Inactivation of the Thymidine Kinase Gene after in Vitro Modification with Benzo(a)pyrene-Diol-Epoxide and Transfer to LTK- Cells as a Eukaryotic Test for Carcinogens

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ABSTRACT

A recombinant plasmid containing the thymidine kinase (TK) gene (pAGO; 6.36 kilobases) was reacted in vitro with (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, an ultimate carcinogenic metabolite of benzo(a)pyrene. The covalent binding of the metabolite to the circular forms of pAGO was visible by a drastic change in their mobility during agarose gel electrophoresis. The 4% modified DNA was only partially restricted by different endonucleases. Modification and limited restriction were correlated to the biological activity by transfer of the plasmid (TK gene), modified and unmodified, to TK-deficient cells. Upon transfection of mouse LTK- cells with modified plasmid or modified TK gene, no or only a few TK-positive cells were obtained, in contrast to the formation of many colonies after transfection with the unmodified plasmid (gene). Benzo(a)pyrene itself and phenanthrene oxide, a weakly reactive but noncarcinogenic chemical, did not induce this effect. The reactive diol-epoxides of noncarcinogenic benzo(a)acridine and carcino- genic benzo(c)acridine showed a weaker but similar decreasing effect on the formation of TK+ clones. This inhibition of transformation efficiency suggests inactivation of the gene by chemical modification. Our experimental approach challenges the repair capacity of the eukaryotic cell and thus renders the strategy suitable not only as a eukaryotic test for carcinogens but also as a tool for the study of carcinogenesis as aberrant gene expression.

INTRODUCTION

The understanding of the molecular biology of chemical carcinogenesis is of crucial interest to cancer research and has been focused in the last few years on investigations of DNA changes. There is strong evidence that the covalent binding of chemical carcinogens to nucleic acid appears to be a prerequisite for their action. Most carcinogens, including polycyclic aromatic hydrocarbons, require metabolic activation before covalent binding takes place. One of the best-studied compounds is the widely distributed carcinogen benzo(a)pyrene 1. Mixed-function oxidases and hydrolyses cooperatively generate an ultimate carcino- genic intermediate, which is most likely the 7,8-diol-9,10-epoxide of benzo(a)pyrene 2 (10). BP-diol-epoxide3 primarily binds covalently to deoxyguanosine residues in DNA (24).

In vitro, modified DNA exhibits different physical, chemical, and biological properties. The correlation between this binding and its effect on cell regulation, i.e., replication, transcription, and translation, could be the key step in the understanding of the mechanism of carcinogenesis. Also, assays based upon these correlations can be used as screening tests for carci- nogens and thus give more insight into the action of carcinogens.

Up to now, the precise effects of modification on the biological activity of DNA have been investigated predominantly in bacterial systems. In the Ames mutagenicity assay (2), a one-base pair deletion at a —G—G—G— sequence of the hisD gene of Salmonella typhimurium restores the gene activity (9). The molecular mechanisms of BP-diol-epoxide-induced mutations have been studied by modification of plasmids expressing or repressing the Escherichia coli galactokinase (galK) gene. Mutations were identified as inactivation (12) or derepression (13) of the galactokinase activity. Binding of BP-diol-epoxide to φX174 DNA rendered the DNA noninfectious (7).

In contrast, no such system has been reported for mammalian cells. A detailed study of the biological effect of binding to eukaryotic DNA and its implication on gene regulation would elucidate much better the mechanism of chemical carcinogen- esis, especially in view of the recent finding that a 1-base pair mutation converts a normal cellular gene to an active oncogene (18, 21).

In the present study, we describe the in vitro modification of eukaryotic DNA and its biological effect. The new assay combines DNA modification with gene transfer and expression studies in eukaryotic cells. We have modified a plasmid containing the viral TK gene by in vitro reaction with BP-diol-epoxide and have investigated the physical and biological properties of the modified DNA.

MATERIALS AND METHODS

Chemicals. Benzo(a)pyrene was purchased from Sigma Chemical Co. Analytically pure sample of BP-diol-epoxide was obtained by unequivocal chemical synthesis according to the procedure described in Ref. 28. The diastereomeric diol-epoxides 4 and 6 were synthesized as described (5). The diastereomeric diol-epoxides 3 and 5 were synthesized in a manner analogous to that described for the synthesis of the diol-epoxides of benzo(a)anthracene (11). Phenanthrene-5,6-oxide was prepared according to the method described in Ref. 8. Structures of the compounds studied are shown in Chart 1.

Preparation of DNA. The plasmid pAGO was isolated from a pAGO-containing strain of E. coli 1106 (803 rrm/T) (3), and supercoiled DNA was isolated after CsCl centrifugation according to standard procedure. To obtain TK gene, pAGO was digested with PvuII (Boehringer Mannheim), and the 2-kilobase fragment containing the complete TK gene was isolated by agarose gel electrophoresis and purified by phenol:chloroform extraction.

Reaction of DNA with Chemicals. The reaction mixture (10 µl) contained TE buffer (10 mM Tris, pH 7.5:1 mM EDTA), 1 µg of pAGO or TK

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3 The abbreviations used are: BP-diol-epoxide, (±)-7ß,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; TK, thymidine kinase; TE, Tris-EDTA.

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DNA, and varying concentrations of chemicals (in 5 µl of dimethyl sulfoxide), as indicated in "Results." After incubation for 2 hr at 37°, 100 µl of TE buffer were added, and the solution was extracted twice with ethyl acetate and ether. For agarose gel electrophoresis and the transfection experiments, aliquots were taken. Alternative to this procedure, the DNA was precipitated with ethanol and dissolved again in TE buffer.

The percentage of modification was determined according to the method of Pulkrabek et al. (17). Aliquots of the solution were submitted to agarose gel electrophoresis. Fig. 1 shows the electrophoretic mobility of the plasmid without treatment (Lane b) and with treatment with BP-diol-epoxide at 4 and 0.4% modification (actual molar ratio of covalently bound BP-diol-epoxide molecules/ DNA, 250 and 25). A drastic change of the migration pattern upon treatment is best seen with molar ratio 250 (Lane d), where it seems that nicked open circular DNA is migrating faster than is unmodified DNA (Lane b). Two faint bands are left in Lane d which might be modified linear and modified covalently closed DNA, both migrating faster than their normal counterparts (Lanes b and c). At 0.4% modification (molar ratio, 25), a slight difference is still visible and the open circular band migrates faster, but no change of the supercoiled band can be seen. After treatment of the DNA with benzo(a)pyrene or phenanthrene oxide, no difference to the untreated control was detectable (Lane g). Electron micrographs of the 4% modification showed partial unwinding of superhelical DNA and, to a lesser extent, induction of loops and

RESULTS

A recombinant plasmid containing the TK gene, pAGO, was reacted in vitro with ±-7,8,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenz(a)pyrene 2, an ultimate carcinogenic metabolite of benzo(a)pyrene 1. After incubation of the DNA, which consisted of supercoiled and open circular form, with different concentrations of BP-diol-epoxide at 37° for 2 hr, the solution was extracted twice with chloroform or ethyl acetate to remove unreacted chemical. An alternative workup procedure of the modification reaction was precipitation and washing of the treated DNA with ethanol to remove any reactive chemical. The percentage of DNA modification was determined by the method of Pulkrabek et al. (17). Aliquots of the solution were submitted to agarose gel electrophoresis. Fig. 1 shows the electrophoretic mobility of the plasmid without treatment (Lane b) and with treatment with BP-diol-epoxide at 4 and 0.4% modification (actual molar ratio of covalently bound BP-diol-epoxide molecules/ DNA, 250 and 25). A drastic change of the migration pattern upon treatment is best seen with molar ratio 250 (Lane d), where it seems that nicked open circular DNA is migrating faster than is unmodified DNA (Lane b). Two faint bands are left in Lane d which might be modified linear and modified covalently closed DNA, both migrating faster than their normal counterparts (Lanes b and c). At 0.4% modification (molar ratio, 25), a slight difference is still visible and the open circular band migrates faster, but no change of the supercoiled band can be seen. After treatment of the DNA with benzo(a)pyrene or phenanthrene oxide, no difference to the untreated control was detectable (Lane g). Electron micrographs of the 4% modification showed partial unwinding of superhelical DNA and, to a lesser extent, induction of loops and
single-strand regions (data not shown).

In another set of experiments, linear TK gene was isolated from pAGO by digestion with PvuI, separation of the fragments by electrophoresis, phenol:chloroform extraction of the 2-kilobase fragment, and ethanol precipitation. This linear piece of DNA, containing the complete structural gene for TK and its promoter, was also modified with BP-diol-epoxide. In this case, the electrophoretic mobility of modified DNA did not differ from that of the untreated control. The biological properties, however, were different to untreated DNA (see below).

The modification of both the plasmid and the gene itself was apparent by their differential susceptibility to restriction endonuclease. For restriction, we chose the enzymes BglII, EcoRI, and PstI, because their cleavage sites are all localized within the TK gene. Fig. 2 shows agarose gel electrophoresis of the digested DNA, unmodified and modified (4%). Unmodified pAGO yields, after linearization with BglII, a single band of 6.36 kilobases (Lane b), with EcoRI 2 fragments (Lane d) and with PstI 3 fragments (Lane f). In contrast, modified pAGO (Lane h) was restricted only partially with BglII, yielding 50% undigested DNA (compare lanes c and h). With EcoRI, around 50% of the DNA was restricted twice, 50% only once to its linear form (Lane e). That the 2 lower bands in Lane e are migrating faster is due to conformational changes and changes of electrostatic environment of the DNA helix after heavy modification by BP-diol-epoxide. With PstI, almost no digestion took place, and around 20% of the DNA were linearized, the rest being uncleaved (Lane g). These digests were repeated overnight and with new enzymes to make sure that the observed difference was not due to inactivated enzyme. Any free carcinogen had been removed prior to the digestions by extensive extraction with different organic solvents. The DNA was precipitated with ethanol prior to digestion to remove organic solvents. The marked insensitivity of modified plasmid toward restriction was also found for modified linear TK gene (data not shown).

The modification pattern was correlated to the biological activity of the TK gene by determination of the transformation efficiency of mouse L-cells deficient in TK activity after gene transfer with pAGO (and isolated TK gene). For the transfection assay, we chose the calcium phosphate method of co precipitation. Upon transfection of LTK cells with the modified plasmid and selection of stable transformants in hypoxanthine-aminopterin-thymidine medium, no TK-positive cells (at the 4 and 0.4% modification) were obtained, in contrast to the formation of many colonies after transfection with the same amount of unmodified gene. Table 1 shows the result of the transfection experiment. Treatment of pAGO with benzo(a)pyrene or phenanthrene oxide had no inhibiting effect on the transformation efficiency (formation of as many TK+ clones as with untreated control DNA). Treatment of the DNA with BP-diol-epoxide, however, either at 4% modification or at 0.4%, did not yield any clone at all under the standard incubation conditions.

Table 1 shows the result of the transfection experiment. Treatment of pAGO with benzo(a)pyrene or phenanthrene oxide had no inhibiting effect on the transformation efficiency (formation of as many TK+ clones as with untreated control DNA). Treatment of the DNA with BP-diol-epoxide, however, either at 4% modification or at 0.4%, did not yield any clone at all under the standard incubation conditions. At a concentration of 0.04%, the DNA exhibited complete activity; no difference to the control DNA was found. The inactivation of linear TK gene versus circular pAGO was markedly lower, though the transformation efficiency was still significantly decreased to one-fourth compared to the control. A clear dose response for the gene modification by the carcinogen was found.

To investigate whether the DNA had been taken up and integrated, we modified the gene for HBsAg and cotransfected this with TK gene. The mixture of hypoxanthine-aminopterin-thymidine-resistant colonies was checked for the presence of HBsAg DNA by DNA hybridization. Fig. 3 shows the southern blot of modified DNA. DNA with 0.4% modification (Slot C) shows a strong band which comigrates with the gene for HBsAg (Slot E). Positive control (Slot E) is DNA of a cell line continuously expressing HBsAg (20).

To investigate the dose range of the system, the concentration range between 4 and 0.4% modification was studied in more detail. The action of BP-diol-epoxide was compared to the action of diol-epoxides derived from benzo(a) - and - (c)acridine to test whether the system would detect all diol-epoxides as mutagenic compounds. This would have been expected by the bay-region theory; the Ames test, however, gave different results (27). In
general, the number of TK\(^+\) clones decreased with increasing amounts of reactive chemical (Chart 2). There was a slight difference in the biological effect of the different diol-epoxides. Although the absolute number of TK\(^+\) clones varied from experiment to experiment, the relative amount of clones was reproducible and constant. The strongest carcinogen, BP-diol-epoxide, provoked the most rapid decrease in TK\(^+\) clones already at a concentration of 0.3 ng of chemical/10 ng DNA (mol/mol, 840) under the given incubation conditions. The diol-epoxides of benzo(a)acridine showed an inhibiting effect on the DNA at 0.8 ng/10 ng of DNA (mol/mol, 1200). The dose range in which all active carcinogens could be detected was found to be between 0.3 and 2 ng of chemical/10 ng of DNA (mol/mol, 450 to 3000). Benzo(a)pyrene and phenanthrene-5,6-oxide were inactive, even at a 10-fold higher concentration (10 ng of chemical/10 ng of DNA) (mol/mol, 15,000).

**DISCUSSION**

The *in vitro* reaction of carcinogens like BP-diol-epoxide, the ultimate carcinogenic metabolite of benzo(a)pyrene, with DNA and its correlation with the biological activity of the modified gene gives more insight into the action of carcinogens on DNA and their effect on gene expression. Several studies on *in vitro* incubation of calf thymus DNA or \(\phi X\) 174 DNA with BP-diol-epoxide have been reported, and the irreversible modification has been interpreted as covalent intercalative binding (4). Our results confirm these data by showing that plasmid pAGO after incubation with BP-diol-epoxide drastically changes its mobility on agarose gel electrophoresis (Fig. 1). In contrast, DNA incubated with benzo(a)pyrene migrates like untreated DNA. Although benzo(a)pyrene can intercalate into DNA, it is not able to form a covalent bond. This demonstrates also that intercalation alone is not sufficient for the observed effects (see below). The influence of the covalent binding on DNA structure depends on the incubation conditions and can vary from unwinding of supercoiled DNA to induction of new supercoils (1). Agarose gel electrophoresis showed both effects, since both the open and the closed circular form of pAGO collapse to a single band after modification (Fig. 1, Lane c).

The question that evolves is whether there are special sites (hot spots) in DNA for chemical modification *in vitro*. Sequence specificity *in vitro* has been observed for modification of DNA with activated forms of aflatoxin B\(_1\) (14). Preferred binding of BP-diol-epoxide with guanine *in vivo* has been described (17, 24). We found, in our experiments, a differential reactivity of modified DNA toward different restriction enzymes: the endonuclease BgIII, with one recognition sequence, AGATCT, cleaved 50% of the modified DNA (Fig. 2, Lane c); EcoRI, with 2 recognition sequences, GAATTC, linearized 100% of the modified DNA and cut 50% at the second site (Fig. 2, Lane e); and Psfl, with 3 recognition sequences, CTGCAG, could linearize only 20% of modified DNA (Fig. 2, Lane g). All of these enzymes cleave between G and A, but the recognition sequence of Psfl contains an additional pair of GC. This could indicate increased modification at GC-rich positions.

The modification pattern of plasmid pAGO containing the TK gene was correlated directly to its biological activity by gene transfer to mouse L-cells that were deficient in TK. Several new methods for gene transfer into eukaryotic cells have been reported (15, 19). We chose the calcium phosphate method, since very low amounts of DNA are already efficient. Upon transfection with the modified gene (plasmid), fewer/no TK cells were obtained, in contrast to the formation of many colonies after transfection with the unmodified gene (plasmid). There was a marked contrast in the activity of modified linear TK gene to modified circular plasmid containing the TK gene (Table 1); after reaction with the same concentration of chemical/DNA, the transforma-
MODIFICATION OF THE TK GENE WITH BP-DIOL-EPOXIDE

...of modified circular plasmid was inhibited more than with modified linear TK gene. It is not yet clear whether circular supercoiled DNA is modified in greater extent or whether the modification occurs at different sites which would be more effective in the inactivation assay. Also, changed conformation could influence a step in the integration process. In any case, the in vitro reaction with supercoiled DNA is more similar to the in vivo situation in which active chromatin is involved.

A bacterial system consisting of modification of phiX174 DNA by BP-diol-epoxide and inhibition of DNA infectivity of E. coli spheroplasts was reported previously (7). For eukaryotic cells, however, no such system has been reported, except in those assays in which carcinogens were directly incubated with mammalian cells. We chose the TK gene for assay, since the transfer of TK gene to LTK- cells (16) has been used as a standard system for gene transfer and expression studies in the last few years. Moreover, a single defined gene is thus involved, the activation or inactivation of which can easily be determined by colony screening. Our results suggest inactivation of the gene by chemical modification. Complete inhibition of colony formation could be also performed using bacterial assays, e.g., modification of the ampicillin resistance gene in a plasmid and following the amount of reactive chemical needed to see an inhibitory effect (compare with Ref. 27).

This cannot only be studied in mouse cells but also in human TK- cells. How much the system will tell us about chemical carcinogenesis as a changing process of gene activation and inactivation will be seen in further studies.

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