STIMULATION OF RAT LIVER α- AND β-TYPE DNA POLYMERASES BY AN HOMOLOGOUS DNA-UNWINDING PROTEIN

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1. Introduction

Stimulation of DNA polymerases by homologous DNA-unwinding proteins is one of the strongest arguments to assign a role to these proteins in the in vivo DNA synthesis [1]. In the field of prokaryotic replication, Molineux et al. [2] have shown in *E. coli* that only DNA polymerase II was stimulated in vitro by the homologous DNA-unwinding protein. Moreover, a significant inhibition of both DNA polymerase I and DNA polymerase III was observed. Nevertheless, Weiner et al. [3] have reported that the DNA-unwinding protein was also absolutely required in the conversion of the single stranded DNA of phage G4 into replicative form, catalyzed by DNA polymerase III holoenzyme. In both cases, optimum of stimulation occured with an amount of unwinding protein sufficient to cover all the single stranded regions of the template DNA.

In the field of eukaryotic replication, DNA-unwinding proteins had been described by Herrick and Alberts in calf thymus [4,5] and by Otto et al. in mouse cells [7]. These proteins are able to stimulate specifically the homologous DNA polymerase α [6,7].

A DNA-unwinding protein was purified to homogeneity from rat liver and its main properties had been studied. In the native state, the protein is a tetramer of 25 000 daltons subunit which is able to unwind the double helix of DNA by specific binding to single stranded portions of the nucleic acid. Here, we describe the in vitro stimulation (and even the inhibition) of rat liver α- and β-type DNA-polymerases by this DNA-unwinding protein.

2. Materials and methods

2.1. *Unwinding protein and enzymes*

Purification of rat liver DNA-unwinding protein (UP) was performed using differential DNA-cellulose affinity chromatography followed by phosphocellulose chromatography as described elsewhere[+].

Rat liver DNA polymerase α was prepared according to the procedure of the Recondo et al. [8,9]. DNA Polymerase β from the same source was isolated by native DNA-cellulose chromatography in a purification procedure partly common with that of unwinding protein.*

The effect of DNA-unwinding protein on the polymerizing activity of α- and β-type DNA polymerases was studied after preincubation of the template with unwinding protein; unless otherwise stated in the legends to the figures, the deoxyribonucleotide incorporation into an acid insoluble product was measured in initial velocity conditions.

2.2. *Nucleic acids and nucleotide*

Poly[d(A–T)] was purchased from Boerhinger Corp. (Germany) and poly(dC)·oligo(dG) prepared by hybridization of poly(dC) with $(dG)_{12-18}$ (from P.L. Biochemical USA) using a ratio of 2 μmol or 4 μmol poly(dC) for 1 μmol oligo(dG), as indicated in

[+] M. Duguet and A. M. de Recondo, manuscript submitted

* Specific activities of α- and β-type DNA polymerases were 18 000 and 3650 units/mg protein respectively. One unit is defined as the amount of DNA polymerase activity required to convert 1 nmol of total nucleotide h into acid-insoluble material, at 37°C, in the presence of poly(dC)·oligo(dG) as a template.

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the legends of the figures. Concentration of the template poly(dC)·oligo(dG) is given as the poly(dC) mononucleotide concentration. [^{3}H] Labelled deoxyribonucleotides were obtained from the Radiochemical Center (England).

3. Results

3.1. *Stimulation of DNA polymerase α*

The ability of rat liver DNA unwinding protein (UP) to stimulate in vitro the deoxyribonucleotide incorporation catalyzed by DNA polymerase α in the presence of poly(dC)·oligo(dG) as a template is strongly dependent of the UP/DNA weight ratio as shown in fig.1. In this case, for a template concentration of 26 μM, the maximum of stimulation is obtained with an UP/DNA ratio of 1.7. This ratio is much smaller than that necessary to cover all the single-stranded regions of the template (UP/DNA ratio of about 10) if we assume a binding of 1 protein subunit (25 000 daltons) per 7 nucleotides, as otherwise determined with single-stranded SV40 DNA. Inhibition observed at high UP/DNA weight ratio may correspond to the blocking of DNA polymerase α sites by unwinding protein on the template [10].

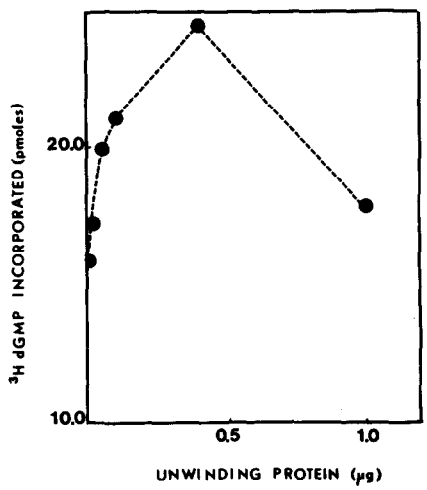

Fig.1. Effects of increasing amounts of unwinding protein on DNA polymerase α activity. Reaction mixture contained, in a total volume of 25 μl, 50 mM Tris–HCl, pH 8.6, 4 mM 2-mercaptoethanol, 3 mM $MgCl_2$, 260 μg/ml bovine serum albumin, 140 μM [^{3}H]dGTP (1.3 μCi/assay) and 26 μM poly(dC)·$(dG)_{12-18}$. Then, 0–1 μg unwinding protein was added and the mixture was incubated for 10 min at 4°C; DNA polymerase α (0.20 units) was added and a new 10 min incubation was performed at 37°C. 20 μl of the mixture were collected on glass fiber filters (Whatman GF/C) immediately dropped in 20 ml of 2% sodium pyrophosphate, 5% perchloric acid solution at 4°C. Filters were washed several times and dried. Incorporated radioactivity was counted in an Intertechnique liquid scintillation counter.

At a given UP concentration, a stimulatory or inhibitory effect is observed as a function of the poly(dC)·oligo(dG) template concentration but also as a function of the length of single stranded regions of this template (fig.2A). The stimulatory effect of the same concentration of UP on DNA polymerase α is detectable above 26 μM template concentration if the initiator template ratio is 1/4 and only above 50 μM template if this ratio is 1/2. An analogous observation has been made for DNA polymerase α and a DNA binding protein from mouse cells [7].

3.2. *Stimulation of DNA polymerase β*

The effect of rat liver DNA-unwinding protein on the polymerizing activity of DNA polymerase β in the presence of poly(dC)·oligo(dG) as a template is very similar to that observed with DNA polymerase α (fig.2B). Nevertheless, unlike the above results, the stimulatory effect of UP is higher when a template with short single stranded regions is used (oligo-(dG)·poly(dC) ratio of 1/2) than with long single stranded template (oligo(dG)·poly(dC) ratio of 1/4). Thus at the same concentration of UP the stimulation of DNA polymerase β appears above 26 μM template when the initiator/template ratio is 1/2 whereas no detectable stimulation of DNA polymerase β was found in the range from 0–100 μM when this ratio was 1/4. These two results are consistent with the hypothetical roles of DNA polymerase β as reparase or as gap-filling enzyme.

Stimulation of DNA polymerase β has also been tested on a double stranded template with few 3′-OH ends: the synthetic polymer poly[d(A–T)]. This template is known to be readily unwound (at 37°C) by rat liver UP at a protein/DNA weight ratio to 10. In this case a 2–5-fold stimulation of activity is observed depending on the template concentration (fig.3). At 20 μM of poly[d(A–T)] the stimulation is maximum for a UP/DNA weight ratio of 3/1 (i.e., smaller than the UP/DNA ratio necessary to unwind

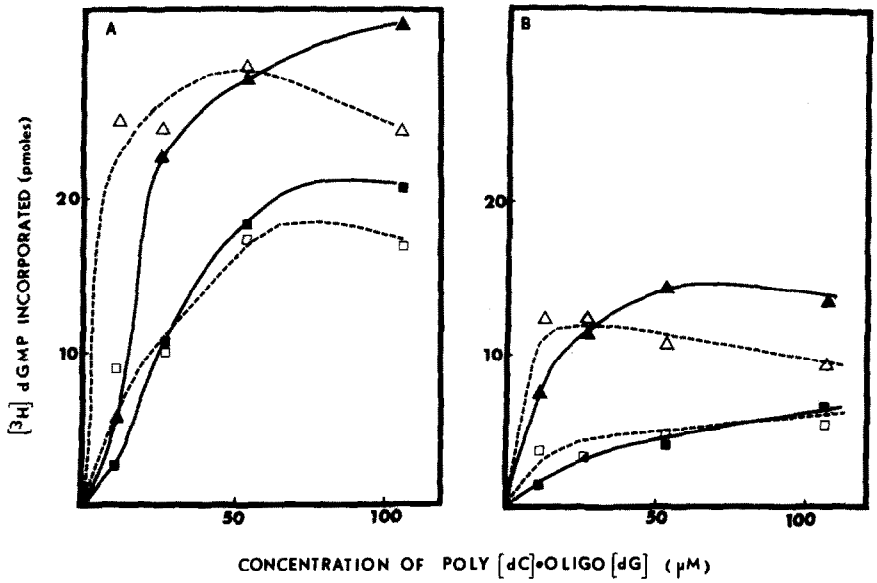

Fig.2. Stimulation of rat liver α- and β-type by rat liver unwinding protein on a poly(dC)·oligo(dG) template-primer. Reaction mixture was the same as that described in the legend of fig.1, excepted that the ratio oligo(dG)/poly(dC) was 1/4 or 1/2 as mentioned below and the concentration of UP was 1 μg/assay. (A) Effect of initiator-template concentration and of the length of single stranded region on DNA polymerase α stimulation. Oligo(dG)·poly(dC) at a ratio of 1/2 with (▲—▲) or without UP (△- -△), and at a ratio of 1/4 with (■—■) or without UP (□- -□). (B) Effect of initiator-template concentration and of the length of single stranded region on DNA polymerase β stimulation. Oligo(dG)·poly(dC) at a ratio of 1/2 with (▲—▲) or without UP (△- -△), and at ratio of 1/4 with (■—■) or without UP (□- -□).

totally the poly[d(A–T)]). On the other hand, we do not observe any inhibition in the low range of poly-[d(A–T)] concentrations (not shown). Kinetics of deoxyribonucleotide incorporation is only linear for 30 min with or without unwinding protein (fig.3B) while only 12% and 4% of the template, respectively, are copied after 60 min incubation.

Since Mg^{2+} concentration affects both DNA polymerase β activity and binding of the UP to DNA, the influence of this ion in stimulation process has been tested (fig.3C). Optimum Mg^{2+} concentration is 2 mM with poly[d(A–T)] alone and 4 mM in the presence of unwinding protein. Yet, stimulation is readily abolished above 10 mM Mg^{2+}. This result is consistent with the stabilization of the double helix by Mg^{2+} which is partially prevented by UP^{+}.

4. Discussion

Stimulation of DNA polymerases by an homologous unwinding protein can be interpreted in two ways. In the first hypothesis, protein may act on the structure of DNA, suppressing thermodynamic barriers to the progression of polymerase molecule along the DNA. This hypothesis is consistent with an optimal UP/DNA ratio smaller than that necessary for complete coating of single stranded portions of DNA or smaller than the UP/DNA ratio able to totally unwind the template.

In the second hypothesis, unwinding protein may form binary complexes with DNA polymerases by direct protein–protein interactions, or ternary complexes with the DNA [11]. This hypothesis implies specific interactions between DNA polymerases and unwinding proteins. Banks and Spanos [12,13] had reported that DNA synthesis of repair type, catalyzed by the unique DNA polymerase of the primitive eukaryote *Ustilago maydis*, was stimulated by homologous unwinding protein, but attempts to detect protein–protein interaction failed. Herrick and Alberts [6] with UP.1 of calf thymus (25 000 daltons),

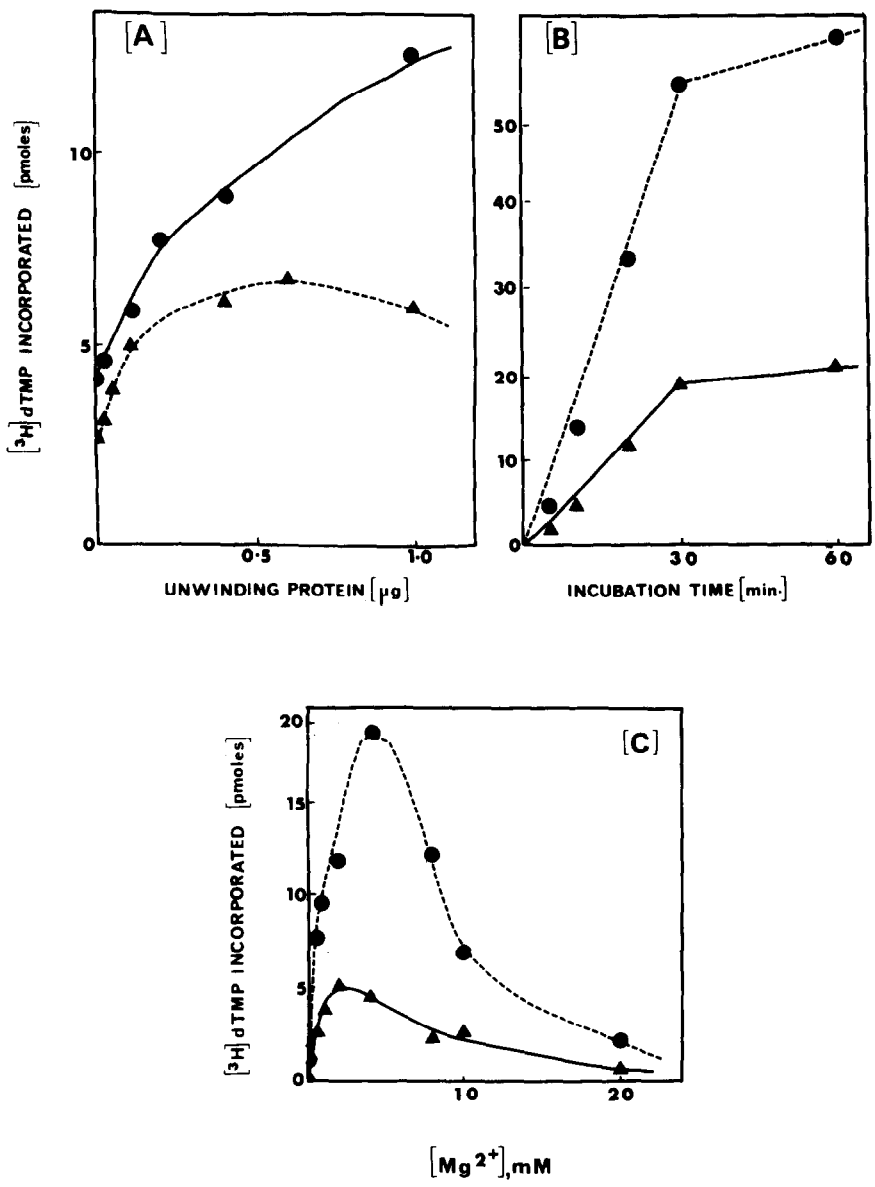

Fig.3. Stimulation of rat liver DNA polymerase β by rat liver unwinding protein on poly[d(A–T)] template. Reaction mixture contained, in total vol. 30 μl, 83 mM Tris–HCl, pH 8.6, 6.6 mM 2-mercaptoethanol, 0–20 mM $MgCl_2$, 2.5 mM KCl, 600 μg/ml bovine serum albumin, 200 μM dATP, 200 μM [^{3}H]dTTP (1.0 μCi/assay) and 20 μM or 50 μM poly[d(A–T)]. Then 0–1 μg unwinding protein was added and the mixture was incubated 10 min at 4°C. Then 2 μl DNA polymerase β (0.25 units) was added and after an incubation time at 37°C indicated below, 25 μl mixture as collected on glass fiber and counted as described in the legend of fig.1. (A) DNA polymerase-β activity at 5 mM Mg^{2+} in the presence of increasing amounts of unwinding protein at 20 μM (▲- - -▲) or 50 μM (●—●) poly[d(A–T)]. Incubation time, 10 min. (B) Time course of polymerization at 5 mM Mg^{2+} and 50 μM poly[d(A–T)] in the presence (●- - -●) or in the absence (▲—▲) of 1 μg unwinding protein. (C) Influence of magnesium ions on DNA polymerase β activity at 50 μM poly[d(A–T)] in the presence (●- - -●) or in the absence (▲—▲) of 1 μg unwinding protein. Incubation time, 10 min.

Otto et al [7] with an unwinding protein from mouse cells (33 000 daltons) had observed a specific stimulation for DNA polymerase α and not for DNA polymerase β from the same source. The phosphorylation of the mouse cells binding protein drastically reduced its effect on the polymerizing activity of DNA polymerase α although it did not interfere with the binding of the protein to single stranded DNA. In contrast, our results show a stimulatory effect of rat unwinding protein (25 000 daltons) on the two homologous DNA polymerases (α- and β-type). This difference might be explained by the extreme sharpness of the stimulation range in regards to the unwinding protein/DNA ratio. Another possibility is that stimulation by rat liver unwinding protein is not strictly specific of one DNA polymerase, but due to action of this protein on DNA structure as well as to direct interaction with the enzymes. This last possibility is supported by the slight stimulation of *E. coli* DNA polymerase I by rat liver unwinding protein observed on poly[d(A–T)] template (not shown). Further experiments are required before a conclusion concerning the specific interaction between mammalian DNA-unwinding proteins and homologous DNA polymerase(s).

Acknowledgements

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