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Genetics

Codon insertion mutants of the adenovirus terminal protein

(cis dominance/negative complementation/temperature sensitivity/DNA replication proteins)

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ABSTRACT **A series of codon insertion mutants was isolated following restriction site-directed linker insertion mutagenesis of the open reading frame for the type 5 adenovirus terminal protein precursor. The conditionally lethal mutant H5*sub*100 bears an insertion mutation upstream of the first AUG in the reading frame, fails to replicate its DNA under nonpermissive conditions, and was assigned to the terminal protein complementation group. These data establish that terminal protein is an essential polypeptide required for DNA replication *in vivo* and indicate that the NH_2-terminal region of the precursor is encoded in an upstream mRNA leader. The extended eclipse period of the viral replication cycle in H5*in*179-infected cells is probably a consequence of delayed onset of DNA replication. Analysis of DNA replication in coinfections with wild-type virus shows that the *in*179 mutation has cis and trans effects. The trans-dominant, negative-complementing *in*179 terminal protein precursor inhibits wild-type DNA replication in a dose-dependent manner. Replication of parental *in*179 templates is not stimulated by an excess of coinfecting wild-type virus, indicating that the mutant terminal protein covalently bound to the *in*179 template in some way interferes with the replication of that template. The implications of these results for the structure and function of the terminal protein are discussed.**

The adenovirus linear duplex DNA genome is covalently linked at each end to a 55-kDa protein (1), referred to as the terminal protein (TP). An 80-kDa precursor of TP (pTP) is covalently bound to replicating DNA (2, 3) and is cleaved to the 55-kDa COOH-terminal fragment (hereafter referred to as the COOH-terminal domain of pTP) during virion maturation (2–4). A serine residue within the COOH-terminal domain is the site of covalent linkage of TP and pTP to the 5′ dCMP residue of each DNA strand (5). The functional significance of the maturation of pTP to TP has not been determined. An open reading frame (ORF), located between viral genome coordinates 23.5 and 29, encodes most or all of pTP (5–7). The pTP ORF sequences are expressed early after infection in mRNA of the E2b transcription unit, which produces several species of mRNAs containing upstream leaders that are spliced to the main RNA bodies (3). It has been suggested that the mRNA leader at coordinate 39 may encode the NH_2 terminus of pTP based on the observation that pTP ORF sequences upstream of the first AUG are highly conserved among different adenovirus serotypes (5, 32).

Evidence from the study of viral DNA replication reconstituted *in vitro* with highly purified viral and host cell factors (reviewed in ref. 8) supports the model that a pTP-dCMP covalent complex serves as primer for DNA replication (1). pTP and the adenovirus 140-kDa DNA polymerase copurify in a tightly bound complex, and both proteins are absolutely required for pTP-dCMP joining (9–11). Heterologous single-stranded templates can direct the joining reaction with residual efficiency (12, 13), and the efficiency of adenovirus duplex templates with covalently bound TPs is reduced by a factor of 25 by treatment with pronase (14, 15). The nonessential but stimulatory role of the template-bound TP in this reaction and the residual activity directed by single-stranded templates have led to the hypothesis that the template-bound TP facilitates binding of the pTP-polymerase complex to a single-stranded region of the origin (12–15).

To investigate structural aspects of the pTP polypeptide and to initiate a genetic analysis of the roles of the pTP and the template-bound TP in viral DNA replication, a series of adenovirus type 5 (Ad5) recombinants with codon insertion mutations in the pTP gene was constructed. The results establish that pTP is an essential polypeptide required for DNA replication *in vivo* and indicate that the mRNA leader at coordinate 39 encodes some or all of the NH_2-terminal region of pTP. Complementation analysis indicates that the mutant pTP of H5*in*179 readily binds to and inactivates at least one essential trans-acting replication factor and also indicates that templates covalently bound to the mutant terminal proteins are inefficient substrates for DNA synthesis.

MATERIALS AND METHODS

Virological Methods. Human KB and 293 cells (16) were grown in monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. An Ad5/Ad2$^+$ ND1 recombinant ($I_0$115.1) was isolated and supplied to us by Munz and Young (17). The left third of its genome derives from Ad5 and the remainder derives from Ad2$^+$ND1, as shown by restriction site polymorphism (17). Virus A6 was constructed by Volkert and Young (18) by ligation of *Eco*RI-digested DNA from *dl*309 (19) and strain 301 (20); it contains *Xba* I restriction sites only at coordinates 3.7 and 29. DNA-TP complexes were purified from virions of A6 as described (18) and were cleaved with the restriction endonucleases *Xba* I and *Cla* I prior to use in transfection assays. 293 cells were transfected using the procedure developed by Graham and van der Eb (21).

Mutagenesis. Supercoiled pPF97 DNA (Fig. 1, line b) was partially digested with either *Hae* III, *Hin*cII, or *Rsa* I, and the products were resolved by electrophoresis in low-melting agarose gels. Full-length linear molecules were extracted from the gel, ligated to *Sma* I (CCCGGG) or *Bam*HI (CGGATCCG) DNA linkers (New England Biolabs), and introduced into competent cells of *Escherichia coli* strain HB101 (22). Large populations of transformants were selected in liquid medium containing 150 μg of penicillin G per ml, grown to stationary phase, and processed to extract plasmid DNA. DNA preparations were digested to completion with the

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Abbreviations: Ad5, type 5 adenovirus; DNA-TP complex, adenovirus DNA covalently linked at both 5′ termini to the 55-kDa terminal protein; TP, 55-kDa terminal protein; pTP, 80-kDa precursor of TP; ORF, open reading frame; URF, unassigned reading frame; wt, wild-type; moi, multiplicity of infection.

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restriction enzyme corresponding to the linker used as mutagen (*Sma* I or *Bam*HI), and the products were resolved by electrophoresis in low-melting agarose gels. Mutagenized DNA migrated as full-length linear molecules, whereas the unmutagenized background of pPF97 DNA (which is resistant to cleavage by *Sma* I or *Bam*HI) migrated as supercoiled and nicked-circular species. Linear DNA was extracted from the gel, recircularized with T4 ligase, and introduced into competent cells of *E. coli* HB101. Transformants were selected on solid medium containing 150 μg of penicillin G per ml, and plasmid DNA from isolated colonies was analyzed to map the linker insertions. DNAs with *Bam*HI insertions were cleaved with *Bam*HI, treated with the Klenow fragment of DNA polymerase I, and either recircularized directly to generate 12-mer insertions (CGGATCGATCCG), which contain a recognition site for *Cla* I (underlined), or ligated to *Sma* I linkers to generate 18-mer insertions (CGGATCCCCGGGGATCCG) with recognition sites for *Bam*HI and *Sma* I. The 36-mer insertion of H5*in*183, CGGATCCCCGAAGCTCCCGGGAGCTTCGGGGATCCG, was constructed at the *Sma* I site of an 18-mer insertion by ligation of an 8-mer *Hin*dIII linker (Boehringer Mannheim) and subsequent digestion with *Hin*dIII, treatment with the Klenow fragment of DNA polymerase I, and ligation to a 6-mer *Sma* I linker. To construct H5*sub*101, the *Cla* I B fragment of pPF344 was ligated to the *Cla* I A fragment of pPF343 (see Table 1), resulting in the deletion of sequences between the adjacent insertion mutations and a net loss of 24 nucleotides. To construct the single codon insertion of H5*sub*100, pPF97 DNA was linearized by partial digestion with *Bgl* I, the cohesive ends were degraded using the 3′ to 5′ exonucleolytic activity of the Klenow fragment of DNA polymerase I in the presence of all four dNTPs, and the blunt-ended molecules were ligated to a *Sma* I (CCCGGG) linker, resulting in a net gain of 3 nucleotides.

Nomenclature. Nucleotide positions of insertions listed in Table 1 correspond to the position of the 5′ nucleotide of the linker in the Ad5 DNA sequence as compiled by Sussenbach (23). Each Ad5 genome coordinate (map unit) is equivalent to 365 nucleotides.

RESULTS

Construction of Terminal Protein Mutants. An Ad5 DNA fragment containing the ORF for pTP was subjected to linker-insertion mutagenesis (Fig. 1, line b) and transferred to pPF337, which contains a cloned fragment of viral DNA extending from coordinates 0 to 42 with restriction site markers in early region 1 (E1) distinguishing it from wild-type (wt) viral DNA. The mutagenized pPF337 derivatives and *Cla* I/*Xba* I-digested DNA-TP complexes from viral strain A6 were transfected into 293 cells (Fig. 1, lines c and d). Several plaques from each dish were picked and analyzed for the presence of pTP mutations and the plasmid-linked markers in E1. The E1 markers were recovered in every plaque tested, indicating that recombination between plasmid DNA and an A6 DNA fragment was necessary to generate plaques in this system. In those transfected cultures in which viable mutants were recovered, the novel restriction sites in the pTP gene were present in 50% or more of the plaques that were analyzed. Conversely, in about 50% of transfected cultures, pTP mutations were not present in any of the plaques tested, strongly suggesting that these mutations had lethal consequences for viral replication. Transfections of DNA bearing apparent lethal mutations gave rise to significantly fewer plaques than wt DNA, suggesting that additional events—for example, marker rescue by the A6 *Xba* I B fragment (coordinates 3.7–29) that was also present in the cotransfection mixture (Fig. 1, line d)—were required to generate these plaques. The results of the mutagenesis experiments are summarized in Fig. 1 and Table 1.

Distribution of Viable Mutants. The pTP ORF overlaps with four other sequence elements of potential importance, three unassigned reading frames (6, 7), and the third segment of the tripartite leader found on most late viral mRNAs (25–27). The location of viable and apparent lethal mutations relative to

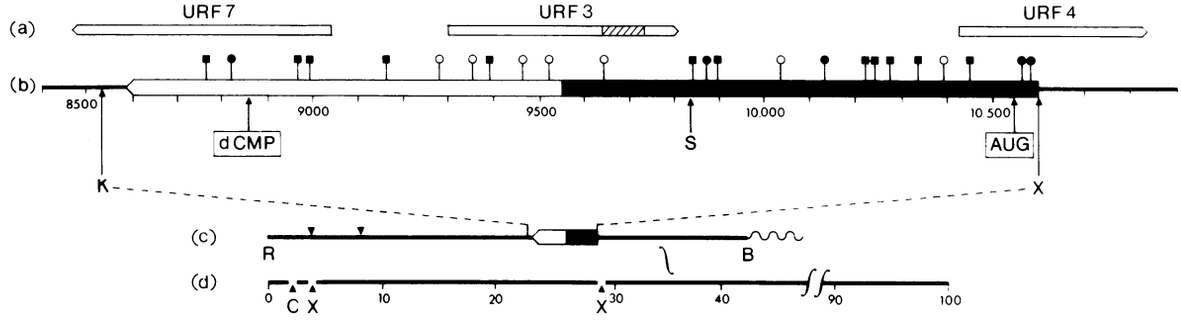

FIG. 1. Construction and location of insertion mutations in the Ad5 pTP gene and their effect on viral replication. The viral DNA insert of pBR322 derivative pPF97 extends from a *Kpn* I site (K) at coordinate 23.5 to an *Xba* I site (X) at coordinate 29 (line b) and contains the entire ORF for the Ad5 pTP. Insertion mutations that conserved the pTP translational reading frame were constructed in pPF97 DNA and transferred to the pBR322 derivative pPF337 (line c), which contains the left 42% (*Bam*HI fragment) of the genome of viral recombinant $I_0$115.1 (17) that was cloned by ligation to an *Eco*RI linker (R) at coordinate 0. To provide unselected genetic markers linked to the pTP insertion mutations, the *Xba* I and *Hin*dIII restriction sites normally present in Ad5 early transcription region E1 were destroyed in pPF337 by filling in their cohesive ends by DNA synthesis *in vitro* using the Klenow fragment of DNA polymerase I (denoted by the triangles above line c). DNA-TP complex from virus strain A6 (18) was cleaved with *Cla* I (C) and *Xba* I (X) as shown (line d) and mixed with a 10-fold molar excess of mutagenized pPF337 DNA that had been linearized with *Eco*RI (line c: ∿∿ = pBR322 DNA), and the mixture was used to cotransfect 293 cells. Duplicate transfected monolayers were overlaid with plaque assay medium and incubated at 32°C or 37°C. Viral DNA derived from isolated plaques was analyzed to establish whether terminal protein mutations were propagated in viral recombinants and to monitor the incorporation of the plasmid-linked markers in early transcription region E1. The corresponding mutagenized pPF337 derivative (Table 1) was sequenced by the Maxam and Gilbert technique (24) to confirm the position and structure of each viable mutation and several lethal mutations. A summary of the positions of viable and apparently lethal mutations and their relationship to known structural elements of pTP and to overlapping DNA sequence elements [unassigned reading frames (URFs)] is shown (lines a and b). Filled squares (■) represent positions of apparently lethal insertions; open circles (○) represent silent mutations; and closed circles (●) represent mutations that result in defective viral replication. See Table 1 for exact positions of the mutations and the sequences inserted. The predicted position of the protease cleavage site is indicated by the transition from the open to filled arrow (line b). Also shown in line b are the positions of the site of covalent linkage of pTP to dCMP (dCMP), the first AUG in the pTP ORF (AUG), and the *Sal* I site used to reconstruct *in*179 (S). The direction of transcription of the various elements is indicated by the arrowheads that point 5′ to 3′. The hatched box within the URF3 element represents the position of the third segment of the tripartite leader of late mRNAs.

Table 1. Nucleotide positions and structures of mutations constructed within the pTP ORF

Nucleotide position	Mutation*	Plasmid	Virus	Phenotype†
10,583 *Hin*cII	+8	pPF436	—	
	+12	pPF437	H5*in*185	rd
10,564 *Bgl* I	+3	pPF423	H5*sub*100	ts
	+5	pPF422	—	
10,448 *Rsa* I	+6	pPF390	—	
10,394 *Hae* III	+12	pPF347	H5*in*178	s
	+18	pPF388	H5*in*181	s
10,334 *Rsa* I	+6	pPF391	—	
10,274 *Rsa* I	+6	pPF392	—	
10,238 *Hin*cII	+12	pPF438	—	
10,223 *Hae* III	+12	pPF234	—	
10,133 *Hae* III	+6	pPF340	H5*in*191	rd
	+12	pPF233	H5*in*179	rd
10,035 *Hae* III	+12	pPF232	H5*in*172	s
	+18	pPF297	H5*in*175	s
9,896 *Hae* III	+12	pPF346	—	
9,872 *Hae* III	+12	pPF345	H5*in*186	rd
9,843 *Hin*cII	+12	pPF439	—	
9,646 *Rsa* I	+6	pPF393	H5*in*180	s
9,523 *Hae* III	+12	pPF231	H5*in*171	s
	+18	pPF296	H5*in*174	s
	+36	pPF389	H5*in*183	s
9,464 *Hin*cII	+12	pPF440	H5*in*187	s
9,389 *Hae* III	+12	pPF344	—	
‡	−24	pPF397	H5*sub*101	s
9,353 *Hae* III	+6	pPF339	H5*in*176	s
	+12	pPF343	H5*in*177	s
	+18	pPF387	H5*in*188	s
9,282 *Hin*cII	+12	pPF441	H5*in*189	s
§ *Hae* III	+12	pPF230	—	
9,164 *Rsa* I	+6	pPF394	—	
8,995 *Hae* III	+12	pPF348	—	
8,969 *Hae* III	+12	pPF229	—	
8,822 *Hin*cII	+12	pPF442	H5*in*190	rd
8,767 *Hae* III	+12	pPF342	—	

*The insertion or deletion size is given in nucleotides. For the sequence of the various mutations, see *Materials and Methods*.
†Designations are replication defective (rd), temperature-sensitive (ts), and silent (s).
‡The DNA sequence between two adjacent *Hae* III sites (nucleotides 9353–9388) was removed and substituted by a 12-base-pair *Cla* I insertion.
§The insertion has not been sequenced and is at either position 9209 or 9225 (*Hae* III sites).

these elements is shown in Fig. 1. Only one of the four mutations that affect pTP and the potential product of URF7 was recovered in a recombinant virus (H5*in*190), and this mutant replicates poorly, yielding <10 plaque-forming units per cell. All of these mutations are clustered near the site of covalent linkage of pTP to viral DNA (5), a region not likely to tolerate structural alterations readily, although inactivation of a putative URF7-encoded polypeptide cannot be excluded as a possible explanation for the sensitivity of this region to mutation.

In contrast, the region of the pTP ORF overlapping URF3 encodes a segment of the pTP polypeptide that is remarkably tolerant to extreme structural modifications. Mutations at four of the five sites tested here were recovered in recombinant viruses. In addition to the standard 2- and 4-codon insertions, insertions of 6 and 12 codons and a substitution mutation leading to a net loss of 8 codons were all recovered in viral recombinants (Table 1). None of these mutations significantly affected viral replication. The presence of the proteolytic processing site and the tolerance of the polypeptide chain to shortening and lengthening imply that this region is in an extended conformation, possibly separating two functional domains in pTP, as suggested (5). It is unlikely that the 11.5-kDa polypeptide tentatively assigned to URF3 (28) is essential for viral replication since silent mutations of potentially disruptive character were distributed throughout the URF3 sequence. Replication of H5*in*180, which bears a 2-codon insertion within the sequence encoding the third segment of the tripartite leader found on most late mRNAs, is indistinguishable from wt, suggesting that the mutation has no deleterious effect on the production or utilization of late mRNAs.

Origin of the NH_2 Terminus of pTP. The region of the pTP ORF overlapping URF4 encodes the first AUG in the pTP reading frame (5–7). To determine if this AUG is the initiation signal for translation of the pTP polypeptide, ORF sequences upstream of the AUG were mutagenized. Codon insertion mutants were recovered at two sites in this region, H5*sub*100 and H5*in*185 (Table 1 and Fig. 1), and both are replication defective. *sub*100 is temperature sensitive, failing to produce infectious particles (not shown) and viral DNA at 39.5°C (Fig. 2). To determine the complementation group of this mutant, viral DNA replication was examined at 39.5°C in single or mixed infections of *sub*100, *in*170 (the phenotypically wt parent constructed from pPF337), and H5*in*186, a mutant bearing an insertion in a region of the pTP gene that does not overlap with other sequence elements (Table 1 and Fig. 1). Viral DNA was extracted at the indicated times, digested with restriction enzymes that differentially cleave the mutant and wt DNAs, and analyzed by Southern blotting (Fig. 2). Both mutants were complemented by *in*170 (lanes 1 and 7), but in coinfections of *sub*100 and *in*186 (lane 4) neither template replicated significantly above the single infection levels (lanes 2, 3, 5, and 6). This test assigns *sub*100 to the pTP complementation group, and establishes that pTP is an essential viral polypeptide required for DNA replication *in vivo*. The position of the *sub*100 mutation indicates that ORF sequences upstream of the AUG are translated; therefore, the NH_2-terminal region of pTP must be encoded in an upstream

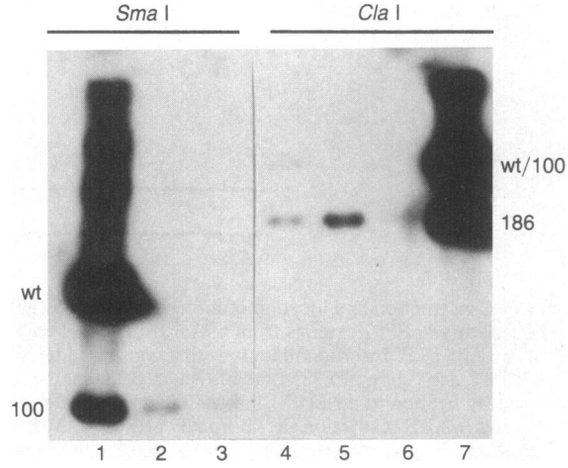

FIG. 2. Complementation analysis of *sub*100 and *in*186. Dishes of 293 cell monolayers were infected singly or with combinations of *sub*100, *in*186, and *in*170 (wt) at multiplicity of infection (moi) = 10 and incubated at 39.5°C. Viral DNA was harvested at 1 hr (lanes 2 and 5) or 16 hr (all other lanes) after infection as described (29), and equal volumes of the Hirt supernatant DNA were cleaved with *Sma* I (lanes 1–3) or *Cla* I (lanes 4–7). Restriction fragments were separated by electrophoresis in a 0.6% agarose gel, transferred to a nitrocellulose filter, hybridized to nick-translated Ad5 *Sal* I D fragment (coordinates 26–27), and autoradiographed. Lane 1, *in*170/*sub*100; lanes 2 and 3, *sub*100; lane 4, *sub*100/*in*186; lanes 5 and 6, *in*186; lane 7, *in*170/*in*186.

mRNA exon, as suggested (5). Although the effect of the *sub*100 mutation on URF4 cannot be determined from this complementation test, earlier analysis showed that the deletion mutation of H5*dl*309 (19), which alters the URF4 reading frame after the first 52 codons (30), has no detectable effect on viral replication.

The remainder of the viable mutations map in regions of the pTP ORF that do not overlap with other sequence elements. Two of the five mutants in these regions are defective, *in*186 (see Fig. 2) and H5*in*179. Characteristics of *in*179 replication are described below.

Reconstruction of H5*in*179. To exclude the possibility that amino acid substitutions in the COOH-terminal domain of pTP arising from spontaneous mutations might contribute to the *in*179 phenotype, a pPF233 (Table 1) DNA fragment extending from coordinates 0 to 27 (*Eco*RI–*Sal* I, Fig. 1, lines b and c) was excised and replaced with the corresponding wt DNA fragment. The resulting plasmid (pPF425) no longer contained the restriction site markers in E1. The phenotypes of the reconstructed mutant (derived from pPF425) and the original mutant (derived from pPF233) were indistinguishable in 293 cells and similar to the phenotype of H5*in*191 (data not shown), a 2-codon insertion mutant constructed at the same *Hae* III restriction site (Table 1). The reconstructed mutant was used in the following studies.

Viral Replication. Replication cycles of *in*179 and strain A6 (phenotypically wt control for *in*179) were compared in KB cells. An extended eclipse period and a reduced final yield of infectious *in*179 particles relative to A6 were observed at all temperatures tested (Fig. 3, only 32°C data are shown). Further characterization of *in*179 was conducted at 32°C since the duration of the lag was longer in absolute time at this temperature.

DNA Replication. The synthesis of *in*179 DNA during infection at 32°C was monitored qualitatively by Southern blot analysis (Fig. 4). The onset of A6 DNA replication occurred about 12 hr after infection (compare lanes 1 and 6), and by 15 hr the copy number had increased significantly (lane 10). In contrast, the *in*179 genome had replicated only slightly at 15 hr (compare lanes 2 and 7) but had replicated extensively by 20 hr [lane 11; less Hirt extract (by a factor of 50) was loaded than in lanes 2 and 7]. Therefore the lag in viral

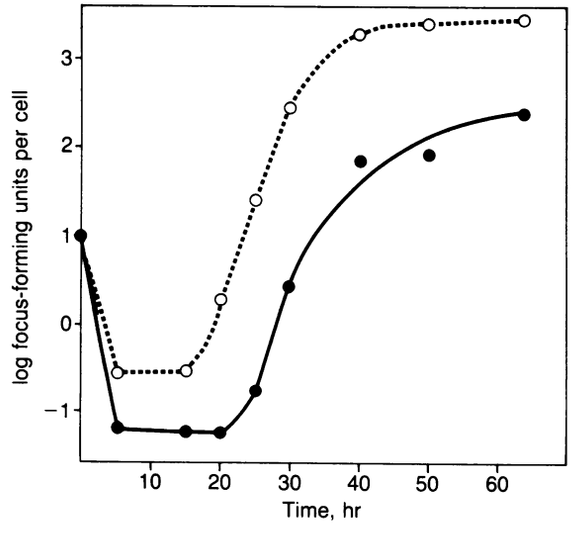

FIG. 3. Replication cycles of *in*179 and A6 at 32°C. Cultures of KB cell monolayers were infected at moi = 10 with *in*179 (●—●) or A6 (○- - -○) and frozen at the times indicated. Virus was released by multiple cycles of freezing and thawing and titrated by indirect immunofluorescence on KB cell monolayers. The number of fluorescent focus-forming units produced per cell was calculated and plotted against time as shown.

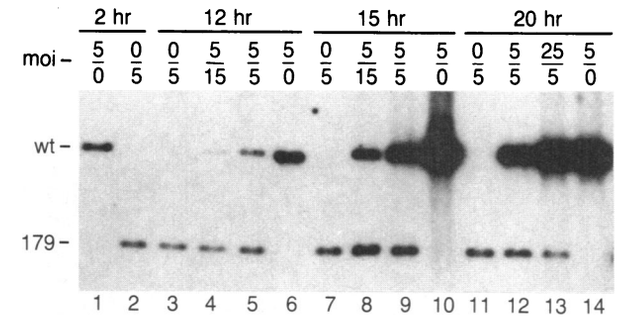

FIG. 4. Complementation of H5*in*179 by A6 at 32°C. Monolayer cultures of KB cells were singly or doubly infected with *in*179 and/or A6 at the indicated multiplicities (moi, expressed as y/x, where y = A6 moi and x = *in*170 moi), and Hirt supernatant DNA was prepared at 2, 12, 15, and 20 hr after infection at 32°C as described (29). Equal volumes of Hirt supernatant DNA were cleaved with *Cla* I, electrophoresed in a 0.6% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized to nick-translated Ad5 *Sph* I F fragment (coordinates 10–15) and autoradiographed. Lanes 1–10 each contain 1/4 of the total yield of Hirt DNA, and lanes 11–14 each contain 1/200 of the total yield of Hirt DNA.

production (Fig. 3) appears to be linked to late onset of *in*179 DNA replication.

Complementation Analysis. The *in*179 DNA replication phenotype was examined in coinfections with A6 (Fig. 4). In coinfections initiated with equal multiplicities of virus (lanes 5, 9, and 12), A6 DNA accumulated to ≈50% of the single infection level (lanes 6, 10, and 14), and *in*179 DNA did not significantly increase in copy number over its single infection level (lanes 3, 7, and 11). In coinfections initiated with a 3-fold excess of *in*179 virus (lanes 4 and 8), a greater reduction in the accumulation of A6 DNA was observed. No reduction in the copy number of A6 DNA was observed in coinfections initiated with 3-fold (not shown) or 5-fold (lane 13) excess A6 virus, and the level of *in*179 DNA was again similar to its single infection level. Therefore, the *in*179 mutation is trans-dominant, demonstrated by the dose-dependent inhibition of A6 DNA replication, and cis-dominant, demonstrated by the inability of A6 to stimulate the replication of *in*179 templates.

Structure of the *in*179 Virion TP. The cis-dominant phenotype of *in*179 was evident from the onset of A6 DNA replication, suggesting that the *in*179 viral particles initiating these infections contained defective DNA templates resulting from the *in*179 mutation. Since the site of this mutation is within the NH_2-terminal domain of pTP, failure to process pTP to the 55-kDa TP species during maturation of virions would allow the intact mutated domain to exert the observed cis-dominant effect on parental templates. To determine if *in*179 virions contained a processed 55-kDa TP, DNA-protein complexes were extracted from purified virions of *in*179, A6, and H2*ts*1, an Ad2 mutant that fails to cleave pTP and several other capsid precursors under nonpermissive conditions (2–4). Purified DNA-protein complexes were iodinated by the chloramine-T procedure, and the covalently associated proteins were removed from the DNA by treatment with DNase I or piperidine and analyzed by polyacrylamide gel electrophoresis (Fig. 5). This analysis indicates that apparently normal cleavage of the *in*179 pTP occurred, as demonstrated by the comigrating polypeptides released from A6 and *in*179 DNA.

DISCUSSION

The reconstitution of adenovirus DNA replication with highly purified pTP and other viral and cellular components has led to the conclusions that pTP (perhaps in a complex with the 140-kDa DNA polymerase) is absolutely required for

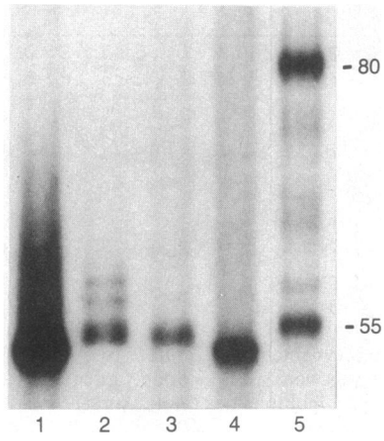

FIG. 5. Proteolytic processing of the H5*in*179 virion TP. DNA-terminal protein complexes from virions of *in*179, A6, and H2*ts*1 grown at 39.5°C were purified as described (2) and labeled *in vitro* with ^{125}I using a chloramine-T procedure (3). Aliquots of labeled material were treated with piperidine (lanes 1 and 4) or DNase I (lanes 2, 3, and 5) and were electrophoresed in a 10% polyacrylamide gel containing $NaDodSO_4$. Shown are the proteins derived from *in*179 (lanes 1 and 2), A6 (lanes 3 and 4), and *ts*1 (lane 5) DNA-TP complexes. The gel was dried and autoradiographed. The molecular mass estimated from standards is shown in kDa.

the initiation of DNA synthesis *in vitro* (14, 15) and that the template-bound TP is nonessential but in some way enhances the efficiency of the template (12, 13). These conclusions are largely supported by the characterization of the pTP mutants described in this report. The conditionally lethal mutant, *sub*100, failed to replicate its DNA at 39.5°C and was assigned to the pTP complementation group, establishing that pTP is absolutely required for DNA replication *in vivo*. This result also established that some or all of the NH_2-terminal region of pTP is encoded in the mRNA leader at coordinate 39, since the *sub*100 insertion maps 5′ of the first AUG in the pTP ORF. In contrast to the recessive mutations of *sub*100 and *in*186, the *in*179 mutation appeared to have dominant effects on DNA replication. The trans-dominant, negative-complementing phenotype of *in*179 suggests that the mutant pTP readily associates with and inactivates an essential replication factor. A likely candidate for this association would be the adenovirus 140-kDa DNA polymerase that copurifies with pTP in a tightly bound complex (9). The resulting inactivity might arise from defective origin binding of the pTP-polymerase complex or the inability to efficiently form pTP-dCMP complexes. The possibility that pTP has an origin-binding activity has been reported (31).

The cis-dominant phenotype of *in*179 indicated that its templates covalently bound to the mutant TP could not support DNA replication efficiently. This may be analogous to the decreased efficiency of templates that are devoid of TP in supporting the initiation reaction *in vitro* (14, 15). The fact that *in*179 templates eventually replicate after a considerable lag period might result from an accumulation of replication factors to a concentration sufficiently high to drive the initiation reaction.

The inability of an excess of coinfecting wt virus to complement the cis-dominant phenotype of *in*179 suggests that the *in*179 parental templates initiating these infections were covalently bound to defective terminal proteins. Therefore, it was surprising to find that *in*179 virion DNA was bound to a 55-kDa TP species, which implied that the mutated domain had been removed by apparently normal processing. The simplest explanation of this result is that although cleavage has occurred, the mutated NH_2-terminal domain remains associated with the COOH-terminal fragment by means of bonds that are labile to the chaotropic reagents used to purify and analyze the molecules. Other explanations require that the *in*179 mutation exert a long-range effect on processing or folding of the COOH-terminal domain.

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