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Adenovirus Preterminal Protein Binds to the CAD Enzyme at Active Sites of Viral DNA Replication on the Nuclear Matrix

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Adenovirus (Ad) replicative complexes form at discrete sites on the nuclear matrix (NM) via an interaction mediated by the precursor of the terminal protein (pTP). The identities of cellular proteins involved in these complexes have remained obscure. We present evidence that pTP binds to a multifunctional pyrimidine biosynthesis enzyme found at replication domains on the NM. Far-Western blotting identified proteins of 150 and 240 kDa that had pTP binding activity. Amino acid sequencing of the 150-kDa band revealed sequence identity to carbamyl phosphate synthetase I (CPS I) and a high degree of homology to the related trifunctional enzyme known as CAD (for carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase). Western blotting with an antibody directed against CAD detected a 240-kDa band that comigrated with that detected by pTP far-Western blotting. Binding experiments showed that a pTP-CAD complex was immunoprecipitable from cell extracts in which pTP was expressed by a vaccinia virus recombinant. Additionally, in vitro-translated epitope-tagged pTP and CAD were immunoprecipitable as a complex, indicating the occurrence of a protein-protein interaction. Confocal fluorescence microscopy of Ad-infected NM showed that pTP and CAD colocalized in nuclear foci. Both pTP and CAD were confirmed to colocalize with active sites of replication detected by bromodeoxyuridine incorporation. These data support the concept that the pTP-CAD interaction may allow anchorage of Ad replication complexes in the proximity of required cellular factors and may help to segregate replicated and unreplicated viral DNA.

Initiation of adenovirus (Ad) replication occurs via a protein priming mechanism involving the precursor of the terminal protein (pTP). The covalent addition of a dCMP residue to pTP at serine 580 provides a 3′ hydroxyl to stimulate DNA chain extension (16, 18, 31). The Ad DNA polymerase and pTP form a heterodimer which binds specifically to the Ad origin of replication (62). Nuclear factors I (NFI) and III (NFIII) further stimulate initiation by interacting with the polymerase complex and the Ad origin DNA (18, 45). The Ad DNA binding protein (DBP) serves to stabilize single-stranded DNA (ssDNA) during replication (58). There is considerable evidence that Ad replication complexes are bound to specific sites on the nuclear matrix (NM) (7, 14, 49, 69). Attachment of these complexes to the NM is mediated through the interaction of pTP (1, 25, 26, 55). Interaction of pTP with the NM may also enhance expression of the late genes (55). Subsequent to Ad replication site formation via pTP, NFI is recruited to the same location (8).

The NM is a highly complex fibrillar protein network which is impervious to treatment with DNase and high salt or lithium-3,5-diiodosalicylate (3, 4, 42). This nuclear infrastructure is made up of lamin polymers, core filaments, and membrane-associated proteins. The NM is known to organize genomic DNA into looped domains via matrix attachment regions (9). The enzymatic activities of transcription, mRNA splicing, and DNA replication are localized to discrete sites on the NM (6, 12, 13, 50). In addition, there is evidence that DNA modification and deoxyribonucleoside triphosphate (dNTP)-synthetic enzymes such as cytosine DNA methyltransferase and thymidine kinase, respectively, are associated with NM compartments (38, 39). The association of large multiprotein complexes of dNTP-synthetic enzymes and replication proteins in eukaryotes has been proposed previously (51). Evidence for the existence of such complexes is suggested by cosedimentation of virus-encoded ribonucleotide reductase and thymidine kinase activities with herpes simplex virus (HSV) Pol (30, 51). Ad and other DNA virus proteins bind to the NM, presumably to harness such cellular activities.

Spherical or doughnut-shaped domains of viral synthesis occur early after Ad infection and are composed of overlapping zones of transcription and replication. Microscopy has demonstrated that these structures contain DBP which partitions with ssDNA (12). Over the intermediate phase of infection, ssDNA storage zones form and are surrounded by peripheral replicative zones which continue to expand during infection (14). Ad pTP and Pol can be detected in the doughnut-shaped peripheral zones, which contain double-stranded DNA, but not in the DBP-containing ssDNA centers (44). It is thought that the peripheral zones containing pTP represent initiation whereas the DBP centers represent elongation (14). The majority of cellular components participating in Ad replication centers have yet to be identified. Therefore, our objective was to identify the protein(s) to which pTP binds at replication sites on the NM.

In this work, we have demonstrated by far-Western blotting and coimmunoprecipitation that Ad pTP binds to the pyrimidine biosynthesis enzyme known as CAD (for carbamyl phosphate synthetase [CPS; EC 2.1.3.2], aspartate transcarbamylase [ATC; EC 2.1.3.2], and dihydroorotase [EC 3.5.2.3]). We found that CAD colocalizes with pTP foci on the NM. Bromodeoxyuridine (BrdU) incorporation experiments have shown that pTP-CAD foci colocalize with active Ad DNA replication centers. These foci were numerous and often became large and pleomorphic late in infection. We suggest that
the pTP-CAD interaction may serve to anchor the viral genome in the proximity of factors required for replication.

**MATERIALS AND METHODS**

**Cell lines, viruses, and antibodies.** HeLa CCL-2 cells (American Type Culture Collection, Rockville, Md.) were used for isolation of pTP binding proteins derived from the NM. Cells were grown in monolayer cultures at 37°C in Dulbecco’s modified Eagle’s medium (GIBCO, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (BIOMED,Logans, Utah). To prepare a pTP extract, HeLa cells were cotransfected with vaccinia virus vvpTP and vvT7 at a multiplicity of infection of 10 (2, 27). Virus was allowed to attach to the cells for 1 h. The infected cells were washed with phosphate-buffered saline (PBS) and then infected with DNase in Dulbecco’s modified Eagle’s medium containing 5% FBS. The cells were harvested at 20 h after postinfection (p.i.). Ad infections of HeLa cells for indirect immunofluorescence experiments were carried out for 15 or 20 h at a multiplicity of infection of 10.

The 3-1A monoclonal antibody recognizing pTP was raised against a carboxy-terminal peptide (PEPPLPGARRPRRRC) and was used for Western blotting and immunoprecipitations (25). A monoclonal antibody (IB6A8) raised against purified pTP was used for immunofluorescence and immunoprecipitation experiments (1). All Western blots were probed with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Southern Bio-technology Associates, Birmingham, Ala.) and with 3% nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (2). The CAD cDNA was then subcloned into the expression vector pET15b (Novagen, Madison, Wis.). The recombinant CAD protein-specific antibody was recognized by David Evans (Wayne State University). A monoclonal antibody directed against the FLAG epitope (DYKDDDDDE) was purchased from Kodak (Rochester, N.Y.). As a positive control against the influenza virus hemagglutinin (HA) epitope (YPFPDVPDYA) was a generous gift from Vincent Kidd (St. Judes Children’s Research Hospital). A monoclonal antibody (Anti-body-1) used to detect BRu incorporation into viral replication sites was purchased from Oncogene Science (Manhasset, N.Y.). Secondary antibodies used for the immunofluorescence experiments were fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and anti-rabbit and Texas red (TXRD)-conjugated goat anti-mouse and anti-rabbit antibodies (Southern Biotechnology Associates).

**Preparation of NM.** The protocol used for preparation of NM involved DNase treatment of HeLa cell nuclei followed by high-salt extraction of soluble proteins and chromatin, as previously described (1, 42). Digestion of the nuclei was carried out at a concentration of 2.5 × 10⁶ nuclei per ml in modified digestion buffer (20 mM Tris-HCl [pH 7.4], 20 mM KCl, 70 mM NaCl, 10 mM MgCl₂ with 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at room temperature, as described by Angeletti and Engler (1) and by Mirkovitch et al. (42). The nuclear pellet was recovered by centrifugation at 2000 × g and 4°C for 10 min in a microcentrifuge for 10 min. The nuclear pellet was then extracted with high-salt buffer (2 M NaCl, 20 mM HEPES [pH 7.4], 20 mM EDTA) for 5 min on ice. The insoluble NM pellet was recovered by centrifugation at 2000 × g and 4°C for 4 min. The pellet was then resuspended in digestion buffer and finally resuspended in 100 µl of buffer with 1 mM PMSF. The protein concentrations of the NM preparations were determined by the method of Bradford (using a kit from Bio-Rad Laboratories, Hercules, Calif.) (11).

**Preparation of pTP-VV extracts, cytoplasmic extracts, and NM.** One-hundred-milliliter-diameter plates of HeLa cells that either were uninfected or infected with recombinant vaccinia virus vvpTP1 and vvT7 at a multiplicity of infection of 7 (Boehringer Mannheim, Indianapolis, Ind.). The probe was then washed with 10 volumes of buffer D (20 mM HEPES [pH 7.5], 5 mM MgCl₂, 0.5 mM dithiothreitol [DTT]) and allowed to swell at 4°C for 10 min. The blots were washed with 10 volumes of buffer D (20 mM HEPES [pH 7.5], 20 gycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF). Bound protein was eluted with a linear KCl gradient, from 100 to 1,000 mM in buffer D, over 30 fractions of 1 ml each. Fractions which contained pTP binding activity were detected by far-Western blotting. The pTP binding activity eluted between 640 and 700 mM KCl. Positive fractions 18 and 19 were pooled for amino acid sequence analysis.

**Amino acid sequence analysis.** A total of 600 µg of protein from the fractions positive for pTP binding (fractions 18 and 19) was separated by loading it in one long slot of an SDS–8% polyacrylamide gel and subjecting it to electrophoresis. The protein was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.) and blocked against 5% dry milk protein in PBS. The blot was rinsed with PBS for 10 min and then with a blocking buffer (0.1% Tween 20 in buffer A). The blot was incubated in a blocking solution with anti-pTP or anti-ATC (CAD) antibodies. The protein was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.) and blocked against 5% dry milk protein in PBS. The blot was rinsed with PBS for 10 min and then with a blocking buffer (0.1% Tween 20 in buffer A). The blot was incubated in a blocking solution with anti-pTP or anti-ATC (CAD) antibodies. The protein was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.) and blocked against 5% dry milk protein in PBS. The blot was rinsed with PBS for 10 min and then with a blocking buffer (0.1% Tween 20 in buffer A). The blot was incubated in a blocking solution with anti-pTP or anti-ATC (CAD) antibodies. The protein was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, Calif.) and blocked against 5% dry milk protein in PBS. The blot was rinsed with PBS for 10 min and then with a blocking buffer (0.1% Tween 20 in buffer A). The blot was incubated in a blocking solution with anti-pTP or anti-ATC (CAD) antibodies.
pcDNA3.1+ clone containing the (His)-HA tag. This construct was named pcDNA-CAD. Similarly, a (His)-FLAG epitope tag was designed as a fusion to pTP. The predicted amino acid sequence of the (His)-FLAG linker is MHHHHHHHHHDDDDDD. The (His)-FLAG linker was constructed with HindIII and EcoRI sites on the ends as follows: top strand, 5'-AGCGCTTTGGCGCA CCATTGGGGCCCACTACCCACACCCACGCTAAAGGGAGAGCGAGG TGoACG-3'; and bottom strand, 5'-ATTTCGCTATCATGTCCTGTTGATCGT GTGATGTTGATGTTGATGTTGCCACTGTTGCCAC-3'. The pcDNA3.1+ vector was digested with HindIII and BamHI and then gel purified. Ad type 2 pTP was excised from pGEM-pTP by digestion with EcoRI and BamHI (New England Biolabs), gel purified, and ligated into the (His)-FLAG double-stranded linker. The resultant product was subjected to HindIII-BamHI digestion followed by ligation into the same sites in pcDNA3.1+. The subsequent clone was designated pcDNA-pTP.

**Immunoprecipitations.** Whole-cell extracts of cells expressing pTP were prepared after a vprTFI-vvT confection. Five micrograms of polyclonal antibody to CAD was allowed to bind to a 70-μl bed volume of CL-4B protein A-Sepharose in a 300-ml total volume of immunoprecipitation buffer (50 mM HEPES [pH 7.5], 250 mM NaCl, 0.05% SDS, 10 mM NaPO4 [pH 7.0], 1 mM NaF, 1 mM AEBSF, and 0.5 mM DTT) for 1 h at 25°C with rotation (2, 37). The beads were washed three times with immunoprecipitation buffer and then resuspended in the same buffer. Fifty micrograms of cell extract was then incubated with a 10-μl bed volume of beads for 2 h at 4°C with rotation. As a negative control, lysates were incubated with beads that were preincubated with mouse immunoglobulins. Immunoprecipitations were washed three times with immunoprecipitation buffer prior to boiling of samples in Laemmli sample buffer and loading them onto an SDS–8% polyacrylamide gel (36). The protein was then transferred to nitrocellulose and probed with either pTP monoclonal antibody (IB6A8) or CAD polyclonal antibody.

The immunoprecipitations with in vitro-translated His-FLAG–pTP and His-HA–CAD were done to further demonstrate a protein–protein interaction between pTP and CAD. In vitro translation labeling of each fusion protein was performed in a 1-ml total volume with 2 μg of plasmid DNA in the presence of 40 μCi of [35S]methionine (10 mCi/mmol; Amersham) and the buffer specified by the manufacturer of the TNT kit (Promega, Madison, Wis.). These immunoprecipitations were done with immunoprecipitation buffer (25 mM HEPES [pH 7.5], 300 mM NaCl, 1 mM EDTA, 0.05% Nonidet P-40, 1 mM AEBSF) as described by Webster et al. (66). Monoclonal antibodies directed against either the FLAG or the HA epitope were used in conjunction with Streptococcus protein G (Sigma) to precipitate the immunocomplexes. Five microliters of each translation product was incubated either alone or in combination in 300 μl of immunoprecipitation buffer containing protein G–anti-FLAG or –anti-HA antibody complexes. Immunocomplexes were electrophoresed on an 8% polyacrylamide gel, and bands were visualized with a model 860 Storm PhosphorImager (Molecular Dynamics).

**Retention of CAD on the NM.** Five hundred micrograms of HeLa cell NM was prepared. The NM pellet was successively extracted for 1 min with 1 M guanidine hydrochloride (GnHCl, Sigma) in accordance with the protocol of Fredman and Engler (25). After each extraction, the NM pellet was redissolved by centrifugation at 15,000 × g for 10 min. The GnHCl was removed by washing the NM with digestion buffer. After each of five washes, an aliquot was taken for analysis. The protein concentration of each aliquot was measured by the Bradford assay (11).

Amino acid sequencing and analysis of peptides. From the 600 μg of protein present in P11 column fractions 18 and 19, we were able to employ phosphocellulose chromatography to further purify high-MW proteins which bound to pTP. Protein fractions eluted from the P11 phosphocellulose column were assayed for pTP binding by far-Western blotting (4). These fractions 18 and 19 showed two major bands in this assay (Fig. 1A). As a negative control for the far-Western blot assay, we used a cytoplasmic extract from HeLa cells that were infected with a vaccinia virus-T7 recombinant that expressed bacteriophage T7 RNA polymerase, rather than pTP; these two high-MW protein bands were not detected when the load material was probed with this cytoplasmic extract. The detected nuclear polypeptides eluted from the column between 640 and 700 mM KCl. The most-intense bands were at approximately 240 and 150 kDa. However, a 120-kDa band and a series of lower-MW bands were also detectable, all of which are consistent with observations in our previous study (1).

Using the portion of the extract that remains soluble after step dialysis, we were able to employ phosphocellulose chromatography to further purify high-MW proteins which bound to pTP. Protein fractions eluted from the P11 phosphocellulose column were assayed for pTP binding by far-Western blotting (4). These fractions 18 and 19 showed two major bands in this assay (Fig. 1A). As a negative control for the far-Western blot assay, we used a cytoplasmic extract from HeLa cells that were infected with a vaccinia virus-T7 recombinant that expressed bacteriophage T7 RNA polymerase, rather than pTP; these two high-MW protein bands were not detected when the load material was probed with this cytoplasmic extract. The detected nuclear polypeptides eluted from the column between 640 and 700 mM KCl. The most-intense bands were at approximately 240 and 150 kDa. However, a 120-kDa band and a series of lower-MW bands were also detectable, all of which are consistent with observations in our previous study (1).

**RESULTS**

Isolation of pTP binding proteins in the NM. Ad DNA replication sites occur in punctate foci in the nucleus (12, 50; for a review, see reference 14). Previous work has also demonstrated that pTP binds tightly to the NM, thereby anchoring the Ad genome to a cytoskeleton-like structure in the nucleus (25, 55). However, because of the insolubility and complexity of this structure, little progress has been made in identifying proteins that bind pTP in replication foci on the NM. In a previous report, we described one approach to solubilizing this structure: treatment of the NM with increasing concentrations of urea; in low concentrations of urea, the nuclear lamins form a precipitate that can be removed by centrifugation (1, 24).

Using the portion of the extract that remains soluble after step dialysis, we were able to employ phosphocellulose chromatography to further purify high-MW proteins which bound to pTP. Protein fractions eluted from the P11 phosphocellulose column were assayed for pTP binding by far-Western blotting (4). These fractions 18 and 19 showed two major bands in this assay (Fig. 1A). As a negative control for the far-Western blot assay, we used a cytoplasmic extract from HeLa cells that were infected with a vaccinia virus-T7 recombinant that expressed bacteriophage T7 RNA polymerase, rather than pTP; these two high-MW protein bands were not detected when the load material was probed with this cytoplasmic extract. The detected nuclear polypeptides eluted from the column between 640 and 700 mM KCl. The most-intense bands were at approximately 240 and 150 kDa. However, a 120-kDa band and a series of lower-MW bands were also detectable, all of which are consistent with observations in our previous study (1).

Amino acid sequencing and analysis of peptides. From the 600 μg of protein present in P11 column fractions 18 and 19, we were able to purify 32 pmol of the 150-kDa band; substantially less of the 240-kDa protein was recovered. After endoproteinase Lys-C digestion and HPLC purification of the peptide fragments of the 150-kDa protein, two peaks were chosen for amino acid sequencing. Peptides designated p70 and p120 gave the following amino acid sequences: p70, FLEEAT- and p120, YDKDDDD. These sequences were compared to those of proteins in the GenBank database by using the GCG software package (Genetics Computer Group, Madison, Wis.) (Fig. 1B). Peptide p120 was an exact match for CPS I (EC 6.3.4.16), while p70 gave the following amino acid sequence: p70, FLEEAT-FLEEAT-FLEEAT-.
p70 against CPS I  p120 against CPS I

Query: FLEATVSGEHPPVLT  Query: EPLFGISGMLITQLGAAGA
CPS I: FLEATVSGQTVVIPVLT  EPSGISGMLITQLGAAGA
p70 against CAD  p120 against CAD

Query: EPLFGISGMLITQLGAAGA  Query: EPLFGISGMLITQLGAAGA
CPS I: EPLFGISGMLITQLGAAGA CAD: RPPVPGICGHQMLALLAOGA

FIG. 1. Fractionation of pTP binding proteins and amino acid sequencing. (A) Eleven milligrams of NME was loaded onto a 5-ml P11 phosphocellulose column. The retained protein was eluted with a linear KCl gradient of 100 to 1,000 mM. Twenty-microgram samples representing loaded NME (Load), flowthrough (FT), or eluted fractions (17 to 21) were separated on an 8% polyacrylamide gel and transferred to nitrocellulose. The blot was then probed with a pTP cytoplasmic extract prepared from cells infected with a vaccinia virus recombinant expressing pTP (vvpTP1). An identical blot of the loaded NME was probed with a T7-vaccinia virus recombinant cytoplasmic extract (Negative Control). Two major bands, with molecular masses of 240 and 150 kDa, were detected with pTP. The positions of molecular mass markers are shown to the left (in kilodaltons). (B) Fractions 18 and 19 were pooled, electrophoresed in batch, and blotted to a polyvinylidene difluoride membrane. The pTP-reactive fractions of molecular size markers are shown to the left (in kilobases). (A) Eleven milligrams of NME was loaded onto a 5-ml P11 phosphocellulose column. The retained protein was eluted with a linear KCl gradient of 100 to 1,000 mM. Twenty-microgram samples representing loaded NME (Load), flowthrough (FT), or eluted fractions (17 to 21) were separated on an 8% polyacrylamide gel and transferred to nitrocellulose. The blot was then probed with a pTP cytoplasmic extract prepared from cells infected with a vaccinia virus recombinant expressing pTP (vvpTP1). An identical blot of the loaded NME was probed with a T7-vaccinia virus recombinant cytoplasmic extract (Negative Control). Two major bands, with molecular masses of 240 and 150 kDa, were detected with pTP. The positions of molecular mass markers are shown to the left (in kilodaltons). (B) Fractions 18 and 19 were pooled, electrophoresed in batch, and blotted to a polyvinylidene difluoride membrane. The pTP-reactive fractions of molecular size markers are shown to the left (in kilobases).

FIG. 2. CPS I is expressed only in HeLa cells. A multitumor Northern blot containing 2 μg of poly(A)+ mRNA per lane was probed with a 0.6-kb fragment of the CPS I gene (nucleotides 3471 to 4077). The blot was hybridized at 42°C for 12 h. The lanes contained the following samples: promyelocytic leukemia (HL-60), HeLa cell, myelogenous leukemia (K562), lymphoblastic leukemia (Molt4), Burkitt's lymphoma (Raji), colorectal carcinoma (SW480), lung carcinoma (A549), and melanoma (G361). CPS I (6.0 kb) is indicated by the upper arrow. The 2.0-kb (β-actin) internal control is indicated by the lower arrow. The positions of molecular size markers are shown to the left (in kilobases).

shown). On a multitumor blot, we found that CPS I mRNA was present only in HeLa cells (Fig. 2) and not in promyelocytic leukemia (HL-60), myelogenous leukemia (K562), lymphoblastic leukemia (Molt4), Burkitt's lymphoma (Raji), colorectal carcinoma (SW480), lung carcinoma (A549), or melanoma (G361) cells. This result is in agreement with previous work indicating that CPS I expression is extremely tissue specific and is found only in hepatocytes and the intestinal mucosa (19, 28).

Because the mRNA expression profile of CPS I did not extend to tissues known to be permissive to Ad infection (such as A549), we reasoned that CPS I itself may not be the NM target for pTP binding. We therefore investigated the hypothesis that the 240-kDa protein is the actual molecule to which pTP binds during infection and that this protein exhibits homology to CPS I. Our homology searches had revealed that the p70 and p120 peptides also exhibit significant similarity to a 240-kDa CPS I-related pyrimidine synthesis enzyme, CPS II, also referred to as CAD (Fig. 1B). CAD contains a CPS domain which exhibits 50% identity and 70% similarity to CPS I (57). CAD has a wide tissue expression profile and is required for cell growth because it directly controls the synthesis of all pyrimidines (61, 68). Therefore, we tested whether CAD was present in NME from HeLa cells, whether it was released as a complex with pTP from the NM, and whether it could bind pTP in vitro.

Release of pTP-CAD complexes from the NM. Our previous work demonstrated that a high-MW pTP-containing complex was released from the insoluble NM by a mechanism that requires ATP hydrolysis and that is inhibitable by compounds known to inhibit tyrosine kinases (1). We therefore tested whether rATP treatment might result in the release of pTP and CAD from the NM as a detectable complex. We found that like pTP, CAD was also released from the NM by rATP treatment (Fig. 3A). Furthermore, under low-SDS conditions (0.1%) without heating, a complex in excess of 300 kDa was recognized by both anti-pTP (3-1A) and anti-CAD (specific for the ATCase domain within CAD) antibodies. This complex appeared to be very sensitive to proteolytic degradation, as indicated by our detection of lower-MW forms (200 and 150 kDa) whose relative abundance varied from experiment to experiment (data not shown). These data are consistent with the formation of a high-MW complex containing pTP and CAD.

pTP binds to an NM band that comigrates with the CAD enzyme. Using a polyclonal antibody directed against CAD, Western blotting was performed in tandem with the pTP far-Western analysis against HeLa cell NME (Fig. 3B). The high-MW band (240 kDa) identified by the pTP far-Western analysis comigrated with the band detected by the CAD antibody. The lower-MW band that reacted with pTP is consistent with a known CAD degradation product (25). A cytoplasmic extract from vaccinia virus-T7-infected HeLa cells was used as a negative control in these NME blotting experiments; this control demonstrated that the pTP antibody did not nonspecifically cross-react with NME protein. We concluded that the CAD enzyme is present in NME and that it may represent the pTP binding protein. Since CAD and CPS I show homology...
only in the CPS domain, it is possible that the pTP binding site lies in this domain.

**CAD is retained on the NM.** In earlier work, Fredman and Engler (25) had demonstrated that pTP remained associated with the NM despite repeated extraction with 1 M GnHCl. In an effort to impose the same criteria on the CAD-NM association, we successively extracted uninfected HeLa cell NM with 1 M GnHCl. Western blotting of the resulting protein fractions was performed with antibody to CAD. As shown in Fig. 4, CAD is retained on the NM after as many as five extractions with 1 M GnHCl. These results are in agreement with the GnHCl-resistant retention of viral proteins such as Ad pTP and cytomegalovirus pp65 on the NM (25, 54). Mammalian CAD is known to be present throughout the cell, including the nuclear lamina and the nucleolus (17). There is also evidence that the CAD equivalent in *Saccharomyces cerevisiae*, URA2, is present in the nuclear structures (46). URA2 contains a putative tripartite nuclear localization signal which has significant similarity to regions in the human CAD gene (47). Therefore, the detection of CAD in the nucleus and in the GnHCl-resistant NM was not surprising.

**pTP and CAD form immunoprecipitable complexes.** In an effort to determine if pTP-CAD complexes form in vivo, immunoprecipitations were performed. Whole-cell lysates were prepared from cells infected with a vaccinia virus recombinant expressing pTP protein. The lysates were incubated with CL-4B (protein A-Sepharose) beads to which the CAD antibody was or was not prebound. The immunoprecipitated complexes were analyzed for both CAD and pTP by Western blotting. Immunoprecipitation of CAD was clearly dependent on the presence of CAD antibody, as shown in the left panel of Fig. 5. No pTP was precipitated from the reaction mixture containing CL-4B beads alone (right panel). However, pTP coimmunoprecipitated with CAD in the presence of CAD antibody. These results suggest that a pTP-CAD complex is formed in cells expressing pTP.

Is the pTP-CAD complex formed by a direct or an indirect interaction? To address this question, we constructed epitope-tagged pTP and CAD open reading frames that could be transcribed from a T7 promoter (Fig. 6A). In vitro-translated pTP and CAD were mixed in equal amounts in immunoprecipitation buffer with protein G-Sepharose beads with or without antibodies directed against either influenza virus HA or the FLAG epitope (Fig. 6B). In this experiment, the CAD fusion was specifically precipitated by the anti-HA antibody and the pTP fusion was precipitated by the anti-FLAG antibody. When incubated together, pTP and CAD were coimmunoprecipitated with either of the two antibodies. These results suggest that there may be a direct interaction between pTP and CAD.
pTP and CAD colocalize in foci on the NM. The work of Pombo et al. (50) and Bridge et al. (13) has demonstrated that Ad replication foci form in a coordinated manner during infection. Immunofluorescence experiments indicate that pTP, Pol, DBP, and Ad DNA colocalize in early viral structures (13, 43, 50). By using confocal and digital sectioning techniques, we visualized pTP sites on the NM with reference to CAD enzyme (Fig. 7). A monoclonal antibody (IB6A8) was used to detect pTP, and a rabbit polyclonal antibody directed at the ATC domain of CAD was used; the use of this ATC-specific antibody eliminated the possibility of cross-reactivity with CPS I present in the infected HeLa cells. Ad-infected cells, harvested at 20 h p.i. and treated with DNase and 2 M NaCl, showed obvious colocalization of pTP and CAD. Both pTP and CAD displayed punctate as well as diffuse nucleoplasmic staining. Negative controls (secondary antibodies alone or pTP antibody against uninfected cells) showed no significant background (data not shown). The pTP-CAD foci numbered from 2 to 20 in a given focal plane; these values are similar to the numbers seen in other studies (50). The spherically shaped structures had a diameter of 1 μm or less at 15 h p.i. and continued to expand and change shape throughout infection. This size is similar to the 1- to 2-μm-diameter prereplicative foci observed in HSV type 1 (HSV-1)-infected cells (20). At 20 h p.i., pTP and CAD colocalized in ring-like structures which are completely consistent with the morphology described by Pombo et al. (50).

pTP-CAD foci colocalize with sites of active DNA replication. Other groups have previously demonstrated that pTP and Pol are present at sites of active virus DNA replication (14, 44, 50). To determine whether our pTP-CAD foci colocalize with sites of DNA synthesis, we pulsed Ad-infected cells with BrdU (Fig. 8). Control samples in which the BrdU pulse was omitted showed no staining when reacted with the monoclonal BrdU antibody (data not shown). We observed ringed pTP foci which colocalized and encircled sites of BrdU incorporation. This phenomenon was observed in most of the infected cells and is consistent with the morphology of Ad replication foci described by Pombo et al. (50). We found that rings of CAD surrounded sites of BrdU incorporation in Ad-infected cells in a manner identical to that of pTP. These results strongly support the interpretation that NM-bound CAD colocalizes with and anchors pTP at active sites of Ad DNA replication.

DISCUSSION

The NM network has been identified as a specialized cellular microenvironment whose domains allow important activities such as DNA replication and modification, transcription, mRNA splicing, kinase activity, and dNTP synthesis (1, 6, 32, 38, 39, 48, 67). Several viruses (such as HSV, cytomegalovirus, simian virus 40, Ad, human papillomavirus (HPV), and Epstein-Barr virus (EBV) encode proteins that appear to bind to the matrix (13, 29, 40, 54, 56, 60, 63); the activities of some of these proteins promote the rearrangement of this structure during the course of the infectious cycle (22). Additionally, we have observed that HPV type 11 E1, E2, E6, and E7 proteins all bind to the NM (59). Ad invades nuclear domains containing these activities in order to complete its replication program and produce viral particles. For example, immunofluorescence studies have demonstrated that pTP, Pol, DBP, and Ad DNA all colocalize in discrete early viral structures (reviewed in reference 14). The formation of Ad replication domains on the
NM occurs through interactions between pTP and other nuclear proteins and was therefore the target of our efforts (25, 44, 55).

In this work, we have utilized a biochemical approach to identify the nuclear protein(s) to which pTP binds during Ad infection. The extreme insolubility of the NM proteins has been an impediment to the identification of components of Ad replication foci. We have circumvented this problem by generating a soluble NME from which nuclear proteins that bind pTP can be identified. Three criteria had to be satisfied in this identification: (i) the target pTP binding site on the NM should be a preexisting component; previous evidence indicated that viral replication proteins are targeted to preexisting NM domains and can bind in the absence of a viral infection (7, 20, 40); (ii) the pTP binding protein should be expressed in cell lines capable of supporting Ad infection; and (iii) the pTP binding protein must be tightly bound to the NM and colocalize with pTP. Based on these criteria, we have provided both biochemical and microscopy experiments that suggest that Ad pTP binds directly to the CAD pyrimidine synthesis enzyme at the sites of active Ad DNA replication on the NM.

The Northern blot analysis of expression of CPS I by tissues supports our conclusion that CPS I is not likely to be the NM target of pTP binding; it is found only in HeLa cells and not in any other cell line tested, including cells known to be permissive to Ad, such as A549 (Fig. 2). In contrast, the CPS I-related enzyme CAD is known to be expressed in the spleen, kidney, heart, lung, brain, adrenal gland, muscle, small intestine, large intestine, and testis (61, 68). In fact, the expression of CAD is correlated with the rate of cell division; CAD levels are two- to fivefold higher in tumor cells than in normal cells and are almost nonexistent in quiescent cells (10, 68). This evidence, coupled with the fact that CAD expression is essential for S phase and is upregulated at the G1/S boundary by c-Myc (10), makes CAD a logical target for pTP binding during Ad infection.

In further analyses, we were able release from the NM by rATP treatment a complex that was recognized by both pTP and CAD antibodies (Fig. 3A). This complex appeared to be very labile in in vitro manipulations. Additionally, we found that the CAD enzyme comigrated with the pTP binding protein detected by far-Western blotting (Fig. 3B). Immunoprecipitation experiments demonstrated that pTP and CAD interact directly (Fig. 5 and 6). Also, the fact that CAD is tightly bound to the NM after extraction with 1 M GnHCl (as is pTP [25]) further qualifies CAD as a potential pTP binding site on the NM (Fig. 4). The CAD enzyme satisfies our initial criteria because it interacts and colocalizes with pTP and because it remains tightly associated with the NM. However, we cannot rule out the possibility that other proteins interact with pTP or may be involved in the complex with CAD.

In addition to the in vitro biochemical methods used to show a direct protein-protein interaction between CAD and pTP, digital confocal fluorescence microscopy was used to show the localization of CAD within the cell nucleus. That CAD was found to be associated with the nucleus and the NM was not unexpected. There is evidence that the yeast homolog of CAD, URA2, is in the nucleus (46) and that it contains a tripartite nuclear localization signal that is conserved in human CAD (47). A study by Chaparian and Evans (17) also showed that mammalian CAD was found in the nucleus. The fact that CAD partitions with the NM may be the result of its high MW and/or protein-protein interactions with other NM components.

By fluorescence microscopy, we found that CAD localization coincided with that of pTP in in situ NM preparations (Fig. 7). The size and distribution of our pTP-CAD foci are in agree-
ment with data from similar studies by others (8, 43, 50). We observed that prior to 15 h p.i., pTP-CAD foci were punctate, small (<1 μm in diameter), and spherical. However, from 15 to 20 h p.i., pTP-CAD foci expanded into large (>1-μm-diameter) rings or perhaps hollow spheres (50). The observation of pTP-CAD toroids at 20 h p.i. is consistent with other work showing that peripheral zones are likely to be the sites of initiation of replication while sites of elongation of viral DNA are thought to be in the center of such structures (14). Accordingly, we found that the highest concentration of BrdU was at the centers of pTP-CAD rings (Fig. 8). All of these morphological characteristics are consistent with data from previous work on Ad replication foci (14, 42). Further, the prereplicative nuclear structures of HSV-1 have a distinct punctate structure similar to those described here (20, 40).

It is possible that CAD, pTP, and other Ad replication proteins are part of a very large multienzyme complex associated with the NM. The work of Ricigliano et al. (53) suggests that DBP participates in the formation of a 650-kDa high-salt-stable complex which retains DNA binding and kinase activities. Cellular proteins such as NFI can also be recruited into Ad replication complexes (8). Cellular DNA replication complexes are known to be large and to be associated in part with the NM; for example, DNA polymerase α can be isolated from the NM as a 100S to 150S megacomplex (64). Among herpesviruses, there is also evidence for the formation of a multienzyme complex containing HSV Pol, ribonucleotide reductase, thymidine kinase, cellular dihydrofolate reductase, and nucleoside diphosphate kinase (30). These examples suggest that we cannot rule out the possibility that pTP and CAD also participate in a larger complex of enzymes. The purpose of such a complex might be twofold: (i) to provide an anchoring point at which to segregate replicated and unreplicated viral DNA within the expanding domains; and (ii) to bring viral replication into proximity with dNTP-synthetic enzymes, either to impose regulation upon them or to take advantage of nucleotide channeling, as has been suggested by Panzet and Ringer (49). The possibility that dNTP-synthetic enzymes are physically linked with the replication machinery has been considered by others (30, 51, 52). It is also possible that pTP binds other components of the NM in addition to CAD.

Recent efforts have defined ND-10 domains and their constituent proteins (such as the promyelocytic leukemia antigen [PML]) as sites of deposition of viral replicative proteins. Indeed, there is evidence that HSV-1 UL29, Ad DBP, and EBV EBNA-5 tend to be found adjacent to ND-10 (22, 40, 60). NM antigens such as B23 may also colocalize with Ad replication proteins (65). However, there is ample indication that ND-10 may not be the target of direct binding of pTP and perhaps other viral proteins. For instance, during viral infection, PML and SP100 are reorganized by the activity of the Ad E4 ORF3 protein and actually are ejected from sites where viral replication proteins are staged (22); redistribution of ND-10 proteins can also be induced by gamma interferon (34). In our experiments, we have not observed the colocalization of PML with pTP, with CAD, or with sites of BrdU incorporation into virus DNA during Ad infection (data not shown).

The use of NM domains for replication and other viral processes is a common phenomenon among DNA viruses such as HSV, simian virus 40, Ad, HPV, and possibly EBV (13, 40, 56, 60). There still remain many questions about how these viruses utilize sites on the NM during their infectious cycles and about the cellular factors required for viral replication. Biochemical characterization of these factors will be easier as new methods for disruption of the insoluble NM complex become available. By using virus proteins as tools to identify important NM domains, we may be able to understand the role that these complex nuclear structures play in regulating important viral and cellular processes.

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