Role of Depurination in Mutagenesis by Chemical Carcinogens

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ABSTRACT

The effect of modifying ϕX174 viral DNA by the chemical carcinogens β-propiolactone, N-acetoxyacetacetylaminofluorene and anti-benzo[α]pyrene diol-epoxide was investigated by transfecting the modified DNA into Escherichia coli spheroplasts. Modification of the DNA in vitro by each of these agents was mutagenic for the ϕX174 amber mutants am3 and am86. Mutagenicity depended on the induction of the "SOS" response in the host spheroplasts. Heating β-propiolactone-treated DNA at neutral pH caused strong inactivation such that the number of lethal hits was increased 40-fold. Sucrose gradient analysis showed the induction of alkali-labile sites in the heated DNA. The "nicked circle assay" with double-stranded ϕX174 DNA showed greater than 70% of these sites to be apurinic sites. Concomitantly with the production of these new sites, a strong increase in the mutation frequency was observed. This mutagenesis also depended upon the induction of the error-prone SOS response in the spheroplasts, as was previously shown to be the case for mutagenesis at putative apurinic sites induced directly by acid-heat treatment. These results suggest that depurination may be of importance to the mechanism of mutagenesis by β-propiolactone and other carcinogens.

INTRODUCTION

To understand the process of chemical carcinogenesis at the molecular level, a detailed knowledge is required of the interaction of chemical carcinogens with cellular macromolecules, in particular with DNA. This interaction, frequently in the form of covalent binding to the DNA, is thought to be a crucial initiating event in carcinogenesis (14). Progress in this field has been hampered by the complex variety of lesions produced in DNA by most of these agents. It has therefore been difficult to assign the mutagenic or carcinogenic effects of an agent to one particular lesion. We have recently developed a combined in vitro-in vivo assay using ϕX174 DNA, which allows the DNA to be treated in vitro and to be introduced into the cell by transfection. This permits us to control both the degree of damage to the DNA and the condition of the host. This technique has allowed us to establish the mutagenicity of apurinic sites (20) in Escherichia coli spheroplasts in which the error-prone SOS response had been induced. No mutagenic response was observed in normal spheroplasts. These results were interpreted to mean that apurinic sites cannot easily be copied during normal DNA replication but are copied to a significant extent during the presumably modified mode of replication in SOS-induced bacteria. In these experiments, apurinic sites were introduced by a combined acid-heat treatment. Although a role for other heat-acid-induced lesions cannot be rigorously excluded, the mutagenicity of apurinic sites is strongly supported by the abolition of mutagenesis by alkaline (20) and by purified apurinic endonuclease. Apurinic sites could also be important for mutagenesis by chemical carcinogens. Modification of purines at N3 and N7 and of pyrimidines at O2 positions stabilizes the N-glycosylic bond, leading to sharply increased spontaneous depurination rates (2, 11, 22). Alternatively, apurinic sites may be created in vivo by N-glycosylases, of which increasing numbers are being discovered (12, 23).

In this paper, we used this ϕX174 transfection system to evaluate the lethal and mutagenic consequences of treatment of ϕX-DNA with the chemical carcinogens BPL, NAAAF, and aBPDE. We report that each of these agents is mutagenic when error-prone repair has been induced. In the case of BPL, we present evidence suggesting that the mutagenic consequences of modification may be enhanced by the creation and bypass of apurinic sites.

MATERIALS AND METHODS

Chemicals. BPL was obtained from Sigma Chemical Co. (St. Louis, Mo.); [3H]aBDPE (565 mCi/mmol) and [3H]NAAAF (710 mCi/mmol) were obtained from Midwest Research Institute, Kansas City, Mo., through the Cancer Research Program of the Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md. 0X-DNA with the chemical carcinogens BPL, NAAAF, and aBPDE. We report that each of these agents is mutagenic when error-prone repair has been induced. In the case of BPL, we present evidence suggesting that the mutagenic consequences of modification may be enhanced by the creation and bypass of apurinic sites.

Bacteria and Bacteriophage. ϕX174 single-stranded viral DNA and RF I DNA were prepared as described previously (8). High-lifter stocks of ϕX am3 and am86 were prepared as described (6). am3 is located at positions 586 to 588 in gene E(D), and am86 is located at positions 4116 to 4118 in gene A (17). E. coli HF4714 (su"

Transfection Procedures. Spheroplasts were prepared from E. coli KT-1 through the lysozyme-EDTA procedure (8, 20). For SOS induction, the bacteria were irradiated in thin layers with UV (80 J/sq m) (254 nm) and incubated for 45 min at 37° prior to their conversion to spheroplasts (20). The details of the transfection procedure have been described (8). In short, 1.0 µg ϕX174 DNA, either normal or carcinogen-modified, was dissolved in 5 ml 20 µm Tris-HCl (pH 8.1). Spheroplasts (5 ml) were added, and the mixture was incubated at 37° for 15 min. At that time, 10 ml prewarmed PAM medium (pH 7.2), containing 10 g/liter nutrient broth, 10 g/liter casamino acids, 10 g/liter glucose, 100 g/liter sucrose, and 0.2% MgSO4 were added, and incubation continued for approximately 90 min. This entire procedure was performed under subdued light. After the mixture was frozen at −20° or
-70° and thawed and 100 µl chloroform had been added, phage titer and reversion frequency of the resultant progeny phage were determined by plating on HF4714 (su+) and HF4704 (su-).

Modification of the DNA. One volume of ϕX174 DNA (0.1 µg/ml in 10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) was modified by adding 0.1 volume of an appropriate dilution of the activated carcinogen. Incubation was at 37° for 1 to 2 hr in the dark to prevent photodecomposition. This DNA was either used directly or stored at -70°. BPL was diluted in 20 mM Tris (pH 8.0) immediately before use. aBPDE was diluted in dimethylformamide, and NAAAF was diluted in dimethyl sulfoxide. Depurination of unmodified ϕX174 DNA was performed at pH 5.00 and 70° as described (20). Heat treatment subsequent to modification was by addition of 10 volumes of 10 mM potassium phosphate buffer (pH 7.0) followed by incubation at 70° for 1 hr in the dark.

Characterizing Heat-induced Sites. Alkali-labile sites were demonstrated by treating the modified and/or heated DNA with an equal volume of 0.2 n NaOH at 37° for 20 min followed by sucrose gradient analysis. Neutral sucrose gradients (5 to 20%) were run for 4.5 hr at 40,000 rpm in a SW 50.1 rotor. The identity of apurinic sites was established by using the "nicked-circle assay" as developed by Kuhnlein et al. (7). The assay measures the conversion of RF I to replicative form II DNA which results in the DNA sticking to nitrocellulose filters upon quick denaturation and renaturation. To measure apurinic sites, they must be hydrolyzed by alkali treatment (30 min, 37°, 0.1 n NaOH) or enzymatically by treatment with apurinic endonucleases. The latter was conducted by incubating 0.5 µ ϕX174 RF I (11,000 cpm 3H per µg) in a final volume of 150 µl containing 25 mM Tris-HCl (pH 7.50), 0.2 mM EDTA, 12.5 mM MgCl2, and 0.005% Triton X-100, with 1 µl apurinic endonuclease purified from HeLa cells (20 units/µl) for 30 min at 37°, terminated by the addition of EDTA to 20 mM. The apurinic endonuclease was kindly provided by Drs. C. M. Kane and S. Linn (5).

RESULTS

Inactivation and Mutagenesis of ϕX174 DNA. The loss of biological activity by treatment of single-stranded ϕX174 DNA with increasing concentrations of BPL, aBPDE, and NAAAF is displayed in Chart 1. These carcinogens interact directly with DNA and do not require metabolic activation (14, 24). The 37% survival levels, at which an average of one lethal hit per molecule is introduced according to Poisson statistics, is attained at approximately 450, 1.2, and 1.3 µM for BPL, aBPDE, and NAAAF, respectively. Adducts formed with aBPDE and NAAAF appear to block DNA replication, and its likely that the number of lethal hits equals the number of adducts per circle (4, 15). For BPL, many adducts might form per lethal event. In Chart 2, a representative mutagenesis experiment is depicted for the reversion of the ϕX174 mutant am3 to wild type. Mutagenesis as a result of heat-acid-catalyzed depurination is included for comparison. The salient feature is that mutagenesis induced by these compounds depends on the induction of the SOS response in the spheroplasts. In these studies, the SOS response was induced by exposing bacteria to UV irradiation prior to their conversion to spheroplasts. In the absence of exposure of ϕX174 DNA to the carcinogens (0 concentration), there was no difference in mutagenesis between normal and SOS-induced spheroplasts. The results of a large number of experiments are averaged in Table 1 for 2 ϕX174 mutants, am3 and am66. To facilitate comparison, the increases in reversion frequencies are expressed per lethal hit. In the case of heat-acid, aBPDE, and NAAAF, this presumably represents one modification per molecule and in the case of BPL it represents several per molecule. DNA sequence studies of this laboratory have shown that am66 is capable of reversion by single base substitutions at any of the 3 positions of the (TAG) amber codon whereas am3 appears to revert exclusively at the middle (adenine) position (9, 10). This discrepancy is presumably due to the fact that am3 is located in the overlap region of genes E and D (17), whereas am66 is not located in such an overlap region. The ability of am66 to revert at the third position is particularly important since the tested chemical carcinogens preferentially modify guanines (3, 21). Indeed, the reversion of am66 occurred more frequently than did the reversion of the am3 mutant. Reversion frequencies vary from experiment to experiment as a consequence of the varying ability of spheroplast preparations to express the SOS phenotype. However,
within each experiment, the relative mutagenicities of the 4 treatments are similar. It can be concluded that heat-acid is generally the most effective in eliciting mutagenesis expressed per lethal hit. This is striking with am3 but is also observed with am86.

Effect of Heat on Survival and Mutagenesis. Several different base modifications, most notably at positions N3 and N7 of purines, destabilize the N-glycosyl bond and increase the rate of depurination (11). To assess whether depurination through this pathway could contribute to mutagenesis by the agents used in this study, we heated the modified φX174 DNA molecules at 70° at neutral pH. This procedure is expected to effectively hydrolyze the labilized bases (11) without causing significant depurination of unmodified bases. For aBPDE- and NAAAF-modified DNAs, no detectable changes in either survival or mutation frequencies could be detected [within normal confidence levels (changes less than 2-fold; results not shown)]. In contrast, in the case of BPL-modified DNA, a strong inactivation occurred as a result of heat treatment (Charts 3 and 4). This inactivation was reproducible in several different experiments and corresponded to the introduction of 3 additional lethal hits for every preexisting lethal hit. It is unlikely that this inactivation was due to increased reaction of the DNA with residual amounts of BPL since the samples were routinely diluted 10-fold before heating, and even overnight dialysis against a large volume of buffer prior to heating did not diminish the heat-induced inactivation. Heating BPL-modified DNA also has a large effect on the reversion frequencies of am3 and am86 (Chart 4). A 7- to 9-fold increase is observed for the 2 mutants.

Analysis of Modified-Heated DNA. Chart 5 shows a neutral sucrose gradient analysis of the BPL-modified φX174 single-stranded DNA. Fractions were collected from the top. Sedimentation is from left to right. Arrow, position of the φX174 single-stranded circle. The DNA was treated with 320 μM BPL. The modified DNA does not shift its position (not shown). Indicated are the profiles obtained with this DNA subsequently heated (●) or alkali treated (○), or both (△). Neither heat, nor alkali, nor their combination affected the position of unmodified DNA (not shown).

Chart 4. Reversion frequencies of BPL-treated am3 and am86 DNA before and after heat treatment as measured in SOS-induced cells. See legend to Chart 3. The increase for control am3 DNA might be due to direct depurination (about one-third of an apurinic site is expected per molecule). Conc., concentration.

Chart 5. Neutral sucrose gradient analysis of BPL-modified φX174 single-stranded DNA. Fractions were collected from the top. Sedimentation is from left to right. Arrow, position of the φX174 single-stranded circle. The DNA was treated with 320 μM BPL. The modified DNA does not shift its position (not shown). Indicated are the profiles obtained with this DNA subsequently heated (●) or alkali treated (○), or both (△). Neither heat, nor alkali, nor their combination affected the position of unmodified DNA (not shown).
Discussion

In this paper, we report that the in vitro modification-transfection system of ϕX174 DNA can be applied to study mutagenesis by chemical carcinogens. All 3 tested carcinogens were found to be mutagenic in this system. In trying to define the lesions specifically responsible for the mutagenic and/or carcinogenic effects of certain agents, this system may offer a number of advantages. The use of single-stranded DNA allows one to analyze the results more directly with respect to the outcome of the interaction of DNA replication with damaged bases with little interference by DNA repair. The use of a phase system allows one to analyze the response in the absence or presence of "inducible error-prone repair." Moreover, in vitro DNA modification opens up the possibility of further in vitro manipulations, like heat, acid, or alkali treatments or enzymatic treatments such as by N-glycosylase or damage-specific endonucleases. To exploit the full potential of this strategy, a basic knowledge of the different kinds of DNA damage and their chemical properties is needed. Such knowledge is becoming increasingly available. A limitation of the system in its present form is that it measures only base substitution mutations, ignoring the potentially important contribution of base addition-deletion mutations.

Mutagenesis by the 3 chemical carcinogens used in this study proved to be SOS dependent. Therefore, the lesions responsible for mutagenesis are not likely to cause direct miscoding but rather to result in termination of normal DNA synthesis. One might therefore speculate on the importance of such intermediates since they are the potential secondary product of a large variety of lesions. Moreover, when SOS has been induced, they are mutagenic in vivo (20) and are copied to measurable extents by DNA polymerases in vitro. In its simplest form, this model (Chart 6) involves cessation of DNA replication at bulky adducts and the resultant induction of an error-prone (SOS) response (18, 19); and, as a consequence, the replication complex may be modified so as to more easily replicate past modified DNA structures. In case of specific adducts, their depurination, either spontaneously or enzymatically by specific N-glycosylases, could provide a much better substrate for error-prone bypass than does the original blocking lesion. Thus, apurinic sites may represent an important intermediate in the production of mutations. We speculate that the presence of polymerase may protect the apurinic site from hydrolysis by endonuclease prior to DNA replication.

To explore whether depurination of labilized bases could play a role in mutagenesis by the agents used here, we subjected the modified DNA to moderate heat treatment at neutral pH. This treatment had only a minor effect on either survival or mutation frequency of the undamaged DNA or the DNA treated with abPDE or NAAAF. In contrast, both survival and mutation frequency of BPL-treated DNA were strongly affected. Two explanations can be advanced for the absence of an effect in the case of abPDE or NAAAF. (a) Since both the bulky adducts produced by these carcinogens and subsequent apurinic sites are likely to block DNA synthesis and therefore constitute lethal lesions, transformation of one into the other would change neither survival nor mutagenicity if the lethal or mutagenic potentials of the 2 types of lesions were approximately equal. (b) Heating the modified DNA might not depurinate the adducted bases very efficiently. abPDE and NAAAF interact predominantly at positions N₂ and C₉ of guanine (3). These modifications do not labilize the glycosyl bond. In the case of abPDE, modification at position N₇ of guanine occurs as a minor product (10 to 20%), and an alkali-labile adenine product has also been detected (6, 16). It is conceivable that depurination of these minor products has minor effects on mutation frequencies, but the sensitivity of the ϕX174 assay is such that changes up to 2-fold cannot be assessed unambiguously.

Significant changes are observed upon heat treatment of such a system (18, 19). We propose a possible mechanism by which apurinic sites function as intermediates in the mutagenesis by many chemical carcinogens, including those that cause a (transient) termination of DNA synthesis. Apurinic sites are attractive as such intermediates since they are the potential secondary product of a large variety of lesions. Moreover, when SOS has been induced, they are mutagenic in vivo (20) and are copied to measurable extents by DNA polymerases in vitro. In its simplest form, this model (Chart 6) involves cessation of DNA replication at bulky adducts and the resultant induction of an error-prone (SOS) response (18, 19); and, as a consequence, the replication complex may be modified so as to more easily replicate past modified DNA structures. In case of specific adducts, their depurination, either spontaneously or enzymatically by specific N-glycosylases, could provide a much better substrate for error-prone bypass than does the original blocking lesion. Thus, apurinic sites may represent an important intermediate in the production of mutations. We speculate that the presence of polymerase may protect the apurinic site from hydrolysis by endonuclease prior to DNA replication.

Table 2

<table>
<thead>
<tr>
<th>Treatment following BPL</th>
<th>No BPL</th>
<th>BPL (1.6 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>589 (ND)</td>
<td>400 (ND)</td>
</tr>
<tr>
<td>Apurinic endonuclease</td>
<td>595 (ND)</td>
<td>740 (0.12)</td>
</tr>
<tr>
<td>Heat</td>
<td>490 (ND)</td>
<td>860 (0.22)</td>
</tr>
<tr>
<td>Alkali</td>
<td>800 (0.17)</td>
<td>770 (0.15)</td>
</tr>
<tr>
<td>Heat + alkali</td>
<td>790 (0.15)</td>
<td>1859 (1.83)</td>
</tr>
<tr>
<td>Heat + apurinic endonuclease</td>
<td>580 (ND)</td>
<td>1875 (1.90)</td>
</tr>
</tbody>
</table>

* ND, nondetectable (less than 0.05 site).
* Numbers in parentheses, calculated numbers of apurinic sites present per molecule.
* Measures alkali-sensitive sites rather than apurinic sites.

FIGURE 6

Table 6. Mutagenesis by chemical carcinogens via depurination.
BPL-modified DNA. Our contention that the increased mutagenesis observed upon heat treatment of BPL-modified DNA is likely to result from depurination is based on the following arguments. (a) Depurination is expected since, of the 4 characterized BPL DNA adducts [i.e., the (2-carboxyethyl) addition product to the N7 position of guanine, the N1 position of adenine, and the N3 positions of both cytosine and thymine], the N7-guanine product is the most frequent (70%) (21), and its depurination has been well established (1, 21). It is important to note, however, that limited depurination of the N7-adenine product has also been reported (1). (b) Heat introduces 3 additional lethal hits for every preexisting lethal hit. Apurinic sites have been shown to be lethal hits (20). (c) BPL-treated DNA is essentially alkali stable. Upon treatment, however, alkali-sensitive sites are observed. Apurinic sites are alkali sensitive (13). (d) Filter-binding studies with apurinic endonuclease show that at least 70% of the heat-induced alkali-sensitive sites have the characteristics of apurinic sites. However, this last argument must be qualified since the experiment was done with double-stranded DNA and the product distributions could be different for double- and single-stranded DNA.

It may prove instructive to make some calculations based on the survival and mutagenesis data given in Charts 3 and 4. It appears that heat treatment of BPL-modified DNA is equally mutagenic for am3 and am86 DNA since, corrected for background reversion frequencies, an approximately equal increase is observed (7- and 9-fold, respectively). However, in terms of absolute increases, am86 is much more mutable than am3. Calculating the mutagenicities per lethal hit before and after heat treatment, one obtains the following values: am3, 8 x 10^-6 (before) and 10 x 10^-6 (after); am86, 23 x 10^-6 (before) and 55 x 10^-6 (after). The analogous data for directly depurinated DNA in the same experiment were 32 x 10^-6 (am3) and 47 x 10^-6 (am86). It thus appears that, for am3, depurination through BPL is less mutagenic than when directly induced (10 x 10^-6 versus 32 x 10^-6). For am86, the values are approximately equal (55 x 10^-6 versus 47 x 10^-6). (A similar result was obtained in another experiment.) Since am86 is capable of reversion at the third (guanine) position but am3 only at the second (adenine) position of the (TAG) amber codon, these data are consistent with the suggestion that direct depurination involves both adenine and guanine residues but that, in contrast, mainly guanines but some adenines are removed via the BPL pathway.

In a recent study, Drinkwater et al. (2) tested a series of chemical carcinogens for their abilities to produce apurinic sites and their relative mutagenicities in a bacterial assay system. The compounds ranged from simple alkylating agents to the activated forms of arylamines and polycyclic hydrocarbons. A positive correlation was demonstrated for the ordering of the tested compounds with respect to the 2 parameters. However, the quantitative correlation between the production of apurinic sites in vitro and the production of missense mutations in Salmonella typhimurium was rather poor, and a direct causal relationship was therefore considered to be unlikely. However, a direct comparison of depurination in vitro with mutagenicity in vivo is unlikely to reflect the real relationship for the following reasons. (a) The per mm comparisons assume the number of modifications of DNA in vitro and in vivo to be identical. In reality, the activated chemical carcinogens will form adducts in vivo with a variety of other compounds not present in vitro, potentially altering the molar concentration. This bacterial “sieving” effect is likely to vary among different carcinogens. Therefore, comparisons are more appropriate on the basis of final DNA modification than on a molar basis. (b) Because only a single Salmonella tester strain was used, the quantitative results depend on the mutational specificity of the agents tested. (c) DNA modification in vivo elicits DNA repair, which is likely to be different for different kinds of damage. Such effects could readily allow agents producing similar amounts of apurinic sites in vitro to show widely different mutagenicities in vivo. (d) Most importantly, mutagenesis at apurinic sites is strongly SOS dependent. Therefore, the ability of the compound to induce the SOS response may be rate limiting in mutagenesis. This will depend both on the qualities of the lesions and on their quantities, which in turn are influenced by repair processes.

The 8x174 system as used in our study has the potential to circumvent each of these problems and should therefore be well suited for the described methods of analysis. The differential results of aBPDE- and NAAAF- compared to BPL-treated DNA, however, demonstrate the limitations of any simplistic modeling. Mutagenesis by these compounds probably occurs through a number of different pathways. Depurination could play a minor role in mutagenesis by some agents but a major role in mutagenesis by compounds like BPL or aflatoxin B1 where the major product is addition at the N7 position of guanine (25). These studies show the potential available when DNA can be treated in vitro, subsequently modified by a variety of means, and then used to transfect host cells of a chosen repair capacity.

REFERENCES

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