Role of Preterminal Protein Processing in Adenovirus Replication

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Received 14 March 1997/Accepted 20 May 1997

Preterminal protein (pTP), the protein primer for adenovirus DNA replication, is processed at two sites by the virus-encoded protease to yield mature terminal protein (TP). Here we demonstrate that processing to TP, via an intermediate (iTP), is conserved in all serotypes sequenced to date; and in determining the sites cleaved in Ad4 pTP, we extend the previously published substrate specificity of human adenovirus proteases to include a glutamine residue at P4. Furthermore, using monoclonal antibodies raised against pTP, we show that processing to iTP and TP are temporally separated in the infectious cycle, with processing to iTP taking place outside the virus particles. In vitro and in vivo studies of viral DNA replication reveal that iTP can act as a template for initiation and elongation and argue against a role for virus-encoded protease in switching off DNA replication. Virus DNA with TP attached to its 5' end (TP-DNA) has been studied extensively in in vitro DNA replication assays. Given that in vivo pTP-DNA, not TP-DNA, is the template for all but the first round of replication, the two templates were compared in vitro and shown to have different properties. Immunofluorescence studies suggest that a region spanning the TP cleavage site is involved in defining the subnuclear localization of pTP. Therefore, a likely role for the processing of pTP-DNA is to create a distinct template for early transcription (TP-DNA), while the terminal protein moiety, be it TP or pTP, serves to guide the template to the appropriate subcellular location through the course of infection.

Adenovirus DNA replicates by a protein-priming mechanism in which the virus-encoded preterminal protein (pTP) and adenovirus DNA polymerase (Adpol) form a stable heterodimer and pTP serves as the protein primer (20, 35, 37, 39). DNA replication is initiated by the covalent attachment of dCMP to pTP, following the binding of pTP-Adpol to the core origin of replication (20, 35, 42). Initiation is enhanced by two cellular factors, nuclear factor I (NFI or CTF1) and nuclear factor III (NFIII or Oct1), and by the virus-encoded, single-stranded DNA binding protein (3, 5, 6, 8, 10, 12, 25, 26, 31, 40). In addition to the incoming pTP, the presence of terminal protein (TP), covalently attached to the 5' end of the DNA, stimulates initiation in in vitro DNA replication assays, a phenomenon due in part to an increased affinity of the incoming pTP-Adpol for TP-linked DNA (29, 30). Initiation of DNA replication is followed by dissociation of NFI from the complex (9), elongation of the daughter strand by Adpol, and strand displacement. The nature of the molecular rearrangements at the origin of replication preceding elongation and the functional contributions of each of the protein components remain to be established. In particular, questions that elude us are whether pTP and Adpol remain associated throughout elongation, and the nature, if any, of the roles of the pTP moieties attached to the displaced and daughter strands (20, 43).

A further complication to the system is that pTP is processed, via an intermediate (iTP), to the mature TP late in infection (39). The protease responsible is a virus-encoded 23-kDa protein that has a specificity (M,L,I)XGX-X, as determined by using synthetic peptides and sequencing of viral proteins (1, 47, 48). All peptide and protein substrates sequenced thus far have the additional constraint of a glycine residue at either the P1 or P1' position, and sites in Ad2 pTP generating iTP and TP that conform to this specificity have been identified (50). The recently elucidated crystal structure of the protease reveals a substrate binding cleft consistent with the proposed specificity and identifies the enzyme as being a member of a novel class of cysteine protease, with an active site made up of His₅₄, Glu₇₁, Cys₁₂₂, and a papain-like fold (13). Adenovirus protease is activated by an 11-amino-acid disulfide-linked peptide, derived from the C-terminus of the virus structural protein pVI (pVIc) (24, 49). A temperature-sensitive mutant, Ad2ts1, has been isolated with a Pro₁₃₉-to-Leu₁₃₉ substitution in the protease, rendering it inactive at nonpermissive temperatures (46, 53). The cocrystal structure of Ad2 protease and pVIc reveals that the peptide contacts the protease through a number of side chains and that the N terminus binds close to Pro₁₃₉ (13), indicating that the defect in Ad2ts1 could be binding to pVIc. This may be significant in view of the fact that the protease is not present in Ad2ts1 virus particles prepared at nonpermissive temperatures (1, 32). A role proposed for the regulation of adenovirus protease by pVIc is that the enzyme must be synthesized in an inactive form to prevent premature processing of precursor molecules which would abrogate assembly (49). It is clear, however, that some substrates can be cleaved outside virus particles, as in the case of cytokeratin 18 (7), raising the question whether cytokeratin 18 and other such substrates are cleaved by a different mechanism. The observation that the rate of cleavage of pTP to iTP is considerably higher than that of iTP to TP suggests that cleavages at the two sites may serve distinct functions, especially since cleavage to iTP but not to TP takes place in insect cells in the absence of pVIc (50).

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A direct consequence of processing of pTP by the protease is that there are two templates for virus DNA replication and transcription in virus infections: TP-DNA, which is the template for early transcription and the first round of DNA replication, and pTP-DNA, which is the template for all subsequent rounds of DNA replication and for transcription of late viral genes. The significance of the two templates in terms of defining their roles in virus infection remains to be established. In particular, it has been shown that both pTP and TP bind strongly to the nuclear matrix, in a manner assumed analogous to that of matrix attachment regions of eukaryotic chromosomes (2, 15, 36). The identities of the proteins in the nuclear matrix to which pTP and TP bind, and indeed whether they bind to the same sites, are not known (2). Immunofluorescence studies reveal that although sites of viral DNA replication and transcription are proximal in infected nuclei, they are distinct (4, 14, 28). Consensus appears to be that the terminal protein moiety, be it TP or pTP, is at least in part responsible for directing adenovirus DNA to appropriate sites in the nucleus during the course of virus infection (2, 15, 36). Extensive mutagenesis of pTP has been carried out and the conclusions from such studies are that regions spanning the molecule are required for matrix attachment, DNA replication, and virus viability (15–17, 27, 33, 34). More recently, domain-mapping experiments reveal that an N-terminal protease-resistant fragment of pTP contacts DNA whereas multiple regions within the C-terminal two-thirds of pTP interact with Adp1 (51). Furthermore, an extreme dependence on the conformation of pTP was demonstrated for both DNA and Adp1 binding. Given that pTP binds to both Adp1 and DNA but TP binds to neither, the templates are also likely to have distinct properties with regard to initiation of DNA replication (50).

Here we demonstrate the conserved nature of pTP processing via an intermediate by showing that iTP is generated in Ad4, and, in so doing, we extend the previously described substrate specificity of human adenovirus proteases. Furthermore, we make use of monoclonal antibodies, raised against pTP, to monitor the processing of both genome-bound and free pTP through the course of wild-type adenovirus type 2 (Ad2) and Ad2ts1 infections. In situ and in vitro assays show that processing of pTP is not required to switch off DNA replication whereas TP-DNA and pTP-DNA templates have distinct properties in vitro. Based in part on immunofluorescence studies with antibodies recognizing epitopes spanning the TP cleavage site, we propose that a likely role for the processing of pTP is to create distinct templates with different subnuclear localizations.

MATERIALS AND METHODS

Cloning and expression of Ad2 and Ad4 proteases. The fragment representing the entire coding region for the 23-kDa protease was copied from Ad4 DNA by PCR by using the primers GCTAGGATCCCATGGCCGCGGGCTCCGGCGAGCAG (primer 1) and CGATGAATTCGTCCCTGCCCGCGGGCCATGCCG (primer 2) (PCR1) or GACAGGATCCATGGCCGCGGGCTCCGGCGAGCAG (primer 3) and primer 2 (PCR2). Both PCR products were gel purified, digested with *Bam*HI and *Eco*RI. PCR1 was cloned into the *Bam*HI-*Eco*RI site of pGEX-2T for expression in *Escherichia coli* as a fusion protein with glutathione-S-transferase. PCR2 was cloned into the *Bam*HI-*Eco*RI site of the transfer vector pVL1393 (Invitrogen). The resulting plasmid was cotransfected into Sf9 cells with linearized AcRP23lacZDNA, and the recombinant baculovirus expressing Ad4 protease was purified as described previously (45, 49).

The glutathione-S-transferase-Ad4 protease fusion protein was expressed and purified by affinity chromatography on a glutathione-agarose column, digested with thrombin, and dialyzed against 50 mM Tris-HCl (pH 8)–1 mM EDTA, and the Ad4 protease was separated from the glutathione-S-transferase by collecting the flowthrough from a second glutathione-agarose column equilibrated with 50 mM Tris-HCl (pH 8)–1 mM EDTA. Ad2 protease was expressed and purified from Sf9 cells infected with recombinant baculoviruses as described previously (49).

Purification of pTP, Adp1, DBP, NFI, and NFII. Ad2 pTP, Ad2 pol, Ad2 DNA binding protein (DBP), and NFI were purified from Sf9 cells infected with recombinant baculoviruses as described previously (5, 42, 45, 51). NFII was expressed as a fusion with glutathione-S-transferase and purified by affinity chromatography on glutathione-agarose and DNA-Sepharose columns (51). Ad4 pTP-expressing baculovirus (38) was obtained from Michael Stanglmaier and Ernst Winnacker (Institut für Biochemie der Universität München, Munich, Germany), and Ad4 pTP was purified with a combination of phenyl Superose and DNA-Sepharose columns as described previously for Ad2 pTP (51). Ad4 pTP was concentrated by centrifugation in a Microcon 30 microconcentrator unit (Amicon) and stored in aliquots at -70°C .

Proteolytic digestions of pTP. Ad2 or Ad4 pTP was digested to iTP and TP with activated, recombinant Ad2 or Ad4 protease as previously described (49). The equivalent of 20 ng of pTP was applied to each lane of a sodium dodecyl sulfate (SDS) gel, and digestion was monitored by Western blotting and probing with the appropriate antibody.

Baculovirus coinfections. Coinfections of Sf9 cells with the recombinant baculoviruses expressing Ad2 or Ad4 pTP and Ad2 or Ad4 protease were carried out in 24-well plates, seeded with 10^6 cells per well. Each well was infected with 200 μl of various ratios of the protease and pTP virus stocks (10^7 PFU/ml). The cells were harvested at 72 h after infection and washed with phosphate-buffered saline (PBS), prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

Cells and virus. HeLa cells were maintained in suspension at a density of 3×10^5 to 6×10^5 cells per ml in Glasgow S-minimal essential medium supplemented with 7% newborn calf serum (52). Ad2 and Ad2ts1 were grown in suspension cultures of HeLa cells, extracted with fluorocarbon, and subjected to titer determination as described previously (23, 49, 52).

Ad2 and Ad2ts1 viruses were purified on CsCl gradients, and DNA complex and cores were prepared and dialyzed extensively against 10 mM Tris-HCl (pH 7.8) (18, 23). To separate protein from the DNA, DNA-protein complex and cores were digested with 100 μg of DNase I (Boehringer Mannheim) in 10 mM Tris-HCl (pH 7.8)–20 mM MgCl_2 –10 mM CaCl_2 –1 mM phenylmethylsulfonyl fluoride for 1 h at 37°C .

To prepare Ad2- and Ad2ts1-infected cell extracts, HeLa spinner cells were infected with the appropriate virus at a multiplicity of infection of 20 PFU/cell, infections were allowed to proceed at 33 or 39°C , and at various times postinfection 200 ml of cells was collected by centrifugation for 15 min at $1,000 \times g$ and washed with PBS. Cytoplasmic and nuclear extracts were prepared from cell pellets (21), with the final volume of each extract being 1 ml for each time point.

Analysis of viral DNA replication. HeLa cells in 50-mm-diameter plates were infected with 20 PFU of purified Ad2 or Ad2ts1 per cell. At various times postinfection, cells were harvested and viral DNA was selectively extracted, denatured with alkali, and spotted onto nitrocellulose filters. The membranes were baked, hybridized to random-primed Ad2 DNA probe, and washed as described previously (19). Viral DNA synthesis was quantified by determining the ^{32}P radioactivity present in each spot by using a PhosphorImager.

SDS-PAGE and immunoblotting. Protein samples were denatured and reduced by boiling in the presence of 1.25% SDS and 0.35 M 2-mercaptoethanol and analyzed by SDS-PAGE and immunoblotting as described previously (49). Tissue culture supernatants containing anti-pTP antibodies (51) were diluted 1:25 and used as primary antisera in immunoblotting. A monoclonal antibody raised against IkB α was purified and used as a control (22, 51). The preparation and use of polyclonal antisera specific for the N-terminal 15 amino acids of pTP have been described elsewhere (49).

In vitro DNA replication assays. In vitro initiation and elongation reactions were carried out with purified recombinant proteins. Purified Ad2 and Ad2ts1 complex or cores or the linearized plasmid pHRI, containing the Ad2 origin of replication (19), was used as the template. Initiation assays were performed with a final volume of 10 μl comprising 25 mM bicine-NaOH (pH 8), 0.2 mg of protease-free bovine serum albumin per ml, 2 mM dithiothreitol, 1 mM MnCl_2 , 0.15 mM ATP, 500 ng of DBP, 20 ng of NFI, 20 ng of NFII, 2 to 50 ng of pTP-Adp1, 50 ng of purified monoclonal antibody, and the appropriate template. [α - ^{32}P]dCTP (Amersham; 2.5 μCi , 3,000 Ci/mmol) was added, and the mixtures were incubated for 1 h at 30°C . The reaction mixtures were then made 10 mM with respect to CaCl_2 , and 1 U of micrococcal nuclease was added to each prior to incubation for a further 30 min at 37°C . Partial elongation assay mixtures contain the chain terminator ddGTP with dATP and dTTP, resulting in complex formations corresponding to the pTP-dCMP and its 26-base elongation product (pTP-26b); the first dG is at nucleotide 26 in Ad2 (23). Here, assays were performed with a final volume of 10 μl comprising 25 mM bicine-NaOH (pH 8), 0.2 mg of protease-free bovine serum albumin per ml, 2 mM dithiothreitol, 5 mM MgCl_2 , 0.15 mM ATP, 35 μM dATP, 35 μM dTTP, 30 μM ddGTP, 500 ng of DBP, 20 ng of NFI, 20 ng of NFII, and 2 to 50 ng of pTP-Adp1. [α - ^{32}P]dCTP (2.5 μCi , 3,650 Ci/mmol) was added, and the reactions were continued for 1 h at 30°C . Initiation and elongation samples were analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography or PhosphorImaging.

Immunofluorescence. HeLa cells, grown on circular glass coverslips, were infected with Ad2 at 5 PFU per cell and incubated at 37°C for times ranging from 8 to 28 h. The cells were fixed in formaldehyde and processed for immunofluorescence as described previously (4). Primary antibodies were previously described monoclonal antibodies raised against pTP (51). The second antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin

FIG. 1. Processing of Ad4 pTP by virus-encoded protease. (A) Purified Ad4 pTP (1 μ g) was incubated at 37°C for 6 h alone (lane 1), with 0.2 μ g of purified Ad4 protease (lane 2), and with 0.2 μ g of purified Ad4 protease in the presence of pVIc (lane 3), and digestion products were analyzed by SDS-PAGE (15% acrylamide) and Coomassie blue staining. (B) Time course of digestion of Ad2 pTP by Ad2 protease (left panel) and of Ad4 pTP by Ad4 protease (right panel). Purified Ad2 or Ad4 pTP (100 ng) was incubated with 20 ng of Ad2 or Ad4 protease in the presence of pVIc at 37°C. At 0, 10, 30 and 120 min, samples equivalent to 20 ng of pTP were removed and analyzed by SDS-PAGE. Proteins were transferred to PVDF membrane and analyzed by Western blotting with a primary antiserum specific for the N-terminal 15 amino acids of Ad2 pTP. (C) Ad4 pTP (10 μ g) was incubated with 2 μ g of Ad2 or Ad4 protease for 6 h in the presence of pVI-CT, digestion products were separated by SDS-PAGE (15% acrylamide) and transferred to PVDF membrane, and the bands corresponding to TP and NT2 were cut out and sequenced. The sequences of the N terminus of NT2 and TP are shown schematically below the bar, representing pTP, and the positions of the digestion products, NT1, NT2, NT1+2, iTP, and TP are indicated. (D) Alignments of the virus-encoded protease cleavage sites in Ad2 and Ad4 pTP. The asterisk marks the glutamine (Q) at P4 in the Ad4 iTP cleavage site, arrows indicate sites cleaved by the protease in each case, dashes represent amino acids deleted in the Ad4 sequence, and vertical lines show amino acids identical in the two proteins.

G (Sera Lab). The cells were examined by laser scanning confocal microscopy (Bio-Rad 500 microscope).

RESULTS

Ad4 pTP is processed at two sites by adenovirus protease.

Ad4 pTP and DNA polymerase genes have recently been sequenced, and the two proteins, expressed in insect cells by using recombinant baculoviruses, have been shown to be functional when assayed in *in vitro* DNA replication assays (38).

The sequences of Ad4 pTP and DNA polymerase are 81 and 77% identical, respectively, to the corresponding sequences of the Ad2 proteins, with the most striking differences in the pTP being a 22-amino-acid deletion immediately to the C terminus of the iTP sites (38) (Fig. 1D), a region that is important for the DNA binding of pTP (50, 51). Examination of the putative iTP processing sites in Ad4 pTP reveals that neither fits exactly into the previously defined adenovirus protease substrate specificity, (M,L,I)XGX-G or (M,L,I)XGG-X; in one of the sites, the P4 position is occupied by a glutamine residue, while in the second, neither the P1 nor the P1' position is occupied by a glycine residue (Fig. 1D). These differences can be explained in three different ways: either there is not an intermediate cleavage product for Ad4 pTP, making its processing distinct from that in the

other serotypes studied to date, or there is processing at one or both of the iTP sites in Ad4 pTP. The latter would either extend the substrate specificity of the Ad2 protease or identify an altered specificity of Ad4 protease compared to that of Ad2 protease. To distinguish between these possibilities and to ascertain whether the marked differences in the sequence in the region of Ad4 iTP sites are related to the lack of requirement for NFI and NFIII in Ad4 DNA replication (41), the processing of Ad4 pTP by Ad2 and Ad4 proteases was investigated.

Ad4 pTP was digested *in vitro* with purified active Ad4 protease for 6 h at 37°C and analyzed by SDS-PAGE. A pTP-to-protease ratio of 5:1 was used, much higher than normal, to maximize processing to NT1 and NT2 for protein sequencing (Fig. 1A). Even with this high concentration of protease, a band of around 35 kDa is present on gels (NT1+NT2), in addition to the expected digestion products, TP, NT1, and NT2. For Ad2 pTP, where the iTP site is cleaved very efficiently, the intermediate product, NT1+NT2, has never been observed (50). Ad2 and Ad4 pTP were incubated with their corresponding proteases in the presence of pVIc for 0 to 120 min, and the samples were analyzed by SDS-PAGE and Western blotting with an antipeptide antiserum raised against the N-terminal 15 amino acids of pTP. The digestion profile (Fig. 1B) reveals that while Ad2 iTP sites are processed to completion within 10 min, processing at the Ad4 iTP site is much less efficient. After 2 h, some NT1+NT2 remains (Fig. 1B). Nevertheless, it is clear that Ad4 pTP is cleaved at one or both of its putative iTP sites. To identify the site, 10 μ g of Ad4 pTP was incubated with Ad4 or Ad2 protease as described for Fig. 1A, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane, and the bands corresponding to NT2 and TP were cut out and analyzed by N-terminal sequencing. Irrespective of whether Ad2 or Ad4 protease was used, the digestion products were identical; the N terminus of Ad4 TP was VFTLRPREDG, indicating cleavage at the conserved site, MRGG-V, while only one of the putative iTP sites, QRGF-G, was cleaved, with the second potential site, MHGRA, remaining intact (Fig. 1C). Cleavage by both Ad2 and Ad4 proteases at the site QRGF-G is significant in that it extends the previously published substrate specificity of adenovirus protease to include glutamine as well as methionine, leucine, and isoleucine at P4. The site, however, is cleaved much less readily than the sites in Ad2 pTP (Fig. 1B), an observation consistent with previous studies indicating that peptides with glutamine at P4 are poor substrates (48). Also of interest is that the second site, MHGR-A, is not processed, even though it is in a surface-accessible region of the protein, meaning that no substrates of adenovirus protease, either protein or peptide, have been identified that do not have a glycine residue at either P1 or P1'. The sequences containing the Ad2 and Ad4 pTP cleavage sites are aligned in Fig. 1D to include the 22-amino-acid deletion, C-terminal of iTP sites, a sequence which has been shown to influence the binding of Ad2 pTP to DNA (51). The main conclusion from the processing of Ad4 pTP is that cleavage does take place at an iTP site, indicating that this intermediate cleavage is conserved among the serotypes studied to date and perhaps that it plays a specific role in virus infection.

Processing of Ad2/4 pTP in recombinant baculovirus- and adenovirus-infected cells. It has been shown previously that in Sf9 cells coinfecting with recombinant baculoviruses expressing Ad2 pTP and protease, pTP is processed to iTP but not to mature TP (49). Thus, in insect cells, processing to iTP can take place in the absence of pVI or pVIc. Given that Ad4 pTP is cleaved with relatively low efficiency at its iTP site (Fig. 1B), Sf9 cells were coinfecting with Ad2 pTP or Ad4 pTP and Ad2 or Ad4 protease. Cells infected with different ratios of the two viruses were harvested 72 h postinfection, and processing of pTP was assessed by SDS-PAGE and Western blotting with

FIG. 2. Digestion of pTP to iTP in baculovirus- and adenovirus-infected cells. (A) Sf9 cells were coinfecting with baculoviruses expressing Ad2 pTP and Ad2 protease (left panel), Ad4 pTP and Ad2 protease (middle, left panel), Ad2 pTP and Ad4 protease (middle, right panel), and Ad4 pTP and Ad4 protease (right panel) at pTP virus-to-protease virus ratios of 10:0, 8:2, 6:4, and 4:6 as indicated. The cells were harvested at 72 h postinfection, washed, and lysed in gel-loading buffer. Proteins were separated by SDS-PAGE (15% acrylamide), transferred to PVDF, and analyzed by Western blotting with the antipeptide serum specific for the N-terminal 15 amino acids of pTP as the primary antiserum. (B) HeLa cells were infected with wild-type Ad2 (left panel) or Ad2ts1 (right panel) at 39°C at a multiplicity of infection of 20 PFU/cell. At 0, 22, 28, and 48 h postinfection, 50 ml of cells was harvested, nuclear extracts were prepared, and 50 μ g of total protein from each sample was analyzed by SDS-PAGE (15% acrylamide) and Western blotting. The primary antiserum was a mixture of monoclonal antibodies specific for pTP. The positions of the pTP and iTP bands are indicated.

the antipeptide serum specific for the N-terminal 15 amino acids of pTP. Consistent with *in vitro* results, much more efficient processing of Ad2 pTP by both Ad2 and Ad4 proteases was observed compared to that of Ad4 pTP (Fig. 2A). Limited processing of Ad4 pTP to iTP took place as indicated by the appearance of an NT1 band when high ratios of protease to pTP baculovirus were used, but no processing to mature terminal protein was observed when blots were probed with a polyclonal antiserum to either Ad2 or Ad4 pTP (data not shown). Thus, Ad4 pTP is processed to a limited extent in Sf9 cells at the iTP site even though the site is cleaved relatively inefficiently *in vitro* (Fig. 1B) and pVIc is not present.

Although pTP has been shown to be processed outside virus particles in Sf9 cells, a more pertinent question is whether such processing takes place in adenovirus-infected cells and, if so, what its function is in the virus life cycle. A pool of monoclonal antibodies raised against pTP provided the required sensitivity to detect pTP from adenovirus-infected nuclear extracts in Western blots. In this instance, we are studying the free pTP and not genome-bound pTP, destined for packaging in virus particles. A time course of infection was carried out for HeLa cells infected with either wild-type Ad2 or Ad2ts1 at permissive (33°C) and nonpermissive (39°C) temperatures. At 39°C, processing of pTP to iTP was observed in cells infected with wild-type virus but not in those infected with Ad2ts1 (Fig. 2B). As in Sf9 cells (Fig. 2A), no processing to TP was observed. At 33°C, an iTP band was visible in cells infected with both Ad2 and Ad2ts1 at 50 h postinfection (data not shown). The implication from these studies is that processing to iTP, like that of cytochrome 18 (7), can take place outside the virus particle. Processing to TP, however, appears to take place only inside the virus particle, where the protease and pVIc are concen-

FIG. 3. Processing of pTP to iTP does not affect virus DNA synthesis. (A) HeLa cells in 50-mm-diameter plates were infected with 20 PFU of purified Ad2 or Ad2ts1 per cell, as indicated, at the nonpermissive temperature, 39°C (left graph), and the permissive temperature, 33°C (right graph). At various times after infection, virus DNA synthesis was assessed by dot blot hybridization with random-primed Ad2 DNA as a probe. Quantification was achieved by determining the ³²P radioactivity present in each spot by PhosphorImager analysis. (B) Ad2 pTP was digested *in vitro* to iTP by incubation at 37°C for 10 min with Ad2 protease in the presence of pVIc. Complete digestion was ascertained by analyzing the equivalent of 0.5 μ g of pTP, or digested sample, on a 15% polyacrylamide gel containing SDS and staining with Coomassie blue (left panel). pTP-Adpol and iTP-Adpol were prepared, and 50 ng of the resulting heterodimers was included in *in vitro* initiation (middle panel) and partial elongation (right panel) assay mixtures. Ad2 DNA complex (TP-DNA) (50 ng) was used as the template in each case. The positions of pTP-dCMP, iTP-dCMP, pTP-26 bases (pTP-26b) and iTP-26 bases (iTP-26b) are indicated.

trated. It is noted that even at 40 h postinfection, a substantial proportion of pTP remains unprocessed in Ad2-infected cells (Fig. 2B). Nevertheless, the possibility that processing of pTP to iTP plays a role in switching off DNA replication during adenovirus infection was investigated.

Viral DNA synthesis in Ad2- and Ad2ts1-infected cells. To determine whether processing of pTP at the iTP site plays a role in switching off DNA replication by depleting the pool of free pTP, viral DNA replication was examined in HeLa cells infected with wild-type Ad2 and Ad2ts1. The time courses of infection were 30 and 60 h at 39 and 33°C, respectively, since infection proceeds more slowly at the lower, permissive temperature. Infected cells were harvested at various times after infection, and viral DNA was selectively extracted (19). Extracted DNA was spotted onto nitrocellulose, and the filter was probed with ³²P-labelled Ad2 DNA followed by quantitation on a PhosphorImager. The graphs in Fig. 3A reveal that for both Ad2- and Ad2ts1-infected cells, the rate of viral DNA synthesis drops after 25 h (39°C) and 42 h (33°C). At each temperature, the rates of synthesis in wild-type- and Ad2ts1-infected cells are not significantly different (Fig. 3A), suggesting that adenovirus protease does not influence the rate of synthesis of viral DNA and does not play a role in switching off DNA replication. Addressing this question from a different standpoint, Ad2 pTP was digested to iTP *in vitro* and its ability to act as a primer in initiation and elongation assays was examined. Previous studies revealed that while iTP binds to

Adpol, it does not bind to DNA. pTP was digested to iTP in vitro (Fig. 3B, left panel) and incubated with Adpol to form the iTP-Adpol heterodimer, which was then assessed in in vitro initiation and elongation assays. As a control, a mock digestion of pTP was carried out in the absence of pVlc, prior to incubation with Adpol. In vitro, iTP acted as an efficient primer in both initiation and partial elongation assays, with the signal in each case being slightly greater than for the unprocessed control. Although these results were obtained in vitro and do not necessarily reflect the in vivo situation, the ability of iTP to function in initiation reactions suggests that the DNA binding properties of pTP may not be important in initiation. They also add further credence to the conclusion that processing of pTP at iTP sites does not serve to switch off virus DNA replication.

Comparison of TP-DNA and pTP-DNA as templates for DNA replication. In previous studies, DNA isolated from Ad2 and Ad2ts1 virus particles was labelled with ^{125}I in a Bolton-Hunter reaction and treated with DNase I, and the labelled proteins were analyzed by SDS-PAGE and autoradiography. A species with lower mobility was observed in Ad2ts1 and, based on its molecular weight, was identified as pTP (39).

Here we digested pTP in vitro to give a mixture of iTP and TP and used the sample as a marker on a Western blot probed with the pooled monoclonal antibodies (Fig. 4A, left panel). Ad2 and Ad2ts1 complexes were prepared as described previously and treated with DNase I, and the proteins were analyzed by SDS-PAGE and Western blotting with pooled monoclonal antibodies raised against pTP as the primary antisera. In agreement with previous studies, it is clear that while TP-DNA is present in Ad2, pTP-DNA is present in Ad2ts1 virions. This provides further evidence that pTP is not processed to iTP in Ad2ts1-infected cells and provides us with sources of TP-DNA and pTP-DNA that can be compared in in vitro replication assays.

Most studies of adenovirus DNA replication have used TP-DNA complex, and it has been shown that the terminal protein moiety in TP-DNA increases the efficiency of DNA replication (29, 30). Although TP-DNA is the template for early transcription and the first round of DNA replication, the template for subsequent rounds of DNA replication is pTP-DNA. Hence, a comparison of the two templates is of value. A 50-ng portion of either TP-DNA or pTP-DNA (in 1 μl of 10 mM Tris-HCl [pH 7.8]) was assayed in initiation reactions at KCl concentrations ranging from 0 to 0.1 M. The graph in Fig. 4B shows the mean results from three independent experiments and indicates that as the KCl concentration is increased above 20 mM, the level of initiation drops rapidly when pTP-DNA is used as the template, whereas when TP-DNA is used, the peak of activity is broader and the level of initiation does not decrease until the KCl concentrations rise above 50 mM. Although this is perhaps not as dramatic an effect, it was reproducible when tested for core and complex and gives some indication that the molecular interactions in the preinitiation complex are distinct when pTP rather than TP is covalently attached to the DNA.

It is well established that the transcription factors NFI and NFIII stimulate the initiation of DNA replication in vitro and in vivo for the TP-DNA template. Here we investigate whether having a pTP moiety at the end of the genome has any effect on the level of stimulation by NFI and NFIII. Under the conditions described above, 50 ng of TP-DNA or pTP-DNA was incubated in an in vitro initiation reaction with 2 or 10 ng of pTP-Adpol in the presence and absence of NFI and NFIII; the reaction products were then visualized by SDS-PAGE and autoradiography (Fig. 4C). NFI and NFIII stimulated initiation to a similar degree, irrespective of whether TP-DNA or pTP-DNA was used as the template.

FIG. 4. Comparison of TP-DNA and pTP-DNA in in vitro initiation assays. DNA complex and cores were prepared from purified Ad2 and Ad2ts1 virus particles as described in the text, and the equivalent of 1 μg of DNA was incubated with DNase I and micrococcal nuclease at 37°C for 1 h. The samples were analyzed by SDS-PAGE and Western blotting with a mixture of monoclonal antibodies specific for pTP as the primary antibodies. In vitro-digested pTP was used as a marker for iTP and TP (left panel); Ad2 and Ad2ts1 complex are in the central panel, and Ad2 and Ad2ts1 cores are in the right panel. (B) In vitro initiation reactions were carried out with 50 ng of Ad2 (TP-DNA) or Ad2ts1 (pTP-DNA) as the template and 20 ng of pTP-Adpol. The ionic strength was varied by including KCl in the reaction mixtures at a range of concentrations from 0 to 0.1 M, the samples were analyzed by SDS-PAGE (12.5% polyacrylamide) and PhosphorImager analysis, and the results of three independent experiments were averaged. The values for each triplicate set were within 5% of each other. The data is shown graphically, with the incorporation of dCMP (10^{-1} fmol) being plotted against KCl concentration (millimolar). (C) Effects of NFI and NFIII on in vitro initiation of DNA synthesis with Ad2 (TP-DNA) or Ad2ts1 (pTP-DNA) as the template (left and right panels, respectively). Reactions, including 50 ng of template and 2 or 10 ng of pTP-Adpol, were carried out in the presence (+) and absence (–) of NFI and NFIII as indicated. The samples were denatured and analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography.

Effects of monoclonal antibodies on DNA replication. To investigate further the differences between the TP-DNA and pTP-DNA templates, a panel of monoclonal antibodies raised against pTP was used. Six of the antibodies were specific for pTP and did not react with the mature TP: 5E3 and 16H11, which recognize epitopes to the C terminus of iTP sites; 7A2 and 44E1, whose epitopes span the TP cleavage site; and two conformational antibodies, 6E2 and 10B1. Characterization of the antibodies revealed that 16H1 inhibited DNA binding of pTP, as did 6E2. The latter, along with the other conformational antibody, 10B1, has also been shown to induce the dissociation of pTP from Adpol in vitro. Two antibodies, 3D11 and 11F11, binding to epitopes contained within the C-terminal 60 amino acids of pTP, were shown to stimulate DNA binding of pTP 10-fold in gel retardation assays (51).

Antibodies (50 ng) were included in in vitro initiation and partial elongation assays in an attempt to ascertain whether any of the pTP-specific antibodies exerts specific effects when pTP-DNA, as opposed to TP-DNA, is the template. Assays were carried out as described above, and incorporation of [^{32}P]dCMP was determined by SDS-PAGE and PhosphorImager analysis. For each reaction, the signal was compared with those of samples where an $\text{IkB}\alpha$ monoclonal antibody or PBS was included as a control. The profiles for initiation and partial elongation reactions were the same, and the latter are shown in Fig. 5A. Only two antibodies, 6E2 and 10B1, had a significant effect when TP-DNA was used as the template. Both antibodies gave a fivefold reduction in the amount of ^{32}P -dCMP incorporated compared to the controls and presumably exerted

FIG. 5. Effect of monoclonal antibodies on in vitro replication with pTP-DNA, TP-DNA, and naked DNA templates. (A) TP-DNA (top panel) or pTP-DNA (bottom panel) (50 ng) and pTP-Adpol (20 ng) were included in in vitro elongation assays with 50 ng of purified monoclonal antibodies included as indicated. In each case, the control (PBS, no antibody) reaction is in the first track. Reactions were carried out as described in the text, and the products were separated by SDS-PAGE and visualized by autoradiography. (B) Linearized plasmid pHR1 (10 ng) containing the Ad2 origin of replication was used as the template (naked DNA). Partial elongation reactions were carried out as described for panel A. (C) Three independent experiments were carried out in which the effects of antibodies 3D11, 6E2, 10B1, 11F11, and 44E1 on incorporation of dCMP in elongation assays were determined for each of the three templates TP-DNA, pTP-DNA, and naked DNA. Reactions were carried out as described for panels A and B, and the samples were analyzed by SDS-PAGE and PhosphorImager analysis. Incorporation of dCMP was calculated, control values were normalized, and means of the three experiments were calculated. The values for each triplicate set were within 10% of each other. The results are represented in the form of a bar chart.

their inhibitory effects by dissociating the pTP-Adpol heterodimer. In the case of the pTP-DNA template, both 6E2 and 10B1 inhibited initiation and partial elongation but to a lesser extent than observed with TP-DNA templates. Furthermore, effects were observed with other antibodies. In particular, antibody 44E1, which reacts only with pTP, gave a twofold reduction in the incorporation of [32 P]dCMP (Fig. 5A), whereas smaller but nevertheless significant inhibition by antibodies 3D11 and 11F11 was observed (Fig. 5C).

To determine whether these template-specific effects were a result of antibodies binding to the pTP moiety covalently attached to the template or whether they reflected differences in conformation between the initiation complexes with TP-DNA and with pTP-DNA, the antibodies were tested with a linearized plasmid, pHR1 (19), containing the inverted terminal repeat of Ad2 as the template in initiation and partial elongation reactions.

Surprisingly, the profile of antibody effects on this naked DNA

template bore a strong resemblance to that of pTP-DNA, but the effects were much more pronounced (Fig. 5B). In particular, a fivefold inhibition was observed for 3D11 and 11F11; a threefold inhibition was observed for 44E1, and a twofold inhibition was observed for 6E2 and 10B1. Also evident was an approximately twofold increase in the incorporation of [32 P]dCMP when antibodies 5E3 and 16H1 were included. Quantitation was carried out for three independent experiments, and the results for those antibodies giving significant inhibition are represented in the form of a bar graph in Fig. 5C.

In conclusion, the effects of antibodies 3D11, 11F11, and 44E1 observed for pTP-DNA templates were amplified when naked DNA templates were used, suggesting that the antibodies are not exerting their effect through the pTP moiety covalently attached to the template but, rather, through interaction with the incoming pTP component of the pTP-Adpol heterodimer.

One explanation, in keeping with the KCl results, is that a more compact preinitiation complex is formed when TP is attached to the 5' end of DNA, in which the primer pTP is less accessible to monoclonal antibodies and the complex is less sensitive to increases in ionic strength. The relevance of this to the in vivo situation remains to be established, but differences between TP-DNA and pTP-DNA as templates for DNA replication in vitro clearly do exist.

Localization of pTP in Ad2-infected cells; two distinct patterns of fluorescence with different monoclonal antibodies.

The subcellular distribution of pTP in Ad2-infected HeLa cells was investigated with a panel of 11 specific monoclonal antibodies raised against pTP (51). Uninfected cells or cells infected with 5 PFU of Ad2 per cell for various times were fixed in formaldehyde, penetrated with detergent, and exposed to a dilution series of tissue culture supernatants expressing each of the monoclonal antibodies. An FITC-conjugated second antibody was used to permit visualization of the antibody locations by fluorescence microscopy. Uninfected cells displayed no cross-reaction with the monoclonal antibodies, with the exception of 11F11, which appeared to cross-react with a cellular protein (data not shown). In infected cells, the 10 remaining antibodies revealed that pTP is present exclusively in the nucleus. Remarkably, however, two distinct subnuclear localizations were observed with the different monoclonal antibodies. At early times, antibodies 7A2 and 44E1 revealed a punctate localization of pTP (Fig. 6A and C), similar to that reported for DBP (4), while the remaining eight antibodies, represented by 3D11 in Fig. 6B and D, revealed that pTP was localized throughout the nucleus. Experiments with dilution series of the various antibodies revealed that the distinct patterns are not a function of different titers or avidities of the antibodies but, instead, suggest that two populations of pTP exist within the nucleus. Interestingly, antibodies 7A2 and 44E1 react with epitopes in the same 15-amino-acid sequence, spanning the TP cleavage site, and digestion of pTP by the adenovirus protease destroys the epitopes recognized by the two antibodies (51). This region of pTP is surface exposed, and both 7A2 and 44E1 were able to immunoprecipitate pTP-Adpol in vitro (51). A more detailed analysis of the subnuclear localization of pTP is required, but since it has been established that pTP binds tightly to the nuclear matrix (15, 36), one interpretation of our results is that the extensive nuclear staining observed with most of the antibodies corresponds to the fraction of pTP bound to the nuclear matrix. Consistent with this notion is that at late times in infection, when pTP is released from the nuclear matrix (2), the patterns observed with 7A2/44E1 and the remainder of the antibodies became similar (Fig. 6E and F). The reason that 44E1 and 7A2 do not react with the bulk of pTP is presumably because the epitope is masked either by another

FIG. 6. Subcellular localization of pTP in HeLa cells infected for 8 h (A and B), 12 h (C and D) and 20 h (E and F) with Ad2 at 37°C. The immunofluorescence pictures are of cells treated with pTP-specific monoclonal antibodies 7A2 (A, C, and E), and 3D11 (B, D, and F), with the pTP-specific fluorescence coming from an FITC-conjugated goat anti-mouse immunoglobulin G.

protein or through a conformational change in pTP. An extension of this argument is that the region spanning the TP cleavage site may be required for attachment of pTP to a particular nuclear structure and that processing of pTP to TP serves to alter the subnuclear localization of the protein.

DISCUSSION

The main aim of this study was to establish a role for processing of adenovirus pTP in virus infection, but a prerequisite for this was to establish whether the cleavages at iTP and TP sites are temporally separated and serve distinct functions in the adenovirus life cycle. Of interest in this regard was the recent sequencing of the pTP and Adpol genes from human Ad4, which revealed significant sequence changes in and around the iTP sites (38). Ad4 DNA replication has no requirement for NFI and NFIII, a property that distinguishes it from other human adenoviruses and must be accounted for by specific differences in the properties of Adpol, pTP, or DBP (38, 41). Here we show that Ad4 pTP is processed at a single iTP site, QRGF-G, albeit at a lower rate than that of Ad2 pTP processing. Cleavage at the QRGF-G site was also carried out

by Ad2 protease, and so processing of Ad4 iTP extends the human adenovirus protease specificity to include a glutamine at the P4 position. Recent sequencing of an ovine adenovirus, OAV287, revealed distinct differences in its genome organization compared to other adenoviruses and also showed that the homology of its protease and pVIc sequences to those of Ad2 were considerably lower than observed for other serotypes (44). Analysis of proteins from mature OAV287 particles indicates that its protease cleaves at sites MRAT-G and NTGW-G in pVII, differing from the established consensus at P2 and P4, respectively (44). Substrate modelling with the crystal structure of Ad2 protease indicates that only substrates with glycine at P2 will fit into the substrate binding pocket (13). It cannot be ruled out, however, that a specific secondary or tertiary structural element may enable alanine to be present at P2 in certain substrates. As yet, it is not known whether the results from OAV287 represent an expansion of the adenovirus protease specificity or a divergence between human and ovine viruses (44).

pTP is packaged into virus particles only when it is covalently linked to the virus DNA; therefore, processing of pTP outside

virus particles can be monitored by observing the fate of non-genome-bound pTP. We have previously shown that adenovirus protease is detected 24 h postinfection, and here we demonstrate that pTP is processed outside virus particles from 28 h postinfection (50). Processing of pTP is not complete, however, and no free TP is detected. Consistent with the results of studies on processing of cytokeratin 18 (7), which also takes place outside virus particles late in infection, we found no processing of pTP in cells infected with Ad2ts1 at nonpermissive temperatures. This is at odds with the notion that the sole defect of Ad2ts1 is packaging of protease into virus particles. Although it has been shown that processing of pTP to iTP does take place in insect cells in the absence of pVIc, it remains to be established whether processing of substrates outside the virus particle, in adenovirus infections, has a requirement for pVI or pVIc. The processing of cytokeratin 18 (7) and free pTP (Fig. 2B) does, however, leave open the possibility that other cellular and virus-encoded proteins are cleaved outside the virus by the protease, especially in light of the requirement for protease activity early after infection (11, 47).

The observation that iTP is produced at about the time when viral DNA synthesis is switched off led us to examine whether the protease plays a role in turning off DNA replication either by depleting the cells of free pTP or by producing a digestion product that inhibits replication. Rates of viral DNA synthesis were compared between Ad2 and Ad2ts1 at the permissive and nonpermissive temperatures. No processing of pTP takes place in Ad2ts1; therefore, if processing of pTP to iTP is required to regulate virus DNA synthesis, differences between rates of DNA synthesis would be expected to occur between Ad2 and Ad2ts1 at nonpermissive temperatures; none were found. This data was supported by the results of an *in vitro* experiment showing that both initiation and partial elongation take place when iTP is the primer. It is noted that previous work indicates that even after processing of pTP to iTP, the N-terminal fragment, NT1, remains bound to iTP (50). iTP, prepared in this fashion, retained its ability to bind to Adpol but no longer bound to DNA, as assessed in gel retardation assays (50). It has been shown that pTP contacts DNA through a protease-resistant N-terminal domain and that it has a regulatory domain within its C-terminal 60 amino acids, but the role of its DNA binding properties remains elusive (51). Here, our *in vitro* data suggests that a stable association between pTP and DNA is not required for initiation and partial elongation, perhaps indicating that the contact points between heterodimer and DNA are primarily through Adpol. We used a pool of pTP-specific monoclonal antibodies to confirm that it is pTP, not iTP or TP, that is covalently attached to DNA in Ad2ts1 particles prepared at nonpermissive temperatures. This data indicates that processing of pTP to TP by protease is not required to release virus DNA from the nuclear matrix, as proposed previously (15), or for packaging of virus DNA into virus particles. Our data does not, however, preclude the possibility that in wild-type Ad2 infections, genome-bound pTP is either partially or completely processed prior to assembly.

Given the differences between pTP and TP in terms of their molecular associations (50), the influence of a pTP molecule covalently attached to the 5' end of the genome on the efficiency of DNA replication was investigated. The overall conclusions are that, as in the case of TP-DNA, initiation is stimulated by NFI and NFIII when pTP-DNA is used as template, but a threefold reduction in initiation was observed at 0.06M KCl for pTP template compared to TP template. This, in combination with the inhibitory effects of three antibodies specific for pTP-DNA template, suggests that when TP is the moiety covalently attached to the end of the DNA, the preini-

tiation complex is more compact and stable than when pTP is present. No difference in efficiency of pTP-DNA complex was observed *in vitro*, but this does not rule out that TP-DNA is required *in vivo* for the first round of DNA replication, where there is a low abundance of template. Perhaps the most attractive explanation for processing of pTP relates to the matrix association properties of TP- and pTP-DNA (2, 15, 36). Both TP-DNA and pTP have been shown to bind tightly to the nuclear matrix, but the identity of their respective sites of attachment is unknown, meaning that a combination of proteolysis and phosphorylation could serve to guide the viral DNA through the nucleus to appropriate sites for early transcription, DNA replication, late transcription, and packaging into the virus (2, 15, 28, 36). Supporting evidence for this hypothesis comes from results of immunofluorescence experiments, which show that monoclonal antibodies recognizing epitopes spanning the TP site bind to pTP only in the foci of viral DNA replication (Fig. 6A), whilst antibodies reacting with other epitopes in the protein recognize pTP localized throughout the nucleus (Fig. 6B). These observations suggest that integrity of TP cleavage site may be a critical determinant of the subnuclear localization of the terminal protein moiety, be it TP or iTP. In a similar vein, it has been noted that the TP moiety of the incoming genome is required for transcriptional activation of some of the viral early genes through two potential activation domains at amino acids 398 to 470 and 549 to 609 (36). It is possible that these domains are exposed in the mature terminal protein but are masked by intramolecular associations in the precursor protein. Such a mechanism would ensure that TP-DNA but not pTP-DNA can serve as the template for early transcription. Also related to matrix attachment is the observation that a large portion of pTP extending from the iTP site to its C terminus appears to contact Adpol (51); thus, the binding of Adpol to pTP could interfere with its matrix attachment region, giving a clear distinction between the properties of pTP in the pTP-Adpol heterodimer, compared to pTP covalently attached to the 5' end of genome. It remains unclear whether Adpol can associate with genome-bound pTP, with one proposal being that the negatively charged polymer extending from Ser₅₈₀ of pTP dissociates Adpol from the heterodimer (51).

We propose that the role of the processing of pTP to TP is to create distinct templates for early transcription and DNA replication (Fig. 7), with the TP-DNA template being directed to a specific subnuclear location, distinct from that of pTP-DNA. Processing to iTP was shown to take place late in adenovirus infection outside the virus, but the purpose of the cleavage at the iTP site, which is conserved in all serotypes studied, including Ad4, is not known. It is possible that the processing at the iTP site serves solely as an intermediate; alternatively, cleavage may be required to remove NT1+NT2, a fragment with the potential to act as a dominant negative.

FIG. 7. Schematic diagram of TP-DNA and pTP-DNA complexes, indicating the tripartite nature of pTP and the fact that the two complexes have distinct properties.

ACKNOWLEDGMENTS

We are indebted to Michael Stanglmaier and Ernst Winnacker for supplying the recombinant baculovirus expressing Ad4 preterminal protein and to Graham Kemp and Paul Talbot for protein sequencing. Thanks are also due to Joanna Destero for many useful discussions, to Catherine Botting and Joanna Destero for comments on the manuscript, and to Bill Blythe for photography.

Work was supported by grants from the Medical Research Council and the Wellcome Trust.

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