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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 precurser 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.
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 Tissue 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 (Hyclone Laboratories, Logan, Utah). To prepare a pTP extract, HeLa cells were coinfected with vaccinia virus recombinants vvpTP1 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 vTF7-3 in the medium containing 5% FBS. The cells were harvested at 20 h postinfection (p.i.). Ad infections of HeLa cells for indirect immunofluorescence experiments were carried out for 15 h or 20 h at a multiplicity of infection of 10. The 3-1A polyclonal antibody recognizing pTP was raised against a carboxyterminal peptide (PEPPPPGGPRRPPRR) and was used for Western blotting and immunoprecipitation experiments (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 developed with 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (2). The pTP antibody was a kind gift from A. Verheyen. The ATP pTP complex-specific antibody was kindly provided by David Evans (Wayne State University). A monoclonal antibody directed against the FLAG epitope (DYKDDDDDE) was purchased from Kodak (Rochester, N.Y.). All other antibodies were raised against the influenza virus hemagglutinin (HA) epitope (YPDPDVPDYA) was a generous gift from Vincent Kidd (St. Judes Children's Research Hospital). A monoclonal antibody (Antibody-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 DNaše 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 mphenylisothiocyanate 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 then washed 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 2,000 × g and 4°C for 4 min. The pellet was then washed twice in the same buffer; 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 whole-cell, cytoplasmic extracts, cytoplasmic and NM extracts. One-hundred-millimeter-diameter plates of HeLa cells that either were uninfected or were infected with the vvpTP1 and vvT7 at a multiplicity of infection of 10 were harvested at 20 h postinfection (p.i.). Ad infections of HeLa cell nuclei followed by high-salt extraction of soluble proteins was assayed by techniques described previously (1). Fifty micrograms of whole-cell extract (WCE) was incubated with 3 mM ribo-ATP (rATP) in phosphorylation buffer (20 mM HEPES [pH 7.5], 5 mM MgCl2, 1 mM DTT, and 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride [AEBSF]). Microchem, Cambridge, Mass.). Two peaks from the HPLC purification, which were designated p70 and p120, were subjected to amino acid sequence analysis.

PCR cloning, and Northern blot analysis of CPS 1 in multiple cell lines. Oligonucleotide primers were designed which reflected the amino acid sequence of peptide p120 (RSQVHEI; residues 1157 to 1162) except that third-base wobble was introduced so that potential naturally occurring isomers would not be excluded. A downstream primer was chosen in a region which was well conserved between CPS 1 and CAD (STGFKP; residues 1352 to 1355). These primers were synthesized as follows: 5′ primer, 5′-AGAGTTCAGCAGA/A/GATG-3′; and 3′ primer, 5′-GGG(T/G)ATCTTAAA(G/T)CC(A/T)GTG-3′. With these primers, a 0.6-kb DNA fragment from the pDR2 HeLa cell cDNA plasmid library (Clontech, Palo Alto, Calif.) was amplified, using the Advantage amplification kit (Clontech). The DNA fragment was cloned into the pGEM-T vector by standard techniques (2). Sequence analysis and comparison of the clone to the Genbank databases confirmed its identity as CPS 1. The 0.6-kb fragment was excised from the gel and ligated into the XbaI-NorI sites of plasmid (pDR2). The construct was excised from the membrane for proteolytic digestion with endoproteinase Lys-C, high-performance liquid chromatographic (HPLC) purification of the resulting peptides, and amino acid sequence analysis performed (by Harvard Microchem, Cambridge, Mass.). Two peaks from the HPLC purification, which were designated p70 and p120, were subjected to amino acid sequencing.

PCR amplification and cloning. A PCR-generated DNA fragment from the pDR2 plasmid library (Clontech, Palo Alto, Calif.) was PCR amplified, using the Advantage amplification kit (Clontech). The DNA fragment was cloned into the pGEM-T vector by standard techniques (2). Sequence analysis and comparison of the clone to the Genbank databases confirmed its identity as CPS 1. The 0.6-kb fragment was excised from the gel and ligated into the XbaI-NorI sites of plasmid (pDR2). The construct was excised from the membrane for proteolytic digestion with endoproteinase Lys-C, high-performance liquid chromatographic (HPLC) purification of the resulting peptides, and amino acid sequence analysis performed (by Harvard Microchem, Cambridge, Mass.). Two peaks from the HPLC purification, which were designated p70 and p120, were subjected to amino acid sequencing.

Plasmid constructs. The immunoprecipitation experiment (see Fig. 6B) was conducted with epitope-tagged CAD and pTP proteins. Both epitope linkers were designed to encode the Kozak consensus translational start sequence (ACGATG) to enhance initiation of translation (35). The predicted amino acid sequence of the (His)6-HA linker was constructed using complementary oligonucleotides with the sequence of the (His)7-HA linker being: MGHHHHHHHVYPYDVPDYASLG. The predicted amino acid sequence of the (His)7-HA linker was constructed using complementary oligonucleotides with the sequence of the (His)7-HA linker being: MGHHHHHHHVYPYDVPDYASLG. The predicted amino acid sequence of the (His)7-HA linker was constructed using complementary oligonucleotides with the sequence of the (His)7-HA linker being: MGHHHHHHHVYPYDVPDYASLG.
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 rATP resulted in the release of pTP-NM protein complexes, some of which were of very high molecular weight (MW) (1). A second strategy for release of proteins from the NM is extraction with 8 M urea. Urea extraction releases a large number of proteins, including the nuclear lamins, which act like intermediate filaments within the nucleus (24). The nuclear lamins can then be isolated by step dialysis against decreasing 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. A two- to three-band pattern of pTP binding was observed in each of the fractions. The most intense bands were at 240 and 600 kDa. 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, FLEAT-RVSOEHPVVLTT; and p120, EPLFGISTGNLITGLAAGA. 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 differed from CPS I by only 2 residues. CPS I is a liver-specific urea cycle enzyme (19, 28).

Northern blot analysis of CPS I tissue expression. Using primers described in Materials and Methods, a DNA fragment of 0.6 kb was amplified from the pDR2 HeLa cell cDNA library. Southern blots of this fragment were done against the GenBank database. The fragment was compared to those of proteins in the GenBank database by using the GCG software package (Genetics Computer Group, Madison, Wis.). None of these showed a significant background (data not shown).

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acid positions 1110 to 1126 and 246 to 264, respectively, of CAD (right). p120 to the related CAD enzyme. Peptides p70 and p120 corresponded to amino acids 288 to 306 (left). The Blast search also revealed the homology of p70 and is the result of a Blast search of the GenBank database. Peptide p70 corre-

Lys-C digestion of the 150-kDa band were determined. The pileup of sequences batch, and blotted to a polyvinylidene difluoride membrane. The pTP-reactive band of 150 kDa was excised and subjected to amino acid sequencing. The amino

acid sequences of two peptides (p70 and p120) obtained by endoproteinase

left (in kilodaltons). (B) Fractions 18 and 19 were pooled, electrophoresed in

flowthrough (FT), or eluted fractions (17 to 21) were separated on an 8%

column. The retained protein was eluted with a linear KCl gradient of 100 to

(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 pTP cytoplasmic extract prepared from cells infected with a vaccinia virus

8 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 posi-

tions of molecular size markers are shown to the left (in kilobases).

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 Con-

The 2.0-kb (β-actin) internal control is indicated by the lower arrow. The posi-

tions 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 posi-

tions 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 promyelo-

cytic leukemia (HL-60), myelogenous leukemia (K562), lym-

phoblastic leukemia (Molt4), Burkitt’s lymphoma (Raji), colore-

cctal carcinoma (SW480), lung carcinoma (A549), or melano-

cma (G361) cells. This result is in agreement with pre-

vious 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 hypoth-

thesis that the 240-kDa protein is the actual molecule to which

pTP binds during infection and that this protein exhibits ho-

mology 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 do-

main 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-

est-MW band (240 kDa) identified by the pTP far-Western analysis comigrated with the band detected by the CAD anti-

body. The lower-MW band that reacted with pTP is consistent with a known CAD degradation product (23). 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 nonspe-

cifically 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.

**FIG. 3.** The CAD enzyme comigrates with the 240-kDa NM band detected by pTP far-Western blotting. (A) Thirty micrograms of supernatant from rATP-treated pTP NM was either heated or not in the presence of Laemmli buffer with 0.1% SDS. Samples were applied to an SDS–8% polyacrylamide gel in duplicate. The resultant halves of the blot were probed with either anti-pTP (αTP) or anti-CAD (αATC) antibodies. The arrow to the right indicates the position of the high-MW pTP- and CAD-containing complex. The positions of molecular mass markers are shown to the left (in kilodaltons). (B) Thirty micrograms of NME was separated on an SDS–8% polyacrylamide gel and blotted to nitrocellulose. A portion of the blot was probed with anti-CAD antibody (αCAD Western). The other lanes were probed with either pTP extract (pTP Far Western) or T7-vaccinia virus cytoplasmic extract as a control for the far-Western blotting (Negative Control). Far-western blot lanes were then developed to show the presence of a pTP interaction with the polyclonal anti-pTP antibody (3-1A). The arrow indicates the 240-kDa band detected by pTP which comigrates with CAD. The positions of molecular mass markers are shown to the left (in kilodaltons).

**FIG. 4.** CAD is tightly associated with the NM. Each lane contains 20 μg of uninfected-HeLa-cell NM which was washed with 1 M GnHCl for the number of times indicated above the blot. The blotted protein was probed with anti-CAD antibody raised against the ATC domain. On the left is indicated the position of the 206-kDa molecular mass marker.

**FIG. 5.** CAD and pTP coimmunoprecipitate from HeLa cell extracts. Fifty micrograms of whole-cell extract prepared from HeLa cells programmed to express pTP was mixed with either protein A-Sepharose beads alone or beads to which anti-CAD (αCAD) antibody was prebound (indicated by plus and minus symbols). Duplicate immunoprecipitation reaction mixtures were applied to an 8% polyacrylamide gel. Protein blotted to nitrocellulose was probed with either αCAD or antibody to pTP (αTP). The positions of molecular mass markers are shown to the left of each blot (in kilodaltons).
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

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**FIG. 6. Coimmunoprecipitation of in vitro-translated pTP-CAD fusions.**

- **A.** The diagram at the top represents the epitope-tagged constructs that were used. The fusion proteins were cotranslationally labeled with [35S]methionine.
- **B.** The products were mixed with either protein G (Prot-G) alone or protein G pre-bound with monoclonal antibody to HA (αHA) or FLAG (αFLAG). The combinations for each reaction are indicated above the gel (by plus and minus symbols). The gel was visualized with a Storm PhosphorImager (Molecular Dynamics). Immunoprecipitation of pTP and CAD proteins is indicated by the arrows. The positions of molecular mass standards are shown to the left (in kilodaltons).

**FIG. 7. pTP and CAD colocalize in foci on the NM.**

HeLa cells were grown on coverslips and infected with Ad for 20 h. The cells were treated with DNase and extracted with 2 M NaCl to create in situ NM. Double staining with anti-pTP monoclonal antibody (IB6A8) and anti-CAD polyclonal antibody specific for the ATC domain was performed. (A) pTP was visualized with TXRD-conjugated goat anti-mouse antibody. (B) CAD was visualized with FITC-conjugated goat anti-rabbit antibody. (C) Merged image of pTP and CAD staining.
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 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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