In vitro complementation as an assay for purification of adenovirus DNA replication proteins

(adenovirus terminal protein/adenovirus DNA binding protein)

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ABSTRACT As an approach to the purification of adenovirus-encoded DNA replication proteins, we have developed in vitro complementation assays that make use of viral mutants defective in DNA replication in vivo. Nuclear extracts prepared from cells infected with H5ts36 or H5ts125, two such mutants belonging to different complementation groups, were found to be defective in viral DNA replication in vitro. However, replication activity could be restored by mixing the two extracts. Replication activity in either extract also could be restored by addition of appropriate replication-deficient fractions purified from cells infected with wild-type adenovirus. By using such assays, H5ts36- and H5ts125-complementing activities were extensively purified. As expected, purified H5ts125-complementing activity consisted of a single major polypeptide, the 72-kilodalton (kDal) adenovirus DNA binding protein. The purified H5ts36-complementing activity consisted of the 80-kDal adenovirus terminal protein precursor and two other major polypeptides with apparent molecular masses of 140 and 65 kDal. Formation of the 80-kDal terminal protein–dCMP complexes, the proposed initial step in adenovirus DNA replication, required components in the purified H5ts36-complementing fraction and a cellular factor(s) but did not require the adenovirus DNA binding protein. The complete in vitro adenovirus DNA replication reaction was reconstituted from the purified H5ts36-complementing activity, the adenovirus DNA binding protein, and an extract from uninfected cells.

The adenovirus genome is a linear, double-stranded DNA molecule whose 5' termini are covalently linked to a virus-encoded protein with a molecular mass of 55 kilodaltons (kDal). The replication of the viral genome has been studied extensively as a model for probing the mechanisms of DNA synthesis that operate in eukaryotic cells (for reviews, see refs. 1–3). Initiation of adenovirus DNA replication takes place at the ends of the genome and involves the formation of a covalent linkage between an 80-kDal terminal protein (the precursor to the 55-kDal protein) and the first nucleotide in the new DNA chain (dCMP) (4–8). A daughter strand is then extended in the 5' to 3' direction displacing one of the parental strands. The displaced parental strand serves as template for the synthesis of the second daughter strand.

We have shown previously that adenovirus DNA replication takes place in a soluble in vitro system that is dependent upon exogenous adenovirus DNA templates (9, 10). This system has provided a means to explore the molecular mechanisms involved in initiation and chain elongation (4–8, 11–13) and to purify and characterize replication proteins (14, 15). Our approach to the latter problem has been to develop in vitro complementation assays that make use of viral mutants defective in DNA replication in vivo. Extracts prepared from cells infected at the nonpermissive temperature with either of two such mutants belonging to different complementation groups (H5ts36 or H5ts125) were found to be defective in DNA replication in vitro. However, replication activity could be restored by mixing the two extracts. Replication activity in either extract also could be restored by addition of appropriate replication-deficient fractions purified from wild-type-infected cells. By using such complementation assays we have obtained purified fractions that contain all of the virus-encoded proteins required for adenovirus DNA replication in vitro.

MATERIALS AND METHODS

Preparation of Nuclear Extracts and Conditions for in Vitro DNA Synthesis. Nuclear extracts from adenovirus 5 (Ad5)-infected HeLa cells were prepared as described (9) with the following minor modifications. Isolated nuclei were extracted with 0.2 M NaCl for 1 hr at 0°C and were centrifuged at 12,000 × g for 20 min. The resulting supernatant was dialyzed overnight against 50 mM Hepes, pH 7.5/50 mM NaCl/10% (wt/vol) sucrose. Extracts from HeLa cells infected with temperature-sensitive mutants (H5ts36 and H5ts125) were prepared in an identical fashion except that the infection was carried out at 40°C with a multiplicity of infection of 60 plaque-forming units/cell. Nuclear extracts from uninfected cells were prepared from cultures maintained at 37°C in the absence of hydroxyurea.

Adenovirus DNA covalently linked to the 55-kDal terminal protein (DNA–protein complex) and deproteinized adenovirus DNA were prepared as described (6, 9, 16). Reaction mixtures for adenovirus DNA replication (100 µl) contained 50 mM Hepes (pH 7.5), 5 mM MgCl2, 0.5 mM dithiothreitol, 2 mM ATP, 25 µM each of dATP, dGTP, dCTP, and dTTP, 25 µCi of [α-32P]dCTP (300 Ci/mmol; 1 Ci = 3.7 × 1010 Bq), 160 ng of Ad5 or Ad2 DNA–protein complex digested with the restriction enzyme HindIII, and 10–50 µl of nuclear extract or purified fraction. Reaction mixtures were incubated at 37°C for 30 min. The in vitro reaction product was isolated and analyzed by agarose gel electrophoresis as described (6).

Conditions for Formation of 50-kDal Terminal Protein–dCMP Complexes. Reaction mixtures for the protein–nucleotide joining reaction (25 µl) contained 25 mM Hepes (pH 7.5), 6 mM MgCl2, 4 mM ATP, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µCi of [α-32P]dCTP (3,000 Ci/mmol), 80 ng of Ad5 DNA–protein complex, and 5–15 µl of nuclear extract or purified fraction. After incubation for 1 hr at 37°C, reaction mixtures were heated to 75°C for 10 min and were centrifuged at 13,000 × g for 2 min. The resulting pellet was resuspended in 30 µl of 40 mM Tris-HCl, pH 8.0/10 mM CaCl2/6 mM MgCl2/10% (vol/vol) glycerol and was

Abbreviations: kDal, kilodalton; PhMeSO4F, phenylmethylsulfonyl fluoride.
incubated with 9 units of micrococcal nuclease (Worthington) for 1 hr at 37°C. The sample then was analyzed by NaDodSO4/polyacrylamide gel electrophoresis as described by Laemmli (17). The gels contained 8% acrylamide/0.21% N,N'-methylenebisacrylamide. After electrophoresis the gels were dried and analyzed by autoradiography.

**Purification of H5ts36- and H5ts125-Complementing Activities.** Extract was prepared from 2.5 × 10^10 Ad5-infected HeLa cells as described above. The extract (containing 1.6 g of protein) was loaded on a DEAE-cellulose column (Whatman DE52; 2.5 × 37 cm) in 50 mM Hepes, pH 7.5/50 mM NaCl/10% (wt/vol) sucrose/0.1 mM PhMeSO4F. The column was washed extensively with the same buffer and then was eluted stepwise with 50 mM Hepes, pH 7.5/10% (wt/vol) sucrose/0.1 mM PhMeSO4F containing increasing concentrations of NaCl (100, 200, or 500 mM). The fraction eluting at 200 mM NaCl (275 mg of protein; 25 ml) contained both the H5ts36- and H5ts125-complementing activities. After dialysis against 50 mM Hepes, pH 7.5/50 mM NaCl/20% (vol/vol) glycerol/0.1 mM PhMeSO4F (buffer A), the active fraction was loaded on a phosphocellulose column (Whatman P-11; 2.5 × 17 cm) and was eluted stepwise with buffer A containing increasing concentrations of NaCl as above. The fraction eluting at 500 mM NaCl (98 mg of protein; 41 ml) contained both complementing activities. This fraction was dialyzed against buffer A and loaded on a column of single-stranded DNA cellulose (2.5 × 4 cm) prepared according to the method of Alberts and Herrick (18). The column was washed with buffer A and was eluted sequentially with buffer A containing 100, 200, 500, or 750 mM NaCl. The fraction eluting at 500 mM NaCl (33 mg of protein; 10 ml) contained the H5ts36-complementing activity and the fraction eluting at 750 mM NaCl (2.5 mg of protein; 6 ml) contained the H5ts125-complementing activity. The H5ts125-complementing fraction was dialyzed against buffer A and stored at −70°C. The H5ts36-complementing fraction was dialyzed against buffer A and loaded on a hydroxyapatite column (Bio-Rad HTP; 1.5 × 4 cm). The column was eluted stepwise with buffer containing 0.5 mM dithiothreitol, 20% (vol/vol) glycerol, 0.1 mM PhMeSO4F, and 50, 150, 250, 350, 450, or 550 mM or 1 M potassium phosphate (pH 7.0). The H5ts36-complementing activity, which eluted at 150 mM potassium phosphate, was dialyzed against 50 mM Hepes, pH 7.5/50 mM NaCl/0.5 mM dithiothreitol/0.1 mM PhMeSO4F/20% (vol/vol) glycerol and stored at −70°C. Both complementing activities were stable on storage for at least 6 months.

**RESULTS**

**In Vitro Complementation in Extracts from Cells Infected with Adenovirus Temperature-Sensitive Mutants.** Genetic studies have defined two complementation groups of adenovirus temperature-sensitive mutants that are defective in viral DNA replication (19, 20). One of these groups includes mutants (e.g., H5ts125) whose lesions map in the gene encoding the 72-kDa adenovirus DNA binding protein (map position 61.6–66.5) (21–25). Mutations belonging to the second complementation group (e.g., H5ts36) map in a large open reading frame that lies between map positions 24.0 and 14.2 (26–28). The nature and function of the gene product(s) encoded in this region are unknown.

Preliminary attempts to demonstrate increased thermolability of replication activities in crude extracts from cells infected at the permissive temperature with H5ts36 or H5ts125 were not generally successful. Therefore, in vitro complementation studies were carried out with extracts prepared from mutant-infected cells propagated at the restrictive temperature. As shown in Fig. 1 (lanes b and c), extracts from H5ts36- or H5ts125-infected cells were defective in DNA replication in the standard in vitro assay. However, replication activity comparable to that observed in extracts of cells infected with wild-type adenovirus (Fig. 1, lane a) was observed when the two mutant extracts were mixed (Fig. 1, lane d). Replication activity could be restored to the H5ts125 extracts by addition of purified adenovirus DNA binding protein (gift of Carl Anderson; ref. 29); f, extract from H5ts125-infected cells (12.5 μl) and fraction of extract from Ad5-infected cells eluting at 200 mM NaCl from DEAE-cellulose; g, extract from H5ts36-infected cells (12.5 μl) and 1 μg of purified adenovirus DNA binding protein.

![FIG. 1. DNA replication in extracts from cells infected with wild-type adenovirus or temperature-sensitive mutants. DNA was synthesized in a standard in vitro reaction mixture (100 μl) containing a-32PdCTP as the labeled deoxynucleoside triphosphate and HindIII-digested Ad2 DNA–protein complex as template. The radioactive DNA product was isolated and analyzed by electrophoresis in a 1.4% agarose gel. In this assay adenovirus DNA replication, initiated at the ends of the genome, results in preferential incorporation of 32P into the terminal HindIII restriction fragments G (2,765 base pairs) and K (1,005 base pairs). The low level of incorporation into other fragments is due to a repair-like reaction (10). The sources of extract were as follows. Lanes: a, extract from Ad5-infected cells (25 μl); b, extract from H5ts36-infected cells (25 μl); c, extract from H5ts36-infected cells (25 μl); d, mixture of extracts from H5ts125- and H5ts36-infected cells (12.5 μl each); e, extract from H5ts125-infected cells (12.5 μl) and 1 μg of purified adenovirus DNA binding protein (gift of Carl Anderson; ref. 29); f, extract from H5ts125-infected cells (12.5 μl) and fraction of extract from Ad5-infected cells eluting at 200 mM NaCl from DEAE-cellulose; g, extract from H5ts36-infected cells (12.5 μl) and 1 μg of purified adenovirus DNA binding protein.](image-url)
cells, to Fig. DEAE-cellulose column. The various fractions (50 μl) were assayed in the presence and absence of extract from H5ts36-infected cells (20 μl) as described in the legend to Fig. 1. O and K, as in the legend to Fig. 1. Lanes: a, extract from Ad5-infected cells (25 μl); b, fraction eluting from DEAE-cellulose at 50 mM NaCl; c, fraction eluting at 100 mM NaCl; d, fraction eluting at 200 mM NaCl; e, fraction eluting at 500 mM NaCl; f, extract from H5ts36-infected cells (20 μl) and fraction eluting at 50 mM NaCl; g, extract from H5ts36-infected cells and fraction eluting at 100 mM NaCl; h, extract from H5ts36-infected cells and fraction eluting at 200 mM NaCl; i, extract from H5ts36-infected cells and fraction eluting at 500 mM NaCl.

as some minor components (Fig. 3, lane a). The 80-kDa polypeptide comigrated with authentic 80-kDa adenovirus terminal protein labeled in vitro with [α-32P]dCTP (data not shown).

The H5ts125-complementing activity copurified with H5ts36-complementing activity through the second column fractionation step (DEAE-cellulose and phosphocellulose). Complete separation of the two activities was achieved at the single-stranded DNA cellulose chromatography step. The most highly purified fraction exhibiting H5ts125-complementing activity contained a single major polypeptide with a mobility slightly greater than the 68-kDa marker by NaDodSO4/polyacrylamide gel electrophoresis (Fig. 3, lane b). As expected, this polypeptide comigrated with authentic 72-kDa adenovirus DNA binding protein purified by other methods (29) and generously provided by Carl Anderson. A minor component with apparent molecular mass of 48 kDa was observed also and probably corresponds to the previously described proteolytic cleavage product of the DNA binding protein (30). The slightly lower estimate for the apparent molecular mass of the DNA binding protein obtained in these studies probably reflects small differences in electrophoresis conditions relative to previous studies. The actual molecular mass of the DNA binding protein derived from nucleotide sequence analysis of the DNA binding protein gene is 59 kDa (25).

**Characterization of the Purified Complementing Activities.** The requirements for formation of the 80-kDa terminal protein–dCMP complex, the proposed initial step in adenovirus DNA replication, were explored in the series of experiments shown in Fig. 4. Extracts or purified fractions, or both, were incubated with adenovirus DNA–protein complex, ATP, and [α-32P]dCTP, and the formation of 32P-labeled 80-kDa protein–nucleotide complex was monitored by NaDodSO4/polyacrylamide gel electrophoresis (5–8). As previously reported (6), extracts from H5ts125-infected cells catalyzed the protein–nucleotide joining reaction to a similar extent as extracts from wild-type-infected cells. On the other hand, extracts from H5ts36-infected cells were devoid of this activity (Fig. 4, lane d). The purified H5ts36-complementing activity catalyzed the formation of the 80-kDa protein–dCMP complex to only a limited extent. Under the usual assay conditions, no 32P-labeled complex was detected (Fig. 4, lane e); however, upon prolonged exposure of the autoradiograms, a faint 32P-labeled 80-kDa band could be observed. The ability of purified H5ts36-complementing activity to support the protein–nucleotide joining reaction was enhanced greatly by addition of H5ts36 extract (Fig. 4, lane f) or extract from uninfected cells (Fig. 4, lane g).

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**Table 1. Purification of H5ts36-complementing activity**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (μl)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear extract</td>
<td>100</td>
<td>1,600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>25</td>
<td>275</td>
<td>25</td>
<td>0.09</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>41</td>
<td>98</td>
<td>8.2</td>
<td>0.08</td>
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<tr>
<td>Single-stranded DNA cellulose</td>
<td>9.6</td>
<td>33</td>
<td>9.6</td>
<td>0.29</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>18</td>
<td>1</td>
<td>2.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

The activity of each fraction was determined by assaying a series of dilutions in standard replication reaction mixtures (50 μl) containing 6 μl of extract from H5ts36-infected cells and supplemented with 1 μg of purified adenovirus DNA binding protein. The DNA product of each reaction was analyzed by agarose gel electrophoresis and the relative incorporation of α-32PdCTP into the terminal restriction fragments of the template was estimated by microdensitometry of autoradiograms of the dried gel. One unit was defined as the complementing activity present in 1 ml of the DEAE-cellulose fraction.
FIG. 4. Requirements for formation of 80-kDal protein–dCMP complexes. The formation of 80-kDal protein–nucleotide complexes was assayed in reaction mixtures (25 μl) containing Ad5 DNA–protein complex as template and [α-32P]dCTP as the only deoxynucleotide triphosphate (see Materials and Methods). The reaction product was analyzed by electrophoresis in an 8% NaDodSO4/polyacrylamide gel. Lanes: a, extract from Ad5-infected cells (deproteinized Ad5 DNA substituted for Ad5 DNA–protein complex); b, extract from Ad5-infected cells (7.5 μl); c, extract from uninfected HeLa cells (7.5 μl); d, extract from H5ts36-infected cells (7.5 μl); e, purified H5ts36-complementing activity (7.5 μl); f, purified H5ts36-complementing activity and extract from H5ts36-infected cells (7.5 μl each); g, purified H5ts36-complementing activity and extract from uninfected HeLa cells (7.5 μl each).

The requirements for the complete in vitro DNA replication reaction were determined also. The purified H5ts36-complementing activity and the purified adenovirus DNA binding protein (H5ts125-complementing activity) were defective in DNA replication when tested alone (data not shown) or in combination (Fig. 5, lane b). However, DNA replication activity comparable to that observed with crude nuclear extracts from wild-type-infected cells was restored when the mixture of H5ts36-complementing activity and DNA binding protein was supplemented with extract from uninfected cells (Fig. 5, lane c). We conclude that the two purified complementing fractions contain all of the virus-encoded components required for adenovirus DNA replication in vitro.

DISCUSSION

The results described in this paper demonstrate that in vitro complementation provides a convenient and sensitive means of assaying adenovirus-encoded DNA replication proteins. Using such assays we have extensively purified proteins present in nuclear extracts from wild-type-infected cells that are capable of restoring DNA replication activity to nuclear extracts from cells infected with adenovirus mutant H5ts36 or H5ts125. The two purified complementing fractions appear to contain all of the virus-encoded proteins required for viral DNA replication in vitro.

As expected, the most highly purified fraction exhibiting H5ts125-complementing activity contained as the major component the 72-kDal adenovirus DNA binding protein. The H5ts125 mutation has been mapped to the DNA binding protein gene by several independent methods (22, 25) and the DNA binding protein isolated from H5ts125-infected cells grown at the permissive temperature has been shown to be thermolabile for binding to single-stranded DNA (21). In addition, previous in vitro studies have demonstrated that purified DNA binding protein can restore replication activity to nuclear extracts or replicating viral chromosomes prepared from H5ts125-infected cells (6, 12, 31). The precise role of the adenovirus DNA binding protein in viral DNA replication is not clear. Our results (ref. 6 and this paper), as well as those of Enomoto et al. (15), indicate that the DNA binding protein is not required for the formation of the terminal protein–dCMP complex, the putative first step in DNA replication. Thus, it seems likely that the DNA binding protein functions during the chain elongation process.

The most highly purified fraction exhibiting H5ts36-complementing activity contained three major polypeptides with apparent molecular masses of 140, 80, and 65 kDal, as well as some minor components. It is probable that the 80-kDal polypeptide represents the precursor form of the adenovirus terminal protein because it comigrated in NaDodSO4/polyacrylamide gels with the authentic 80-kDal precursor labeled in vitro. The nature of the 65- and 140-kDal polypeptides and their role, if any, in viral DNA replication are not yet clear. The 65-kDal polypeptide may well be a proteolytic cleavage product of the 80-kDal protein. A proteolytic fragment of similar size (62 kDal) has been described (32). The results of Enomoto et al. (15) suggest a possible function for the 140-kDal protein. These investigators have purified adenovirus replication proteins using assays different from that described in this paper. The major components present in their most highly purified fraction are the 80-kDal terminal protein and a 140-kDal polypeptide. The purified fraction contains a DNA polymerase activity which Enomoto et al. suggest may reside in the 140-kDal polypeptide or a complex of the 140- and 80-kDal polypeptides. We have observed DNA polymerase activity in the purified H5ts36-com-
plementing fraction, but its properties must yet be characterized.

The locus of the H5ts36 mutation has been mapped to the region of the adenovirus genome between 18.5 and 22 map units (26). This region falls within a large (120 kDal) open reading frame that maps between 24.0 and 14.2 map units and lies outside the sequences that encode the 80-kDal terminal protein (map positions 28.9–23.5) (27, 28, 33). The gene product corresponding to the 120-kDal open reading frame has not yet been identified; however, the 140-kDal polypeptide present in the purified H5ts36-complementing activity clearly represents the best candidate.

The results of previous studies (4–8, 15) strongly suggest that the first step in adenovirus DNA replication involves the formation of a covalent complex between the 80-kDal terminal protein and dCMP. The purified H5ts36-complementing fraction catalyzes this reaction but requires a cellular factor(s) for optimal activity. Further work will be required to define this cellular factor and to determine whether components in the H5ts36-complementing factor other than the 80-kDal terminal protein are required for the protein–nucleotide joining reaction. The complete in vitro DNA replication reaction can be reconstituted from the H5ts36-complementing fraction, the adenovirus DNA binding protein (H5ts125-complementing fraction), and extract from uninfected cells. It should now be possible by further fractionation to purify all of the components involved in replication, both viral and cellular, and to characterize their mechanisms of action.

Note Added in Proof. Recently, Lichy et al. (34) reported separation of the 80-kDal terminal protein and the 140-kDal polymerase. Both components were required for optimal formation of terminal protein–dCMP complexes.

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