

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

PETER C. ANGELETTI AND JEFFREY A. ENGLER*

*Department of Biochemistry and Molecular Genetics, Schools of Medicine and Dentistry,
University of Alabama at Birmingham, Birmingham, Alabama 35294-0005*

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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 deoxynucleoside 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 6.3.5.5], 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

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Genetics, Room 460, Basic Health Sciences Building, University of Alabama at Birmingham, Birmingham, AL 35294-0005. Phone: (205) 934-4734. Fax: (205) 934-0758. E-mail: Jengler@bmg.bhs.uab.edu.

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 (FBS; HyClone Laboratories, Logan, Utah). To prepare a pTP extract, HeLa cells were coinfectd 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 overlaid with Dulbecco's modified Eagle's 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 or 20 h at a multiplicity of infection of 10.

The 3-1A polyclonal antibody recognizing pTP was raised against a carboxy-terminal peptide (PEPLPPGARPRRRRC) 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 Biotechnology Associates, Birmingham, Ala.) and developed with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (2). The CAD polyclonal antibody and the CAD ATC region-specific antibody were kindly provided by David Evans (Wayne State University). A monoclonal antibody directed against the FLAG epitope (DYKDDDDDE) was purchased from Kodak (New Haven, Conn.). Monoclonal 12CA5 antibody raised against the influenza virus hemagglutinin (HA) epitope (YPYDVPDYA) was a generous gift from Vincent Kidd (St. Jude's Children's Research Hospital). A monoclonal antibody (Antibody-1) used to detect BrdU 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 2.5×10^6 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 $2,000 \times g$ and 4°C 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 $2,000 \times g$ and 4°C for 4 min. The pellet was then washed several times in digestion buffer and finally resuspended in 100 μ l 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 extracts, cytoplasmic extracts, and NME. One-hundred-millimeter-diameter plates of HeLa cells that either were uninfected or were coinfectd with the vvpTP1 and vvt7 viruses (multiplicity of infection of 7 for each virus) were resuspended in hypotonic buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM dithiothreitol [DTT]) and allowed to swell on ice for 15 min. The cells were lysed by 10 strokes of a tight-clearance Dounce homogenizer. Preparation of the whole-cell extract was similar to preparation of a nuclear extract except that the post-Dounce lysate was adjusted to 100 mM NaCl and incubated on ice for 1 h (15). Soluble protein was recovered by centrifugation at $15,000 \times g$ and 4°C for 10 min. Cytoplasmic supernatants were recovered from nuclei immediately after Dounce homogenization by centrifugation at $2,000 \times g$ and 4°C for 10 min and clarified by an additional centrifugation at $15,000 \times g$ and 4°C for 4 min (15).

NM extract (NME) was prepared by extraction of NM with 8 M urea (1, 5, 24). First, the NM was washed in digestion buffer containing 1 mM PMSF. The NM pellet was extracted with a threefold-volume equivalent of disassembly buffer [8 M urea, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.6), 1 mM EDTA, 0.1 mM MgCl₂, 1% β -mercaptoethanol, 1 mM PMSF]. The collected NME supernatant was then step dialyzed for 2 h in 1,000 volumes of the same buffer with 4 M urea followed by successive 2-h dialysis steps in disassembly buffer containing 2 M, 1 M, and then 0.1 M urea at 4°C. The final dialysis step was in 1,000 volumes of buffer B (20 mM NaCl, 20 mM HEPES [pH 7.5], 5 mM MgCl₂, 1 mM DTT, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSEF; Calbiochem, La Jolla, Calif.) overnight at 4°C. The NME was clarified by centrifugation at $15,000 \times g$ and 4°C for 4 min.

Complex release assay. Phosphorylation-dependent release of pTP-CAD complexes was assayed by techniques described previously (1). Fifty micrograms of pTP-NM was incubated with 3 mM ribo-ATP (rATP) in phosphorylation buffer (30 mM NaCl, 15 mM Tris-HCl [pH 7.0], 3 mM MgCl₂, and 1 mM DTT with 1 mM AEBSEF) for 1 h at 25°C. Supernatants recovered by centrifugation were

resuspended in Laemmli buffer with 0.1% sodium dodecyl sulfate (SDS) (36). Samples (either heated or unheated) were then electrophoresed on an SDS-8% polyacrylamide gel. The protein was transferred to nitrocellulose and probed with anti-pTP or anti-ATC (CAD) antibodies.

pTP far-Western assay. The pTP far-Western blotting experiment was performed as previously described (1). Protein samples were heated in Laemmli denaturing buffer and electrophoresed on an SDS-8% polyacrylamide gel (36). The proteins separated by size in the gel were then transferred to nitrocellulose and blocked against 5% dry milk protein in PBS. The blot was gently rocked at 4°C overnight with 1 mg of cytoplasmic extract from cells infected with vvpTP1 and vvt7 in 5 ml of PBS with 1% dry milk protein and 2 mM AEBSEF. Negative-control blots were probed with vvt7 cytoplasmic extract alone. The blot was rinsed with PBS for 10 min at room temperature. The subsequent development of the blot was performed in the same manner as a Western blot (2).

Fractionation of NM proteins. Eleven milligrams of NME isolated from uninfected HeLa cell nuclei was diluted into Dignam buffer and passed over a 5-ml P11 phosphocellulose column at a rate of 0.5 ml/min (21). The column was washed with 10 volumes of buffer D (20 mM HEPES [pH 7.5], 20% glycerol, 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 μ g 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) (2, 41). The blot was rinsed with PBS three times. A strip was cut from the blot and was developed for determination of pTP interaction as described for the far-Western blot. The remaining section of the blot was rinsed with sterile deionized H₂O and stained for protein with Ponceau S (Sigma Chemical Co., St. Louis, Mo.; final concentration, 0.5%) (2, 41). The bands reacting with pTP were identified by lining up the two sections of the blot. The 150-kDa reactive band 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, cloning, and Northern blot analysis of CPS I in multiple cell lines. Oligonucleotide primers were designed which reflected the amino acid sequence of peptide p120 (RVSQEH; 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 I and CAD (STGFKIP; residues 1352 to 1358). These primers were synthesized as follows: 5' primer, 5'-AGAGTTTCTCAGGA(A/G)CAT-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 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 I. The 0.6-kb fragment with excised by *Apal*-*NotI* digestion. Twenty-five nanograms of probe DNA was labeled with 20 μ Ci of [α -³²P]dATP (3,000 Ci/mmol) by random hexamer labeling (Boehringer Mannheim, Indianapolis, Ind.). The probe was then heat denatured for 5 min at 100°C. To obtain a CPS I mRNA expression profile, multitissue and multitumor blots (Clontech) were probed. The blots were incubated in Expresshyb solution (Clontech) at 42°C for 2 h with agitation. Hybridization was carried out at 42°C for 12 h with agitation. The blots were also probed with β -actin as an internal standard. The Northern blots were then washed with $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.05% SDS for 40 min at 50°C followed by a wash with $0.1 \times$ SSC-0.1% SDS for 40 min at 50°C (2). The dried blots were visualized with a model 860 Storm PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

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 (AC CATGG) to enhance initiation of translation (35). The predicted amino acid sequence of the (His)₇-HA linker is MGHHHHHHHHVYPYDVPDYASLG. The (His)₇-HA linker was constructed using complementary oligonucleotides with *EcoRI* and *NotI* sites on the ends as follows (with underlining indicating the start site for translation of the modified gene): top strand, 5'-AATTCGCGCACCA TGGGGCCACCATCACCATCACCATCAGCTATCCTTATGAGCCATCCCT GACTATCCAGCCTGGGC-3'; and bottom strand, 5'-GGCCGCCACCGC TGGCATAGTCAGGGACGTCATAAGGATAGACGCTGATGGTGTATGGT GATGGTGGCCCATGGTGCAGCG-3'. The top and bottom strands were mixed in equimolar amounts, heated to 70°C for 10 min, and slowly cooled to room temperature. The pcDNA3.1+ plasmid (Invitrogen, San Diego, Calif.) was digested with *EcoRI* and *NotI* (New England Biolabs, Beverly, Mass.) and gel purified. The double-stranded linker was subcloned into pcDNA3.1+. The full-length human CAD cDNA was then excised from pCAD6 by *NotI*-*Apal* (New England Biolabs) digestion (33). The CAD cDNA was then subcloned into the

pcDNA3.1+ clone containing the (His)₇-HA tag. This construct was named pcDNA-CAD. Similarly, a (His)₇-FLAG epitope tag linker was designed as a fusion to pTP. The predicted amino acid sequence of the (His)₇-FLAG linker is MGHHHHHHHYKDDDD. The (His)₇-FLAG linker was constructed with *Hind*III and *Eco*RI sites on the ends as follows: top strand, 5'-AGCTTGGCGCA CCATGGGGCACCATACCATACCATACGACTACAAGGACGACGA TGACG-3'; and bottom strand, 5'-AATTCGTCATCGTCGTCCTTGTAGT CGTGATGGTGATGGTGATGGTCCCCATGGTGC-3'. The pcDNA3.1+ vector was digested with *Hind*III and *Bam*HI and then gel purified. Ad type 2 pTP was excised from pGEM-pTP by digestion with *Eco*RI and *Bam*HI (New England Biolabs), gel purified, and ligated to the (His)₇-FLAG double-stranded linker. The resultant product was subjected to *Hind*III-*Bam*HI 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 vvpTP1-vvT7 coinfection. Five micrograms of polyclonal antibody to CAD was allowed to bind to a 70- μ l 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% Nonidet P-40, 0.1% Triton X-100, 0.005% 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- μ l bed volume of beads for 2 h at 4°C with rotation. As a negative control, lysates were incubated with beads lacking antibody. Immunoprecipitates 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 50- μ l total volume with 2 μ g of plasmid DNA in the presence of 40 μ Ci 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 μ l 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 guanidinium hydrochloride (GnHCl; Sigma) in accordance with the protocol of Fredman and Engler (25). After each extraction, the NM pellet was recovered by centrifugation at 15,000 \times g and 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); this was followed by Western blot analysis of 20 μ g of protein with antibody raised against the CAD enzyme.

Immunofluorescence of *in situ* NM. HeLa cells were seeded on coverslips and allowed to grow to 60% confluence. The cells were then infected with Ad type 2 at a multiplicity of infection of 10. At 15 or 20 h p.i., the cells were harvested, extracted, and fixed in accordance with the protocol of Pombo et al. (50). Infected cells were pulsed with 1 mM BrdU for 20 min prior to harvesting (48, 50). Adherent cells were washed with PBS. The cells on the coverslips were immediately extracted *in situ* with 0.5% Triton X-100 in CSK buffer (10 mM HEPES [pH 7.5], 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM PMSF). The cells were rinsed with CSK buffer, incubated with 0.1- μ g/ml DNase in CSK buffer for 10 min at 25°C, and then extracted with 2 M NaCl in CSK buffer on ice for 5 min. The remaining cells were fixed with 4% paraformaldehyde in CSK buffer. Alternatively, cells were fixed after washing with 0.5% Triton X-100 prior to DNase treatment; this protocol gave identical results. Coverslips were pre-blocked with 5% FBS in PBS for 30 min. Primary antibodies to CAD or pTP were diluted 1:50 and 1:25 for the anti-BrdU antibody in PBS with 1 mM PMSF. Coverslips were inverted on individual beads of antibody solutions for 2 h at 4°C and then rinsed in PBS for 10 min. Secondary antibodies bearing FITC or TRXR were incubated in the same manner at dilutions of 1:100 each. Coverslips were mounted in Vectashield antifade medium (Vector Laboratories, Burlingame, Calif.). Optical sections of 0.5 μ m in diameter were obtained with a model 2000 confocal microscope (Molecular Dynamics) equipped with a 100 \times oil immersion objective. Images were captured with a Silicon Graphics workstation and stored as Adobe 3.0 files (Adobe Systems, Mountain View, Calif.). Alternatively, an Olympus IX70 microscope with a 100 \times objective, equipped with digital confocal functions, was used. Images were captured with IPLab Spectrum software (Signal Analytics, San Jose, Calif.), and digital sections (of 1 μ m in thickness) were obtained with the power microtome function. As negative controls, infected cells were probed with secondary antibodies alone or uninfected cells were stained

with either polyclonal or monoclonal anti-pTP antibodies followed by secondary antibodies. None of these showed a significant background (data not shown).

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; 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 at 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 μ g 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, FLEET-RVSEQHPVVLT; 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. Blast searches done against the GenBank database indicated that the 0.6-kb DNA fragment was CPS I. Multitissue Northern blots were probed with the 0.6-kb CPS I fragment to obtain a profile of CPS I expression. Normal tissues (spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes) expressed no detectable CPS I mRNA (data not

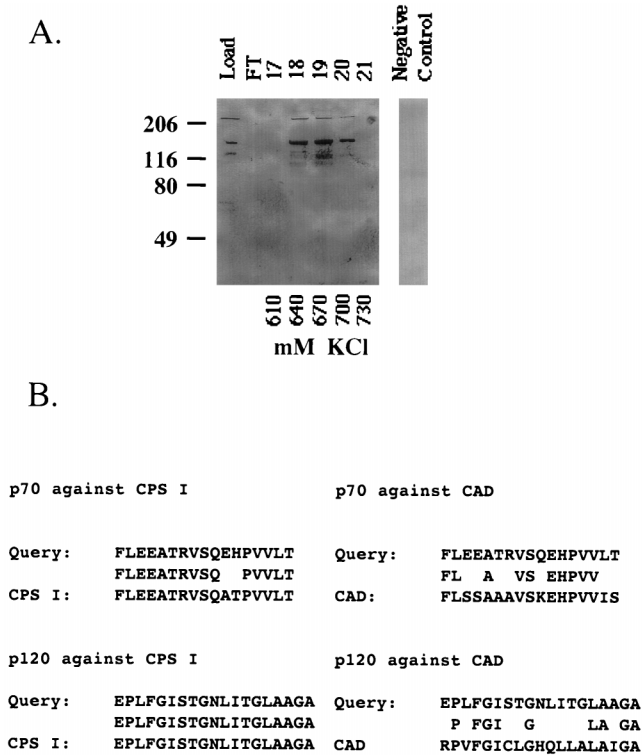


FIG. 1. Fractionation of pTP binding proteins and amino acid sequencing. (A) Eleven milligrams of NME was loaded onto a 5-ml P11 phosphocellulose column. The retained protein was eluted with a linear KCl gradient of 100 to 1,000 mM. Twenty-microgram samples representing loaded NME (Load), flowthrough (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 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 Lys-C digestion of the 150-kDa band were determined. The pileup of sequences is the result of a Blast search of the GenBank database. Peptide p70 corresponded to amino acids 1151 to 1167 of CPS I, and p120 corresponded to amino acids 288 to 306 (left). The Blast search also revealed the homology of p70 and p120 to the related CAD enzyme. Peptides p70 and p120 corresponded to amino acid positions 1110 to 1126 and 246 to 264, respectively, of CAD (right).

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,

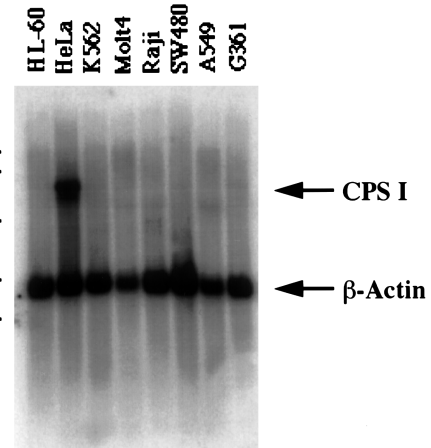


FIG. 2. CPS I is expressed only in HeLa cells. A multitumor Northern blot containing 2 μ g of poly(A)⁺ mRNA per lane was probed with a 0.6-kb fragment of the CPS I gene (nucleotides 3471 to 4077). The blot was hybridized at 42°C for 12 h. The lanes contained the following samples: promyelocytic leukemia (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).

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 highest-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 (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 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

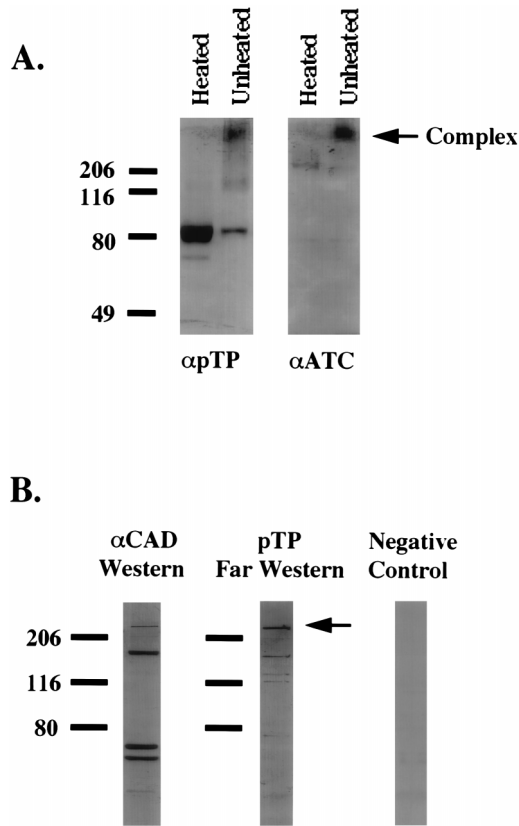


FIG. 3. The CAD enzyme comigrates with the 240-kDa NM band detected by pTP far-Western blotting. (A) Thirty micrograms of supernatant from rATP-treated pTP NM was either heated or not in the presence of Laemmli buffer with 0.1% SDS. Samples were applied to an SDS-8% polyacrylamide gel in duplicate. The resultant halves of the blot were probed with either anti-pTP (α pTP) or anti-CAD (α ATC) 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 (α CAD 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).

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 GdnHCl. In an effort to impose the same criteria on the CAD-NM association, we successively extracted uninfected HeLa cell NM with 1 M GdnHCl. 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 GdnHCl. These results are in agreement with the GdnHCl-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,

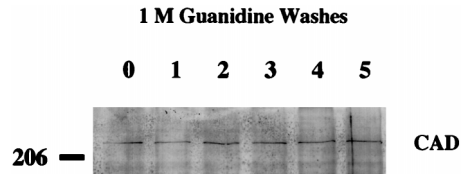


FIG. 4. CAD is tightly associated with the NM. Each lane contains 20 μ g of uninfected-HeLa-cell NM which was washed with 1 M GdnHCl for the number of times indicated above the blot. The blotted protein was probed with anti-CAD antibody raised against the ATC domain. On the left is indicated the position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

the detection of CAD in the nucleus and in the GdnHCl-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.

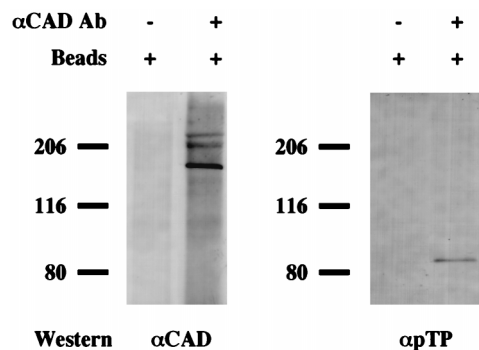


FIG. 5. CAD and pTP coimmunoprecipitate from HeLa cell extracts. Fifty micrograms of whole-cell extract prepared from HeLa cells programmed to express pTP was mixed with either protein A-Sepharose beads alone or beads to which anti-CAD (α CAD) antibody was prebound (indicated by plus and minus symbols).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

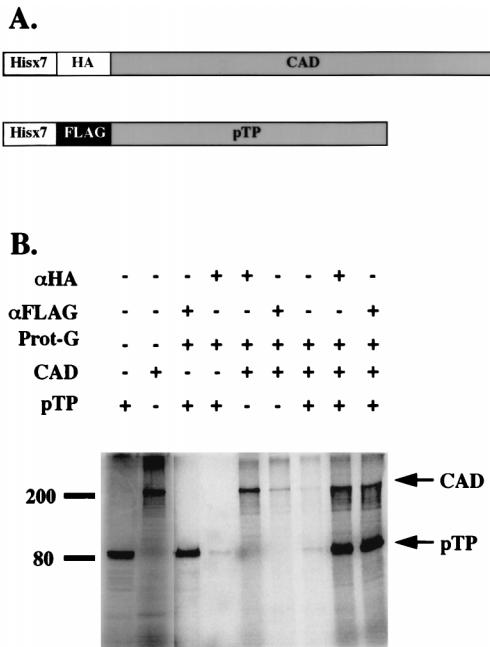


FIG. 6. Coimmunoprecipitation of in vitro-translated pTP-CAD fusions. (A) The diagram at the top represents the epitope-tagged constructs that were used. The fusion proteins were cotranslationally labeled with [³⁵S]methionine. (B) The products were mixed with either protein G (Prot-G) alone or protein G prebound 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).

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

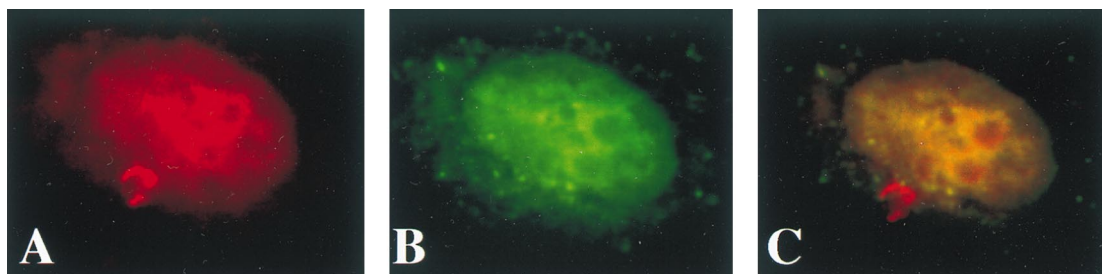


FIG. 7. pTP and CAD colocalize in foci on the NM. HeLa cells were grown on coverslips and infected with Ad for 20 h. The cells were treated with DNase and extracted with 2 M NaCl to create in situ NM. Double staining with anti-pTP monoclonal antibody (IB6A8) and anti-CAD polyclonal antibody specific 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.

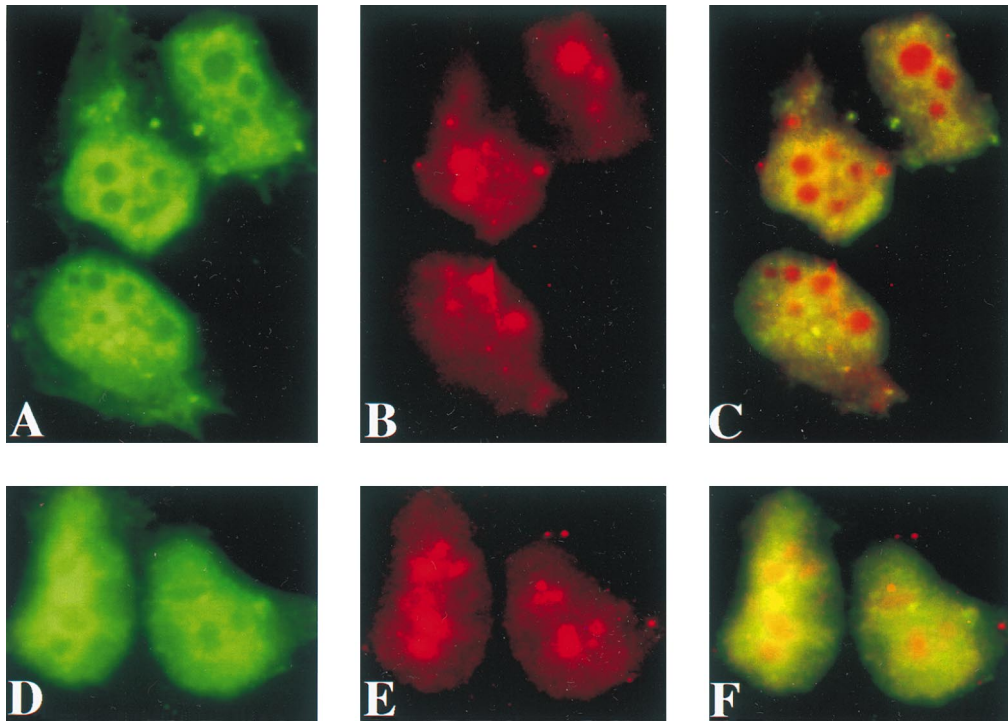


FIG. 8. pTP and CAD colocalize with sites of active replication on the NM. Cells infected with Ad for 20 h were pulsed with BrdU for 20 min. Cells were double stained either with anti-pTP polyclonal antibody (A) and anti-BrdU monoclonal antibody (B) or with anti-CAD polyclonal antibody (D) and anti-BrdU monoclonal antibody (E). Polyclonal antibodies to the CAD ATC domain and pTP were reacted with FITC-conjugated goat anti-rabbit IgG, while the monoclonal antibody to BrdU was detected with TXRD-conjugated goat anti-mouse IgG. (C) Merged image of pTP and BrdU staining. (F) Merged image of CAD and BrdU 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 to 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 Panzeter 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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