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Biochemistry

Adenovirus DNA replication *in vitro*: Purification of the terminal protein in a functional form

(adenoviral terminal protein/eukaryotic DNA polymerase)

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ABSTRACT **The 80,000-dalton form of the adenovirus (Ad) terminal protein (pTP) has been purified from Ad-infected HeLa cells. pTP was assayed by its ability to form a covalent complex with dCMP. The protein copurified with an activity that is essential for *in vitro* Ad DNA replication (Ad protein activity) as well as with a DNA polymerase activity that was distinguished from those of HeLa cell DNA polymerases α, β, and γ. The Ad protein-associated DNA polymerase activity was detected with activated DNA but not with poly(rA)·oligo(dT) as template and was insensitive to aphidicolin and sensitive to *N*-ethylmaleimide. The Ad protein, DNA polymerase, and pTP-dCMP complex-forming activities sedimented in a glycerol gradient as a single peak with an apparent molecular size of 180,000 daltons. $NaDodSO_4$/polyacrylamide gel analysis of the glycerol gradient fraction showed major bands of 80,000 and 140,000 daltons. The 80,000-dalton band was identified as pTP by comparison of its tryptic peptide map with that of the 55,000-dalton form of the terminal protein, which was purified from Ad virions.**

The adenovirus (Ad) terminal protein is a viral gene product synthesized as an 80,000-dalton precursor (pTP) that is cleaved late in infection to a 55,000-dalton protein (TP) (1–3). The TP is found in mature Ad virions covalently linked to the 5′ terminus of each strand of the Ad genome (4–6). The protein is linked to DNA via a phosphodiester bond joining the β-OH of a serine residue to the 5′-OH of the terminal deoxycytidine residue (2, 7). In virions of the protease-deficient Ad mutant (Ad2ts1) grown at nonpermissive temperature, the terminal protein is found in the pTP form (3). The pTP was originally identified as the form of the terminal protein linked to the termini of Ad DNA synthesized in an *in vitro* DNA replication system prepared under conditions that prevented the expression of late viral genes (2).

All available evidence supports a role for the terminal protein in the initiation of Ad DNA replication. Rekosh *et al.* (4) hypothesized that the protein might initiate replication by forming a covalent complex with dCMP providing a 3′-OH end that could be used as a primer for subsequent DNA polymerase action. The finding that nascent DNA chains synthesized *in vivo* or *in vitro* are linked to protein (8–12) is consistent with this model. In addition, the species pTP–dCMP, which the model predicts as an intermediate in Ad DNA replication, has been identified as a product formed *in vitro* in the presence of dCTP (13). The formation of this product did not require the presence of other deoxynucleotides and was not inhibited by 2′,3′-dideoxynucleoside triphosphates (ddNTPs), suggesting that elongation is not a prerequisite for the attachment of protein to dCMP. The TP may also have a role in making the Ad DNA molecule an active template for replication; only Ad DNA with intact terminal protein is efficiently replicated *in vitro* (10, 14–16).

In this report, we describe the purification of pTP from extracts of Ad-infected HeLa cells. It was previously shown that an *in vitro* Ad DNA replication system was reconstituted in reaction mixtures containing nuclear and cytoplasmic extracts from uninfected cells, Ad DNA binding protein (Ad-DBP), Ad DNA–protein complex (Ad DNA-pro), and an Ad protein fraction (10). The Ad protein fraction was purified by using an assay designed to score for Ad-coded or induced proteins involved in the replication of Ad DNA. We now describe a procedure for the isolation of this Ad protein fraction that gives greater yield, purification, and stability than was previously obtained. The purified Ad protein fraction contained an 80,000-dalton protein as a major component. This component was identified as pTP by comparison of its tryptic peptides with those of the 55,000-dalton terminal protein isolated from Ad DNA-pro. The Ad protein fraction copurified with an activity assayed by the synthesis of pTP–dCMP. The Ad protein fraction also copurified with a DNA polymerase activity that was distinguished from HeLa cell DNA polymerases α, β, and γ by both its chromatographic and enzymatic properties and may represent a novel DNA polymerase implicated in Ad DNA replication.

MATERIALS AND METHODS

Materials. All preparations of extracts, Ad2 DNA-pro, and other reagents were as described (10, 13, 16).

DNA Polymerase Assay. DNA polymerase α was assayed with nicked salmon sperm DNA in the presence of $MgCl_2$ (7.5 mM). Poly(rA)·oligo(dT) and $MnCl_2$ (0.5 mM) were used in the assays for DNA polymerases β and γ (17).

Assay for Synthesis of pTP–dCMP. Reaction mixtures (0.05 ml) were 25 mM Hepes, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/3 mM ATP/100 μM aphidicolin/0.5 μM [α-^{32}P]dCTP (410 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) containing 5 μg of bovine serum albumin, 0.2 μg of Ad DNA-pro, nuclear extract (30 μg of protein) from uninfected HeLa cells, and the Ad protein fraction at various stages of purification. pTP–dCMP was detected by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (13) and quantitated by excising the band from the gel and assaying the Cerenkov radiation. Background radioactivity was determined by averaging the radioactivity in regions of the gel immediately above and below the pTP–dCMP.

Assay for Ad Protein Activity. Reaction mixtures (0.05 ml) were 25 mM Hepes, pH 7.5/5 mM $MgCl_2$/4 mM dithiothreitol/3 mM ATP/40 μM each of dATP, dCTP, and dGTP/4 μM [^{3}H]dTTP (3000–4000 cpm/pmol) containing 10 μg of bovine serum albumin, 0.5 μg of Ad DBP, nuclear (9.4 μg of protein)

Abbreviations: Ad, adenovirus; Ad DNA-pro, adenovirus DNA with terminal protein covalently bound to each 5′ end; Ad-DBP, adenovirus-coded DNA binding protein; pTP, 80,000-dalton terminal protein; TP, 55,000-dalton terminal protein that is covalently bound to the 5′ ends of Ad DNA; ddNTP, 2′,3′-dideoxynucleoside triphosphate; Ara-CTP, cytosine β-D-arabinofuranoside-5′-triphosphate.

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and cytoplasmic (40 μg of protein) extracts from uninfected HeLa cells, 0.1 μg of Ad DNA-pro, and the Ad protein fraction at various stages of purification; 1 unit incorporated 1 nmol of [^{3}H]dTMP into acid-insoluble material in 60 min at 30°C.

Purification of the Ad Protein Fraction. Crude cytoplasmic extract prepared from (2.5 × 10^{10}) Ad2-infected HeLa cells (14) was centrifuged at 105,000 × *g* for 60 min, adjusted to 50 mM NaCl/1 mM dithiothreitol/1 mM EDTA (Ad cytosol, 170 ml) and applied to a DEAE-cellulose column (6 cm × 18 cm) equilibrated with buffer A [25 mM Tris·HCl, pH 7.5 (4°C)/1 mM dithiothreitol/1 mM EDTA/20% (vol/vol) glycerol/0.01% Nonidet P-40]/containing 50 mM NaCl. The column was washed with 1 liter of 50 mM NaCl in buffer B [10 mM sodium phosphate, pH 6.0/1 mM dithiothreitol/1 mM EDTA/10% sucrose/0.01% Nonidet P-40/20% glycerol (vol/vol)] and eluted with 0.2 M NaCl in buffer B. Fractions containing Ad protein activity were pooled (DEAE eluate, 365 ml) and applied to a phosphocellulose column (2.9 cm × 16 cm) equilibrated with 0.15 M NaCl in buffer B. The column was washed with 200 ml of the equilibration buffer and eluted with a 600-ml linear gradient of 0.15–1.0 M NaCl in buffer B. The Ad protein fraction eluted at 0.4 M NaCl. The peak fractions were combined (phosphocellulose eluate, 92 ml), dialyzed against 0.1 M NaCl in buffer B, and applied to a column of denatured DNA-cellulose (2.6 cm × 8 cm) equilibrated with 0.15 M NaCl in buffer B. The column was washed with 90 ml of 0.15 M NaCl in buffer B and eluted with a 250-ml linear gradient of 0.15–0.6 M NaCl in buffer B. Ad protein activity eluted at 0.36 M NaCl. The peak fractions were pooled (denatured DNA-cellulose eluate, 46 ml), dialyzed against 0.1 M NaCl in buffer B, and applied to a column of native DNA-cellulose (1.5 cm × 5.5 cm) equilibrated with 0.1 M NaCl in buffer B. The column was washed with 17 ml of 0.1 M NaCl in buffer B and eluted with an 80-ml linear gradient of 0.1–0.6 M NaCl in buffer B. Ad protein activity eluted at 0.24 M NaCl. The peak fractions were pooled (native DNA-cellulose eluate, 19 ml) and a portion was adsorbed to a small phosphocellulose column and concentrated by stepwise elution with buffer C [25 mM sodium phosphate, pH 6.0/1 mM EDTA/1 mM dithiothreitol/0.01% Nonidet P-40/10% glycerol (vol/vol) /0.5 M NaCl]. The peak fractions were combined and a portion (0.2 ml) was layered on top of a 15–35% glycerol gradient in buffer C. Centrifugation was performed at 48,000 rpm for 20 hr at 4°C in an SW50.1 rotor. Fractions (0.12 ml) were collected from the bottom of the tube (glycerol gradient fraction).

Tryptic Peptide Mapping. Ad DNA-pro purified as described from virions (16) was concentrated (0.8 ml containing 260 μg of DNA) and digested with 100 units of micrococcal nuclease for 60 min at 37°C. Another 100 units of micrococcal nuclease was added, and incubation was continued for an additional hr. The digests and glycerol gradient fraction of Ad protein were precipitated with trichloroacetic acid and washed with ether. These two samples and Ad-DBP as a nonhomologous control (18) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis; regions containing the protein bands of interest were excised, radioiodinated, and digested with trypsin (19). After digestion, the soluble material was removed and lyophilized; the residue was dissolved in 0.1 ml of H_2O and lyophilized. The samples were dissolved in 20 μl of buffer I [acetic acid/pyridine/water (10:1:89)] and 5 μl of each fraction was spotted onto cellulose-coated thin-layer chromatography plates. Electrophoresis was carried out in buffer I at 1 kV for 70 min. The plates were dried and the peptides were chromatographed in a second direction in buffer II [*n*-butanol/pyridine/acetic acid/water (75:60:15:60)] for 5 hr.

RESULTS

Purification of the Ad Protein Fraction. The procedure used for the isolation of the Ad protein fraction is summarized in Table 1. The conditions used stabilized and greatly increased the recovery of Ad protein activity in comparison with previous results (10). The most highly purified fraction represented a purification of at least 1000-fold over the crude extract.

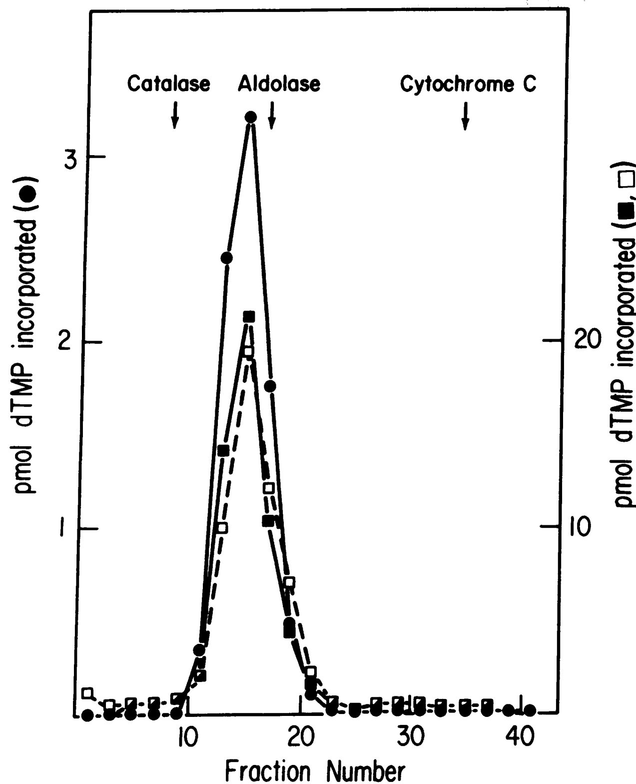
Glycerol gradient centrifugation, the final step in the purification of the Ad protein activity, yielded a single peak sedimenting with an apparent molecular size of 180,000 daltons relative to marker proteins (Fig. 1*A*). DNA polymerase activity assayed with activated DNA as template cosedimented with the Ad protein activity. pTP–dCMP-forming activity, which copurified with the Ad protein activity on each of the four columns used (data not shown), also cosedimented with the Ad protein activity (Fig. 1*B*). $NaDodSO_4$/polyacrylamide gel electrophoresis of the glycerol gradient fractions (Fig. 2) showed major protein bands at 80,000 and 140,000 daltons that coincided with the peak of Ad protein activity and pTP–dCMP-forming activity.

Both DNA-dependent and independent ATPase activities were detected in the Ad protein fraction through native DNA-cellulose chromatography. These activities were separated from the Ad protein activity during glycerol gradient centrifugation. The glycerol gradient fraction was free of endonuclease activity

Table 1. Purification of Ad protein

	Ad protein activity			DNA polymerase activity: Without aphidicolin		DNA polymerase activity: With aphidicolin	
Fraction	Total protein, mg	Total units, nmol	Specific activity, nmol/mg	Total units, nmol	Specific activity, nmol/mg	Total units, nmol	Specific activity, nmol/mg
Ad cytosol	2,240	737	0.33	2,330	1.04	673	0.30
DEAE-cellulose	657	788	1.2	1,580	2.40	604	0.92
Phospho-cellulose	79.1	448	5.7	1,040	13.1	338	4.26
Denatured DNA-cellulose	5.98	230	38.5	204	34.2	170	28.5
Native DNA-cellulose	3.78	151	40.0	168	44.4	153	40.6
Glycerol gradient	<0.11	55	>500	45	>410	53	>480

DNA polymerase was assayed with nicked salmon sperm DNA in the presence and absence of 100 μM aphidicolin. One unit of DNA polymerase catalyzed the incorporation of 1 nmol of dTMP into acid-insoluble material in 20 min at 30°C. A portion of the native DNA-cellulose fraction was subjected to glycerol gradient centrifugation. The values reported 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. The protein content of the glycerol gradient fractions was estimated from a densitometric scan of a Coomassie blue-stained gel using bovine serum albumin as the standard.



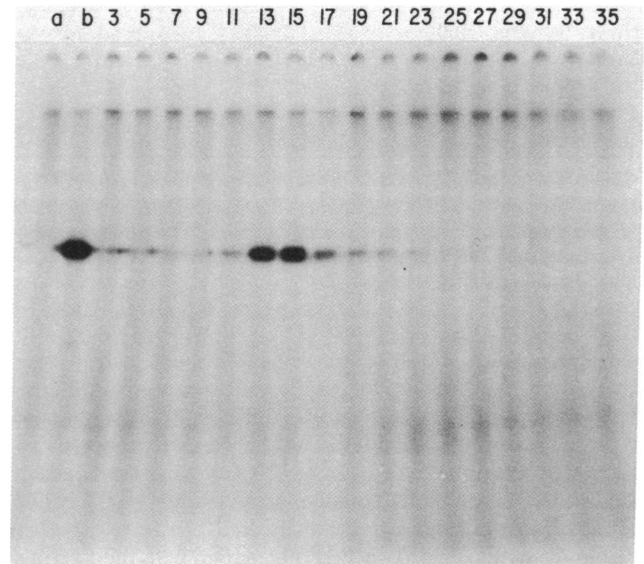


FIG. 1. Glycerol gradient centrifugation of Ad protein fraction. (A). Assay for Ad protein and DNA polymerase activities. The native DNA-cellulose column fraction was concentrated on a phosphocellulose column, and an aliquot was sedimented through a 15–35% glycerol gradient. One microliter of each gradient fraction used for the Ad protein assay (●) and 4 μl was used for the DNA polymerase assays. DNA polymerase was assayed with nicked salmon sperm DNA in the presence (■) and absence (□) of 100 μM aphidicolin. Positions of marker proteins in a parallel gradient are indicated. (B). Assay for pTP–dCMP formation. Aliquots (0.5 μl) of fractions from the same glycerol gradient were assayed for synthesis of pTP–dCMP. Lanes a, no gradient fraction; b, 0.5 μl of native DNA-cellulose fraction; 3–35, glycerol gradient fraction assayed.

assayed with single- and double-stranded circular φX174 DNA.

Characterization of the DNA Polymerase Activity in the Ad Protein Fraction. The Ad protein activity copurified with DNA polymerase α activity on DEAE-cellulose chromatography and phosphocellulose chromatography. Denatured DNA-cellulose chromatography separated the DNA polymerase activity into a major peak, identified as polymerase α by its sensitivity to aphidicolin and template specificity, and a minor peak, that coincided with Ad protein activity (Fig. 3). The latter DNA polymerase copurified with the Ad protein fraction through subsequent chromatography on native DNA-cellulose and sedimentation through a glycerol gradient (Fig. 1A). DNA polymerase γ, assayed by activity on poly(rA)·oligo(dT), was detected in the denatured DNA-cellulose eluate but not in subsequent fractions.

The DNA polymerase activity in the native DNA-cellulose fraction was compared with that of DNA polymerases α, β, and γ (Table 2). The Ad protein-associated DNA polymerase resembled polymerase α in its template preference; it efficiently used

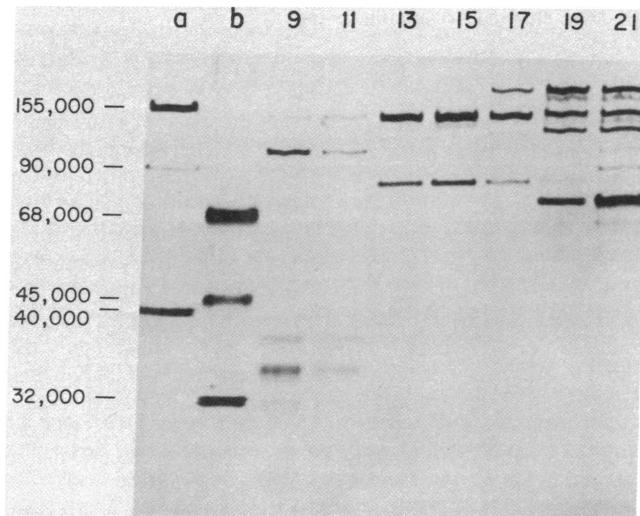


FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of glycerol gradient fractions. Lanes: a and b, size standards [*Escherichia coli* RNA polymerase (40,000, 90,000, 155,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (45,000 daltons), and carbonic anhydrase (32,000 daltons)]; 9–21, aliquots (18 μl) of glycerol gradient fractions. Protein bands were visualized by silver staining (20).

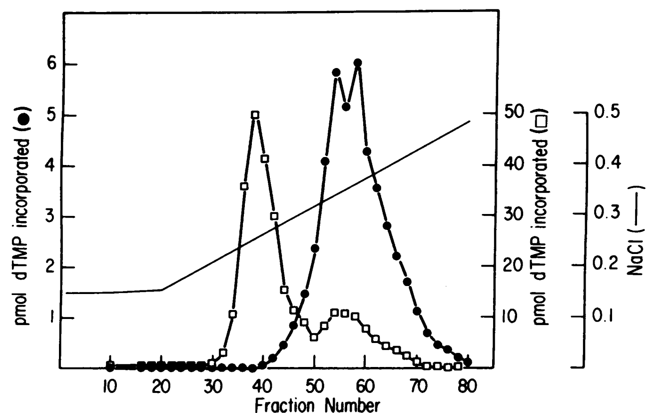


FIG. 3. Denatured DNA-cellulose chromatography of pooled phosphocellulose fraction. Aliquots of the fractions were assayed for Ad protein (●) and for DNA polymerase activities (□) as described in the legend to Fig. 1.

Table 2. Comparison of the properties of DNA polymerases α, β, and γ with those of Ad protein-associated polymerase

Polymerase	Activated DNA	Assay, % of maximum: poly(rA)·oligo-$(dT)_{12-18}$	Assay, % of maximum: poly(rA)·oligo-$(dT)_{12-18}$/ *N*-ethylmaleimide (10 mM)
DNA α	100	2	<1
DNA β	21	100	75
DNA γ	6	100	<1
Ad protein-associated	100	3	<1

The native DNA-cellulose column fraction was used for assay of the Ad protein-associated DNA polymerase. HeLa cell DNA polymerases α, β, and γ were purified as described (17). The value of 100% for DNA polymerases α, β, and γ corresponded to the incorporation of 21.2, 17.2, and 29.3 pmol of dTMP, respectively.

activated DNA as a template but was inactive on poly(rA)·oligo(dT). It also resembled polymerase α in its sensitivity to *N*-ethylmaleimide, NaCl, and cytosine β-D-arabinofuranoside-5'-triphosphate (Ara-CTP) (Table 3). However, the Ad protein-associated polymerase was more sensitive to ddTTP and resistent to aphidicolin, unlike DNA polymerase α. Aphidicolin at 100 μM inhibited α polymerase activity $\approx$ 90%, while the Ad protein polymerase was unaffected.

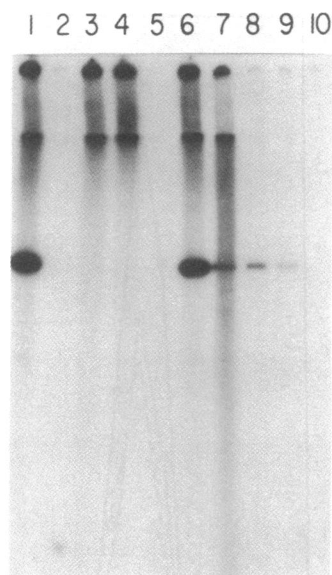
Requirements for pTP–dCMP Formation with the Purified Ad Protein Fraction. Formation of pTP–dCMP was observed with the Ad protein fraction in combination with Ad DNA-pro, $MgCl_2$, and [α-^{32}P]dCTP. No pTP–dCMP was detected when any one of these components was omitted from the reaction mixture or when Ad DNA-pro was replaced by deproteinized Ad DNA (Fig. 4). pTP–dCMP formation was stimulated by the addition of ATP or nuclear extract from uninfected cells, and the effect of the addition of both was greater than additive. Stimulation by nuclear extract was heat labile, suggesting that protein present in the nuclear extract facilitated the formation of pTP–dCMP. Aphidicolin (100 μM) did not inhibit formation of pTP–dCMP.

The 80,000-Dalton Protein in the Purified Ad Protein Fraction is pTP. The 55,000-dalton protein (i.e., TP) was isolated from Ad DNA-pro by extensive digestion with micrococcal nuclease followed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The 55,000–dalton band was compared with the 80,000-dalton band present in the purified Ad protein fraction. Regions of gel containing the bands of interest were excised. The proteins in the gel slices were iodinated and digested with trypsin, and the resulting peptides were fractionated by electrophoresis followed by ascending thin-layer chromatography. The map derived from the 80,000-dalton protein showed extensive homology with that of TP (Fig. 5 *A* and *B*). In contrast, a map of Ad-DBP showed no such homology (Fig. 5*C*).

Table 3. Effect of inhibitors on DNA polymerase α and Ad protein-associated polymerase

Inhibitor	Concentration	dTMP incorporated, pmol: Polymerase α	dTMP incorporated, pmol: Ad protein polymerase
None		25.9	28.4
ddTTP	13.3 μM	26.4	8.9
	133 μM	21.1	6.1
Aphidicolin	10 μM	9.7	28.6
	100 μM	2.7	26.8
Ara-CTP	100 μM	10.6	15.2
	1 mM	7.7	9.2
N-Ethylmaleimide	10 mM	0.7	0.7
NaCl	100 mM	3.2	4.5

The native DNA-cellulose column fraction was used for assay of the Ad protein-associated DNA polymerase. A mixture of DNA polymerase α and the Ad protein polymerase gave additive incorporation of dTMP. The addition of aphidicolin to such reactions diminished dTMP incorporation due to DNA polymerase α.



	1	2	3	4	5	6	7	8	9	10
Ad-protein	+	+	−	+	+	+	+	+	+	−
Nuclear Extract	+	+	+	+	+	+	+	−	−	−
$MgCl_2$	+	−	+	+	+	+	+	+	+	+
ATP	+	+	+	+	+	+	−	+	−	−
Ad DNA-Prot	+	+	+	−	−	+	+	+	+	+
Ad DNA	−	−	−	+	−	−	−	−	−	−
Aphidicolin	+	−	−	−	−	−	−	−	−	−
pTP-dCMP formed (fmol)	1.5	<0.1	<0.1	<0.1	<0.1	1.4	0.2	0.2	0.1	<0.1

FIG. 4. Requirements for pTP–dCMP synthesis with the purified Ad protein fraction. The compositions of the reaction mixtures are described below the gel lanes.

DISCUSSION

We have described a method for the purification of pTP in a functional form from extracts of Ad-infected HeLa cells. Two assays were used to identify pTP. The Ad protein assay was devised to detect Ad-coded or induced proteins involved in Ad DNA replication *in vitro*. Formation of pTP–dCMP was assumed to be a more specific, although a more time-consuming, assay for pTP. Both assays identified an activity that purified as a single peak through four different chromatographic columns and a glycerol gradient centrifugation. As the most highly purified fraction alone supported formation of pTP–dCMP, it contained pTP. Tryptic peptide analysis of the 80,000-dalton protein band present as a major component in this fraction showed it to be structurally related to TP. Therefore, the 80,000-dalton protein present in the glycerol gradient fractions is pTP.

The glycerol gradient fraction also contained a major component of 140,000 daltons. Since the Ad protein fraction sedimented as a protein with an apparent molecular size of 180,000 daltons, the active form of Ad protein may consist of pTP complexed with the 140,000-dalton protein or, alternatively, may be a dimer of pTP. The distribution of the 140,000-dalton band across the glycerol gradient was similar to that of the 80,000-

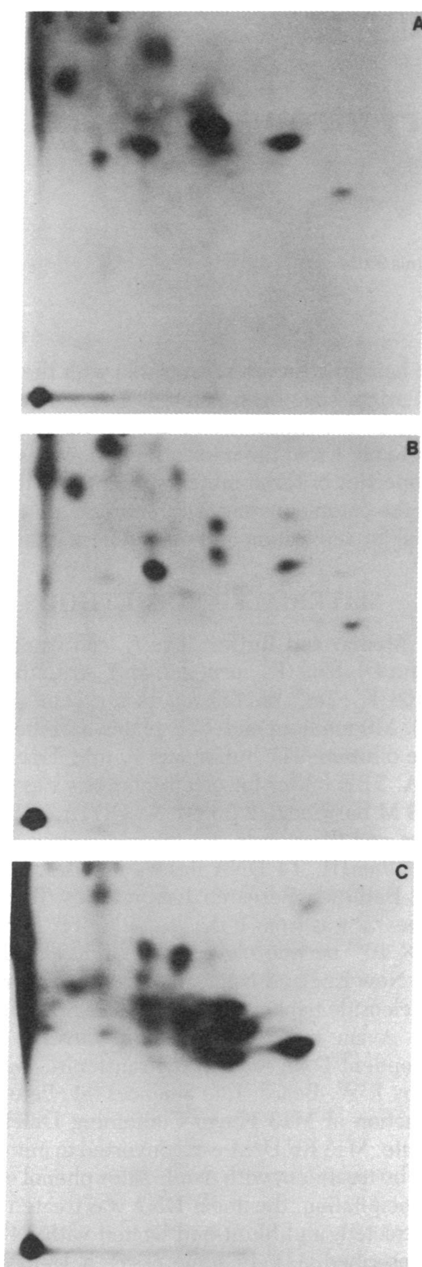


FIG. 5. Tryptic peptide mapping. High-voltage electrophoresis was in the horizontal direction with the anode on the left, and thin-layer chromatography was carried out in the vertical direction. (A) pTP (80,000-dalton protein from Ad protein fraction). (B) TP (55,000-dalton terminal protein isolated from virion). (C) Ad-DBP.

dalton band; the molar ratio of these proteins, estimated from a densitometric scan of a Coomassie blue-stained gel was ≈1:1. These results are consistent with the complexing of pTP with the 140,000-dalton band. The glycerol gradient fraction contains several minor components that are probably contaminants.

The Ad protein fraction copurified with a DNA polymerase activity. The Ad protein-associated polymerase was chromatographically distinct from HeLa cell DNA polymerases α, β, and γ. The polymerase resembled DNA polymerase α in its template preference and in its sensitivity to N-ethylmaleimide, Ara-CTP, and NaCl (21, 22). However, it was insensitive to aphidicolin, a potent inhibitor of polymerase α, and was more sensitive than polymerase α to inhibition by ddTTP. The origin of this DNA polymerase is unknown. Since no Ad-coded DNA polymerase has been observed, this polymerase may be one of the host DNA polymerases altered by interaction with pTP. An interaction between a host and viral gene product has been observed in bacteriophage T7 DNA polymerase (23). It is possible that the DNA polymerase activity resides in the 140,000-dalton protein that copurifies with pTP, although the possibility that pTP itself or one of the minor bands has polymerase activity cannot yet be ruled out.

The Ad protein fraction formed pTP–dCMP, a putative intermediate in Ad DNA replication, in the presence of Ad DNA-pro without the addition of any other protein fractions. The reaction was stimulated at least 10-fold by the addition of nuclear extract from uninfected cells and ATP. Since the Ad protein fraction is free of Ad-DBP after denatured DNA-cellulose chromatography, it seems likely that Ad-DBP is not required for the attachment of dCMP to pTP. The sedimentation of pTP–dCMP in a glycerol gradient was found to be identical to that of the Ad protein activity, suggesting that no dissociation of subunits occurs immediately after linkage to dCMP (data not shown).

Further studies of the Ad protein fraction should aim to resolve the active components. Identification of the associated DNA polymerase activity with respect to its host or viral origin, as well as the localization of the activity to a particular subunit, would clarify its role in Ad DNA replication.

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