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DNA BINDING PROTEINS FROM CALF THYMUS WITH AN ENHANCED ABILITY TO STIMULATE DNA POLYMERASE α IN VITRO

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SUMMARY

We have isolated from calf thymus glands a new class of single-stranded DNA binding proteins (DBP) specifically endowed with the ability to stimulate DNA polymerase α activity in vitro. Such result was attained by using a partially new purification procedure and a functional assay, i.e. stimulation of DNA polymerase α on poly(dAT) throughout purification. We observed stimulations up to 30-fold of DNA polymerase α on poly(dAT) at a protein:DNA ratio of 1:1, that is much below the saturating level. Such stimulation is specific for DNA polymerase α. These results indicate a possible direct functional interaction between our DBP, polymerase α and the template.

INTRODUCTION

In bacteria and bacteriophages specific DNA binding proteins (DBP) have been shown to be required for chromosome DNA replication (1). In vitro studies indicate that one of the probable roles of DBP is that of extending the single-stranded DNA, thus easing the advancement of DNA polymerase. On the other hand, experiments on the stimulation of DNA polymerase in vitro by several DNA binding proteins indicate a certain degree of specificity with regard to the type of DNA polymerase stimulated. In any case, the stimulations observed are relatively modest and not very reproducible (2). This is also true for the DBP purified from various eukaryotic sources (see Results and Discussion) and for the DBP isolated from calf thymus glands (3,4,5). Since in vivo these proteins are likely to exist in complexes with other proteins (6) or smaller molecules, it is possible that different purification procedures might yield products with similar physicochemical properties but with different functional characteristics. For example, different levels of phosphorylation or acetylation or a limited protease digestion could produce significant effects on functional activities (2,7).

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Since the purpose of this work was that of isolating a particular class of DBP specifically endowed with the ability to stimulate DNA polymerase, we tried to purify the DNA binding proteins from calf thymus using a milder purification procedure and a functional assay, i.e. the ability of DBP to stimulate DNA polymerase α in vitro on poly(dAT).

MATERIALS AND METHODS

Purification procedure

100 g of calf thymus glands from 9-week-old calves were processed as described by Herrick and Alberts (3) up to the two (DS-DNA cellulose and SS-DNA cellulose) columns linked in series. After this step our procedure was different. The material bound to the above SS-DNA cellulose was eluted in three steps with two column volumes (120 ml) of Buffer A (Tris-HCl pH 8 20 mM; NaCl 50 mM; EDTA 1 mM; DTT 0.1 mM; glycerol 5%) containing 0.25 M, 0.5 M and 1.0 M NaCl, respectively. The three eluates were then processed separately and identified as 0.25 M, 0.5 M and 1.0 M fractions. Starting from this step, samples were assayed for both stimulation of DNA polymerase α activity and binding to SS-DNA under the conditions described below.

The three eluates from the SS-DNA cellulose column were precipitated with 75% saturated $(NH_4)_2SO_4$ and resuspended in 10 ml of Buffer A + 0.1 M NaCl. The samples were then applied to a 3 ml DEAE-cellulose column equilibrated with the same buffer. In all three cases DNA binding and polymerase stimulatory activity were found in the flow-through. The flow-throughs were dialyzed against Buffer HA (KPO_4 pH 7.5 10 mM; NaCl 50 mM; DTT 0.1 mM; glycerol 5%) and applied to a hydroxyapatite (HA) column (vol.: 5 ml; DNA grade HPT, Bio-Rad) equilibrated with the same buffer. Proteins were eluted with a gradient (25 ml + 25 ml) 10 mM - 500 mM KPO_4 in Buffer HA. The fractions most active in stimulating DNA polymerase α on poly(dAT) were pooled, concentrated by dialysis against solid sucrose to a final volume of 1 ml and fractionated on a Sephadex G-50 column (1.5 cm x 60 cm). The active fractions were pooled, concentrated against solid sucrose, dialyzed against Buffer HA + 50% glycerol and stored at -25°C.

For the sake of comparison the so-called UP1 proteins Acidic (A), Basic (B) and Mixed (M) fractions (3) were also purified from 100 g of glands following the procedure described by these authors.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Samples were run on a slab-gel apparatus. Gels (1.5 mm x 10 cm) 8% polyacrylamide with 5% polyacrylamide stacking were prepared in Tris-HCl-SDS buffer according to Laemmli (8). 2-5 µg of DBP per well were applied. Protein standards used for molecular weight calibration were: Phosphorylase B (94000); Bovine Serum Albumin (67000); Ovoalbumin (43000); Carbonic Anhydrase (30000); Soybean Tripsin-Inhibitor (21000).

DNA polymerase α assay

DNA polymerases α and β purified according to Spadari and Weissbach (9) were provided by S. Spadari and G. Pedrali-Noy. The reaction mixture contained in 100 µl final: KPO_4 pH 7.5, 5 mM; $MgCl_2$ 5 mM; DTT 0.5 mM; dATP 100 µm; ^{3}H-dTTP 20 µM, 1000 cpm/pmol; poly(dAT) (Serva) 10 µg/ml; DBP, in the indicated amounts, and DNA polymerase α or β 0.25 and 1.0 units, respectively.

The reaction was started by adding the DNA polymerase to the otherwise complete mixture. Incubation was performed at 35°C for 30 min. 50 µl samples were taken at 15 and 30 min and the radioactivity incorporated into acid-precipitable material was measured. In the reactions with activated CT-DNA the above conditions were used with the following changes: dCTP, dGTP, dATP 100 µM each; dTTP 20 µM, 1000 cpm/pmol; activated CT-DNA (10) 12 µg/ml.

DNA filter binding assay

The retention by DNA binding proteins of single-stranded and double-stranded DNA on cellulose nitrate filters was measured according to Carrara et al. (11). 0.1 µg of ØX-^{3}H DNA (50000 cpm/µg) (SS) or SPP1-^{3}H DNA (25000 cpm/µg) were used per filter.

RESULTS AND DISCUSSION

Starting from the assumption that stimulation and binding to SS-DNA might not be necessarily coincident properties of the same protein, we developed a purification procedure aimed at isolating proteins peculiarly endowed with the capacity to stimulate DNA polymerase α on suitable duplex DNA templates *in vitro*. The template we chose for the assay was poly(dAT), the alternating co-polymer that, in aqueous solution, can assume various configurations, some of which might mimic the structure of a replicative fork (2). The double-stranded structure of this polymer is relatively weak so that the destabilization of the duplex region by a DBP at physiological temperature is facilitated. On the other hand, the opening of the double-stranded structure ahead of a progressing DNA polymerase is certainly an important factor (but probably not the only one) in the stimulation of enzyme ac-

TABLE I - Purification of DBP from calf thymus

1)	Total homogenate
2)	Low speed centrifugation
3)	Polyethylene-glycol/2M NaCl partition
4)	DNA free supernatant applied to DS-DNA cellulose and SS-DNA cellulose columns (connected in series)
5)	SS-DNA cellulose column eluted in three steps with Buffer A + 0.25 M, 0.5 M and 1.0 M NaCl, respectively
6)	Assay of the three eluates for SS-DNA binding to filters and stimulation of DNA polymerase α
7)	Application of the three eluates to DEAE cellulose columns; binding proteins recovered in the flow-through
8)	Application of DEAE flow-through to hydroxyapatite columns
9)	Elution with K-phosphate buffer gradient (10-500 mM) Assay fractions for SS-DNA binding and DNA polymerase α stimulation
10)	Pool of fractions stimulating DNA polymerase α , concentration and gel filtration on G-50 column
11)	DNA polymerase α stimulatory fraction pooled, concentrated and stored at -25°C

tivity particularly for DNA polymerase α, which has a strict requirement for a single-stranded template structure.

Purification procedure

The purification procedure of the binding proteins from calf thymus glands is schematically shown in Table I. The dextran sulfate washing step of the SS-DNA cellulose used by Herrick and Alberts (3) was eliminated since it could be too strong and remove some less tightly bound proteins; also, this compound is a potent inhibitor of DNA polymerase α and even trace amounts would interfere with the subsequent assays. Proteins bound to the SS-DNA cellulose were eluted and further purified as described in Materials and Methods. Fig. 1 shows the elution profiles of the 0.25 M, 0.5 M and 1.0 M NaCl fractions. It is evident that the major peaks of stimulatory activity do not coincide with the peak of affinity for SS-DNA. Stimulation of DNA polymerase is not due to any aspecific activation of the template, as proven by the fact that no detectable degradation of poly(dAT) during the reaction was observed. On the contrary, the elution profiles of DNA polymerase α stimulating activity and SS-DNA binding from the last column (Sephadex G-50) were superimposed (data not shown). At this stage the proteins were highly purified and free of any detectable enzymatic activity. It should be observed that the overall yield of our procedure (see Table II) is about 5 times lower than that of the UP1 proteins purified according to Herrick and Alberts (3). The general properties of DBP are summarized in Table II.

SDS-Polyacrylamide Gel-Electrophoresis

SDS-polyacrylamide gel-electrophoresis was performed as described in Materials and Methods. For the sake of comparison, we also analyzed in parallel the three fractions A (Acidic), B (Basic) and M (Mixed) of the UP1 DBP (3). Results are shown in Fig. 2. The 0.25 M purified fraction is composed of 4 polypeptides with MW of approximately 22000, 24000, 25000 and 27000. The 0.5 M fraction contains the 24000 and 27000 polypeptides and only traces of the 25000 one. The 1.0 M fraction contains a 33000 polypeptide and traces of others, with MW ranging from 30000 to 40000. As to the UP1 pattern, the (A) fraction is composed of two polypeptides of 22000 and 25000, the (B) fraction has only one polypeptide with MW 24000 while, as expected, the (M) fraction contains all three polypeptides 22000, 24000 and 25000. Thus our 0.25 M

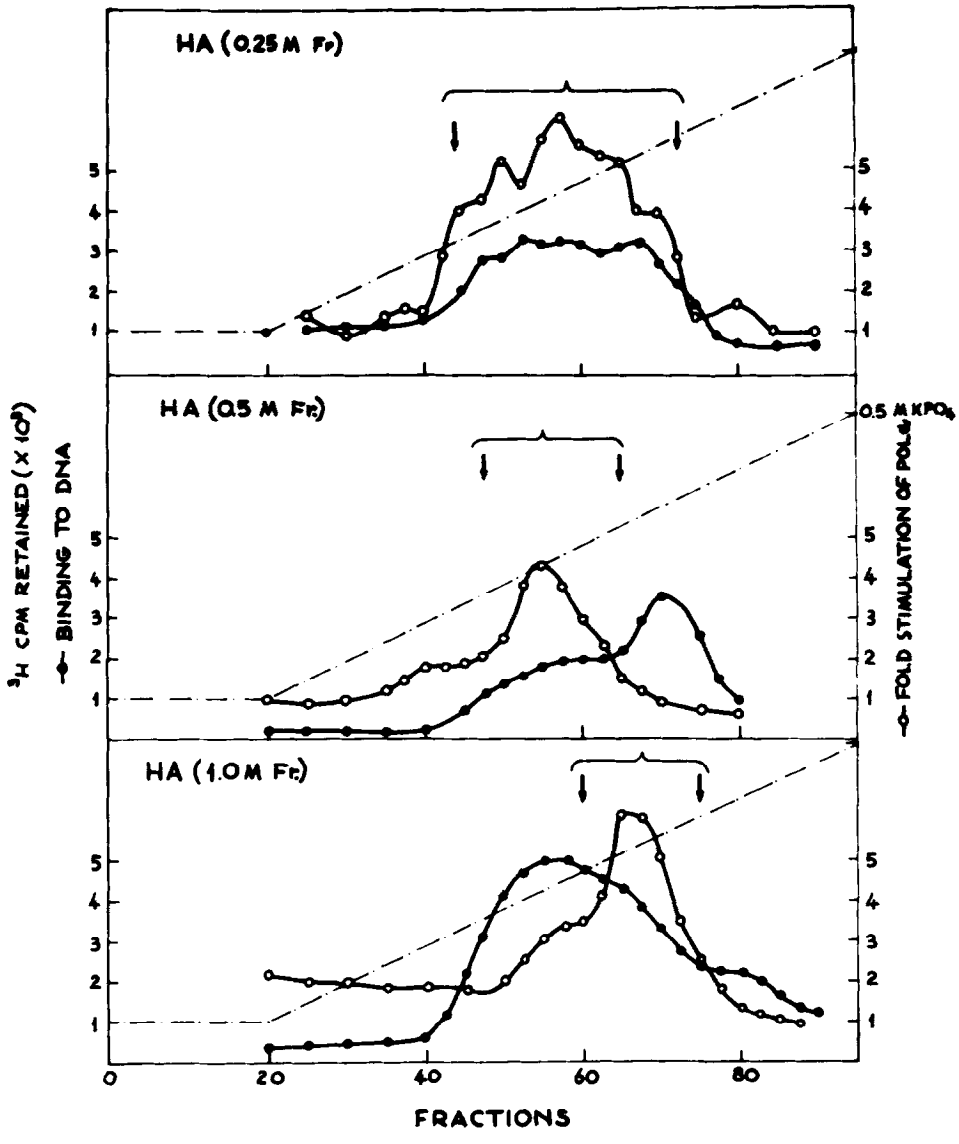

Fig. 1 - Hydroxyapatite columns of the three DBP fractions (0.25 M, 0.5 M, 1.0 M). DNA polymerase α stimulation assay was performed as described in Materials and Methods. Possible "activation" of DNA by the fractions tested was checked separately and was found to be negative. Also, the active fractions were free of any polymerase activity (data not shown). Binding assays were performed as described in Materials and Methods. 5 µl per fraction were assayed for their ability to retain 0.1 µg ^{3}H-ØX DNA on nitrocellulose filters. The fractions between arrows were pooled and further purified.

and 0.5 M fractions resemble the UP1 (M) proteins, but contain one additional polypeptide of 27000. Also, the relative proportion of the various components is different from that of UP1 (M). On

TABLE II - Properties of DNA binding proteins from calf thymus

Final yield	0.5-0.7 µg/100 g tissue (0.25 M and 0.5 M Fr.) 0.2-0.3 µg/100 g tissue (1.0 M Fr.)
Molecular weight	multiplicity of forms: 22000-27000 (0.25 M and 0.5 M species) and higher 33000 (1.0 M species)
Exonuclease-endonuclease activity	none
DNA polymerase activity	none
DNA binding	specific for SS-DNA
Duplex (polydAT) destabilization	yes
DNA polymerase stimulation	Different for the three species (0.25 M, 0.5 M, 1.0 M). Specific for DNA polymerase α; maximum on poly(dAT), lower on activated CT-DNA

the contrary our 1.0 M fraction is totally different and probably resembles the proteins eluting at higher ionic strength from the SS-DNA column, observed by Herrick and Alberts (3) but not further characterized.

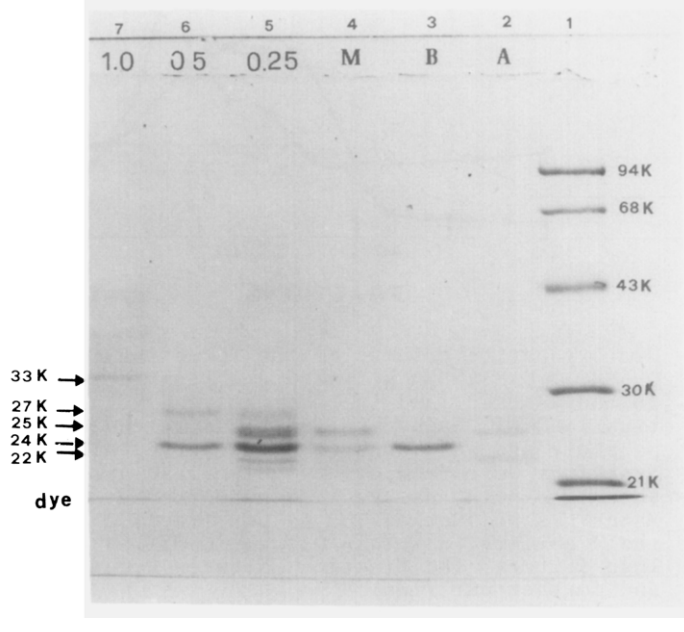

Fig. 2 - SDS-polyacrylamide gel electrophoresis performed as described in Materials and Methods. Lanes, from right to left: 1) MW standards; 2) UP1 (A); 2) UP1 (B); 3) UP1 (M); 4) 0.25 M fr.; 5) 0.5 M fr.; 6) 1.0 M fr.

TABLE III - Stimulation of DNA polymerase α and β by DBP from calf thymus

DNA template	dNMP incorporated in 30 min (pmol/assay) -DBP	+0.25M fr.	+0.5M fr.	+1.0M fr.
		DNA polymerase α		
poly(dAT)	1.3	37.9 (29.2)	19.5 (15)	10.5 (8.1)
Activated CT-DNA	12.3 -	13.2 (1.1)	43.0 (3.5)	61.5 (5)
		DNA polymerase β		
poly(dAT)	2.1	3.8 (1.8)	2.8 (1.3)	2.8 (1.3)

Number in brackets represents fold stimulation

The data for activated DNA vary from one activated DNA preparation to another. The reported stimulations were obtained with a protein:DNA ratio of 1:1. At higher ratios no further stimulation is observed

For other experimental details see Materials and Methods

Stimulation of DNA polymerase α in vitro

The UP1 proteins we have purified give only modest stimulation of polymerase α on a poly(dAT) template. More precisely, using a 10:1 w/w ratio between protein and DNA (nearly saturating conditions) we observed a 2-3 fold stimulation by UP1 (M) and (B) species while species (A), under the same conditions, did not stimulate but was in fact slightly inhibitory. No significant stimulation by any of the UP1 species was observed on activated or denatured CT-DNA (data not shown). These data are basically in agreement with those reported by other authors on the same or on other DBP, even if such reports are often contradictory and obtained under different experimental conditions (2,5,12,13,14,15).

On the other hand, the DBP purified with our procedure exhibit a pattern of stimulation of polymerase α on poly(dAT) which is strikingly different. With the 0.25 M fraction stimulations up to 30-fold are obtained at a protein:DNA ratio of 1:1 w/w. Under the same conditions, the 0.5 M and the 1.0 M fractions give 15-fold and 9-fold stimulations, respectively (see Table III). In all three cases, no significant increase is observed at higher protein:DNA ratios. Therefore we found maximum stimulation at protein:DNA ratios which are less than stoichiometric. This in-

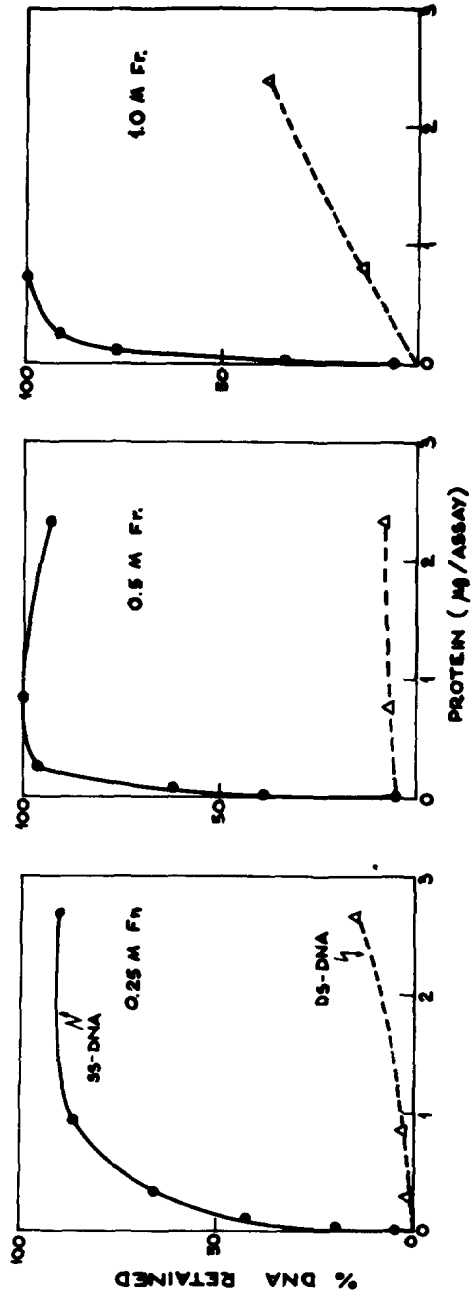

Fig. 3 - DNA filter binding assay on the three purified DBP fractions (0.25 M; 0.5 M; 1.0 M) was performed as described in Materials and Methods. 0.15 µg of ^{3}H-ØX DNA (single-stranded) and 0.15 µg of ^{3}H-SPP1 DNA (double-stranded) per assay were used. In the absence of DBP about 5% of the single-stranded DNA was retained by filters. The lowest protein concentration corresponds to 1:3 (w/w) protein:DNA ratio.

dicates that the effect we observed is not simply due to an extending effect on the DNA. Other factors are probably involved and the specificity of stimulation for DNA polymerase α suggests at least a functional interaction between the DBP and the enzyme. In fact, no significant stimulation was observed on DNA polymerase β (Table III) and E.coli polymerase I (data not shown). As to the activity of DNA polymerase α on activated CT-DNA we observed some stimulation with the 0.5 M and the 1.0 M fractions (but not with the 0.25 M fraction); the extent of stimulation is however somewhat variable and seems to depend on the state of activation of DNA which is a rather vaguely controlled parameter.

Why are our 0.25 M and 0.5 M fractions more effective in stimulating DNA polymerase α than the UP1 (B) and (M) fractions? We would like to propose a number of possible explanations.

The SDS-gel electrophoresis (Fig. 2) shows that our 0.25 M and 0.5 M fractions share three bands with the UP1 (M) and (B) fractions, namely the 22000, 24000 and 25000 polypeptides. However, they contain one additional band of approximately 27000. Herrick and Alberts (3) have suggested that such multiplicity of forms could arise from a limited exopeptidase digestion of an original polypeptide precursor. It is therefore possible that the 27000 protein represents the undigested (or less digested) form which is specifically endowed with the capacity of stimulating the polymerase α. Alternatively, by virtue of our specific assay we might have selected a subclass of DBP carrying a specific chemical modification or a particular combination of unmodified proteins possibly forming an active complex with the DNA template-primer structure.

In any case, these data clearly indicate that the stimulation of DNA polymerase α by DBP is not simply related to their ability to bind SS-DNA and to destabilize duplex DNA in vitro. In fact the 0.25 M, the 0.5 M and the 1.0 M fractions can lower the melting temperature of poly(dAT) to the same extent (data not shown); in addition, their affinity for SS-DNA, as measured by the filter-binding assay (see Materials and Methods) is comparable (see Fig. 3). Actually, if any difference can be envisaged between the three types, it seems to suggest that the species with more affinity for DNA are the least stimulatory. In this context, it is clear that the possibility of a physical interaction between the binding proteins we have isolated and DNA polymerase α should be care-

fully investigated. Furthermore, we have recently observed (data to be published) that the stimulatory effect on polymerase α of the DBP described here is more than additive with the stimulation produced by a DNA-dependent ATPase isolated in our laboratory from HeLa cells (16). Also this finding points to a functional interaction between the proteins involved in DNA replication.

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