Adenovirus Early Region 4 Is Required for Efficient Virus Particle Assembly

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H2d/807, a defective deletion mutant of human adenovirus type 2 lacking parts of early regions 3 and 4 and all of late region 5, was severely defective for virus particle assembly on HeLa cells, producing about 1% of the normal yield of particles. On Vero cells, H2d/807 produced only 5% as many particles as wild type, while on W162 cells, a Vero cell derivative which supports the growth of early region 4 mutants, H2d/807 produced nearly 40% of the wild-type level of particles. Two other defective deletion mutants, H2d/802 and H5d/1021, which lack parts of early region 3 and which are incapable of making fiber, the product of late region 5, were wild type for virus assembly. These data suggest that the cause of the assembly defect of H2d/807 is the lack of a diffusible early region 4 product. H2d/807-infected Vero cells accumulated nearly wild-type amounts of viral late proteins in the nucleus and cytoplasm. Thus, the defect of the mutant in assembly on Vero cells is not due to a general lack of late proteins. Finally, the fact that H2d/802 and H5d/1021 make wild-type amounts of virus particles suggests that fiber is not essential for adenovirus assembly.

Early region 4 (E4) occupies the righthand 9% of the human adenovirus genome. S1 analysis has detected as many as 24 distinct E4 mRNA species during the early phase of a productive adenovirus type 2 (Ad2) infection (31), and 16 potential protein products of E4 have been identified by *in vitro* translation of E4 mRNA (30). So far, seven protein-coding regions (open reading frames, ORFs; see Fig. 2) in E4 have been identified by sequencing E4 DNA and E4 cDNA clones coupled with analysis of E4 mRNA by S1 mapping (15, 18, 32).

Several genetic studies have been undertaken to assign functions to the various E4 products (6, 7, 17, 29, 35, 36). In addition to a number of deletion and insertion mutants which have wild-type phenotypes, three mutants in E4 with aberrant phenotypes have been reported. Two of these mutants (H2d/808 and H5d/366) have large deletions entirely within E4 (17, 35). Both are defective for growth on normal adenovirus hosts and are grown with the help of a complementing cell line, W162, which contains E4 DNA (34). These mutants are defective for viral DNA accumulation and late mRNA and protein synthesis, fail to inhibit host cell protein synthesis, and overproduce the 72-kilodalton (kDa) DNA-binding protein, a product of E2. The third mutant (H5d/355) carries a smaller deletion which is localized to an ORF expected to encode a 34-kDa product. This mutation has less severe effects on viral DNA accumulation and late protein synthesis (17). In addition to these three mutants with lesions entirely within E4, one mutant (H2d/807) with a large deletion not entirely within E4 has been described (7). Deletion 807 affects E3 and late region 5 (L5), as well as E4.

In this study, we utilized H2d/807 and two overlapping deletion mutants, H2d/802 and H5d/1021, to further characterize E4. Both H2d/802 and H5d/1021 remove sequences in

E3 and L5, but leave E4 intact. We show that both of these deletions are wild type for DNA and late protein synthesis, whereas H2d/807, as previously reported (7), is wild type for DNA synthesis but is defective in late protein synthesis on HeLa cells. Additionally, we show that H2d/807 is defective for virus particle assembly on both HeLa and Vero cells, but is nearly normal on the complementing cell line W162. Both H2d/802 and H5d/1021 are wild type for assembly. We conclude that an adenoviral E4 product is involved in virion assembly.

MATERIALS AND METHODS

Cells and viruses. HeLa cells, Vero cells, and W162 cells (34) were grown in monolayer culture in autoclavable Eagle minimum essential medium (MEM; Flow Laboratories, Inc., McLean, Va.) containing 10% calf serum. 293 cells were grown as monolayers in MEM plus 10% fetal calf serum (MEM10).

The isolation, purification, and partial characterization of H2d/807 have been described previously (7). The isolation of H2d/802 was described in the same reference. The construction and isolation of H5d/1021 is described in detail in the Results.

Viral infections of cells were performed by adding virus directly to the cell culture media, incubating at 37°C for 2 h, and then replacing the media. The multiplicity of infection was normally between 5 and 50 PFU per cell. The titers used for wild-type virus were determined on HeLa or 293 cells; all plaque experiments were done in medium containing 5% fetal calf serum.

Purification of deletion mutant virions. The purification of H2d/807 was as described previously (7), except the second discontinuous CsCl gradient was omitted. The purification of H2d/802 and H5d/1021 followed this same protocol, with the addition in some cases of a fourth equilibrium CsCl centrifugation. In one series of experiments, an Ad2-simian virus 40 hybrid derived from Ad2ND3 (19) was used as the helper virus for H2d/802, instead of H5ts147. This hybrid virus contains about 60% of the simian virus 40 genome in place of

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the Ad2 DNA between 80.5 and 85.7 map units (m.u.); thus its genome is about 4% larger than that of Ad2 (unpublished data).

For all three deletion mutants, purified virus was dialyzed into sterile Ad storage buffer (0.25 M sucrose, 0.02 M Tris hydrochloride [pH 7.4], 0.001 M MgCl_2 , 0.5% *n*-butanol), adjusted to an A_{260} of less than 1 with the same buffer, and stored at 4°C; this was done to prevent aggregation of virus particles. The level of contamination of these stocks by helper virus was estimated by titration as described previously (7), except titrations were done on HeLa cells. Less than 0.5% of the virus particles in the H2d/807 stocks were helper virus, the H2d/802 stocks contained about 1% helper, and the H5d/1021 stock contained less than 0.3% helper virus. The specific infectivity (PFU per milliliter per A_{260}) for deletion mutant stocks was assumed to be the same as for the wild-type stocks prepared in parallel, as discussed previously (7). This number was always on the order of 10^9 PFU/ml per A_{260} .

Analysis of virion DNA. DNA was prepared from purified virions in Ad storage buffer as previously described (7). Purified DNA was digested with restriction enzymes (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; New England BioLabs, Inc., Beverly, Mass.) in enzyme excess in buffer as recommended by the supplier. Digests were analyzed on 1.4% agarose gels as described previously (35).

For DNA sequencing, 15-base oligonucleotide primers were synthesized. The primer for sequencing deletion 807 was 5'-GACATGATTCCTCGA-3', corresponding to nucleotide numbers 29778 to 29792 in the consensus Ad2 sequence (24). The primer for deletion 802 was 5'-GCCACTGTTAGGGCG-3', corresponding to nucleotides 31373 to 31359. The primers were purified as described previously (1). Sequencing was done by a modification (26a, 33) of the Sanger dideoxynucleotide method (27), using 1 μg of purified virion DNA and 30 ng of primer and omitting the restriction digestion step. Interpretation of ambiguous residues was aided by comparison with the known sequence of Ad2.

Radiolabeling with [^{35}S]methionine. To label proteins for analysis on gels, about 3×10^5 cells in a well of a 24-well cluster dish were starved for 1 h at 23 h postinfection (p.i.) in 0.5 ml of MEM containing 5% the normal level of methionine plus 2% calf serum (MEM2Cmet/20). The starved cells were labeled for 1 h in 0.2 ml of the same medium containing 0.01 to 0.02 mCi of [^{35}S]methionine per ml and then chased for 30 min in 1 ml of MEM10. The cell monolayers were lysed in situ with 0.5 ml of protein gel loading solution (PLS; 0.05 M Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.004% bromophenol blue, 1% 2-mercaptoethanol) and stored frozen. The same protocol was used to make extracts for immunoprecipitation analysis, except the [^{35}S]methionine concentration was 0.05 to 0.1 mCi/ml.

For analysis of virus particle assembly, about 10^6 cells in a 60-mm-diameter dish were labeled at 24 h p.i. for 5 to 6 h in 1 ml of MEM2Cmet/20 containing 0.05 to 0.1 mCi of [^{35}S]methionine per ml and then chased for 15 min in 5 ml of MEM10. The cells were fractionated into cytoplasmic and nuclear fractions as described below.

Immunoprecipitations. Rabbit antiserum raised against SDS-denatured purified Ad2 fiber protein was from C. Anderson. Cell extracts in PLS were boiled and centrifuged for 2 min at $12,000 \times g$, and the supernatants were adjusted to 10% Nonidet P-40 and 0.15 M NaCl. Excess antiserum was added, and the samples were incubated overnight at

4°C. Then excess Pansorbin (Calbiochem-Behring, La Jolla, Calif.) was added and allowed to react for 1 h at 4°C. The Pansorbin had been prewashed in RIPA buffer (0.15 M NaCl, 0.002 M EDTA, 0.01 M Tris hydrochloride [pH 7.4], 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS). The precipitates were collected by centrifugation at $12,000 \times g$ for 1 min, washed five times with RIPA buffer, and then suspended in 0.05 ml of PLS. The samples were boiled and centrifuged, and the supernatants were examined on an SDS-polyacrylamide gel.

Cell fractionation. Cells were fractionated as described previously (35), except the sucrose cushion was omitted and centrifugation was at $1,000 \times g$ for 5 min. The supernatant is the cytoplasmic fraction; the pellet is the nuclear fraction.

Preparation and analysis of cytoplasmic and nuclear DNA. DNA was prepared from nuclei as described previously (35). Cytoplasmic fractions were mixed with an equal volume of 0.04 M Tris hydrochloride (pH 7.4)–0.002 M EDTA–1.2% SDS–2 mg of pronase per ml, and DNA was isolated as for nuclear fractions. Yeast tRNA (10 to 20 μg) was added as a carrier to the cytoplasmic DNA fractions. Nucleic acid was precipitated with ethanol (37) and redissolved in deionized water. Analyses by restriction digestion-agarose gel electrophoresis and by dot-blot hybridization were as described previously (35).

SDS-polyacrylamide gel electrophoresis. The gel system was as described previously (35), except in some of the immunoprecipitation experiments the gel was 17.5% acrylamide–0.077% bisacrylamide. For the analysis of different volumes of radioactive proteins, a constant load volume was maintained by adding PLS. Samples were vortexed, boiled for 2 min, and then briefly centrifuged just before loading. Gels were run at constant voltage until the dye front was near the bottom.

Gels were impregnated with 2,5-diphenyloxazole for fluorography with glacial acetic acid as described previously (5). The dried gel was used to expose Kodak XAR-5 X-ray film at -70°C .

Sucrose gradient analysis of virus particles. The protocol used for sucrose gradient analysis of virus particles was essentially as described previously (11), with a few modifications. The cytoplasmic fractions of cell extracts were adjusted to 0.02 M EDTA and 0.001 M phenylmethylsulfonyl fluoride and were stored at 4°C. The nuclear pellets were suspended in 0.3 ml of 0.05 M Tris hydrochloride (pH 8.0)–0.01 M EDTA–0.3 M ammonium sulfate, adjusted to 0.001 M phenylmethylsulfonyl fluoride and then homogenized with 25 strokes in a 1-ml-capacity Dounce homogenizer and stored at 4°C. Aliquots of the cytoplasmic and nuclear samples to be analyzed by gel electrophoresis were diluted 10-fold into PLS and stored frozen.

Linear 25 to 40% sucrose gradients containing 0.2 M NaCl, 0.05 M Tris hydrochloride (pH 8.0), and 0.01 M EDTA were gently overlaid with up to 0.3 ml of sample and centrifuged in an SW41 rotor at 26,000 rpm for 105 min at 4°C. Gradients were fractionated by collecting drops from the bottom of the tube. Aliquots were diluted at least fivefold in deionized water and then counted in Triton-toluene fluor (8 g of Omnifluor [New England Nuclear Corp., Boston, Mass.] dissolved in 2 liters of toluene:1 liter of Triton X-100 [Sigma Chemical Co., St. Louis, Mo.]) at a final aqueous content of about 10%.

CsCl gradient analysis of virus particles. Peak fractions from the sucrose gradients were pooled and dialyzed against 0.15 M NaCl–0.01 M Tris hydrochloride (pH 8.1)–0.5% *n*-butanol to remove sucrose. Aliquots of dialyzed samples

FIG. 1. Scheme for the construction of H5dl1021. (a, b, c, d, e) Structures of plasmids used. An *SpeI*-*HindIII* fragment of pHS88 (a, hatched box) and a *BsmI*-*XbaI* fragment of pBN27 (b, filled box) were combined with a piece of pBR322 to create pHF19 (c). The large *AatII*-*KpnI* fragment of pHF19 was ligated to the small *AatII*-*KpnI* fragment of pEcoRIBAd5 (d, hatched box plus solid line) to make pHFΔXS (e). (f) Ad5 DNA showing the *EcoRI* sites and fragments. (g) H5dl1021 DNA made by ligating an *EcoRI* fragment of pHFΔXS to Ad5 *EcoRI*-A, decorated to illustrate the origins of the various portions of the genome. The *BclI* site shown is Ad2 specific. Boxes represent Ad DNA; lines represent pBR322 DNA. Ad DNA is drawn to scale; plasmid DNA is not. The scale used in panels a through e differs from the scale used in panels f and g. Decorated boxes are fragments used in the construction of H5dl1021; open boxes are unused fragments. Solid lines are plasmid sequences which do become part of pHFΔXS; broken lines are plasmid sequences which do not. Positions of Ad restriction sites are labeled in map units (1 m.u. is 1% of the Ad genome length). Pertinent plasmid *AatII* sites are labeled. The hybrid *XbaI*/*SpeI* site is no longer cut by either enzyme.

were then adjusted to a density of 1.34 g/ml with CsCl and centrifuged to equilibrium at 45,000 rpm in a Sorvall TV865 rotor. A small sample of ^{32}P -labeled Ad2 virions was included as an internal standard (7). Gradients were fractionated by collecting drops from the bottom of the tube. The refractive index of every second or third fraction and the radioactivity of each fraction were measured.

RESULTS

Construction and isolation of H5dl1021. Deletion 1021 was constructed to remove E3 and L5, yet leave E4 intact, to serve as a control for E4 function when compared with H2dl807. Plasmid pHS88, consisting of the Ad2 *HindIII* F fragment cloned into pBR322, was digested with *HindIII* and *SpeI* (Fig. 1a). Plasmid pBN27 (from E. Bridge), containing the Ad5 *BamHI*-*NdeI* fragment from 60.1 to 86.5 m.u. cloned in pBR322, was digested with *XbaI* and *BsmI* (Fig. 1b). (The locations of the *BsmI* sites at 71.5 and 73.5 m.u. in Ad5 were mapped for this work.) The digested plasmids were mixed, extracted with phenol and chloroform, precipitated with ethanol, and then redissolved and ligated in the presence of *XbaI*, *BsmI*, and *HindIII*. Plasmid pBR322 which had been digested with *BsmI* and *HindIII* was added,

and the mixture was extracted, precipitated, redissolved, and then ligated in the absence of restriction enzymes. This ligated DNA was used to transform *Escherichia coli* HB101 (21), and the plasmid pHF19, with the structure shown in Fig. 1c, was isolated. Plasmid pHF19 was digested with *AatII* and *KpnI*, as was plasmid pEcoRIBAd5 (Fig. 1d), which contains the Ad5 *EcoRI* B fragment cloned in pBR322 (4). These digested plasmids were mixed, ligated, and used to transform *E. coli*, and the plasmid pHFΔXS was isolated (Fig. 1e).

Next, Ad5 DNA with the terminal proteins intact (Ad5 DNA-protein complex) was prepared from virions as described previously (25). The Ad5 DNA-protein complex was digested with *EcoRI* (Fig. 1f) and centrifuged on a sucrose gradient (26). Fractions were collected from the bottom of the tube and dialyzed, and samples were digested with pronase and examined on an agarose gel. Fractions lacking the Ad5 *EcoRI* C fragment were used in the next step (the Ad5 *EcoRI* A and B fragments were not resolved in this gradient).

The sucrose gradient fractions lacking the Ad5 *EcoRI* C fragment were ligated to *EcoRI*-digested pHFΔXS. Ligated material (1 to 2 μg) was used to transfect each dish of 293 cells (16), which had been infected with H5ts147 at a

FIG. 2. Map of the deletions. The line at the bottom depicts the right-hand end of Ad2, marked off in map units. The sequences removed by deletions 807, 802, and 1021 are indicated by the black bars. The endpoints of deletions 807 and 802 are given in nucleotide numbers, and the positions of the restriction sites which were fused to make deletion 1021 are given in map units, below the bars. The arrows depict E3 and E4, the body of L5 mRNA, and part of the body of L4 mRNA. Arrowheads correspond to polyadenylation sites. P indicates promoters. The open boxes depict the regions encoding the proteins pVIII, E3 19 kDa, and IV (the capsid protein fiber) and E4 ORFs 1, 2, 3, 4, 3/4, 6, and 6/7. The nucleotide positions on the Ad2 genome (and Ad5 map coordinates, in some cases) of some of the indicated features are as follows: L4 polyadenylation site, 28223 (78.0 m.u.); E3 19-kDa protein coding sequence start and end, 28812 (79.7 m.u.) and 29289 (80.1 m.u.), respectively; L5 splice acceptor site and fiber coding sequence start, 31030; fiber-coding sequence end, 32776; L5 polyadenylation site, 32798; E4 polyadenylation site, 32802; E4 ORF 6/7 end, 32916; ORF 6/7 internal splice acceptor, 33192; ORF 6/7 internal splice donor, 33904; ORF 6/7 AUG, 34077; ORF 6 end, 33195; ORF 6 AUG, 34077; ORF 4 end, 34000; ORF 4 AUG, 34342; ORF 3/4 end, 34000; ORF 3/4 internal splice acceptor, 34802; ORF 3/4 internal splice donor, 34606; ORF 3/4 AUG, 34706; ORF 3 end, 34358; ORF 3 AUG, 34706; ORF 2 end, 34705; ORF 2 AUG, 35095; ORF 1 end, 35148; ORF 1 AUG, 35532; E4 cap site, 35609 to 35614. The end of the genome is at 35931.

multiplicity of infection of 10^{-3} (titer determined at 32.5°C); or the ligation mixture was cotransfected onto 293 cells with 0.04 or 0.4 μ g of H5ts147 DNA-protein complex. Plaques were selected at 37°C. These were screened as described previously (7), and a stock containing a mixture of helper virus and H5d/1021 was isolated. The expected structure of the H5d/1021 genome is shown in Fig. 1g. Deletion 1021 DNA should be chimeric, consisting mainly of Ad5 DNA, with a small piece of Ad2 DNA from 90.8 to 93.5 m.u., but with no viral sequences corresponding to 79.7 to 90.8 m.u.

Mapping of deletions. The deletions in H2d/802 and H2d/807 have been previously characterized by heteroduplex mapping (7). To better map these deletions, we subjected purified deletion mutant virion DNA preparations to digestion with a variety of restriction enzymes, and the products were analyzed on agarose gels (data not shown). The left end of deletion 802 was found to fall between the *Bgl*II site at 78.4 m.u. and the *Hind*III site at 79.7 m.u.; the right end of deletion 802 mapped between the *Sph*I site at 86.8 m.u. and the *Mst*II site at 87.2 m.u. For deletion 807, the left end mapped between the *Xho*I site at 82.9 m.u. and the *Mst*II site at 83.4 m.u., while the right end fell between the pair of *Bgl*II sites at 94.9 and 95.7 m.u.

Using this mapping information along with the known sequence of Ad2, oligonucleotides were synthesized and used to sequence across the deletions. The deletion endpoints at the nucleotide level are shown in Fig. 2. In agreement with previous results, deletion 807 was found to lack the promoter-distal halves of E3 and E4 and was completely deleted for L5, which encodes the viral capsid protein fiber. Deletion 802 removed almost all of E3, and entered L5, removing the first 210 nucleotides of the 1,747-nucleotide-long fiber-coding sequence, but it did not enter E4. H2d/802 is thus incapable of making a wild-type fiber protein, although it could conceivably make a peptide containing a substantial portion of the fiber amino acid sequence.

The mutant H5d/1021 was engineered to have the deletion shown in Fig. 2. This structure was confirmed by heteroduplex and restriction mapping. Some of the mapped sites are shown in Fig. 1g. The presence of the *Bcl*I site at 91.5 m.u. confirmed that the DNA from 90.8 to 93.5 m.u. is Ad2 derived; the rest of the molecule had the expected Ad5 restriction sites. Note that the Ad5 *Xba*I site which defines the left endpoint of deletion 1021, at 79.7 m.u., falls substantially downstream of the pVIII terminator and the L4 polyadenylation site (about 900 and 600 nucleotides, respectively) (9), and the deletion therefore should not affect L4 expression. Indeed, H5d/1021-infected cells produce normal levels of pVIII and 100K (data not shown). The right endpoint of deletion 1021 is the Ad2 *Spe*I site at 90.8 m.u.; thus, deletion 1021 destroys all but the last 132 nucleotides of the fiber-coding sequence and does not affect E4.

Both deletion 1021 and deletion 802 remove most of E3, and neither deletion mutant could produce fiber, but both mutants are wild type for E4. Deletion 807 removes all of L5 and parts of E3 and E4. Since viruses carrying large deletions in E3 display no mutant phenotype when grown in cell culture (4, 7, 19), any aberrant phenotypes exhibited by H2d/807 but not by H2d/802 or H5d/1021 must be caused by the removal of E4 sequences by deletion 807.

Purity of deletion mutant virus stocks. As discussed in a previous report (7), it is important that the purified deletion mutant virus stocks not be highly contaminated with helper virus, to permit the phenotypic characterization of the mutants. Titration of the deletion virus stocks provides one estimate of the degree of helper virus contamination. The mapping results (Fig. 2) indicated that all three deletion mutants are incapable of producing wild-type fiber. The degree of helper virus contamination of the deletion mutant stocks can also therefore be estimated from the level of wild-type fiber produced in mutant-infected cells.

To quantitate the level of wild-type fiber in mutant infec-

tions, we immunoprecipitated ^{35}S -labeled infected cell extracts with an antiserum directed against SDS-denatured fiber. The immunoprecipitates were examined by SDS-polyacrylamide gel electrophoresis and fluorography. The left panel of Fig. 3 shows a comparison of the immunoprecipitates from H2d/807- and H5d/1021-infected cell extracts with a dilution series of immunoprecipitates from Ad2- and Ad5-infected cell extracts. The right panel of Fig. 3 shows a similar experiment comparing H2d/802 with Ad2 and Ad5. The fiber observed in the H2d/807- and H5d/1021-infected cell extracts was the Ad5 type, derived from the residual H5ts147 helper virus in these stocks. The helper for the particular H2d/802 stock used in this experiment was an Ad2-simian virus 40 hybrid, and the contaminating fiber observed was consequently the Ad2 type. The fiber signal observed in each deletion lane was compared with the signals in the appropriate wild-type dilution series. The relative fiber levels in the mutant-infected cell extracts are compared with the level of helper virus in these mutant stocks (as determined by titration) in Table 1. H2d/807- and H5d/1021-infected cells had nearly equal levels of fiber, at most 0.1% of the wild-type level. H2d/802-infected cells had a slightly higher level of fiber contamination, around 0.3% of the wild-type level. All three deletion mutants produced three- to fivefold less fiber than would have been predicted from the level of helper contamination determined by titration. Even assuming that the degree of helper contamination of these deletion mutant stocks is as estimated by titration, at the usual multiplicity of infection of 10 PFU per cell used for experiments, the multiplicity of infection of

TABLE 1. Comparison of the level of fiber in infected cell extracts and level of contamination of virus stocks with helper

Virus	% of wild-type fiber ^a	% Helper contamination ^b
H2d/802	0.1–0.3	0.78
H2d/807	0.03–0.1	0.15
H5d/1021	0.03–0.1	0.3

^a Determined by a visual comparison of the wild-type and mutant fiber signals in the gels in Fig. 3.

^b Determined by titration.

helper virus is not more than 0.078. This seems low enough to permit the phenotypic characterization of the mutant viruses without interference from helper virus.

The possible production of fiber-related polypeptides not comigrating with authentic fiber (cross-reacting material) in the mutant infections is also addressed by the gels shown in Fig. 3. No fiber cross-reacting material was observed in any lane, although the fiber signals in the Ad2 and Ad5 1/3,000-dilution lanes (0.033% of the wild-type level of fiber) were plainly visible. Clearly, no significant amounts of fiber cross-reacting material were produced by the mutants. There was some nonspecific precipitation of other viral proteins evident in the mutant lanes, but these proteins were also present in the wild-type lanes; keep in mind that much larger fractions of the deletion extracts were loaded than of the wild-type extracts. In other experiments, in which equal fractions of extracts were examined, the intensities of these bands in mutant and wild-type lanes were the same (data not shown).

Accumulation of DNA in mutant-infected cells. The accumulation of DNA in H2d/802-, H2d/807- and Ad2-infected HeLa cells was investigated. Infected cells were fractionated into nuclear and cytoplasmic compartments at 40 h p.i., and DNA was isolated. The amount of viral DNA in the nucleus was analyzed by restriction enzyme digestion and gel electrophoresis (Fig. 4) or by dot hybridization with adenovirus DNA as the probe (data not shown). The analyses agree and demonstrate that both deletion mutants accumulated wild-type amounts of viral DNA in the nucleus by 40 h p.i. This result is consistent with previous data (7), which suggested that H2d/807 is normal for DNA replication.

The amount of viral DNA in the cytoplasm of these cells was analyzed on the same gel (Fig. 4). While H2d/802 DNA accumulated to near wild-type levels, H2d/807 DNA was strikingly reduced in the cytoplasm. Additionally, in cells confectioned with both deletion mutants or with H2d/807 and Ad2, the H2d/807-specific restriction fragment (arrow) appeared at wild-type levels in the cytoplasm. This complementation by H2d/802 and by Ad2 of the defect in the accumulation of H2d/807 DNA in the cytoplasm is evidence that it is the lack of an E4 product and not the lack of a *cis*-acting site which is responsible for that defect.

Viral DNA accumulation in Ad2- and H2d/807-infected Vero cells was also examined. As in HeLa cells, the mutant accumulated normal amounts of viral DNA in the nucleus, suggesting that viral DNA replication was normal, but accumulated reduced amounts in the cytoplasm.

Particle production by the mutants. Adenovirus particles are known to accumulate in the cytoplasm at late times in infection (13), and this seemed likely to be the reason that Ad2 DNA accumulated in the cytoplasm in the experiments above. To measure adenovirus particle production, infected cells were labeled from 24 to 30 h p.i. with [^{35}S]methionine, fractionated into nuclear and cytoplasmic compartments,

FIG. 3. Immunoprecipitation of fiber in deletion mutant-infected cells. HeLa cells were labeled with [^{35}S]methionine 24 to 25 h after infection with H2d/802, H2d/807, H5d/1021, Ad2, or Ad5, and whole-cell extracts were immunoprecipitated with an antiserum directed against SDS-denatured fiber. Variable amounts of the immunoprecipitates from Ad2- and Ad5-infected cell extracts were compared with the immunoprecipitates from deletion mutant-infected cell extracts on SDS-polyacrylamide gels. The numbers above the wild-type lanes indicate the fractions of the wild-type samples loaded in those lanes. Left panel, H2d/807 and H5d/1021 compared with Ad2 and Ad5 on a 10% gel. Right panel, H2d/802 compared with Ad2 and Ad5 on a 17.5% gel. The positions of the Ad2- and Ad5-type fibers are indicated.

FIG. 4. DNA in deletion mutant infections. Nuclear and cytoplasmic DNAs were isolated from H2d/802-, H2d/807-, H2d/802 plus H2d/807-, Ad2-, or Ad2 plus H2d/807-infected HeLa cells at 40 h p.i., digested with *Sst*I, electrophoresed on an agarose gel, and visualized by staining with ethidium bromide and illuminating with UV light. Left panel, Nuclear DNA. Right panel, Cytoplasmic DNA. Lane U, DNA from uninfected cells. The identities of the Ad2 *Sst*I restriction fragments are marked between the two panels. Deletion 807 converts *Sst*I-B to a novel fragment (arrow) which runs between *Sst*I-C and -D. Deletion 802 fuses *Sst*I-B and -J to create a novel band which runs between *Sst*I-B and -C. The faint bands above *Sst*I-A and just above *Sst*I-B are present in uninfected-cell cytoplasms.

and analyzed on sucrose gradients. A representative set of sucrose gradient profiles are shown in Fig. 5a to f for H2d/807-, H2d/802-, and Ad2-infected HeLa cells. Figure 6 shows characterization in CsCl gradients of the sucrose gradient peaks (data shown only for Ad2).

Two peaks were observed consistently in sucrose gradients of the nuclear fraction of cells infected with either H2d/802 or Ad2 (Fig. 5b and c). The lower peak (NV) cosedimented with ³²P-labeled marker Ad2 virions (marker not shown), and this material had the same buoyant density as marker virions (1.35 g/ml) in CsCl gradients (Fig. 6a). The upper peak (IM) had the characteristics in both sucrose gradients (Fig. 5b and c) and CsCl gradients (Fig. 6b, peak at 1.315 g/ml) of the major nuclear intermediate in virus assembly, as previously described (13). There was sometimes a minor third peak above the major intermediate peak (Fig. 5b, unlabeled arrow), which probably corresponds to another intermediate (10).

In sucrose gradients of cytoplasms from H2d/802- or Ad2-infected cells (Fig. 5e and f), the major peak (CV) cosedimented with marker virions (marker not shown); this material also cosedimented with marker virions in CsCl gradients (Fig. 6c). A small intermediate peak (IM) was also seen, often running as a shoulder on the main peak as in Fig. 5e.

In contrast, the H2d/807 nuclear and cytoplasmic profiles showed essentially no virus particles or intermediates detectable above the uninfected cell background (Fig. 5a and d). Cytoplasmic virus at a few percent the wild-type level would have been visible in these gradients. The lack of

cytoplasmic DNA in H2d/807 infections of HeLa cells is therefore due to a failure to assemble virus particles, and assembly is defective at a step before assembly of the nuclear intermediates.

Table 2 summarizes many sucrose gradient profiles, measuring cytoplasmic virus production by deletion viruses and Ad2 on three cell lines: HeLa, Vero, and W162. H2d/807 was defective for virus assembly on both HeLa and Vero cells, producing about 1 and 5% as much cytoplasmic virus as Ad2 on those cell lines, respectively. However, on W162 cells, which contain Ad5 DNA corresponding to all of E4 plus parts of E3 and L5, H2d/807 produced nearly 40% as many particles as Ad2. This is about seven times the level of particles produced on the Vero cell line, from which W162 cells were derived. This is further evidence that the virus assembly defect of H2d/807 can be complemented in *trans*, in this instance by viral DNA stably incorporated into a cell line. Furthermore, this implicates E4 as the complementing genetic region, since W162 cells are known to complement viruses defective in E4 (34), while the E3 and L5 regions present in W162 cells lack promoters and thus should not be expressed. It should be noted that in HeLa cells, H2d/807 exhibits a defect in late protein synthesis (7) which presumably contributes to the defect of the mutant in particle production. However, no striking late protein synthetic defect was observed in H2d/807-infected Vero cells, and gross underaccumulation of capsid proteins probably does not contribute to the assembly phenotype in those cells.

In contrast to H2d/807, both H2d/802 and H5d/1021 were nearly normal for particle assembly on HeLa cells, and H2d/802 was also normal on Vero and W162 cells (H5d/1021 was not tested on these lines). This reinforces the conclusion that the assembly defect of H2d/807 is due to the absence of an E4 product. This also suggests that the fiber protein is not essential for particle production, unless the small amount of fiber contributed by the helper virus contaminants in the H5d/1021 stock (about 0.1% of the wild-type level) is sufficient.

The nuclear extracts were also examined in these experiments. Quantitation of the nuclear peaks was made difficult by the relatively high base line of radioactivity and by the substantial overlap of the nuclear virion and intermediate peaks (Fig. 5b and c). Consequently, no quantitation of the profiles of the nuclear sucrose gradients is reported. Qualitatively, on HeLa cells, the nuclear profiles of H2d/802 and H5d/1021 had a smaller nuclear virion peak and a larger intermediate peak than wild type, although both mutants had nearly wild-type levels of cytoplasmic virus. This may represent some mild derangement in the assembly of virus particles in the nucleus of HeLa cells infected with H2d/802 and H5d/1021, which does not result in reduced accumulation of virus in the cytoplasm.

Accumulation of viral proteins in the nucleus. Since protein synthesis occurs in the cytoplasm, but adenovirus assembly occurs in the nucleus, a plausible explanation for the defect in virus assembly of H2d/807 is that viral proteins do not accumulate normally in the nucleus. To test this hypothesis, we examined the levels of radioactive viral proteins in the nuclear and cytoplasmic compartments of Vero cells infected with either Ad2 or H2d/807. Vero cells support approximately normal levels of late protein synthesis by H2d/807, and most late viral proteins accumulated to wild-type or nearly wild-type levels in both compartments in mutant-infected cells (Fig. 7). As expected, there was very little fiber in either compartment, and in addition polypeptide III seemed to be somewhat underrepresented in the

FIG. 5. Sucrose gradient analysis of virus particle production. HeLa cells were labeled with [^{35}S]methionine 24 to 30 h after infection with H2d/807, H2d/802, or Ad2, and nuclear (a, b, c) and cytoplasmic (d, e, f) fractions were analyzed on sucrose gradients. Gradients were fractionated from the bottom. Arrows denote peaks. NV, Nuclear virions. IM, The major Ad assembly intermediate. CV, Cytoplasmic virions.

H2d/807-infected cells. The underrepresentation was not very severe: the level of polypeptide III in H2d/807-infected Vero cells was only about two- to threefold less than that in Ad2-infected Vero cells in either compartment, as determined by comparing dilutions of the wild-type extracts with the mutant extracts on SDS-polyacrylamide gels (data not shown). This small difference in the levels of viral late proteins in the nuclei of H2d/807- and Ad2-infected Vero

cells was far less than the nearly 20-fold difference in the levels of cytoplasmic virus particles made in the two types of infections. It seems that the underaccumulation of virus particles in the cytoplasm of Vero cells by H2d/807 is not simply explained by a commensurate underaccumulation of viral late proteins in the nucleus. It is also worth noting the slight overaccumulation of the E2 72-kDa DNA-binding protein in the nucleus of mutant-infected cells.

FIG. 6. CsCl gradient analysis of nuclear and cytoplasmic virions and nuclear intermediates. Samples of the ^{35}S -labeled peaks from the Ad2 sucrose gradients (Fig. 5c and f) were centrifuged to equilibrium in CsCl along with ^{32}P -labeled Ad2 virions. Symbols: $\diamond$, ^{35}S counts per minute; $\blacksquare$, $10 \times$ ^{32}P counts per minute. Density is shown by the broken line. Arrows indicate the expected location in the gradients for virions (1.35 g/ml) or for intermediates (1.315 g/ml).

TABLE 2. Measurement of cytoplasmic virus production by deletion mutants

Virus	Cell type								
	HeLa			Vero			W162		
	CV ^a	SEM	n ^b	CV	SEM	n	CV	SEM	n
H2d/802	72	6.4	12	70	25	4	108	26	4
H2d/807	1.0	0.3	14	5.4	0.7	11	39	4.1	10
H5d/1021	99	29	2	NT ^c			NT		

^a Average level of cytoplasmic virus, expressed as percentage of wild type.^b n, Number of experiments.^c NT, Not tested.

DISCUSSION

We have shown that H2d/807 DNA accumulates in the nuclei of infected HeLa and Vero cells as well as that of wild-type Ad2 but that the mutant is defective in the accumulation of cytoplasmic DNA in both cell types. This lack of cytoplasmic DNA is evidently due to the lack of cytoplasmic virus particles in H2d/807-infected cells. The underaccumulation of cytoplasmic virus particles is due to a block in virion assembly at a step before the formation of the nuclear intermediates. We conclude that the defect in virus assembly in H2d/807 infections is due to the lack of a diffusible E4 product, because of the three following observations. (i) H2d/802 and H5d/1021, which contain an intact E4, are nondefective for particle assembly; (ii) W162 cells, which contain E4 DNA and complement defective E4 mutants, also complement the assembly defect of H2d/807; (iii) the defect in cytoplasmic DNA accumulation by H2d/807 is complemented in *trans* both by H2d/802 and by Ad2.

The accumulation of viral proteins in the nuclei of H2d/807- and Ad2-infected cells was investigated to see whether the defect of the mutant in assembly might be due to a lack of viral proteins in the nucleus. H2d/807-infected Vero cells accumulated wild-type or nearly wild-type nuclear levels of all viral proteins identified in Fig. 7, except fiber (which is deleted in the mutant) and the 72-kDa DNA-binding protein (which was overaccumulated). Thus, while some protein critical for assembly may have escaped detection, the 20-fold reduction in particle production by H2d/807 compared with that of Ad2 on Vero cells is not due to a general failure to accumulate viral proteins in the nucleus.

The molecular basis for the particle assembly defect of H2d/807 is not yet clear. The phenotype might be the result of the lack of an E4 product which participates directly in the process. Alternatively, the effect of the mutation might be an indirect consequence of a primary defect in another process in infected cells. For example, alterations in the stoichiometry of viral late proteins, such as the two- to threefold reduction in the level of polypeptide III, might have large effects on the efficiency of assembly. Abnormalities in the kinetics of accumulation of late proteins or in the nuclear level of the 72-kDa DNA-binding protein might also affect assembly. However, particularly in Vero cells in which such effects of the 807 mutation are small, it seems most likely that E4 has some role in particle assembly separate from its role in regulating gene expression.

The H2d/807 protein synthetic defect on HeLa cells is reminiscent of the defect in late protein synthesis seen in E1B mutants (2, 7, 20). It has been shown that there is an association in vivo between a product of the E4 ORF 6 region, the 25-kDa protein, and a product of the E1B region,

the 58-kDa protein (28), and so it is not surprising that mutants in these two disparate early regions should behave similarly in some respects (3, 22). It would be of interest to know whether E1B mutants are normal for particle assembly, as this might help to determine whether the assembly defect of H2d/807 is independent of the defect of the mutant in late protein synthesis.

It came as a surprise that H5d/1021 and H2d/802 were wild type for particle assembly despite the absence of the fiber gene, since previous studies with temperature-sensitive (*ts*) mutants in fiber had suggested that fiber was essential for virion assembly (8, 12, 14). One possible explanation of this discrepancy is that the small amount of contaminating fiber observed in our deletion mutant infections, less than 0.3% of the wild-type level, is sufficient to allow normal assembly. Preliminary experiments (data not shown) have demonstrated that the virus particles produced during infection of W162 cells by H2d/807 or H2d/802 contain less than 10% of the wild-type amount of fiber. Evidently, the contaminating fiber level in our deletion mutant infections is not sufficient for assembly of particles with the wild-type fiber stoichiometry. It is possible that fiber is required in substoichiometric amounts for some function necessary for assembly and that the contaminating fiber level in deletion infections is sufficient for this purpose. A plausible alternative explanation is that the *ts* fiber protein, when denatured at the nonpermissive temperature, acquires the ability to interfere with as-

FIG. 7. Accumulation of late proteins in the nucleus of H2d/807-infected cells. Vero cells were labeled with [³⁵S]methionine 24 to 30 h after infection with H2d/807 or Ad2, and nuclear and cytoplasmic fractions were analyzed by electrophoresis on an SDS-10% polyacrylamide gel followed by fluorography. The positions of the viral proteins are indicated on the right side of the figure. U, Uninfected cell proteins.

sembly. A similar explanation has been invoked to explain the discrepancy in behavior of *ts* and deletion mutants in E2 (23).

Apparently, the E4 sequences deleted in H2d/807 have multiple effects on the phenotype of the virus. On HeLa and Vero cells, the lack of this portion of E4 results in a defect in virus assembly. On HeLa cells, the lack of this region additionally results in a defect in late protein synthesis (7). H2d/807 lacks all of ORFs 6, 6/7, and 7, is missing all of the ORF 4 part of ORF 3/4, and lacks about one-third of ORF 4. The deletion of some subset of these ORFs is probably responsible for the defects exhibited by H2d/807. Higher-resolution genetic dissection of E4 is now under way, using overlapping deletions confined to E4, and this may make possible the specific assignment of functions to individual ORFs.

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