REACTIONS OF CALF THYMUS DNA POLYMERASES α AND β WITH NATIVE DNA DAMAGED BY THYMINE STARVATION OR BY METHYL METHANESULPHONATE TREATMENT OF *ESCHERICHIA COLI* CELLS

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1. Introduction

We have demonstrated that native DNA from *Escherichia coli* CR-34 cells containing breaks due to incomplete excision repair is replicated in vitro more efficiently by calf thymus DNA polymerase β than α [1]. Here, two other native DNAs damaged by thymine starvation or methyl methanesulphonate (MMS) treatment of *E. coli* cells were used as templates. It was found that the amounts of DNA polymerase β products were greater than those of DNA polymerase α, and exceeded the content of the single stranded fraction in the template. This suggests that DNA synthesis by DNA polymerase β is accompanied by displacement of non-replicated strands.

2. Materials and methods

Escherichia coli KMBL 1787 thy⁻ polA⁻ cells and terminal deoxynucleotidyl transferase (TdT) were kindly offered by B. W. Glickman and F. J. Bollum, respectively; MMS was from Merck (Schuchardt); activated calf thymus DNA was prepared as in [2]; other compounds were described in [1].

2.1. *DNA polymerases*

Calf thymus DNA polymerases α and β were isolated according to [3] and [4], respectively. The first enzyme was further purified on hydroxylapatite and Sephadex G-100 columns. One unit of DNA polymerase incorporates 1 nmol labelled dNTP into the acid-precipitable material at 37°C within 60 min, under the conditions described in fig.1 with 125 μg activated DNA/ml as template. Two assays for TdT [1], estimating an incorporation of 5 and 0.5 pmol substrate, respectively, showed that DNA polymerase β was free from TdT. In the first assay, the reaction was carried out for 1 h at 37°C, under optimal conditions for TdT [3]: 125 mM potassium cacodylate (pH 7.2), 5 mM $MgCl_2$, 0.3 mM DTT, 1 mg BSA/ml, 25 μg/ml oligo(dT)$_7$, 1.25 mM d[^{3}H]ATP (33 dpm/pmol) and DNA polymerase β (132 units/ml). In the second assay the substrate incorporation was measured for 6 h under the conditions used in the assay for DNA polymerase β (fig.1), except that 80 μM d[^{3}H]GTP (500 dpm/pmol) was the only substrate and poly(dA)$_{300}$ (125 μg/ml) was used instead of DNA. The assay for exonucleases [1] measured acid-soluble ^{3}H-labelled material and showed that under the conditions applied for polymerization (fig.1), DNA polymerase α split 45 nucleotide pmol/5 μl assay from sonicated [^{3}H]DNA after 2 h, whereas DNA polymerase β split 25 nucleotide pmol/50 μl assay after 6 h. The assay for endonucleases [1] using agarose gel electrophoresis showed that the average number of incisions after 2 h reaction with DNA polymerase β under the conditions applied in fig.1 was 0.4/Col E1 [^{3}H]DNA molecule. Both polymerases were free from phosphatases; this was demonstrated by an assay in which each of the four 79 μM d[^{3}H]NTPs (1000 dpm/pmol) was incubated with DNA polymerase α and β, respectively, under the conditions used in fig.1 and analysed by thin-layer chromatography in isobutyric acid–concentrated NH_4OH–water (66:1:33, by vol.).

2.2. *DNA from thymine-starved cells (TS-[^{3}H]DNA)*

An overnight culture of *E. coli* KMBL 1787 thy$^-$ polA$^-$ cells grown on TB1 medium containing thymine was diluted with medium (1:50) supplemented with [^{3}H]Met-thymidine and grown to 2–6 × 10^7 cells/ml. Cells washed with 10 mM $MgSO_4$ were suspended in an equal volume of M9 medium [5] deprived of thymine and supplemented according to [6]. Cells were incubated for 40 min at 37°C, washed with 0.1 M EDTA in 0.15 M NaCl (pH 8.0), suspended in the same solution and treated with lysozyme (1 mg/ml) and SDS (1%, w/v) for 1.5 h at 37°C, then with pancreatic RNase (0.2 mg/ml) plus RNase T1 (20 units/ml) for 1 h. Pronase (1 mg/ml) was then added and the mixture was dialysed overnight at 37°C against 10 mM EDTA in 0.15 M NaCl (pH 8.0). TS-[^{3}H]DNA (8×10^5 dpm/μg) was isolated by centrifugation in CsCl (1.7 g/cm^3) in a Ti 40 Beckman rotor. As determined by centrifugation in neutral and alkaline sucrose gradients [7] with phage T7 [^{14}C]DNA, the mean M_r of native and denatured TS-[^{3}H]DNA were 92×10^6 and 18×10^6, respectively.

2.3. *The single-stranded fraction in TS-[^{3}H]DNA*

This was measured using nuclease S1. Reaction mixtures as in [8] contained in 200 μl, 5 μg of either denatured *E. coli* [^{3}H]DNA (8×10^5 dpm/μg) or of native TS-[^{3}H]DNA (4×10^4 dpm/μg) and were incubated with 10 units nuclease S1 at 37°C for 20 min. The acid-soluble ^{3}H-labelled material was then collected and counted in Bray's scintillator, assuming that the acid-soluble material from the sample with denatured DNA represents 100% DNA; the amount of the acid-soluble material obtained in case of native TS-[^{3}H]DNA was 3.2%.

2.4. *DNA from MMS-treated cells (MMS-DNA)*

An overnight culture of *E. coli* KMBL 1787 thy$^-$ polA$^-$ cells grown on TB_1OX medium [9] was diluted and grown to 5–12 × 10^7 cells/ml, as for TS-[^{3}H]DNA. Cells were washed with 85 mM NaCl in 0.5 mM $MgSO_4$, suspended in an equal volume of 0.1 M citric acid in 0.2 M Na_2HPO_4 (pH 7.0) and shaken with 0.02% MMS at 37°C for 20 min [10]. Washed cells were treated with lysozyme, SDS and pronase, and then dialyzed overnight at 37°C, as for TS-[^{3}H]DNA. MMS-DNA was purified by phenol extraction and ethanol precipitation; it was then treated with pancreatic RNase (0.2 mg/ml) plus RNase T1 (20 units/ml) for 1 h, this being followed by phenol extraction, ethanol precipitation and dialysis. Native and denatured MMS-DNAs centrifuged in a Beckman L5-75 ultracentrifuge with an UV scanner had mean M_r of 8.2×10^6 and 2.6×10^6, respectively [11].

3. Results and discussion

Culturing of *E. coli* thy$^-$ polA$^-$ cells under conditions of thymine starvation or in the presence of MMS causes mutations, loss of cell viability and single-stranded breaks in DNA [10,12–14]. In agreement with these data, centrifugation in neutral and alkaline sucrose gradients demonstrated that TS-[^{3}H]DNA and MMS-DNA isolated from cells which lost 95% and 85% of viability, respectively, contained 3 and 2 single-stranded breaks/molecule. 3′-OH DNA ends formed by the incision of a damage-containing strand represent a potential primer for DNA polymerases.

The extensive replication of TS-[^{3}H]DNA and MMS-DNA by DNA polymerase α reached a plateau after 1 h (fig.1). Further addition of the enzyme after 2 or 6 h incubation did not change the yields of these reactions. In contrast, dTMP incorporations into these DNAs by DNA polymerase β were not complete even after 6 h. At this time, for TS-[^{3}H]DNA and MMS-DNA, they were, respectively, 18- and 24-times greater than the incorporations catalyzed by DNA polymerase α (fig.1).

The above reactions were carried out under optimal conditions for each enzyme. Fig.2 shows the results obtained when the same buffer and the optimal salt concentrations, or if the same buffer and the same salt concentration were used. These suboptimal conditions partly inhibited both polymerases: when using activated calf thymus DNA (125 μg/ml), these suboptimal conditions gave, respectively, 94% and 75% of the optimal α polymerase activity, and 48% and 64% of the optimal β polymerase activity, determined as described under fig.1. Nevertheless, the yields of the reactions of DNA polymerase β with TS-[^{3}H]DNA and MMS-DNA were distinctly higher than those of DNA polymerase α.

Differences between the polymerases in replication of native damaged DNAs could not be caused by different mechanisms for by-passing the damage, e.g., an apurinic site present in a non-incised template DNA strand. The dTMP incorporations during replication of thermally denatured TS-[^{3}H]DNA (fig.1) and thermally denatured MMS-DNA (not shown) catalyzed

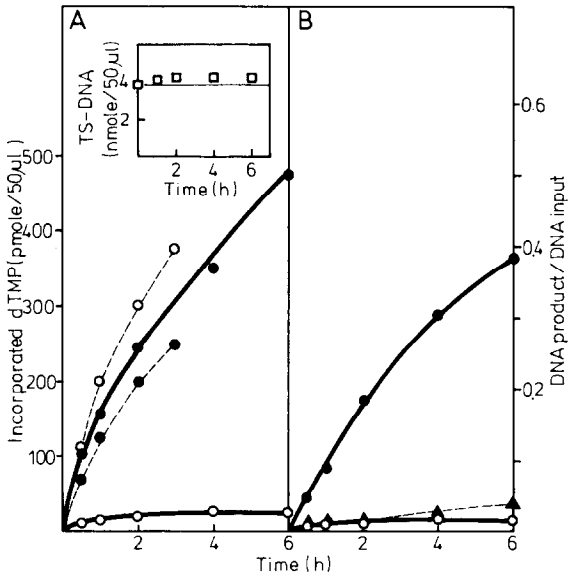
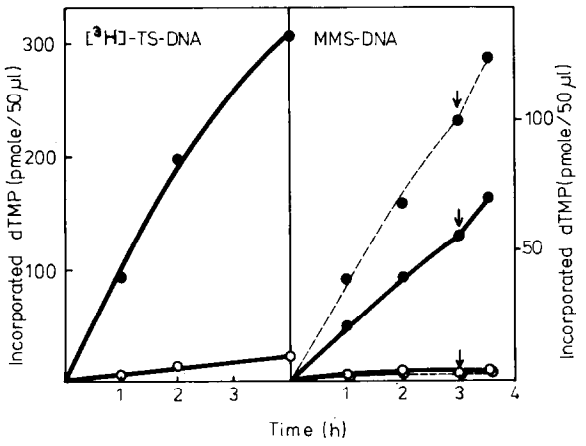


Fig.1. Replication of TS-[^{3}H]DNA, denatured TS-[^{3}H]DNA and MMS-DNA by DNA polymerases α and β. Reaction mixtures for DNA polymerase α contained: 33 mM phosphate buffer (pH 7.2), 1.3 mM DTT, 8 mM $MgCl_2$, 25 µg/ml TS-[^{3}H]DNA (4 × 10^4 dpm/µg) ((A), ○——○), MMS-DNA ((B), ○——○) or thermally denatured TS-[^{3}H]DNA ((A), ○- -○), 158 µM dATP, dCTP, dGTP, and d[^{14}C]TTP (10 dpm/pmol) (A), or d[^{3}H]TTP (250 dpm/pmol) (B) and 145 units enzyme/ml. Reaction mixtures for DNA polymerase β contained: 33 mM Tris–HCl buffer (pH 8.5), 1.3 mM DTT, 8 mM $MgCl_2$, 0.1 M NaCl, 25 µg TS-[^{3}H]DNA/ml ((A), ●——●), MMS-DNA ((B), ●——●) or denatured TS-[^{3}H]DNA ((A), ●- -●), 132 units enzyme/ml and substrates as above. (▲- -▲) Reaction of DNA polymerase β with dATP and d[^{3}H]TTP substrates only. Reaction mixtures were incubated at 37°C, and acid-precipitable ^{14}C- or ^{3}H-labelled materials were measured [1]. Insert: TS-[^{3}H]DNA content in the acid-precipitable material.



by DNA polymerases β were not higher than those observed for DNA polymerase α with the same DNAs.

The maximal amount of the DNA polymerase α product synthesized on TS-[^{3}H]DNA (108 pmol/50 µl assay) was comparable with the amount of single-stranded fraction in this template (121 pmol/50 µl assay) (see section 2). The amount of DNA synthesized by DNA polymerase β was very much higher (1880 pmol/50 µl assay).

The DNA polymerase β product was evidently synthesized on a DNA template, since elimination of 2 or 3 substrates from the reaction mixture reduced the d[^{3}H]TMP incorporation to 8% and 1%, respectively, of that in the presence of 4 substrates (fig.2B). The high efficiency of the DNA polymerase β reaction with damaged DNAs was not due to TdT contamination: for pure calf thymus TdT the incorporation of dNTP into MMS-DNA was 0.4% of the dGTP incorporation with poly(dA)$_{300}$ as primer. This conclusion is in agreement with the negative results of the assays for TdT in the DNA polymerase β preparation (see section 2).

Likewise, nucleases were not responsible for formation of extra single-stranded templates because the amount of DNA formed by DNA polymerase β (fig.1) was ~65-times greater than that degraded by exonucleases (see section 2). Furthermore, the synthesis of the ^{14}C-labelled product by DNA polymerase β proceeded without degradation of the ^{3}H-labelled template (fig.1).

These results extend our data showing that DNA polymerases α and β differ in their reaction with DNA containing breaks, although they incorporate similar amounts of precursors into native, undamaged *E. coli* DNA [1]. They suggest also that, like those catalyzed by *E. coli* and phage T5 DNA polymerases [15–18], the DNA polymerase β reaction proceeds with strand displacement. The possible strand displacement ability of DNA polymerase β represents an important difference between DNA polymerases α and β.

Fig.2. Replication of TS-[^{3}H]DNA and MMS-DNA by DNA polymerases α and β under the same buffer conditions and under the same buffer and salt conditions. Reaction mixtures for DNA polymerase α (○) and β (●) were as in fig.1 except that: the same buffer, 33 mM Tris–HCl (pH 7.8), (——) or the same buffer, 33 mM Tris–HCl (pH 7.8) and salt, 0.04 M NaCl, (- - -) conditions were used for both enzymes; (↓) a fresh sample of enzyme was added.

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