Activity of Carcinogens That Bind to the C8 Position of Guanine Residues in an Assay Specific for the Detection of \(-2\) Frameshift Mutations in a Defined Hot Spot

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In this paper we describe a reversion assay specific for the detection of \(-2\) frameshift mutations occurring within short stretches of alternating GC sequences. We have compared a series of chemical carcinogens that all bind covalently to the C8 position of guanine residues for their potency in inducing revertants in this assay. Large variations in potency are found within the list of compounds that were tested. The most potent chemicals tested induce the reversion frequency by a factor of \(10^5\) over background, whereas others only increase it by two orders of magnitude. These differences are discussed in terms of the conformational changes that the different C8-guanine adducts induce in DNA.

Introduction

The ultimate carcinogen \(N\)-acetoxy-\(N\)-2-acetylaminofluorene (\(N\)-Aco-AAF), a model for the strong liver carcinogen \(N\)-2-acetylaminofluorene (AAF), binds covalently to DNA, yielding primarily -AAF adducts at the C8 position of guanine (1). The spectrum of mutations induced by -AAF adducts in \(E.\ coli\) has been studied in our laboratory using a forward mutation assay based on inactivation of the tetracycline resistance gene carried by plasmid pBR322 (2,3). More than 90\% of the mutations induced by -AAF adducts are frameshift mutations, and many of them are located at specific sequences, the so-called mutation hot spots (2,3).

Two classes of mutation hot spots have been defined on the basis of the local sequence where they occur: a) -1 frameshift mutations within runs of guanines: Induction of mutations within such sequences requires a functional umuD/C locus (3) and can be ascribed to a slipped-strand mispairing event occurring during replication (4). This pathway is referred to as the slippage pathway. b) -2 frameshift mutations within short stretches of alternating GC sequences: This mutation pathway was originally discovered at the NarI sequence (GCGGCC \(\rightarrow\) GC GCC) (2) and extended to other sequences such as the BssHII sequence (5). The induction of mutations within these sequences is \(UmuD/C\) independent (3). This pathway is referred to as the NarI mutation pathway. Additional differences in genetic requirements of the two pathways have recently been reported (6).

Because we were interested in the mechanisms that are involved in the NarI mutation pathway, we developed a reversion assay specific for the detection of \(-2\) frameshift mutations within short stretches of alternating GC sequences (5,7). This assay has been used to compare the mutagenic potency of several bulky carcinogens that are known to bind covalently to the C8 position of guanine residues. There are large differences in the potency with which the chemicals induce \(-2\) frameshift mutations. These results are discussed in terms of the conformational changes that are induced in the DNA helix upon binding of the chemicals.

Design of a Mutation Assay Specific for Detecting \(-2\) Frameshift Mutations within Alternating GC Sequences

The mutation assay is based upon the reversion of a specific mutation that inactivates the tetracycline resistance gene carried on plasmid pBR322 (5). The reversion event calls for the loss of two base pairs (CpG or GpC dinucleotide) located within a GCGGC sequence in order to restore the wild-type sequence of the tetracycline resistance gene (Fig. 1). The target for the mutation assay is located on a multicopy plasmid pX2 derived from pBR322. This plasmid contains two
Additional base pairs (GpC) inserted by genetic engineering between base pairs 435 and 436 within a NarI site in the tetracycline resistance gene (5). Its phenotype is ampicillin resistant, tetracycline sensitive. This plasmid is stably maintained in E. coli strains by selective growth on plates containing ampicillin. The assay detects plasmids that have undergone the specific loss of the two additional base pairs to yield the wild-type tetracycline resistance gene. Since the mutational event is umuC/D independent (3,5) and occurs by the specific loss of two base pairs within a short run of alternating GC sequences, it is representative of the NarI mutation pathway. The selection is based on the ability of the revertant to grow on plates containing tetracycline. In this assay, the product of the reversion event is a dominant allele, and the target is multiple since pBR322 is a multicopy plasmid (average of 20 copies per bacterium).

Restriction enzyme analysis of the plasmid DNA contained in a collection of individual revertant colonies obtained after treatment with mutagens showed that the tetracycline-resistant phenotype was associated with the loss of the BssHII restriction site (Fig. 1) (7). This result indicates that the mutation assay is indeed specific for the detection of -2 frameshift mutations within a short alternating GC sequence.

### Chemical Carcinogens That Bind Covalently to the C8 Position of Guanine Residues

We have selected several chemical carcinogens (Fig. 2) known to form covalent adducts at the C8 position of guanine residues in order to compare their mutagenic potency in inducing -2 frameshift mutations in a sequence of alternating GC base pairs.

Compound I, II, III and IV (Fig. 2) are reactive derivatives of the aromatic amine AAF, a strong rat liver carcinogen. All four compounds are known to form the majority of their adducts at the C8 position of the guanine residues in DNA (80 to 96% of the total adducts formed) (1,8–10).

Compound V and VI (Fig. 2) both belong to the family of so-called food mutagens (11), which form during pyrolysis of proteins. Compound V, N-Acetoxy-N-2-acetylaminofluorene (N-AcO-AAF), is a model ultimate metabolite (12) of the parent compound GluP3, a chemical found in pyrolysates of L-glutamic acid (13). Compound V forms mostly C8 guanine adducts (12). Compound VI, NO2-IQ, is the nitro derivative of IQ, a strong mutagen found in the charred surface of fish and beefsteak (14). NO2-IQ does not react chemically with DNA as such but requires metabolic activation in vivo. It has been proposed that activation of NO2-IQ to a chemically reactive mutagen in bacteria proceeds via a nitroreductase-catalyzed step followed by an O-acetyltransferase-catalysed step (15).

Compound VII, 4-NQO, is another carcinogenic nitro compound (16) that requires metabolic activation, probably by the same route as NO2-IQ. In vivo, NQO is known to form several adducts whose relative propor-
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Comparative Mutagenic Potency of Chemical Carcinogens That Bind Covalently to the C8 Position of Guanine Residues

Several chemical carcinogens (Fig. 2) known to bind covalently to position C8 of guanine residues in DNA were tested for their potential to induce −2 frameshift mutations in the assay described above. Since the NarI mutation pathway is umuD/C independent (3,5), we performed the mutation experiments in strain GW2100, a umuC mutant (18). Plasmid pX2, which carries the target for the mutation assay, was introduced by a transformation step into a permeable derivative of strain GW2100 (7) and stably maintained in this strain by growth in media containing ampicillin. The use of a permeable derivative of strain GW2100 was necessary to allow the penetration of the chemicals.

Briefly, the protocol for the mutation assay is as follows. Log-phase bacteria are treated for 30 min in phosphate buffer with several concentrations of the test chemical dissolved in dimethyl sulfoxide or ethanol (final concentration 5%). After treatment, the bacteria are plated on ampicillin plates for determining numbers of survivors and grown at 37°C for a 90 min expression period before plating on tetracycline plates for determining numbers of revertants.

Large differences in reversion frequencies are found among the chemicals tested (Table 1). The background reversion frequency is low (= 5 × 10⁻⁵). The curve of reversion frequency versus concentration for most compounds has a broad maximum (data not shown). In order to compare the mutagenic potency of the different chemicals, we have defined a mutation induction factor (MIF) as the ratio of the maximal reversion frequency for a given mutagen over the background reversion frequency (Table 1). The concentration of the mutagen needed to achieve the maximal reversion frequency and survival at this dose are reported in Table 1.

Although all the mutagens that we tested bind covalently to the C8 of guanine residues, they exhibit very different mutagenic potencies. Three mutagens (N-AcO-AAF, N-AcO-AAFF, and NO₂-IQ) appeared to be extremely efficient (MIF ≥ 50000) in this assay. Two chemicals (N-AcO-AGluP3 and NQO) had low efficiency (MIF ≈ 300), and two chemicals (N-AcO-AAIF and N-OH-AF) had intermediate efficiency (MIF between 1000 and 5000).

The chemical concentration at the maximal reversion frequency ranged from 5 × 10⁻⁴ M (N-OH-AF) to ~ 2 × 10⁻³ M (N-AcO-AGluP3), possibly reflecting differences in reactivity with the medium and or with the DNA, penetration into the cells, and metabolism. Survival at the maximal reversion frequency was about 2 to 8% except for N-OH-AF and N-AcO-AGluP3, which were less toxic (~ 50% survival).

Relationship between Mutagenic Potency and Local Conformation of the Adduct

A forward mutation assay based on the inactivation of the tetracycline resistance gene on plasmid pBR322 was developed in our laboratory several years ago (2). In this assay, covalent adducts are formed in the plasmid DNA by treatment with a mutagen in vitro, and the modified plasmids are introduced into SOS-induced bacteria. Among the transformants selected on ampicillin containing plates, tetracycline sensitive mutants are detected by their inability to grow on plates containing tetracycline (19). Using this assay, AAF adducts were found to be very efficient in inducing −2 frameshift mutations at the NarI site in pBR322 (3). Indeed, among the mutants sequenced in this forward mutation assay, a high proportion (i.e., 12/36 = 33%) are −2 frameshift mutations occurring at one of the three NarI sites in the mutation target (Table 2). With the same assay, AF adducts (20) and AAFF adducts (21) were found to induce NarI mutations, but at a lower frequency (Table 2). On the other hand, no −2 frameshift mutations were detected among a similar number of mutations induced by NQO (P. Daubercies, S. Galillé, Zouitana, M.-H. Loucheux, and B. Bailleul, personal communication) or AGluP3 adducts (22) (Table 2). Therefore, there is qualitative agreement between the efficiency of a mutagen in inducing NarI mutations in the forward mutation assay and in inducing reversions

| Compound                  | Maximal reversion frequency | Concentration at maximal reversion frequency | Survival at maximal reversion frequency, % | MIF*  |
|---------------------------|-----------------------------|---------------------------------------------|-------------------------------------------|-------|
| N-AcO-AAF(I)              | 2-3 × 10⁻⁴                  | 3 × 10⁻⁴ M                                  | 8                                         | ≈ 50,000 |
| N-AcO-AAFF(II)            | 2-3 × 10⁻⁴                  | 3 × 10⁻⁴ M                                  | 2                                         | ≈ 50,000 |
| N-AcO-AAIF(III)           | 2-3 × 10⁻⁵                  | 5 × 10⁻⁴ M                                  | 3                                         | ≈ 5,000  |
| N-OH-AF(IV)               | 6 × 10⁻⁶                    | 5 × 10⁻⁵ M                                  | 50                                        | 1,200  |
| N-AcO-AGluP3(V)           | 1-2 × 10⁻⁶                  | 1-2 × 10⁻⁸ M                                | 40                                        | 300    |
| NQO (VII)                 | 8 × 10⁻⁷                    | 2-3 × 10⁻⁴ M                                | 6                                         | 160    |
| NO₂-IQ (VI)               | 1-2 × 10⁻⁴                  | 4 × 10⁻⁴ M                                  | 1.5                                       | 30,000 |

*The mutation induction factor (MIF) is defined as the ratio of the maximal reversion frequency over the background reversion frequency (5 × 10⁻⁵).
in the assay described in this paper. The assays differ in important respects: forward mutations versus reversions and in vitro versus in vivo treatment of the mutation target. Nevertheless, the data from the reversion assay permit the prediction that NO2-IQ would be efficient in inducing NarI mutations in the forward mutation assay.

Single adduct mutagenesis experiments have shown that NarI mutagenesis results from the processing of an AAF adduct bound specifically to the internal guanine residue of the NarI sequence (23). Circular dichroism spectra of short helices containing the NarI site have indicated that binding of AAF adducts to the internal guanine residue induces a local Z-DNA-like conformation (24). We have hypothesized that the -2 frameshift event results from the processing of this Z-like conformation (3,25). In the cascade of events leading to the conversion of a small alternating CpG sequence from the B to the Z conformation, one can distinguish two steps: the anti to syn conversion of the guanine residue and the conformational change of the phosphate-sugar backbone from the B type to the Z type conformation. In this scheme, the driving force is the conversion of the guanine residue from anti to syn. At least two conditions have to be met to produce local Z-DNA structures within B-DNA: the anti to syn conversion of a guanine residue triggered by covalent binding to C8, and a proper sequence context in the neighborhood of the added guanine (i.e., alternating CpG or TpG sequences). The local sequence requirements are not fully clear, however, since there might also be a sequence requirement for the formation of the two B/Z junctions that exists when a Z-like region is surrounded by B-DNA.

It is tempting to relate the ability of a C8-G adduct to trigger the anti to syn conformational change to its efficiency in inducing -2 frameshift mutations within short alternating CpG sequences (Table 2). NMR and CD studies have shown the AAF and AAFF adducts, but not AAIF nor AF adducts, favor the anti to syn conversion of the added guanine residue (8,10). Moreover, it has been shown that AAF and AGluP3 adducts facilitate the B to Z conversion of modified poly dCpG (25–27). On the other hand, AF and NQO adducts have little or no effect on the B to Z conversion of modified poly dCpG (25,28). Therefore, the data currently available tend to support our hypothesis that the induction of -2 frameshift mutations is related to the changes from anti to syn and from the B-conformation to the Z-conformation. AGLuP3 appears to be an exception in that it is efficient in promoting the B to Z conversion (27) but poor in inducing NarI type mutations in the reversion assay (28). A possible explanation for this discrepancy is that deacetylation of AGLuP3 adducts, which has been reported to occur in the presence of divalent ions such as Zn2+ or Co2+, leads to the formation of GluP3 adducts rather than AGLuP3 (12). By analogy to the differences between AAF and its deacetylated form AF (3,20,25), these deacetylated GluP3 adducts would be expected to induce neither the B to Z transition nor the -2 frameshift mutations. For steric reasons, the presence of an N-acetyl group appears to be critical in favoring the anti to syn conversion in the case of C8-AAF adducts, and perhaps for N-arylamino-C8-guanine adducts in general.

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Table 2. Correlation between the induction of NarI mutations and local conformation of the adducts.

| Adduct     | Number of NarI mutants | Total number of mutants | Proportion of NarI mutants |
|------------|------------------------|-------------------------|---------------------------|
| AAF        | 12                     | 36                      | 33%                       | ++ +                     |
| AF         | 22                     | 23                      | 9%                        | +                        |
| AAIF       | 1                      | 24                      | 4%                        | +                        |
| AGluP3     | 0                      | 26                      | <4%                       | + + +                    |
| NQO        | 0                      | 31                      | <3%                       | -                        |

*All data are obtained in strain AB1157 except for AAF, which is a compilation of data obtained in the strain AB1157 and an isogenic \(uvrA\) strain (AB1886).

\(^{(a)}\) (++) anti to syn conformation or B to Z transition is highly favored; (+) anti to syn conformation or B to Z transition is slightly favored; (-) anti to syn conformation or B to Z transition is not favored.

dCpG (25–27). On the other hand, AF and NQO adducts have little or no effect on the B to Z conversion of modified poly dCpG (25,28). Therefore, the data currently available tend to support our hypothesis that the induction of -2 frameshift mutations is related to the changes from anti to syn and from the B-conformation to the Z-conformation. AGLuP3 appears to be an exception in that it is efficient in promoting the B to Z conversion (27) but poor in inducing NarI type mutations in the reversion assay (28). A possible explanation for this discrepancy is that deacetylation of AGLuP3 adducts, which has been reported to occur in the presence of divalent ions such as Zn2⁺ or Co2⁺, leads to the formation of GluP3 adducts rather than AGLuP3 (12). By analogy to the differences between AAF and its deacetylated form AF (3,20,25), these deacetylated GluP3 adducts would be expected to induce neither the B to Z transition nor the -2 frameshift mutations. For steric reasons, the presence of an N-acetyl group appears to be critical in favoring the anti to syn conversion in the case of C8-AAF adducts, and perhaps for N-arylamino-C8-guanine adducts in general.

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