Digestion of damaged DNA by the T7 DNA polymerase-exonuclease

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We have investigated the 3′–5′-exonuclease activity of phage T7 DNA polymerase for its usefulness as an approach for the detection of lesions in DNA. Unlike the T4 DNA polymerase-exonuclease, which is commonly used to map the position and frequency of lesions in very small DNA fragments, T7 DNA polymerase-exonuclease is able to hydrolyse almost completely the large fragments from *Kpn*I-restricted mammalian DNA. However, we found that the exonuclease was also able to hydrolyse DNA containing several kinds of lesions: cyclobutane

pyrimidine dimers, thymine glycols, and mono-adducts of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen and 5′-methylisopsoralen. Modifications of the reaction conditions did not significantly alter the extent of hydrolysis. These properties distinguish the T7 DNA polymerase-exonuclease from the T4 DNA polymerase-exonuclease and make the T7 DNA polymerase-exonuclease unsuitable for detecting several types of lesions in DNA.

INTRODUCTION

We sought to develop a new, broadly applicable assay for the presence of DNA lesions, induced *in vivo*, in mammalian genes. The approach would be to use a single-strand specific exonuclease that will completely digest lesion-free restriction fragments, but whose progression will be blocked at DNA lesions, resulting in only partial digestion of fragments containing lesions. The undigested fragments can then be collected on a nitrocellulose membrane and detected by hybridization with a radioactive probe complementary to one end of a single-stranded DNA fragment of interest. The choice of probe location would determine which of the two DNA strands would be examined. Since the DNA fragments detected would be only those containing a lesion, it should be possible to discern differences between samples with very low lesion frequencies. Such sensitivity is not possible with currently used assays that rely on endonucleases to specifically incise DNA at the sites of lesions (e.g. [1]).

The 3′–5′-exonuclease activity of phage T4 DNA polymerase is inhibited to a substantial degree by lesions in DNA produced by u.v. C radiation [2], *N*-acetoxy-2-acetylaminofluorene [3], psoralens plus u.v. A [4] and the *N*²-guanine adduct of 4-nitroquinoline 1-oxide [5]. Thus, the position and frequency of lesions in a homogeneous population of 5′-end-labelled single-stranded DNA have been determined by electrophoretic resolution, in a gel, of the small fragments remaining after incubation with T4 DNA polymerase in the absence of deoxynucleotide triphosphates (e.g. [2]). However, this enzyme is not suitable for detecting lesions in large DNA fragments [6]. We found that the 3′–5′-exonuclease activity of T7 DNA polymerase, which can digest both single- and double-stranded DNA [7], rendered *Kpn*I-restricted mammalian DNA almost completely acid soluble. However, the T7 DNA polymerase-exonuclease was also able to bypass many different types of lesions, as documented in this report.

MATERIALS AND METHODS

Exonuclease reactions

Phage T4 DNA polymerase and native phage T7 DNA

polymerase holoenzyme were purchased from United States Biochemicals (Cleveland, OH, U.S.A.). The T4 exonuclease reaction was performed as described [2]. The T7 exonuclease reaction was performed in 20 mM Tris/HCl (pH 7.5 for double-stranded substrates, pH 7.0 for single-stranded substrates), 10 mM MgCl₂ and 5 mM dithiothreitol. The absence of endonuclease or exonuclease in buffers was confirmed by incubation with supercoiled plasmid DNA or linear [³H]DNA respectively. T7 DNA polymerase was confirmed free of endonuclease by incubation with supercoiled plasmid DNA. Unless otherwise stated, all exonuclease reactions were run to completion (at least 6 h). Products were precipitated by mixing with 10 µg of carrier DNA and ice-cold 5% (w/v) trichloroacetic acid and assayed for radioactivity by scintillation counting.

U.v. irradiation, OsO₄ and psoralen treatments

Samples (1 ml) of DNA (5 µg/ml) were irradiated with u.v. C as described [8].

Linear pZH-2 plasmid was treated with 0.5% OsO₄ as described [8] to produce approx. 0.6 thymine glycol lesion per kbp, or about 1.5 per strand. Treated and undamaged control plasmid were 5′-end-labelled with ³²S using T4 polynucleotide kinase (Bethesda Research Laboratories kit).

Linearized plasmid was incubated with 17 µM or 34 µM [³H]4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) (HRI Associates, Concord, CA, U.S.A.) for 30 min at 37 °C, then irradiated at 4 °C with 240 or 160 J/m² u.v. A respectively, as described [9]. Unreacted HMT was removed by chloroform extraction. Cross-linked DNA was separated from DNA without cross-links by alkaline isopycnic CsCl centrifugation [9]. DNA containing only mono-adducts was dialysed, concentrated and renatured by incubation overnight at 55 °C.

Linearized plasmid or restricted Chinese hamster ovary (CHO) cell DNA was incubated with 16 or 32 µM [³H]5-methylisopsoralen (5-MIP) (HRI Associates), then irradiated with up to 29 kJ/m² u.v. A at 4 °C. Unbound 5-MIP was removed by chloroform extraction. Specific radioactivities of [³H]HMT (14.5 Ci/mmol) and [³H]5-MIP (6 Ci/mmol) were used to estimate adduct frequencies.

Abbreviations used: u.v. A, ultraviolet A; u.v. C, ultraviolet C; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; 5-MIP, 5-methylisopsoralen; CHO, Chinese hamster ovary; CPD, cyclobutane pyrimidine dimer; *DHFR*, dihydrofolate reductase gene.

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RESULTS AND DISCUSSION

Hydrolysis of undamaged DNA

Incubation of *Kpn*I-restricted CHO [3 H]DNA (average size 20–25 kb) with T4 DNA polymerase, at a ratio of up to 200 units of enzyme per μ g of DNA for 2 h at 37 °C (completion), rendered up to 80 % of the DNA acid-soluble. Similar results were obtained when the enzyme was added at several times during the reaction. A 'background' level of 20 % undigested DNA is clearly unacceptable in an assay to be used to detect a few percent of damaged DNA fragments in a population of undamaged fragments. However, using the T7 DNA polymerase, at an enzyme-to-DNA ratio of 10 units/ μ g of DNA, we could reliably digest 95–99 % of single- or double-stranded *Kpn*I-restricted CHO or linearized plasmid DNAs to acid solubility within 6 h. We then assessed the inhibition of T7 DNA polymerase-exonuclease by lesions produced by several different DNA-damaging agents, using an enzyme-to-DNA ratio of 10 units/ μ g of DNA throughout, unless otherwise specified.

U.v. C radiation

Irradiation of DNA with u.v. C reportedly inhibits hydrolysis by T7 DNA polymerase-exonuclease [10]. To determine if the exonuclease is blocked by u.v.-induced cyclobutane pyrimidine dimers (CPDs), we first determined the frequency of CPDs in a 14 kb *Kpn*I fragment of the dihydrofolate reductase gene (*DHFR*) in CHO DNA, using T4 endonuclease V to incise DNA at the sites of CPDs [11]. Another sample of the same DNA was used as substrate for T7 DNA polymerase-exonuclease. The reaction was run to completion and we detected the undigested DNA fragments, bound to a nitrocellulose membrane (slot blot), with a 32 P-labelled DNA probe complementary to the 5' end of the *Kpn*I fragment (pZH-4) [12]. By comparing the intensity of the signal with that obtained with an undigested control, and assuming a Poisson distribution of lesions, we calculated the frequency of exonuclease-blocking lesions present in one strand of the *DHFR* fragment. At u.v. doses of 5, 10 and 20 J/m 2 we

found the exonuclease-blocking lesion frequency to be 0.0084, 0.007 and 0.035 per DNA fragment respectively, or 0.9 %, 0.5 % and 1.4 % of that of the CPD frequency respectively (results not shown).

The very slight inhibition of T7 DNA polymerase-exonuclease caused by u.v. C radiation indicates that only a minor fraction of the principal lesions induced [CPDs and pyrimidine (6-4) pyrimidone photoproducts] can block the exonuclease, and/or a minor u.v.-induced lesion, such as thymine glycol [13], may block the enzyme efficiently. To test specifically for blockage by thymine glycol we treated linearized plasmid (pZH-2, 4.5 kb) with OsO $_4$ to produce thymine glycols at a predicted frequency of 1.5 per strand [8]. This DNA was then 5'-end-labelled with 35 S and digested with T7 DNA polymerase-exonuclease. Only 10 % of the 35 S label in the OsO $_4$ -damaged DNA remained acid-insoluble.

At the u.v. C doses described above thymine glycol would be expected to occur at a frequency of far less than 1 % of the CPD frequency [8]. Therefore, it is most likely that the exonuclease is blocked, to various degrees, by subsets of one or more kinds of u.v. C-induced lesions, possibly dependent upon the nucleotide sequence context of the lesion.

Psoralen plus u.v. A

HMT produces both mono-adducts and interstrand cross-links in DNA upon exposure to u.v. A (for review see [14]). CHO *Kpn*I-restricted DNA treated with [3 H]HMT (17 μ M) and various doses of u.v. A (200–1200 J/m 2) to yield 0.3–0.8 adduct per kbp retained most of the acid-insoluble label after incubation with T7 DNA polymerase-exonuclease. This inhibition is probably due to interstrand cross-links (see results with mono-adducts below). However, we were specifically interested in the inhibition by psoralen mono-adducts, since a sensitive assay for cross-links was already available [15].

Inhibition in plasmids containing only mono-adducts of [3 H]HMT (see the Materials and methods section) was measured by retention of acid-insoluble label after incubation with the enzyme. We found that 0.7 adduct per strand in linearized pZH-1 (13.2 kb) inhibited the exonuclease activity 21 % when the plasmid was double-stranded and 11 % when the plasmid was single-stranded. Similarly, 1 adduct per strand in pZH-2 (4.5 kb) inhibited the enzyme by 20 % and 7 % in double- and single-stranded DNA respectively. Doubling the concentration of enzyme in the reaction mixture from 10 to 20 units/ μ g of DNA did not significantly increase the extent of digestion.

We also examined blockage of the T7 DNA polymerase-exonuclease by a monofunctional psoralen, 5-MIP. As shown in Figure 1, the inhibition increases with increasing adduct frequency. Doubling the concentration of enzyme in the reaction from 10 to 20 units/ μ g of DNA did not affect the release of label (tested at an adduct frequency of 2.2 per kbp). The mean size of *Kpn*I-restricted CHO DNA is about 20–25 kbp, so that almost all of the fragments contained more than one 5-MIP adduct. It is likely that the increased inhibition of the exonuclease in response to increased adduct frequency is due to adduct frequencies being much greater than one per fragment, so the fraction of all adducts bypassed in any strand decreases with increasing adduct frequency. In a small plasmid substrate, 1.2 [3 H]5-MIP adducts per kbp in linear pZH-2 (2.7 adducts per strand) inhibited the exonuclease slightly. Only 5 % of the [3 H]5-MIP remained acid soluble after exonuclease digestion, compared with approx. 50 % in the CHO DNA containing this level of [3 H]5-MIP adducts (Figure 1).

We next examined the kinetics of digestion of control DNA and the release of [3 H]HMT mono-adducts from pZH-2 over a

Figure 1 Inhibition of T7 DNA polymerase-exonuclease by 5-MIP in CHO DNA

Results are shown of three independent experiments to test the blockage of T7 DNA polymerase-exonuclease by 5-MIP adducts. *Kpn*I-restricted CHO DNA was treated with 16 μ M or 32 μ M [3 H]5-MIP and different doses of u.v. A, as described in the Materials and methods section. Adducts per kbp were calculated based on the specific radioactivity of [3 H]5-MIP.

Figure 2 Time course of T7 DNA polymerase-exonuclease digestion of ^{35}S -labelled undamaged linear plasmid DNA, or linearized pZH-2 DNA with an average of 1 $[^3\text{H}]$ HMT mono-adducts or 2.7 $[^3\text{H}]$ 5-MIP adducts per strand

Reactions were stopped by chilling on ice and addition of 20 mM EDTA.

6 h time course. The kinetics of release of $[^3\text{H}]$ 5-MIP adducts was essentially identical to the digestion of undamaged DNA, while the release of $[^3\text{H}]$ HMT mono-adducts proceeded at a slightly slower rate (Figure 2).

Reaction conditions

Varying the pH from 7.0 to 8.0 did not greatly affect the extent of digestion of undamaged DNA produced by the exonuclease (see also [7]). It also did not affect the inhibition of the enzyme by thymine glycol or HMT mono-adducts in plasmid, or 5-MIP adducts in CHO DNA. Raising the reaction temperature from 37 °C to 41 °C similarly reduced the exonuclease activity on both undamaged and 5-MIP-treated plasmid.

We found the exonuclease activity to be very sensitive to Mg^{2+} concentration in the range 0.16–0.22 mM, and inhibition of the enzyme by 5-MIP adducts in plasmid was similarly affected by Mg^{2+} concentration. Replacing Mg^{2+} with Mn^{2+} had no significant effect.

T7 DNA polymerase-exonuclease is inhibited by Na^+ ions [7]. Over the range 0–100 mM Na^+ we found that the digestion of undamaged DNA and the release of HMT mono-adducts from plasmid were similarly inhibited.

Other exonucleases

We considered the use of other exonucleases in the proposed

assay. λ phage exonuclease (5'–3') and *Escherichia coli* exonuclease III (3'–5') both require double-stranded substrates [17,18]. This makes them unsuitable for our assay since both strands of an undamaged DNA fragment must be digested. Additionally, λ exonuclease is only partially inhibited by adducts formed by 5-MIP [19], *cis*-platinum and the acridine mustard ICR-170 [20], and exonuclease III has an abasic-site endonuclease activity [18] and can also terminate at undamaged sites [21]. We tested *E. coli* exonuclease I, a 3'–5' exonuclease specific for single-stranded DNA and found, in preliminary experiments, that this enzyme could suitably digest undamaged *Kpn*I-restricted CHO DNA, but was inhibited only slightly by u.v. C-induced lesions or HMT mono-adducts.

In order to be useful for our assay, an exonuclease must be able to hydrolyse large fragments of single-stranded or single- and double-stranded DNA, and be inhibited to a substantial degree by lesions which occur in the DNA. We found that T7 DNA polymerase-exonuclease meets the first criterion but fails in the second for the DNA lesions tested here. Other characterized exonucleases also fail to meet these criteria. There may exist a suitable exonuclease yet to be discovered and characterized.

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REFERENCES

- Terleth, C., van de Putte, P. and Brouwer, J. (1991) *Mutagenesis* **6**, 103–111
- Doetsch, P. W., Chan, G. L. and Haseltine, W. A. (1985) *Nucleic Acids Res.* **13**, 3285–3304
- Fuchs, R. P. D. (1983) *J. Mol. Biol.* **177**, 173–180
- Boyer, V., Moustacchi, E. and Sage, E. (1988) *Biochemistry* **27**, 3011–3018
- Panigrahi, G. B. and Walker, I. G. (1990) *Biochemistry* **29**, 2122–2126
- Huang, W. M. and Lehman, I. R. (1972) *J. Biol. Chem.* **247**, 3139–3146
- Hori, K., Mark, D. F. and Richardson, C. C. (1979) *J. Biol. Chem.* **254**, 11598–11604
- Spivak, G., Leadon, S. A., Vos, J.-M., Meade, S., Hanawalt, P. C. and Ganesan, A. K. (1988) *Mutat. Res.* **193**, 97–108
- Islas, A. L., Vos, J.-M. and Hanawalt, P. C. (1991) *Cancer Res.* **51**, 2867–2873
- Adler, S. and Modrich, P. (1979) *J. Biol. Chem.* **254**, 11605–11614
- Bohr, V. A. and Okumoto, D. S. (1988) in *DNA Repair: A Laboratory Manual of Research Procedures* (Friedberg, E. C. and Hanawalt, P. C., eds.), pp. 347–366, Marcel Dekker, Inc., New York
- Mellon, I., Spivak, G. and Hanawalt, P. C. (1987) *Cell* **51**, 241–249
- Beer, M., Stern, S., Carmalt, D. and Mohlhenrich, K. M. (1966) *Biochemistry* **5**, 2283–2288
- Hearst, J. E., Issacs, S. T., Kanne, D., Rapoport, H. and Straub, K. (1984) *Q. Rev. Biophys.* **17**, 1–44
- Vos, J.-M. and Hanawalt, P. C. (1987) *Cell* **50**, 789–799
- Reference deleted
- Little, J. W. (1967) *J. Biol. Chem.* **242**, 679–686
- Rogers, S. G. and Weiss, B. (1980) *Methods Enzymol.* **65**, 201–211
- Ostrand, E. A., Karty, R. A. and Hallick, L. M. (1988) *Nucleic Acids Res.* **16**, 213–227
- Mattes, W. B. (1990) *Nucleic Acids Res.* **18**, 3723–3730
- Linxweiler, W. and Horz, W. (1982) *Nucleic Acids Res.* **10**, 4845–4859