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Total purification of a DNA-dependent ATPase and of a DNA-binding protein from human cells

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We have purified to near homogeneity the major DNA-dependent ATPase from human cells. The pure enzyme has a mol. wt. of 68 000 and a minimum specific activity of ~150 U/mg. When the properties of the pure enzyme are compared with those of a less purified preparation, significant differences are observed both in structure and in function. These can be ascribed to the interaction of the ATPase with a DNA-binding protein (mol. wt. 28 000) that we can also purify to near homogeneity from the same cells and which is present in the less purified preparations of the ATPase. The ability of the less purified ATPase to stimulate DNA polymerase α in helicase fashion is probably due to the presence of the DNA-binding protein.

Key words: DNA-dependent ATPase/DNA-binding protein/stimulation of DNA polymerase α

Introduction

We have described the isolation and some of the properties of the major DNA-dependent ATPase activity from HeLa cells (Cobianchi *et al.*, 1979, 1982). We report here the total purification of this enzyme and its main physico-chemical properties. We also describe the purification of a DNA-binding protein which partially co-purifies with the ATPase and whose association with it might account for some of the functional properties observed in earlier, less pure preparations of the ATPase.

Results

The purification of the DNA-dependent ATPase is summarized in Table I. The enzyme binds tightly to DNA-cellulose. Figure 1 shows the elution profiles of the proteins and of the enzyme activity from the single-stranded DNA-cellulose columns with the potassium phosphate gradient (A) followed by the NaCl gradient (B).

Figure 2 shows the elution profile from the ATP-agarose column. The shadowed area is Fraction IV. The ATPase in Fraction IV is ~20% pure as judged by SDS-polyacrylamide gel electrophoresis (see later).

Electrophoretically homogenous enzyme was obtained by running Fraction IV on a non-denaturing polyacrylamide gel. The experiment is shown in Figure 3; the gel slices containing ATPase activity were cut out and applied directly onto an SDS-polyacrylamide slab gel (Banks *et al.*, 1979); a single protein band is observed with a mol. wt. of ~68 000. The same band is visible also in the electropherogram of Fraction IV (ATP-agarose column) where it constitutes about one fifth of the total protein (see Figure 3). From the experiment shown in Figure 3 the specific activity of the pure enzyme can be indirectly calculated. In fact the microdensitometric scanning of the SDS-polyacrylamide electrophoresis of Fraction IV shows that the ATPase band (68 000) is ~15% of the total protein. From this value, knowing the amount of enzyme units applied to the gel, the specific activity of the ATPase band can be calculated. On this basis a minimum estimate of 150 U/mg is obtained.

Physical and catalytic properties of the pure enzyme

Pure enzyme was eluted from native gel slices as described earlier, concentrated against solid PEG 6000 and stored at −25°C in 50% glycerol. Under these conditions the ATPase proved stable for at least 2 months.

Sedimentation in glycerol gradient yielded an *s* value of 5.3S which is significantly lower than the *s* value of the less purified enzyme (*s* = 6.4S) (Cobianchi *et al.*, 1979). This suggests that the active form of the pure enzyme is a monomer of ~68 000 and that in the less pure preparation this polypeptide is physically associated with an unidentified factor. The pure enzyme shows maximum activity in sodium-phosphate buffer at pH 8; potassium-phosphate buffer and Tris-HCl buffer give lower activities (80% and 50%, respectively). Optimum $MgCl_2$ concentration is 2 mM. K_m for ATP (and dATP) is 0.5 mM; K_m for single-stranded DNA (ϕX DNA) is 0.5 μM; no difference is observed between different circular or linear single-stranded DNAs. ATPase activity with double-stranded DNA cofactors (ϕX RFI and ϕX RFIII DNAs), tested at limiting DNA concentrations, is ~15% of that on single-stranded DNA, while in the absence of single-stranded DNA cofactor the residual activity is <5%. Some of these kinetic parameters differ from those observed with the less purified preparations of the enzyme (Cobianchi *et al.*, 1979, 1982); it is possible that binding with the above mentioned factor (or with others) interferes with the ATPase activity.

We have previously shown (Cobianchi *et al.*, 1979, 1982) that the less purified ATPase is able to partially unwind short DNA duplexes in an ATP-dependent fashion. This reaction entails the sliding of the enzyme along the DNA molecule (Abdel-Monem *et al.*, 1976). The possibility that the pure ATPase might also slide along single-stranded DNA was tested by measuring the rate of ATP hydrolysis as a function of the size of DNA cofactor. In fact, if the ATP hydrolysis is coupled to the migration of the enzyme, a drop in activity is

Table I. Purification of the DNA-dependent ATPase

Fraction	Volume (ml)	Total protein (mg)	Total activity (U[a])	Specific activity (U/mg)
Crude extract	400	7200	–	–
DEAE filtrate	650	1820	117	0.064
Single-stranded DNA-cellulose	13	7.5	25	3.84
ATP-agarose	10	0.76	13	17.1

The various steps of purification are described in detail in Materials and methods.
[a]One Unit is the amount of enzyme which hydrolyzes 1 μmol of ATP in 20 min at 37°C.

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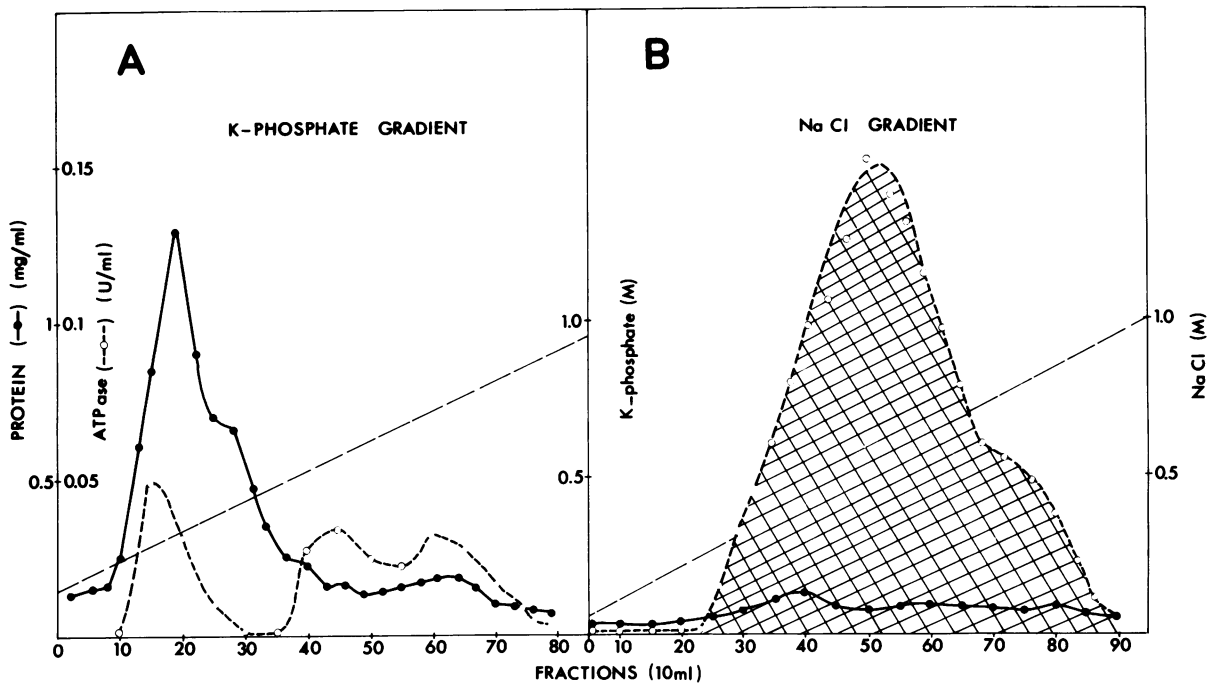

Fig. 1. Single-stranded DNA-cellulose column. Serial elutions with potassium phosphate gradient **(A)** and NaCl gradient **(B)**. Experimental conditions are as described in Materials and methods. Fractions under the shadowed area were pooled (Fraction III).

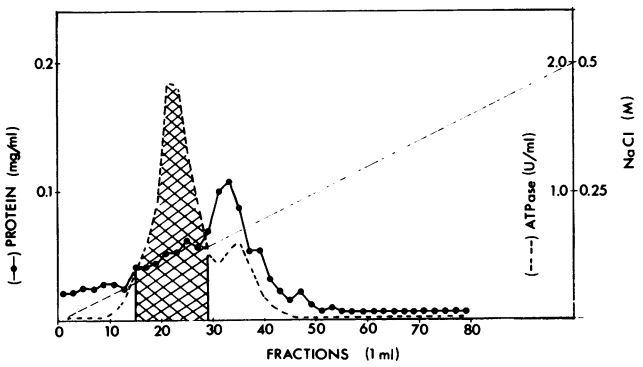

Fig. 2. ATP-agarose column. Experimental conditions as described in Materials and methods. Fractions under the shadowed area were pooled (Fraction IV).

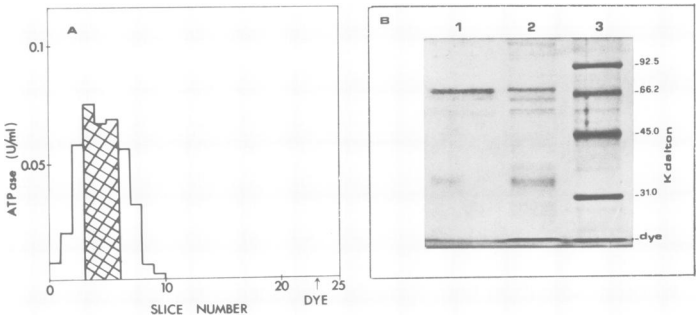

Fig. 3. Preparative non-denaturing polyacrylamide gel electrophoresis of Fraction IV. Samples were run in duplicate in two parallel lines. 25 slices (4 mm wide) were cut from one lane, the enzyme was extracted as described in Materials and methods and the activity assayed (see **Panel A**). Gel slices corresponding to the peak of ATPase activity (shadowed area) were cut from a parallel lane, boiled for 1 min in the SDS-PAGE sample buffer and put directly into the wells of a SDS-polyacrylamide slab gel prepared and run as described in Materials and methods. The results are shown in **Panel B** (**lane 1**: native gel slice containing ATPase activity; **lane 2**: Fraction IV; **lane 3**: markers).

expected on short molecules. The results of these experiments are reported in Table II. No difference in the V_{max} and K_m values was observed between 40 000 bp and 500 bp single-stranded DNA. With short oligodeoxynucleotides, oligo-$(dT)_{12-18}$, oligo$(dA)_{12-18}$ and oligo$(dG)_{12-18}$ a significant drop in the V_{max} was observed (down to 33%, 20% and 4.5%, respectively). K_m values for oligo(dT) and oligo(dG) are similar to that for SPP1 DNA, while that for oligo(dA) is higher. These results do not permit any clear cut conclusions to be drawn since the influence of base composition on activity is unknown; however, they suggest that some limited sliding might in effect occur, comparable to the limited extent of unwinding carried out by the less pure enzyme.

Effect of inhibitors

ATPase activity is strongly inhibited by the non-hydrolysable ATP analogue, adenosine 5′-[γ-thio]triphosphate (ATP-[γ-S]) while adenyl-5′-yl imidodiphosphate (ADP-[NH]P) has not significant effect. The results are shown in Table III.

Binding to DNA

The binding to DNA was measured by the DNA filter-binding assay as described in Materials and methods. The results are shown in Table IV. The ATPase seems to bind to the same extent to single- and double-stranded DNA at least within the sensitivity limits of this method.

Functional properties of ATPase

Pure ATPase has no other detectable enzymatic activity; assays for polymerase, nuclease and topoisomerase activities were all negative (data not shown). Surprisingly, the pure enzyme lacks the capacity to stimulate DNA polymerase α on poly[d(AT)] and to unwind partial DNA duplexes; however, this capacity is present in less pure preparations (Cobianchi *et al.*, 1979, 1982). We find that the DNA polymerase stimulatory activity separates from the ATPase upon elution

Table II. Effect of DNA cofactor size on ATPase activity

Single-stranded-DNA cofactor	K_m (μM)	V_{max} (U/ml)
Single-stranded SPP1 DNA 40 000 bp	0.42	10
Single-stranded-SPP1 DNA 500 bp	0.42	10
oligo(dT)$_{12-18}$	0.44	3.3
oligo(dA)$_{12-18}$	2.0	2.0
oligo(dG)$_{12-18}$	0.57	0.45

K_m and V_{max} values were determined at limiting DNA concentrations. 500-bp single-stranded-DNA was obtained by sonication of native DNA followed by denaturation; oligodeoxynucleotides were purchased from P-L Biochemicals Inc. and were heated for 2 min at 60°C before use.

Table III. Effect of ATP[γ-S] and ADP[NH]P on ATPase activity

Conditions	% Activity
ATPase (complete mixture[a])	100
ATPase (complete mixture) + ATP[γ-S] 2 μM[b]	84
ATPase (complete mixture) + ATP[γ-S] 10 μM	54
ATPase (complete mixture) + ATP[γ-S] 100 μM	23
ATPase (complete mixture) + ATP[γ-S] 1.6 mM	5
ATPase (complete mixture) + ADP[NH]P 1.6 mM	96

[a]The ATP concentration in the assay is 400 μM as described in a previous paper (Cobianchi *et al.*, 1982).
[b]Final concentration in the assay.

Table IV. Binding of ATPase to DNA

Fraction	μg Protein/assay	% DNA retained on filters	
		single-stranded	double-stranded
Fraction IV	0.25	52	57
	0.5	67	62
	1.25	69	85
	2.5	83	94
Pure enzyme	0.04	7	11
	0.08	16	17
	0.16	38	36

of the DNA-cellulose column with a potassium phosphate gradient. The stimulatory factory (Fraction A, see Materials and methods) elutes at ~0.3 M potassium phosphate while the ATPase is eluted only by the NaCl gradient. As described previously (Cobianchi *et al.*, 1979, 1982), if the DNA-cellulose column is eluted directly with a NaCl gradient the stimulatory factor elutes with the ATPase at 0.6 M NaCl. The stimulatory activity of the less pure ATPase (and also its unwinding capacity) are presumably due to the association of a distinct stimulatory factor with the enzyme. Whether this factor is physically associated with the ATPase or simply, under certain conditions, co-purifies with it, is still an open question. The sedimentation data reported above, however, support the first possibility.

Properties of the DNA polymerase stimulatory factor

The DNA polymerase α stimulatory factory, when purified as described in Materials and methods (Fraction B, Figure 4), appears to be at least 90% pure as indicated by the fact that only one protein band with a mol. wt. of 28 000 is visible on SDS-polyacrylamide gel electrophoresis (Figure 4 insert).

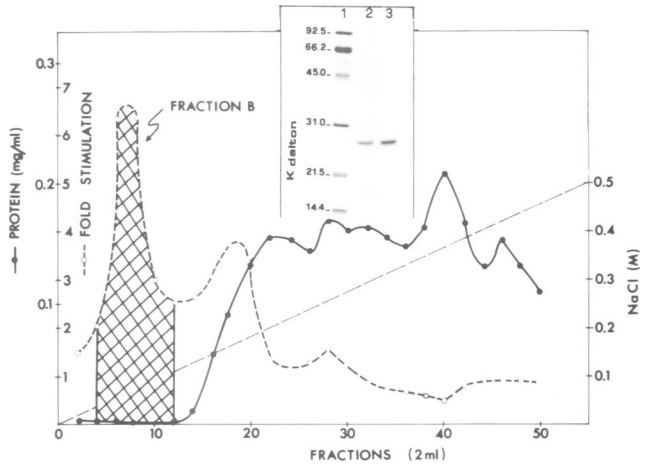

Fig. 4. Purification of the DNA polymerase α stimulatory factor on a DEAE-cellulose column. For experimental details see Materials and methods. Fractions 3–12 (Fraction B) were pooled, concentrated with solid PEG 6000 and stored at −25°C in 50% glycerol. Fractions 13–22, although stimulating, were discarded, being contaminated with nuclease and polymerase activity. The insert shows the SDS-PAGE profile of Fraction B. **Lane 1**: mol. wt. markers; **lanes 2** and **3**: Fraction B: 2 μg, 5 μg. The small band at 66 000 is due to traces of BSA from the tube coating.

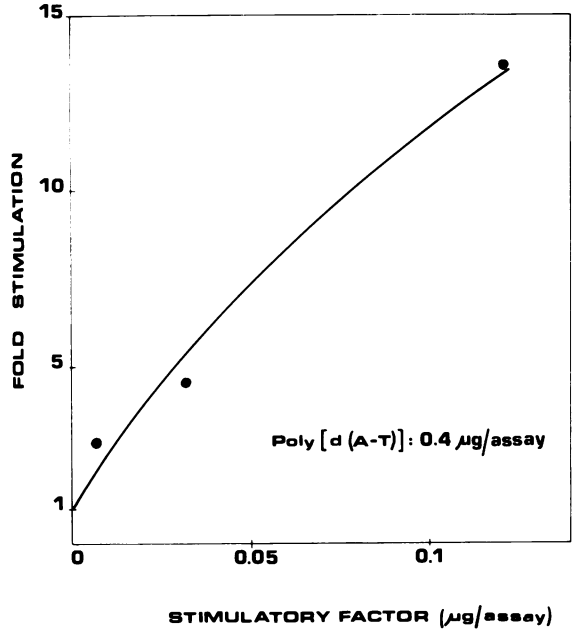

Fig. 5. Stimulation of DNA polymerase α on poly[d(AT)] by the stimulatory factor (Fraction B, concentrated). Stimulation assays are as described in Materials and methods.

Fraction B is highly stimulatory for polymerase α on poly[d(AT)] template (see Figure 5). Stimulation occurs at very low protein to DNA ratios, thus an effect on the template structure only seems unlikely. The stimulatory factor has no enzymatic activity (polymerase, nuclease, ATPase, topoisomerase, etc.). We are presently investigating whether this factor is related to the helix destabilizing proteins (mol. wt. 24 000–27 000) purified from calf thymus (Riva *et al.*, 1980). As expected, the factor binds to single-stranded DNA but, unlike the calf thymus protein, it binds equally well (or better) to double-stranded DNA. The experiment was performed by using the DNA filter-binding assay as described in Materials and methods; the results are reported in Table V.

It is interesting to observe that the mol. wt. of the ATPase

Table V. Binding of the DNA polymerase stimulatory factor to DNA

	μg Protein/assay	% DNA retained on filters	
		single-stranded	double-stranded
Fraction B	0.08	13	23
	0.16	24	40
	0.32	46	56

monomer, as deduced from the *s* value and from SDS-PAGE, and that of the stimulatory factor add up to ~100 000, a value in good agreement with the mol. wt. of the partially purified ATPase (110 000) reported previously (Cobianchi *et al.*, 1979).

Discussion

We have described the properties of the major DNA-dependent ATPase from HeLa cells after partial purification (Cobianchi *et al.*, 1979, 1982). This enzyme exhibited a number of features which pointed to a possible role in DNA replication. The partially pure ATPase is able to stimulate strongly DNA polymerase α (the replicative enzyme in mammalian cells) on poly[d(AT)] template. Also the enzyme showed an ATP-dependent duplex-unwinding activity with a 3′ to 5′ polarity on the unwound strand. On this basis the enzyme was classified as a DNA helicase (Falaschi *et al.*, 1980). However, when the enzyme is purified to near homogeneity, we observe a loss of these properties and quite surprisingly we also observe a significant reduction in its mol. wt. The 'disassemblement' of the enzyme probably takes place on single-stranded DNA-cellulose upon elution with potassium phosphate. We propose that during this step a 'cofactor' of the enzyme is lost, which is essential for eliciting stimulation of polymerase α and duplex unwinding. So far we have been unable to identify unambiguously this cofactor or to reassemble a structure reacquiring the unwinding ability. However, from the potassium phosphate eluate we can recover and completely purify a DNA-binding protein (mol. wt. 28 000) which might be the cofactor; the mol. wt. of this protein roughly corresponds to the observed decrease in the mol. wt. of the ATPase upon elution from the single-stranded DNA-cellulose column. This 28 000 protein exerts a powerful stimulatory effect on DNA polymerase α (see Figure 5) at very low protein/DNA ratios. It is worth recalling that the 'classic' single-stranded DNA-binding proteins (Riva *et al.*, 1980) enhance the rate of DNA polymerase α on single-stranded DNA only at rather high protein/DNA ratios, in agreement with the contention that they play a 'structural' role on the template, stretching it in front of the incoming polymerase by stoichiometric cooperative binding of a high number of protein molecules. Thus, template-saturating conditions are observed, in those cases, at 10/1 protein/DNA ratios, whereas we observed significant effects at a ratio of 0.2. At such ratios only a very small fraction of the DNA template is covered with the protein, as can be inferred by simple estimates on the size of a 28 000 mol. wt. protein (see also Table V). This leads us to rule out the possibility that the stimulatory effect is achieved by stoichiometric binding and indicates a more specific interaction with the catalytic properties of the α-polymerase. Similarly, a direct association of the 28 000 mol. wt. protein with the ATPase might be responsible for the overall functional properties observed in the less pure ATPase preparations. We are presently trying to confirm this hypothesis by *in vitro* reconstitution experiments. If this model should prove correct, then a pattern similar to that described in bacterial multienzyme replication systems would emerge where the interaction of different molecules leads to the formation of functional complexes with helicase and polymerase properties. At present the involvement of this DNA-dependent ATPase (with or without an associated cofactor) is simply a matter of speculation; it may be relevant to point out that the turnover number of the pure ATPase is ~1000 (ATP molecules hydrolyzed/enzyme molecule/min) and on this basis an estimation of ~3 x 10^5 enzyme molecules/cell can be obtained. If a role of the ATPase in DNA replication is hypothesized, this number should be compared with the number of growing forks for replication in the human genome (in the order of 10^5).

Materials and methods

Purification of the ATPase

44 g (frozen cell paste) of HeLa cells grown as previously described (Cobianchi *et al.*, 1982) were suspended in 400 ml of 0.35 M potassium phosphate buffer pH 7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA containing protease inhibitors (phenylmethanesulfonyl fluoride, PMSF (Sigma), 1 mM; $Na_2S_2O_5$, 10 mM; Pepstatin (Sigma), 1 μg/ml. The first two inhibitors were added to all solutions through the whole purification).

The suspension was sonicated for three 5-s intervals and centrifuged for 20 min at 12 000 *g*. To completely remove DNA, the supernatant (Fraction I, 400 ml, 7200 mg of protein) was filtered through a 5 cm diameter x 25 cm DEAE-cellulose column, equilibrated with the extraction buffer, which was then washed with the same buffer. The flow-through and the washing were pooled (Fraction II, 650 ml, 1820 mg of protein). In order to decrease the ionic strength, Fraction II was diluted to the following final conditions: 0.15 M potassium phosphate buffer pH 7.5, 0.5 mM DTT, 1 mM EDTA, 20% glycerol (v/v) and applied at a rate of 1 column volume/h to a single-stranded DNA-cellulose column (2.5 cm diameter x 25 cm; 5.9 mg DNA/g cellulose, dry weight) equilibrated with the same buffer. The column was washed with 120 ml of application buffer. No DNA-dependent ATPase activity was found in the flow-through and in the washing.

The proteins were eluted (elution rate 100 ml/h) with a 1000 ml linear gradient from 0.15 M to 1.0 M potassium phosphate pH 7.5, 0.5 mM DTT, 1 mM EDTA, 20% glycerol; 10 ml fractions were collected. Very little (~10%) DNA-dependent ATPase activity was eluted by this gradient. The bulk of activity was eluted between 0.4 M and 0.7 M NaCl in a subsequent 1000 ml linear gradient from 20 mM to 1 M NaCl, in 50 mM potassium phosphate pH 7.5, 0.5 mM DTT, 1 mM EDTA, 20% glycerol (100 ml/h). The peak fractions were pooled, concentrated with solid PEG 6000 and dialyzed against 20 mM potassium phosphate buffer, 0.5 mM DTT, 1 mM EDTA, 20% glycerol (Fraction III, 15 ml, 7 mg of protein). An ATP-agarose column (0.8 cm diameter x 10 cm) was prepared and equilibrated with the dialysis buffer. In order to avoid loss of activity due to the low protein concentration, the packed column was washed with three volumes of buffer containing bovine serum albumin (BSA) 2 mg/ml, then washed with buffer containing 2 M NaCl and finally re-equilibrated with the original buffer. In addition, all glassware, tubings and tubes for fraction collection were coated with protein by the method described by De Jong *et al.* (1981). Fraction III was applied to the ATP-agarose column, the column was washed with two volumes of application buffer and the enzyme was eluted with a 100 ml linear gradient from 0 to 0.5 M NaCl in the same buffer. Application, washing and gradient elution were performed at 5 ml/h and 1 ml fractions were collected. The active fractions were pooled, concentrated with solid PEG 6000, dialyzed against 50 mM potassium phosphate buffer pH 7.5, 0.5 mM DTT, 1 mM EDTA, 50% glycerol (v/v) and stored at −25°C. (Fractions IV, 10 ml, 0.76 mg protein.)

Purification of a DNA-binding protein which stimulates DNA polymerase α

When the single-stranded DNA-cellulose column was eluted with a potassium phosphate gradient (see previous section) a protein peak containing a stimulatory factor for DNA polymerase α on poly[d(AT)] template eluted between 0.2 M and 0.4 M potassium phosphate. The peak fractions were pooled, concentrated with solid PEG 6000 and dialyzed against 20 mM potassium phosphate buffer pH 7.5, 0.5 mM DTT, 1 mM EDTA, 20% glycerol (Fraction A, 22 ml, 180 mg of protein). A DEAE-cellulose column (1.5 cm diameter x 12 cm) was prepared and equilibrated with the dialysis buffer. Fraction A was applied at a rate of 20 ml/h, the column was washed

with two volumes of the same buffer and the bound proteins were eluted with a 200 ml linear gradient from 0 to 0.5 M NaCl in the same buffer. Elution rate was 20 ml/h and 4 ml fractions were collected. The DNA polymerase-stimulating factor eluted at the very beginning of the gradient between 50 mM and 100 mM NaCl. The active fractions were pooled, concentrated with PEG 6000, dialyzed against 20 mM potassium phosphate pH 7.5, 0.5 mM DTT, 1 mM EDTA, 50% glycerol and stored at −25°C (Fraction B, 1.8 ml, 300 μg of protein).

Non-denaturing polyacrylamide gel electrophoresis

The procedure described by Banks *et al.* (1979) was followed with only minor modifications (polyacrylamide concentration was lowered from 3.5% to 2.5%). Elution of proteins from gel slices and transfer of protein bands with associated enzyme activity to SDS-polyacrylamide slab gels was also performed according to these authors.

SDS-polyacrylamide gel electrophoresis

Samples were run on slab gels (1.5 mm x 15 cm) containing 10% polyacrylamide with 5% polyacrylamide stacking in Tris-HCl-SDS buffer; gels were prepared, run and stained with Coomassie blue according to Laemmli (1970).

Assay of ATPase activity

DNA-dependent ATPase activity was measured as already described (Cobianchi *et al.*, 1982). One unit of ATPase is the amount of enzyme which hydrolyzes 1 μmol of ATP in 20 min at 37°C in the presence of single-stranded DNA.

Stimulation of DNA polymerase α on poly[d(AT)]

DNA polymerase α, purified according to Spadari and Weissbach (1979) up to the phosphocellulose column, was a generous gift of S.Spadari. The reaction mixture was as previously described (Cobianchi *et al.*, 1982). After adding the stimulatory factor in the appropriate amounts, the reaction was initiated by adding the DNA polymerase (0.25 − 0.5 units). Incubation was performed at 37°C, samples were taken at various times and the radioactivity incorporated into acid precipitable material was measured.

DNA filter-binding assay

The retention of single-stranded or double-stranded DNA on nitrocellulose filters by DNA-binding proteins was measured as described by Carrara *et al.* (1977). 0.1 μg of φX [^{3}H]DNA (50 000 c.p.m./μg) (single-stranded) or *Pst*I cut φX [^{3}H]RFI-DNA (48 000 c.p.m./μg) (double-stranded) were used per filter.

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