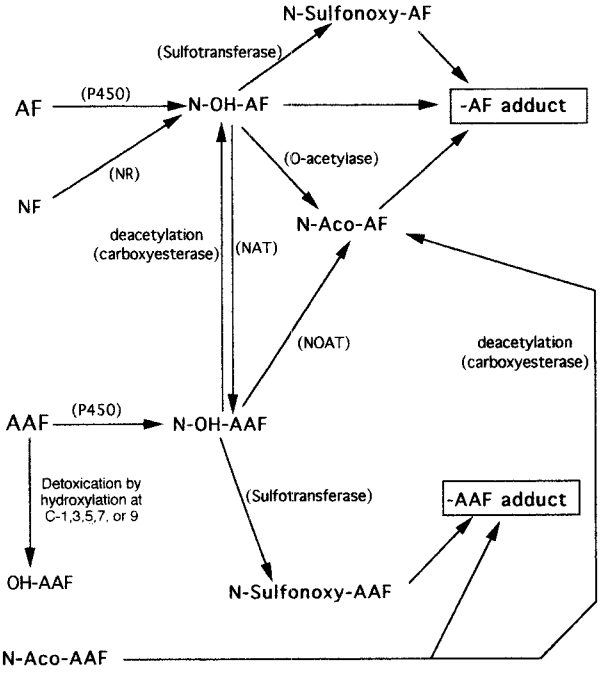

Figure 2. Metabolism of AAF, AF, and related compounds (figure based on Heflich and Neft (*1*)). Compounds are abbreviated as follows: AAF: *N*-2-(acetylamino)fluorene; AF: *N*-2-aminofluorene; NF: 2-nitrofluorene; N-Aco-AAF: *N*-acetoxy-*N*-2-(acetylamino)fluorene; N-Aco-AF: *N*-acetoxy-*N*-2-aminofluorene; N-OH-AAF: *N*-hydroxy-*N*-2-(acetylamino)fluorene; OH-AAF: several C-hydroxylated derivatives of AAF; N-OH-AF: *N*-hydroxy-*N*-2-aminofluorene. Enzyme activities are given in parentheses: P450: cytochrome P450 monooxygenases; NR: nitroreductase; NOAT: *N*,*O*-acyltransferase.

Kinds and Proportions of Adducts. After rats are exposed to AAF or its *N*-hydroxy derivative, two kinds of adducts are found on the C8 position of guanine in liver DNA: AAF adducts, which retain the acetyl group, and AF adducts, which represent the deacetylated form of the compound (*20*). The principal site of adduct formation in naked DNA treated with N-Aco-AAF is also the 8-position of guanine (*19*, *21*, *22*). Binding to the 8-position yields the principal adduct *N*-(2′-deoxyguanosin-8-yl)-2-AAF, and binding to the amino group of guanine yields 3-(2′-deoxyguanosin-N^2-yl)-AAF; about 85% of the adducts are bound to C8 and 15% to N^2 (*23*). When DNA is treated with N-OH-AF, the lesions formed are specifically AF adducts on the C8 position of guanine (*19*). Treatment of DNA with N-OH-AF therefore permits biological and conformational effects of AF adducts to be compared with those of AAF adducts formed by treatment with N-Aco-AAF.

DNA Conformational Alterations. The binding of AAF to a random sequence of DNA can be described by an insertion−denaturation model, also called a base-displacement model (*21*, *22*, *24*−*29*). The model, sup-

ported by evidence from formaldehyde unwinding, the pattern of hydrolysis by endonuclease S_1, linear dichroism, circular dichroism, reaction with specific antibodies, thermal stability, and nuclear magnetic resonance, proposes that a guanine residue carrying an AAF adduct rotates from the *anti* conformation to the *syn* conformation and is shifted outside the double helix. In its place, the fluorene ring is inserted into the DNA, causing a localized disorganization of the DNA structure (*21*, *22*, *25*, *26*, *29*–*36*). The deformation of the helix depends on the local sequence context and extends an average of three to eight base pairs centered around the adduct (*37*, *38*).

Closely related aromatic amines differ from AAF in the nature of the conformational changes that they bring about in DNA. Unlike the insertion and denaturation caused by AAF adducts, AF adducts cause duplex DNA to adopt two interchangeable conformations: a major conformation described by an outside binding model (*25*), in which the fluorene moiety remains outside the helix, and a family of minor conformers in which the AF moiety is stacked within the helix disrupting the G-AF:C base pair (*39*–*41*). In outside binding, AF protrudes in the major groove, leaving the Watson–Crick base pairing of G-AF with cytosine intact (*25*, *39*–*41*). Although not demonstrated experimentally, computations have suggested that both *syn* and *anti* conformations of the G-AF adduct are energetically possible (*39*, *42*).

AAIF adducts do not alter the helical structure of DNA as severely as AAF adducts do. Indeed, the decrease in melting temperature caused by AAIF is less than that caused by AAF (*30*). Lower susceptibility of AAIF-modified DNA than AAF-modified DNA to endonuclease S_1 digestion (*26*) and formaldehyde unwinding (*30*, *31*) also indicate that AAIF does not cause as extensive local denaturation as AAF. Linear dichroism measurements suggest that the AAIF adduct is oriented at 60° with respect to the helix axis, placing it in the groove (*22*). On the basis of these lines of evidence, it was suggested that AAIF fits an outside binding model, the bulkiness of the iodine atom preventing insertion and denaturation like that caused by AAF (*22*). However, minimized energy calculations on a short $(dGdC)_3$ duplex suggest a different conformation in which the fluorene is at the helix interior and the modified guanine is in *syn*, yet still stacked with the adjacent cytosine in one direction. Like the outside binding model, this model explains the lesser conformational alteration of DNA by AAIF than by AAF on the basis of the iodo group sterically hindering the complete insertion of the fluorene ring and the displacement of the guanine residue (*43*).

Thus, while AAF, AAIF, and AF all form adducts at the C8 position of guanine, they have different effects on DNA conformation (*25*). AAF adducts distort the structure of DNA by inserting into the helix and causing the extrusion of the guanine residue. The insertion causes a localized denaturation of the DNA that disrupts base pairing and hinders DNA replication. In contrast, AAIF adducts cause lesser distortion of DNA. AF adducts cause little or no distortion, remaining outside the helix or inserting without disrupting the B-DNA structure. Figure 3 summarizes the effects of AAF and AF adducts on DNA conformation, as described by the insertion–denaturation and outside binding models, respectively.

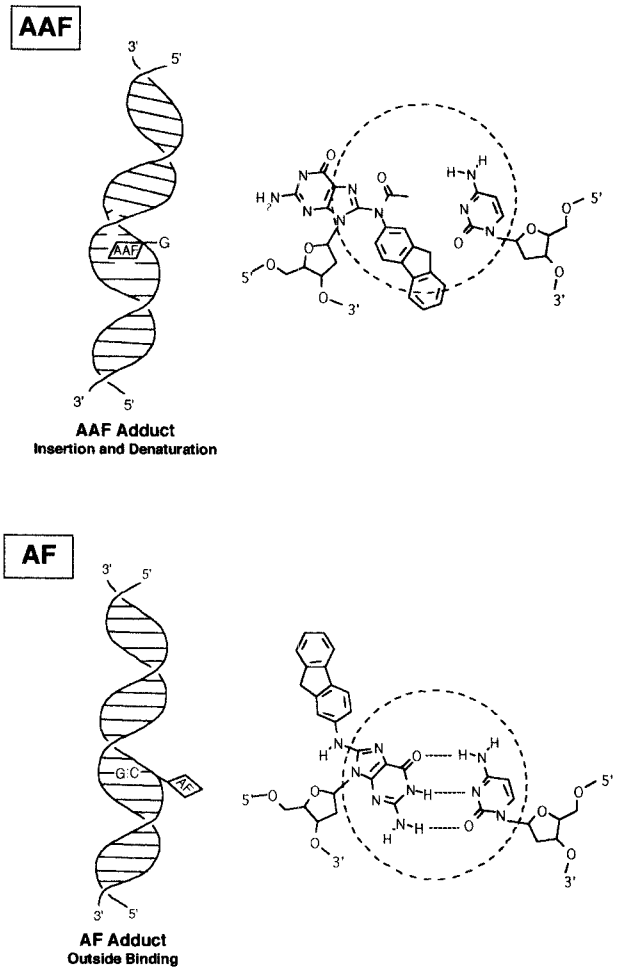

Figure 3. Effects of AAF and AF adducts on DNA conformation. The binding of AAF to DNA is described by an insertion–denaturation model in which the fluorene ring is inserted into the helix displacing the adducted guanine. The major conformation of DNA containing an AF adduct is described by an outside binding model in which the fluorene ring remains outside the helix in the major groove. An AAF adduct on the C8 position of guanine causes the guanine moiety to rotate from the *anti* to the *syn* conformation and prevents it from base pairing with the complementary strand of DNA. An AF adduct on the C8 position of guanine, when remaining in the *anti* conformation, does not cause a major conformational alteration and does not prevent base pairing.

Mutagenicity of AAF, AF, and AAIF

Spectra of Forward Mutations in the *tet* Gene in *E. coli*. The spectra of mutations induced by adducts of AAF (*18*), AF (*20*), AAIF (*44*), and several other compounds (*45*–*47*) have been analyzed in an assay developed by Fuchs and colleagues (*48*) based on the detection of forward mutations in the tetracycline-resistance gene (*tet*) of plasmid pBR322 in *E. coli*. The *tet* gene is 1188 base pairs long and contains *Bam*HI and *Sal*I restriction sites that delimit a 276-base-pair segment of DNA. This fragment can be removed from the plasmid with restriction enzymes and serves as the target for mutagenesis in the forward mutation assay. Plasmid DNA is treated with the mutagen of interest *in vitro*. In some experiments, mutagenized plasmids have been used directly to transform host bacteria in which the effects of the adducts are evaluated. A genetic method called an interplasmidic recombination assay (*18*, *20*) permits mutants that carry a mutation in the *Bam*HI–*Sal*I fragment to be distinguished from those that carry a mutation elsewhere in the *tet* gene. In other studies, the

Table 1. Forward Mutations Induced by AF, AAF, and AAIF Adducts in the Tetracycline-Resistance Gene (*tet*) of pBR322 in *E. coli*

mutations	AF[a]	AAF[b]	AAIF[c]
classes of point mutations			
frameshift mutations	6/23 (26)[e]	32/34 (94)	14/24 (58)
base-pair substitutions	17/23 (74)	2/34 (6)	10/24 (42)
frameshift mutations			
+1 frameshifts	0/6 (0)	2/32 (6)	3/14 (12)
−1 frameshifts	4/6 (17)	13/32 (38)	9/14 (38)
−2 frameshifts	2/6 (9)	12/32 (35)	2/14 (8)
−3 deletions[d]	0/6 (0)	5/32 (15)	0/14 (0)
base-pair substitutions			
transitions	1/17 (4)	1/2 (3)	1/10 (4)
G:C→T:A transversions	14/17 (61)	0/2 (0)	5/10 (21)
G:C→C:G transversions	1/17 (4)	0/2 (0)	4/10 (17)
A:T→T:A transversions	1/17 (4)	1/2 (3)	0/10 (0)
sequence context			
run of ≥3 bases	9/23 (39)	7/34 (21)	11/24 (46)
$(GCG)_3$ repeat	0/23 (0)	5/34 (15)	0/24 (0)
−2 at a *Nar*I site	2/23 (9)	12/34 (35)	1/24 (4)
other sequences	12/23 (52)	10/34 (29)	12/24 (50)

[a] Data from Bichara and Fuchs (*20*). [b] Data from Koffel-Schwartz et al. (*18*). [c] Data from Hoffmann and Fuchs (*44*). [d] Though not frameshifts in a strict sense, −3 mutations are pooled with frameshifts because they involve deletions of small numbers of base pairs and may arise by similar mechanisms. [e] Values in parentheses are the percentage (%) of all mutations.

target restriction fragment was isolated from the remainder of the chemically treated plasmid and ligated into reconstructed plasmids so that only the target fragment contains adducts. In either case, plasmid DNA that contains adducts in the target sequence is introduced into SOS-induced *E. coli*, selecting transformants on the basis of the ampicillin resistance conferred by the plasmid. Testing transformants for growth on medium with and without tetracycline permits the identification of mutants by their tetracycline-sensitive phenotype. The mutants are characterized by DNA sequence analysis.

After treatment of pBR322 with N-Aco-AAF, there is a dose-dependent increase in the frequency of tetracycline-sensitive mutants, which may become as high as 3% of the transformants in SOS-induced bacteria (*18*, *48*). AAF adducts are converted into mutations at an average frequency of about one mutation per 1000 adducts (*20*). Almost all the mutations occur at G:C base pairs (*18*). Since AAF reacts only with guanine residues in DNA, this observation suggests that mutagenesis is targeted at the sites of the modified bases. More than 90% of the mutations induced by AAF are frameshift mutations (*18*), including additions and deletions of a single G:C base pair, deletions of two adjacent base pairs, and a few three-base-pair deletions (*18*, *48*). In contrast to AAF adducts, AF adducts induce primarily base-pair substitution mutations in the *tet* forward mutation assay (*20*). About 85% of the mutants recovered after treatment of pBR322 with N-OH-AF contained base-pair substitutions, primarily G to T transversions (*20*). In the same assay, AAIF induces frameshift mutations and base-pair substitutions at approximately equal frequencies (*44*). The frameshifts were mainly −1 frameshifts, but −2 and +1 frameshifts were also detected. The substitutions were almost all transversions at a G:C base pair. The mutation spectrum of AAIF is thus intermediate between those of AAF and AF.

The mutation spectra of AAF, AF, and AAIF are summarized in Table 1. The most striking difference is between AAF and AF, in that the ratio of substitutions to frameshifts was 17:6 for AF (*20*), 10:14 for AAIF (*44*), and 2:32 for AAF (*18*). Rather than being strictly intermediate, the mutation spectrum of AAIF has distinctive properties (*44*). AAIF induces G:C to T:A and G:C to C:G transversions in approximately equal numbers, whereas AF induces G:C to T:A transversions almost exclusively. The frameshift mutations induced by AAIF differ from those induced by AAF, in that AAIF induces principally −1 frameshifts (*44*), whereas AAF induces a high frequency of both −1 frameshifts and −2 frameshifts (*18*).

The mutations induced by AAF are not distributed randomly among guanine residues. Rather, some sites are mutated at much higher frequencies than others (*18*, *48*). AAF-induced *tet* mutations occur largely in two sequence contexts: runs of a single base (*e.g.*, GGGGG) and the alternating G and C residues of the *Nar*I restriction site 5′GGCGCC3′ (*18*). The mutations in the monotonous runs are −1 frameshifts, while those in the *Nar*I sequence are −2 frameshifts (*18*). Thus, the mutation spectrum of AAF reveals two kinds of hotspots: those in which the same base (*i.e.*, G) is repetitive and those in which a dinucleotide (*i.e.*, GpC) is repetitive. The most common mutational event in both kinds of hotspots is the frameshift in which one unit of the repetition is deleted (*18*). The mutation spectrum of AF does not show the pronounced hotspots that are seen with AAF. While AF-induced frameshifts occur preferentially at sequences similar to those of AAF-induced mutations, the transversions that represent the majority of AF-induced mutations are more randomly distributed among the guanine residues (*20*). Like AAF-induced mutations, AAIF-induced mutations occur preferentially at repetitive sequences (*44*), and the extent to which a sequence is a hotspot depends on the length of the repeat. The longest run in the target fragment in the *tet* assay is five guanines, and this site accounts for one-fourth of the mutations induced by AAIF (*44*).

Explanation of Hotspots. The occurrence of hotspots could theoretically be explained by selection bias, differential repair, or unequal distribution of premutational lesions in the gene. None of these possibilities applies to the AAF hotspots in *tet*. Frameshift mutations at all sites in the target sequence confer the tetracycline-sensitive phenotype, eliminating selection bias as a factor. A mutant (*uvrA*) that lacks nucleotide excision repair, which is the principal repair pathway for AAF adducts, does not show mutable sites differing from those in a repair-proficient strain (*18*); such a difference in mutation spectrum would be expected if hotspots were ascribable to preferential repair of adducts in less mutable sites. Finally, the distribution of AAF adducts in the *Bam*HI−*Sal*I fragment has been mapped by a procedure that measures the blockage by AAF adducts of the 3′ to 5′ exonuclease activity of T4 DNA polymerase (*49*). Double-stranded fragments were labeled at one 5′ end, digested by the enzyme, and analyzed on sequencing gels. In the absence of AAF adducts, the enzyme completely digests the DNA, but in the presence of AAF adducts, fragments are left undigested; the size of the fragments depends on the position of the adducts in the DNA. The DNA modification spectrum determined by this procedure indicates that all guanine residues are subject to reaction with N-Aco-AAF, with differences in the frequency of adduct formation depending on the local sequence. The distribution of mutations induced by AAF does not correspond to the distribution of AAF adducts in DNA, in that guanine residues in hotspots exhibit the

same mean reactivity with N-Aco-AAF as guanine residues at other sites (*49*). Only 19% of the AAF adducts are found at the sites of mutational hotspots, but these adducts produce 89% of the mutations (*49*).

Thus, the data on hotspots, DNA repair, and adduct distribution support the conclusion that the distribution of mutations among highly mutable and less mutable sites in DNA is determined by the processing of DNA damage in particular sites and not by selection bias, differential repair, or the distribution of adducts. When a premutational lesion occurs in a mutation-prone sequence, it is converted into a mutation much more efficiently than when it occurs at other sites. We shall return to the processing of premutational lesions in hotspots shortly.

Forward Mutation Spectrum of AAF in the *E. coli lacI* Gene. The spectrum of forward mutations induced by N-Aco-AAF in the *lacI* gene of *E. coli* contained 44% typical point mutations comparable to those analyzed in the *tet* gene (*50*). The remaining 56% was comprised of gains or losses of four base pairs in the spontaneous $[TGGC]_3$ hotspot (26%), other deletions (21%), other duplications (2%), complex mutations (1%), and mutants that were not readily characterized (6%). The large insertions and deletions represent a potentially important class of AAF-induced mutations, and they require further study.

Among the typical point mutations (*i.e.*, not including those in the unique *lacI* $[TGGC]_3$ hotspot), the spectrum in *lacI* is consistent with that in *tet*. Of AAF-induced point mutations in *lacI*, 25% were base-pair substitutions, 25% were −1 frameshifts, and 50% were −2 frameshifts (*50*). The higher proportion of base-pair substitutions in *lacI* than in *tet* may be explained by AF adducts forming in the *in vivo* treatment of bacteria with N-Aco-AAF but not in the *in vitro* treatment of plasmid DNA. As shown in Figure 2, some of the N-Aco-AAF may be deacetylated in the bacteria before it reacts with DNA, giving rise to AF adducts (*51*). The −1 frameshifts in *lacI* are not as strongly associated with runs of a single base as they are in *tet*, though runs are still hotspots. All the −2 frameshifts were in regions of alternating purine and pyrimidine bases, 96% of them involving the loss of a GpC dinucleotide and 4% the loss of a GpT dinucleotide. Thus, the *lacI* system supports the same conclusion as the *tet* assay with respect to −2 frameshifts: the induction of −2 frameshifts is a prominent and characteristic part of AAF mutagenesis and is strongly associated with hotspots at alternating repetitions of G:C base pairs (*50*).

Reversion Assays of AAF and Related Compounds. The Ames assay detects mutations by selecting for histidine-independent revertants in histidine-requiring strains of *Salmonella typhimurium*. AAF, when metabolically activated by a rat liver homogenate, is mutagenic in strains TA1538 (*hisD3052 rfa ΔuvrB*), TA98 (*hisD3052 rfa ΔuvrB* pKM101), TA100 (*hisG46 rfa ΔuvrB* pKM101), and TA1537 (*hisC3076 rfa ΔuvrB*) (*52*). Its activity is greatest in reverting the *hisD3052* allele in strains TA1538 and TA98, indicating the induction of frameshift mutations at a site of alternating G:C base pairs (GCGCGCGC). The *hisC3076* allele in TA1537 reverts by frameshift mutations in a monotonous run (GGGGG), and the *hisG46* allele in TA100 reverts by base-pair substitutions.

Colony hybridization analysis has been used to determine the sequence of AAF-induced revertants of the *hisD3052* allele, which lacks one base pair relative to the wild-type sequence (*53*). Every mutation in 600 AAF-induced revertants was a −2 deletion that restores the correct reading frame in the hotspot sequence GCG-CGCGC. The Ames assay thus confirms the *tet* and *lacI* forward mutation spectra in showing the induction of −2 frameshifts at sites of alternating G:C base pairs. AAF was much less effective in inducing reversion of the base-pair substitution allele *hisG46* in strain TA100 by transversions (86%) and transitions (14%) (*53*). Like AAF, N-Aco-AAF (*52*), N-OH-AAF (*52*), and AAIF (*54*) induce reversion of the *hisD3052* allele.

Reversion is readily measured in pBR322 using mutants isolated in the *tet* forward mutation assay (*18*) or constructed so as to have a specific sequence at the mutant site (*55*). Mutant plasmids may be treated *in vitro* (*55*–*57*) or in a bacterial host (*4*, *11*, *58*), and revertants are detected by a positive selection for the tetracycline-resistant phenotype. Mutants used in the reversion assay give rise to revertants by the predicted mutational events. For example, analysis of the size of restriction fragments has shown that a plasmid that has an additional guanine in a run of G:C base pairs reverts by a −1 frameshift, and a plasmid that contains a −1 frameshift in the same run reverts by a +1 frameshift (*58*). Sequence analysis of 15 revertants of the plasmid with the extra guanine revealed that 14 were −1 frameshifts that restore the wild-type sequence and that one had a −1 frameshift immediately 5′ to the run of guanine residues (*57*). In the construction of plasmid pX_2, a CpG dinucleotide was inserted in a *Nar*I sequence (GGCGCC) in the *tet* gene, creating a *Bss*HII restriction site (GGCGCGCC). When strains containing this plasmid revert to tetracycline resistance, the *Bss*HII site disappears and the *Nar*I site is restored, indicating that a GpC dinucleotide has been deleted (*55*). Thus, the *tet* reversion assay specifically detects particular classes of frameshift mutations (+1, −1, or −2) through the use of appropriate pBR322 mutants.

N-Aco-AAF effectively reverts the *tet* mutation in plasmid pX_2 (*4*). The reversion assay thus confirms the *tet* forward mutation assay (*18*) in showing that AAF adducts are potent inducers of −2 frameshift mutations in short alternating GpC sequences (*4*, *55*). Like N-Aco-AAF, reactive forms of other fluorene compounds that make adducts on the C8 position of guanine induce reversion in pX_2 (*4*). *N*-Acetoxy-*N*-2-(acetylamino)-7-fluorofluorene (N-Aco-AAFF) is as potent as AAF in pX_2, causing a maximum revertant frequency 50 000 times higher than the spontaneous frequency of 5×10^{-9}. In contrast, AF adducts formed by treating bacteria with N-OH-AF cause a maximum increase of only 1200-fold. N-Aco-AAIF is intermediate in potency between N-Aco-AAF and N-OH-AF (*4*).

Relationship of Mutagenesis to DNA Conformation. AAF, AF, and AAIF all induce targeted mutations as a consequence of their adducts at the C8 position of guanine. As shown in Table 1, however, they differ in the spectrum of mutations that they induce. Thus, closely related compounds that cause different conformational changes in DNA (*25*) also differ in mutagenicity (*18*, *20*, *44*). The greater effectiveness of AAF than of AF or AAIF in inducing −2 frameshift mutations in alternating GpC sites may be ascribed to the greater distortion of DNA triggered by AAF adducts, which cause guanine to rotate from the *anti* to the *syn* conformation (*25*). The structural distortion severely hinders replication and undoubtedly provides a better substrate for

mutational processing into frameshift mutations than does DNA containing the less disruptive AF or AAIF adducts. Adducts that distort the local DNA conformation less severely, such as AF or AAIF, are more apt to induce base-pair substitutions.

Elucidation of Mechanisms of Mutagenesis by AAF and AF

Genetic and molecular approaches have been used to explore mechanisms of frameshift mutagenesis by aromatic amines. We shall first summarize genetic functions that play a role in mutagenesis by AAF and AF and then review effects of these adducts on DNA replication. The discussion of replication hindrance and the genetic control of mutagenesis leads to an analysis of mechanisms underlying the induction of −1 frameshift mutations in monotonous runs of bases and −2 frameshift mutations in alternating GpC sequences. Though the two kinds of frameshifts have different genetic requirements, both may be explained by slippage models.

Genetic Control of −1 and −2 Frameshift Mutagenesis by AAF and AF. Many aspects of mutagenesis in *E. coli* depend on the SOS system, a complex of coordinated responses to DNA damage controlled by the *recA*$^+$ and *lexA*$^+$ genes (reviewed in ref 59). SOS functions are induced (*i.e.*, activated) by exposing the bacteria to such genotoxic agents as ultraviolet light (UV). The mutagenicity of AAF adducts (*18*) and AF adducts (*20*) in the *tet* forward mutation assay depends on SOS functions of the host, in that the mutagenic responses are much greater in irradiated bacteria than in unirradiated bacteria. Though involved in mutagenesis by both AAF and AF, the SOS system does not control the mutagenicity of the two compounds in the same way. The *umuDC*$^+$ genes, controlled by the SOS system, are required for the mutagenicity of AF; AF mutagenesis, like that of UV, is strongly suppressed in a *umuC* mutant even under SOS-induced conditions (*20*). In contrast, AAF adducts are mutagenic in a *umuC* mutant, but the mutation spectrum is altered, in that −1 frameshifts in monotonous runs are essentially eliminated (*18*). Most AAF-induced mutations in the *umuC* strain are −2 frameshifts in *Nar*I sites (*18*).

Reversion assays have confirmed the difference in genetic requirements between −1 and −2 frameshift mutations. The induction by AAF of −1 frameshifts in a run of guanine residues requires SOS induction (*56*) and *umuDC*$^+$ gene function (*57*). The activated form of RecA protein (RecA*) is required for cleavage of both the LexA repressor and the UmuD$^+$ gene product (*56*, *57*). Thus, the −1 frameshift mutation pathway has the same genetic requirements as mutagenesis by UV (reviewed by ref 60). In contrast, the induction by AAF of −2 frameshift mutations in plasmid pX_2 requires SOS induction but does not depend on *umuC*$^+$ gene function (*55*, *56*). The induction of −2 frameshifts in alternating G:C base pairs does not require RecA* except for cleaving the LexA repressor. These findings led to the proposal that this pathway of mutagenesis involves an unknown SOS gene named *npf* for the *Nar*I processing factor (*57*). The role of RecA in −2 frameshift mutagenesis is distinct from that in −1 frameshift mutagenesis. Wild-type RecA and RecA430 proteins exert an inhibitory effect on −2 frameshift mutagenesis that is alleviated by the presence of activated RecA (RecA*), RecA730, or RecA495 proteins, or by the absence of RecA (*57*).

Replication Hindrance by Adducts and Translesion Synthesis. Bulky adducts in DNA can block replication. Survival therefore depends on the ability of cells to remove such lesions by DNA repair processes, such as nucleotide excision repair (reviewed in ref 59), or to bypass them during DNA replication. The hindrance of replication and bypass of lesions are important for mutagenesis because mutations arise during the processing of damaged DNA. Figure 4 shows different mechanisms by which cells can survive despite chemical lesions in their DNA. The mechanisms fall into two categories: damage avoidance (reviewed in ref 59) and translesion synthesis (TLS). The former, which uses the information in the nondamaged strand to circumvent the effect of the adduct, includes postreplication recombinational repair and DNA polymerase strand switching, also called lesion-induced strand switching. These processes tend to be error-free. In contrast, TLS involves the polymerase inserting a nucleotide directly opposite the adduct and then extending the polynucleotide chain (reviewed in ref 59). The process by which the polymerase proceeds through the adduct in TLS may be either error-free or error-prone. Frameshift mutations arise in TLS when the elongation step proceeds from a slipped mutagenic intermediate at the site of the adduct. The hindrance of replication is thus a key step in the mutagenicity of bulky chemical adducts because it leads to TLS, in which mutational errors are introduced.

AAF adducts in bacteriophage M13 DNA block replication by several DNA polymerases *in vitro*: T7 DNA polymerase, Sequenase 2, T4 DNA polymerase, and *E. coli* DNA polymerase I (*61*). AF adducts are much less effective than AAF adducts as replication-blocking lesions (*61*). The retardation of T7 DNA polymerase by an AF or AAF adduct in a synthetic template/primer has been quantified by single-turnover kinetics (*62*). The polymerase showed no retardation before reaching the site of the adduct, but the rate of incorporation of dCTP opposite a guanine carrying an AAF adduct was 4×10^6 times slower than that opposite an unmodified guanine. AF adducts also slowed the polymerase but less severely than AAF adducts, in that the incorporation of dCTP opposite an AF adduct was 5×10^4 times slower than opposite an unmodified guanine (*62*). Retardation of DNA synthesis is not limited to slower base insertion opposite an adduct; rather, the processivity of the polymerase is reduced even after passing the adducted base (*62*).

While AF adducts typically impede replication less than AAF adducts, the local sequence context affects the extent to which an adduct blocks replication. AF adducts in some sites, such as G_3 of the sequence $5'G_1G_2CG_3CC3'$, hinder replication much as AAF adducts do (*63*). The nucleotide incorporated by T7 DNA polymerase at a guanine residue carrying an AF adduct was usually correct (*i.e.*, dCTP), but some misincorporation does occur. The most frequently misincorporated nucleotide was dATP (*62*), thus predicting the induction of G to T transversions as seen in the forward mutation spectrum of AF (*20*).

Comparing the efficiencies with which plasmids containing adducts and unmodified plasmids transform bacteria provides indirect evidence whether the adducts block replication *in vivo*. The transforming activity of pBR322 containing AAF adducts is less in *uvrA* and *polA* strains than in a strain with wild-type DNA repair, though the mutation spectrum does not differ appreciably

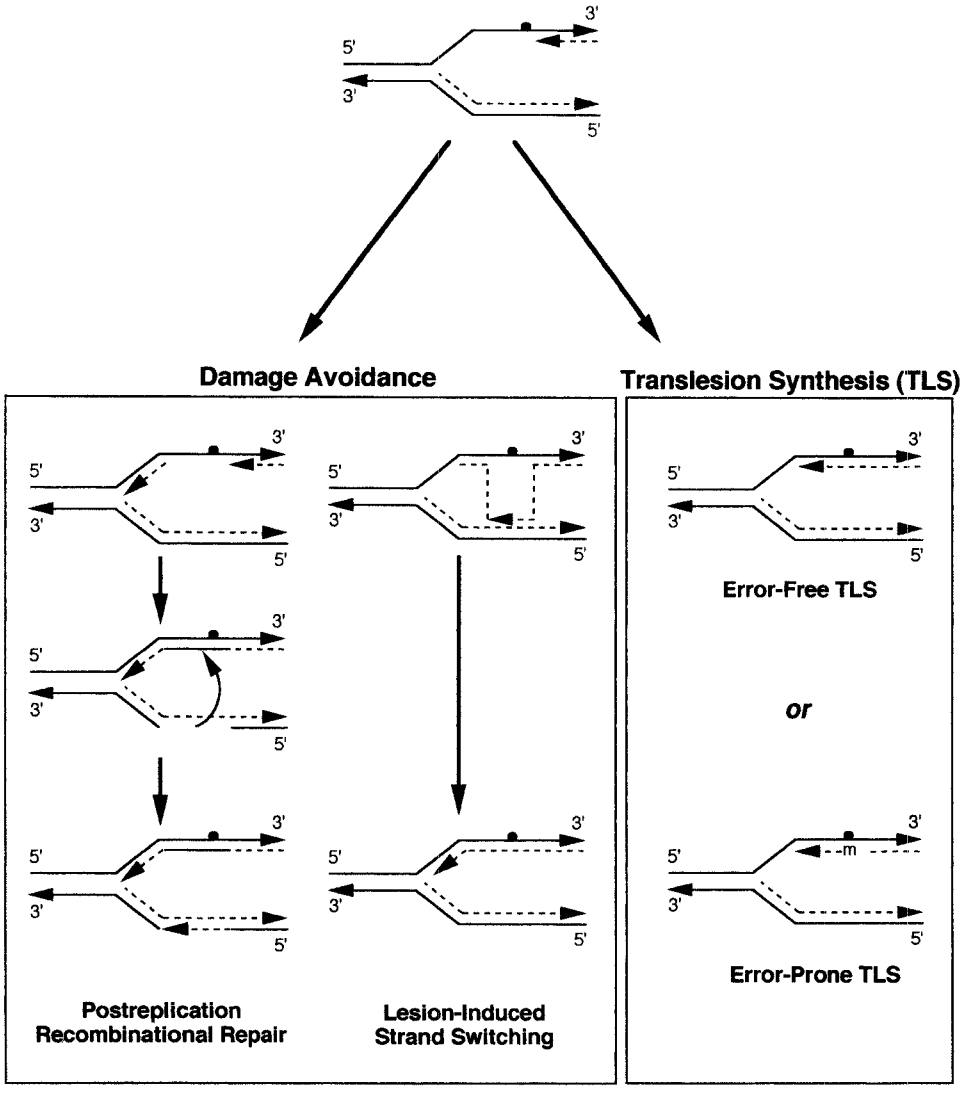

Figure 4. Strategies by which cells tolerate chemical adducts in DNA. Cells survive despite the presence of bulky adducts that block DNA replication through processes of damage avoidance and translesion synthesis (TLS). Damage avoidance, including postreplication recombinational repair and lesion-induced strand switching, tends to be error-free, whereas TLS may be either error-free or error-prone. The figure shows elongation from a mutagenic intermediate (m) in TLS.

between wild-type and *uvrA* strains (*18*). The difference in transforming efficiency is ascribable to potentially lethal AAF adducts being subject to nucleotide excision repair (*18*, *64*). In a wild-type strain, about 60 AF adducts are required per lethal hit, compared to 20 AAF adducts (*64*). Moreover, DNA containing AF adducts has roughly equal transforming activity in wild-type and *uvrA* strains (*20*), despite the fact that *uvrABC* excinuclease acts on both AAF adducts and AF adducts (*64*). In the absence of excision repair, the blockage of replication by AAF adducts causes a reduction in transforming activity, while AF adducts, which do not efficiently block replication, are tolerated (*64*). The difference in the efficiency with which AAF adducts and AF adducts block replication is related to their effects on DNA conformation. AAF adducts cause a major alteration in local DNA conformation, described by the insertion–denaturation model, whereras AF adducts cause little structural alteration, compatible with an outside binding model (*25*).

AAF adducts that block replication cause strand loss in plasmid DNA (*65*). Heteroduplex pBR322, carrying a single mismatch as a genetic marker in the *tet* gene, was used to explore effects of AAF adducts in a single strand of the double-stranded plasmid. The genetically labeled plasmids were introduced into a *mutS uvrA* strain that cannot correct the marker mismatch or excise the adducts. The adduct-bearing strand was lost in over 80% of the transformants, compared to a background frequency of less than 20% loss of a strand marker in the absence of adducts (*65*). When adducts were present in both strands, the transformants contained the genetic information of one strand or the other but not both, suggesting that the strand in which the bypass of blocking lesions occurs first is conserved (*65*). The frequency of forward mutations in the *tet* gene was 10 times lower when AAF adducts were present in only one strand than when both strands contained adducts (*65*). The reduction in mutagenic efficiency undoubtedly reflects the loss of the adducted strand.

Strand segregation analysis has been used to explore the relationship between mutagenesis and the bypass of AAF and AF adducts in a run of three guanine residues (*66*). Single AAF or AF adducts were built into a heteroduplex plasmid that has an insertion of three base pairs in the opposite strand serving as a strand marker directly across from the adduct. The presence of the two strands was monitored by a colony hybridization assay in a *mutS uvrA* strain. Retention of the marker from both strands indicated that replication had proceeded through the adduct. Thus, TLS, in which replication

proceeds through the adduct, could be distinguished from strand loss and damage avoidance mechanisms.

When the SOS system was not induced, TLS occurred less than 1% of the time for AAF adducts but 70% of the time for AF adducts, thus correlating with the efficiency of the two adducts as blocking lesions. Induction of the SOS response did not alter the probability of TLS at an AF adduct but increased the frequency of TLS at AAF adducts to about 13% (*66*). The increase in TLS with SOS induction was accompanied by a proportional increase in the frequency of −1 frameshift mutations. Both TLS and mutagenesis depended on *umuDC*$^+$ gene function. Rather than causing a decrease in polymerase fidelity as sometimes thought, the induction of the SOS response causes a shift from the processing of the AAF adduct by an error-free damage avoidance pathway to a pathway of TLS. Frameshift mutations arise in TLS at hotspots when a polymerase elongates a DNA chain from a mispaired primer and template. The error rate per TLS seems to be constant and has been measured as about 0.15, meaning that about 15% of elongation was from a slipped intermediate and about 85% was from a nonslipped primer and template (*66*). In sequences other than the hotspots, TLS proceeds from the properly paired intermediate.

Mechanisms of Induction of −1 Frameshift Mutations in Monotonous Runs of Bases. Plasmids containing a single AAF adduct on different bases in a run of guanine residues have been used to evaluate the effect of an adduct's position on −1 frameshift mutagenesis (*67*). The restoration of *lacZ* gene function by a −1 frameshift mutation was monitored by the formation of blue rather than white colonies on Xgal indicator medium. Plasmids with an AAF adduct on one of the guanine residues of the sequence 5′CCCG$_1$G$_2$G$_3$3′ revealed that an adduct on G$_3$ was 10-fold more likely to give rise to a −1 frameshift mutation than an adduct on G$_2$ and 100 times more likely than an adduct on G$_1$ (*67*). Mutagenesis was SOS-dependent, and the mutations induced by adducts on G$_3$ were targeted in the run of guanines (*67*). Mutations induced by adducts on G$_1$ or G$_2$ were mostly localized to the run of cytosine residues 5′ to the guanine run; these mutations can be thought of as semi-targeted (*67*). Similarly, the frequency of −1 frameshift mutations induced by AAF adducts on G$_4$ of the sequence 5′CG$_1$G$_2$G$_3$G$_4$3′ was 600 times higher than that induced by adducts on G$_1$ (*67*). The results show that adducts at the 3′ end of a run are more mutagenic than those at the 5′ end.

The importance of the adduct's being at the 3′ end of the run has been confirmed with the sequence 5′CG$_1$G$_2$G$_3$-XT3′. The frequency of −1 frameshift mutations, monitored by the blue/white *lacZ* system, was 20−200 times higher when an AAF adduct was at the 3′ end of the run than when it was at the 5′ end (*68*). When an adduct was on G$_3$, mutagenesis was greatest when the base immediately 3′ to an adducted guanine (base X) was A, followed by G, C, and T in that order (*68*). The length of the run also influenced the mutagenicity of an AAF adduct, in that the frequency of −1 frameshift mutations was 167 × 10^{-4} for 5′CGGGGT3′, 76 × 10^{-4} for 5′CGG-GT3′, and only 14 × 10^{-4} for 5′CGGT3′ (*68*).

These results are best explained by a slippage model for −1 frameshift mutagenesis (*67*, *68*). Figure 5, from Napolitano et al. (*68*), shows slippage-mediated frameshift mutagenesis caused by an AAF adduct in a monotonous run of guanines. The first step leading to

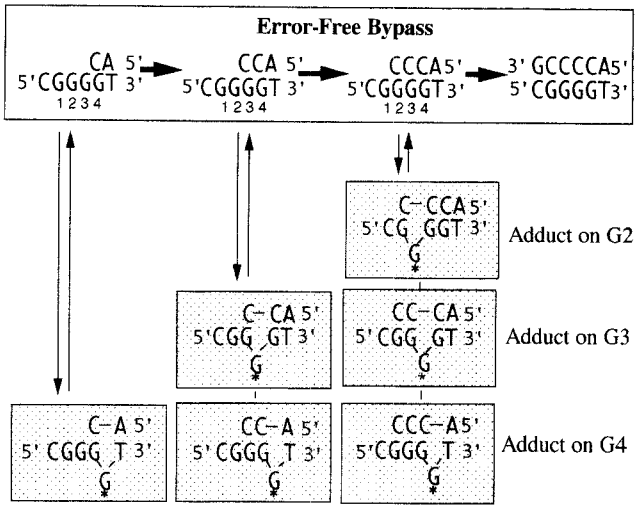

Figure 5. Slippage model for −1 frameshift mutagenesis induced by an AAF adduct at various positions in a run of guanine residues (figure from Napolitano et al. (*68*)). A slipped mutagenic intermediate is formed after the incorporation of cytosine opposite the adducted guanine. The AAF adduct stabilizes the slippage, and elongation from the slipped intermediate leads to a −1 frameshift mutation in the new strand.

mutagenesis is the insertion of a nucleotide opposite the AAF adduct during DNA synthesis. This step does not entail an error, in that a cytosine residue is inserted across from the adduct. The adduct, however, hinders elongation of the new strand, increasing the likelihood of slipped mispairing. Retardation of the polymerase occurs not only at the site of the adduct but continues for several subsequent nucleotides (*62*), thereby creating an opportunity for semi-targeted mutations slightly downstream from the adduct site (*67*). In slipped mispairing, the cytosine that was correctly inserted opposite the adduct pairs with a guanine 5′ to the adduct-bearing guanine. The influence of the position of the adduct and length of the run on mutation frequency is explainable on the basis of the number and stability of potential slipped mutagenic intermediates increasing when there are more guanine residues 5′ to the adducted guanine (*68*).

The applicability of slippage models to induced frameshift mutagenesis depends on how adducts influence the formation and stability of transient slipped mutagenic intermediates. To explore whether AAF adducts affect the stability of mispaired intermediates, heteroduplexes were constructed to simulate the slipped structures (*69*). As shown in Figure 6, the adducted heteroduplexes contained only two cytosine residues in the complementary strand opposite an AAF adduct in a run of three guanines. Heteroduplexes with a bulged guanine were compared with homoduplexes having all three cytosines. An AAF adduct on any of the three guanines destabilized the homoduplex, as indicated by a melting temperature (T_m) about 10 °C lower than that of the unmodified homoduplex. The unmodified heteroduplex was also less stable than the unmodified homoduplex, having a T_m about the same as that of the AAF-modified homoduplexes. The AAF-modified heteroduplexes, however, were more stable than the unmodified heteroduplex, having a T_m only slightly lower than that of the unmodified homoduplex. Thus, the T_m values indicate that AAF stabilizes the heteroduplex DNA.

Chemical probes were used as an independent source of evidence on whether AAF adducts can stabilize a

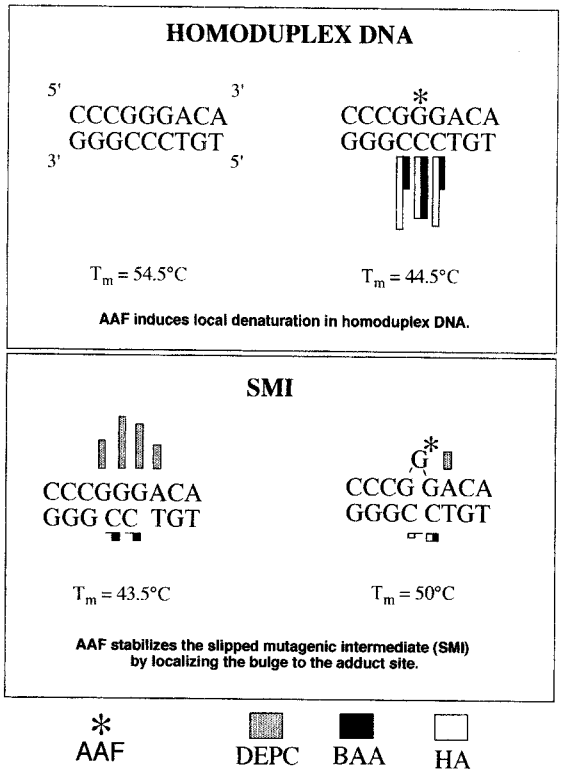

Figure 6. Evidence from melting temperatures and chemical probes that AAF adducts stabilize slipped mutagenic intermediates (see text for details).

simulated slipped mutagenic intermediate (*69*). The results are summarized in Figure 6. Hydroxylamine (HA) and bromoacetaldehyde (BAA) were used as probes for cytosine, and reactivity with these reagents was taken as evidence of denaturation of the helix. The probes reacted with an AAF-modified homoduplex but not an AAF-modified heteroduplex, indicating that the adducted DNA is stabilized by the mispairing (*69*). Diethyl pyrocarbonate (DEPC), used as a probe for guanine, reacted with all three guanine residues of the unmodified heteroduplex, indicating transient single strandedness delocalized over the three guanines. Reduced DEPC reactivity of the AAF-modified heteroduplex suggested that the bulge was now restricted to the adducted base and that the mispaired structure as a whole was stabilized (*69*). Thus, the analysis with chemical probes is consistent with the T_m analysis in suggesting that AAF adducts stabilize a heteroduplex that mimics a slipped mutagenic intermediate.

Analysis of a heteroduplex by nuclear magnetic resonance spectroscopy (NMR) has provided further evidence that an AAF adduct can stabilize a frameshift mutagenesis intermediate (*70*). The heteroduplex consisted of a 10-mer and an 11-mer that are complementary except for a guanine residue at position 6 of the 11-mer. The two oligonucleotides therefore pair normally if the extra guanine is bulged. The bases of the AAF-modified heteroduplex remained paired at 30 °C, while the unmodified heteroduplex was denatured (*70*).

Taken together, these results indicate that AAF adducts both increase the opportunity for slipped mispairing by hindering a DNA polymerase and stabilize the slipped mutagenic intermediates. AAF adducts cause localized distortion in DNA, and the formation of a slipped structure lessens the destabilization. AAF is apparently accommodated more readily by the slipped DNA than by the nonslipped helix. A highly displaced equilibrium exists in repetitive sequences, with normal pairing greatly predominating over slipped mispairing; an AAF adduct seems to shift the equilibrium significantly toward the slipped state. The properties of −1 frameshift mutagenesis, hindrance of polymerases, and stabilization of slipped mutagenic intermediates provide strong support for the proposal that AAF adducts induce −1 frameshift mutations by a slippage mechanism.

Mechanisms of Induction of −2 Frameshift Mutations in Alternating GpC Sequences. The induction by AAF of −2 frameshift mutations in which a GpC dinucleotide is deleted from a *Nar*I site (5′$G_1G_2CG_3$CC3′) could in principle be caused by an adduct on any of the three guanine residues. To determine whether the three guanines are equally important for mutagenesis, plasmids were constructed so as to contain a single AAF adduct at a defined position in the *Nar*I hotspot. When the three plasmids were individually introduced into *E. coli* by transformation, only the plasmid bearing the adduct on G_3 gave rise to −2 frameshift mutations (*71*). The same specificity, in which only the AAF adduct on position G_3 induces −2 frameshifts, has been observed using an *in vitro* replication system in which plasmid vectors containing a simian virus 40 origin of replication and a single AAF adduct were replicated in a human cell extract (*72*, *73*).

To define the *Nar*I hotspot better, plasmids were constructed so as to contain all possible combinations of bases N_a and N_b flanking the GpC dinucleotide repeat in the target sequence 5′N_aGCGCN_b3′ (*74*). All 16 sequences were highly mutable by AAF adducts introduced randomly into the plasmids. The plasmids could theoretically detect +1, +4, −5, and −2 frameshifts in and near the target by means of the blue/white *lacZ* system in *E. coli*. Almost 90% of the mutations were targeted −2 frameshifts in which a GpC dinucleotide was deleted between bases N_a and N_b. The remainder were mostly +1 frameshifts in a nearby run of guanine residues. Varying nucleotide N_a at the 5′ end of the target had little effect on mutagenesis, but varying the 3′ flanking nucleotide N_b strongly affected mutagenesis (*74*). Mutagenesis was greatest when N_b was cytosine, as in the *Nar*I sequence, or adenine. Mutagenesis was intermediate when N_b was guanine and least when N_b was thymine.

The substantial alteration of DNA conformation by an AAF adduct, which causes guanine to rotate from *anti* to *syn*, apparently increases the likelihood of −2 frameshift mutagenesis (*1*, *37*, *38*). The tendency of alternating GpC sequences to undergo a transition from B-DNA to Z-DNA (*75*) and the observation that AAF adducts favor the B to Z transition in synthetic polynucleotides (*76*) led to speculation that the formation of Z-DNA is involved in the induction by AAF of −2 frameshift mutations at *Nar*I sites. It was proposed that destabilization of the B-structure of DNA triggers a localized transition from B-DNA to Z-DNA that promotes the mutational process by making the site a better substrate for proteins that process the premutational lesions into mutations (*18*, *55*). An accumulation of evidence, however, indicates that −2 frameshift mutagenesis in short sequences of alternating base pairs is intimately associated with replication, and this association favors a slippage model similar to that proposed for −1 frameshift mutagenesis.

In plasmid replication in *E. coli*, a single AAF adduct near the origin of replication in the lagging strand is more than 20 times more likely to give rise to a −2 frameshift

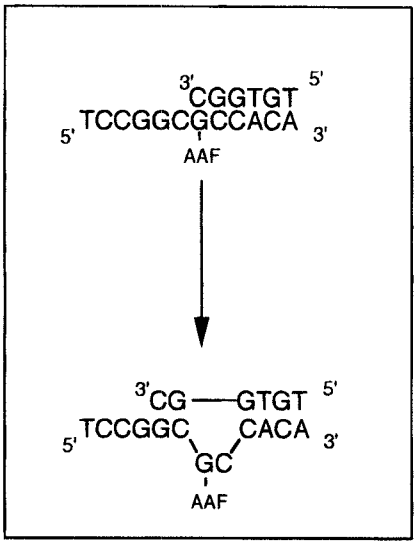

Figure 7. Slippage model for the induction of a −2 frameshift mutation by an AAF adduct on the central guanine of the hotspot sequence 5′GGCGCC3′ (figure based on Koffel-Schwartz and Fuchs (*74*)). The mechanism is similar to that shown in Figure 5 for −1 frameshift mutagenesis, but the slipped mutagenic intermediate involves a dinucleotide bulge in the alternating GpC sequence.

mutation than a single adduct in the leading strand (*77*). The strand bias directly links −2 frameshift mutagnesis by AAF to replication. The observation that an AAF adduct on G_3 of the sequence 5′$G_1G_2CG_3CC$3′ is strongly mutagenic while adducts on G_1 or G_2 are not (*71*) is also best explained by slippage in replication, because only adducts on G_3 can give rise to a slipped mutagenic intermediate that leads to −2 frameshift mutations. This intermediate is shown in Figure 7. If an AAF adduct on G_3 stabilizes a bulge of G_3C, the G_2C dinucleotide on its 5′ side can engage in slipped mispairing with the newly synthesized strand. This view of the *Nar*I hotspot is reinforced by the finding that bases on the 3′ side of the G_3C dinucleotide strongly modulate mutagenesis, while those on the 5′ side of G_2C have little influence (*74*). A polymerase encounters the 3′ base immediately before reaching the adduct, so this base is well-situated to modulate TLS and/or the stability of slipped mutagenic intermediates (*74*).

A slippage model similar to that proposed for AAF-induced −1 frameshift mutations (*67*, *68*) can explain −2 frameshift mutagenesis by AAF (*53*, *72*−*74*). Figure 7, based on Koffel-Schwartz and Fuchs (*74*), shows slippage-mediated −2 frameshift mutagenesis caused by an AAF adduct on the central guanine of the hotspot sequence 5′GGCGCC3′. The mutation pathway begins with the hindrance of replication by the adduct. The incorporation of a nucleotide opposite the adducted base (*62*) and progression of the polymerase past the adduct (*62*, *78*) are substantially retarded relative to DNA synthesis on an undamaged template. Hindered replication increases the opportunity for slipped mispairing in repetitive sequences during TLS. Slippage in alternating GpC sequences produces a family of possible slipped structures (*53*) all of which share the common feature of having a GpC dinucleotide in an unpaired bulge.

Evidence that AAF adducts stabilize the slipped intermediates of −2 frameshift mutagenesis (*79*), as they do those of −1 frameshift mutagenesis (*69*, *70*), has been obtained by analyzing a heteroduplex that simulates a −2 frameshift intermediate. The heteroduplex consisted of a 12-mer carrying the *Nar*I sequence $G_1G_2CG_3CC$ and a 10-mer that was complementary to it except for having only G_1G_2CC in place of the *Nar*I sequence. An AAF adduct on G_3 in the 12-mer raised the T_m relative to that of the unmodified heteroduplex, and NMR analysis showed the modified helix to be strongly stabilized by the presence of the adduct (*79*). Stabilization of the slipped mutagenic intermediate by the adduct increases the likelihood that elongation will occur from a slipped structure rather than a properly aligned structure. Elongation from a slipped mutagenic intermediate, with the GpC bulge remaining unreplicated, leads to −2 frameshift mutation.

Conclusions

The mutagenicity of AAF in bacteria is characterized by the induction of −1 frameshift mutations in monotonous runs of a single base and −2 frameshift mutations in regions of alternating G:C base pairs (*18*, *50*, *53*). AAF adducts on the C8 position of guanine cause a localized distortion of the helical structure of DNA, described by an insertion−denaturation model (*25*). The related compounds AF and AAIF cause less distortion in DNA (*25*, *32*) and are less effective than AAF in inducing −2 frameshift mutations (*20*, *44*; Table 1). The frameshift mutagenicity of AAF is closely associated with the localized DNA denaturation caused by the AAF adduct and its hindrance of replication. Processes of translesion synthesis (TLS) enable replication to bypass the adduct, but mutations arise as a consequence. The processing of AAF adducts into frameshift mutations depends on the SOS system; the induction of −1 frameshifts requires UmuDC protein and activated recA protein, whereas −2 frameshifts depend on still unidentified SOS functions.

The slippage model proposed by Streisinger and colleagues (*12*, *13*) has long dominated thinking on the nature of frameshift mutations in repetitive sequences. Most observations on the frameshift mutagenicity of chemicals can be encompassed by variations on the Streisinger model (*9*, *53*, *67*, *68*, *74*, *80*). Slippage mechanisms can explain the induction of both −1 and −2 frameshift mutations by AAF. The key steps in the induction of frameshift mutations in repetitive sequences are the insertion of a correct nucleotide opposite the adduct, retardation of the DNA polymerase, formation and stabilization of a slipped mutagenic intermediate, and chain elongation from the slipped structure.

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