Differential inhibition of rat liver DNA polymerases *in vitro* by direct-acting carcinogens and the protective effect of a thiol reducing agent

John Y. H. CHAN and Frederick F. BECKER*

Section of Experimental Pathology, Department of Pathology, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, TX 77030, U.S.A.

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The direct-acting carcinogens acetoxyacetylaminofluorene, methylnitrosourea, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were tested for their ability to inhibit rat liver DNA polymerase- α , - β , and - γ activity *in vitro*. DNA polymerase- α was the most sensitive, polymerase- β was the most resistant, and polymerase- γ exhibited an intermediate response. When the reactions were reassayed in the presence and absence of dithiothreitol, a thiol reducing agent, it was shown that the inhibition by carcinogens was generally reversible with increasing dithiothreitol, except that polymerase- β recovered only 80–90% of control values. These and binding data suggest that DNA polymerase- β , the putative repair enzyme, is highly resistant to carcinogen damage. This resistance may contribute to the retention of normal function and fidelity of the repair enzyme during carcinogen exposure *in vivo* and to normal cellular repair.

Many chemical carcinogens undergo metabolic activation *in vivo* to form electrophilic reactants that bind covalently to cellular macromolecules, including proteins, DNA and RNA (Gronow, 1980; Grunberger & Weinstein, 1979). The effect of these bindings is often to produce functional changes in the macromolecules (Gronow, 1980; Grunberger & Weinstein, 1979; Pulkrabek *et al.*, 1979; Guzzo & Glazer, 1976). However, it is not clear which of these targets is the critical one in the process of malignant transformation. It is generally assumed that the interaction of activated metabolites with nuclear DNA and the subsequent intervention of repair mechanisms may play a crucial role in the carcinogenic sequence. It has also been shown previously that treatments of animals (Kaufman *et al.*, 1979) or cells with carcinogens can induce unscheduled DNA synthesis (San & Stitch, 1975). Thus the normal excision–repair machinery appears to be unaffected by the carcinogen treatment and has been shown to be error-free (Lieberman, 1976).

As part of a programme to elucidate the molecular mechanisms of DNA damage and repair during chemical carcinogenesis, we have shown that the putative repair enzyme, DNA polymerase- β , was

not altered during AAF-induced rat liver carcinogenesis (Chan & Becker, 1979). Similarly, no alteration in repair glycosylases (Teebor *et al.*, 1977) was found, supporting the concept that the normal cellular repair synthesis appears to be unaffected during chemical carcinogenesis.

In the present study, additional data show that the purified polymerase- β from rat liver, unlike the other two forms of eukaryotic polymerase, polymerase- α and - γ , is relatively resistant to inhibition *in vitro* by direct carcinogens. The extent of inhibition of these enzymes by direct carcinogens was related to the amount of thiol reducing group present in the reaction mixture.

Materials and methods

Materials

[³H]dTTP (64 Ci/mol) was obtained from Schwarz–Mann (Orangeburg, NY, U.S.A.). Poly-(rA)·oligo(dT) was obtained from Miles Laboratories (Elkhart, IN, U.S.A.). Iodoacetamide was from Calbiochem (LaJolla, CA, U.S.A.); acetoxy-AAF was prepared from *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF) (purchased from New England Nuclear, Boston, MA, U.S.A.) by Dr. Dan Stout of our laboratories. MNU was purchased from ICN Pharmaceuticals (Plainview, NY, U.S.A.). MNNG was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Abbreviations used: AAF, 2-acetylaminofluorene; acetoxy-AAF, acetoxyacetylaminofluorene; MNU, methylnitrosourea; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

* To whom reprint requests should be addressed.

Purification of DNA polymerases

DNA polymerases were isolated from 24 h-regenerating rat liver as described previously (Chan & Becker, 1979). In brief, DNA polymerase- α was isolated from cytoplasm and was subsequently purified by DEAE-cellulose (DE-23), phosphocellulose (P11), DEAE-cellulose (DE-52), hydroxyapatite and AcA-34 gel-filtration chromatography. DNA polymerase- β and - γ were isolated from the nuclei of normal or 24 h-regenerating rat livers as described by Chan & Becker (1979). In brief, the nuclei were extracted with 2.0 M-NaCl, and the solubilized enzyme fractions were subsequently purified by chromatography on DEAE-cellulose DE-23, phosphocellulose P11, hydroxyapatite and Sephadex G-100.

The specific activities of the purified DNA polymerase- α , - β and - γ were 1530, 6500 and 699 units/mg of protein. Purified DNA polymerase- α (6–8S) was characterized by its low pH (pH 7.0–7.4) and low salt (0.025 M-NaCl) requirement for optimal activity as well as extreme sensitivity to *N*-ethylmaleimide inhibition. With 0.1 mM-*N*-ethylmaleimide, its activity was inhibited by 90–95%. DNA polymerase- β (3–4S) required an alkaline pH (pH 8.6) and higher salt concentration (0.05–0.1 M-NaCl) for optimal activity and was insensitive to *N*-ethylmaleimide inhibition. With 0.1 mM-*N*-ethylmaleimide, only 0–20% inhibition of its activity occurred.

DNA polymerase- γ (6–7S) utilized poly-(rA)-oligo(dT) exclusively and had its optimal activity at pH 7.5 and 0.1 M-NaCl. The general properties of this enzyme fraction were in agreement with properties for most mammalian DNA polymerases reported previously (Bollum, 1975; Brun & Chapeville, 1977; Weissbach, 1977).

DNA polymerase assay

DNA polymerase- α and - β were assayed by the procedure of Chan *et al.* (1977). The final reaction mixture contained 50 mM-Tris/HCl (pH 8.6 for polymerase- β and pH 7.4 for polymerase- α optimal assay), 6 mM-magnesium acetate, 2 mM-dithiothreitol, 50 μ g of activated calf thymus DNA/ml, 100 μ M each of dATP, dCTP and dGTP, 20 μ M-dTTP, 1 μ Ci of [3 H]dTTP, and enzyme in 0.2 ml final volume. The reaction mixture for the assay of DNA polymerase- γ contained 50 mM-Tris/HCl (pH 7.5), 0.5 mM-MnCl₂, 100 mM-KCl, 2 mM-dithiothreitol, 50 μ g of poly(rA)-oligo(dT)/ml, 100 μ M-[3 H]dTTP (1 μ Ci) and enzyme in 0.2 ml final volume (Chan & Srivastava, 1976).

After 30 min incubation at 37°C (30°C for polymerase- γ assay), the reaction was stopped by adding 1 ml of 10% (v/v) trichloroacetic acid and 3% (w/v) sodium pyrophosphate, containing 100 μ g

of yeast RNA as carrier. The acid-insoluble material was collected on a GF/C filter and washed with 50 ml of cold 5% trichloroacetic acid. DNA synthesis was measured by the incorporation of [3 H]-dTMP into acid-insoluble material. One unit of enzyme activity incorporated 1 nmol of [3 H]dTMP in 1 h.

Reactions of carcinogen with enzyme

DNA polymerase- α , - β and - γ were treated with acetoxy-AAF (dissolved in dimethyl sulphoxide), MNU (in 0.1 M-cacodylate, pH 7.0) and MNNG (in water) for 2–4 h at room temperature. The samples were then dialysed extensively against 0.01 M-Tris/HCl (pH 8.0)/45% (v/v) glycerol/0.1 mM-EDTA/1 mM-dithiothreitol (TGED buffer) for 24 h to eliminate unchanged carcinogen. The samples were then assayed for inhibition of polymerase activity. Enzyme samples, not treated with carcinogen, were treated by identical procedures and served as controls (100% activity). Enzymes were dialysed overnight against TGE buffer (TGED buffer minus dithiothreitol) for experiments on the effect of dithiothreitol or iodoacetamide.

Results

Inhibition of rat liver DNA polymerase activities by increasing amounts of carcinogens

Increasing concentrations of the agents acetoxy-AAF, MNU and MNNG were separately incubated with DNA polymerase- α , - β and - γ for 4 h at 25°C in the presence of TGED buffer. After exhaustive dialysis against TGED buffer, the various polymerases were then assayed for the effect of carcinogen reaction.

With acetoxy-AAF (Fig. 1a), DNA polymerase- α was the most sensitive to the carcinogen inhibition, whereas polymerase- β was only slightly sensitive, and the effect on polymerase- γ was intermediary. With 7 mM-acetoxy-AAF, polymerase- α , - β and - γ activities were 10%, 85% and 30% of their control values respectively.

With MNU as the reacting compound (Fig. 1b), DNA polymerase- α , - β and - γ activities were also inhibited in a similar manner. However, the concentration of drug required to give the same degree of inhibition was greatly different. With a dose of 290 mM-MNU, the activities were 5%, 78% and 30% of control values for polymerase- α , - β and - γ respectively.

When the polymerases reacted with 2 mM-MNNG, another potent methylating compound, DNA polymerase- α , - β and - γ activities detected were 16%, 86% and 66% of their control values respectively (Fig. 1c).

Fig. 1. Inhibition of rat liver DNA polymerase activities by increasing concentrations of direct-acting carcinogens. Increasing amounts of (a) acetoxy-AAF, (b) MNU and (c) MNNG were made to react with 1–10 units of DNA polymerase- α , - β and - γ for 4 h at room temperature. The samples were then dialysed overnight against TGED buffer and assayed in triplicate for the remaining DNA polymerase activities: ●, DNA polymerase- α ; ■, DNA polymerase- β ; △, DNA polymerase- γ . Vertical bars indicate ranges for triplicate samples.

Fig. 2. Protective effect of the thiol reducing agent dithiothreitol on the inhibition of DNA polymerase activities by direct carcinogens

Samples (1–10 units) of DNA polymerase- α (●), - β (■) and - γ (△) were dialysed separately against TGE buffer overnight. Increasing amounts of dithiothreitol were then added. Samples were immediately treated with (a) 5.3 mM-acetoxy-AAF, (b) 145 mM-MNU and (c) 2 mM-MNNG for 4 h at room temperature. Samples were assayed for DNA polymerase activity after overnight dialysis against TGED buffer. C, control samples with no carcinogen. Vertical bars indicate range for triplicate samples.

Effect of dithiothreitol on the inhibition of rat liver DNA polymerases by carcinogen

Previously it has been suggested that the inhibition *in vitro* of a DNA-modifying enzyme, DNA methylase, by the carcinogen MNNG was sensitive to the presence of a thiol reducing agent (Cox, 1980). Therefore we next determined if the inhibition of the various eukaryotic DNA polymerases could be reversed by the addition of a thiol-group reducing compound, dithiothreitol. Purified rat liver DNA polymerase- α , - β and - γ fractions were separately dialysed against TGE buffer to eliminate dithiothreitol originally present in the samples. Then increasing amounts of dithiothreitol were added to samples of dialysed polymerases. These samples

were then treated with carcinogen for 4 h at 25°C as described above. After dialysis overnight against TGED buffer, the samples were then assayed for polymerase activities.

As shown in Fig. 2, the inhibition *in vitro* of different species of eukaryotic DNA polymerases by all three carcinogens was affected by the presence of dithiothreitol. With 5.3 mM-acetoxy-AAF, and in the absence of dithiothreitol, the activities of all three polymerases were the lowest; polymerase- α , - β and - γ activities were 27%, 60% and 30% of their control values respectively. With 5 mM-dithiothreitol and 5.3 mM-acetoxy-AAF, the activities of polymerase- α , - β and - γ were 54%, 81% and 108% of their control values respectively (Fig. 2a).

When the enzymes were treated with 145 mM-MNU in the presence of increasing concentrations of dithiothreitol, similar results were obtained (Fig. 2b). In the absence of dithiothreitol, the activities of polymerase- α , - β and - γ were 25%, 75% and 40% of their controls respectively. With 5 mM-dithiothreitol, DNA polymerase- α , - β and - γ activities were 27%, 90% and 76% of their control values respectively.

With MNNG (Fig. 2c), a pattern similar to that for acetoxy-AAF was obtained. In the absence of dithiothreitol, the activities of polymerase- α , - β and - γ after reaction with 2 mM-MNNG were 18%, 60% and 40% of their controls respectively. At 5 mM-dithiothreitol, the activities were 70%, 82% and 100% for polymerase- α , - β - γ respectively. It is apparent that the thiol reducing agent dithiothreitol protects the polymerases from the inhibitory effect of the three carcinogens tested. However, the degree of protection differed greatly for the different forms of polymerase, e.g. polymerase- β recovered earliest (to 80% of its original value), polymerase- γ followed, and DNA polymerase- α required the highest concentrations of dithiothreitol for protection. In general, the greatest effect was on DNA polymerase- γ .

Inhibition in vitro of DNA polymerases by iodoacetamide

Since the above data indicate the protective role of dithiothreitol, we then tested the inhibitory effect of iodoacetamide, a compound known to react with the thiol groups of proteins. DNA polymerase- α , - β and - γ were assayed after incubation with increasing amounts of iodoacetamide. With 30 mM-iodoacetamide, the percentages of polymerase activity remaining were 15%, 78% and 45% for polymerase- α , - β and - γ respectively (Fig. 3). The inhibition of the three polymerases by iodoacetamide reflects the relative effects on these enzymes of the direct carcinogens, i.e. polymerase- α is inhibited more than polymerase- γ , which is inhibited more than polymerase- β . The results suggest that these polymerases contain various thiol groups and that these carcinogens react with the enzymes and affect these thiol groups in a manner similar to the inhibition by iodoacetamide.

Binding in vitro of acetoxy-[14 C]AAF to DNA polymerase- α and - β

To determine if the differential extent of inhibition of the various enzymes by the carcinogens is related to the amount of carcinogen bound, we measured the effect of an increasing amount of acetoxy-[14 C]AAF on DNA polymerase- α and - β for 4 h, with subsequent processing as described above. Portions of each sample were analysed separately for degree of inhibition and the amount of acetoxy-[14 C]AAF bound, as shown in Fig. 4. DNA

Fig. 3. Effect of iodoacetamide on rat liver DNA polymerase- α , - β and - γ

Increasing concentrations of iodoacetamide were treated with 1–10 units of DNA polymerase- α (●), - β (■) and - γ (Δ) for 15 min on ice. After treatment, the samples were assayed for DNA polymerase activities as described in the Materials and methods. Vertical bars indicate ranges for triplicate samples.

Fig. 4. Correlation between amount of acetoxy-[14 C]AAF bound to rat liver DNA polymerase and the degree of inhibition

Increasing amounts of acetoxy-[14 C]AAF were made to react with constant amounts of DNA polymerase- α and - β (2 units and 5 units respectively). After dialysis, the samples were assayed separately for activities and the amounts of trichloroacetic acid-precipitable 14 C radioactivity. Vertical bars indicate ranges for triplicate samples.

polymerase- α was inhibited greatly, whereas polymerase- β was relatively insensitive. However, the amount of labelled carcinogen bound, as determined by acid-precipitable radioactivity, increased with increasing concentrations of carcinogen. With 7 mM-

acetoxy-AAF, 10% of DNA polymerase- α and 80% of polymerase- β activity remained, although approximately equal amounts of carcinogen were found to be associated with both polymerase- α and - β acid-precipitable protein fractions.

Discussion

The inhibition of these rat liver DNA polymerases by iodoacetamide indicates that they contain thiol groups. Further, the protective effect of dithiothreitol suggests that the carcinogens tested achieved their inhibitory effect by affecting the crucial thiol sites. The results of binding studies with acetoxy-[^{14}C]AAF revealed that the different extents of inhibition of polymerase- α and - β were not due to differences in each fraction's ability to react with the carcinogen molecules. Rather, the aggregate findings suggest that the relative sensitivity of DNA polymerase- α is due to the location of the thiol group(s) at the active, catalytic site. Examples of nucleophilic centres present in protein that may be attacked by activated carcinogens include the sulphur of methionine and cysteine, the N-1 and N-3 atoms of histidine, the C-3 and possibly O-4 atoms of tyrosine, and uncharacterized sites of attack in tryptophan (Gronow, 1980). The binding *in vitro* of esters of carcinogenic aromatic amines and amides with methionine was demonstrated by Lotlikar *et al.* (1966). The binding of lysine, glycine, β -alanine and δ -aminovalic acid by MNNG (McCalla & Reuvers, 1968), as well as the reactions of nitrosourea with lysine amino, serine hydroxy and cysteine thiol groups, were also reported (Cheng *et al.*, 1972; Brown & Wold, 1973; Twu & Wold, 1973). Thiol-group inhibitors that sterically hinder the access of substrate to the active site of the enzyme and thus partially inhibit activity have been reported. However, it has also been shown that the thiol group itself plays no part in the substrate binding or in catalysis (Torchinskii, 1974).

Several explanations are possible for the partial inhibition of DNA polymerase- β . It may be that the thiol group(s) are not located at the active site of this enzyme, but participate either in maintaining an active conformation or in binding allosteric effectors (Torchinskii, 1974). Another possibility is that the inactivation is modified by the conformation and thus the availability of the thiol group(s) at the active site.

However, one other explanation exists that the data apparently support. The consistent degree of inhibition of polymerase- β by the three carcinogens, approx. 20% in the presence of increasing concentrations of dithiothreitol from 1 to 5 mM, the rapidity with which the activity is restored by dithiothreitol, and the failure of higher concentrations of dithiothreitol (5 mM) to restore 100%

activity, suggest the possible presence of two subspecies of DNA polymerase- β .

The resistance of polymerase- β to the inhibition *in vitro* by direct carcinogens is in accord with the proposed concept that it is a component of the excision-repair system and with our previous results *in vivo* that demonstrated it to be unaltered during carcinogenic exposure to *N*-2-acetylaminofluorene (Chan & Becker, 1979). Other components of this system, such as the repair glycosylases (Teebor *et al.*, 1977), are also resistant to alteration, and preliminary results show that DNA polymerase- β treated *in vitro* with carcinogen appears to be faithful when assayed for the incorporation of non-complementary nucleotides into a poly(dA-dT) template (J. Y. H. Chan & F. F. Becker, unpublished work).

It is noteworthy that a much higher concentration of MNU is required to inhibit the polymerase activities compared with that of the other two carcinogens. However, it has been shown that 32.2 mg of MNU/ml (318 mM) was required to methylate 3.5% of the poly(dA-dT), or to cause 70% inhibition of its template activity (Abbott & Saffhill, 1977), whereas it only took 0.5 mM-acetoxy-AAF to cause a 10% binding to poly(dC-dG) template (Harvan *et al.*, 1977). Similarly, a very low dose of MNNG (32 $\mu\text{g}/350 \mu\text{l}$; 0.62 mM) will cause more than 94% inhibition *in vitro* of DNA methylase activity, whereas a similar dose of MNU has no effect. It is apparent that MNU reacts less effectively than MNNG and acetoxy-AAF. The results obtained for the inhibition of DNA polymerases by the three different carcinogens are therefore in accordance with that by other macromolecules. However, the adducts responsible for the inhibition are unknown at present.

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