

Adenovirus DNA replication *in vitro*: Identification of a host factor that stimulates synthesis of the preterminal protein–dCMP complex

(adenoviral terminal protein/stimulating factor)

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ABSTRACT A protein factor that participates in the formation of a covalent complex between the 80,000-dalton precursor of the adenovirus (Ad) terminal protein (pTP) and 5'-dCMP has been isolated and characterized. This 47,000-dalton protein, isolated from nuclear extracts of uninfected HeLa cells, has been designated nuclear factor I. It is free of detectable DNA polymerase α , β , and γ activities. In the presence of Ad DNA-prot, the Ad-protein fraction (containing the pTP and the Ad-associated DNA polymerase), ATP, Mg^{2+} , and dCTP, nuclear factor I stimulates formation of the pTP–dCMP complex. Addition of the Ad DNA binding protein (Ad DBP) renders the formation of the pTP–dCMP complex completely dependent on the addition of nuclear factor I. When Ad DNA-prot is replaced with ϕ X174 single-stranded circular DNA, pTP–dCMP complex formation requires only the Ad-protein fraction; Ad DBP and ATP are inhibitory and nuclear factor I has no effect on this reaction. This suggests that the initiation reaction observed with Ad DNA-prot in the absence of Ad DBP occurs at single-stranded DNA sites. In the presence of Ad DBP, these sites are blocked thus creating a requirement for nuclear factor I in pTP–dCMP complex formation.

Soluble extracts of adenovirus (Ad)-infected HeLa cells catalyze replication of the 35-kilobase linear duplex Ad genome (1–3). In this system, Ad DNA replication requires the following additions: Ad DNA covalently linked to a 55,000-dalton protein at each 5' terminus (Ad DNA-prot), the four dNTPs, ATP, and Mg^{2+} .

Two Ad-encoded fractions that are essential for Ad DNA replication have been isolated from the cytoplasm of infected cells. One is the 72,000-dalton Ad DNA binding protein (Ad DBP) (2, 4, 5) and the other is the 80,000-dalton preterminal protein (pTP) that is processed by an Ad-encoded protease (6, 7) to give the 55,000-dalton terminal protein. The pTP copurifies with a DNA-dependent DNA polymerase that is present only in Ad-infected cells (8, 9). This polymerase can add dNMPs to the 3'-hydroxyl end of DNA-primed DNA templates and also adds 5'-dCMP to the β -hydroxyl group of a seryl residue of pTP (10, 11). We have separated the pTP from the Ad-associated DNA polymerase by urea treatment followed by urea/glycerol gradient centrifugation (9). This DNA polymerase resembles DNA polymerase α in many respects but differs in its insensitivity to aphidicolin, its sensitivity to 2',3'-dideoxythymidine triphosphate and its ability to form the pTP–dCMP complex.

It has previously been shown that nuclear fractions are required for formation of the pTP–dCMP complex as well as for Ad DNA elongation (3, 12). In this report, we describe the isolation and characterization of a protein isolated from extracts of

uninfected nuclei. This protein, which we have called nuclear factor I, stimulates dNMP incorporation in the Ad-DNA replication system and, under certain conditions, stimulates or is completely required for pTP–dCMP complex formation.

MATERIALS AND METHODS

Materials. The preparation of extracts and the purification of proteins and Ad2 DNA-prot were as described (2, 3, 8, 12).

Complementation Assay for Nuclear Factor I. Reaction mixtures (50 μ l) contained 25 mM Hepes/NaOH (pH 7.5), 5 mM $MgCl_2$, 4 mM dithiothreitol, 3 mM ATP, 4 μ M [3H]dTTP (specific activity, 2,000–3,000 cpm/pmol), 10 μ g of bovine serum albumin, the Ad-protein phosphocellulose fraction (2 μ g of protein), Ad DBP (1 μ g), cytoplasmic extracts (40 μ g of protein) from uninfected HeLa cells, 0.1 μ g of Ad DNA-prot, and nuclear extract or fractions at various stages of purification. One unit of factor I catalyzed the incorporation of 1 nmol of dTMP into acid-insoluble material in 60 min at 30°C.

Assay for DNA Polymerases. DNA polymerase α was assayed with activated calf thymus DNA (13) in the presence of 7.5 mM $MgCl_2$. DNA polymerases β and γ were assayed with poly(rA)·oligo(dT)_{12–18} in the presence of 0.5 mM $MnCl_2$ (14).

Assay for Formation of the pTP–dCMP Complex. Reaction mixtures (50 μ l) contained 25 mM Hepes/NaOH (pH 7.5), 5 mM $MgCl_2$, 4 mM dithiothreitol, 3 mM ATP, 0.5 μ M [α - ^{32}P]dCTP (specific activity, 400–410 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), 10 μ g bovine serum albumin, the glycerol gradient fraction of the Ad-protein preparation (0.002 unit), 0.1 μ g of Ad DNA-prot, and nuclear extract or fractions at various stages of purification. Processing of samples, electrophoresis, and autoradiography were as described (12). The pTP–dCMP complex was quantitated by excising the band from the gel and counting Cerenkov radiation or by densitometric analysis of autoradiograms using an RFT scanning densitometer (model 2950). Under the above conditions, 0.1–0.15 fmol of dCMP was incorporated into pTP–dCMP complex by the Ad-protein fraction in the absence of any other protein fractions. One unit of factor I catalyzes the incorporation of 1 pmol of [α - ^{32}P]dCTP into pTP–dCMP complex in 60 min at 30°C. This value reflects the amount of pTP–dCMP complex formed above that observed with the Ad-protein fraction alone.

Purification of Nuclear Factor I. The following purification

Abbreviations: Ad, adenovirus; Ad DNA-prot, Ad DNA covalently linked to a 55,000-dalton terminal protein to each 5' end; Ad DBP, adenovirus-encoded DNA binding protein; pTP, 80,000-dalton precursor to the terminal protein; SSB, *Escherichia coli* single-stranded DNA binding protein; ss(c), single-stranded circular DNA.

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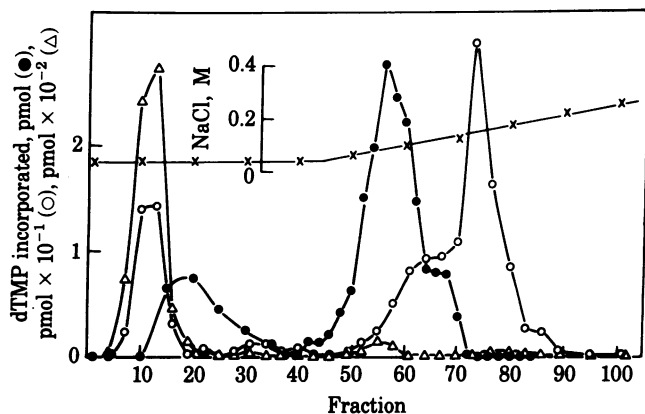


FIG. 1. Second DEAE-cellulose column chromatography. The first DEAE-cellulose fraction was dialyzed and then chromatographed on a second DEAE-cellulose column. Aliquots of each fraction were assayed for complementing activity (●) and for DNA polymerase activities with activated calf thymus DNA (○) and poly(rA)-oligo(dT)₁₂₋₁₈ (Δ). DNA polymerase activity recovered in the flow-through fractions was resistant to *N*-ethylmaleimide and aphidicolin at concentrations of 10 mM and 100 μM, respectively. DNA polymerase activities that were detected with poly(rA)-oligo(dT)₁₂₋₁₈ and activated calf thymus DNA and eluted at 0.08 and 0.15 M NaCl, respectively, were sensitive to *N*-ethylmaleimide (10 mM).

scheme started with 5×10^{10} HeLa cells. Nuclei were isolated as described by Challberg and Kelly (1), washed three times with 25 mM Tris·HCl, pH 7.5/10% sucrose, and suspended in 25 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/10% sucrose/0.35 M NaCl, and the suspension was stirred slowly for 30 min at 0°C. Crude nuclear extracts (40 ml) were prepared by successive centrifugations at $12,000 \times g$ for 20 min and $105,000 \times g$ for 40 min. The high-speed supernatant was adjusted to 0.2 M NaCl by addition of buffer A (25 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/20% glycerol) and applied to a DEAE-cellulose column (3.2×12 cm) equilibrated with buffer A/0.2 M NaCl. Material that was not adsorbed by the column (73.5 ml) was dialyzed against buffer B (25 mM Tris·HCl, pH 8.5/1 mM EDTA/1 mM dithiothreitol/20% glycerol/10% sucrose/0.01% Nonidet P-40)/0.03 M NaCl and applied to a second DEAE-cellulose column (2.6×9 cm) equilibrated with buffer B/0.04 M NaCl. The column was washed with 120 ml of the same buffer and eluted with a 400-ml linear gradient of 0.04–0.4 M NaCl in buffer B. The major peak of activity eluted at 0.08 M NaCl (see Fig. 1). Peak fractions (second DEAE-cellulose eluate, 50.5 ml) were combined and applied to a phosphocellulose column (1.4×5 cm) equilibrated with buffer B/0.15 M NaCl. The column was washed with 20 ml of the same buffer and eluted with an 80-ml linear gradient of 0.15–1.0 M NaCl in buffer B. The complementing activity eluted at 0.3 M NaCl (with a minor shoulder at 0.45 M NaCl). The major peak fractions were pooled (phosphocellulose eluate, 10 ml), dialyzed against buffer B/0.09 M NaCl and applied to a denatured DNA-cellulose column (1.4×5 cm) equilibrated with buffer B/0.1 M NaCl. The column was washed with 20 ml of the same buffer and eluted with an 80-ml linear gradient of 0.15–0.6 M NaCl in buffer B. Complementing activity eluted at 0.25 M NaCl. Peak fractions were pooled (denatured DNA-cellulose eluate, 8 ml) and a portion was adsorbed on a small phosphocellulose column and concentrated by stepwise elution with buffer C (25 mM Tris·HCl, pH 8.5/1 mM EDTA/1 mM dithiothreitol/10% glycerol/0.01% Nonidet P-40/0.5 M NaCl). Peak fractions were pooled and 0.2 ml of the concentrate was layered onto a 15–35% glycerol gradient (4.8 ml) in buffer C. Centrifugation was carried out at 48,000 rpm for 20 hr at 4°C

in an SW 50.1 rotor. Fractions (0.125 ml) were collected from the bottom of the tube.

RESULTS

Purification of Nuclear Factor I Complementing Activity from Uninfected HeLa Nuclei. The distribution of DNA polymerase activities was followed during the purification. As determined by template preference and sensitivities to *N*-ethylmaleimide and aphidicolin, DNA polymerase β was present in the second DEAE-cellulose flow-through fractions (Fig. 1) while most of the nuclear factor I complementing activity ad-

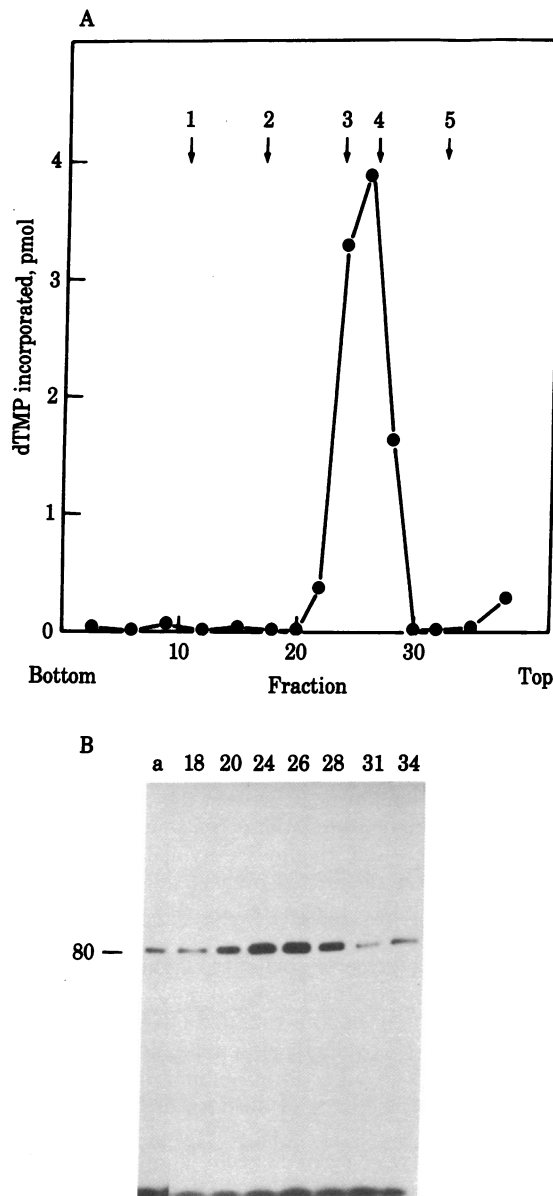


FIG. 2. Glycerol gradient centrifugation of nuclear factor I activity. (A) Assay of complementing activity. The denatured DNA-cellulose fraction was concentrated by adsorption to and elution from phosphocellulose. An aliquot was sedimented through a 15–35% glycerol gradient, and an aliquot (2 μl) of each gradient fraction was assayed for complementing activity; the positions of marker proteins in a parallel gradient are indicated by arrows: 1, catalase; 2, aldolase; 3, bovine serum albumin; 4, ovalbumin; 5, cytochrome c. (B) Assay of the stimulating activity for pTP-dCMP formation. Aliquots (2 μl) of the glycerol gradient fractions were assayed for their ability to stimulate formation of pTP-dCMP complex by Ad-protein fraction. Lanes: a, no gradient fraction present; 18, 20, 24, etc., glycerol gradient fraction assayed. 80, molecular mass (kilodaltons).

Table 1. Purification of nuclear factor I

Fraction	Total protein, mg	Complementing activity		Stimulating activity (pTP-dCMP formation)	
		Total units	Specific activity, units/mg of protein	Total units	Specific activity, units/mg of protein
0.35 M NaCl nuclear extract	348.0	128.0	0.38	58.4	0.17
First DEAE-cellulose eluate	166.0	78.2	0.47	28.1	0.17
Second DEAE-cellulose eluate	31.3	59.6	1.90	10.7	0.34
Phosphocellulose eluate	5.31	26.1	4.92	5.21	0.98
Denatured DNA-cellulose eluate	1.20	14.8	12.3	5.09	4.24
Glycerol gradient fraction	0.15	12.1	80.7	4.54	30.8

A portion of the denatured DNA-cellulose fraction was subjected to glycerol gradient centrifugation. The values summarized here are those expected if the entire fraction were subjected to the glycerol gradient procedure, assuming that the yield and purification would be the same.

sorbed to DEAE-cellulose and eluted at 0.08 M NaCl. Nuclear factor I complementing activity was separated from DNA polymerases α and γ by chromatography on denatured DNA-cellulose. Nuclear factor I activity eluted at 0.2 M NaCl; DNA polymerases α and γ eluted in the flow-through fractions and at 0.28 M NaCl, respectively (data not shown).

Properties of Nuclear Factor I Complementing Activity. Centrifugation of the denatured DNA-cellulose fraction in 15–35% glycerol gradients as the final step of the purification procedure yielded a single peak of complementing activity sedimenting at 3.4 S, which corresponds to a native molecular mass of 47,000 daltons (Fig. 2A).

NaDodSO₄/polyacrylamide gel electrophoresis of glycerol gradient fractions revealed the presence of a major band of 47,000 daltons. Analysis by the silver staining procedure of Oakley *et al.* (15) showed that the amount of the 47,000-dalton band corresponded with the level of Ad DNA-prot replication complementing activity detected (data not shown).

As previously reported (12), nuclear extracts increased the rate and yield of pTP-dCMP complex formation. The activity that stimulated pTP-dCMP complex formation copurified with the Ad DNA replication complementing activity (Table 1). Glycerol gradient fractions that contained complementing activity also stimulated pTP-dCMP complex formation (Fig. 2B).

Both the complementing activity in the Ad DNA-prot replication system and the stimulating activity for pTP-dCMP complex formation were sensitive to *N*-ethylmaleimide treatment. Both activities were unaffected by heat treatment at 55°C for 15 min but were totally inactivated by heat treatment at 90°C for 2 min (data not shown).

Nuclear factor I (glycerol gradient fraction, 20 ng of protein) contained no detectable nuclease (<3 pmol was rendered acid soluble with single- or double-stranded DNA and there was no conversion of PSM2 covalently closed double-stranded circular superhelical DNA to other forms), topoisomerase I, ATPase (<20 pmol in the presence or absence of DNA), RNA polymerase (<0.2 pmol), or DNA polymerase (<0.5 pmol) activities. All reactions were carried out for 60 min at 30°C.

Effects of Nuclear Factor I on pTP-dCMP Complex Formation. The effect of nuclear factor I on pTP-dCMP complex formation was examined using Ad DNA-prot as template (Table 2). In the absence of Ad DNA-prot, Mg²⁺, or the Ad-protein fraction, no detectable pTP-dCMP complex was observed in either the presence or the absence of nuclear factor I. In the presence of Ad DNA-prot and the Ad-protein fraction, low levels of the pTP-dCMP complex were formed. This reaction was stimulated by ATP and by the addition of nuclear fraction I.

The addition of Ad DBP altered the response of the system to nuclear factor I. In the presence of Ad DNA-prot and the Ad-protein fraction with or without ATP, Ad DBP addition reduced pTP-dCMP complex formation below detectable levels. Under

these conditions, pTP-dCMP complex formation was completely dependent on the addition of nuclear factor I. This reaction was further stimulated by ATP addition.

Characterization of the Effect of Nuclear Factor I on pTP-dCMP Complex Formation. The rate of pTP-dCMP complex formation in the presence of nuclear factor I was linear up to 60 min (data not shown) and proportional to the amount of nuclear factor I added in both the presence and the absence of Ad DBP (Fig. 3).

The presence of nuclear factor I did not alter the nucleotide specificity of complex formation. Maximal stimulation of pTP-dCMP complex formation by nuclear factor I required the presence of ATP (optimal concentration, 3 mM) in both the presence and the absence of Ad DBP. Among the adenine derivatives tested, ADP could substitute for ATP at an efficiency of 80%; adenosine 5'-[γ -thio]triphosphate and adenosine 5'-[β , γ -imido]triphosphate could not substitute for ATP; dATP could substitute for ATP at an efficiency of 10% while AMP was inactive (data not shown).

Escherichia coli single-stranded DNA binding protein (SSB) did not substitute for Ad DBP; pTP-dCMP complex formation in the presence of the Ad-protein fraction and nuclear factor I was inhibited by SSB addition (Fig. 4). The rate of pTP-dCMP complex formation observed with factor I in the presence of Ad DBP was reduced 50% by the addition of *E. coli* SSB. The effects of SSB and Ad DBP on initial rate of pTP-dCMP complex formation were markedly influenced by the order of addition and by temperature of assembly of the protein-nucleic acid complex (unpublished observations).

pTP-dCMP Complex Formation Using ϕ X174 Single-stranded Circular [ss(c)] DNA. Ikeda *et al.* (16) have shown that pTP-dCMP complex formation occurs on single-stranded DNA

Table 2. Requirements for pTP-dCMP complex formation

Ad-protein fraction	Addition			pTP-dCMP formed, fmol
	Factor I	Ad DBP	ATP	
+	+	+	+	0.93
+	+	+	–	0.43
+	–	+	±	<0.05
–	+	+	+	<0.05
+	+	–	+	0.33
+	–	–	+	0.15
+	+	–	–	0.13
+	–	–	–	0.07

Reactions were carried out as described in *Materials and Methods* except that Ad DBP (1 μ g) and nuclear factor I (glycerol gradient fraction, 10 ng) were added where indicated. No detectable pTP-dCMP complex was observed in the absence of Ad DNA-prot, the Ad-protein fraction, or MgCl₂.

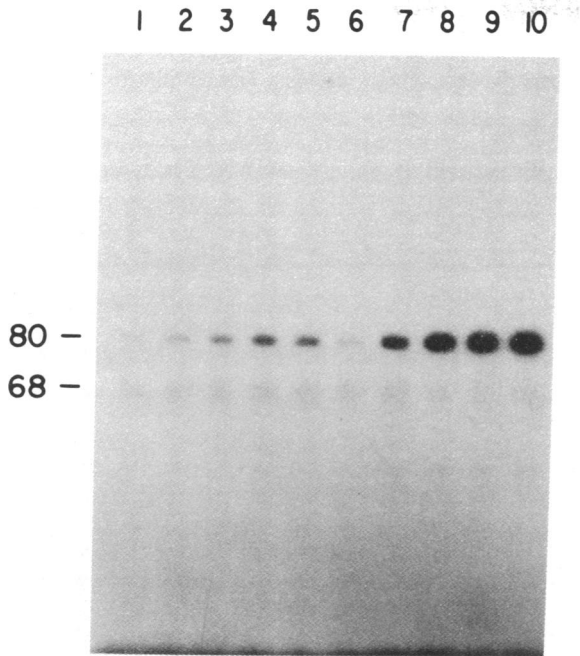


FIG. 3. Influence of amount of factor I on pTP-dCMP complex formation. Reactions were carried out with various amounts of factor I in the absence (lanes 1-5) or presence (lanes 6-10) of Ad DBP (1 μg). Lanes: 1 and 6, no factor I added; 2 and 7, 2.5 ng, 3 and 8, 5 ng, 4 and 9; 10 ng, 5 and 10, 15 ng, respectively, of factor I (glycerol gradient fraction). Numbers on the left indicate molecular masses in kilodaltons.

but on no other duplex DNA other than Ad DNA-prot. The addition of ATP or nuclear extracts abolished pTP-dCMP complex formation on φX174 ss(c) DNA. The formation of pTP-dCMP

complex by the Ad-protein fraction was completely inhibited by Ad DBP (Fig. 5). Nuclear factor I had no effect on pTP-dCMP complex formation in the absence of Ad DBP or on the inhibition of complex formation caused by Ad DBP with φX174 ss(c) DNA. φX174 duplex DNAs (covalently closed double-stranded circular and linear) did not support pTP-dCMP complex formation either in the presence or absence of factor I (data not shown).

DISCUSSION

A protein that is required for the replication of Ad DNA-prot in a system containing the Ad-protein fraction, Ad DBP, and cytosol from uninfected cells has been purified from nuclear extracts of HeLa cells. This protein, nuclear factor I, was free of detectable DNA polymerase activities. Glycerol gradient centrifugation yielded a single peak of activity sedimenting with an apparent molecular mass of 47,000 daltons. In addition to this complementing activity for Ad DNA replication, factor I also stimulated the formation of the initiation complex, pTP-dCMP. These two activities appear to be an intrinsic property of factor I, since both activities copurified on chromatography with a number of adsorbents, cosedimented in glycerol gradients, and showed similar sensitivities to *N*-ethylmaleimide and heat.

It was previously reported that reaction mixtures containing cytosol from Ad-infected cells and either nuclear extracts from uninfected cells or DNA polymerase β preparations supported the replication of Ad DNA-prot (3, 17). We now believe that the latter observation was due to the presence of factor I in the DNA polymerase β preparations used in those experiments. More extensively purified HeLa DNA polymerase β preparations failed to replace the nuclear fraction or nuclear factor I in the *in vitro* Ad DNA replication system.

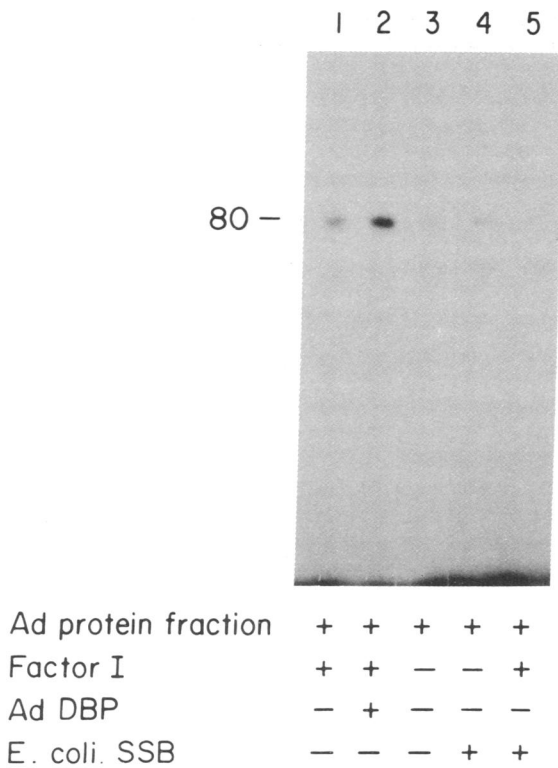


FIG. 4. Substitution of Ad DBP by *E. coli* SSB. Reactions were carried out in the presence of factor I (glycerol gradient fraction, 10 ng), Ad DBP (1 μg), and *E. coli* SSB (1 μg) as indicated. 80, molecular mass (kilodaltons).

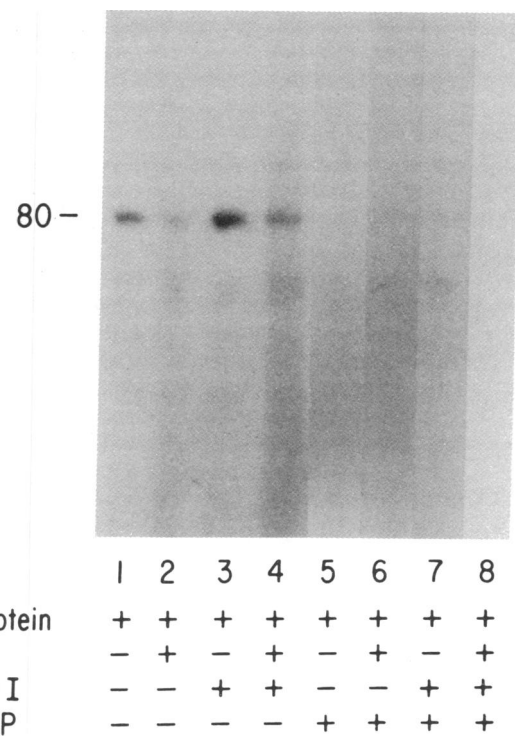


FIG. 5. pTP-dCMP complex formation using a single-stranded DNA template. Reaction mixtures contained φX174 ss(c) DNA (76.8 pmol of nucleotide) in lieu of Ad DNA-prot. Where indicated, Ad-protein fraction, Ad DBP (0.6 μg), factor I (glycerol gradient fraction, 10 ng), and 3 mM ATP were present. 80, molecular mass (kilodaltons).

With Ad DNA-prot as the template, factor I could replace nuclear extracts in supporting pTP-dCMP formation. The extent of this reaction depended on the presence of ATP and Ad DBP. In the absence of factor I, Ad DBP inhibited pTP-dCMP formation. Under these conditions, complex formation was completely dependent on factor I and ATP further stimulated this reaction.

In contrast to observations with Ad DNA-prot, pTP-dCMP complex formation on single-stranded DNA requires only the Ad-protein fraction. This reaction is unaffected by nuclear factor I and inhibited by Ad DBP. These results suggest that, in the absence of Ad DBP, complex formation may be dependent on regions of Ad DNA-prot that are single-stranded. If these single-stranded regions of Ad DNA-prot are complexed with Ad DBP, the initiation reaction can occur only in the presence of nuclear factor I. This mechanism predicts that agents that bind to single-stranded regions should inhibit complex formation in the absence of nuclear factor I. This is indeed observed with *E. coli* SSB. Since SSB cannot replace Ad DBP in the nuclear factor I-dependent initiation reaction, Ad DBP must also play a specific role in this reaction.

Nuclear factor I stimulated pTP-dCMP complex formation in the presence of Ad DBP efficiently, while crude nuclear extracts stimulated the same reaction as much as 10-fold in the absence of Ad DBP (8, 12). These observations suggest the presence of a cellular binding protein or factor that can replace Ad DBP. Stimulation of pTP-dCMP complex formation by nuclear factor I required high concentrations of ATP; however, the role played by ATP in the initiation reaction is unclear.

Nuclear factor I, in addition to its role in initiation, acts coordinately with Ad DBP and the Ad-protein fraction to synthesize DNA chains ranging in size up to 25% of full-length Ad DNA. In the presence of another nuclear fraction, full-length Ad DNA is synthesized.

Although the initiation reaction in the Ad DNA replication system is highly specific, the subsequent elongation process

with its dependence on host proteins may be more general. Thus, by defining the mechanism of action of these proteins, we hope to gain insight into host-DNA replication reactions.

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