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Genetics

Characterization of mutations induced by 2-(*N*-acetoxy-*N*-acetyl)aminofluorene in the dihydrofolate reductase gene of cultured hamster cells

(chemical carcinogenesis/mutagenesis/deoxyguanosine adduct/CHO cells)

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ABSTRACT **To determine the types of alterations in gene structure that are induced by the carcinogen 2-(*N*-acetoxy-*N*-acetyl)aminofluorene, we used this compound to generate mutations at the dihydrofolate reductase (DHFR) locus (*DHFR*) in Chinese hamster ovary cells. Twenty-nine independent enzyme-deficient mutants were isolated. A profile of the 26-kilobase (kb)-long gene was obtained by Southern blot analysis of the mutant and parental DNAs digested with *Bst*EII/*Kpn* I. Hybridization to a mixed probe of 10 *DHFR* genomic and cDNA fragments revealed 12 bands that scan 34 kb. Twenty-one DHFR$^-$ clones (72%) contained small mutations (changes <100 base pairs in size). Large or small deletions involving various parts of the gene occurred in eight of the mutants (28%). A large deletion (>35 kb) with 5′ and 3′ breakpoints mapping to approximately the same location was noted in four mutants. One mutant has undergone a deletion of 550–900 bp that eliminated the first coding exon. Concomitantly, a chromosomal event (either translocation, insertion, or inversion) has separated the 5′ flank from the body of the gene. In another mutant, four deletions have occurred at the *DHFR* 5′ end and internally. Restriction fragment length polymorphism analysis of the mutant DNAs with exon-specific probes localized three mutations. One mutant has lost a *Taq* I (TCGA) site, and another has lost a *Sac* I (GAGCTC) site. In a third, a GC→TA transversion has created a *Bst*EII (GGTNACC) site. Finally, we used HPLC to determine the ratio of acetylated (12%) to deacetylated (88%) 2-aminofluorene adducts formed in the parental cells. A correlation between the mutational specificities and the conformational changes induced by the two types of DNA adducts is discussed.**

Exposure of animals to carcinogens results in a series of phenotypic changes in emerging cell populations leading eventually to malignant neoplasia (for review, see ref. 1). Some of the events responsible for these changes are presumed to involve mutation. Oncogenes isolated from human and animal tumors are capable of inducing malignant transformation upon transfection into cultured cells. Alterations in either the expression or the function of particular oncogenes are implicated in the development of neoplasia. Tumor cells can display a variety of different types of mutations in cellular oncogenes. Examples are point mutation (2), translocation (3), amplification (4), and insertion (5).

Information learned about the activation of cellular oncogenes emphasizes the importance of studying chemical carcinogens as mutagens. That carcinogens are mutagenic has been well established in bacteria (6). Several quantitative assays have been developed to evaluate the mutagenic potential of carcinogens in animal cells (7–9). However, less is known about the spectrum of mutations inducible by chemical carcinogens. Molecular biological approaches for characterizing the physical nature of induced genetic changes is possible in cultured mammalian cells for any selectable locus that has been cloned and mapped.

Some studies using DNA probes for Southern (10) blot analysis of carcinogen-mutagenized animal cells have been reported. For instance, analysis of benzo[*a*]pyrene (11) and 3-methylcholanthrene (12) diol epoxide-induced hypoxanthine phosphoribosyltransferase-deficient (HPRT$^-$) hamster cells showed either no structural alterations (11) or large deletions (12). It was acknowledged, though, that the deletion mutants (3 out of 22) might be spontaneous in origin rather than induced (12). In another study, ethyl methanesulfonate was used to generate adenine phosphoribosyltransferase-deficient (APRT$^-$) Chinese hamster cell lines, starting with an APRT$^+$ hemizygote (13). Both induced and spontaneous *APRT*$^-$ alleles were compared. This chemical created single base-pair (bp) changes or other small alterations (<50 bp). To date, no gross structural rearrangements in selectable genes have been characterized at the DNA level after exposure of mammalian cells to a carcinogen.

We are interested in learning the types of gene alterations inducible by chemical carcinogens and the DNA sequence changes engendered in point mutants. We have used a model mammalian gene encoding dihydrofolate reductase (DHFR) to characterize mutations induced in cultured Chinese hamster ovary (CHO) cells by the carcinogen 2-(*N*-acetoxy-*N*-acetyl)aminofluorene (AAAF). AAAF reacts predominantly at the C-8 position of deoxyguanosine residues, yielding two types of adducts, acetylated {2-[*N*-(deoxyguanosin-8-yl)-*N*-acetyl]aminofluorene (dG-AAF)} and deacetylated [2-(deoxyguanosin-8-yl)aminofluorene (dG-AF)]. These adducts are known to affect DNA conformation differently (14–17). Both types of adducts were formed in the DNA of treated CHO cells. DHFR$^-$ mutants are readily selectable in a line of CHO cells that is hemizygous for this locus (18), and probes for the entire region containing the 26-kilobase (kb)-long gene are available (19). DNA from DHFR$^-$ mutants induced by AAAF was examined by Southern blotting and several different types of mutations were found, including base substitutions and complex rearrangements.

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Abbreviations: DHFR, dihydrofolate reductase; *DHFR*, genetic locus for DHFR; APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; AAAF, 2-(*N*-acetoxy-*N*-acetyl)aminofluorene; AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; RFLP, restriction fragment length polymorphism; bp, base pair(s); kb, kilobase(s).

MATERIALS AND METHODS

Materials. Restriction endonucleases, DNA polymerase I, and T4 DNA ligase were from New England Biolabs. Enzymes used to digest or modify nucleic acids were RNase A, *Escherichia coli* alkaline phosphatase, spleen phosphodiesterase II (Sigma); calf intestinal alkaline phosphatase (Boehringer Mannheim); and venom phosphodiesterase I (Pharmacia/P-L Biochemicals). Unlabeled AAAF and [G-^{3}H]AAAF were obtained from the National Cancer Institute Chemical and Radiochemical Repositories, respectively. [^{3}H]Deoxyuridine and α-^{32}P-labeled deoxynucleotides were from New England Nuclear. [^{3}H]Methotrexate was from Amersham. Nitrocellulose filter sheets (BA85) were from Schleicher & Schuell. For HPLC, a Waters μBondapak C_{18} column (3.9 mm × 30 cm) was used. Bacterial medium was from Difco; tissue culture medium was from GIBCO. All other biochemicals were from Sigma.

Mutagenesis and Selection. Three separate mutagenesis experiments were performed using a DHFR$^+$ hemizygote cell line, UA21 (16). This clone carries one wild-type allele; the other was entirely deleted by γ irradiation (DHFR$^{+/\Delta}$). Stock AAAF solutions were made in 100% ethanol. In the first experiment, 10^7 cells were exposed to 7.1 μM AAAF for 2.5 hr. The cells were then trypsinized, counted, and plated on eighteen 150-mm dishes. After 6 days, the cells from each dish were separately challenged with [^{3}H]deoxyuridine (20) to select DHFR$^-$ clones. Individual colonies were isolated, and the cells were tested for glycine, thymidine, and hypoxanthine auxotrophy. Only one mutant per dish was analyzed further. Alternatively, mutants were selected in the fluorescence-activated cell sorter as described (21). Clones displaying the appropriate phenotype were assayed for failure to bind [^{3}H]methotrexate (22). The subsequent two mutagenesis experiments were performed similarly except that 16 or 20 individual cultures were grown from small inocula and treated with AAAF. In the third experiment, the cells were exposed to AAAF in suspension rather than as monolayers.

Characterization of Mutant DNA. Preparation of genomic, plasmid, and fragment DNAs and methods for subcloning, restriction digestion, gel electrophoresis, Southern blot analysis, and nick-translation have been described (19). For the initial screening of mutant DNA by Southern blotting, a mixed probe of 10 fragments (the "polyprobe") derived from *DHFR* recombinant plasmids (19) was prepared (Fig. 1). Components of the mixed probe were as follows: A, pMH8 3.4-kb *Bgl* II fragment; B, pB6-14 1.7-kb *Bam*HI–*Sac* I; C, pB6-14 1.6-kb *Eco*RI; D, pB61H1 1.8-kb *Hin*dIII; E, pB61H3 0.9-kb *Hin*dIII; F, pB6-7 3.2-kb *Bam*HI–*Hin*dIII; G, pB6-7 2.1-kb *Hin*dIII; H, pDCH14 1.1- and 0.28-kb *Pst* I; and I, pB13-6 0.7-kb *Bam*HI. Exon-specific probes used in the RFLP analysis were pMB5 0.55-kb *Sac* II (exon I), pMB5 0.7-kb *Sac* II–*Bst*EII (exon II), pE11-15 1.45-kb *Ava* I–*Eco*RI (exon III), pB6-14 1.0-kb *Eco*RI–*Bam*HI (exon IV), pDCH6 0.23-kb *Sac* I–*Kpn* I (a cDNA clone containing exons IV and V, bp 262–489 in ref. 23; the *Kpn* I site is a cloning site), and pDCH14 1.1- and 0.28-kb *Pst* I (exon VI). Since cDNA for the protein-coding portion of the *DHFR* gene has been cloned and sequenced (23), cleavage sites in the exons are known. The parental and mutant DNAs were digested with the following enzymes for analysis of restriction fragment length polymorphism (RFLP): *Taq* I (exons I and III); *Bst*NI (exons I, III, V, and VI); *Hae* III (exons I, III, and V); *Sca* I, *Eco*RI, and *Xmn* I (exon II); *Hph* I, *Mbo* II, and *Msp* I (exon III); *Sac* I (exons III and IV); *Ava* II and *Kpn* I (exon VI). To obtain a probe for the 5′ flank of the *DHFR* gene, we cloned a 1.5-kb *Hin*dIII fragment located 10.4 kb upstream of exon I from the cosmid clone cH2 (24). The plasmid, designated pHH15, is devoid of repeated sequences. A 1.5-kb *Sma* I–*Eco*RI fragment (5′ portion) and a 0.85-kb *Hin*dIII–*Sma* I fragment (3′ portion) were purified from the 7.3-kb *Sma* I insert on another cH2 subclone, designated pH2Sm7. The 3′ *Sma* I site is located 468 bp upstream of the initiation codon (25). Filters were rehybridized with different probes. Dehybridizations were performed in 50% (vol/vol) formamide containing 3× SSC (0.45 M NaCl/0.045 M sodium citrate, pH 7.0) at 70°C for 30 min.

Analysis of Deoxynucleoside Residues Modified by [G-^{3}H]AAAF. UA21 cells were grown in two 150-mm dishes (5 × 10^7 cells). Growth medium containing 10% fetal bovine serum was removed and replaced by serum-free medium. [G-^{3}H]AAAF (232 mCi/mmol; 1 Ci = 37 GBq) was added at a concentration of 7.1 μM. The cells were then incubated for 2.5 hr and harvested, and the DNA was prepared. At this carcinogen dose, the binding level was 0.1 mmol of AAAF per mol of nucleotide. The DNA was enzymatically hydrolyzed (26) and extracted with 1-butanol (27). The adduct sample was solubilized in 100% methanol, combined with UV markers, and analyzed by HPLC (28).

RESULTS

We performed three separate carcinogen experiments in which AAAF was used to induce DHFR$^-$ CHO clones. The conditions used for mutagenesis yielded about 35% survival of the treated cells. The induced frequency of mutation was 1.5×10^{-5}; the spontaneous rate is 1.3×10^{-7} (29). Our initial approach for characterizing the mutants was to digest genomic DNA with *Kpn* I and *Bst*EII and analyze the digests by Southern blotting and hybridization to the nick-translated mixed probe (polyprobe). These enzymes create 12 resolvable bands scanning 34 kb of the locus. An example of the mixed probe screening is depicted in Fig. 1. If the DNA banding pattern of a mutant was identical to that of the parental cell line UA21, it was designated as carrying a small lesion, possibly a point mutation, deletion, or insertion. Sequence changes of 100 bp or less are below the resolution limit of this technique. Table 1 lists the 29 AAAF-induced DHFR$^-$ clones analyzed; of these, 21 were categorized as having small mutations.

Mutagenesis by AAAF also generated structurally different types of lesions in the *DHFR* locus. Three mutants with gross alterations in the gene are shown in Fig. 1. A complete deletion (>34 kb) has occurred in DF17. Another mutant, DF20, has lost the 4.4-kb *Kpn* I fragment and a new band at 2.3 kb is evident. The 3.4-kb band containing the 5′ end of the gene is missing in DF27. The latter two mutants were further characterized (Figs. 2 and 3, respectively). It is apparent that exon V has been eliminated from DF20, because cDNA sequences for this portion of the coding region do not hybridize (Fig. 2, probe A). The *Kpn* I site, located 229 bp upstream from the 5′ end of exon V (29), is retained in the mutant. Thus, one end of the deletion is near the 3′ boundary of intron IV. Probe C of Fig. 2 shows that the deletion terminates within the 5′ end of intron V.

The screening of DF27 indicated a rearrangement at the 5′ end of the *DHFR* gene. More detailed examination (Fig. 3) showed that a deletion had occurred, since an exon I-specific probe (probe C) failed to hybridize to DF27 DNA. Rehybridization of the filter with three other probes from this region revealed that the deletion was relatively small. The maximum size of the deletion is about 900 bp, since a *Bgl* II site at bp −720 and a *Pst* I site at bp +206 are still present in DF27 DNA. The minimum size is ≈550 bp, the size of probe C. The sequences 5′ and 3′ of the deletion are not colinear as in the case of DF20. Cutting with enzymes (*Kpn* I, *Bgl* II, *Hin*dIII, *Hin*cII, *Eco*RI, *Bst*EII) that surround the deletion site yielded a pattern that cannot be reconciled with the wild-type map (Fig. 3 and data not shown). Hence, a disruption of the *DHFR* gene has occurred in DF27 concom-

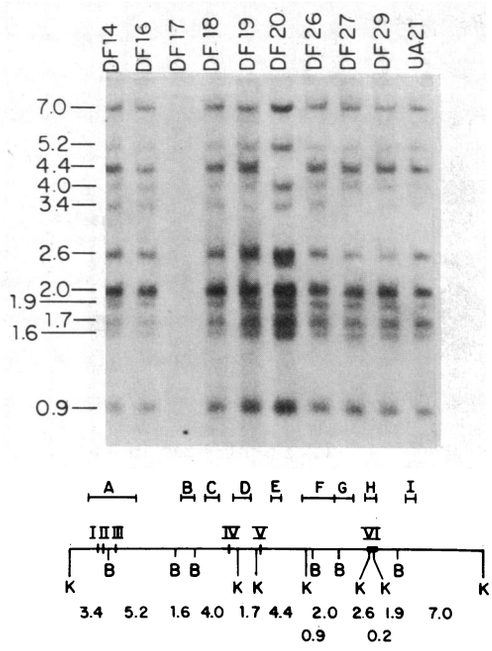


FIG. 1. (*Upper*) Example of screening the entire *DHFR* gene in DNA samples from AAAF-induced DHFR⁻ mutants. The mutant and parental DNA (18 μg of each) were digested by *Kpn* I (30 units per digest) at 37°C for 2 hr. The concentration of NaCl was raised from 6 mM to 150 mM and *Bst*EII (20 units) was added to each sample. Digestion was allowed to proceed at 60°C for 1.5 hr. Samples were then applied to a 0.8% agarose gel and electrophoresed at 25 mA for ≈20 hr. Southern blotting conditions have been described (19). Mutant designations are given above each gel lane. The sizes of restriction fragments (in kb) are shown at left. The 0.2-kb *Kpn* I fragment that hybridizes to probe H is visible only after a long exposure time and is omitted. (*Lower*) A map of the 34-kb sequence scanned by the mixed probe, showing the location of *Kpn* I (K) and *Bst*EII (B) cleavage sites. The mixed probe is represented by lines labeled A–I, drawn above the *DHFR* fragments to which they hybridize. Components of the mixed probe are detailed in *Materials and Methods*. The six exons are represented as black bars identified by numerals I–VI. Exon VI has been drawn larger in size than the others in order to indicate that there are about 2 kb of untranslated sequences at the 3′ end of the gene.

itant with and close to the deletion. A map of the *DHFR* 5′ end of UA21 is shown in Fig. 3 and is compared with that of DF27. The region 5′ of the gene in the mutant is depicted as being attached to a different segment of chromosome. The remainder of the gene, which is anomalously joined to another region, is drawn below. The mutant may have undergone a translocation moving either the 5′ flank or the body of the gene to a new location. Alternatively, we cannot rule out a large insertion or inversion.

In the first set of mutants isolated (Table 1), four (DF21, DF11, DF13, and DF91) appeared to carry a similar large deletion. Analysis of three of them is shown in Fig. 4.

Table 1. Structurally different types of mutations induced in *DHFR* by AAAF

Type of mutation	Number of mutants			
	Exp. 1	Exp. 2	Exp. 3	Total
Small*	6	6	9	21
Deletions	4†	2	0	6
Disruptions‡	1	1	0	2

*Single bp changes or small (<100 bp) insertions or deletions.
†Induced or spontaneous mutants (see *Discussion*).
‡Translocation, large insertion duplication, or inversion.

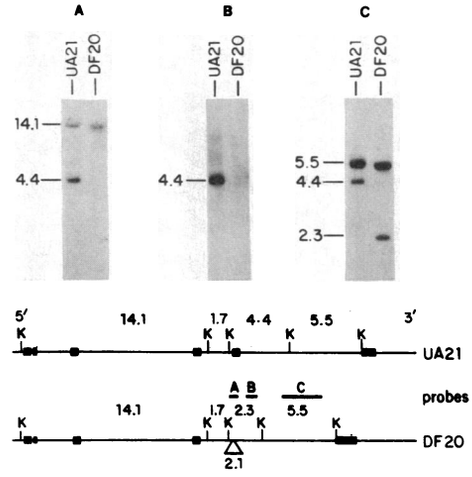


FIG. 2. (*Upper*) Southern blot analysis of deletion mutant DF20. The parental UA21 and mutant DF20 DNAs were digested with *Kpn* I. Conditions were the same as described in *Materials and Methods* and the legend to Fig. 1. The single filter was hybridized serially with each different probe (identified above each autoradiograph): A, pDCH6 0.23-kb *Sac* I–*Kpn* I fragment (cDNA containing sequences of exons IV and V); B, pB61H3 0.9-kb *Hin*dIII fragment; and C, pB6-7 3.2-kb *Bam*HI–*Hin*dIII fragment. (*Lower*) Maps of the wild-type UA21 and mutant DF20 genes. Location of *Kpn* I (K) sites and areas corresponding to probes A–C are indicated.

Screening with the mixed probe revealed that the mutants retained only the *DHFR* 3′ end and that a new band at 5.2 kb was created. In each mutant, the deletion arose within the 2.0-kb *Bst*EII fragment and has extended 5′ more than 35 kb, since pHH15 (probe A) failed to hybridize. The single new band, detected by sequences from intron V (probe B),

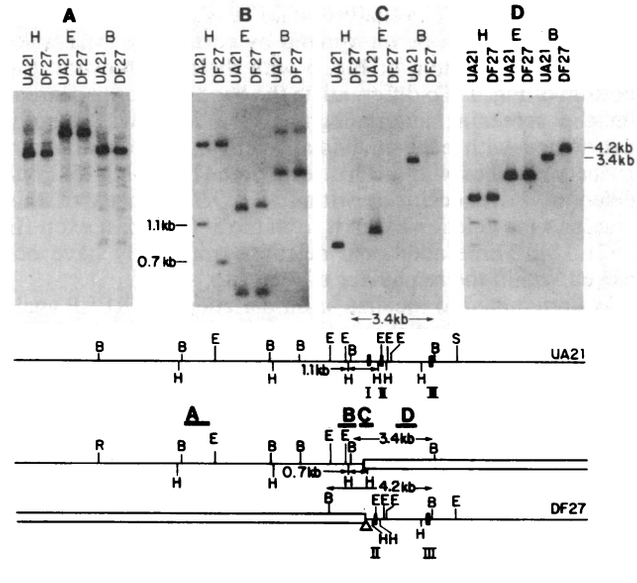


FIG. 3. (*Upper*) Southern blot analysis of the gene-disruption mutant DF27. Mutant and parental DNAs (18 μg of each) were digested with *Hin*cII (H, 24 units), *Eco*RI (E, 40 units), and *Bgl* II (B, 40 units). Conditions were as described for Fig. 1. The single filter was serially hybridized with each different probe: A, pH2Sm7 1.5-kb *Sma* I–*Eco*RI fragment (5′ flank); B, pH2Sm7 0.85-kb *Hin*dIII–*Sma* I fragment (5′ flank); C, pMB5 0.55-kb *Sac* II fragment (exon I); and D, pMB5 1.1-kb *Bst*EII–*Ava* I fragment (intron II). (*Lower*) Restriction maps of the wild-type UA21 *DHFR* 5′ end and of the 5′ flanking region and gene of DF27 (depicted on two lines in order to indicate that these segments are not colinear; see *Results*). Lettered bars represent probes A–D; restriction sites are abbreviated (H, E, B) as above.

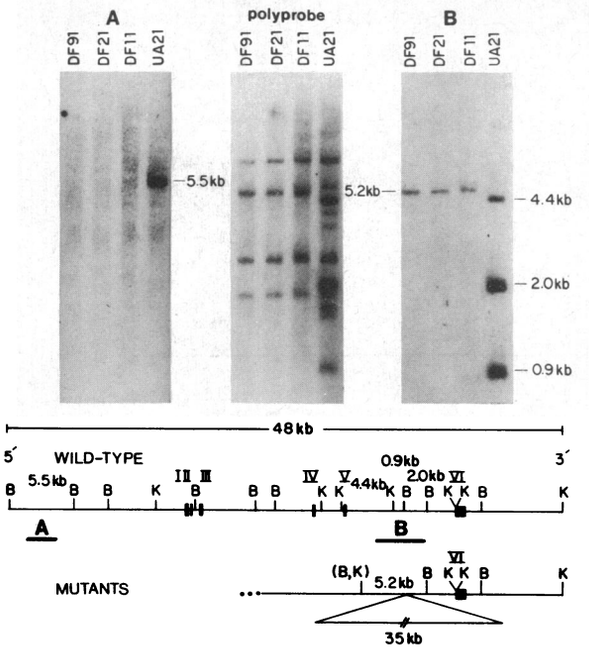
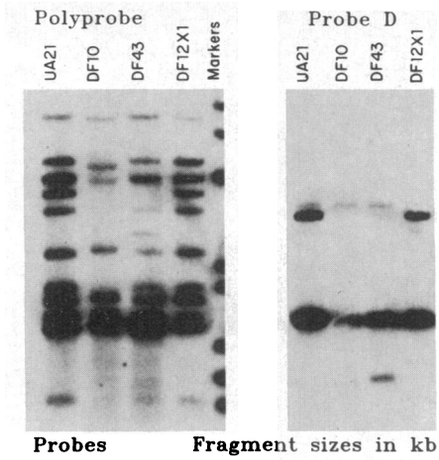


FIG. 4. (*Upper*) Southern blot analysis of deletion mutants DF91, DF21, and DF11. Digestion and other conditions were as for Fig. 1. Probes: A, pHH15 1.5-kb *Hin*dIII fragment; the mixed probe (polyprobe; see *Materials and Methods*); and B, pB6-7 3.2-kb *Bam*HI–*Hin*dIII fragment. (*Lower*) Restriction maps. B, *Bst*EII; K, *Kpn* I.



Probes	Fragment sizes in kb	
	UA21	DF10
pHH15	5.5	–
A	3.4, 5.2	–
B	1.6	1.6
C	4.0	–
D	4.0, 1.7	1.7
E	4.4	–
F	4.4, 0.9, 2.0	4.8
G	2.0, 2.6	4.8, 2.6
H	2.6, 0.2, 1.9	2.6, 0.2, 1.9
I	7.0	7.0

FIG. 5. (*Upper*) Southern blot analysis of the gene-disruption mutant DF10 and of mutants DF43 and DF12X1. Digestion and other conditions were as for Fig. 1. The filter was hybridized serially with the mixed probe (shown), with each component of the mixed probe (probe D shown), and with the 1.5-kb *Hin*dIII fragment of pHH15. (*Lower*) Summary of results. Letters under the heading for probes correspond to those in Fig. 1 and represent fragments detailed in *Materials and Methods*.

suggests that the deletion may have the same 5′ and 3′ endpoints in these four mutants.

Another type of structurally rearranged mutant, DF10, was isolated from the same mutagenesis experiment as the four deletion mutants. The analysis of DF10 is summarized in Fig. 5. The results can be interpreted by correlating the results shown at the bottom of Fig. 5 with the map shown at the bottom of Fig. 1. To differentiate the bands that are apparent on the screening autoradiograph, the filter was serially hybridized with each individual component of the mixed probe, as well as with the upstream probe from pHH15. Four deletions have occurred within the *DHFR* locus of this mutant. One deletion extends from the 5′ flank past exon III (>21 kb). Three additional deletions apparently have occurred within the remainder of the gene.

We attempted to localize small mutations by RFLP analysis. Designations of these mutants isolated from the three experiments are as follows: DF11-1, DF31, DF51, DF41, DF12X1, and DF81 (experiment 1); DF14, DF16, DF18, DF19, DF26, and DF29 (experiment 2); and DF30, DF31-1, DF32, DF35, DF40, DF42, DF43, DF44, and DF47 (experiment 3). DNA of these mutants was digested with 12 enzymes that each cut at least once in the *DHFR* exons (see *Materials and Methods*). Analysis of two clones carrying small mutations (DF43 and DF12X1) is shown in Fig. 5. From screening with the mixed probe, it was apparent that DF43 no longer yielded the 4.0-kb *Bst*EII–*Kpn* I fragment containing exon IV. A new, 3.0-kb band appeared in the lane containing DNA of this mutant. Reprobing the filter with intron IV sequences (probe D) revealed a new 1.0-kb fragment. This additional band accounts for the original size of the 4.0-kb piece, thus ruling out a deletion. Digestion of DF43 DNA with *Kpn* I alone followed by Southern blotting showed the normal 14.1-kb fragment (data not shown) indicating that a *Bst*EII site and not a *Kpn* I site had been created in the mutant. If a new restriction site were positioned 1.0 kb upstream from the 3′ *Kpn* I site of the 4.0-kb fragment, it would lie within exon IV. The sequence at the 5′ end of this exon is AGGGAACCA (23) (the consensus splice site is underlined). The sequence GAA encodes a glutamic acid residue and defines the reading frame. A GC→TA transversion at this sequence generates a *Bst*EII (GGTNACC) cleavage site. This single bp change creates a TAA codon signaling chain termination.

RFLP analysis localized the lesions in two other DHFR$^-$ mutants. DF11-1 has lost a *Taq* I site (TCGA) in exon I (data not shown). A GC→TA transversion at this site would create the sequence TAG, another chain termination codon. DF26 has lost a *Sac* I site (GAGCTC) in exon III (data not shown).

As mentioned above, the carcinogen AAF binds to guanine in DNA, yielding mainly two types of adducts. In an effort to rationalize the various types of mutations obtained in CHO cells at the *DHFR* locus, we performed HPLC analysis of enzymatically hydrolyzed DNA (26) from UA21 cells incubated with [G-^{3}H]AAAF under the same conditions used for mutagenesis. Both acetylated and deacetylated adducts were found in these cells. Of total modified nucleosides, 12% were dG-AAF and 88% were dG-AF (data not shown).

DISCUSSION

We have examined the nature of lesions induced by the carcinogen AAAF in the well-characterized *DHFR* locus of CHO cells. As a selectable marker, the *DHFR* gene has several advantages. We are evaluating mutations engendered in an endogenous, constitutively expressed gene in chromatin. We isolated mutants starting with a *DHFR*$^{+/\Delta}$ hemizygote cell line, UA21 (18). Physical analysis of the single remaining allele is unambiguous. We can score for deletion events because no genes essential for viability are

closely linked to this gene (18). By Southern blot analysis using a mixed probe, we are able to scan a continuous 34-kb region containing the *DHFR* locus. We screened 29 mutants and found that 8 carry deletions and gene disruptions. The rest contain either point mutations or small (<100 bp) deletions or insertions.

The collection of AAAF-induced DHFR$^-$ mutants is heterogeneous, and their genotypes are distinct from those of spontaneous mutants (29). Fuscoe *et al.* (30) have analyzed the nature of spontaneous and UV-induced mutations at the *HPRT* locus of CHO cells. UV mutagenesis increased the frequency of HPRT$^-$ clones 20-fold. Deletion mutations were found in 1 out of about 10 spontaneous or UV-induced mutants. Recently, five spontaneous *DHFR*$^-$ mutants have been isolated; none of them carry deletions (29). Three spontaneous mutations have been sequenced. Each mutation has created a different single bp change that abolishes mRNA splicing at exon V.

This system may be able to demonstrate specificity with respect to the types of mutations that are inducible by particular chemical carcinogens. It is evident that AAAF is able to induce both point mutations and gene rearrangements. The disruption events induced by AAAF can be quite complex, as indicated by the deletion plus translocation in DF27 and the multiple deletions in DF10. We have performed a similar analysis of mutants induced by benzo[*a*]pyrene diol epoxide and found virtually all point or small mutations (unpublished results).

Regarding the mutational specificity of AAAF, four mutants were isolated in experiment 1 that appear to carry a similar large deletion, one that leaves only about 20% of the *DHFR* gene remaining at the 3′ end (Fig. 4). The mutagenesis protocol in that experiment rules out the possibility that these identical mutants arose from a single AAF-induced progenitor because the treated culture was split immediately after exposure to the carcinogen and all four mutants were cloned from separate dishes. Another possibility is that they arose as sister colonies from a spontaneous mutant clone that preexisted in the population. Although this possibility cannot be eliminated, it is unlikely on statistical grounds; the mutagenized culture yielded a mutant frequency 100-fold greater than the usual spontaneous frequency. The third possibility is that this deletion represents a hot spot for AAAF-induced deletion, the extent of the deletion being governed by some aspect of chromatin structure. If this were the case, one might have expected to see this deletion reappear in the two subsequent experiments that generated 18 more mutants. Thus, the origin of these deletion mutations remains unclear.

Both AAF and AF bind to the C-8 position of guanine. The acetylated deoxyguanosine adduct causes a major conformational distortion in DNA structure (16, 31). The adduct displaces the guanine ring out of the helix, and the fluorene residue is intercalated. However, the deacetylated deoxyguanosine adduct causes only small changes in DNA structure (15, 17). Normal DNA conformation is maintained, and the fluorene residue is situated in the major groove. Using a forward mutation assay in bacteria, Koffel-Schwartz *et al.* (32) and Bichara and Fuchs (33) have demonstrated that the mutational specificity of the two adducts is different. They found that dG-AAF induced frameshift mutations, whereas dG-AF adducts induced mainly base substitutions. Because the acetylated and deacetylated adducts produce different conformational changes and show different mutational specificities, we analyzed the ratio of these products in the DNA of mutagenized CHO cells. We found that the ratio of dG-AAF and dG-AF adducts formed in UA21 DNA is similar to the ratio of gross- and small-lesion *DHFR* mutants isolated after AAAF treatment. It is not feasible to ascertain the ratio of adducts during the period in which the treated cells recover and replicate. Nonetheless, there may be a correlation between the conformational alterations induced in DNA by dG-AAF and dG-AF and the types of mutations we have identified at the *DHFR* locus.

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