

Initiation of Adenovirus DNA Replication: Detection of Covalent Complexes Between Nucleotide and the 80-Kilodalton Terminal Protein

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We have previously shown that the 5'-terminal deoxycytidine residue of each nascent adenovirus 5 DNA strand synthesized *in vitro* is covalently linked to the 80-kilodalton (kd) terminal protein precursor via a phosphodiester bond to a serine residue in the protein. When extracts prepared from adenovirus 5-infected cells are incubated with [α - 32 P]dCTP as the only added deoxynucleoside triphosphate, complexes consisting of nucleotide covalently linked to the 80-kd protein can be detected. The nucleotide moieties present in such complexes include d(pC) and d(pCpA), the 5'-terminal nucleotide and dinucleotide of adenovirus 5 DNA, respectively, as well as some longer oligonucleotides. The formation of these complexes requires the presence of adenovirus DNA containing the attached 55-kd terminal protein and ATP. Extracts from H5ts125-infected cells which are defective in DNA replication catalyze complex formation to the same extent as extracts prepared from wild-type infected cells; thus, the presence of the adenovirus-coded 72-kd DNA-binding protein is apparently not required. Most, if not all, of the 80-kd protein-nucleotide complexes that are formed are noncovalently bound to the input viral DNA. These observations are consistent with the protein-priming model for the initiation of adenovirus DNA replication.

The adenovirus 5 genome is a linear duplex DNA molecule containing about 35,000 base pairs (8). The nucleotide sequences at each end of the genome are identical for the first 103 base pairs (18). In addition, the 5' end of each DNA strand is covalently linked via a phosphodiester bond [deoxycytidyl-(5'→O)-serine] to a protein of 55,000 molecular weight (5, 16).

The replication of adenovirus DNA is initiated at the molecular termini and proceeds by a strand displacement mechanism (13, 25; Kelly, *in A. S. Kaplan (ed.), Organization and replication of viral DNA*, in press). Recent speculations concerning the mechanism of initiation have centered on the possible role(s) of the terminal protein (1, 16). Analysis of adenovirus DNA replication *in vitro* has revealed that the 55-kilodalton (kd) protein attached to the parental DNA strands is essential for efficient replication (2, 3). It has also been demonstrated that the 5' ends of nascent strands synthesized *in vitro* or *in vivo* are covalently linked to an 80-kd protein which represents the precursor to the mature 55-kd terminal protein (1, 4, 19). The latter observation is consistent with a model in which the primary initiation event in adenovirus DNA replication is the formation of an ester linkage between the α -phosphoryl group of dCTP and

the β -OH of a serine residue in the 80-kd terminal protein precursor (1). It is envisioned that this event takes place as a part of a concerted reaction in which the free 80-kd terminal protein associates with the terminus of the parental genome. Such an association might be mediated by interactions with specific terminal nucleotide sequences and/or interactions with the terminal protein attached to the parental DNA. As a result of these interactions, the 3'-OH of the dCMP residue attached to the 80-kd protein is positioned in such a way that it can serve as a primer for subsequent chain elongation.

In this paper, we report further *in vitro* studies on the protein-nucleotide joining reaction. We show that when extracts from virus-infected cells are incubated with dCTP as the only added deoxynucleoside triphosphate, covalent 80-kd protein-nucleotide complexes can be detected. The nucleotide moieties present in such complexes include d(pC) and d(pCpA), the 5'-terminal nucleotide and dinucleotide of adenovirus 5 DNA, respectively, as well as some longer oligonucleotides. Formation of the complexes requires the presence of the adenovirus DNA-55-kd terminal protein complex and ATP, but does not require the adenovirus 72-kd DNA-binding protein (DBP). Most, if not all, of the 80-kd

protein-nucleotide complexes that are formed are bound by noncovalent bonds to the input viral DNA template. These results are consistent with the basic elements of the protein priming model.

Recently, Lichy et al. (14) reported the formation of similar 80-kd protein-nucleotide complexes in extracts of adenovirus 2-infected cells.

MATERIALS AND METHODS

Preparation of nuclear extracts and conditions for forming protein-nucleotide complexes. Nuclear extracts from Ad5-infected HeLa cells were prepared as previously described (2). Extracts from H5ts125-infected HeLa cells were prepared in an identical fashion, except that the infection was carried out at 40°C. To remove small molecules, 0.4 ml of nuclear extract was passed over a column (0.8 by 15 cm) of Sephadex G-25 in 10% sucrose, 50 mM HEPES (pH 7.5). Material eluting in the void volume of the column was collected.

Adenovirus 5 DNA covalently linked to the 55-kd terminal protein (DNA-protein complex) was isolated from purified virions as described previously (2). Deproteinized adenovirus 5 DNA was prepared by incubating the DNA-protein complex in 1.0% sodium dodecyl sulfate (SDS) with pronase (at 1 mg/ml) at 37°C for 2 h. The resulting solution was extracted with phenol and dialyzed against 10 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA.

Reaction mixtures for the protein-nucleotide joining reaction contained (in 0.1 ml) 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 0.5 mM dithiothreitol, 2 mM ATP, 2 to 10 μM [α -³²P]dCTP (400 Ci/mmol), 300 ng of adenovirus 5 DNA-protein complex, and 50 μl of nuclear extract. To reduce a background resulting from a repair-like reaction (3), the extract was incubated at 37°C for 30 min immediately before use. Such treatment had no effect on the replication activity of the extract. After incubation of the reaction mixture at 37°C for 15 min, EDTA was added to a concentration of 25 mM, and macromolecules were precipitated with trichloroacetic acid. The precipitate was washed with ether, redissolved in 100 μl of 10 mM NaOH, 0.1% SDS, and re-precipitated with trichloroacetic acid. The acid-insoluble material was then collected and analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli (12). Gels contained either 7.5 or 10% acrylamide. After electrophoresis, the gels were dried, and radioactivity was located by autoradiography.

RESULTS

Formation of 80-kd protein-nucleotide complexes. We previously reported that the 5'-terminal deoxycytidine residue of each nascent adenovirus DNA strand synthesized *in vitro* is covalently linked to an 80-kd terminal protein by a phosphodiester bond to a serine residue (1). This protein is a precursor to the 55-kd protein attached to the 5' end of mature adenovirus DNA strands (1, 4, 19). To determine whether the nucleotide-protein joining reaction can occur in the absence of significant DNA chain

elongation, extracts from virus-infected cells were depleted of endogenous nucleotides by gel filtration and incubated with [α -³²P]dCTP, ATP, and adenovirus 5 DNA containing the attached 55-kd protein. Analysis of the reaction product by SDS-polyacrylamide gel electrophoresis revealed the presence of a ³²P-labeled protein which migrated with an apparent molecular weight of 80,000 (Fig. 1A and B, lane a). The yield of this protein was greatly reduced when [α -³²P]dATP was substituted for [α -³²P]dCTP (Fig. 1A, lane b). The ³²P-labeled 80-kd protein was eluted from the gel and incubated in 50 mM NaOH at 70°C for 1 h. We previously demonstrated that these conditions are sufficient to hydrolyze the adenovirus protein-DNA linkage, yielding a DNA chain terminated by a 5' phosphoryl group (5). Analysis of the resulting hydrolysate by two-dimensional thin-layer chromatography on PEI cellulose revealed radioactive products which comigrated with

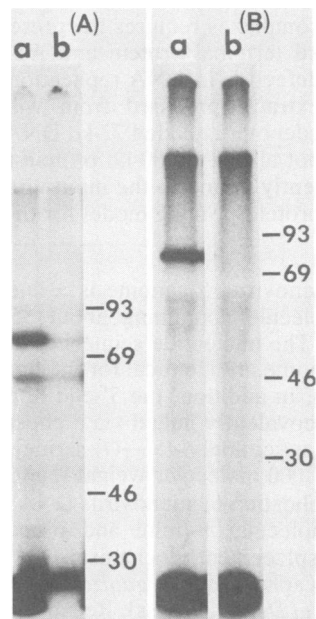


FIG. 1. SDS-polyacrylamide gel electrophoresis of protein-nucleotide complexes formed in the presence of a single added [α -³²P]deoxynucleoside triphosphate. (A) Nuclear extract from adenovirus 5-infected HeLa cells was incubated with 2 μM [α -³²P]dCTP (a) or [α -³²P]dATP (b). Reaction mixtures were analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel as described in the text. (B) Nuclear extract from adenovirus 5-infected HeLa cells was incubated with [α -³²P]dCTP and 300 ng of adenovirus 5 DNA-protein complex (a) or deproteinized DNA (b). Reaction mixtures were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. The positions of marker ¹⁴C-proteins electrophoresed in another lane on the same gels are indicated on the right. The numbers refer to the $M_r \times 10^{-3}$ of each ¹⁴C-labeled protein.

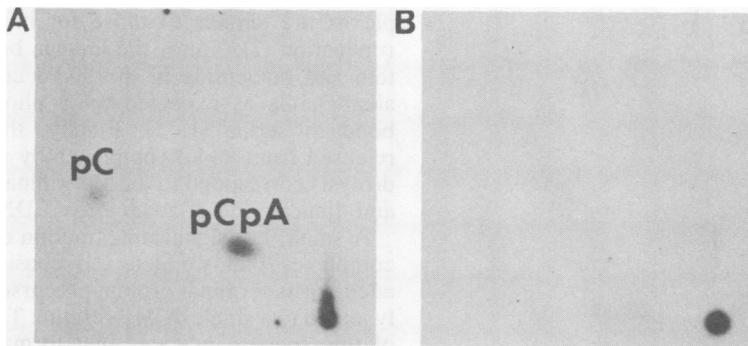


FIG. 2. Identification of the nucleotides covalently bound to the 80-kd protein. The protein-nucleotide joining reaction was carried out as described in the text. The reaction mixture was passed over a Sepharose-2B column and the material eluting in the void volume of the column was electrophoresed on a 10% polyacrylamide gel in the presence of SDS. After autoradiography of the gel, the major radioactive species, with an apparent M_r of 80,000, was eluted from the gel (15), combined with dCMP and d(pCpA), and NaOH was added to a concentration of 50 mM. An aliquot of the resulting sample was heated to 70°C for 60 min and neutralized with HCl (A). As a control, an identical aliquot was neutralized with HCl before incubation at 70°C for 60 min (B). Each sample was then applied to a PEI cellulose thin-layer plate. Separation was performed in the first dimension (bottom to top) by chromatography in 0.5M LiCl and in the second dimension (right to left) by chromatography in 1.0 M acetic acid. Markers were located under UV light, and radioactivity was detected by autoradiography.

d(pC) and d(pCpA), as well as some material near the origin which may represent longer oligonucleotides (Fig. 2). We conclude that a fraction of the labeled 80-kd protein detected in these experiments represents the adenovirus terminal protein precursor covalently joined to a single nucleotide (dCMP). The presence of 80-kd terminal protein molecules yielding d(pCpA) (and perhaps longer oligonucleotides) upon alkaline hydrolysis is presumably a consequence of limited chain elongation resulting from incomplete removal of endogenous deoxynucleoside triphosphates (the 5'-terminal nucleotide sequence of adenovirus 5 DNA is pCpApT. . .).

The formation of 80-kd protein-nucleotide complexes is completely dependent upon the presence of adenovirus DNA containing the attached 55-kd terminal protein; no detectable 80-kd protein-nucleotide complexes were formed when reaction mixtures were incubated in the presence of deproteinized adenovirus DNA (Fig. 1B, lane a versus lane b). The reaction also requires ATP or an ATP generating system (data not shown).

Role of the adenovirus DBP in the formation of protein-nucleotide complexes. Adenovirus encodes a 72-kd single-stranded DBP that is known to be involved in viral DNA replication (6, 22, 23). To determine whether this protein plays a role in the formation of the 80-kd protein-nucleotide complex, extracts were prepared from cells infected at the restrictive temperature with H5ts125, a well-characterized mutant carrying a temperature-sensitive lesion in the DBP gene (23). It has been previously shown that such

cells contain greatly reduced levels of 72-kd DBP (7, 23). As shown in Fig. 3, extracts from H5ts125-infected cells were defective in adenovirus DNA replication *in vitro*, but replication activity could be restored by addition of purified DBP. When tested for the ability to carry out complex formation, the mutant extracts were found to be nearly as active as wild-type extracts; moreover, the amount of 80-kd complex synthesized by mutant extracts was not increased by addition of purified DBP (Fig. 4). Thus, the adenovirus DBP is apparently not required for the formation of the 80-kd protein-nucleotide complex.

Association of the 80-kd terminal protein-nucleotide complex with adenovirus DNA. When the product of the protein-nucleotide joining reaction was analyzed by gel filtration on Sepharose-2B, it was found that most of the labeled 80-kd protein-nucleotide complex eluted in the excluded volume together with the input viral DNA (Fig. 5, lanes a and b). DNase I treatment of the reaction product before gel filtration resulted in a shift of the labeled 80-kd protein-nucleotide complex to the included fractions (Fig. 5, lanes c and d). These results suggest that most, if not all, of the 80-kd protein-nucleotide complex that is formed is bound to the viral DNA.

DISCUSSION

It has been previously shown that nuclear extracts from adenovirus-infected cells are capable of carrying out the replication of added viral DNA template (2, 3). The results presented here



FIG. 3. DNA replication in extracts from adenovirus 5- or Ad5ts125-infected cells. Adenovirus 2 DNA-protein complex was digested with the restriction enzyme *Hind*III as described previously (3) and incubated in a standard *in vitro* replication reaction (2) with nuclear extract from either adenovirus 5- or H5ts125-infected HeLa cells. The reactions were terminated by the addition of EDTA to 25 mM and SDS to 1%. The resulting solution was incubated with pronase at 1.0 mg/ml for 2 h at 37°C and extracted with phenol. The DNA was collected by ethanol precipitation and analyzed by electrophoresis on a 1.4% agarose gel as described previously (2). The positions of the terminal *Hind*III restriction fragments of adenovirus DNA are indicated by the letters G and K. The dark bands immediately above and below fragment K in lane a are probably single strands arising from more than one round of displacement synthesis on fragments G and K, respectively (10). Lane a, nuclear extract from adenovirus 5-infected cells; lane b, nuclear extract from Ad5ts125-infected cells; lane c, nuclear extract from Ad5ts125-infected cells plus 1.0 μ g of purified adenovirus DBP (17).

indicate that, in the presence of a single added deoxynucleoside triphosphate, dCTP, such extracts catalyze the formation of 80-kd covalent protein-nucleotide complexes. Several lines of evidence suggest that the protein moiety in these complexes is identical to the 80-kd terminal protein precursor previously detected at the 5' termini of nascent adenovirus DNA strands synthesized *in vitro* or *in vivo* (1, 4, 19). First, the apparent molecular weight of the complexes is the same as that of the terminal protein precursor released from nascent strands by DNase digestion (1, 4, 19). Second, the requirements for the formation of 80-kd protein-nucleotide com-

plexes are similar to those for *in vitro* DNA replication (2). Third, the linkage between protein and nucleotide in the 80-kd complexes is alkali labile as expected for a phosphodiester bond to serine (1, 5). Finally, the products released from 80-kd complexes by alkaline hydrolysis correspond to the 5'-terminal nucleotide and dinucleotide of adenovirus 5 DNA (18).

A small, but significant, fraction of the 80-kd complexes that we detected consisted of the adenovirus terminal protein precursor covalently joined to a single dCMP residue. The presence of this species indicates that formation of the protein-nucleotide linkage does not require prior chain elongation. This finding is consistent with the hypothesis that the protein-nucleotide joining reaction is the first event in the initiation of new adenovirus DNA chains; however, confirmation of this hypothesis will clearly require more direct studies. In preliminary pulse-chase experiments (not shown), we were not able to chase more than a small fraction of the 32 P-labeled material formed during a brief preincubation into newly synthesized viral DNA. This result suggests that under the experimental conditions currently employed most of the 80-kd protein-nucleotide complexes that are formed

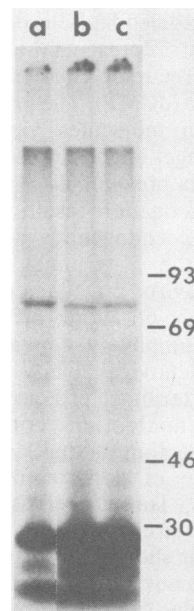


FIG. 4. SDS-polyacrylamide gel electrophoresis of protein-nucleotide complexes formed with extracts from wild-type or H5ts125-infected cells. The protein-nucleotide joining reaction was carried out as described in the text. Lane a, nuclear extract from adenovirus 5-infected cells; lane b, nuclear extract from Ad5ts125-infected cells; lane c, nuclear extract from Ad5ts125-infected cells plus 1.0 μ g of purified adenovirus DBP (17).

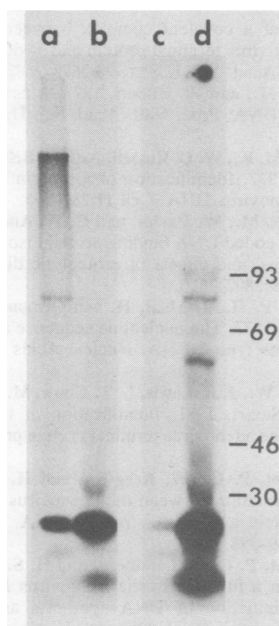


FIG. 5. Gel filtration of the 80-kd protein-nucleotide complex. Nuclear extract from adenovirus 5-infected cells was incubated at 37°C for 15 min with [α - 32 P]dCTP and adenovirus 5 DNA-protein complex. A sample of the reaction mixture was incubated for an additional 15 min in the presence of 50 μ g of DNase I per ml. The DNase-treated sample and an untreated sample were each passed over a column (0.8 by 15 cm) of Sepharose-2B in 50 mM HEPES (pH 7.5), 25 mM NaCl, 0.5 mM MgCl₂, 0.2 mM dithiothreitol, and 2% sucrose. The material eluting in the void volume and the included volume of each column was collected, precipitated with 10% trichloroacetic acid, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The positions of marker 14 C-labeled proteins electrophoresed in another lane on the same gel are indicated on the right. The numbers refer to the $M_r \times 10^{-3}$ of each 14 C-labeled protein. Lane a, excluded fractions of untreated sample; lane b, included fractions of untreated sample; lane c, excluded fractions of DNase-treated sample; lane d, included fractions of DNase-treated sample.

are not substrates for subsequent chain elongation. The reason for this is not yet understood.

Lichy et al. (14) also detected the formation of 80-kd protein-nucleotide complexes in extracts from adenovirus 2-infected cells. The requirements for complex formation reported by these investigators were essentially identical to those described here. Complexes formed in the presence of aphidicolin, an inhibitor of DNA polymerase α , were found to contain exclusively dCMP. In agreement with our results, Lichy et al. were unable to quantitatively chase preformed 80-kd protein-nucleotide complexes into elongated DNA chains.

The formation of the 80-kd protein-nucleotide complexes is dependent upon the presence of adenovirus DNA with attached 55-kd terminal protein molecules. This finding is consistent with the hypothesis that the protein-nucleotide joining reaction requires the prior association of the 80-kd terminal protein precursor with the terminus of the viral genome and that this association is mediated, at least in part, by interactions with the 55-kd protein. Our data also indicate that once the joining reaction occurs, the 80-kd protein-nucleotide complexes that are formed remain bound to the viral genome.

The only virus-coded protein, other than the terminal protein, that is known to be directly involved in DNA replication is the 72-kd adenovirus DBP (6, 22, 23). This protein binds strongly and cooperatively to single-stranded DNA (20, 22). The precise role of DBP in viral DNA replication is not yet clear. Recent *in vitro* studies are most consistent with a requirement for DBP in chain elongation (9, 11); however, earlier investigations provided some evidence that DBP may also play a role in initiation (21, 24). Our studies with H5ts125, a mutant which produces a defective DBP, confirm previous work indicating that adenovirus DNA replication *in vitro* is completely dependent upon the presence of DBP (9, 11). The protein-nucleotide joining reaction, on the other hand, does not appear to require DBP, at least at the concentrations that are necessary to support DNA replication. We cannot rule out the possibility that the joining reaction requires low levels of the DBP since we cannot be certain that the extracts derived from H5ts125-infected cells are completely free of DBP. This caveat aside, our results support the view that the DBP functions during DNA replication at some step subsequent to the protein-nucleotide joining reaction.

The ability to detect radioactively labeled covalent 80-kd protein-nucleotide complexes provides a simple assay which can be used for the purification of the components involved in the joining reaction. The availability of such purified components should, in turn, make it possible to study the mechanism of the reaction and to further define its relationship to viral DNA replication.

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