Arrest of DNA Elongation by DNA Polymerases at Guanine Adducts on 4-Hydroxyaminoquinoline 1-Oxide-modified DNA Template

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ABSTRACT

In vitro modification of M13 phage single-stranded DNA with 4-hydroxyaminoquinoline 1-oxide (4HAQO) resulted in four kinds of adducts: three guanine adducts, QG, QGn, and QGm; and one adenine adduct, QA. The reaction products were analyzed on a DNA-sequencing gel. DNA elongation by DNA polymerase I was arrested at putative guanine adducts on the template in three ways: at one base prior to guanine; at positions opposite to guanine; and at one base beyond guanine. Similar patterns of elongation arrest were also obtained with the mammalian DNA polymerases \( \alpha \) and \( \beta \). In contrast to guanine adducts, the adenine adduct, QA, might lack the capacity to arrest DNA chain elongation by DNA polymerases.

INTRODUCTION

4HAQO is a proximate form of the potent carcinogen, 4-nitroquinoline 1-oxide (16). Binding of 4HAQO to nucleic acids is achieved by the action of some species of aminopyrine-TRNA synthetase via an active intermediate, aminopyrine-4HAQO (17). Four kinds of quinoline:purine base adducts have been identified chromatographically either in vitro or in vivo; one 4HAQO-adenine adduct (QA) and three 4HAQO-guanine adducts (QG, QGn, and QGm) (18). The structures of QA and QG are currently determined as 3-(N\(^x\)-adenyl)-4-aminoquinoline 1-oxide (18) and N-(guanyl-8-yl)-4-aminoquinoline 1-oxide, respectively. The structures of QG and QGn are still under investigation. Like UV-induced pyrimidine dimers, these 4HAQO adducts can be excised effectively in mammalian cells as well as in Escherichia coli and repaired correctly by an excision repair mechanism (4, 15). The replication of DNA containing damages left unrepaired may be an important process for inducing mutation or initiation of carcinogenesis. Although 4NQO provides an excellent model for studying carcinogenesis (8), there is little information concerning the replication of DNA-carrying 4HAQO adducts. In the present work, we studied the synthesis of DNA on a 4HAQO-modified M13 phage DNA template, using DNA polymerase I and calf thymus DNA polymerases \( \alpha \) and \( \beta \), by analyzing the products on sequencing gel.

MATERIALS AND METHODS

Chemicals. [methyl-\(^{3}H\)]dCTP (20 Ci/mmol) and [\( \alpha ^{-32}P \)]dATP (800 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Unlabeled deoxyribonucleoside triphosphates were obtained from Boehringer/Mannheim, Federal Republic of Germany. \([\text{H}]\)4HAQO was a generous gift from Dr. Y. Kawazoe, Nagoya City University.

Enzymes and DNAs. DNA polymerase I Klenow fragment of Es. coli was obtained from P-L Biochemicals, Inc., Milwaukee, WI. Calf thymus DNA polymerases \( \alpha \) and \( \beta \) were purified as described previously (19). To prepare the template primer, M13mp7 DNA (0.5 \( \mu \)g/ml) was mixed with the same volume of synthetic primer, 5'CCCAGTCACGACGTT3' (0.1 M\( \mu \)g/ml; obtained from P-L Biochemicals), heated at 55° for 20 min in the presence of 50 mM NaCl and 10 mM Tris-HCl (pH 7.5), and cooled slowly. HDH-1 was purified from calf thymus as described by Plank and Wilson (13).

Modification of M13 DNA with 4HAQO. M13 phage DNA was treated with 4HAQO using seryl-AMP, which was synthesized according to the method of Berg (1). The reaction mixture (0.5 ml) contained 0.2 or 1 mM 4HAQO, 2 mM seryl-AMP, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0), and 100 \( \mu \)g of M13 DNA. Reaction was carried out at 37° for 15 min. The unbound carcinogens were removed by successive extractions with phenol and ether. The DNA was precipitated by ethanol, and the precipitate was washed once with ethanol.

The extent of the binding was measured using 0.025 ml of the reaction mixture containing \([\text{H}]\)4HAQO (28 Ci/mol) and 5 \( \mu \)g of M13 DNA in a parallel experiment. The radioactivity in DNA was measured as described previously (18).

Chromatographic Analysis of 4HAQO:Base Adducts. \([\text{H}]\)4HAQO-modified DNA was mixed with 1 mg of unlabeled 4HAQO-modified DNA and hydrolyzed in 0.1 ml of 0.2 N HCl at 70° for 1 hr. The acid hydrolysates were subjected to paper chromatography with the following solvent systems: ethanol:water (4:1, v/v); and n-butyl alcohol/formic acid:water (77:10:13, v/v). The relative ratios of the adducts (QG, QA, QGn, and QGm) were estimated as reported previously (18).

DNA-sequencing Reactions. Deoxyribonucleotide-sequencing reactions were routinely carried out according to the method of Sanger et al. (14) using M13mp7 DNA primed with oligodeoxynucleotide and \( \alpha^{-32}P\)labeled deoxyribonucleoside triphosphates. Four reaction products of adenine, guanine, thymine, and cytosine (14) were usually run as sequence controls for the reaction products on modified templates. In some experiments, the sequencing reactions were also done using modified templates as indicated.

DNA Polymerase Reaction on the Modified Templates. The reaction mixture (10 \( \mu \)l) for DNA polymerase I (Klenow fragment) contained 0.5 \( \mu \)g M13 mp7 DNA template primer; 5 mM Tris-HCl (pH 7.6); 40 mM NaCl; 1 mM dithiothreitol; 5 mM MgCl\(_2\); 40 \( \mu \)M concentrations each of dTTP, dGTP, and dCTP; 5 \( \mu \)Ci of \([\alpha ^{-32}P]dATP\) (6.25 \( \mu \)M); 1 \( \mu \)g of bovine serum albumin; and 0.2 unit of enzyme. Incubation was carried out at 25° for

1 This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: 4HAQO, 4-hydroxyaminoquinoline 1-oxide; 4NQO, 4-nitroquinoline 1-oxide; 4NO, 4-nitroquinoline 1-oxide; HDH-1, helix-distabilizing protein 1; RF1, replicative form I.
4 M. Tada, K. H. Kohda, and Y. Kawazoe. Structure of QA, one of the DNA adducts formed in cells treated with 4-nitroquinoline 1-oxide, manuscript in preparation.
5 QA and QGn were identified as novel guanine adducts named QA and QGn.

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20 min; then, unlabeled 0.5 mM dATP was added to the final concentration of 50 μM. Incubation was carried out for another 20 min (chase incubation); then the reaction was terminated by adding 1 μl of 0.2 M EDTA and 3 μl of 0.3% bromophenol blue and xylene cyanol in formamide (dye mixture). The mixtures were heated at 100° for 10 min immediately before electrophoresis.

The reaction mixture for DNA polymerase α (10 μl) contained 1 μg of M13mp7 DNA template primer; 1 μg of HDP-1; 50 mM Tris-HCl (pH 7.5); 5 mM MgCl₂; 4 mM dithiothreitol; 40 μM concentrations each of unlabeled dGTP and dTTP; 5 μCi of [α-32P]dATP (6.25 μM); 1 μg of bovine serum albumin, and 2 μl of 10S DNA polymerase α of calf thymus (1 unit). Incubation was carried out at 30° for 40 min followed by another 40 min of chase incubation.

For the reaction of DNA polymerase β, 100 mM NaCl was added to the mixture for DNA polymerase α, and 0.8 unit of DNA polymerase β from calf thymus was used.

Electrophoresis. After boiling for 10 min with the dye mixture, samples were electrophoresed at 1400 V for 60 min on 8% polyacrylamide gel (30 x 40 x 0.05 cm) prepared in 0.05 M Tris-boric acid (pH 8.5) containing 7 M urea and 1 mM EDTA. After electrophoresis, the gel was autoradiographed with a Fuji RX film (Fuji Photo Film Co., Japan), using an intensifying screen at −70°, overnight.

RESULTS

Decrease in the Template Activity of M13 DNA by Adducts with 4HAQO. The single-stranded circular DNA of M13mp7 phage was modified with 4HAQO and annealed with oligodeoxynucleotide as described in "Materials and Methods." By the modification, the template activity of M13 DNA for DNA polymerase I of E. coli was drastically decreased; to one-thirtieth of the control M13 DNA when one adduct was formed at every 165 deoxynucleotides; and to one-sixteenth when one adduct was formed at every 74 deoxynucleotides (Chart 1A). Decrease in the template activity was also observed with calf thymus DNA polymerase α but to a lesser extent (Chart 1B). Analysis of acid hydrolysate of 4HAQO-modified M13 DNA by paper chromatography showed that 77.3% of all of the adducts were guanine adducts (QG, QGh, and QGm) and the remainder (22.7%) was the adenine adduct (QA). Among guanine adducts, QGh was most prominent, amounting to almost one-half of all of the adducts formed (47.3%), while QG was 16.4% and QGm was 13.7%.

Reaction Products on the 4HAQO-modified M13 DNA. The reaction products formed by DNA polymerases on 4HAQO-modified M13 DNA were analyzed by sequencing gel electrophoresis. The exact positions of elongation arrest were determined by comparing the arrest bands with lanes of dideoxynucleotide-sequence reactions using untreated DNA according to the method of Sanger et al. (14). As shown in Fig. 1, the arrest bands occurred exclusively at or near guanine bases on the template. The positions of these bands were further confirmed by dideoxynucleotide-sequencing reactions with the 4HAQO-modified DNA template; elongation arrest was visualized on autoradiograms as unusual bands occurring commonly in all adenine, guanine, cytosine, and thymine lanes (data not shown). Assuming that all these arrest bands were due to guanine adducts, they can be classified into 3 categories, as summarized in Chart 2: at one nucleotide prior to guanine; at the position opposite to guanine; and at one nucleotide beyond guanine. It is also seen in Fig. 1 that these arrested products remained at the same position after chasing for 90 min in the presence of an excess amount of unlabeled dATP. This result indicates that the 3' hydroxyl ends of the arrested products could hardly be used as primers for further elongation by polymerase.

Eukaryotic DNA polymerase α shows a lower processibility of elongation reaction on natural or synthetic templates compared with E. coli DNA polymerase I (6). This may be due, at least in part, to the specific arrest of DNA polymerase α at the "arrest sequence" such as the guanine cluster (20). This "natural" arrest could be partly overcome by adding single-stranded DNA binding protein, HDP-1 (2). The processibility of the reaction of 10S DNA polymerase α from calf thymus was also improved by adding HDP-1 (data not shown), although the strong arrest bands just before the guanine cluster were still prominent (Fig. 2, Lanes 1 and 2). Analysis of the reaction products by DNA polymerase α on the sequencing gel revealed that the pattern of elongation arrest was similar to that observed with E. coli DNA polymerase I.

In Fig. 3, the products of calf thymus DNA polymerase β were shown. It was evident that DNA polymerase β also stopped at one nucleotide prior to putative guanine adducts, although faint arrest bands were sometimes observed at the positions opposite...
guanine. For an unknown reason, the reaction products of DNA polymerase β were too short to analyze beyond position 25 (Fig. 3).

In Chart 2, the arrest sites of 3 enzymes are summarized: (a) all 3 enzymes were strongly arrested at positions 6 and 9 at one base prior to guanine adducts of positions 7 and 10. The guanine adducts at positions 31, 32, and 35 also showed a similar pattern; (b) at guanine adducts at positions 14, 26, 42, 47, and 50, the elongation by E. coli enzyme arrested at exactly the opposite positions of the adducts. This second type of arrest was also observed with calf thymus DNA polymerase α at positions 14 and 26. The third type of arrest, one nucleotide beyond adduct, was observed at position 15 with both E. coli DNA polymerase I and calf thymus DNA polymerase α, and at position 40 with E. coli enzyme. The arrest bands at positions 13, 14, 23, 24, 25, and 38 could not be classified definitely because these bands were at guanine clusters on the template. They might belong to either the first type, second type, or a mixture of the 2 types.

On the other hand, no arrest bands which could be attributed to adenine adducts were observed in this system. The arrest bands at adenine positions 22, 30, 34, 40, and 45 may be due to the guanine adducts at the positions next (5') to these bands on the templates, because other adenine residues, e.g., positions 17 and 22, did not arrest the reaction. However, the possibility of arrest at the opposite position of adenine adducts (second type) could not be completely ruled out.

DISCUSSION

In this report, we demonstrated that 4HAQO:base adducts blocked DNA elongation in an unusual way. The arrest band was always observed at every guanine base on the template. However, the modes of arrest at these putative guanine adducts differed from one to the other. The arrest modes could be classified into 3 categories: at one nucleotide prior to guanine; at the position opposite to guanine; and at one nucleotide beyond guanine. These arrest modes might reflect the different natures of adducts. It has been established that 3 kinds of guanine adducts, QG, QG5, and QG6, are formed when nucleic acids are modified with 4HAQO in vitro or when cells are treated with 4HAQO in vivo (18). At present, however, it is not possible to correlate these 3 kinds of guanine adducts to 3 different arrest modes, since we cannot estimate which kind of adduct is most frequent at given guanine bases on DNA sequence.

The arrest modes by 4HAQO-guanine adducts are different from those of pyrimidine dimers induced by UV irradiation at which the elongation terminates one nucleotide before the first (3') pyrimidine in a dimer (11). We also confirmed this using the M13 DNA system (data not shown). Rather, the 4HAQO-guanine adducts resemble those of another chemical carcinogen, 2-aminofluorene. Recently, Moore et al. (12) showed that elongation by E. coli DNA polymerase I arrests one nucleotide before acetylated 2-aminofluorene adducts at C-8 of guanine but incorporates an additional nucleotide opposite the deacetylated aminofluorene adducts. It is conceivable, therefore, that the nature of the adducts may influence the template-enzyme interaction, resulting in various stopping patterns.

In the present study, it is obvious that the arrest bands were much more intense at the —TGC— sequence than the —GG— or —GGGG— sequences on the template. A model experiment using polydeoxyguanulate-polydeoxycytidylate duplex and polydeoxyguanulate-deoxycytidylate alternating copolymer showed that 4HAQO adducts were formed 5 times as efficiently in the latter as in the former (data not shown). Therefore, different intensities of arrest bands might reflect the different efficiencies in adduct formation of guanine bases depending on neighboring base sequences.

It is remarkable that neither E. coli DNA polymerase I nor calf thymus DNA polymerase α stopped at adenine adducts which accounted for 23% of the total 4HAQO:base adducts on M13 phage single-stranded DNA. Although some arrest bands were observed at the positions opposite adenine (Chart 2), these bands could be ascribed to adjacent (5') guanine adducts on template.

Mutations of T4 phage induced by 4NQO have been shown to be largely G → A-T transition, and alteration in A-T site is rather rare (5). The bias in favor of guanine as a target of point mutation by 4HAQO has been attributed to the higher rate of modification of guanine than of adenine (9). The present results suggesting that the adenine adduct does not block DNA elongation may raise another possibility; the 4HAQO-adenine adduct could be read efficiently as adenine by polymerases in an almost normal way, and consequently the error frequency is rather low. The 3'-end of DNA arrested at guanine adducts must be further elongated to fill gaps by an unknown process which might be error prone, leading to mutagenesis or carcinogenesis.

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REFERENCES


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Fig. 1. Analysis of reaction products by E. coli DNA polymerase I with the 4HAQO-modified template. Reactions were performed with E. coli DNA polymerase I Klenow fragment using 4HAQO-modified DNA. Reaction products were electrophoresed as described in "Materials and Methods." Lanes 1 to 3, 1 adduct per 165 deoxynucleotides; Lanes 4 to 5: 1 adduct per 74 deoxynucleotides; Lanes 6 to 9, no modification control. Chasing reactions were performed for the indicated times in the presence of 50 nM unlabeled dATP. The base sequence of template DNA was deduced from parallel runs of sequencing reactions.

Fig. 2. Analysis of reaction products by calf thymus DNA polymerase α with 4HAQO-modified template. Reactions for DNA polymerase α were carried out as described in "Materials and Methods." Two µl (Lanes 1 and 3) and 5 µl (Lanes 2 and 4) of 10S DNA polymerase α (1 unit/µl) from calf thymus were used. Lanes 1 and 2 are control runs with unmodified DNA. Lanes 3 and 4 show the patterns of products with 4HAQO-modified template (1 adduct per 74 deoxynucleotides). The base sequence of the template strand is shown.

Fig. 3. Analysis of reaction products by calf thymus DNA polymerase β with 4HAQO-modified template. Reactions for DNA polymerase β were performed as described in "Materials and Methods" using control DNA (Lanes 1 to 3) and 4HAQO-modified DNA (Lanes 4 to 6). Three different fractions of DNA polymerase β were used: first peak (Lanes 1 and 4); second main peak (Lanes 2 and 5); and third peak (Lanes 3 and 6) from a native DNA-cellulose column (7). Approximately 1.5 units of DNA polymerase β were used in each reaction. Lanes 7 to 10 are DNA sequencing controls. Other conditions including electrophoresis are described in "Materials and Methods."
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