Analysis of the Adenovirus Type 5 Terminal Protein Precursor and DNA Polymerase by Linker Insertion Mutagenesis

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A series of adenovirus type 5 precursor terminal protein (pTP) and DNA polymerase (Ad pol) genes with linker insertion mutations were separately introduced into the vaccinia virus genome under the control of a late vaccinia virus promoter. The recombinant viruses were used for overexpression of the mutant genes in HeLa cells. In total, 22 different mutant pTP and 10 different Ad pol vaccinia virus recombinants were constructed, including some that expressed carboxyl-terminus-truncated forms of both proteins and one that produced the mutant H5ts149 Ad pol. To investigate the structure-function relationships of both proteins, extracts from cells infected with the recombinant viruses were tested for in vitro complementation of the initiation and elongation steps in adenovirus DNA replication. The results were in accordance with those of earlier in vivo experiments with these insertion mutants and indicate that multiple regions of both proteins are essential for adenovirus DNA replication. The carboxyl termini of both pTP and Ad pol were shown to be essential for proper functioning of these proteins during initiation of adenovirus DNA replication. Three different DNA replication-negative pTP mutations were shown to have residual activity in the initiation assay, suggesting not only that pTP is required for initiation but also that it may play a role in DNA replication after the deoxyribonucleotide step.

For replication of its linear duplex DNA genome, which initiates at the origins of DNA replication that are located at the DNA termini, adenovirus (Ad) makes use of a protein-priming mechanism. Initiation of Ad DNA replication requires, in addition to several host factors (33, 34, 38), three virus-coded proteins: a DNA-binding protein (DBP), a DNA polymerase (Ad pol), and the precursor form of the terminal protein (pTP) (for reviews, see references 23 and 53). pTP and Ad pol copurify from Ad-infected cells as a tight, noncovalent bimolecular complex (12, 29, 50). In the initiation step, formation of a covalent complex of pTP with dCMP is catalyzed by Ad pol. Elongation of the pTP-dCMP primer occurs via a strand displacement mechanism in which Ad pol has a highly processive mode of action. This enzyme can translocate through long stretches of duplex DNA and is therefore ideally suited for Ad DNA synthesis. The only other protein that is required for the elongation reaction is DBP (14, 30). This protein might facilitate efficient displacement synthesis by interacting directly with Ad pol or by changing the conformation of the DNA template.

Late in infection, the amino-terminal part of the 87-kDa pTP is removed by an Ad-encoded protease to yield the 55-kDa terminal protein (TP) (5, 8, 49). The functional significance of this cleavage has not yet been established. The TP or pTP that is attached to the parental strand is needed for efficient initiation of Ad DNA replication (8, 19, 49) and probably facilitates unwinding of the DNA in the replication origin. The pTP-Ad pol complex is thought to bind to residues 9 through 18 in this region (10, 24, 25, 39, 54).

Most of our knowledge of pTP and Ad pol originates from functional studies with purified proteins in an in vitro Ad DNA replication system (6). Although this has led to a consistent model of Ad DNA replication, many details of the structure-function relationships of these proteins remain to be solved. Progress in this field has especially been hampered by the low amounts of pTP and Ad pol that are produced in Ad-infected cells. The mRNAs for pTP and Ad pol are both synthesized from the same promoter at 75 map units (m.u.) and arise from differential splicing of the primary early region 2 transcript. They share common leaders at 75, 68, and 39 m.u. that are spliced onto the main coding exon of pTP or Ad pol (48). For Ad type 2 (Ad2), the leader at 39 m.u. was demonstrated to encode three amino acids (Met-Ala-Leu) that constitute a common N terminus for pTP and Ad pol (46). This leader appeared to be essential for production of functional pTP and Ad pol in COS cells (35, 45). Transient expression systems have also been used to identify the nuclear localization signals of pTP and Ad pol (58, 59) and to map regions in both proteins that are essential for Ad DNA replication (9, 16). Complete genes, assembled by ligation of a synthetic oligonucleotide encoding the three amino acids from the m.u. 39 leader to the splice acceptor sites of the Ad5 pTP and Ad5 pol genes, have been used for the construction of recombinant vaccinia viruses which overexpress pTP and Ad pol, respectively, after infection of eukaryotic cells (51). The recombinant proteins produced were shown to be functionally active in Ad DNA replication.

For the analysis of the function of Ad pol in Ad DNA replication, a limited number of temperature-sensitive DNA replication-negative virus mutants, like the Ad pol mutant H5ts149, were available (18, 50). Additional Ad pol and pTP virus mutants have been generated by mutagenesis of both genes in vitro and reintroduction of the mutated genes into the Ad genome (17, 41). In our studies, a 12-bp synthetic oligonucleotide was inserted into multiple restriction sites...
within the pTP and Ad pol coding regions. A large number of these mutations were classified as lethal in the sense that no viable virus could be recovered. These lethal mutations are expected to have a negative effect on essential functions of pTP and Ad pol in virus multiplication. Since pTP and Ad pol both are essential for Ad DNA replication, the majority of these mutant proteins were thought to be affected in the initiation and/or elongation step of this process. Further investigation of these lethal mutations might therefore directly contribute to our understanding of Ad DNA replication. Since the expressed wild-type (wt) pTP or wt Ad pol gene, which is located in the genome of vaccinia virus recombinants, is not directly accessible to mutagenesis, we have reinserted each mutated gene fragment separately into a recombinant vector (pATA-pTP or pATA-pol). We have constructed several recombinant vaccinia virus recombinants by insertion of mutant pTP or Ad pol genes into the thymidine kinase (tk) gene of the vaccinia virus genome by homologous in vivo recombination. The use of a strong vaccinia virus late promoter allowed efficient production of these mutant proteins and subsequent characterization by in vitro assays for initiation and elongation of Ad DNA replication.

MATERIALS AND METHODS

Enzymes. The conditions for restriction enzyme digestions and DNA ligation were those recommended by the manufacturers except when described otherwise. All enzymes used were supplied by Boehringer Mannheim or Gibco/BRL.

Plasmids. The plasmids pPX2186 and pKH2288, which contain the main open reading frame of pTP and part of the Ad pol open reading frame, respectively, have been described earlier (41). The recombination vectors pATA-pTP and pATA-pol (51) that were used for construction of recombinant vaccinia viruses contain the complete pTP and Ad pol genes, respectively, under the control of a modified promoter of the vaccinia virus 11-kDa gene (3, 20). All plasmid DNAs were multiplied in Escherichia coli HB101 and isolated by the alkaline lysis method (4).

Construction of mutant pTP and Ad pol recombination vectors. The construction of pTP and Ad pol genes with codon insertion mutations has been described earlier (41). The mutated versions of the recombination vectors pATA-pTP and pATA-pol were constructed as follows. After digestion of pATA-pol with Smal and KpnI (unique sites at AdS positions 6586 and 8534, respectively) as used in reference 52) and of pATA-pTP with PstI and Nael (unique sites at AdS positions 8404 and 10470, respectively), the large vector-containing fragments were isolated. Mutated PstI-Nael pTP and Smal-KpnI Ad pol gene fragments were isolated from plasmids pFX2186 and pKH2288, respectively, and ligated to the corresponding recombination vector fragments, thereby generating a mutated version of the respective recombination vector (Fig. 1). After transformation of E. coli HB101, the desired clones were selected. Electrophoresis and visualization of DNA fragments in agarose gels, ligations, and transformations were all performed according to standard procedures (32).

The carboxyl-terminal (C-terminal) deletion derivatives pTP-CA17, Ad pol-CA195, and Ad pol-CA266 were constructed by ligation of the XbaI linker 5'-d(CGCTAGCTCA GACTAG)-3' into HpaII-linearized pATA-pTP and pATA-pol plasmids, respectively. The added oligonucleotide is self-complementary and contains a TAG translational stop codon in all reading frames.

5'-G C G T A G T C T A G A C T A G -3'
3'-GAT C A G A T C T G A T C G C 5'

Linearization of the plasmids was performed by digestion with HpaII in the presence of ethidium bromide to limit cleavage to once per molecule, as described previously (41). After ligation, removal of excess linkers, and transformation of E. coli HB101, colonies were screened for plasmids that carried an insertion in the C-terminal region of the pTP or Ad pol gene. The plasmids pTP-C32, pTP-C79, pTP-C130, and pTP-C205 were constructed by insertion of the XbaI linker in the ClaI site of linker insertion mutants pTP-ins639, pTP-ins590, pTP-ins541, and pTP-ins467 (these mutants are named for the codon in or after which the ClaI linker has been inserted) as described before (41). The added linker sequences led to insertion of a maximum of six foreign amino acids in front of the in-frame stop codon.

In addition to the mutants listed above, two pATA-pTP mutants in which the region between the linker insertions of mutants pTP-ins235 and pTP-ins243 was deleted [pTP-D(235-243)] or duplicated [pTP-dup(235-243)] were constructed. Furthermore, a pATA-pol plasmid containing the H5ts149 mutation at AdS position 7563 (42) was constructed.

Cells and viruses. Recombinant and wt vaccinia viruses (WR strain) were grown in HeLa cells maintained in suspension culture on suspension minimal essential medium (Flow Laboratories, Inc.) containing 5% fetal calf serum at a density of 2 × 10^5 to 5 × 10^6 cells per ml. Monolayers of human tk^-143 and rabbit kidney RK13 cells were cultured in Eagle minimum essential medium (Flow Laboratories) supplemented with 10% fetal calf serum. Virus stocks were produced in RK13 cells. Cells and medium were collected at 42 h postinfection (hpi) and subjected to three cycles of freeze-thawing. After a low-speed centrifugation step, the supernatant was used as virus stock for all further experiments. Titers of wt and mutant virus stocks were measured by plaque assay on tk^-143 cells.

FIG. 1. Cloning protocol used to obtain the pTP and Ad pol recombination vectors for synthesis of mutant proteins. The mutated 2,066-bp Nael-PsrI pTP gene fragment was isolated from pPX2186 linker insertion mutants (the inserted linker, which contains a recognition site for ClaI [C] is indicated) and cloned at the corresponding location of the pATA-pTP plasmid. Mutated Ad pol recombination vectors were generated by insertion of the mutated 1,948-bp KpnI-Smal fragment obtained from pKH2288 linker insertion mutants at the corresponding location of pATA-pol, E, EcoRI; K, KpnI; N, Nael; P, PstI; S, Smal; Tk-L and Tk-R, segments of the vaccinia virus genome (boxed) containing parts of the tk gene (shaded boxes). Open arrows indicate directions of transcription.

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Construction and selection of recombinant vaccinia viruses. Transfer of the mutant pTP or Ad pol genes from the reconstructed pATA-pTP or pATA-pol recombination vector to the vaccinia virus genome was achieved by homologous recombination after calcium phosphate cotransfection as described by Hänggi et al. (20). Briefly, tk" 143 cells were infected with vaccinia virus mutant ts7 (11) and transfected with a mixture of 100 ng of wt vaccinia virus DNA isolated from purified virus as described by Mackett et al. (31), 100 ng of the appropriate recombination vector DNA, and 3 μg of sheared calf thymus DNA (CT DNA). The recombinant vaccinia viruses, in which the tk gene had been inactivated by insertion of the foreign gene, were selected for by using bromodeoxyuridine. The plaques obtained were subsequently screened by infection of RK13 cells with these plaques and then analyzed by hybridization of total DNA extracted from these cells with labeled pTP or Ad pol gene fragments (see below) as probes. Positive recombinants were selected and used for generation of high-titer viral stocks after plaque purification on tk" 143 cells.

Transfer of mutant genes to the expected location in the vaccinia virus genome was confirmed by restriction enzyme analysis of DNAs from these vaccinia virus recombinants. Total cellular DNA from recombinant vaccinia virus-infected RK13 cells was prepared as follows. The infected cells were washed twice with phosphate-buffered saline (PBS) and lysed in a buffer containing 0.5% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 10 mM EDTA. After an overnight digestion with 100 μg of proteinase K per ml at 37°C, the solution was extracted once with phenol and twice with phenol-chloroform-isomyl alcohol (25:24:1), and nucleic acid was precipitated with ethanol. Isolated DNA was redissolved in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), cleaved with the appropriate restriction endonucleases, and electrophoresed in 0.8% agarose gels. Generally, about 2 μg of DNA was used in each digestion. For Southern blot analysis, DNAs from gels were transferred to nitrocellulose (47) and hybridized with a tk-, pTP-, or Ad pol-specific DNA probe. These probes were generated by random priming (13) in the presence of [α-32P]dCTP under the conditions described by the manufacturer (Pharmacia, Uppsala, Sweden). As templates for random priming, pATA18 plasmid cut with PvuII (contains vaccinia virus tk gene fragments), the 2,186-bp PstI-XhoI pTP gene fragment (Ad5 genome between positions 5793 and 8259), and the 2,466-bp XhoI-XhoI Ad pol gene fragment (Ad5 genome between positions 5793 and 8259) were used. Results were visualized by autoradiography at ~80°C with Fuji RX film and an intensifying screen.

Analysis of mutant proteins. For labeling experiments, 3-cm plastic dishes (Nunc, Roskilde, Denmark) were seeded with 2 × 106 RK13 cells in Eagle minimal essential medium supplemented with 10% fetal calf serum and l-glutamine. After incubation overnight, the confluent cells were rinsed once with PBS containing 0.5 mM MgCl, and infected with 5 PFU of vaccinia virus. At 20 hpi, cells were starved for 1 h with methionine-free medium (Flow Laboratories; no. 16-222-49) and labeled for 2 h with 10 μCi of [35S]methionine (Amersham; SJ235; specific activity, >1,000 Ci/mmol) in 1 ml of methionine-free medium. The infected cells were washed once with PBS, harvested by being scraped into 1 ml of PBS, and collected by low-speed centrifugation. The cell pellets were disrupted by heating for 3 min at 100°C in Laemmli sample buffer, and labeled proteins were analyzed by electrophoresis in SDS–10% polyacrylamide gels (27). After electrophoresis, the gels were soaked for 1 h in 1 M sodium salicylate, dried, and exposed to Fuji RX film. Prestained protein markers were run in parallel to determine the molecular weights of the labeled proteins. The relative amounts of mutant pTP and Ad pol proteins were measured by determining the intensities of the protein bands by densitometric scanning of the fluorographs. The total amounts of protein in the extracts were measured by the method of Flores (15).

For investigation of subcellular distribution of the produced proteins, RK13 cells from 3-cm dishes were labeled at 20 hpi with [35S]methionine as described above and chased for 1 h with methionine-containing medium, after which cytosol and nuclear extract were prepared according to a published protocol (44). Proportional fractions of these extracts were applied to the same SDS–10% polyacrylamide gel. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography as described above.

Preparation of cellular and nuclear extracts. For infection with vaccinia virus, HeLa cells grown in 50 ml of suspension culture (5 × 106 cells per ml) were spun down, rinsed once with PBS–0.5 mM MgCl, and infected with 5 PFU of virus in 5 ml of PBS–0.5 mM MgCl2 per cell. After incubation for 1 h at 37°C, fresh medium was added to the cells. Extracts were prepared from vaccinia virus-infected HeLa cells at 21 hpi. The cells were washed twice with cold PBS–0.5 mM MgCl2, suspended in 0.5 ml of hypotonic buffer, swollen by incubation for 15 min on ice, and disrupted by incubation for 4 min in a Branson 1200 sonicator bath. Completion of homogenization was monitored by microscopy. All further procedures were performed at 4°C. The nuclei were spun down by centrifugation for 8 min at 800 × g. Cytosol was obtained after centrifugation of the supernatant for 15 min at 15,000 rpm in an Eppendorf centrifuge. The nuclear pellet was resuspended in 60 μl of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-succrose buffer, and nuclear proteins were extracted by addition of 35 μl of 1 M NaCl–10 mM dithiothreitol (DTT) followed by occasional vortexing for 60 min at 0°C. Nuclear extract was obtained after centrifugation for 10 min at 15,000 rpm in an Eppendorf centrifuge. The total amounts of protein in the extracts were measured by the method of Flores (15). Both cytosol and nuclear extracts were supplemented with an equal volume of 40% glycerol–20% sucrose–2 mM EDTA–2 mM DTT–0.02% Nonidet P-40 and stored at −20°C.

Immunoprecipitation of pTP, Ad pol, and pTP-Ad pol complex. Immunoprecipitation of pTP and Ad pol from extracts of infected RK13 cells was used for identification of expressed pTP and Ad pol proteins. For this purpose, antisera directed against peptides representing the C-terminal amino acids of pTP or Ad pol were used (a gift from Philip R. E. Branton). Cytosol and nuclear extracts of vaccinia virus-infected cells were prepared from 3-cm dishes as follows. At 20 hpi, cells were labeled with [35S]methionine as described above, washed twice with cold PBS, and incubated for 10 min on ice with 200 μl of hypotonic buffer (20 mM HEPES-KOH [pH 7.5], 5 mM KCl, 0.5 mM MgCl2, 0.5 mM DTT, 0.18% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) containing 1% Triton X-100. Cytosol fraction was obtained after centrifugation of the supernatant for 1 min at 15,000 rpm in an Eppendorf centrifuge. The pellet was suspended in 50 μl of HEPES-sucrose buffer (50 mM HEPES-KOH [pH 7.5], 10% sucrose) and extracted by addition of 20 μl of 1 M NaCl–10 mM DTT and incubation for 1 h on ice. Nuclear extract was obtained after centrifugation for 3 min at 15,000 rpm in an Eppendorf centrifuge.
The two fractions were combined and adjusted to 0.5 mM MgCl₂-0.5 mM phenylmethanesulfonyl fluoride (a total volume of 600 µl of extract from 10⁶ cells). From this extract mixture, 50 to 150 µl was used for immunoprecipitation with 10 to 30 µl of the appropriate specific antiserum. After incubation for 1 h on ice, antigen-antibody complexes were collected by addition of 30 µl of protein A-Sepharose beads (Pharmacia) (1:1 mixture in PBS) and incubation for 2 h at 4°C in an end-over-end mixer and were pelleted by a short centrifugation step. The Sepharose beads were washed three times with 1 ml of 10 mM Tris-HCl (pH 7.6)-150 mM NaCl-2 mM EDTA-0.2% Triton X-100 and once with 1 ml of 10 mM Tris-HCl (pH 7.6). The beads were suspended in 50 µl of Laemmli buffer and boiled for 5 min, and after centrifugation, the supernatant was analyzed by SDS-PAGE followed by fluorography.

Assays for in vitro initiation and elongation of Ad DNA replication. Cytosol and nuclear extracts from infected HeLa cells were prepared as described above and combined, giving 1.0 ml of extract from 2.5 × 10⁷ cells. This cell extract was used in the in vitro assays described below.

Initiation of Ad DNA replication was monitored as described (37) by measuring the amount of [α-32P]dCTP incorporated into the pTP-dCMP initiation complex. Reaction mixtures (50 µl) contained 100 ng of Ad5 DNA-TP complex, 10 µl of Ad pol extract, 15 µl of pTP extract, 2.5 µg of DBP, and 1 µl of nuclear extract from unfected HeLa cells. After incubation with 5 µCi of [α-32P]dCTP (Amersham; 400 Ci/mmol) for 1 h at 37°C, labeled initiation complexes were analyzed by SDS-PAGE. The formation of pTP-[α-32P]dCMP complex was detected by autoradiography after SDS-PAGE and dehydroxylation of the gel. Elongation was monitored by measuring the incorporation of label into the origin-containing terminal DNA fragments of the Ad genome essentially as described before (37). The DNA-TP complex of Ad5 virus was prepared as described before (42) and digested with XhoI. Incubation under DNA replication conditions results in almost exclusive labeling of the origin-containing XhoI fragments B and C. Reaction mixtures (15 µl) contained 0.1 µl of nuclear extract and 1 µl of cytosol (both from uninfected HeLa cells), 40 ng of XhoI-digested Ad5 DNA-TP complex, 2 µl of Ad pol extract, 2 µl of pTP extract, 1.5 µg of DBP, and 1 µl of nuclear extract from hematopoietic HeLa cells. After incubation at 37°C for 1 h, the reaction products were resolved by electrophoresis in 1% agarose gels containing 0.1% SDS. After electrophoresis, the gels were partially dried and autoradiographed at −80°C with Fuji RX film and an intensifying screen.

For both the initiation and elongation assays, the specific reaction was measured by densitometry of the autoradiograms or by excising the labeled products from the dried gel and counting the Cerenkov radiation in a scintillation counter.

DNAPolymerase assay. Cytosol from vaccinia virus-infected RK13 cells was prepared from 3-cm dishes as described above. The DNA polymerase activities of the lysates were determined as described before (26) by using activated CT DNA as template. Reaction mixtures (50 µl) contained 0.1 µg of activated CT DNA, 0.5 µCi of [α-32P]dCTP, and 2 µl of Ad pol RK13 extract. The Ad pol activity was determined by including 100 µM aphidicolin in the reaction mixture to suppress host DNA polymerase α activity. After incubation for 30 min at 30°C, the radioactivity in trichloroacetic acid-precipitable material was counted in a scintillation counter. One unit of DNA polymerase activity corresponds to 1 nmol of dCMP incorporated per 20 min at 30°C.

RESULTS

Construction of vaccinia virus recombinants. Previously, we described the linker insertion mutagenesis of the Ad5 pTP and Ad pol genes (41). To express the mutant genes in the vaccinia virus system, each mutated gene fragment was first separately transferred into a recombination vector (pATA-pTP or pATA-pol; see Materials and Methods). This was done for a set of 12 pTP and 7 Ad pol mutant genes containing linker insertions. Also, two pATA-pTP mutants in which the region between the linker insertions of mutants pTP-ins235 and pTP-ins243 was deleted or duplicated, respectively, were constructed. Further, a pATA-pol plasmid containing the H5ts149 mutation was constructed. The 587-bp NruI-ScaI fragment (Ad5 positions 7146 through 7733) of the latter plasmid was sequenced to confirm the presence of the H5ts149 mutation (42). These mutated recombination vectors were subsequently used to transfer the mutant genes into the vaccinia virus genome by employing the methods described in Materials and Methods. This resulted in a number of different vaccinia virus recombinants, each containing one of the set of 14 pTP and 8 Ad pol mutants. In a separate set of mutant genes, an XbaI linker containing translational stop codons was inserted at different places into the pATA-pTP and pATA-pol plasmids. Eight different pATA-pTP mutants and two different pATA-pol mutants, each bearing an insertion of the XbaI linker within the C-terminal region of its respective genes, were obtained. The resulting recombination vectors were used to build vaccinia virus recombinants that produce C-terminus-truncated versions of pTP and Ad pol.

Analysis of DNA isolated from RK13 cells infected with the constructed vaccinia virus recombinants demonstrated the expected integration of the pTP or Ad pol gene within the tk gene of the vaccinia virus genome. For this purpose, we used the restriction enzymes XbaI and BclI, which cut within the vaccinia virus tk locus and generate a 1,216-bp fragment which spans the recombination site. The size of this XbaI-BclI fragment, which can be detected by hybridization with a tk probe (for wt vaccinia virus DNA; Fig. 2A, lane 1), therefore identifies the inserted DNA segment. Neither of the two enzymes cleaves within the inserted wt pTP or wt Ad pol gene. Therefore, insertion of the pTP gene results in an XbaI-BclI fragment of about 3,400 bp (Fig. 2A, lanes 2 through 4), whereas an XbaI-BclI fragment of about 4,900 bp indicates integration of the Ad pol gene (lanes 5 through 7). Additional digestions of these virus DNAs showed that all recombinant vaccinia viruses contained the insertion-specific additional cleavage site (ClaI or XbaI) at the expected location of the mutant pTP or Ad pol gene. An example of such an analysis is shown in Fig. 2B for five pTP and four Ad pol insertion mutants (see legend of Fig. 2 for more details).

Analysis of mutant proteins and quantitation. Infection with the different recombinant vaccinia viruses resulted in large quantities of wt and mutant pTP and Ad pol. The relative levels of expression and the stabilities of the mutant protein products were determined by measuring the amounts of these proteins after continuous labeling for 2 to 8 h with [35S]methionine relative to the total protein contents of the isolated extracts. This was done by scanning the pTP- and Ad pol-specific bands after fluorography of SDS gels as described in Materials and Methods. Examples for wt pTP and a number of pTP mutants are shown in Fig. 3A. Cells infected with recombinant vaccinia virus containing a wt or mutant pTP gene synthesized a major component of about 80
kDa that was absent in cells infected with wt vaccinia virus. This band was positively identified as being pTP by using immunoprecipitation with antiserum against pTP (results not shown). Several of the mutant pTPs exhibit slightly slower migrations during electrophoresis in SDS gels, which are probably due to structural changes in the protein induced by the insertion mutation. The experiments described above revealed that the mutant pTPs were expressed with yields 50 to 150% that of wt pTP, depending on the construct employed. The same variation in yields applies to the wt and mutant Ad pol proteins. These 120-kDa proteins were expressed in similar relative quantities (mutant Ad pol is about 50 to 150% the amount of wt Ad pol [results not shown]), but the production of Ad pol on a molecular base was lower than with pTP, as can also be judged from the relative activities of wt pTP and wt Ad pol extracts in our in vitro Ad DNA replication system (see below). The relative and total amounts of mutant pTP and Ad pol proteins (compared with the total amounts of protein in the extracts) were consistent in different infections with the same virus stock. Five pTP and two Ad pol C-terminal deletion mutants tested were identified by coimmunoprecipitation with wt Ad pol or wt pTP, respectively (Fig. 3B shows results with some Ad pol mutants). Their production levels were also comparable to those of the wt proteins. Exceptions were the three pTP mutants with C-terminal deletions larger than 205 amino acid residues (CA327, CA366, and CA428), which were undetectable in cell extracts (results not shown). This last observation might be due to a reduced stability in HeLa cells of truncated pTPs with more-extended C-terminal deletions.

Subcellular distribution of mutant pTP and Ad pol proteins. The majority of wt pTP was shown to be present in the nucleus (Fig. 4, lanes 4 and 5). To investigate whether the nuclear localization of the pTP mutants differed from that of the wt protein, we isolated nuclear and cytoplasmic fractions of infected cells after pulse-labeling with [35S]methionine. Comparison of the distribution of wt and mutant pTP species showed no difference with respect to their relative abundances in nuclei (70%) and cytosol (30%) (data not shown). Interestingly, this was also the case for mutant pTP-ins378, which bears an insertion near the nuclear localization signal of pTP (amino acid residues 380 to 391 [58]). This is illustrated in Fig. 4. A similar analysis for wt Ad pol and the different Ad pol mutants showed that these proteins were mainly present (70%) in the cytosol (data not shown).

DNA polymerase activity. The incorporation of [α-32P]dCTP in nicked CT DNA was used as an assay for basal DNA polymerase activity. No pTP, DBP, or nuclear factors are required for this reaction. The crude cytoplasmic extracts from vaccinia virus-infected cells showed a significant increase in both total (data not shown) and aphidicolin-resistant DNA polymerase activities. The aphidicolin-resis-
The labeled bands shown). Among the tested Ad pol mutants, only the ins130 mutant and H5ts149 showed only 30 to 40% of the activity of wt Ad pol in this assay. At the permissive temperature (32°C), the H5ts149 mutant had 90% of the wt Ad pol activity (results not shown). Further experiments with this mutant will be described elsewhere (36a). Activities of the remainder of the mutants showed no significant difference from the activities of extracts from wt (strain WR) vaccinia virus-infected cells.

In vitro Ad DNA replication. As established by labeling with [35S]methionine (see above), considerable amounts of the various mutant pTP or Ad pol proteins were produced during infection with the vaccinia virus recombinants. We decided to use total cell extracts for the in vitro assays to exclude possible effects from unequal cytosol-nucleus distribution for the different mutant proteins, as described by Zhao and Padmanabhan (58). In addition, this approach enabled us to use extracts of essentially the same composition for the pTP and Ad pol mutants. Since each reaction contained the same volume of extract, the obtained values were corrected for the amount of pTP or Ad pol that was present in the extract.

Initiation. The formation of a pTP-[α-32P]dCMP initiation complex was determined by autoradiography after electrophoresis on an SDS-polyacrylamide gel (Fig. 6). In some lanes, this complex shows a slightly aberrant migration behavior, probably due to the mutations in the pTP moiety. The labeled bands were cut from the gel, and counted values were corrected for the nonspecific background of the gel. The pTP mutants ins318 and ins467 were approximately as active as wt pTP in this assay. The mutant ins639 retained 60% of wt activity, whereas mutants dup(235-243), ins378, and ins541 had a reduced but clearly detectable activity that was 5 to 10% of wt pTP activity. The other tested mutations completely abolished the priming activity of pTP. Among the tested C-terminus-truncated pTPs, only pTP-CA17 (55% of wt activity) and to a lesser extent pTP-CA32 (8% of wt activity) functioned in the initiation reaction. Among the tested Ad pol mutants, only the ins130 mutant and H5ts149 showed a considerable priming activity (respectively, 40 and 30% of wt Ad pol activity). The remainder of the Ad pol mutants showed less than 5% of the priming activity of wt Ad pol (ins379 and ins549) or were essentially inactive (ins243, ins353, ins366, ins593, Ad pol-CA195, and Ad pol-CA266).

Elongation. The DNA-TP complex of Ad5 virus was digested with XhoI and used as template for Ad DNA replication. Incubation under the appropriate conditions resulted in almost exclusive labeling of the origin-containing
**FIG. 7.** Activities of different pTP and Ad pol linker insertion mutants for Ad-specific DNA elongation in vitro. As template for in vitro Ad DNA replication, an XhoI digest of Ad5 DNA-TP complex was used. Positions of some of the XhoI fragments are indicated. Specific Ad DNA replication results in replication of the origin-containing XhoI B and C fragments. The pTP (A) or Ad pol (B) mutants used are indicated below the lanes. (C) As standards, a series of dilutions of wt pTP (2.5, 0.5, 0.1, and 0.02 µl) and Ad pol (2.5, 0.5, and 0.1 µl) extracts were used. pTP only wt pTP extract with no Ad pol added; Ad pol, only wt Ad pol extract with no pTP added. At higher concentrations, single-stranded DNAs originating from a second round of replication (ssB, ssC) are visible. WR, wt vaccinia virus extract; +, pTP-dup(235-343); Δ, pTP-d(235-243).

XhoI fragments B and C. The labeled XhoI B and C bands were cut from the gel, and their radioactivity was counted. The low level of incorporation in the internal XhoI fragments (fragments A and D are visible in Fig. 7) is due to a repair-like reaction (7). Therefore, the amount of specific DNA synthesis was corrected for nonspecific incorporation in the XhoI D band. The higher background obtained with Ad pol mutants extractive defective in Ad DNA replication may be due to a higher level of basal cellular (not Ad-specific) DNA polymerase activity in these extracts. Only reaction mixtures containing both functional pTP and Ad pol were active for specific replication of Ad DNA. Omission of pTP or Ad pol leads to less than 1% incorporation compared with the complete replication system (Fig. 7C). A serial dilution of the extracts shows that both wt pTP and wt Ad pol are produced in large amounts (a 30- to 60-fold overexpression compared with extract from Ad-infected cells). The elongation activities obtained with pTP mutants ins318 and ins467 and the wt pTP were similar, whereas mutant ins378 showed only 35% of wt pTP activity. Mutant ins639 retained 55% of wt pTP activity.

Among the tested C-terminus-truncated pTPs, only pTP-CA17 was fully active. The remainder of the mutations completely abolished the priming activity of pTP. Among the tested Ad pol mutants, only ins130 and the H5ins149 mutant showed significant activity in this assay (respectively, 60 and 45% of wt activity).

**Interaction between pTP and Ad pol.** To probe the effect of the insertion mutations on the interaction between pTP and Ad pol, the ability of mutant pTP or Ad pol proteins to compete with the wt protein during Ad DNA replication in vitro was studied. For this purpose, extracts containing wt pTP (0.5 µl) or wt Ad pol (2.5 µl) were mixed with extracts containing replication-defective mutant Ad pol or mutant pTP proteins, respectively. After 15 min at 0°C, the remaining components of the Ad DNA replication system (including extract containing wt Ad pol or wt pTP, respectively) were added, and Ad DNA replication was monitored as usual (see Materials and Methods). An example of such a competition experiment for the mutant pTP-ins590 is shown in Fig. 8. The presence of extract from wt vaccinia virus-infected cells (WR) did not inhibit Ad DNA replication, while preincubation with 5.0 µl of pTP-ins590 extract almost completely abolished DNA replication activity. The extracts containing other pTP or Ad pol mutants did not inhibit Ad DNA replication except for those of pTP-ins111 (50% inhibition with 5.0 µl) and Ad pol-ins243 (30% inhibition with 5.0 µl).

**DISCUSSION**

In this study, we describe the construction of several vaccinia virus recombinants by separate insertion of mutated pTP and Ad pol genes within the tk gene of the vaccinia virus genome behind a strong vaccinia virus promoter. This system allows expression of pTP and Ad pol in cells derived from the healthy human host and produces functional proteins (51). The mutant pTP and Ad pol proteins were tested for their abilities to stimulate Ad DNA replication in a partially reconstituted system; this was done in order to investigate the structure-function relationships of the Ad5 pTP and DNA polymerase. The results of the initiation and elongation assays are displayed graphically in Fig. 9 as percentages of the wt activity level for each tested mutant. Our findings show that the majority of the tested pTP and Ad pol mutants are defective in Ad DNA replication. The results are consistent with previously described observations with these mutants in vivo (41). All mutations that appeared to be lethal for virus multiplication in vivo were shown to affect the corresponding mutant protein in DNA replication. This fact in itself is a satisfactory explanation for the lethal phenotype of the virus mutants. The results obtained with the different mutants in vitro will be discussed below in more detail.

**pTP mutants.** The N-terminal part of pTP constitutes the precursor-specific part of this protein, which suggests a
distinct function for this region. Recently, it has been demonstrated that pTP mediates tight attachment of Ad DNA to the nuclear matrix (43). The N-terminal region is supposed to be involved in this specific Ad DNA-nuclear matrix interaction that is probably important for virus multiplication. An essential function for the pTP N-terminal region is in agreement with earlier reports that mutations in this region affect pTP functioning in vivo (17, 41) and in vitro (35, 36). Interestingly, the sequence motif YSRLRYT (codons 104 through 110), which was recently reported to be conserved among all DNA-terminal proteins studied so far (21), is also located in this region. This short patch of conserved amino acid sequence was shown to play an essential role in functioning of the phage PRD1 terminal protein during formation of the initiation complex. The results from this study directly show that mutations located near this conserved sequence (ins111, ins120, and ins126) are able to block pTP priming activity in the in vitro DNA replication system. A mechanism in which arginine residues from this region are directly involved, like that proposed for the PRD1 system (21), might also be applicable for Ad pTP.

The function of the processing of pTP late in infection still remains puzzling. It is clear that scission of pTP might be an effective means of generating Ad DNA-pTP complex destined for packaging into virions. Furthermore, processing might also be necessary for efficient release of virus particles from the nucleus. In this respect, it is interesting to note that virion DNA of a recently constructed replication-defective Ad mutant with a single point mutation at codon 243 of pTP was shown to lack a terminal protein (41a). Two other mutations, ins235 and ins243, were previously suspected to be lethal for virus production in vivo because of possible disruption of the proteolytic processing site of pTP that might be located in this region (41). Since processing of pTP takes place late in the Ad infection cycle and the Ad DNA-pTP complex isolated from the protease-deficient mutant Ad2ts1 is as active in DNA replication as the Ad DNA-TP complex (8, 49), a direct, measurable effect on DNA replication is not expected. Both pTP mutants were shown to be severely affected for initiation of Ad DNA replication, just like the two mutants in which the region between the ins235 and ins243 linker insertions was deleted or duplicated. However, on the basis of the in vitro studies described here, we cannot exclude the possibility that some of these mutants might also be affected in the processing of pTP to TP. It has been demonstrated that the secondary structures of the local amino acids at the cleavage site play an important role in the cleavage specificity of the Ad protease (55). This was established by using synthetic polypeptides as a substrate for the protease. However, this approach did not reveal which amino acid residues constitute the processing site of pTP. In this special case, the folding of a local subdomain of pTP, which cannot be mimicked by a relatively short polypeptide, may be necessary. The mutant pTPs described here may therefore be useful for in vitro processing studies which could determine the precise location of the cleavage site. The vaccinia virus expression system provides a convenient source for these mutant proteins.

The mutant ins378, which bears an insertion near the pTP nuclear localization signal (amino acid residues 380 to 391) (58), has reduced priming activity but is not defective for nuclear transport (Fig. 4). This indicates that the ins378 mutation leaves the nuclear localization signal intact and also does not perturb the local structure of the pTP, which is necessary for exposing the nuclear localization signal to the surface of the protein. The apparent lethality of this mutation in vivo can thus be attributed solely to the DNA replication defect.

As for the mutants ins318 and ins467, both were shown to retain full activity in DNA replication. This is in accordance with earlier results with these mutants in vivo (41). Besides their functioning as a positive control for the used recombinant vector (pATA-pTP), this observation further indicates that our earlier in vivo analysis of pTP mutants (41) was highly selective for fully functional mutants. The latter may also apply to the case of the ins639 mutant. This mutation was earlier reported to be lethal for production of virus in vivo (41), although the mutant pTP has significant activity in both initiation and elongation assays. Because the mutation is located in the area of the Ad genome where the pTP and Ad pol genes overlap, it also disrupts the reading frame for Ad pol and thus may affect this protein as well. This Ad pol mutation might therefore contribute to the lethality of the ins639 mutation for virus growth. The construction of a recombinant vaccinia virus that expresses an Ad pol gene with this mutation will be necessary to solve this question. Another possible explanation is that the ins639 pTP mutant is affected in a function that is not apparent in the in vitro assays used here.

The mutants ins541 and ins590, which are both defective for elongation of Ad DNA replication, bear insertions in the neighborhood of the serine 580 residue that are covalently coupled to the Ad genome during initiation. The amino acid sequence surrounding this serine residue is perfectly conserved in different Ad serotypes, suggesting that this region of the protein plays an important role during the initiation step of Ad DNA replication. The presence of pTP mutant ins590 was shown to interfere with Ad DNA replication in vitro by the wt pTP-Ad pol complex (Fig. 8), which suggests that its binding capacity for Ad pol is not affected. Interestingly, mutant ins541 showed considerable activity in the initiation assay, whereas it was completely inactive in the elongation assay. Similar results obtained with deletion mutant pTP-Δ32 and mutant pTP-Δ235-243 suggest a function for pTP in Ad DNA replication after formation of the pTP-dCMP initiation complex. This might simply be related to the dissociation of Ad pol from the initiation complex or may point at a direct involvement of pTP in chain elongation, as suggested earlier on the basis of immunological studies (40).

In addition to the insertion mutants, some C-terminal lesions were examined to investigate the function of the C terminus of pTP. The mutant with the smallest deletion (17 amino acids) was shown to be fully active in both initiation and elongation. This is rather surprising, because the very high conservation of the deleted region among different Ad serotypes suggests that it is essential for proper functioning of pTP. In vivo analysis of this pTP-CA17 mutant will be necessary to demonstrate a potential function for this region. The pTP-CA32 mutant had reduced priming activity but displayed no activity in the elongation assay. Deletion of 79 or more amino acid residues resulted in complete abolishment of both activities, as might be expected, because this directly affects the domain of pTP that contains the dCMP-serine residue. The fact that some of the linker insertion mutations led to a marked change in mobility of the pTP (Fig. 3A) suggests that the conformation of this protein is easily disturbed by changes in the primary structure. This might account for the fact that most of the pTP mutants are defective for DNA replication. This hypothesis is supported by the experiments of Schaeck et al. (43), which showed that
several mutations spread across the entire pTP gene affect function of pTP in nuclear matrix association and transcription of viral genes.

**Ad pol mutants.** Besides its essential role in replication of the linear Ad genome, Ad pol is also interesting to study because it shares many characteristics with other DNA polymerases. Among DNA polymerases from diverse eukaryotic and prokaryotic organisms, six regions of homology which are generally thought to be involved in essential functions of this enzyme have been detected (56). Five of these blocks of homology are present in the primary structure of Ad pol (indicated by I to V in Fig. 9), which suggests that this enzyme shares typical features like structural organization and mechanism of action with DNA polymerases in general. The phenotypes of the different Ad pol mutants described here will be discussed in relation to the results from comparable in vitro studies with Ad2 pol codon insertion mutants that have been expressed in COS cells (9, 10).

The mutant ins130, which bears an insertion located within the N terminus of Ad pol upstream of the first methionine codon in the long open reading frame of Ad pol, retained significant activity in Ad DNA replication and was fully functional for replication with nicked CT DNA as template. This is in accordance with earlier results from in vivo studies (41) and results of others with the comparable Ad2 pol mutant, 69A, which was shown to behave exactly like wt Ad pol in all in vitro assays (9, 10).

The initiation-defective mutant ins243 has an insertion in a putative metal-binding Zn finger motif (H-X-C-X2-C-X7-H-X-C-X2-C-X7-H [Ad-conserved Cys or His residues are underlined]) starting at codon 226 of Ad pol. There is still no direct evidence for the existence of a Zn finger in Ad pol, but the possible involvement of this potential metal-binding domain in the binding of DNA and its involvement in DNA polymerase function deserves our attention. Although the ins243 mutant is severely affected in Ad DNA replication, it retains full capacity for aphidicolin-resistant DNA synthesis with activated CT DNA as template. This proves that it is at least possible to separate this basal polymerase activity from a specific function in initiation of Ad DNA replication. A similar Ad2 pol mutant, called 7S (9), with an insertion in the same Zn finger motif, also is totally inactive in initiation and elongation of Ad DNA replication in vitro. Notably, gel retardation studies indicated that this Ad pol mutant does not bind to Ad origin DNA, although it was shown to retain full activity in poly(dA) synthesis and is capable of associating with pTP as well as nuclear factor I (9, 10). The suggestion that this Zn finger motif is an important component of the DNA-binding domain of Ad pol (10) may also apply to our ins243 mutant.

In addition to the six conserved homology blocks mentioned above, three additional conserved regions that are involved in exonuclease 3'→5' proofreading activity in the DNA polymerase of *E. coli* and φ29 have been identified (1). These regions are located between amino acid residues 275 and 589 in Ad pol (Fig. 9), with conserved region IV (amino acid residues 388 to 428) containing the middle exonuclease 3'→5' block. Comparison with the known tertiary structure of the *E. coli* DNA polymerase 1 3'→5' exonuclease domain suggests that the homologous region of Ad pol might be folded in a similar domain that comprises all three exonucleases 3'→5' blocks and conserved region IV. Five of the Ad pol mutants described here bear linker insertions that are located within (ins353, ins366, ins379, and ins549) or near (ins593) this region. All these mutants, earlier shown to be lethal for virus production in vivo (41), were severely affected in activity in Ad DNA replication, which again indicates an important function of this region. The thermosensitive DNA replication mutant H5ts149, which has a point mutation in codon 411 (41), maps within conserved region IV. Previous in vitro studies with extracts isolated from H5ts149-infected cells have already indicated that this Ad pol mutant at nonpermissive temperatures is affected in priming activity in Ad DNA replication (18, 50). Accordingly, extracts isolated from cells infected with our H5ts149

![Diagram](http://jvi.asm.org/DownloadedFrom)
vaccinia virus recombinant showed essentially the same defect. Although preliminary results indicate that some differences with respect to stability of the expressed product are to be expected (in accordance with earlier data [18]), this vaccinia virus recombinant will allow us to produce sufficient H5s149 Ad pol for a more-extensive analysis of the interesting region IV mutant. Two Ad2 pol mutants, 20S and 9A, earlier described by Chen and Horwitz (9), are comparable (bear an insertion at the same position) to our mutants ins549 and ins366, respectively. The Ad2 pol insertion mutant 20S is also defective in Ad DNA replication. However, Ad2 pol mutant 9A, which is comparable to our mutant ins366, was shown to be only slightly defective. We can ascribe this discrepancy in results only to the different nature of the inserted sequence (20S, Tyr→Ser change and insertion of the sequence Arg-Ile-Arg-Asp; ins366, insertion of the sequence Pro-Ser-Met-Gly).

None of the Ad pol mutants described here or elsewhere (9) were defective at the stage of elongation alone. This indicates that the affected mechanism for formation of the pTP-dCMP initiation complex may be closely related to the mechanism utilized for synthesis of nucleotide-DNA primer bonds during elongation. Because both activities are associated with the same domains of the Ad pol molecule, mutations in Ad pol might directly influence pTP deoxyribonuclease activity by interference with the necessary polymerase activity. This view is supported by the studies of Bernard et al. (2) and Joung et al. (22) which showed that the most-conserved sequence of DNA polymerases in general, conserved region I, is essential for protein-primed initiation of DNA replication by the DNA polymerases of φX and Ad. Another explanation for the high frequency of Ad pol mutants that are defective at the initiation level might be the obvious complexity of the involved protein-protein contacts and interactions with substrate and DNA template that are necessary for efficient initiation of Ad DNA replication.

Interaction between pTP and Ad pol. A tight complex between pTP and Ad pol forms immediately when extracts obtained from cells infected separately with pTP- or Ad pol-producing recombinant vaccinia viruses are mixed. The protein-protein interaction between pTP and Ad pol is likely to be essential for proper functioning of the complex in initiation and/or elongation of Ad DNA replication. This was suggested by coimmunoprecipitation studies with Ad2 pol mutants (10). However, until now, no distinct sequence motifs that may be involved in the association between both proteins, like regularly spaced leucine residues, have been identified in the primary structures of pTP and Ad pol (28). In our hands, coimmunoprecipitation of mutant proteins as part of the pTP-Ad pol complex did not reveal quantitative binding differences, not even for the C-terminus-truncated pTP and Ad pol mutants. This may indicate that the tight interaction between both proteins consists of multiple contact sites, as was suggested for TP and DNA polymerase from bacteriophage φ29 (57). Perturbation of, e.g., one single contact site may not suffice to suppress binding activity. The discrepancy between our results and those of Chen et al. (10) can be explained by the much fewer stringent conditions that were used in our studies. This stresses the fact that the conditions that have to be used for a useful (discerning) coimmunoprecipitation assay do not represent the in vivo situation and that the results from such studies must therefore be interpreted with extreme care. Our competition experiments, however, enabled us to compare directly the binding capacities of DNA replication-defective mutant pTP and Ad pol proteins (within the pTP-Ad pol complex) and their wt equivalents under Ad DNA replication conditions. The results suggest that complex formation for the majority of these pTP and Ad pol mutants is relatively weak, so that they are easily displaced by their wt equivalent. This defect in pTP-Ad pol interaction might explain the inability of the mutants to support Ad DNA replication. Only a few mutants (pTP-ins111, pTP-ins590, and Ad pol-ins243) were shown to interfere with Ad DNA replication in vitro by wt pTP-Ad pol complex. Thus, these mutants are the best candidates in which to look for mutations that affect specific functions of pTP and Ad pol in Ad DNA replication other than the correct interaction between them.

Together with earlier experiments that have been performed by others with mutated pTP and Ad pol constructs that are transiently expressed in COS cells (9, 10, 16, 36, 58), this study demonstrates the effectiveness of the in vitro approach in probing the mechanism of Ad DNA replication. The results obtained again emphasize the fact that the replication of Ad DNA requires a complicated set of interactions between the proteins that are involved in initiation and elongation of this process. This is most clearly shown by the pTP and Ad pol proteins: only small alterations at specific sites of these proteins are allowed. Most of the mutations tested inactivate one or more of the functions that are essential for proper functioning of these proteins. Up to now, it has proved to be very difficult to obtain pTP or Ad pol mutants that are affected exclusively in a single function. The results obtained by others (9, 10, 16, 36, 43) point in the same direction. Therefore, it is not yet possible to ascribe separate domains of pTP and Ad pol to particular functions in the DNA replication.

Although the construction of the vaccinia virus recombinants is rather elaborate, this approach has the advantage that interesting mutants can be expressed in high quantities for extensive biochemical, biophysical, and structural studies. Since all other proteins that are necessary for Ad DNA replication are now available in purified form, it should now be possible with the aid of the set of mutant proteins described here to investigate in detail the mutual interactions between pTP and Ad pol and the other replication proteins and to characterize their precise functions and mechanisms of action in adenovirus DNA replication.

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