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# Tyrosine Kinase-Dependent Release of an Adenovirus Preterminal Protein Complex from the Nuclear Matrix

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**Adenovirus (Ad) replicative complexes form at discrete sites on the nuclear matrix (NM) through the interaction of Ad preterminal protein (pTP). The NM is a highly salt-resistant fibrillar network which is known to anchor transcription, mRNA splicing, and DNA replication complexes. Incubation of rATP with NM to which pTP was bound caused the release of pTP as a pTP-NM complex with a size of 220 to 230 kDa; incubation with 5′ adenylylimidodiphosphate (rAMP-PNP) showed no significant release, indicating that rATP hydrolysis was required. With NM extracts, it was shown that a pTP-NM complex which was capable of binding Ad origin DNA could be reconstituted in vitro. A number of high-molecular-weight NM proteins ranging in size from 120 to 200 kDa were identified on Far Western blots for their ability to bind pTP. rATP-dependent release of pTP from the NM was inhibited in a dose-dependent fashion by the addition of tyrosine kinase inhibitors, such as quercetin, methyl-2,5-dihydroxycinnamate, or genistein. NM-mediated phosphorylation of a poly(Glu, Tyr) substrate was also significantly abrogated by the addition of these compounds. rATP-dependent release of Ad DNA termini bound to the NM via pTP was also blocked by the addition of these inhibitors. These results indicate that a tyrosine kinase mechanism controls the release of pTP from its binding sites on the NM. These data support the concept that phosphorylation may play a key role in the modulation of pTP binding sites on the NM.**

Initiation of adenovirus (Ad) DNA replication involves a protein-priming mechanism which requires the interaction of three viral proteins and two host proteins with the origin (14, 15, 53). The Ad DNA polymerase and the precursor to the terminal protein (pTP) form a heterodimer which specifically binds to the origins found at each end of the linear DNA genome. A dCMP residue then becomes covalently linked to pTP through the serine at position 580 in the protein. This nucleotide provides a 3′ hydroxyl group to begin synthesis of a daughter Ad DNA strand. Nuclear factors I and III, which enhance initiation, are cellular proteins that bind to the Ad origin between bases 18 to 40 and 40 to 52, respectively (46). Ad DNA-binding protein stabilizes the single-stranded regions of the origin during synthesis. It has become clear that pTP mediates attachment of the Ad origin to specific sites on the nuclear matrix (NM) (7, 49, 60). Replication studies done by Fredman and colleagues (23, 24) showed that pTP mutants (p233, p340, and p344) were wild type in in vitro replication assays but were defective for replication in vivo. These results pointed to a critical role for pTP, potentially in the formation of replicative complexes at the NM. Other studies report that the pTP association with the NM is also important for transcription of the early genes (49). We were therefore interested in understanding the nature of the interaction between pTP and the NM.

The NM is a complex protein network in the nucleus which can be revealed by DNase treatment followed by high-salt or lithium-3,5-diiodosalicilate extraction (3, 4, 36). The nuclear infrastructure consists of internal core filaments, lamina, and integral membrane proteins which, together, provide the structural integrity of the nucleus. Apart from providing structural support, the NM appears to define discrete regions for enzyme activity (2). Virtually all genomic DNA is organized into supercoiled loops which are attached to the NM via matrix attachment regions. It is thought that matrix attachment regions coincide with origins of replication (9). The NM is involved in many important cellular activities, such as transcription, mRNA splicing, mRNA transport, chromatin organization, and DNA replication (6, 28, 35, 49). Therefore, it is not surprising that sites of Ad replication and transcription are discretely localized to foci in the nuclear substructure (11, 37). The interaction of Ad pTP with the NM may link these cellular processes with the viral life cycle.

The integrity and function of the NM is modulated by posttranslational modifications. Changes in the activity and morphology of the nucleus throughout the cell cycle require the NM to be implicitly dynamic. There is ample evidence that NM proteins are the targets of posttranslational activities, such as phosphorylation, methylation, isoprenylation, proteolysis, and perhaps glycosylation (22, 27, 34, 35). The well-characterized NM proteins, lamins, serve as examples of most of these processes. Phosphorylation appears to be a major control mechanism of the NM. Lamin proteins contain two $p34^{cdc2}$ phosphorylation sites which play a direct role in polymer dynamics (39). Nuclear disassembly is driven by the hyperphosphorylation of lamin filaments (21). Dessev et al. (17) described a protein kinase activity which is associated with the NM and which is directed toward lamin and inhibitable by quercetin. In these studies, solubilization of lamin complexes from the nucleus was shown to be dependent on an endogenous kinase activity (16). Similarly, the phosphorylation state of Rb appears to control its binding to an NM component (p84) (18). Rb has also been shown to directly bind lamin A and C (33). There is also evidence of both casein kinase II and tyrosine kinase activity associated with the NM (40, 54). We reasoned that these NM

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kinase activities may play a role in controlling the binding of pTP-NM complexes or pTP itself to the NM in a manner similar to that for other NM proteins. Here, we report the presence of a tyrosine kinase activity which is associated with the human cell NM and which controls the release of prereplicative pTP complexes from the NM.

## MATERIALS AND METHODS

**Cell lines, viruses, and antibodies.** HeLa cells were used for the analysis of the pTP-NM interaction. Cells were grown in monolayer cultures at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Ad infections were done at a multiplicity of infection of 10. Virus was allowed to bind to the cells for 1 h in 4 ml of Dulbecco's modified Eagle's medium without fetal bovine serum. The infected cells were washed with phosphate-buffered saline (PBS) and then given 8 ml of Dulbecco's modified Eagle's medium with 5% fetal bovine serum. The cells were harvested at 15 h postinfection (p.i.). High-level expression of pTP was achieved by utilizing a recombinant vaccinia virus containing Ad type 5 pTP (vvpTP1; 23) under the control of a T7 promoter. A second virus containing the T7 RNA polymerase (vvT7; 25) was coinfected into the cells. The cells were infected at a multiplicity of infection of 10 for each virus and harvested at 20 h p.i.

A rabbit polyclonal antibody (3-1A) recognizing the pTP was raised to a carboxy-terminal peptide (PEPPLPPGARPRRRC) which was conjugated to keyhole limpet hemocyanin. The 3-1A antibody was used at room temperature for developing Western blots (immunoblots) at a dilution of 1:1,000. The polyclonal antibody used for the detection of human lamin proteins was the generous gift of Robert Goldman (Northwestern University School of Medicine). A goat anti-rabbit immunoglobulin G to which alkaline phosphatase was conjugated (Southern Biotechnology Associates, Birmingham, Ala.) was used as the secondary antibody. A monoclonal antibody (MAb) (IB6A8) was raised to purified pTP and was used for the supershift of gel-shifted pTP-DNA complexes.

**Preparation of NM.** The protocol used for the preparation of NM was similar to that described by Mirkovitch et al. (36). Uninfected or vvpTP1- or Ad-infected HeLa cells were fractionated and nuclei were recovered by the techniques described by Challberg and Kelly (13). Typically, five to seven plates (100-mm diameter) of confluent cells were harvested. Isolated nuclei were resuspended in 500 μl of digestion buffer (20 mM Tris-HCl [pH 7.4], 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and subjected to DNase I digestion for 10 min at room temperature at a final concentration of 0.1 μg/ml (36). For Southern blot analysis of Ad DNA ends, Ad-infected nuclei were incubated with *Hin*dIII enzyme at a concentration of 1 U/μl in the appropriate buffer for 2 to 3 h at 37°C. The nuclei were then resuspended in digestion buffer with 0.1% digitonin and incubated at room temperature for 5 to 10 min. The nuclear pellet was recovered by centrifugation at 10,000 × *g* in a microcentrifuge. The nuclear material was then extracted with high-salt buffer (2 M NaCl, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 20 mM EDTA) for 5 min on ice. The resultant nuclear pellet was washed several times in digestion buffer and finally resuspended in 100 μl of buffer with 1 mM PMSF. Protein concentrations of the NM preparations were determined by the method of Bradford (Bio-Rad, Richmond, Calif.) (10). Generally, concentrations of 5 to 10 μg/μl were achieved.

**Preparation of cytoplasmic extracts and nuclear matrix extracts (NME).** One-hundred-millimeter-diameter plates of HeLa cells which were coinfected with the vvpTP1 and vvT7 viruses as previously described were resuspended in hypotonic buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 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. The nuclear material was pelleted at 2,000 × *g* for 10 min at 4°C in a microcentrifuge. The cytoplasmic supernatant was recovered and clarified by an additional centrifugation at 15,000 × *g* for 4 min (13).

NME was prepared by either an rATP incubation or an 8 M urea extraction of NM. The development of the rATP NME was based on previous work which showed that phosphorylation of many NM proteins caused their solubilization (16, 21, 22, 26). The buffer (30 mM NaCl, 15 mM Tris-HCl, [pH 7.0], 3 mM $MgCl_2$, and 1 mM DTT) (26) was combined with 1 U of creatine kinase per ml and 12.5 mM creatine phosphate for rATP regeneration (49). β-Glycerophosphate was included to reduce phosphatase activity (52). Typically, 500 μg of NM was incubated in the phosphorylation buffer with 3 mM rATP for 1 h at 25°C. The supernatant was then recovered by centrifugation in a microcentrifuge. The protein concentrations, excluding that of creatine kinase, were usually 0.5 to 1 μg/μl. The 8 M urea NME was prepared in a manner similar to that described by Fey and Penman (20). The nuclear pellet was washed several times in digestion buffer containing 1 mM PMSF. High-salt NM was then prepared by the previously mentioned techniques. The NM pellet was extracted with a threefold-volume equivalent of disassembly buffer (8 M urea, 20 mM MES [morpholineethanesulfonic acid] [pH 6.6], 1 mM EDTA, 0.1 mM $MgCl_2$, 1% β-mercaptoethanol, 1 mM PMSF). The supernatant was collected by centrifugation at 15,000 × *g* for 4 min. The NME was then step dialyzed for 2 h in 1,000 volumes of the same buffer with urea at 4, then 2, 1, and 0.1 M successively at 4°C. The final dialysis was in 1,000 volumes of buffer B [20 mM NaCl, 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; Calbiochem, La Jolla, Calif.)] overnight at 4°C. To remove the fluffy white precipitate that contained NM intermediate filament protein and that formed during dialysis, the NME was again clarified by centrifugation at 15,000 × *g* for 5 min. The NME protein concentrations were usually on the order of 1 μg/μl.

**Labeling of pTP.** One-hundred-millimeter-diameter plates of HeLa cells coinfected with vvpTP1 and vvT7 were incubated for 30 min in 4 ml of methionine minus Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, Md.) at 17 h p.i. Labeling of pTP was carried out for 2 h in the presence of 0.05 μCi of $^{35}$S-Trans label (Amersham, Arlington Heights, Ill.) per ml in a total volume of 4 ml. Specific labeling of pTP was possible because of the fact that vaccinia virus inhibits host cell protein synthesis and that pTP contains 20 methionine residues. The infected cells were harvested and pTP-NM was prepared as described above.

**Release of a pTP-NM complex.** Release of pTP complexes was assayed. Fifty micrograms of pTP-NM was incubated with 3 mM rATP in 30 μl of phosphorylation buffer containing 1 mM DTT for 1 h at room temperature in the presence of 1 mM PMSF. Protein released to the supernatant was recovered from the reaction mixture by centrifugation at 15,000 × *g* for 5 min. The supernatant was resuspended in Laemmli buffer with either 2 or 0.1% sodium dodecyl sulfate (SDS) (31). In order to analyze the native pTP complexes, the samples were either boiled or not and loaded onto an 8% polyacrylamide gel. The protein was transferred to nitrocellulose and probed with the anti-pTP or anti-lamin antibodies.

**pTP Far Western assay.** The pTP Far Western experiment was adapted from the protocol described by Carriere et al. (12). Twenty micrograms of whole NM, rATP NME, or 8 M urea NME was heated in Laemmli denaturing buffer and electrophoresed on a 10% polyacrylamide gel. The protein was then transferred to nitrocellulose and blocked against 5% dry milk protein in PBS. The blot was gently rocked at 25°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 AEBSF. The blot was rinsed with PBS for 10 min at room temperature. The subsequent development of the blot was the same as that for a Western blot.

**NM tyrosine kinase activity assay.** Fifty micrograms of pTP-NM was incubated in phosphorylation buffer with or without 10 μg of the tyrosine kinase substrate poly(Glu, Tyr) at 4:1 (Sigma, St. Louis, Mo.) and 5 mCi of [γ-$^{32}$P]ATP (47). β-Glycerophosphate at 50 mM was included in the reactions to inhibit endogenous phosphatase activity (54). Incubations were done with or without quercetin (296 μM), methyl-2,5-dihydroxycinnamate (2,5-MeC) (52 μM), or genistein (130 μM) in the presence of 1 U of creatine kinase per ml and 12.5 mM creatine phosphate (47, 51, 52). These concentrations of each inhibitor were those which had shown maximal inhibition of pTP release (see Fig. 5). The reaction was carried out for 1 h at 25°C. The supernatants were subjected to electrophoresis on a 0.1% SDS–8% polyacrylamide gel and then blotted to nitrocellulose and probed for the presence of pTP. Radioactive counts incorporated into the test substrate because of phosphorylation by the putative tyrosine kinase were visualized and quantitated with a PhosphorImager model 400E (Molecular Dynamics, Sunnyvale, Calif.).

**rATP-dependent release of pTP from NM.** $^{35}$S-labeled pTP bound to NM was prepared from HeLa cells programmed with vvpTP1. The pTP-NM was then incubated in phosphorylation buffer and β-glycerophosphate with 3 mM rATP or 5′ adenylylimidodiphosphate (rAMP-PNP) in the presence of an rATP regenerating system for 1 h at 25°C. The rATP incubations were done in the presence of increasing concentrations of quercetin, 2,5-MeC, or genistein. Supernatants were collected and subjected to electrophoresis on an 8% polyacrylamide gel containing 0.1% SDS. The gel was then vacuum dried. The release of $^{35}$S-labeled pTP from the NM was visualized and directly quantitated with a PhosphorImager (Molecular Dynamics). In all cases, a single radioactively labeled 80-kDa band which was confirmed to be pTP by Western blot was released (data not shown). The quantitation of the release of unlabeled pTP with Western blots developed with a biotinylated secondary antibody and $^{35}$S-labeled streptavidin gave comparable results (data not shown). We also found that 2 M NaCl- or lithium-3,5-diiodosalicilate-extracted NM gave similar results for quantitative pTP release (data not shown). For the purpose of quantitation, triplicate or quadruplicate samples for each concentration of inhibitor were averaged, standard deviations were calculated, and the data were plotted with Cricket Graph III (Computer Associates International, Inc., Islandia, N.Y.).

**Southern blot analysis of rATP-released Ad DNA termini.** HeLa cells infected with Ad type 5 at a multiplicity of infection of 10 were harvested at 15 h p.i. The nuclei were recovered and subjected to *Hin*dIII digestion for 2 to 3 h. High-salt NM was then prepared. Fifty micrograms of Ad-NM was incubated in phosphorylation buffer in the presence of 3 mM rATP or rAMP-PNP and an rATP regeneration system. Duplicate rATP-containing samples were incubated with concentrations of the tyrosine kinase inhibitors which were maximally inhibitory to pTP release (see Fig. 5). The reactions were carried out at 25°C for 1 h. To ensure that the DNA fragments were not being nonspecifically trapped, the Ad-infected NM was washed twice with the same 2 M NaCl buffer used to prepare the NM (49). The recovered supernatants and pellets were then digested with proteinase K (10 μg/ml) for 3 h at 45°C. The DNA samples were heated to 100°C for 5 min and snap cooled on ice. The DNA was then dot blotted to two nitrocellulose filters with a vacuum transfer apparatus (1). The blots were then

carefully placed on a Whatman 3MM filter soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 10 min. The blots were laid on top of another Whatman 3MM filter soaked in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.0]) for 5 min (1). The DNA was then cross-linked to the nitrocellulose with a UV Stratalinker 1800 set on automatic mode (Stratagene). The blots were then rinsed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and put into hybridization bags along with an appropriate amount of prehybridization solution (5× SSC, 5× Denhardt's solution, 0.1% SDS, 0.1 mg of salmon sperm DNA per ml, 1 mM EDTA, 0.01 M sodium phosphate) for 2 h at 70°C. Two oligonucleotides were chosen for the analysis of the Ad DNA ends: 823-842 (5′-TTCTCCGGAGCCGCCTCACC-3′, adjacent to E1a) and 35,683-35,702 (5′-CCCAGAAAACCGCACGCGAA-3′, adjacent to E4). Twenty picomoles of each probe was end labeled with 20 mCi of [γ-$^{32}$P]ATP by using 10 U of T4 polynucleotide kinase (Pharmacia, Piscataway, N.J.) The reaction was carried out for 30 min at 37°C and stopped by heat treatment at 100°C for 5 min. Each of the labeled oligonucleotides was added to separate plastic bags containing hybridization solutions and the identical nitrocellulose blots. The blots were heated to 70°C for 10 min and then were allowed to cool slowly in a water-filled container overnight with shaking. The blots were then washed thoroughly with two changes of 2× SSC for 1 h at 25°C and visualized and quantitated with a PhosphorImager (Molecular Dynamics) (1, 23).

**pTP-NM gel shift assay.** Gel shift assays were performed with a double-stranded oligonucleotide spanning the first 18 bp of the Ad type 5 origin of replication. The top (5′-CATCATCAATAATATACC-3′) and bottom (5′-GGTATATTATTGATGATG-3′) strands were synthesized on an Applied Biosystems 394 DNA synthesizer. The procedure for pTP gel shift was similar to that of Kusukawa et al. (30). Twenty picomoles of the top strand was 5′ end labeled with T4 polynucleotide kinase (Pharmacia) and [γ-$^{32}$P]ATP (20 μCi). The unlabeled bottom strand was annealed in a 10-fold excess to the top strand. The annealing was carried out at 70°C for 5 min, and it was followed by a slow cool to room temperature (29). The double-stranded DNA (dsDNA) oligonucleotide was then desalted with an S-300 microspin column (Pharmacia). The final volume was adjusted to give a concentration of 1 pmol/μl of the dsDNA 18-mer. One picomole of end-labeled dsDNA 18-mer was incubated with either no protein, 10 μg of HeLa cytoplasm, 10 μg of NME, or 10 μg of pTP extract from a vvpTP1 infection. Supershift was performed by the addition of 1 μl of purified anti-pTP MAb to a reaction mixture with pTP and the oligonucleotide. Competition of pTP from the radiolabeled probe was achieved by the addition of 300 pmol of unlabeled dsDNA 18-mer. Experimental conditions contained either 5 μg of rATP NME or 8 M urea NME incubated with 10 μg of pTP extract and the dsDNA 18-mer. Reactions were carried out for 30 min at 25°C in a total volume of 30 μl containing 25 mM HEPES (pH 7.4), 20 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 1 μg of poly(dI)-poly(dC). The samples were then supplemented with DNA gel loading buffer (0.025% bromphenol blue, 0.025% xylene cyanol, and 20% Ficoll) and separated by electrophoresis on a 5% polyacrylamide gel containing 0.1% SDS and 0.5× Tris-borate-EDTA buffer. The gel was vacuum dried to Whatman 3MM paper with a gel dryer (Bio-Rad, Hercules, Calif.). The gel-shifted bands were visualized with a PhosphorImager (Molecular Dynamics).

## RESULTS

Previous work has shown that pTP binds tightly to the NM through the course of infection (24, 49). However, there has been little progress in the identification of the NM protein(s) to which pTP binds or in the understanding of the mechanisms which regulate this interaction. For this reason, we investigated the factors that affect the binding of Ad DNA ends to the NM.

**Release of Ad DNA ends from the NM requires rATP hydrolysis.** While screening for reagents that released Ad DNA ends or pTP proteins from the NM, we observed that added rATP could release a sizeable fraction of the bound Ad DNA (Fig. 1A). The release of Ad DNA termini in the presence of 3 mM rATP was measured by DNA dot blotting of the rATP-released material probed with $^{32}$P-labeled oligonucleotides specific for Ad DNA ends; the Ad end-specific counts were quantitated on a PhosphorImager and expressed as a percentage of the total Ad DNA termini that was bound in high-salt-extracted NM prior to treatment. The smaller amount of release observed in the absence of rATP might be attributed to endogenous rATP that remained in the NM preparation. This release of Ad DNA ends required rATP hydrolysis, as shown by the lower release observed when NM from Ad-infected cells was incubated with 3 mM rAMP-PNP.

To confirm this result, NM from cells infected with a vaccinia virus recombinant that expresses high levels of pTP and

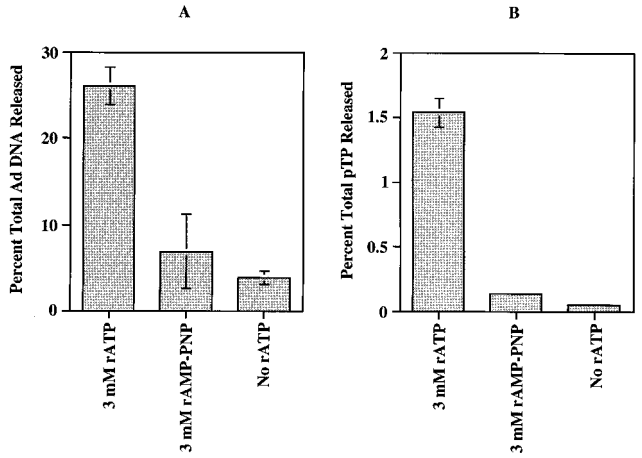


FIG. 1. Release of pTP or pTP-bound Ad DNA termini from the NM requires the hydrolysis of rATP. (A) Fifty micrograms of $^{35}$S-labeled pTP bound to NM was incubated with either 3 mM rATP, 3 mM rAMP-PNP (a nonhydrolizable rATP analog), or buffer alone (no rATP). Supernatants were electrophoresed on an 8% polyacrylamide gel. The gel was vacuum dried to Whatman 3MM paper and pTP was visualized with a PhosphorImager (Molecular Dynamics). Triplicate samples were prepared for each condition. (B) Release of Ad DNA termini from the NM was analyzed by Southern blotting under conditions identical to those described for panel A. Supernatants were dotted to nitrocellulose in triplicate and probed with the left-end oligonucleotide (823-842). Hybridization was visualized with a PhosphorImager (Molecular Dynamics). In both graphs, release was quantitated, averaged, and expressed as a percentage of the total pTP or Ad DNA. Error bars show standard deviations.

that was labeled with [$^{35}$S]methionine was extracted with high NaCl and treated with rATP as described in Materials and Methods. In this case, the amount of an 80-kDa $^{35}$S-labeled pTP protein band released by this treatment was quantitated on a PhosphorImager, and the amount of pTP released was expressed as a percentage of the total pTP in the NM prior to treatment; there were no other $^{35}$S-labeled proteins in this size range released by rATP treatment. In this case, rATP-mediated release was also detectable but at a lower level (1.5%) compared with that of the total pTP protein present. The most likely explanation for this difference is that at high levels of vvpTP1 expression, the rATP-releasable pTP binding sites are oversaturated and that the majority of the pTP protein binds elsewhere in the NM, perhaps at lower-affinity sites that are not capable of release with rATP. The higher percentage of pTP release from NM in a normal Ad infection may reflect a specific preference for binding of Ad DNA to the rATP releasable sites. The nonhydrolyzable rATP analog (rAMP-PNP) had virtually no effect on release of pTP from the NM. Taken together, these results indicate that rATP hydrolysis is a prerequisite to release of pTP of Ad DNA termini.

**rATP-dependent release of a pTP-NM complex.** Supernatants from incubations of pTP-NM with 3 mM rATP were separated by electrophoresis under fully denaturing conditions (either 2 or 0.1% SDS with heating) or under low SDS conditions without heating. Duplicate sets of samples were probed with specific antibodies for the presence of pTP or nuclear lamins A and C (Fig. 2). Under low SDS conditions (0.1%) without heating, a complex with a size of approximately 220 to 230 kDa that was recognized by an anti-pTP antibody (3-1A) was detected; this antibody did not cross-react with the control HeLa cell NM protein. Since pTP has a molecular mass of 80 kDa, formation of a pTP complex with a size of 220 to 230 kDa would suggest that the cellular component(s) of the complex would total approximately 140 to 150 kDa. The pTP complex

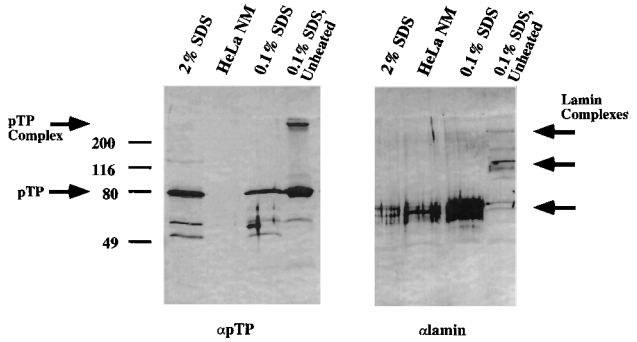


FIG. 2. A 220- to 230-kDa pTP-NM complex is released from the NM upon incubation with rATP. Each panel contains duplicate supernatant samples which were separated by electrophoresis on the same gel. Each half of the resultant Western blot was probed with either anti-pTP antibody (3-1A) (left) or anti-lamin antibody (right), as indicated. Samples were loaded onto the gel either under fully denaturing conditions (2 or 0.1% SDS Laemmli buffer with heating) or under low SDS conditions (0.1% SDS without heating). Uninfected NM (HeLa NM) was included as a control for anti-pTP antibody cross-reaction. The arrows on the left indicate the 220- to 230-kDa pTP complex and the 80-kDa pTP. The arrows on the right panel point to the detected lamin multimers. The numbers on the left are kilodaltons.

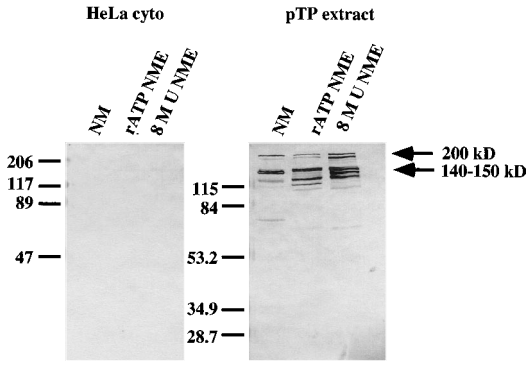


FIG. 3. pTP binds directly to high-MW NM proteins in vitro. Twenty micrograms of HeLa NM, rATP NME, and 8 M urea (U) NME (each) was loaded in duplicate and separated by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS and transferred to nitrocellulose. The identical halves of the blot were incubated either with uninfected HeLa cytoplasm (cyto) or with pTP cytoplasmic extract from HeLa cells infected with a pTP-expressing recombinant vaccinia virus (vvpTP1) as indicated. The blots were then developed for the presence of pTP with the polyclonal anti-pTP (3-1A) antibody. The negative control incubation (HeLa cyto) showed no background. The high-MW NM bands which showed pTP binding activity are indicated by the arrows. The top arrow shows a doublet near 200 kDa. The lower arrow indicates the group of bands which center around 140 to 150 kDa. The numbers to the left of the blots are kilodaltons.

could be disrupted by boiling in 2% SDS Laemmli buffer, indicating that the interaction may be noncovalent, although the presence of a sulfhydryl bond between pTP and the putative cellular protein(s) cannot be ruled out. This pTP complex could be recapitulated in vitro with $^{35}$S-labeled NME prepared by either rATP incubation or 8 M urea extraction (data not shown). Taken together, these experiments defined the presence of a soluble and stable pTP interaction with one or more NM proteins.

Under identical conditions with the same extracts, complexes of nuclear lamins could also be identified with an anti-lamin antibody. The lamin complexes visualized were consistent with the monomer, dimer, and tetramers observed by Moir et al. (38). However, the lamin complexes did not comigrate with the pTP complex, suggesting the lamins are not a direct component of the observed high-molecular-weight (high-MW) pTP complex.

**pTP binds directly to high-MW NM proteins in vitro.** A Far Western blot was used to determine whether the NM proteins released by rATP or by 8 M urea (and subsequently dialyzed to remove urea, as described in Materials and Methods) contained proteins capable of binding pTP (Fig. 3). NM was prepared from uninfected HeLa cells, and 20 μg of whole NM and rATP NME and 8 M urea NME were separated by electrophoresis and immobilized on a nitrocellulose filter. As expected, the anti-pTP antibody (3-1A) used to detect pTP interactions with cellular proteins on this blot did not cross-react with any bands on the left side of the blot, which was incubated with HeLa cytoplasmic extract that lacked pTP. The right side of the same blot was incubated with a cytoplasmic extract from HeLa cells infected with the vaccinia virus that expressed pTP (vvpTP1); when probed with the anti-pTP antibody, a number of high-MW bands were detected. There was a doublet near 200 kDa which bound pTP. The darkest bands were at about 140 to 150 kDa. Two other lower-MW bands with sizes of 30 and <20 kDa seemed to be present in whole NM and not in the extracts (data not shown). The fact that there were numerous proteins which bound pTP may imply (i) that there is more than one type of pTP binding site among the proteins that constitute the NM, (ii) that the cellular protein to which pTP binds is subject to partial proteolytic degradation (perhaps as a consequence of extraction) and that pTP can bind to one or more of these degradation products, or (iii) that all of these proteins may be involved in a larger complex. The sizes of the strongly reacting bands (140 to 150 kDa) would be consistent with the size of the complex seen in Fig. 2.

**A tyrosine kinase controls release of pTP from the NM.** In order to understand the dynamics of the pTP-binding protein, we chose to determine the mechanism of the rATP-dependent release of pTP. Dessev et al. (17) had previously observed that quercetin inhibited an NM kinase activity. Using this observation as a starting point, we confirmed that our NM prepara-

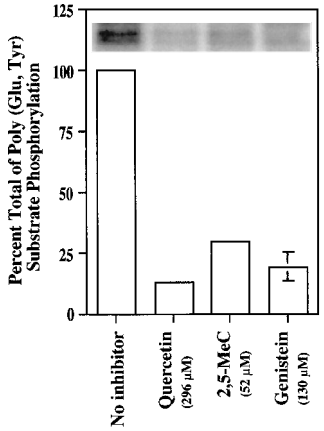


FIG. 4. Tyrosine kinase inhibitors block NM-mediated phosphorylation of an exogenous poly(Glu, Tyr) substrate. Fifty micrograms of pTP-NM was incubated with 5 μCi of [γ-$^{32}$P]ATP, 10 μg of a tyrosine kinase substrate, and the tyrosine kinase inhibitors as described. The supernatants from each reaction were recovered and separated by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The inset shows the representative poly(Glu, Tyr) phosphorylated bands which correspond to the bar graph. The samples were quantitated in triplicate, and background phosphorylation in lanes without the poly(Glu, Tyr) substrate was subtracted. Inhibition was measured as a percentage of the total poly(Glu, Tyr) phosphorylation. Quercetin, 2,5-MeC, and genistein were included at final concentrations of 296, 52, and 130 μM, respectively. The error bar shows the standard deviation.

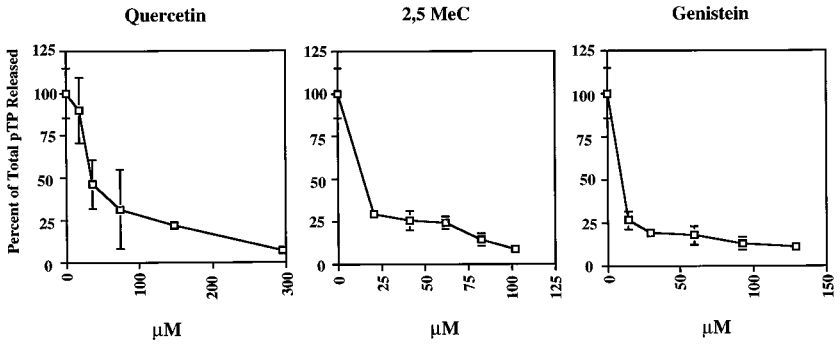


FIG. 5. Dose dependence of the inhibition of pTP release from the NM. Fifty micrograms of pTP-NM was incubated with 3 mM rATP and various concentrations of tyrosine kinase inhibitor. The concentrations of the inhibitors used were as follows: quercetin, 0, 18.5, 36.9, 73.9, 147.8, and 296 μM; 2,5-MeC, 0, 20.6, 41.2, 61.8, 82.4, and 102 μM; and genistein, 0, 14.8, 29.6, 59.2, 92.5, and 129.5 μM. Supernatants were electrophoresed on 10% polyacrylamide gels containing 0.1% SDS which were then dried to Whatman 3MM paper. Release of pTP was quantitated with a PhosphorImager (Molecular Dynamics). Quadruplicate samples were prepared for each concentration point, and the standard deviations of the means for each dose are shown by the bars above and below the point; where no bars are shown, the standard deviation of the mean was too small to be apparent on the scale of the graph. Inhibition was measured as a percentage of the total amount of pTP released with rATP alone.

tions contained a tyrosine kinase activity and determined that quercetin, 2,5-MeC, and genistein each significantly inhibited phosphorylation of a synthetic poly(Glu, Tyr) substrate (Fig. 4). Phosphorylation of the substrate in the presence of quercetin, 2,5-MeC, or genistein was reduced to 13, 30, or 19% of the control (respectively). The robust phosphorylation of intrinsic NM proteins (as determined by the inclusion of [$\gamma$-$^{32}$P] rATP with the NM) was decreased by these inhibitors (data not shown). Interestingly, pTP itself was not phosphorylated in these assays. These results indicate that the mechanism of pTP release is through phosphorylation of NM proteins and not through phosphorylation of pTP itself and may involve a cascade of kinase reactions.

The results in Fig. 5 show that rATP-dependent pTP release from the NM is inhibited by compounds in a dose-dependent manner. Each concentration point represents the mean of quadruplicate samples. pTP release was almost entirely abrogated with each of the tyrosine kinase inhibitors. The point of half-maximal inhibition of pTP release was at a concentration of 35 μM for quercetin, 15 μM for 2,5-MeC, and 11 μM for genistein. The $K_i$ of genistein reported by Teraoka et al. (57) for rat liver NM tyrosine kinase was 10 μM, similar to the inhibitory concentration observed here for rATP-dependent pTP release. No inhibitory concentration data for quercetin or 2,5-MeC have been reported for NM tyrosine kinases, but the ranges of inhibitory concentrations of these drugs reported for other tyrosine kinases are 5 to 100 μM (for quercetin; 48, 58) and 0.8 to 5 μM (for 2,5-MeC; 47, 50). The sigmoidal profile of the inhibition dose curves was similar to that of typical tyrosine kinase-dependent functions (50, 57).

The presence of a 30-kDa tyrosine kinase associated with rat liver NM and capable of autophosphorylation has been reported previously (40, 57). We have also detected a single 30-kDa band in our NM preparations by Western blotting with an anti-phosphotyrosine MAb (data not shown), but there is no evidence at present about this 30-kDa protein representing the previously reported tyrosine kinase or about the role that this tyrosine kinase might play in the release of pTP from the NM.

**Tyrosine kinase-dependent release of pTP-bound Ad DNA termini from the NM.** In order to demonstrate that this tyrosine kinase-dependent mechanism was relevant to a normal Ad infection, we tested the release of Ad DNA termini from the NM in the presence of the inhibitors (Fig. 6). The Ad DNA, which is covalently linked to pTP upon replication, serves as an indicator of the presence of pTP. In these experiments, NM was prepared from Ad-infected cells at 15 h p.i., the Ad and the chromosomal DNAs were cleaved with *Hin*dIII, and the supernatant from after the treatment was probed for release of the Ad DNA ends by dot blotting, with oligonucleotides specific for the right or left ends of the Ad genome being used. Incubation of this NM preparation with 3 mM rATP released 25% of the Ad DNA ends from the NM compared with the control lacking rATP. Quercetin, 2,5-MeC, and genistein effectively blocked release of both left and right Ad DNA ends. In a result similar to the results shown in Fig. 1, 3 mM rAMP-PNP caused essentially no release of the left Ad DNA end (Fig. 6), implicating rATP hydrolysis in this process. All of these results are consistent with the inhibition of pTP release observed with NM prepared from vvpTP1-infected cells. The apparent difference in the intensities of the radioactive signals for the left and right Ad ends in the dot blot is probably due to differences in the relative efficiencies of hybridization of the oligonucleotides used to identify the left and right Ad ends.

To identify the maximal percentage of pTP-bound Ad DNA releasable with rATP, we incubated Ad-NM with increasing

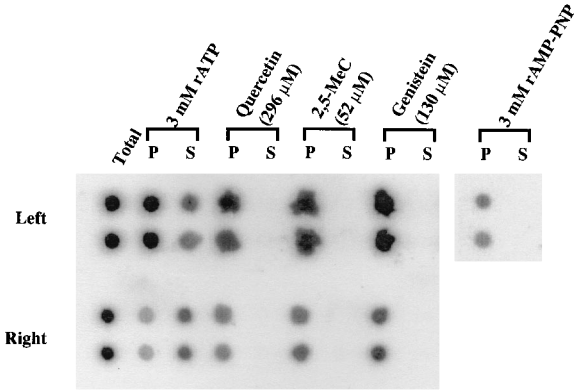


FIG. 6. Tyrosine kinase-dependent release of pTP-bound Ad DNA termini from the NM. Ad-infected HeLa cell NM was incubated with 3 mM rATP and with or without quercetin (296 μM), 2,5-MeC (52 μM), genistein (130 μM), or AMP-PNP (3 mM) as indicated. Samples of total Ad NM (Total) are indicated. Pellets (P) and supernatants (S) recovered after the 1-h reactions are indicated. Duplicate samples for each condition were blotted to nitrocellulose and probed with oligonucleotides representing the left (823-842) and right (35,683-35,702) ends of the genome.

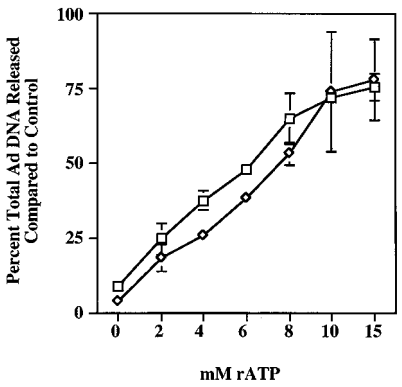


FIG. 7. The maximum pTP-bound Ad DNA released from the NM by rATP treatment. Fifty micrograms of Ad-infected HeLa NM was incubated with 0, 2, 4, 8, 10, or 15 mM rATP for a period of 1 h. Release at each rATP concentration was assayed in triplicate. The samples were washed twice with 2 M NaCl buffer. Supernatants and pellets were blotted to nitrocellulose and probed with the Ad genome right-end or left-end probe. The mean counts of right (squares) or left (diamonds) Ad DNA termini released into the supernatant for each concentration were represented as percentages of the total counts in the control sample.

concentrations of rATP. Southern blot analysis was performed with an Ad left end- or right end-specific oligonucleotide probe (823-842 and 35,683-35,702, respectively). Release of Ad DNA was expressed as a percentage of the total Ad DNA in the control sample and graphed as a function of rATP concentration (Fig. 7). The resulting plot began to plateau at between 10 to 15 mM rATP, and nearly 75% of the bound Ad DNA ends could be released at the highest concentrations; oligonucleotide probes specific for left or right ends gave similar results, suggesting that either Ad end was equally able to be released by the rATP treatment and that one Ad end was not preferentially trapped in the NM. That 100% of the ends could not be released may be due to the trapping of the remaining ends inside the NM, the inefficient cutting of the Ad DNA by *Hin*dIII, or the binding of the remaining 20% of Ad ends to other sites in the NM that are not susceptible to release with rATP.

**A pTP-NM protein complex is capable of binding Ad origin DNA.** Ad origin-specific DNA binding activity of pTP-NM complexes was tested to show that the pTP-NM complexes were functional in the formation of pTP-DNA complexes. The results of this pTP gel shift-supershift assay, originally developed to study the interactions of pTP and Ad polymerase (56), is shown in Fig. 8. The control lanes with no protein, HeLa cytoplasm, or NME alone did not show a gel shift. Supershift of the pTP-specific complex with a purified pTP MAb verified the involvement of pTP in the origin binding activity. The origin DNA binding activity was competed with by the addition of a 300 M excess of unlabeled dsDNA 18-bp oligonucleotide, indicating specificity for origin binding. The addition of either rATP NME or 8 M urea NME to the pTP gel shift reaction also caused a significant supershift. These results indicate that the supershifted pTP-NM complex is still capable of binding the Ad origin. These data present the possibility that the pTP-NM interaction may be closely involved in initiation complex formation.

## DISCUSSION

In uninfected cells, the NM is thought to organize sites of cellular replication and transcription (9). The Ad genome has also been shown to localize to the NM through an interaction mediated by pTP (7, 49). The studies reported here focused on understanding the mechanisms which influence the attachment of the pTP binding sites to the NM. Previous work had suggested a number of kinases that affect the integrity of the NM (17, 39, 54, 57). We have provided evidence here that one or more tyrosine kinase-mediated phosphorylation events may also be an important mechanism for controlling the attachment of at least one NM protein to which pTP binds and that this process requires rATP hydrolysis. Further supporting the hypothesis that tyrosine kinase activity controls pTP release is the fact that the magnitude of the inhibition of poly(Glu, Tyr) phosphorylation (Fig. 4) closely reflected the repression of pTP release (Fig. 5) at the same concentrations of inhibitor. The similar response of poly(Glu, Tyr) substrate phosphorylation and pTP release in the presence of inhibitors further suggests a relationship between tyrosine kinase activity and release of pTP binding sites.

Interestingly, there appeared to be a great difference in the percent total pTP released from vvpTP1-infected NM (1.5%) versus that from Ad-infected NM (26%) at 3 mM rATP. This difference suggests that there may be different functional sites for pTP, some of which are sensitive to release by rATP. The quantity of pTP from a vvpTP1 infection would easily exceed the amount of pTP produced during an Ad infection and would most likely saturate the number of sites available for pTP-mediated binding of Ad genomes (24). Since there is a higher fraction of total Ad DNA ends released by rATP, this class of binding sites may be preferred during Ad infection and attachment of Ad DNA to the NM. Release of Ad termini continued to increase with increasing rATP concentration (Fig. 8). These concentrations of rATP are higher than what might be expected within the nucleus (43) and may overestimate the actual concentration needed for release because of the inefficiency of *Hin*dIII digestion of DNA attached to the NM, the trapping of the DNA, or the loss of some component in the salt-stripped NM preparation.

The rATP-dependent release of a 220- to 230-kDa NM complex that contains pTP means that the complex could be isolated either from an NM preparation of vaccinia virus-in-

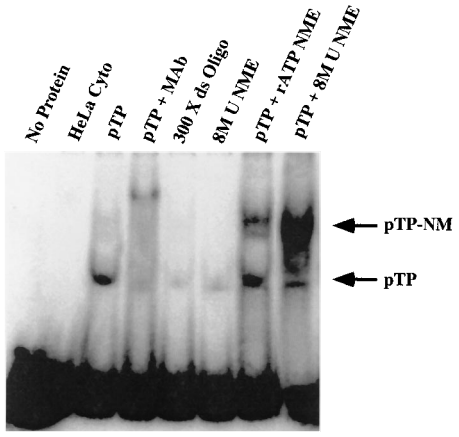


FIG. 8. A pTP-NM complex is capable of binding the Ad origin of replication. Ten micrograms of pTP extract was incubated with a dsDNA 18-mer oligonucleotide which spans the pTP binding site on the Ad type 5 origin of replication. The reaction mixtures were incubated for 30 min at 25°C, and the protein-DNA complexes were resolved on a 5% polyacrylamide gel containing 0.1% SDS. Control lanes contained the probe with no protein, HeLa cytoplasmic (Cyto) extract, pTP alone, pTP with 1 ml of anti-pTP MAb, pTP with a 300 M excess (X) of unlabeled 18-mer, or 5 μg of 8 M urea (U) NME alone. As indicated, the experimental lanes contained pTP and 5 μg of either rATP NME or 8 M urea NME. The pTP arrow designates the pTP-specific gel shift. The pTP-NM arrow indicates the pTP-NM supershifted complex.

fected cells that overexpressed pTP (Fig. 2) or could be reconstituted in vitro from uninfected cell NME and exogenous pTP. The presence of the bound NM proteins did not interfere with the ability of pTP to interact with Ad origins, a crucial requirement for initiation of Ad DNA replication (Fig. 3 and 8). The Far Western blot revealed that pTP was capable of binding a series of high-MW NM bands with sizes of between 120 to 200 kDa. It is not clear at present whether these high-MW proteins identified by Far Western blotting represent a series of NM proteins, either in a complex or acting as independent binding sites, or rather represent proteolytic digestion products of one or a few large NM proteins. Binding to these different sites on the NM could result in different regulatory or other outcomes for the genomes located there during the course of viral infection.

There is evidence to suggest that tyrosine kinases have a significant role in the regulation of transcription and DNA replication in the nucleus (19, 59). A number of tyrosine kinases, such as wee and Ab1, are linked to nuclear events (59). Serine- and threonine-phosphorylating kinases in addition to casein kinase II are also known to be NM associated (17, 55). It is possible that the tyrosine kinase-dependent release of pTP-NM complexes involves a cascade of kinase activities. This possibility is supported by experiments showing an rATP-dependent release of lamin protein, which is not known to be a substrate for tyrosine phosphorylation (38) (Fig. 2). It was also possible to detect kinase activity which was reduced but not ablated in the presence of the tyrosine kinase inhibitors. It is possible that this activity is a normal part of the cell cycle regulation of the NM structure and may have no direct effect on the replication or packaging of Ad DNA during infection.

It is also not clear what the normal role of the cellular proteins that constitute these binding sites might be within the NM. One might imagine that they might play either a structural role (perhaps as a site for cellular DNA replication or a means to concentrate replication factors) or an enzymatic role (providing such required cofactors as purines or pyrimidines). The rATP-dependent release of pTP from the NM could be a marker for one or more steps of nuclear lamina disassembly that are mediated by phosphorylation that occurs at the G2/M boundary. Supporting this possibility is the evidence that rAMP-PNP and quercetin both block nuclear lamina disassembly (16, 51). In addition, quercetin and genistein, used in the present study, are known to block the cell cycle at G2/M (41, 44, 47). These results are consistent with a tyrosine kinase-dependent control of nuclear disassembly at G2/M. Although there is no evidence to suggest that the tyrosine kinase-mediated release of pTP from the NM plays any direct role in Ad infection, linkage of cellular kinase activity with the viral life cycle could be a means to control the release of replicated genomes for packaging.

There is a growing body of evidence that cellular and viral proteins required for transcription and replication might be amassed at discrete sites on the NM where they are eventually to be used (2, 5, 8, 11, 42). That these sites exist is suggested by indirect immunofluorescence experiments that showed that Ad replication proteins are present at punctate sites within the nucleus (11, 24, 42), perhaps within large complexes of NM proteins. Localized concentrations of viral factors may be critical for the occurrence of successful Ad DNA replication (32). For example, it has been shown that the Ad replication protein, DNA-binding protein, is part of a 650-kDa, high-salt, stable complex which contains both DNA binding and kinase activities (45). The fact that pTP associates tightly with one or more NM proteins implies that they might have influence on replication complex formation. We are currently pursuing purification of the NM proteins which bind pTP.

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