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A DNA DEPENDENT ATPase FROM HeLa CELLS

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The purification and the properties of the major DNA dependent ATPase from HeLa cells are described. This enzyme is present in the nucleus and in the cytoplasm in approximately equal amounts. It has a Mr of about 110000 dalton and it hydrolyzes ATP (and dATP) to ADP+Pi only in the presence of single-stranded DNA. The enzyme shows an ATP dependent unwinding activity on DNA duplex, with a 3' to 5' polarity of the unwound strand. Under certain conditions the enzyme is able to stimulate the activity of DNA polymerase α on appropriate DNA templates. Such stimulation is synergistic with that exerted by a DNA binding protein from calf thymus.

INTRODUCTION

The DNA dependent ATPases of prokaryotes are demonstrably involved in several aspects of DNA replication (1-4), recombination and repair (5,6) of these organisms. Several other proteins have properties of DNA dependent ATPase but the lack of genetic evidence does not allow an unambiguous assignment of functions (7,8). A number of these proteins has been described in different eukaryotic systems (9-15), however only in very few cases their function can be assigned unambigously.

We have focused our attention on a DNA dependent ATPase from human cells which has properties suggestive of possible functions in DNA replication. Preliminary reports on this enzyme have been published (16,8).

MATERIALS AND METHODS

Radioactive isotopes were products of the Radiochemical Center (Amersham). Snake venom phosphodiesterase was from Worthington Corp. (Freehold, N. J.). Single-strand DNA cellulose was prepared according to Litman (17). PMSF was a product of Sigma Corp. (Saint Louis, Mo); N. Crassa

Abbreviations used are; BSA: bovine serum albumin; DBP, DNA binding protein; DTT: dithiothreitol; EDTA: ethylene-diamino-tetraacetate; EGTA: ethyleneglycol-(β -aminoethyl-aether)-N,N'-tetraacetate; PEI: poly-ethylene-imino; PMSF: phenyl-methyl-sulfonyl fluoride.

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endonuclease was from Boehringer Corp. (Mannheim, B.R.D.). DNA binding proteins were purified from calf thymus (18).

Assay of ATPase activity. The incubation contained, in a final volume of 25 µl: Potassium phosphate buffer 50 mM, pH 6.5, DTT 1 mM, BSA 200 µg/ml, sodium pyrophosphate 1.25 mM, $MgCl_2$ 2 mM, [^{3}H]ATP 0.4 mM, $4x10^7$ cpm/µmole, fd phage DNA 10 µg/ml and between 0.0002 and 0.002 unit of ATPase. Samples were incubated for 20 min at 35°C; the reaction was stopped by chilling to 0°C and adding 1 µl of 0.5 M EDTA; 2.5 µl were applied to a PEI cellulose (Macherey-Nagel, Düren, B.R.D.) thin layer sheet and developed in 1 M formic acid and 0.25 M LiCl. ADP spots were cut out and counted in a liquid scintillation counter. One unit of ATPase is the amount of enzyme which hydrolyzes 1 µmole of ATP to ADP in 20 min at 35°C.

Cells. HeLa cells were grown in suspension in minimal essential medium (Joklyk modified) (Gibco Corp., Paisley, Scotland) without calcium and sodium bicarbonate, at 37°C and harvested at $6x10^5$ cells/ml. Cells centrifuged at 2000xg for 10 min were washed twice with PBS buffer (Gibco), frozen in liquid nitrogen and stored at -70°C.

[^{3}H]RNA-DNA and [^{3}H]DNA-DNA partial duplexes. They were prepared by incubating ØX 174 DNA with B. subtilis RNA polymerase and with the large fragment of DNA polymerase I of E. coli ("Klenow polymerase", Boehringer Corp., Mannheim, B.R.D.), as described by Hoffmann-Berling et al. (19).

Other assays. Stimulation of DNA polymerase α on different templates was measured as already described (16).

Purification of the enzyme. 1)A frozen cell paste (20g) was suspended in 250 ml of 0.35 M potassium phosphate buffer pH 7.5, 1 mM DTT, 1 mM EDTA, 150 µg/ml PMSF; the suspension was sonicated for three 5 sec intervals and centrifuged for 20 min at $12x10^3$ xg. The supernatant is Fraction I (200 ml, 1400 mg protein). 2)DEAE cellulose filtration: fraction I was applied to a 300 ml DEAE cellulose column equilibrated with the extraction buffer. The column was washed with 180 ml of the same buffer; the filtrate (Fraction II, 300 ml, 1100 mg) was collected. 3)Ammonium sulfate concentration: to fraction II, 150 g of solid ammonium sulfate were slowly added with stirring for a period of 15 min at 0°C; the precipitate was collected with 15 min centrifugation at $15x10^3$ xg and resuspended in 80 ml of 20 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM EDTA, 0.5 mM EGTA, 20% (v/v) glycerol, 0.2 M NaCl and dialysed against the same buffer (Fraction III; 90 ml, 180 U, 0.27 U/mg). 4)DNA cellulose chromatography: fraction III was applied to a 40 ml single stranded DNA-cellulose column equilibrated with the dialysis buffer. The column was washed with 60 ml of the same buffer and eluted with 80 ml of the same buffer made 0.6 M in NaCl; the eluate (80 ml) is Fraction IV (68 U, 1.7 U/mg). 5)Phosphocellulose chromatography: fraction IV was dialyzed against 20 mM potassium phosphate buffer pH 7.5, 1 mM DTT, 0.1 mM EDTA, and applied to an 8 ml phosphocellulose column equilibrated with the same buffer. The column was washed with 16 ml of the same buffer and eluted with an 80 ml linear gradient made in the same buffer but with potassium phosphate concentration ranging between 0.1 M and 0.6 M. The eluted activity shows a peak around 0.2 M phosphate. The pool of the active fractions (16 ml) was concentrated to 2 ml by poly-ethylene glycol 6000 dialysis (Fraction V, 20 U, 3.6 U/mg). 6)Sephadex G-75 filtration: fraction V was dialyzed against the same buffer used in the previous step for equilibration of the phosphocellulose, and applied to a 200 ml column of Sephadex G-75. The filtration was carried out with the same buffer. The activity elutes as a single peak. The active fractions were

pooled and concentrated to 1 ml as in the previous step and stored in 50% glycerol at -20°C. (Fraction VI, 18 U, 12.5 U/mg).

RESULTS AND DISCUSSION

DNA dependence is first observed in the ammonium sulfate fraction; from this step onward purification is about 50-fold with 10% yield; the final step removes an RNAse H activity and a DNAse activity highly specific for poly [d(A-T)].

Properties of the enzyme. The enzyme shows an absolute requirement for the presence of single stranded DNA whether closed circles (ØX 174) or linear molecules (SPP1, L-strand); in its absence the activity is reduced to less than 5%. The Km for single-stranded DNA, as well as for activated DNA, poly [d(A-T)] and native DNA, is of the order of 1.2 μM; the rate of activity at saturating DNA concentration is 67%, 55%, 22% respectively for the latter molecules compared to single stranded DNA. The enzyme splits ATP into ADP and Pi. A divalent cation is required for hydrolysis, though in its absence a 25% residual activity is observed. Optimum $MgCl_2$ concentration is 2 mM. The optimum pH is close to 6; the activity at pH 8 is reduced to approximately 60%. Potassium phosphate or Tris-HCl support enzyme activity to the same degree. The Km for ATP is 0.25 mM; dATP is as good a substrate as rATP; the other three rNTP's are not hydrolyzed by the enzyme. The isoelectric point is 5.5. By combination of sedimentation in glycerol gradient and gel filtration on G-100 (20), a Mr of 110000 dalton was estimated, with a Stokes radius of 40 Å. Preincubation of the enzyme with N-ethyl maleimide causes an 80% inactivation. The ATPase activity remains linear for about two hours in the standard assay conditions.

The enzyme preparation seems free of any obvious contaminating activity; thus no DNA or RNA polymerase, no deoxyribonuclease activity whether endo or exonuclease, whether on native or single-stranded DNA were observed (16); neither nicking nor untwisting activity on supercoiled circular DNA were observed; also no gyrase or topoisomerase II-like activity are measurable; ligase, RNase, and RNAse H are also absent.

We found equal amounts of ATPase in the nuclei prepared according to Challberg and Kelly (21), and in the corresponding cytoplasm fraction. No DNA dependent ATPase activity was found in mitochondria prepared according to Scovassi et al. (22).

Unwinding of partial duplex structures. In order to test the presence of

Table I. Effect of ATPase on susceptibility of short double-stranded stretches of DNA to single-strand specific nucleases

CONDITIONS	A		B	
	N. crassa endonuclease		Snake venom phosphodiesterase	
	[^{3}H] DNA made acid soluble		[^{3}H] DNA made acid soluble	
	pmol	%	pmol	%
+ATP,-ATPase,-nuclease	0.01	0.1	0.01	0.1
+ATP,+ATPase,+nuclease	0.50	3.8	1.69	13.0
-ATP,+ATPase,+nuclease	0.08	0.6	0.39	3.0
+ATP,-ATPase,+nuclease (boiled substrate)	3.50	27.0	1.69	13.0

The 60 min incubation contained the standard reagents except that 130 pmol of [^{3}H] DNA-DNA molecules were present. The radioactive DNA accounts for 10% of cold DNA; 0.30 U of *N. crassa* endonuclease (A) or 0.32 U of snake venom phosphodiesterase (B) were also present, together with 0.006 U of ATPase.

helicase activity (7) we constructed [^{3}H] RNA-DNA and [^{3}H] DNA-DNA partial duplexes as described in Materials and Methods. When [^{3}H] RNA-DNA hybrids were incubated with ATPase in the presence of pancreatic and T1 ribonucleases no obvious unwinding activity was observed (data not shown).

[^{3}H] DNA-DNA partial duplex structures, obtained by elongating RNA primers with *E. coli* DNA polymerase I, did not give evidence of unwinding when incubated with the ATPase and, afterwards, analyzed in neutral sucrose gradients. We looked therefore for conditions in which even partial transient opening of the duplex could be measured. Since the conditions for our ATPase activity are incompatible with those of S1 DNase digestion (7) we incubated the [^{3}H] DNA-DNA hybrids with ATPase in the presence of either *Neurospora crassa* endonuclease or snake venom phosphodiesterase. As the data of Table I show, in these conditions, a fraction of the radioactive material became susceptible to the single-strand specific nucleases, and this effect was dependent on the presence of ATP. Thus, in analogy with the *E. coli* helicase I (7), and with the helicase described in *Lilium* spermatocytes (U-protein) (10), our enzyme is able to expose a partial duplex to the action of single-strand specific nucleases in an ATP dependent fashion. As to the polarity with which such an unwinding occurs, the presence of RNA primers on the 5' end, which have already been shown to be insensitive to any unwinding action by our ATPase, would suggest a 3'to 5' polarity. This contention is

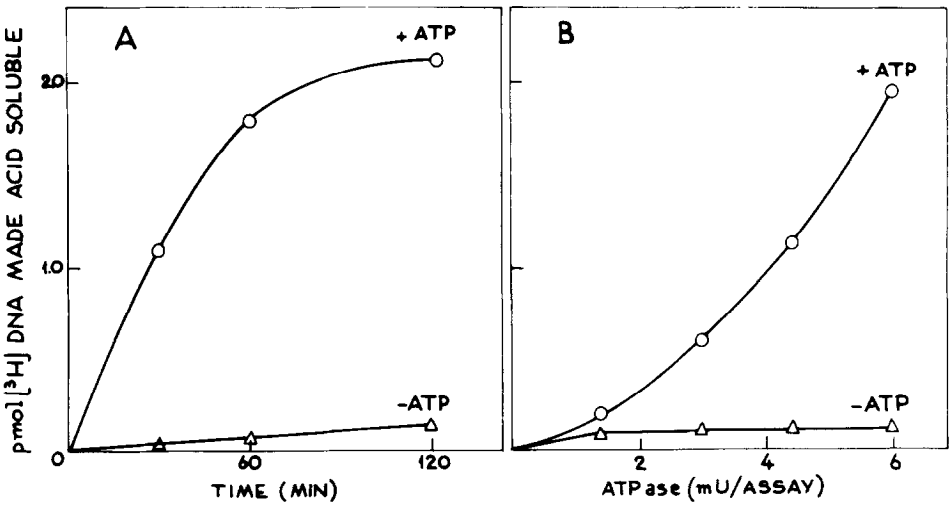

Figure 1: Properties of the unwinding reaction: sensitization of [^{3}H] DNA-DNA hybrid to the snake venom phosphodiesterase by ATPase action. (A) Time course of the reaction. (B) Effect of ATPase concentration. Experimental conditions as in Table II.

confirmed by the results obtained in presence of snake venom phosphodiesterase, an enzyme known to degrade only short single-strand stretches exonucleolytically from the free 3'-OH ends. As shown in Table I (B) 13% of the native hybrid is hydrolyzed by the phosphodiesterase, in the presence of ATPase, the same figure as that obtained with the denatured hybrid; this confirms that the ATPase makes portions of the duplex susceptible to single-strand specific nucleases, only in the presence of ATP and starting from the 3'OH end. Unwinding increases with time and with increasing amounts of ATPase (see Fig. 1). Thus we may conclude that our enzyme has a limited helicase activity and that the unwinding probably occurs with a 3'→5' polarity with respect to the unwound strand.

Stimulation of DNA polymerase α. In order to determine whether our ATPase could ease the advancement of DNA polymerases into double-stranded structures, templates other than DNA "activated" with pancreatic DNase should be chosen since, when copying the latter, the DNA polymerase runs into 5' P ended duplex stretches, which cannot be unwound by our ATPase. In fact, incubating gapped DNA with an amount of DNA polymerase α able to polymerize 100 nmole of total nucleotides in 30 min, no stimulation was observed by the addition of up to 0.01 unit of ATPase. In view of the polarity of our helicase a possible enhancement of the DNA polymerase rate would be expected

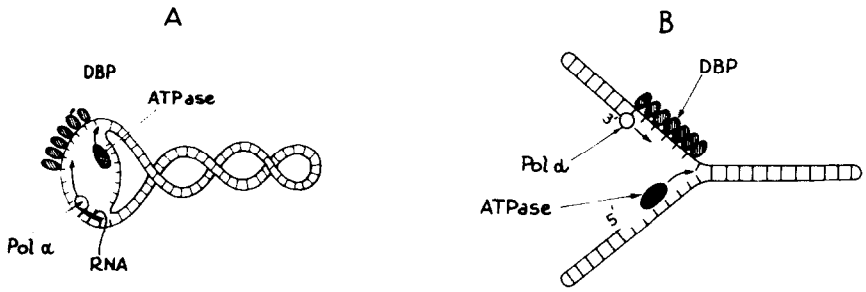

Figure 2: Model for the concerted action of DNA polymerase, ATPase and DBP on RNA primed supercoiled DNA (A)and on poly[d(A-T)] (B)

only at fork-like stuctures, such as those obtained by utilizing supercoiled circular DNA duplexes primed by bacterial RNA polymerases (see Fig. 2 A). We prepared such primed DNA duplexes, both with BK virus DNA, and with the RFI of ØX 174. As Table II shows, these molecules can in fact act as templates for DNA polymerase α and the presence of ATPase enhances the rate of polymerization by a factor of 4.

Another molecule which can assume configurations mimicking a fork-like structure is the poly [d(A-T)] copolymer (see Fig. 2 B). This molecule is a relatively poor template for DNA polymerase α, being copied, at a rate 1/10 of that of eroded or gapped DNA. The presence of ATPase can stimulate by a factor as great as 30 fold the rate of α polymerase on this structure (see Fig. 3 A).

Synergistic action of ATPase and mammalian DNA binding proteins. DNA binding proteins are also known to help in the advancement of the growing fork in bacteria and in higher organisms (8); in our laboratory we have isolated from calf thymus a single-stranded DNA binding protein which specifically stimu-

Table II. Stimulation of α-polymerase activity on primed supercoiled DNAs

Conditions	p mol [^{3}H] dTMP incorporated	Stimulation factor
BK DNA, -ATPase	1.5	-
BK DNA, +ATPase	4.3	2.8
ØX174 RFI, -ATPase	2.7	-
ØX174 RFI, +ATPase	10.9	4.0

0.82 μg of BK with 2 mU of ATPase, or 0.73 μg of ØX174 RFI with 0.5 mU of ATPase were used together with 1 U of polymerase. DNA polymerase α activity was measured as described in Material and Methods.

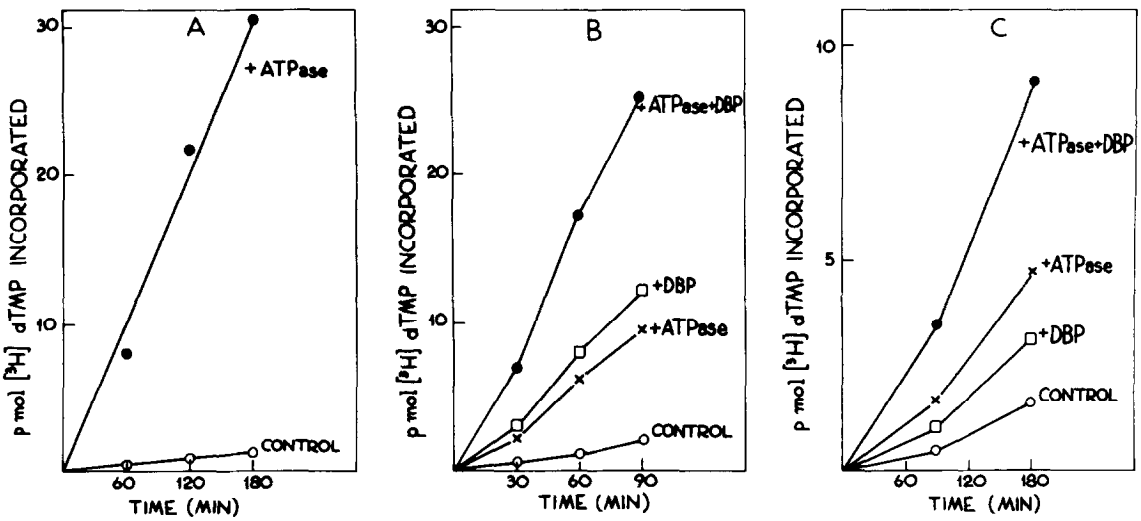

Figure 3: Stimulation of DNA polymerase α activity by ATPase and synergism with DBP. A: poly[d(A-T)] template; 0.75 U of polymerase, 1 mU of ATPase and 0.45 μg of poly[d(A-T)] were used. B: the same except that 1.5 U of polymerase 0.5 mU of ATPase and 0.05 μg of DBP were present. C: primed BK DNA template; 0.82 μg DNA, 4.5 U of α polymerase, 5 mU of ATPase and 0.07 μg of DBP were present.

lates the DNA polymerase α of animal cells (18). In view of the specificity of stimulation for DNA polymerase α it seems likely that the binding proteins somehow recognize and interact with the polymerase molecule; a mode of action like that shown in Fig. 2 can thus be inferred. In these conditions, one may envisage that the ATPase sitting on the lagging strand could act synergistically with the DNA binding protein. In fact, as Fig. 3 C shows, the binding protein and the ATPase added together to primed BK DNA, give a greater stimulation of DNA polymerase α than the sum of the stimulations given by either molecule separately. In the conditions reported in Fig. 3 C up to 6% of the length of BK DNA is copied at the longest time (3 hr). Sedimentation analysis showed that the newly synthesized DNA is associated to whole BK molecules in neutral conditions; in alkaline gradients the product is separated from the bulk of the template DNA and its size corresponds to approximately 6% of the length of the BK duplex, indicating that the product consists of a single chain per template, and that the synthesis is not due to the elongation of occasional nicks on the BK molecule.

Also on poly[d(A-T)] the action of the ATPase and of the binding proteins gives more than additive stimulation (see Fig. 3 B).

More work is obviously necessary for the characterization of the mode of action of the ATPase and of its possible interaction with other molecules acting on DNA. Also, the possible presence of factors which might be responsible at least in part for the activities just described and whose absence may explain the reduced stimulatory capacity of some enzyme preparations (to be published) cannot be ruled out.

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