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Biochemistry

Replication of adenovirus DNA-protein complex with purified proteins

(adenoviral DNA replication/adenovirus DNA binding protein/5′-terminal protein/eukaryotic DNA polymerase)

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ABSTRACT **A protein fraction isolated from the cytosol of adenovirus-infected HeLa cells, which contained DNA polymerase α, catalyzed adenoviral DNA replication in the presence of adenovirus DNA binding protein, eukaryotic DNA polymerase β, ATP, all four dNTPs, and $MgCl_2$. DNA replication started at either end of exogenously added adenoviral DNA and was totally dependent on the presence of terminal 55,000-dalton proteins on the DNA template. The replication of adenovirus DNA in the system was sensitive to aphidicolin and retained nearly all the properties of DNA replication that occur *in vivo* or *in vitro* with crude extracts. The 5′-ends of the newly synthesized adenovirus DNA strands were covalently linked to an 80,000-dalton protein linked to dCMP. DNA synthesized with purified proteins was only 25–50% the length of parental viral strands. Addition of cytosol extracts from uninfected HeLa cells to reaction mixtures containing purified proteins gave full-length adenoviral DNA strands.**

At present, the only eukaryotic system that initiates DNA synthesis *in vitro* with exogenous DNA is the adenovirus (Ad) DNA replication system described by Challberg and Kelly (1). Nuclear extracts isolated from Ad-infected HeLa cells support DNA synthesis when the template used is Ad DNA having a 55,000-dalton protein covalently linked to both 5′ ends of the DNA (Ad DNA-pro). Further proof of the replicative nature of this system has been obtained by using a conditional lethal mutant of Ad that codes for a temperature-sensitive DNA binding protein (Ad-DBP; ref. 2). Cells infected with this mutant support Ad DNA replication at 32°C but not at 39°C, and extracts of cells infected with this mutant behave identically *in vitro* (3, 4). These results suggest that the Ad-DBP plays an important role in the replication of Ad DNA.

We have previously reported the separation of the Ad DNA-pro replication system into a cytosol extract from Ad-infected cells and a nuclear extract from uninfected cells (5). Under specified conditions, the nuclear extract was replaced by preparations of DNA polymerase β. We here describe the purification of a protein fraction from the cytosol extract of Ad-infected HeLa cells that supports Ad DNA synthesis in the presence of Ad-DBP and cytosol and nuclear extracts from uninfected cells. The nuclear extracts from uninfected cells can be replaced by the addition of DNA polymerase (pol) β. In the presence of a purified Ad-protein fraction (which contains pol α), pol β, Ad-DBP, ATP, all four dNTPs, $MgCl_2$, and Ad DNA-pro (or restriction fragments of Ad DNA containing the 5′ proteins), DNA synthesis is initiated at either end of the viral DNA. This synthesis is completely dependent on the presence of terminal 55,000-dalton proteins on the template. The 5′ end of newly synthesized Ad DNA is covalently linked to an 80,000-dalton protein. The DNA synthesized by using purified protein components was not full length but could be completely elongated by addition of cytosol extracts from uninfected cells. Fragments of Ad DNA-pro generated by restriction enzymes (*Xba* I, *Hpa* I, *Bam*HI) can be used as template; in these cases, the DNA fragments containing protein at their 5′ ends were specifically replicated and yielded products that were the length of the DNA fragment copied. DNA fragments devoid of terminal protein were not replicated.

MATERIALS AND METHODS

Preparations and Isolations. [^{14}C]Thymidine-labeled Ad DNA-pro from Ad2, DNA polymerases from HeLa cell nuclei, and HeLa cell cytosol and nuclear extracts were prepared as described (1, 4, 5), except that the nuclear extracts were prepared by extracting washed HeLa cell nuclei with 0.3 M NaCl and then diluting the extract to 0.2 M NaCl, passing it through a DEAE-cellulose column to remove nucleic acid, and concentrating it with 70% ammonium sulfate. Cytosol from Ad-infected HeLa cells was prepared as described (1). The Ad-protein fraction and the Ad-DBP (>95% homogeneity) were isolated as follows. Ad-infected HeLa cell cytosol (142 ml; 1.85 g total proteins; Ad-protein, 667 units per mg; Ad-DBP, 93 units per mg) was prepared from 3×10^{10} cells infected with Ad2 (1). The cytosol was adjusted to 50 mM NaCl/1 mM dithiothreitol/1 mM EDTA and applied to a DEAE-cellulose column (4 cm × 24 cm) equilibrated with buffer A [25 mM Tris·HCl, pH 7.5 (4°C)/1 mM dithiothreitol/1 mM EDTA/20% (vol/vol) glycerol] containing 50 mM NaCl. The column was washed with 600 ml of 50 mM NaCl in buffer A and eluted with 1.5 liters of a linear gradient of NaCl (0.05–0.4 M) in buffer A. The Ad-protein fraction and Ad-DBP were co-eluted at 0.1–0.15 M NaCl and pooled (DEAE-cellulose fraction—205 ml, 230 mg total protein; Ad-protein, 1100 units per mg; Ad-DBP, 385 units per mg). The DEAE-cellulose fraction was applied to a phosphocellulose column (1.6 cm × 10 cm, equilibrated with 0.1 M NaCl in buffer A), the column was washed with 100 ml of 0.2 M NaCl in buffer A, and the product was eluted with 300 ml of a linear gradient of NaCl (0.2–1.0 M) in buffer A. The Ad-protein fraction and Ad-DBP were eluted at 0.39–0.55 M NaCl (peaks of Ad-protein and Ad-DBP activities were 0.45 M and 0.5 M, respectively) and pooled (phosphocellulose fraction—48 ml, 37 mg total protein; Ad-protein fraction, 2000 units per mg; Ad-DBP, 1000 units per mg). The phosphocellulose fraction was dialyzed against buffer A containing 0.1 M NaCl and applied to a denatured DNA-cellulose column (2 cm × 5 cm) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 40 ml of 0.15 M NaCl in buffer A, and the product was eluted with 70 ml of a linear gradient of NaCl (0.15–1.5 M) in buffer A. The Ad-protein fraction, which eluted at 0.3 M NaCl,

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Abbreviations: Ad, adenovirus; Ad DNA-pro, adenovirus DNA covalently linked to a 55,000-dalton protein at the 5′-ends of each strand; Ad DNA, deproteinized adenovirus DNA; Ad-DBP, adenovirus-coded DNA binding protein; pol, DNA polymerase.

Table 1. Replication of Ad DNA-pro with various protein fractions

Conditions	dTMP incorporated, pmol A	B
Complete	4.20	4.90
− HeLa cell cytosol	5.49	3.55
− HeLa cell nuclear extract	0.35	0.24
− HeLa cytosol and nuclear extracts	0.17	0.13
− Ad-infected HeLa cell cytosol	0.15	—
− Purified Ad-protein fraction	—	0.08
− Ad DNA-pro	0.12	0.01
− Ad DNA-pro; + Ad DNA (0.1 μg)	0.39	0.09
− Ad-DBP	0.92	0.31
− ATP	0.96	0.51

(Column A) Reaction mixtures (0.05 ml) were as described in *Materials and Methods* and contained Ad-DBP (2.4 μg), Ad-infected HeLa cell cytosol (13 μg), HeLa cell cytosol (36 μg), and nuclear extract (13 μg). (Column B) Same as for column A except that purified Ad-protein fraction (0.42 μg) was used in place of Ad-infected HeLa cell cytosol. All reactions were carried out at 30°C for 60 min.

was pooled and dialyzed against buffer B [25 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/1 mM EDTA/0.1 M NaCl/50% (vol/vol) glycerol] for 12 hr. This fraction (DNA cellulose) contained, in a volume of 5 ml, 1.3 mg of total protein, 7100 units per mg; the activity before dialysis was 14,000 units per mg. The Ad-DBP was eluted at 0.8 M NaCl, pooled, and dialyzed against buffer B (8 ml, 3.8 mg total protein, 4500 units per mg). The Ad-protein fraction and Ad-DBP were stored at −70°C. Purified Ad-DBP, up to 5 μg (maximum tested), contained no pol, nuclease, or ATPase activities (±DNA).

Conditions for *in Vitro* Ad DNA Replication. Reaction mixtures (0.05 ml) contained 25 mM Hepes buffer, pH 7.5 (30°C)/4 mM dithiothreitol/4.5 mM $MgCl_2$/3 mM ATP/40 μM each of dATP, dGTP, and dCTP/4 μM [^{3}H]dTTP (4000 cpm/pmol)/bovine serum albumin (0.2 mg/ml)/20 mM NaCl/0.1 μg of Ad DNA-pro. To assay the Ad-protein fraction, HeLa cell cytosol (36 μg), HeLa cell nuclear extract (13 μg), and Ad-DBP (2.4 μg) were added to the reaction mixture; to assay the Ad-DBP, Ad-infected HeLa cell cytosol (13 μg) was added in place of Ad-DBP. The amount of dTMP incorporated into acid-insoluble material after incubation for 60 min at 30°C was measured; one unit of activity was defined as 1 pmol of dTMP incorporated under these conditions.

Table 2. Requirements for Ad DNA-pro replication with purified proteins

Conditions	dTMP incorporated, pmol
Complete	4.10
− Ad DNA-pro	0.02
− Ad DNA-pro; + Ad DNA (0.07 μg)	0.24
− Ad-protein fraction	0.01
− Ad-DBP	0.11
− Ad-DBP; + *E. coli*-DBP (1 μg)	0.14
− pol α and pol β	0.52
− ATP	0.27
− ATP; + dATP (3 mM)	0.53
− $MgCl_2$	0.01
+ CTP, GTP, and UTP (0.1 mM)	4.23
+ Aphidicolin (10 μM)	1.32
+ Aphidicolin (100 μM)	0.45

Reaction mixtures (0.05 ml) as described in *Materials and Methods* contained Ad DNA-pro (0.07 μg), Ad-protein fraction (0.42 μg), Ad-DBP (2.4 μg), and HeLa cell pol α (0.02 unit) and pol β (0.05 unit); reactions were incubated at 30°C for 90 min.

Table 3. Influence of HeLa cell DNA polymerases on Ad DNA-pro replication

Addition	dTMP incorporated, pmol
pol α (0.02 unit) and pol β (0.05 unit)	4.10
None	0.52
+ pol α (0.02 unit)	0.46
+ pol β	
0.025 unit	1.97
0.05 unit	3.51
0.10 unit	3.59
+ pol γ (0.04 unit)	0.54
+ pol α and pol γ	0.53
+ pol β and pol γ	3.77
+ pol α, pol β, and pol γ	3.83

Reaction mixtures (0.05 ml) as described in *Materials and Methods* contained Ad DNA-pro (0.07 μg), Ad-protein fraction (0.42 μg), and Ad-DBP (2.4 μg); reactions were incubated at 30°C for 90 min. Varying the concentrations of pol α (up to 0.12 unit) or pol γ (up to 0.1 unit) had no further effects.

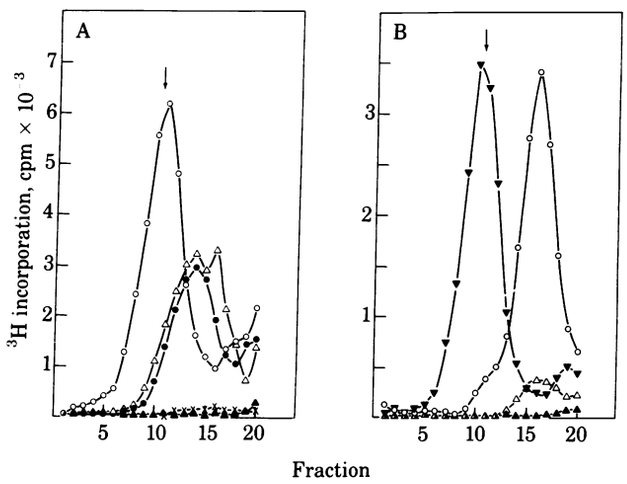

FIG. 1. Alkaline sucrose gradient sedimentation of Ad DNA synthesized *in vitro*. (*A*) Products were formed with purified Ad-protein fraction in the presence of Ad-DBP and HeLa cell cytosol and nuclear extract as described in the legend to Table 1. Reactions were incubated at 30°C for 60 (●) and 120 (○) min. Reactions in the absence of HeLa cell cytosol (△) or nuclear extract (×), or of purified Ad-protein fractions (▲) were carried out at 30°C for 120 min. Synthesis was terminated by the addition of 3 μl of 0.5 M EDTA and 0.1 ml of 0.45 M NaOH. After removal of 20 μl for quantitation of dTMP incorporation, the remaining solution was layered onto a 5–20% alkaline sucrose gradient (4.9 ml; 0.3 M NaOH/10 mM EDTA/1 M NaCl) and centrifuged at 45,000 rpm in a Beckman SW 50.1 rotor for 2.5 hr at 4°C. [^{14}C]Ad2 DNA (5 × 10^3 cpm) was used as internal marker. Arrow indicates the position of the 34S marker [^{14}C]Ad-DNA single strands. Direction of sedimentation was from right to left. In this experiment, [^{3}H] recovery varied between 87% to 95%. (*B*) Reactions were incubated for 2 hr at 30°C and subjected to alkaline sucrose gradient centrifugation as described above. Reaction mixtures (0.05 ml) contained Ad DNA-pro, Ad-protein fraction, Ad-DBP, pol α, and pol β (○); Ad DNA-pro, Ad-protein fraction, Ad-DBP, pol α, and pol β plus 36 μg of cytosol extract of HeLa cells (▼); Ad DNA-pro, Ad-DBP, pol α, and pol β plus cytosol extract (i.e., lacking the Ad protein fraction) (△); Ad DNA-pro, Ad-DBP, pol α, and pol β plus cytosol extract of HeLa cells (i.e., lacking the Ad protein fraction) (▲). In these experiments, the recovery of label in DNA was as described above.

RESULTS

Requirements for Ad DNA-pro-Dependent DNA Synthesis. In the presence of purified Ad-DBP, cytosol and nuclear extracts from uninfected cells, the addition of cytosol extract from Ad-infected cells resulted in dNMP incorporation (Table 1). This reaction required ATP, Ad-DBP, and nuclear extracts from uninfected cells; no activity was detected when Ad DNA-pro was omitted or replaced by Ad DNA. Under these conditions, the reaction was linear up to 120 min; the addition of 6.5, 13, 26, and 39 μg of cytosol protein from Ad-infected cells resulted in the incorporation of 2.28, 4.26, 7.51, and 6.5 pmol of dTMP, respectively. This system was used as the assay for the isolation of Ad-coded proteins.

Requirements for Ad DNA-pro Replication with Purified Ad-Protein Fraction. The purified Ad-protein fraction, isolated as described in *Materials and Methods*, catalyzed maximal dNMP incorporation in the presence of the components described in Table 1 (B). When the purification procedure used for the isolation of the Ad-protein fraction was carried out on extracts prepared from uninfected HeLa cells, no complementing activity was detected. Thus, the reaction required other Ad-coded proteins or Ad-modified proteins in addition to Ad-DBP.

Characterization of Purified Ad-Protein Fraction. The Ad-protein fraction was stable at −70°C; in contrast, 24 hr at 0°C or 1 week at −20°C resulted in 50% loss of activity. The Ad-protein fraction treated with *N*-ethylmaleimide or heated for 5 ration was markedly reduced (Table 2). The incorporation was restored by the addition of pol α plus pol β. Ad-DNA synthesis observed with the purified components was totally dependent min at 60°C did not support dNMP incorporation (data not given). The purified Ad-protein activity sedimented as a single activity peak (70% yield) of approximately 160,000 daltons when subjected to glycerol gradient centrifugation in the presence of 0.6 M NaCl. After $NaDodSO_4$/polyacrylamide gel electrophoresis, no 160,000-dalton protein was found, but at least 3 major polypeptides (90,000, 52,000, and 38,000 daltons) were detected.

The Ad-protein fraction was examined for a number of enzymatic activities. The preparation contained pol α (0.025 unit per μg of protein) but no detectable pol β or pol γ activities (with 2 μg of protein). The preparation contained both DNA-dependent and DNA-independent ATPase activity. No nicking closing activity, DNA ligase, exonuclease, DNA gyrase, or Ad-DBP were detected. This preparation contained no endonuclease activity in the presence of double-stranded DNA; endonuclease was detected with single-stranded DNA. With 2 μg

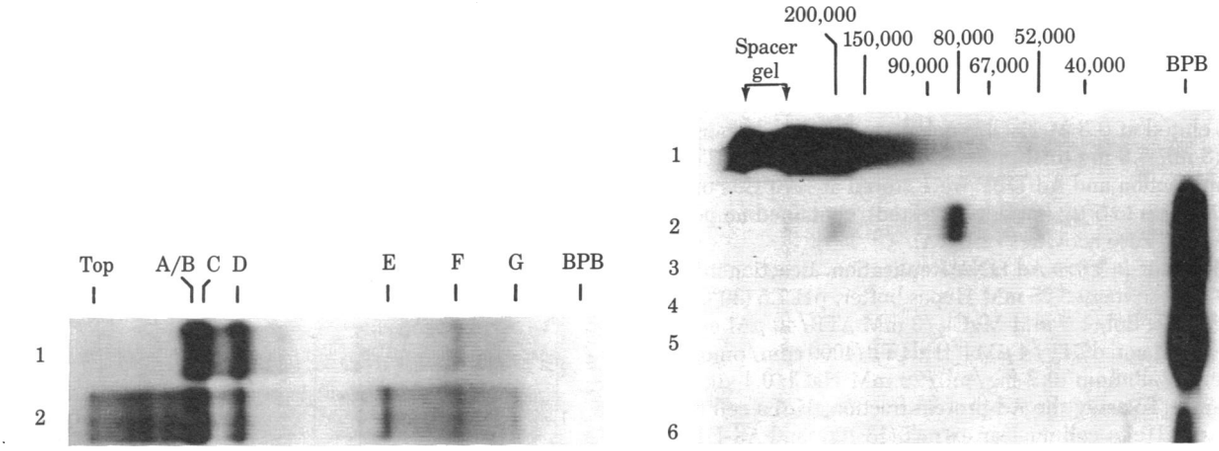

FIG. 2. Nature of the protein covalently linked to the 5′ end of Ad DNA products synthesized by using purified proteins. Reaction mixtures (0.15 ml) as described in *Materials and Methods*, except that they contained 4 μmol of [α-^{32}P]dCTP, dATP, dTTP, or dGTP (410 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and 40 μmol each of the 3 other unlabeled dNTPs; Ad DNA-pro (0.3 μg); purified Ad-protein fraction (1.26 μg); Ad-DBP (7.2 μg); pol α (0.06 unit); and pol β (0.15 unit), were incubated at 30°C for 120 min. Reactions were terminated by heating at 60°C for 10 min, and the mixtures were cooled in an ice-bath. *Hpa* I (2 units) was added, and the mixtures were incubated for 120 min at 37°C. (*A*) Agarose gel electrophoresis of *Hpa* I fragments of ^{32}P-labeled Ad DNA products. Aliquots (10 μl) were diluted by addition of 4 vol of 10 mM Tris·HCl, pH 7.5/5 mM EDTA/0.1% $NaDodSO_4$ and this solution was extracted with an equal vol of phenol/chloroform (1:1). ^{32}P-labeled products were precipitated with 2 vol of ethanol in the presence of φX174 DNA (1 μg) and 0.1 M ammonium acetate (pH 7.5); this procedure was repeated twice. The precipitates were dissolved in 30 μl of Tris·acetate buffer (pH 7.9; 50 mM Tris·base/40 mM sodium acetate/1 mM EDTA) containing bromphenol blue and xylene cyanol, each at 0.2 mg/ml, and 10% (vol/vol) glycerol. The [^{32}P]dCMP-labeled product (3×10^4 cpm) was applied to an agarose gel (lane 1). Another aliquot (10 μl) was incubated with proteinase K (0.2 mg/ml)/0.1% $NaDodSO_4$ at 37°C for 60 min. The mixtures were diluted by addition of 4 vol of 10 mM Tris·HCl, pH 7.5/5 mM EDTA/0.1% $NaDodSO_4$, and the ^{32}P-DNA was ethanol precipitated as described above. [^{32}P]dCMP-labeled products (6×10^4 cpm) were applied to the agarose gel (lane 2). [5′-^{32}P]*Hpa* I-Ad2-DNA fragments (1.5×10^4 cpm) were used as markers and are identified as fragments A–G. The agarose slab gels (1.2%; 10 cm × 15 cm × 0.3 cm) were prepared and electrophoresed in Tris·acetate buffer at 50 V for 4 hr. Radioautography was performed by exposing gels with intensifying screens for 12 hr (Kodak XR-5 film). (*B*) $NaDodSO_4$/polyacrylamide gel electrophoresis of the protein covalently linked to the 5′ end of the Ad DNA product. Aliquots (40 μl) of [^{32}P]dCMP-labeled Ad DNA products digested with *Hpa* I were precipitated with 10% trichloroacetic acid in the presence of 10 μg of bovine serum albumin; the trichloroacetic acid precipitation was repeated twice. The precipitate was washed with ether, dissolved in 50 μl of sample buffer [10 mM Tris·HCl, pH 6.8/1% $NaDodSO_4$/10% (vol/vol) glycerol/0.005% bromphenol blue/10% (vol/vol) 2-mercaptoethanol] and heated at 90°C for 20 min. The entire sample (5×10^5 cpm) was applied to the gel (lane 1). Aliquots (80 μl) of *Hpa* I-digested [^{32}P]dNMP-labeled products were digested with pancreatic DNase I (50 μg/ml) at 37°C for 2 hr. Half of each sample was precipitated with 10% trichloroacetic acid, and the precipitate was dissolved and denatured as described above. Acid-insoluble materials (5×10^4 cpm in each case) labeled with [^{32}P]dCMP (lane 2), [^{32}P]dAMP (lane 3), [^{32}P]dTMP (lane 4), or [^{32}P]dGMP (lane 5) were applied to the gel. The other half of each sample was incubated with proteinase K (0.2 mg/ml) at 37°C for 60 min and then treated with 20 μg of bovine serum albumin and trichloroacetic acid (10%). The acid-precipitated materials were treated as above. The entire sample (1×10^4 cpm) prepared from [^{32}P]dCMP-labeled material was applied to the gel (lane 6) which consisted of a 0.1% $NaDodSO_4$/10% polyacrylamide gel (10 cm × 15 cm × 0.1 cm) with a 1-cm spacer gel. Gel electrophoresis was carried out at 50 V until the marker dye reached the bottom of the gel. In these experiments, 10 μg of *E. coli* RNA polymerase and 5 μg of albumin were used as markers (150,000, 90,000, 67,000, 40,000 daltons) and were stained with amido black 10B after radioautography. Radioautography was performed by exposing gels with intensifying screens at −70°C for 24 hr (Kodak XR-5 film).

of protein, 10% of input ϕX174 DNA was nicked after 60 min at 30°C.

DNA Synthesis with Purified Ad-Protein Fraction. When extracts from uninfected cells were omitted, dNMP incorporation was markedly reduced (Table 2). The incorporation was restored by the addition of pol α plus pol β. Ad-DNA synthesis observed with the purified components was totally dependent on Ad DNA-pro, Ad-protein fraction, Ad-DBP, purified pol β, ATP, all four dNTPs, and $MgCl_2$. The reaction was markedly decreased when Ad DNA-pro, Ad-DBP, or ATP was replaced by Ad DNA, *Escherichia coli* DBP, or dATP, respectively. The reaction was inhibited by aphidicolin, pancreatic DNase I (10 μg/ml), or RNase A (10 μg/ml) (data not given) but was not affected by addition of UTP, GTP, or CTP. The replication of the Ad DNA-pro was not affected by the addition of pol γ (Table 3). The activity observed with pol β in the absence of added pol α may be due to the presence of pol α in the Ad-protein fraction. Treatment of pol β with *N*-ethylmaleimide abolished its ability to support dNMP incorporation; similar treatment of the Ad DNA-pro did not alter its ability to act as a template.

Nature of Ad DNA Products Formed with Purified Components. The synthesis of full-length Ad DNA has been observed with crude fractions from Ad-infected cells in the presence of ATP and Ad DNA-pro (1, 4). Similar observations have been made with partially purified fractions. The product formed in reaction mixtures containing purified Ad-protein fraction Ad-DBP, Ad DNA-pro, and nuclear and cytosol extracts from uninfected cells was full length, as judged by alkaline sucrose gradient centrifugation (Fig. 1*A*). This synthesis required 60–120 min of incubation at 30°C. Omission of the uninfected cytosol extract from such reaction mixtures resulted in the synthesis of Ad DNA approximately 25–50% of the full size. Ad DNA products formed in reaction mixtures containing added pol α and pol β in place of uninfected extracts were not full length (Fig. 1*B*). The addition of cytosol extract from uninfected cells resulted in full-length Ad DNA formation. The use of T4 DNA ligase in place of the uninfected cytosol extract had little effect on the size of the Ad DNA product.

The size of the [^{32}P]dTMP-labeled Ad DNA products formed with purified components was also examined by alkaline agarose gel electrophoresis. DNA chains formed after 120 min of incubation were 25–50% of full-length Ad DNA. The omission of pol β resulted in a marked decrease in synthesis and elongation (data not given).

[α-^{32}P]dCTP-labeled Ad DNA products were digested with *Hpa* I and subjected to neutral agarose gel electrophoresis; *Hpa* I-E and G fragments are derived from the left- and right-end terminal Ad DNA-pro fragments, respectively (Fig. 2*A*). These two fragments were not detected in gels (lane 1) but were observed when the *Hpa* I DNA fragments were treated with proteinase K before phenol extraction and then subjected to gel electrophoresis (lane 2). These observations suggest that the 5′ ends of the newly synthesized Ad DNA are covalently linked to proteins that prevent these fragments from penetrating into the gel. Thus, to gain more information on the linkage and size of the protein(s) linked to DNA, the labeled fragments obtained by *Hpa* I digestion were further digested by using pancreatic DNase I and then subjected to $NaDodSO_4$/acrylamide gel electrophoresis (Fig. 2*B*). A major band of 80,000 daltons and a minor band of 52,000 daltons were detected (lane 2). These bands disappeared after proteinase K treatment (lane 6). Without DNAse I digestion, however, all of the [^{32}P]dCMP-labeled material stayed at the top of the gel (lane 1). No labeled protein bands were detected when [α-^{32}P]dATP-, [α-^{32}P]dTTP-, or [α-^{32}P]dGTP-labeled Ad DNA products (lanes 3–5) were synthesized and then subjected to exhaustive DNase I digestion before the $NaDodSO_4$/acrylamide gel electrophoresis. Thus the 80,000- and 52,000-dalton proteins are labeled solely by linkage to dCMP.

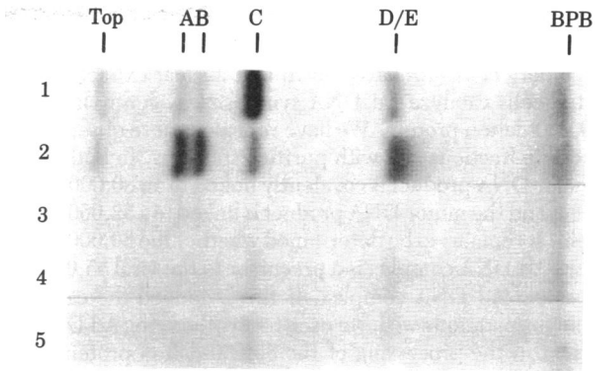

FIG. 3. Selective replication of the 5′-terminal protein-containing fragments by purified proteins. A reaction mixture of 25 mM Hepes buffer, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/10 mM NaCl/Ad DNA-pro at 7 μg/ml/restriction endonuclease *Xba* I at 20 units per ml was incubated at 37°C for 60 min. Aliquots (10 μl) were added to the complete replication system (40 μl), as described in the legend to Table 2, with the exception that 2×10^4 cpm/pmol of [α-^{32}P]dTTP, 0.42 μg of purified Ad-protein fraction, 0.02 unit of pol α, and 0.05 unit of pol β were present. Mixtures were incubated at 30°C for 60 min. Reactions were terminated by the addition of 0.1% $NaDodSO_4$, and the mixtures were then incubated with proteinase K (0.2 mg/ml) at 37°C for 60 min. ^{32}P-labeled material was precipitated with ethanol, dissolved in Tris·acetate buffer, and applied to the gel. Products formed in the complete system (lane 1), in the absence of Ad-DBP (lane 2), in the absence of the purified Ad-protein fraction (lane 3), lacking pol β (lane 4), or lacking both pol α and pol β (lane 5) were applied to a 1.2% agarose gel (10 cm × 15 cm × 0.3 cm) in Tris·acetate buffer. Electrophoresis and radioautography were performed as described in the legend to Fig. 2.

The cleavage of Ad DNA-pro by *xba* I results in the formation of five fragments (A–E), two of which retain the terminal 55,000-dalton proteins (C and E). The others accounting for 84% of the DNA, contain no protein. M. S. Horwitz and H. Ariga (personal communication) have shown that extracts from Ad-infected cells replicate only *xba* I-C and E DNA fragments, each of which contains the terminal protein. This selectivity was also observed with the Ad DNA synthesizing system containing purified components (Fig. 3). This unique specificity depended on the addition of Ad-protein fraction, Ad-DBP, pol β, and ATP. All synthesis was blocked by aphidicolin.

DISCUSSION

The replication of Ad DNA is governed by cellular proteins and Ad-coded proteins. Both the *in vitro* and the *in vivo* mechanisms of replication proceed continuously via strand displacement from either end of the template. Ad DNA isolated from virions contains 55,000-dalton proteins covalently linked to both 5′ ends (6, 7). The nucleotides at the ends of Ad2 DNA adjacent to the proteins have been sequenced (8), and it has been shown that dCMP is covalently linked to the 55,000-dalton protein (9).

The nature of the initiation reaction involved in the replication of Ad DNA has been partially elucidated by *in vitro* studies. It is clear that the covalently linked 5′-proteins are essential. The Ad DNA-pro complex cannot be replaced by deproteinized DNA or DNA isolated by procedures leading to protein denaturation [treatment with phenol, $NaDodSO_4$, or heat (70°C)]. Horwitz and Ariga have found that restriction enzyme fragments from Ad DNA-pro complex can be replicated. This reaction, however, occurs only on those DNA fragments

containing 5′ proteins. This specificity was observed with crude extracts, as well as with the purified system described above.

Challberg *et al.* (10) have shown that nuclear extracts of Ad-infected cells catalyze Ad DNA synthesis covalently linked to an 80,000-dalton protein. We have repeated these observations with crude fractions and with purified fractions. In both cases, the major DNA product is covalently linked to an 80,000-dalton protein, and the minor DNA product is linked to a 52,000-dalton protein. It remains to be determined whether the 80,000-dalton protein-Ad DNA complex is a precursor to the viral 55,000-dalton protein-Ad DNA complex. If this is indeed the case, the protein preparations we have used for synthesizing Ad DNA are deficient in the processing of the 80,000-dalton protein·DNA complex to the Ad DNA-pro complex found in virions.

We have shown that both crude fractions and the Ad-protein fraction catalyze the formation of an 80,000-dalton protein·dCMP covalent complex. Both systems catalyze the selective addition of dCMP to an 80,000-dalton protein. This reaction is specific for dCTP, stimulated by ATP, and unaffected by aphidicolin (J. Lichy and J. Hurwitz, unpublished results).

The ensuing elongation of protein-primed chains is dependent on Ad-DBP, DNA polymerase α (inferred from the aphidicolin sensitivity), DNA polymerase β, and the purified Ad-protein fraction. The products formed with the purified protein components were not full-length and required the addition of cytosol from uninfected HeLa cells to form full-length Ad DNA. Ad DNA products have also been examined in the electron microscope. We have detected a difference in replicating intermediates (D. Reinberg and J. Ikeda, unpublished results). Products formed with the purified proteins contained DNA branches that were double stranded; some replicating molecules contained two branched duplex structures. Products formed with purified proteins plus the cytosol extracts of HeLa cells contained displaced single-stranded DNA at growing forks; in this case, forks were visible only on replicating DNA molecules. The latter finding is more in keeping with the replication pattern seen *in vivo*.

The nature of the elongation reaction catalyzed by pol α and pol β in Ad DNA replication remain to be further examined. The sites at which ATP is required in the replication reaction remain to be elucidated. It may be that a DNA helicase activity facilitates the unwinding of the duplex as the replication fork moves down the duplex DNA. Our finding that the Ad-protein fraction contains DNA-dependent ATPase activity may have some bearing on this process. The further purification of the Ad- and host-coded proteins involved in Ad DNA replication should help to resolve these questions.

Note Added in Proof. Recent experiments suggest that the DNA polymerase β preparations that support Ad DNA replication have been resolved into multiple protein fractions.

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