Evidences for the function of DNA polymerase- β in unscheduled DNA synthesis

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ABSTRACT

The activities of DNA polymerase- α and - β isolated from pig spleen were determined at different temperatures and in the presence of different concentrations of inhibitors. The results were compared with parallel estimations of replicative DNA synthesis and UV-induced repair synthesis in spleen cells.

In respect to pCMB and aCTP, polymerase- α is more sensitive than polymerase- β and similarly is replication more sensitive than repair. Repair synthesis and the activity of polymerase- β decreases at temperatures higher than 40°C with nearly no increase between 37° and 40°C whereas both replication and the activity of polymerase- α are greatly stimulated at elevated temperatures with optima of 45°C (polymerase- α) and 41°C (replication).

The results favour the hypothesis that polymerase- β is involved in repair synthesis.

INTRODUCTION

Nuclei of higher eukaryotes contain two main species of DNA dependent DNA polymerase, designated polymerases- α and - β (1). These enzymes have been distinguished first by their sedimentation coefficient and later on the basis of inhibition by sulfhydryl reagents and intracellular localisation. Polymerase- α is stimulated in proliferating cells, regenerating tissue and in tumors (2-5), it generally seems to increase in activity during the S-phase of the cell cycle whereas polymerase- β appears to be little or not regulated (6). This evidence together with the lower ability of polymerase- β to use oligoribonucleotides instead of oligodeoxyribonucleotides as a primer (7) led to the suggestion that polymerase- α is responsible for replicative DNA synthesis while polymer-

ase- β might be involved in DNA repair (8).

Unfortunately it is not possible to solve this problem by the use of mutants as it was done successfully with bacterial cells, because in eukaryotes such mutants are not yet available. One possible way towards a solution of this problem is to study cellular DNA synthesis under conditions which favour the activity of one or the other of the two enzymes. For instance, it was described previously that α - and β -polymerase exhibit different temperature optima (9,10).

In this paper we compared both DNA replication and repair of spleen cells with the activity of DNA polymerases, isolated from the same cells, using temperature changes and inhibitors of DNA synthesis with the hope to establish a correlation between the effect on one or the other polymerase with that on replication or repair.

MATERIALS AND METHODS

Ficoll, hydroxyurea, DNA (salmon sperm), aC (cytosine-arabinoside), aCTP (cytosinearabinoside triphosphate) NEM (N-ethyl maleimide) and deoxyribonuclease I (beef pancreas) were obtained from Sigma; pCMB (p-chloromercuribenzoate) from Serva; unlabelled deoxynucleotide triphosphates from Boehringer, Mann.; medium 199 and newborn calf serum from Flow Laboratories; Urografin from Schering AG and Periston from Bayer; (^3H)thymidine was purchased from New England Nuclear and all other radiochemicals from the Radiochemical Centre Amersham.

Assays

DNA polymerase was determined by the filter precipitation technique (11). One enzyme unit catalyzes the incorporation of 1 nmol dTMP into activated DNA in 60 min at 37°C (12). The standard assay-mixture contained in a total volume of 125 μl : 0.05 M Tris-HCl; 5 mM MgCl_2 ; 1 mM EDTA; 1 mM 2-mercaptoethanol; 50 μM each of dATP, dGTP, dCTP and dTTP; 9.25 K Bq (^3H)dTTP (=0.25 μCi); 13 μg activated

salmon sperm DNA and 5 to 25 μ l of enzyme preparation. The pH was 8.0 in all assays with polymerase- α , whereas all experiments with polymerase- β were done at a pH of 9.2.

After 15 min of incubation at 37°C, 100 μ l aliquots were spotted onto Whatman GF/C filters, these were dropped into 10 % trichloroacetic acid, washed extensively with 5 % trichloroacetic acid and finally with ethanol/ether (1/1). The radioactivity on the dried filter was measured in a toluene-based scintillation-cocktail.

Preparation of the Spleen Cell Suspension

Spleens were removed from pigs immediately after slaughtering and kept in Periston until working up (about 30 min). Homogenized tissue, freed from connective fibers and suspended in Parker's medium was passed through a nylon filter (100 μ m pore size). We found this method to be superior to the trypsinisation technique in respect to simplicity and yield of intact cells. After washing the cells by repeated centrifugation and resuspension, further purification of cells was achieved by flotation on a Ficoll-Urografin-mixture (13). The white cells obtained were washed once with Peristone and twice with isotonic NaCl. For the experiments the cells were suspended in Parker's medium supplemented with 10 % (v/v) newborn calf serum and adjusted to a density of 5×10^6 cells/ml. Trypanblue exclusion indicated 99.5 % viable cells.

Semiconservative DNA Synthesis

The spleen cell suspension was incubated at 37°C with 74 KBq/ml (3 H)thymidine (= 2 μ Ci/ml) for 60 min (the specific activity was 1.85 TBq/mmol or 50 Ci/mmol). After precipitation in 6 % perchloric acid and hydrolysis of the precipitate at 100°C for 30 min, the absorbance at 260 nm of the soluble material was measured and its radioactivity counted. The amount of semiconservative synthesis was expressed in terms of incorporated radioactivity per one unit of optical density.

Repair Synthesis

After 30 min preincubation of the spleen cell suspension with 2×10^{-3} M hydroxyurea the cells, suspended

in isotonic NaCl, were irradiated in petri dishes with 40 J/m^2 UV light of 254 nm. Centrifugation and resuspension of the cells in Parker's medium was followed by 90 min incubation in the presence of $2 \times 10^{-3} \text{ M}$ hydroxyurea and 74 KBq/ml (^3H)thymidine. The ratio of incorporated radioactivity versus optical density was determined as described above and the value of the unirradiated control was subtracted from that of the corresponding UV-irradiated sample to obtain the net amount of UV-induced unscheduled DNA synthesis.

RESULTS

Enzymes

DNA polymerases were isolated from the nuclear pellet (polymerase- β) or from the supernatant (polymerase- α) of a cell homogenate prepared from fresh pig spleens. For the purpose of our study it was necessary to purify the enzymes to such an extent as to obtain enzyme preparations well separated and of sufficient purity to avoid unwanted side-reactions in the assay. Details of isolation and subsequent purification of the enzymes are described elsewhere (14).

The enzymes were enriched more than 50-fold by ammonium sulfate fractionation and chromatography on DEAE Sephadex and DNA Cellulose; classification according to Weissbach (1) was done by the criteria of sedimentation behaviour and sensitivity to inhibitors. Physical and chemical properties like sedimentation constant, pH- and salt optima, requirements of template, primer, Mg^{++} and deoxytriphosphates were all in good agreement with the properties of other mammalian polymerases- α and - β already described in the literature.

It is noteworthy that polymerase- α splits into two enzyme-fractions when eluting from DEAE Sephadex. A similar effect was demonstrated with yeast DNA polymerases (11,15) as well as with other mammalian enzymes (16,17). The data in this paper are obtained using the major fraction which elutes first from DEAE Sephadex (65 % of the overall α -activity), but we have run all experiments in parallel with both subspecies. With the exception of the different

behaviour in chromatography, we have not been able to find any significant difference neither in the general enzymatic properties of these two fractions, nor in their response to different temperatures or inhibitors.

DNA Synthesis at Different Temperatures

The activity of DNA polymerase- α and - β at different temperatures were compared with the rate of cellular DNA synthesis (fig. 1). The temperature dependence of repair synthesis and polymerase- β is identical within experimental error; elevated temperature causes a rapid decline of both. In repeated experiments, UV-induced thymidine incorporation always had its maximum at 39°C. Polymerase- α and semiconservative DNA synthesis are stimulated remarkable around 40°C, the latter decreases sharply at temperatures above 41°C, probably due to the inhibition of reactions other than that catalysed by DNA polymerase- α . The values given in fig. 1 for semiconservative synthesis are averages of 4 independent tests done with cells from different spleens. All experiments showed a maximum of thymidine incorporation at 41°C. The data of the corresponding polymerase-assays in contrast varied with respect to the maximum of enzyme activity from 43 to 47°C.

In order to estimate repair synthesis, we blocked DNA replication with 10^{-3} M hydroxyurea. This treatment reduced thymidine incorporation from 16800 to 870 counts.min⁻¹ (5.2 % of the original amount) in a typical experiment at 37°C. The absolute values of the remaining synthesis in the presence of hydroxyurea were relatively constant between 21 and 50°C and did not follow the temperature curve of replication nor of repair. Its biological significance is unclear and we cannot decide if it is some form of repair or merely residual semiconservative synthesis. UV-induced repair synthesis was determined by calculating the difference between irradiated and unirradiated samples both in the presence of hydroxyurea.

DNA Synthesis in the Presence of Chemical Inhibitors

In a similar manner we have compared the response of cellular and cell free DNA synthesis to inhibitors (table 1).

Figure 1: DNA synthesis at different temperatures. All values are expressed in relation to standard conditions (= 100 % at 37°C) and are averages from 2 - 4 independent experiments; standard deviation is indicated by bars.
 A: Relative activity of DNA polymerase-α —○— and DNA polymerase-β ---x--- assayed by 15 min incubation of about 0.5 units of enzyme (100 % varies from 1800 to 2600 counts.min⁻¹ according to different enzymes and experiments).
 B: Semiconservative synthesis of spleen cells —○—, (100 % = 25 000 counts.min⁻¹/17 μg DNA in a typical experiment). Repair synthesis of spleen cells ---x---, (100 % = 4000 counts.min⁻¹/17 μg DNA in a typical experiment).

Unfortunately the assortment of chemicals with different effect on α- and β-polymerases is rather small. Sulfhydryl reagents like pCMB and NEM have the serious disadvantage of reacting with numerous proteins of the cells and the effect of those agents on thymidine incorporation does not

Table 1: Cellular and cell-free DNA synthesis in the presence of chemical inhibitors

*	DNA polymerase		**	Semicon- servative synthesis	Repair- synthesis
	α	β			
-	100	100	-	100	100
10^{-5} M NEM	88	110	10^{-6} M NEM	89	100
10^{-4} M NEM	68	97	10^{-5} M NEM	10	20
10^{-3} M NEM	15	106	10^{-4} M NEM	6.5	20
			10^{-3} M NEM	6	20
10^{-6} M pCMB	76	94	10^{-6} M pCMB	76	64
$2 \cdot 10^{-6}$ M pCMB	50	84	10^{-5} M pCMB	34	49
$4 \cdot 10^{-6}$ M pCMB	15	83	10^{-4} M pCMB	9	36
$8 \cdot 10^{-6}$ M pCMB	7	74			
10^{-5} M aCTP	65	94	$2 \cdot 10^{-6}$ M aC	19	64
10^{-4} M aCTP	48	82	$2 \cdot 10^{-5}$ M aC	5	51
10^{-3} M aCTP	21	68	$2 \cdot 10^{-4}$ M aC	4	45

* Standard assay, supplemented with the appropriate amount of inhibitor and lacking 2-mercaptoethanol in the experiments with NEM and pCMB.

**Standard assay, as described in the Material and Methods section except that all incubations and preincubations were carried out in Hanks balanced salt solution supplemented with the indicated concentration of inhibitor.

only reflect their action on the polymerases. Furthermore, in vivo the actual concentration of the inhibitor is unknown since the addition of a definite amount of agent to the medium gives no information on the concentration in the cell and even less on the concentration at the site of replication.

On the other hand, aCTP has the advantage of a rather specific reaction with the polymerases but cannot penetrate the cell membrane. Therefore it was necessary to apply aC to the cells and to ascertain in a control experiment that aCTP is produced under the conditions used. Using H^3 -labelled aC, we found that within the first hour only about 0.1 %

of the added aC penetrates into the cell and from that, only some 1 - 10 % are metabolized into aCTP. Therefore, an initial concentration of 2×10^{-5} M aC would result in about 2 pmol aCTP per 10^7 cells, i.e. about 10^{-6} M aCTP in the cell sap.

However, assuming that the triphosphates are probably not homogeneously distributed in the cells but rather are concentrated in relatively small compartments, tenfold or even hundredfold higher local concentrations could be expected. Probably, the inhibition of replication by the drug also depends on the relation of aCTP and of regular deoxytriphosphates, particularly of dCTP, which might favour the resistance of the polymerases in the assay system compared to the enzyme reaction in vivo.

Considering all these problems, a direct comparison of cellular and cell-free systems at the same concentration is useless. All we can do is to select a concentration where a particular enzyme is strongly inhibited (e.g. polymerase- α at 8×10^{-6} M pCMB or at 10^{-3} M aCTP) and another concentration which inhibits one of the cellular synthesis (e.g. semiconservative DNA synthesis at 10^{-4} M pCMB or at 2×10^{-6} M aC) and to calculate the ratio of inhibition at these concentrations.

This kind of examination demonstrates that in case of pCMB and aC/aCTP repair synthesis and polymerase- β are equally active at concentrations where both semiconservative synthesis and polymerase- α are strongly depressed (table 1).

Repair synthesis is reduced by very low concentrations of NEM to 20 % of its original value, but then remains constant even at rather high concentrations of the inhibitor. The reason for this strange concentration dependence is unknown.

DISCUSSION

Recently many data characterizing and distinguishing eukaryotic DNA polymerases were summarized and the four types of enzymes (designated polymerase α, β, γ and mt) compared and described in some detail (1). The aim of the

present communication was to contribute to the elucidation of the biological functions of DNA polymerase- α and - β . Whereas much evidence supports the hypothesis that polymerase- α is essential for replication, the role of polymerase- β is less clear, although observations concerning the amount of this enzyme in different stages of cell stimulation and proliferation support a possible function in repair synthesis (18,19).

Experiments reported here are in accord with the assumption that polymerase- α is involved in DNA replication while polymerase- β takes part in DNA repair reactions. Concentrations of aCTP or pCMB which almost totally inhibit polymerase- α and DNA replication have little effect on both polymerase- β and unscheduled DNA synthesis. Conversely, temperatures above 39°C cause a severe and rapid inactivation of polymerase- β activity and repair synthesis which are effected in a highly parallel fashion. Polymerase- α is much less sensitive to heat inactivation. The relative temperature sensitivity of polymerase- β reported here is in accordance with data obtained with β -polymerase from different sources (9) and might explain the pronounced temperature sensitivity of repair synthesis observed here and in other systems (20).

We have used UV-irradiation to stimulate repair reactions because of its known ability to induce long patch repair (21). Much longer stretches of the DNA have to be reconstituted in this case than after a damage induced by γ -rays or by methylation and the polymerization steps possibly involving DNA polymerase- β are probably dominating the overall repair reaction. Our results do of course not exclude that polymerase- β participates also in other types of repair. They do not exclude as well any minor involvement of polymerase- α in repair synthesis, but recently it has been shown that brain neurons are able to perform UV-repair in the absence of polymerase- α (22). A disadvantage of our determination of repair synthesis is the necessity to block replication by means of hydroxyurea. Although it is well known that this drug inhibits the reduction of ribonucleotides to deoxyribonucleotides and does not interfere with

the polymerase itself (we could not detect any inhibition of our enzyme preparations by hydroxyurea up to 10^{-1} M) the total mechanism of its action remains unclear and therefore some form of interaction between hydroxyurea and other inhibitors used in this work cannot be excluded. More information could possibly be obtained by the use of isolated nuclei instead of intact cells. This would avoid many uncertainties and errors caused by biological barriers and allow direct comparison of applied concentrations of inhibitors. In such experiments Butt et al. (23) demonstrated that nuclei which had lost all measurable polymerase- α are able to perform normal DNA repair, but these nuclei still had some replicative DNA synthesis left. Whether this arises from residual polymerase- α firmly bound to the chromatin or from an ability of polymerase- β to participate in DNA replication is unclear.

Rodent cells lose most of their excision repair capacity during development but retain still normal amounts of polymerase- β , indicating that this enzyme has probably some other functions too. In fact, rodent DNA polymerase- β differ in some properties from most other mammalian enzymes (24), e.g. only the rodent β -enzyme has to be supplemented by another protein factor to obtain the fully active polymerase. The question arises how eukaryotes like yeasts, protozoa and plants which are known to lack polymerase- β (25,26) are able to perform repair synthesis. It should be mentioned that many lower eukaryotes contain a DNA polymerase of high molecular weight which exhibits a 3' - 5' exonuclease activity (27,28,29,30,31). This enzyme might be a good candidate for a polymerase involved in repair. It is noteworthy that small amounts of a similar enzyme were recently found also in some mammalian cells (32).

Note added in proof: The sensitivity of replication and bleomycin-induced repair in permeabilized cells to araCTP and NEM has been investigated recently (33) and the results agree well with our data.

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