INDUCIBLE DNA POLYMERASE IN CULTURED RAT FIBROBLASTS TREATED WITH SKIN CARCINOGEN MITOMYCIN C

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1. Introduction

DNA polymerases are involved in the filling of excision [1,2] and postreplication [3,4] single-strand gaps in DNA in ultraviolet-irradiated bacteria. Gap-filling with the constitutive DNA polymerase is an error-free process that cannot be the source of ultraviolet-induced mutations [5].

It has been reported that heat treatment of *tif* strain of *E. coli* causes the appearance of a mutagenic DNA polymerase responsible for the fixation of premutational lesions during heat-induced 'SOS repair' [6]. An inducibility has been shown for minor branch of postreplication repair in *E. coli* [7], *Micrococcus luteus* [8] and in human fibroblasts [9]. The question remains: Is inducible repair in mammalian cells coupled with the induction of a DNA polymerase with a reduced fidelity in repair replication?

Mitomycin C (MMC) studied extensively for its mutagenic [10] and carcinogenic [11] properties can induce in *E. coli* the synthesis of X protein [12]. This property usually correlates with the ability of a chemical to induce SOS repair [5]. In connection with the question above the observation of an increased DNA polymerase activity in extracts from MMC-treated mammalian cells [13,14] is of interest. We therefore investigated the origin and reaction properties of the DNA polymerase in cells affected by MMC.

2. Materials and methods

All experiments were with an established line, RNF-22, of new-born rat fibroblasts. The history of the line and the culture conditions will be described [15]. MMC (Calbiochem) in fresh growth medium was added to monolayer cultures up to 0.1 or 0.2 μg/ml and cells were incubated at 37°C for 24 h. In some experiments either 10^{-4} M puromycin (Serva) or 25 μg/ml cycloheximide (Koch Light) or 100 μg/ml caffeine (pharmaceutical grade) were added with or without MMC. Cells were washed with saline, treated with 0.25% trypsin-0.02% EDTA (1:1), harvested by centrifugation and the pellet rewashed with saline. The cells were lysed by sonication at 35 kHz in an USDN-1 dispergator at 10–18°C in 1.5–4 ml/100 mg wet cells of 0.02 M potassium phosphate buffer, pH 7.5, containing 10 mM β-mercaptoethanol, 20% (v/v) ethylene glycol and 0.05 M KCl. Cell debris was separated by centrifugation at 15 000 × g and discarded. The supernatant was dialysed against the same buffer and stored at −10°C.

Standard DNA polymerase assay was in vol. 50 μl containing 0.05 M Tris–HCl, pH 7.9, 8 mM $MgCl_2$, 0.4 mg/ml BSA, 100 μM of each dNTP, 1–2 μCi [^{3}H]dATP or [^{3}H]dTTP and 6.25 μg salmon-sperm DNA activated with DNAase I (DNA_a) or 4 μg DNA denatured with 0.1 M NaOH at 25°C (DNA_d). The mixture with synthetic template, poly(dA)·$(dT)_{12-18}$-(pdA·odT), contained 50 μM dTTP and dCTP, 1 μCi [^{3}H]dTTP or 10 μCi [^{3}H]dCTP and 6 μg pdA·odT, other components being the same as in assays with DNA templates. With a polyribonucleotide as template the reaction mixture contained 0.05 M Tris–HCl, pH 8.5, 0.5–10 mM $MnCl_2$, 0.4 mg/ml BSA, 5 or 100 μM dTTP, 1 μCi [^{3}H]dTTP and poly(rA)·$(dT)_{12-18}$ 1:1 (0.4 μg) or 20:1 (4 μg). After incubation at 37°C for 1–3 h, the polynucleotides were collected onto filter paper

and washed with 5% trichloroacetic acid and ethanol. Radioactivity was counted in a toluene scintillator in a Nuclear Chicage Mark II scintillation counter.

Poly(rA) was from Reanal and pdA·odT, $(dT)_5$ and $(dT)_{12-18}$ from PL Biochemicals. Unlabeled dNTPs were from Merck, Calbiochem and Serva, ^{3}H-labeled dNTPs from Amersham.

3. Results and discussion

In the standard assay the specific DNA polymerase activity of sonicated extracts increased with increased extract dilution (fig.1), indicating the presence of an endogenous inhibitor. Inhibition of DNA polymerase activity at high protein concentration in the assay fell when Mg^{2+} or template·initiator concentrations were increased. There is reason to believe that inhibitory components interact with divalent ions and polynucleotide. These components may be removed by chromatography through cellulose to which single-stranded DNA was complexed. Samples thus obtained were, however, unstable when stored, whereas untreated extracts retained activity for at least 2 months when stored at −10°C. The data presented here were obtained with cell extracts diluted to give the highest specific activity in standard assays with different templates (<0.5 mg/ml total protein in the reaction mixture).

The data in fig.1 and table 1 show that treatment

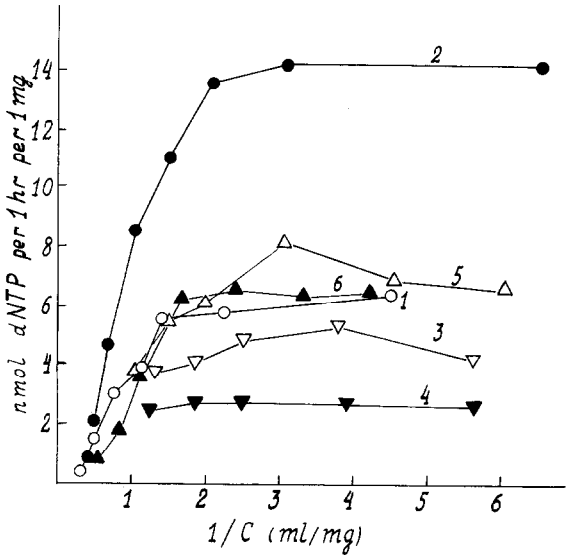

Fig.1. Specific DNA polymerase activity of rat fibroblast extracts in standard assay with activated DNA. C, protein content in the assay. The different curves are from differently treated cells of the same batch: 1, without treatment; 2, mitomycin C (MMC, 0.2 μg/ml); 3, puromycin (10^{-4} M); 4, puromycin + MMC; 5, caffeine (100 μg/ml); 6, caffeine + MMC.

Table 1
Effect of mitomycin C (MMC) on the level of DNA and poly(dA) replicative activity in rat fibroblasts

Treatment of cells	Spec. act. cell extracts[a] with different templates (%)			
	DNA_a	DNA_d	pdA·odT	BMBApdA·odT[b]
Without treatment	100	100	100	100
MMC (0.2 μg/ml)	197 ± 16	195 ± 35	50 ± 14	58 ± 9
Puromycin (10^{-4} M)	65 ± 3	79 ± 10	209 ± 18	138 ± 4
Puromycin + MMC	48 ± 4	89 ± 17	32 ± 8	55 ± 10
Cycloheximide (25 μg/ml)	102 ± 17	–	196	133
Cycloheximide + MMC	102 ± 11	–	21	14
Caffeine (100 μg/ml)	101 ± 8	–	100 ± 6	92 ± 10
Caffeine + MMC	93 ± 2	–	81 ± 7	94 ± 17

[a] The total protein content in the assay probe was not more than 0.5 mg/ml
[b] The poly(dA) was alkylated by treatment of pdA·odT with 7-Br-methylbenzanthracene (50 μg/ml, 60 min, pH 7.5, 37°C)

of cells with MMC resulted in a 2-fold increase of DNA polymerase activity with DNA_a as template (approx. 7000–15 000 pmol dNTPs incorporated/h. mg protein). The same effect was found using DNA_d (700–1400 pmol/h.mg), in agreement with observations with human cells [14]. However, with the template·initiator pdA·odT, activity decreased significantly after MMC treatment (700–350 pmol/h.mg). It should be noted that when the template poly(dA) is treated with 7-bromomethylbenzanthracene, there is a 1.5-fold decrease of dTTP incorporation with enzyme from both normal and treated cells.

The discrepancy between the results with natural and synthetic templates may indicate a change in the DNA polymerase make-up of cells after MMC treatment. MMC apparently brings about the synthesis, or activation, of an enzyme which much prefers DNA_a and DNA_d to pdA·odT. In addition, the drug inhibits the synthesis of the constitutive enzyme with a lower preference for DNAs, or inactivates it. To distinguish between these alternatives the following experiments were performed.

The effect of the protein-synthesis inhibitor puromycin (PM) in the presence and absence of MMC was examined. The level of pdA·odT-dependent DNA polymerase activity per mg total protein was higher in PM-treated cells than in untreated ones (table 1), so the constitutive pdA·odT-adapted enzyme seems to be a relatively 'long-term' protein. The fall in this activity with MMC was retained in the presence of PM, suggesting that MMC inactivates this enzyme directly.

On the other hand, addition of PM totally blocked the rise in the level of DNA-dependent DNA polymerase activity produced by MMC treatment (fig.1 and table 1). Similar results were obtained with cycloheximide, suggesting that the increase in DNA-dependent activity after MMC treatment is due to translation of newly formed or pre-existing mRNAs. This agrees with the proposition that MMC induces the synthesis of DNA-preferring DNA polymerase.

Caffeine (100 μg/ml), which itself had no effect on the levels of constitutive DNA polymerase activity, suppressed completely the effects of MMC (both inhibition and induction) as is seen in fig.1 and table 1. The suppression of MMC-induced DNA_d-dependent DNA polymerase activity by caffeine has been reported [14] but the mechanism of action of caffeine is not yet clear. We should like to emphasize three manifestations of its action:

(i) Suppression of irradiation and chemical carcinogenesis [16,17].

(ii) Suppression of postreplication repair [18,19] that is partly inducible [9].

(iii) Suppression of MMC-induced alterations in the DNA polymerase spectrum of cells reported here.

MMC-induced and constitutive activities do not belong to the low molecular weight type of mammalian DNA polymerases (β-polymerase or terminal deoxynucleotidyl transferase) because:

(1) On Sephadex G-100 gel filtration of extracts from MMC-treated or untreated cells the active material eluted as a single peak corresponding to mol. wt $\geq$ 120 000.

(2) dTTP incorporation did not exceed 1.5 pmol/h.mg in assays with poly(rA)·$(dt)_{12-18}$ and was indistinguishable from zero with $(dT)_5$ without template polynucleotide, whether treated or untreated cells were used.

(3) In both cases 10 mM *N*-ethylmaleimide (NEM) inhibited >90% the activities in assays with DNA_a.

These data led us to conclude that MMC treatment affects the spectrum of high molecular weight DNA polymerases of the α-type (classification [20]). Different forms of α-polymerases that differ in reaction properties have been found in chick-embryo fibroblasts [22].

No difference in sensitivity of constitutive and MMC-induced activities to variations of ion concentrations (K^+, NA^+, Mg^{2+}, Mn^{2+}) were observed in standard assays with DNA_a template. DNA polymerases from MMC-treated and untreated cells show a slight difference in sensitivity to 10 mM NEM; residual DNA_a-dependent activity in the presence of NEM being 3 times higher for the enzyme from untreated cells. Contrary to this, the inducible activity is more resistant to the endogenous inhibitor mentioned above; in concentrated cell extracts (2–3 mg/ml total protein in the reaction mixture) the apparent K_m for DNA_a of the enzyme from MMC-treated cells was 1/3 that of the controls. Besides, spermidine at 0.5 mM did not affect the MMC-induced DNA_a-dependent activity but inhibited the constitutive activity by 30–35% in standard assays; the

inhibitory effect completely disappeared with a 10-fold increase of DNA_a content in the assay. Spermidine as well as NEM had similar effects on both diluted cell extracts and enzymes eluted from DNA-cellulose. The relations between MMC-induced and constitutive DNA polymerases in their sensitivity to spermidine were reversed in the assay with DNA_d.

A difference between the inducible and constitutive DNA polymerases was found in the rate of incorporation of incorrect precursor (dCTP) into pdA·odT modified by 7-bromomethylbenzanthracene. In the controls the frequency of primer-dependent dCTP incorporation did not exceed the experimental error (<0.2% of dTTP incorporation), while after MMC treatment 2.3 ± 0.3% incorrect incorporation was found, indicating that the inducible DNA polymerase has a lower fidelity in selection of complementary nucleotides in the template·initiator system.

Thus, it may be concluded that treatment of rat fibroblasts with MMC results in suppression of (a) constitutive DNA α-polymerase(s) and induction of synthesis of another DNA α-polymerase with a higher affinity for primer DNA and lower fidelity. Another possible interpretation is that the changes in the properties of DNA-dependent DNA polymerase after treatment with MMC are due to the induction of an 'X-like' protein affecting the primer duplex. Isolation and purification of the enzyme are required for the elucidation of this point. However, the data obtained allow one to suggest an inducible repair, as in bacterial mutagenesis, as a mechanism of chemical mutagenesis in mammalian cells. This is of special interest in connection with the assumption that somatic mutation is a prerequisite in chemical carcinogenesis [22,23].

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