ASSOCIATION OF ADENOVIRUS TYPE 2 EARLY PROTEINS WITH A SOLUBLE COMPLEX THAT SYNTHESIZES ADENOVIRUS DNA IN VITRO

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Summary: A soluble Ad2 DNA synthesizing complex was prepared from Ad2-infected KB cell nuclei and purified by exclusion chromatography on a Bio-Gel A-50m column. The purified complex was able to synthesize DNA from all regions of the virus genome, as indicated by EcoRI restriction endonuclease analysis of in vitro labeled DNA. Experiments were performed to identify Ad2-induced early polypeptides present in the complex. Ad2-infected and mock-infected cells were labeled with [^{35}S]methionine 7-10 h postinfection, then incubated for 8 h to allow the ^{35}S-labeled early polypeptides to become associated with the complex. The polypeptides in the purified complex and each of the cell fractions were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography. The major components of the purified complex were the 73K DNA binding phosphoprotein and 11K, two adenovirus 2-induced early polypeptides. The 11K has a preferred nuclear location. Small quantities of other Ad2-induced early proteins, 21K, 15K, and possibly 8.3K were also associated with the complex.

Yamashita et al. (1) described a soluble complex from adenovirus type 2 (Ad2)-infected cells, that synthesizes exclusively Ad2 DNA by a semiconservative mechanism, and elongates nascent viral DNA chains into full length (31S) DNA molecules. The partially purified complex contains cellular DNA polymerases α and γ, plus other enzymes possibly involved in DNA replication (2). Thus, this complex may represent, at least in part, a multienzyme complex that replicates Ad2 DNA, and is a useful system to study the mechanism of eukaryotic DNA replication.

At least 3 Ad-coded early proteins are required for viral DNA replication, i.e. there are 3 complementation groups of DNA-negative mutants (3-6) (early Ad2 proteins are synthesized before the initiation of virus DNA replication at 6-7 h postinfection). Identification and characterization of these early proteins are important to understanding viral DNA replication. In this report we further characterize the soluble Ad2 DNA replication complex, and show that several early proteins are present in the complex.

MATERIALS AND METHODS

Virus infection and labeling of Ad2 early proteins. KB cells grown in minimal essential medium (MEM) were infected with 200 PFU/cell of Ad2 (1, 2). Mock-infected cells were prepared in a similar manner. Cycloheximide (25 µg/ml) was added at 1 h postinfection (p.i.) and cytosine arabinoside (20 µg/ml) at 4 h p.i. Treatment of cells with cycloheximide prior to labeling of protein enhances the synthesis of Ad2 early proteins, and cytosine

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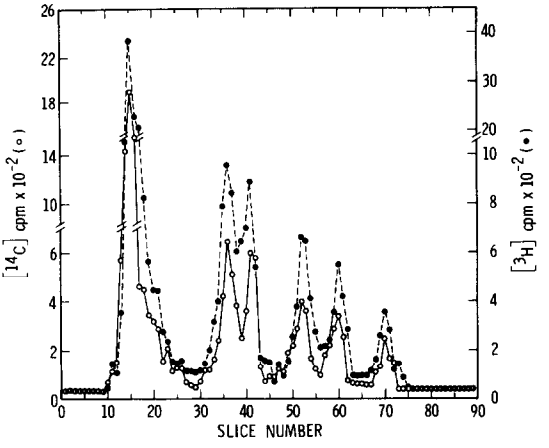


Figure 1. Co-electrophoresis of fragments generated by EcoRI cleavage of ^{3}H-labeled DNA synthesized in vitro by the purified soluble Ad2 DNA replication complex, and of in vivo ^{14}C-labeled Ad2 virion DNA. DNA synthesized in vitro by the purified complex was labeled with [^{3}H]TTP for 60 min. The DNA was purified by phenol-chloroform extraction (22) followed by centrifugation through neutral sucrose gradients (1). DNA sedimenting in a 31S peak (about 80% of the labeled DNA synthesized) was pooled. Virion DNA was labeled in vivo with [^{14}C]thymidine and purified as described (21). In vivo and in vitro labeled DNA were mixed, cleaved with EcoRI restriction endonuclease, and co-electrophoresed through 1.4% agarose gels (22).

period and thus maintains the cells in the early stage of infection (7). At 7 h p.i., cells were suspended at 7x10^{5} cells/ml in warm methionine-free MEM containing cytosine arabinoside but lacking cycloheximide. Cells were labeled with [^{35}S]methionine (25 μCi/ml) from 7-10 h p.i., washed, suspended at 3.5x10^{5} cells/ml in complete MEM, without drugs, and incubated at 37°C to 18 h p.i. to allow the formation of the Ad2 DNA replication complex.

Purification of the Ad2 DNA replication complex. Nuclei and replication complex were prepared as described (1,2). Cells were swollen in reticulocyte standard buffer [RSB: 10 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1.5 mM $MgCl_2$] for 30 min at 4°C, then disrupted with a tight-fitting Dounce homogenizer. The extract was layered over 5 ml of 25% sucrose-RSB in a 50 ml centrifuge tube; nuclei were collected by centrifugation at 800 x g, resuspended in RSB, and washed by recentrifugation through the sucrose cushion. Nuclei were suspended at 10^{7} nuclei/ml in TK buffer [50 mM Tris-HCl (pH 7.9)-25 mM KCl] and lysed by adjusting the suspension to 3 mM dithiothreitol-2 mM Na_2HPO_4-1 mg/ml sodium heparin, and incubating at 4°C for 30 min. To reduce the viscosity, DNA was sheared by gently passing the suspension 3X through a 18 gauge syringe needle. Insoluble material was removed by centrifugation at 45,000 x g for 30 min. The supernatant ("crude complex") was dialyzed at 4°C against TK buffer containing 3 mM dithiothreitol-0.1 mM EDTA-30% glycerol. The crude complex was purified by filtration through a Bio-Gel A-50m column(1x90 cm). The excluded fraction which contained endogenous DNA polymerase activity is designated the "purified complex".

RESULTS

Characterization of the DNA synthesized in vitro by the soluble Ad2

Table 1. Distribution of radioactivity in EcoRI fragments of ^{3}H-labeled DNA synthesized in vitro by the Ad2 DNA replication complex

EcoRI Fragments	Distribution of Radioactivity (%)		
	[^{3}H]TTP in vitro††	[^{14}C]Thymidine in vivo†	[^{32}P]Phosphate in vivo*
A	53.0	51.0	58.5
B	14.2	14.6	11.9
C	10.8	11.3	9.9
D	8.7	10.0	7.5
E	7.7	7.6	6.3
F	5.3	5.2	5.2

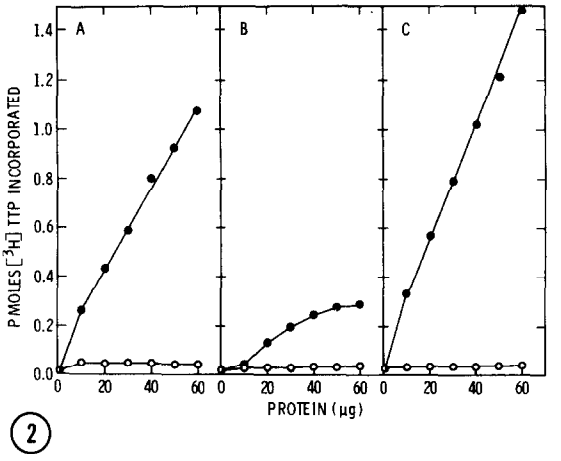
†† DNA was synthesized in vitro by the endogenous DNA polymerase in the purified soluble Ad2 DNA replication complex.

† Ad2 DNA was labeled in vivo with [^{14}C]thymidine.

* Data from Pettersson et al. (22)

arabinoside inhibits the replication of Ad2 DNA during the protein labeling DNA replication complex. Some of the properties of the complex have been described (1,2). We wished to characterize the DNA synthesized in vitro by the purified complex. DNA was labeled in vitro with [^{3}H]TTP, extracted, mixed with ^{14}C-labeled Ad2 virion DNA, and cleaved with EcoRI restriction endonuclease. The resulting EcoRI fragments were resolved on the basis of size by agarose gel electrophoresis. As shown in Fig. 1, the ^{3}H-DNA synthesized by the complex was present in all 6 fragments, and the distribution of radioactivity corresponded very well to virion ^{14}C-DNA fragments. The fraction of radioactivity in each restriction fragment corresponded closely with the fraction of the virus genome represented by the fragment (Table 1). These data indicate that DNA from all portions of the virus genome is synthesized by the purified complex and that little, if any, cell DNA is synthesized.

Identification of Ad2 early proteins associated with the purified complex. Early proteins were labeled with [^{35}S]methionine (in the presence of cytosine arabinoside), and then were allowed to become associated with the replication complex by incubating cells for 8 h without cytosine arabinoside. As shown in Fig. 2C, the crude complex prepared by this protocol had as much endogenous DNA polymerase activity as a complex harvested 18 h p.i. from cells not labeled or treated with drugs (Fig. 2A). The complex isolated from



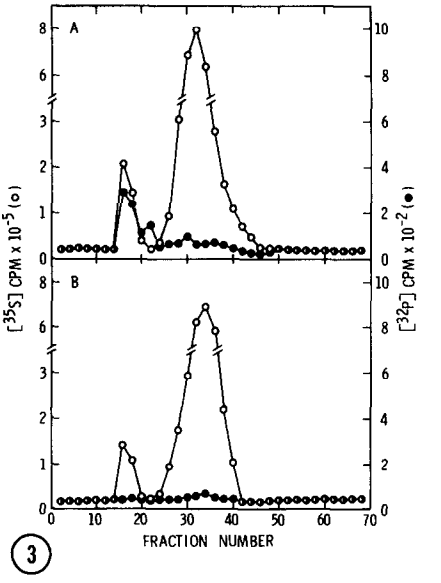


Figure 2. Endogenous DNA polymerase activity in crude soluble Ad2 DNA replication complex from infected (●) and mock-infected (O) cells. A) Cells harvested 18 h postinfection; not treated with drugs and not labeled with [^{35}S]methionine. B) Cells labeled at 7-10 h postinfection with [^{35}S]methionine as described in Materials and Methods, and harvested at 10 h postinfection. C) Cells labeled with [^{35}S]methionine, at 7-10 h postinfection, then harvested at 18 h postinfection. Endogenous DNA polymerase activity was assayed as described by Yamashita et al. (1).

Figure 3. Endogenous DNA polymerase activity in Bio-Gel A-50m excluded (purified Ad2 DNA replication complex) and included (nucleoplasm) fractions. A) Infected cells. B) Mock-infected cells.

cells at 10 h p.i., the end of the labeling period, had much less polymerase activity (Fig. 2B). Very little polymerase was detected in complexes from mock-infected cells. The crude ^{35}S-labeled infected and mock-infected complexes were purified by filtration through Bio-Gel A-50m. The infected cell complex had the majority of the endogenous DNA polymerase activity in the excluded fraction (Fig. 3A), whereas the mock-infected complex had little polymerase activity in any fraction (Fig. 3B). These results suggest that our protocol for labeling early proteins does not adversely affect the formation of the Ad2 DNA synthesizing complex.

Figure 4 illustrates the ^{35}S-labeled polypeptides present in a total cell extract of infected and mock-infected cells and in the purified complex. The following early polypeptides are seen in infected whole cell extracts (lanes A): DBP (a DNA binding protein of 73,000 daltons), 21K, 19K, 15K,

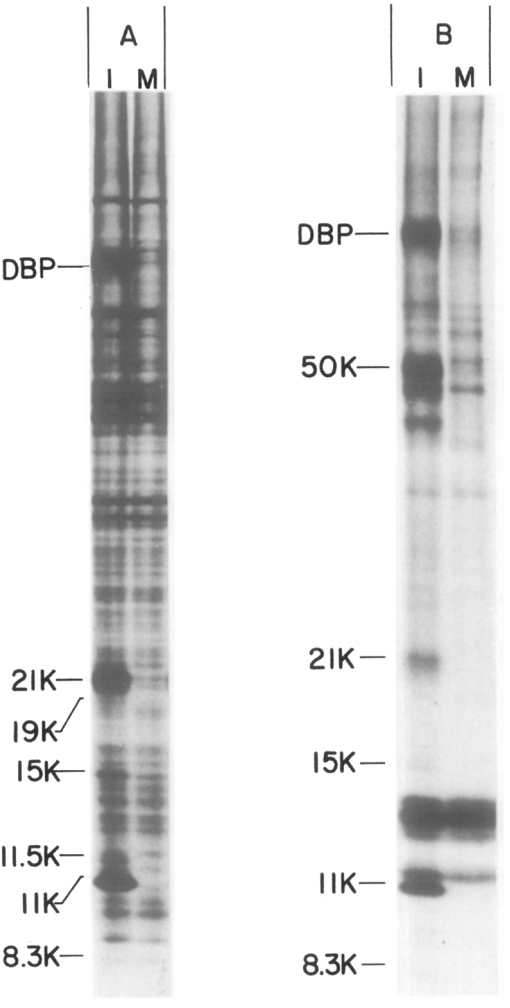


Figure 4. Autoradiogram illustrating Ad2-induced early polypeptides present in the purified soluble Ad2 DNA replication complex. ^{35}S-labeled infected (I) and mock-infected (M) cell complexes, and indicated cell fractions, were prepared as described in Materials and Methods. As described elsewhere (7), equal counts of each fraction were electrophoresed through 27 cm 5-20% gradient polyacrylamide sodium dodecyl sulfate slab gels. Proteins were visualized by autoradiography. Lanes A: whole cell extracts. Lanes B: purified soluble Ad2 DNA replication complex passed through two Bio-Gel A-50m columns. The radioactivity (cpm) in each of the fractions was as follows. Cytoplasm: I, 272×10^6; M, 285×10^6. Crude soluble complex: I, 35.5×10^6; M, 46.5×10^6. Insoluble nuclear material: I, 51×10^6; M, 52.2×10^6. Nucleoplasm: I, 24×10^6; M, 21.4×10^6. Purified soluble complex: I, 1.1×10^6; M, 1.3×10^6.

11.5K, 11K, and 8.3K. The purified soluble Ad2 DNA replication complex contained major amounts of DBP and 11K, and minor quantities of 21K, 15K, and possibly 8.3K (not shown). These early polypeptides remained associ-

ated with the complex after purification through a second Bio-Gel A-50m column (lanes B), suggesting that they may be stably associated with a large complex. The infected-cell-specific polypeptides of 40-50,000 daltons present in the purified complex (lanes B) are the known sub-species (protease breakdown products) of DBP, as indicated by immunoprecipitation by monospecific antiserum (8) against highly purified DBP (not shown).

DISCUSSION

We have shown that all regions of the Ad2 genome are synthesized in vitro by the purified Ad2 DNA replication complex. Our results, together with those of Yamashita et al. (1) and Arens et al. (2), are consistent with the possibility that the soluble complex may represent a multienzyme complex that synthesizes Ad2 DNA in vivo. The major virus-induced early polypeptides in the purified complex were DBP and 11K, raising the possibility that these play a role in Ad2 DNA replication. DBP is a virus-coded (9-11) early phosphoprotein (12-14) that binds to single-stranded DNA. It is virtually certain that DBP functions in Ad2 DNA replication, because (i) the temperature sensitive mutant of Ad5, H5ts125 (4), defective in Ad5 DNA synthesis (15,16), is mutated in the DBP structural gene (15-17), and (ii) fluorescent antibody studies indicate that DBP is localized in the cell nucleus at the time of Ad2 DNA replication (8). The 11K is localized in the cell nucleus (18, 19, unpublished data), consistent with a role in Ad2 DNA replication.

Small quantities of 21K, 15K, and possibly 8.3K were also present in the soluble complex, suggesting that these may also function, perhaps catalytically, in virus DNA replication. The 21K protein is an early glycoprotein (20; Jeng, Wold, and Green, unpublished results). The 21K is probably virus-coded, because it is immunoprecipitated by antisera against Ad2-transformed rat cells (Wold and Green, unpublished results), and because similar-sized polypeptides are produced by cell-free translation of Ad2-specific early mRNA (10). We emphasize that because the complex contained only a small fraction of 21K, 15K, and 8.3K, we cannot exclude non-specific association of these polypeptides with the purified complex.

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