DIFFERENTIAL EFFECTS EXERTED BY RNA POLYMERASES IN VITRO ON THE DNA POLYMERASES I AND II FROM BHK-21/C13 CELLS

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1. Introduction

Two major species of replicative DNA-dependent DNA polymerase have been identified in a variety of proliferating tissues and cell lines. These can be separated by density-gradient centrifugation into species with sedimentation coefficients of 3.3 S and 6–8 S. The apparent subcellular distribution and in vitro characterisation of both species have recently been reviewed [1]. Such species have been identified, partially purified and characterised from total cell extracts of logarithmically growing BHK-21/C13 (baby hamster kidney) cells [2]. In addition, the cellular content of the 6–8 S species has been shown to bear a direct and positive correlation with the degree of cell proliferation not only in BHK-21/C13 cells [3,4], but also in several other tissues and cell lines [5–10]. The amount of the 3.3 S enzyme appears in comparison to be relatively independent of the rate of cell proliferation [3–10]. In the light of present concepts concerning the initiation by RNA of DNA replication in both prokaryotic and eukaryotic organisms [11–16], experiments were carried out to investigate the possible in vitro initiation of DNA synthesis by both BHK cell DNA polymerase I (6–8 S) and BHK cell DNA polymerase II (3. 3 S), using a denatured DNA template, the polyribonucleotide primer being produced in vitro by the action of either *Escherichia coli* RNA polymerase or BHK cell RNA polymerases. These experiments revealed that the activities of BHK cell DNA polymerases I and II were stimulated by the inclusion RNA polymerases when assayed in the presence of a denatured DNA template but in the absence of ribonucleoside 5′-triphosphates. This paper describes the nature of the stimulatory effect.

2. Materials and methods

2.1. *Materials*

BHK cell DNA polymerases I and II were prepared by the method of Craig and Keir [2], and BHK cell DNA-dependent RNA polymerases A and B by the method of Cooper and Keir [17]. *E. coli* RNA polymerase purified by the method of Chamberlin and Berg [18] with an additional glycerol gradient step, was a gift from Dr J. O. Bishop, Department of Genetics, University of Edinburgh. Pancreatic deoxyribonuclease (ribonuclease-free), and calf thymus DNA type V were obtained from the Sigma London Chemical Company Ltd., London, UK; deoxyribonucleoside 5′-triphosphates from Boehringer Mannheim GmbH, Mannheim, Germany; [methyl-^{3}H]thymidine 5′-triphosphate from the Radiochemical Centre, Amersham, UK; ovalbumin from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA; bovine serum albumin (BSA; fraction V from bovine plasma) from Armour Pharmaceutical Company Ltd., Eastbourne, UK.

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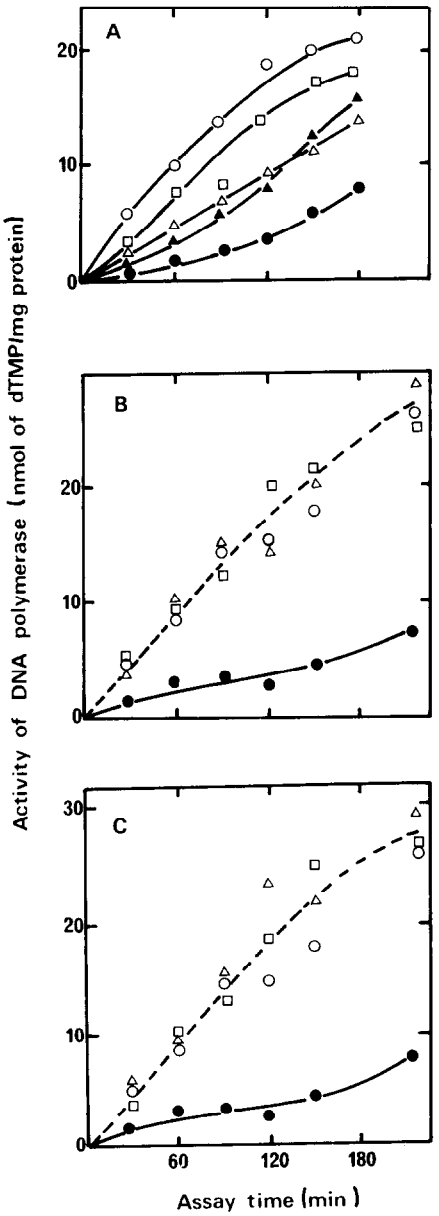
2.2. *Assay for DNA polymerase activity*

Assays for BHK cell DNA polymerases I and II were carried out as described by Craig and Keir [2], with modifications as indicated in the legends to figs. 1 and 2.

DNA polymerase activity was expressed as nmol of dTMP residues incorporated per mg of protein.



2.3. *Heat-denaturation*

The calf thymus DNA and, where appropriate, protein samples were denatured by heating at 100°C for 10 min, followed by immediate cooling to 0°C in an ice-water bath.

2.4. *Treatment of protein preparations with deoxyribonuclease*

Pancreatic deoxyribonuclease was added to 200 μl aliquots of the proteins under investigation to give a final concentration of 7.5 μg/ml. Incubation was then carried out at 37°C for 30 min in the presence of 5 mM $MgCl_2$ and 50 mM Tris–HCl buffer, pH 7.5 at 37°C. A parallel series of incubations was carried out in the absence of deoxyribonuclease. Samples were then denatured by heating at 100°C for 10 min. It was found that if deoxyribonuclease was added to greater concentrations, then in the presence of high protein concentrations (for example, BSA at 2 mg/ml), deoxyribonuclease activity was retained even after the heat denaturation treatment.

3. Results

In the following series of experiments all DNA polymerase assays were carried out with measurements

Fig. 1. Stimulation of DNA polymerase I by protein. Assays (100 μl) used conditions optimal for DNA polymerase I, with heatdenatured calf thymus DNA (25 μg) replacing 'activated' DNA as the primer-template, and in the presence of additional proteins as cited below. Prior to the addition of DNA polymerase I and deoxyribonucleoside 5'-triphosphates, the other components were incubated in 80 μl for 3 min at 37°C, followed by immediate cooling in an ice-water bath. DNA polymerase I (1.8 μg/assay) and triphosphates were then added, the assays were reincubated at 37°C, and subsequently treated as standard DNA polymerase assays [2]. The additional proteins when present, were in the following amounts: BSA 40 μg; BHK RNA polymerase A, 8.9 μg; BHK RNA polymerase B, 5.5 μg; *E. coli* RNA polymerase, 7.3 μg.
A: ●, DNA polymerase I alone; ○, plus BSA; □, plus BHK RNA polymerase A; ▲, plus BHK RNA polymerase B: △, plus heat-denatured BHK RNA polymerase B.
B: ●, DNA polymerase I alone: △, plus BSA and BHK RNA polymerase A; □, plus BSA and heat-denatured BHK RNA polymerase A; ○, plus BSA.
C: ●, DNA polymerase I alone; △, plus BSA and *E. coli* RNA polymerase; □, plus BSA and heat-denatured *E. coli* RNA polymerase; ○, plus BSA.

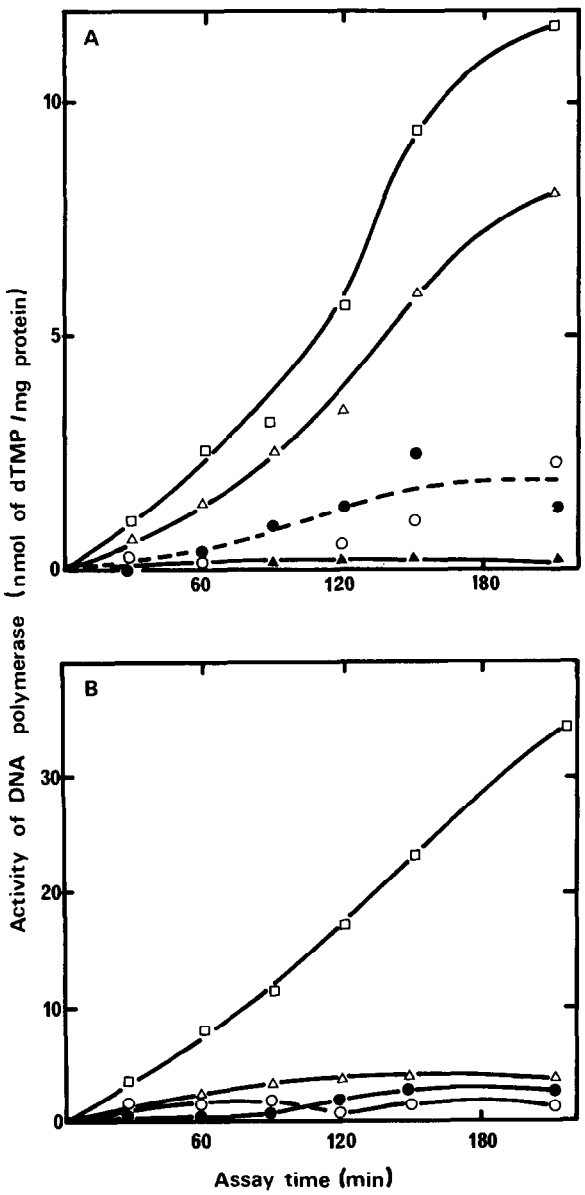


Fig. 2. Stimulation of DNA polymerase II by RNA polymerase. The DNA polymerase was assayed as described in fig. 1 but with 0.45 μg of polymerase II and under optimal conditions [2]. A: •, DNA polymerase II alone; □, plus BHK RNA polymerase A; △, plus BHK RNA polymerase B; ▲, plus heat-denatured BHK RNA polymerase A; ○, plus BSA. B: •, DNA polymerase II alone; □, plus BSA and *E. coli* RNA polymerase; △, plus BSA and heat-denatured *E. coli* RNA polymerase; ○, plus BSA.

being made over a time course using a denatured DNA primer-template, and in the absence of ribonucleoside 5′-triphosphates, as described in the legend to fig. 1.

3.1. *Stimulation of DNA polymerase I*

Fig. 1a clearly demonstrates a 2–3-fold stimulation of the activity of DNA polymerase I in the presence of BHK cell RNA polymerase A or B. However a similar stimulation also occurred not only on the addition of BSA, but also in the presence of heat-denatured RNA polymerase B. Such stimulation was also observed in the presence of heat-denatured BHK RNA polymerase A or of heat-denatured *E. coli* RNA polymerase. Figs.

Table 1

Effects of protein additions to DNA polymerase I on its activity in the standard assay

Added protein and the preliminary treatment	Total protein per assay (μg)	Stimulation factor
BHK RNA polymerase A:		
(i) heated	11.0	3.2
(ii) DNase, then heated	11.0	3.1
BHK RNA polymerase B:		
(i) heated	8.0	2.6
(ii) DNase, then heated	8.0	3.3
E. coli RNA polymerase:		
(i) heated	9.0	2.6
(ii) DNase, then heated	9.0	2.4
Ovalbumin:		
(i) heated	42.0	2.4
(ii) DNase, then heated	42.0	2.5
BSA:		
(i) heated	42.0	2.9
(ii) DNase, then heated	42.0	2.8
DNase:		
heated in glycerol buffer	5.0	2.7

* The activity of the DNA polymerase I alone was 0.27 nmol of dTMP residues incorporated/mg of protein in 180 min at 37°C. The assay (100 μl) included 1.9 μg of purified DNA polymerase I and 25 μg of heat-denatured calf thymus DNA as template. Conditions were otherwise standard [13].

1b and 1c, confirm the stimulatory effect exerted by BSA, and also demonstrate the absence of any summation of the stimulatory effects when the assays were carried out in the presence of both BSA and RNA polymerases in either a native or heat-denatured form.

The continued stimulatory activity after heat-denaturation excluded the possibility that the stimulation might have been due to template modification by deoxyribonuclease contaminants. However, although the observations suggest that the stimulation of DNA polymerase I might be due to a general, protective effect of protein, the possibility remained that oligodeoxyribonucleotide contaminants in the added protein fractions could be annealing to the denatured DNA template, thereby providing additional 3′-hydroxyl termini which in turn could initiate further synthesis of DNA. In order to test such a hypothesis, a number of proteins were incubated with pancreatic deoxyribonuclease, heat-denatured to destroy the deoxyribonuclease activity, and finally tested for stimulatory activity over an incubation period of 180 min. The results (table 1) confirmed that all added proteins, whether simply heat-denatured or treated with deoxyribonuclease prior to heat-denaturation, stimulated the action of DNA polymerase I to a similar extent. All proteins that had been exposed to the preliminary incubation with deoxyribonuclease were free from nuclease activity after heat-denaturation, as assessed by their failure to release acid-soluble radioactivity from native ^{3}H-labelled DNA substrate from *Bacillus subtilis*.

3.2. *Stimulation of DNA polymerase II*

The activity of purified BHK cell DNA polymerase II on a denatured DNA template is at best poor [2]. However, in the presence of either of the BHK cell RNA polymerases or of *E. coli* RNA polymerase, a substantial stimulation was apparent (figs. 2a,b), the former RNA polymerases promoting a 2–3-fold stimulation, and the latter a 10-fold stimulation. Under identical conditions BSA did not stimulate DNA polymerase II, nor did any of the RNA polymerases after heat-denaturation. All BHK cell RNA polymerase preparations were free from contaminating exo- and endodeoxyribonuclease activity: *E. coli* RNA polymerase was free from exonuclease but was not tested for endodeoxyribonuclease activity. Furthermore, all RNA polymerase preparations were free from DNA polymerase activity.

The possibility that the deoxyribonucleoside 5′-triphosphates contained ribonucleoside 5′-triphosphate contaminants, thereby allowing limited transcription by the native RNA polymerases and hence providing an oligoribonucleotide as a primer for the DNA polymerase, was also considered. However, when UTP, ATP, CTP and GTP, were added (0.02 mM each) during the time course, no further stimulation was observed. Moreover, under our standard assay conditions, DNA polymerase II, unlike DNA polymerase I, could not utilise an RNA-primed DNA template [19]. Thus the stimulation of DNA polymerase II activity that we have observed is *not* (i) a function of protein concentration; (ii) due to the presence of oligodeoxyribonucleotide initiators (which would not be destroyed by heat denaturation); (iii) due to template modification by deoxyribonuclease activity; (iv) due to the presence of DNA polymerising activity in the RNA polymerase fractions; (v) due to RNA-primed initiation of DNA synthesis.

4. Discussion

Investigation of the apparent stimulation of BHK cell DNA polymerases I and II in the absence of ribonucleoside 5′-triphosphates, clearly demonstrates a specific stimulation by both prokaryote and eukaryote RNA polymerases of DNA polymerase II on a denatured primer-template, as opposed to an apparently non-specific stimulation by protein, in either a native or denatured form, of DNA polymerase I.

The apparent inability of BHK cell DNA polymerase II to read regions of single-strandedness on DNA, has already been documented for the BHK enzyme [2], and a similar enzyme from KB cells [20]. Thus it seems reasonable to speculate that the stimulation of this activity by RNA polymerases could be due to a direct protein–protein interaction, resulting in the formation of a more stable polymerising complex on the DNA template.

BHK cell DNA polymerase I consists of a heterogeneous group of DNA polymerase activities of relatively high molecular weight [2,3]. Whether all these activities contain a common DNA polymerising subunit(s) suitably modified by other as yet unspecified subunits remains unresolved. However, the apparently general stimulation by protein is consistent with the existence

of a complex subunit structure, high protein concentrations reducing the rate of dissociation of such a structure under the conditions of assay, and hence giving rise to the apparent stimulatory effect of added protein. Whether such observations have any bearing on the true in vivo situation remains open to question.

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