Cyclin-Dependent Kinase Regulation of the Replication Functions of Polyomavirus Large T Antigen

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The amino-terminal portion of polyomavirus (Py) large T antigen (T Ag) contains two phosphorylation sites, at T187 and T278, which are potential substrates for cyclin-dependent kinases (CDKs). Our experiments were designed to test whether either or both of these sites are involved in the origin DNA (ori DNA) replication function of Py T Ag. Mutations were generated in Py T Ag whereby either or both threonines were replaced with alanine, generating T187A, T278A, and double-mutants (DM [T187A T278A]) mutant T Ags. We found that the Py ori DNA replication functions of T278A and DM, but not T187A, mutant T Ags were abolished both in vivo and in vitro. Consistent with this finding, it was shown that the ori DNA binding and unwinding activities of mutant T278A Py T Ag were greatly impaired. Moreover, whereas wild-type Py T Ag is an efficient substrate for phosphorylation by cyclin A-CDK2 and cyclin B-cdc2 complexes, it is phosphorylated poorly by a cyclin E-CDK2 complex. In contrast to mutant T187A, which behaved similarly to the wild-type protein, T278A was only weakly phosphorylated by cyclin B-cdc2. These data thus suggest that T278 is an important site on Py T Ag for phosphorylation by CDKs and that loss of this site leads to its various defects in mediating ori DNA replication. S- and G₂-phase-specific CDKs, but not a G₁-specific CDK, can phosphorylate wild-type T Ag, which suggests yet another reason why DNA tumor viruses require actively cycling host cells.

Polyomavirus (Py) large T antigen (T Ag) is a nuclear phosphoprotein which mediates many different steps in the viral life cycle. It functions in initiating DNA replication both in vivo (13) and in vitro (36, 43) and regulates as well transcription from the early region of the viral genome (4). Its important biochemical properties include sequence-specific origin DNA (ori DNA) binding (5, 28, 50), hexamer formation (52), and DNA unwinding (53). In addition to its DNA helicase (48, 53) and ATPase (14, 48) activities, Py T Ag is capable of inducing local distortions at the Py origin in an ATP-binding dependent manner (1, 26). Since Py T Ag is a phosphoprotein (46), it is not surprising that some of its functions are regulated by its state of phosphorylation. We previously showed that the replication functions of Py T Ag are affected both negatively and positively by this modification, such that mild phosphatase treatment stimulates its replication capability while more extensive removal of phosphates from T Ag greatly reduces its ability to support origin DNA replication in vitro (54).

Simian virus 40 (SV40) T Ag, the homolog of Py T Ag, has similar biochemical activities (reviewed in reference 11) and has also been shown to be regulated by phosphorylation (10, 42). SV40 T Ag's function in viral DNA replication requires phosphorylation of a single residue, T124, a site which can be phosphorylated by cdc2 kinase (31–33, 35). Mutation of this site compromises the function of SV40 T Ag in the initial steps in sequence-specific unwinding of the origin (32, 35), a function that is carried out by two hexamers of T Ag assembled at the origin in a nucleotide-dependent manner (6, 30).

A comparison of the phosphorylation sites on Py and SV40 T Ags reveals both similarities and differences. Both T Ags contain two distinct clusters of phosphorylation sites, and in each case, one of these is located within the vicinity of a

nuclear localization signal (NLS) and the origin binding domain (for sites in Py T Ag, see references 3 and 17). In fact, these regions are highly conserved between the two T Ags. In that region of Py T Ag, there is a potential site at T278 that conforms well to the canonical cyclin-dependent kinase (CDK) consensus site ([S/T]PX[R/K]) (reference 49 and references therein) and that is directly N terminal to one of its two NLSs as well as its origin DNA binding domain (44) (Fig. 1). The second cluster of phosphorylation sites within Py T Ag is located further upstream within the amino-terminal of the polypeptide, while in SV40 T Ag, the second phosphorylated region is at its C terminus. Within the more N-terminally located group of Py T Ag phosphorylation sites is another site, at T187, which more loosely qualifies as a CDK site; T187 is also adjacent to a second NLS identified in Py T Ag (44).

The importance of CDKs lies principally in their timed regulation of passage through the cell cycle, as well as in their relationship to the various checkpoints that have evolved to ensure faithful replication and segregation of DNA, thereby maintaining the integrity of the genome (reviewed in references 16, 37, and 41). While there is a wealth of evidence indicating that in mammalian cells different cyclin-kinase complexes are required for different stages in the cell cycle, it is also the case that at any stage in the cycle there is ample supply of a given kinase. This finding suggests that different CDK complexes have different substrates as targets, which has been borne out experimentally (see, for example references 8, 18, 19, and 55). It is thus of interest to determine the effects of CDKs on the replication functions of Py T Ag. It is also of interest to determine not only if the consensus CDK sites on Py T Ag are functional but also which complexes are capable of phosphorylating them.

MATERIALS AND METHODS

Mutant Py T Ag constructs and recombinant baculoviruses. pPyLT is a cDNA expressing Py T Ag which has been cloned into a pBR322 plasmid (56). For the construction of mutant T Ags, the sequences between the *EcoRI* sites on the plasmid pBR322 component of pPyLT were eliminated. Site-directed mutagenesis was carried out on the Py T Ag cDNA fragment between the *AvaI* site at

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FIG. 1. Locations of potential CDK sites on Py T Ag. Functional domains of Py T Ag are shown, with locations of the presumptive CDK sites at T187 and T278 indicated. Below are shown sequences surrounding these residues, as well as the CDK site in SV40 T Ag. LT, large T.

nucleotide (nt) 1018 and the *EcoRI* site at nt 1562. Six oligonucleotide primers were used for the reaction. The mutant T187A was constructed by using 5' ATCCCCAACCCGAGTTCT 3' and 5' GATACAGGCGCTCTGGGG 3' as one of the two pairs of the primers; the other pair was 5' CCCCCAGAGCGCCTGTAT 3' and 5' GCATTGGAATTCGGGCTG 3'. PCR was used to generate mutations in the Py fragment spanning nt 1018 to 1562 as the template, followed by replacement into the parental Py vector. Thus, the ACT sequence coding for threonine at amino acid 187 was changed into GCG, which codes for alanine. To generate the mutant T278A, ACG (threonine) was changed to GCG (alanine). The primers used for this reaction were 5' TTAGGTGGCGCTGCATTGA 3' and 5' TTCAATGCAGCGCCACCTA 3'. A double mutant (DM) containing both changes at sites 187 and 278 was also constructed by substituting the *Ava-SacI* (nt 1018 to 1379) fragment of T278A with its counterpart in T187A. After mutagenesis, the T Ag sequences were recovered by replacing the *EcoRI* fragment back in the original direction into the plasmid. The mutations were each confirmed by sequencing of the entire fragment (nt 1018 to 1562) containing the changes. Wild-type and mutant Py T Ag cDNAs were moved into pCDNA3, which contains the cytomegalovirus (CMV) promoter for protein expression studies. Mutant Py cDNAs were also used to make recombinant baculoviruses by exchanging the fragment between the *BlpI-EcoRI* restriction sites (nt 1080 to 1562) for the same fragment in the wild-type Py T Ag baculovirus vector, pEV55LT, to create the viruses expressing mutant proteins. Baculogold viral genomic DNA (Pharmingen) was used for the generation of recombinant baculoviruses.

Purification of proteins. *Spodoptera frugiperda* Sf9 insect cells, maintained in TC100 medium supplemented with 10% fetal bovine serum, were plated at 90% confluency per 150-mm-diameter plate and infected with recombinant baculoviruses vEV55PyT, vEV55T187A, vEV55T278A, and vEV55TDMA. The cells were extracted 48 h postinfection, and the extracts were passed over a polyclonal antibody (PAb) F5 antibody column and eluted with a synthetic peptide corresponding to the F5 epitope in Py T Ag as previously described (53). To generate different CDK-cyclin complexes, Sf9 cells were coinfectd with pairs of baculoviruses expressing hemagglutinin epitope (HA)-tagged CDK2 (HA-CDK2) and cyclin A, HA-CDK2 and cyclin E, or HA-CDC2 and cyclin B. The complexes were immunopurified from the infected cells by using the anti-HA antibody 12CA5 column and subsequently eluted off the column by using a synthetic 12CA5 peptide as described previously (55). His-tagged retinoblastoma protein (pRB) was immunopurified from a baculovirus provided by S. Dowdy, using Ni^{2+} -nitrilotriacetic acid agarose beads (Qiagen) and eluted with 300 mM imidazole.

DNA replication. (i) **In vivo.** Mouse 3T6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. DNAs encoded wild-type and mutant Py T Ags as well as viral sequences extending from nt 4634

through the early region and extending past the viral T Ag coding sequences. They thus contain the entire viral regulatory region, including the early promoter and viral replication origin and Py T Ag coding sequences. To reduce possible "poison sequence" effects (29), they were excised from the plasmid vector by digestion with *Bam*HI and recircularized with T4 DNA ligase such that they lack any vector plasmid sequences. Recircularized DNAs were transfected into rapidly growing mouse 3T6 cells by the DEAE-dextran method. As a control, Py ori DNA (pBE102 [21]) containing nt 5022 to 1562 of Py strain A2 inserted into a pML vector was transfected into cells without Py T Ag. After 48 h, cells were extracted and plasmid DNA was partially purified, linearized with *Bam*HI, and digested with *Dpn*I. The digested products were separated by agarose gel electrophoresis, transferred to GeneScreen Plus filters, and probed with P^{32} end-labeled linearized Py LT1 DNA. The blots were exposed to X-ray film.

(ii) **In vitro.** Conditions for in vitro DNA replication of Py ori DNA were essentially the same as described previously (36, 43). Murine FM3A cell extracts were prepared and used as published elsewhere (36). Reaction mixtures containing FM3A extracts, Py T Ags at indicated amounts, Py ori DNA (pBE102; 300 ng), and other replication components were incubated at 33°C for 3 h. Aliquots (5 μ l) of reaction mixtures were then subjected to acid precipitation and liquid scintillation counting to determine the extent of incorporation of radioactivity into acid-insoluble material. Stability of the T Ag protein in the replication assay was ascertained by immunoprecipitating the reaction mixtures with protein G-Sepharose coupled to antibody F5, followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on an 8% polyacrylamide gel, and Western blotting with a mixture of Py T Ag-specific antibodies.

Western blot analysis. 3T6 cells were cotransfected with plasmids pBE102 and pCDNA3, containing wild-type and mutant cDNAs, respectively, by the Ca_2PO_4 coprecipitation method. The cells were lysed at 40 h postinfection in buffer containing 50 mM Tris-HCl (pH 8), 100 mM NaCl, 2 mM EDTA (pH 8), 0.1% SDS, 0.5% Nonidet P-40, and 0.1% aprotinin (Sigma). The reaction mixture was incubated on ice for 30 min. The lysate was then centrifuged for 10 min at 13,000 rpm and was rocked with antibody F5 beads at 4°C overnight. The beads were washed four times with TENG buffer (Tris, EDTA, 10% glycerol, 0.5% Nonidet P-40, 150 mM NaCl).

Protein kinase assay. In vitro protein kinase assays were carried out at 30°C for 30 min. The proteins were incubated in reaction mixtures containing kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl_2 , 1 mM dithiothreitol, 0.4 M ATP, 25 μ Ci of [$\gamma\text{-}^{32}\text{P}$]ATP, various CDK complexes). Reaction mixtures were resolved on SDS-8% polyacrylamide gels, which were silver stained and subjected to autoradiography.

DNase I footprinting assay. A Py origin-containing DNA fragment was prepared by first gel purifying a 2,056-bp fragment from plasmid p373.A2 (51) that had been cut at nt 187 by *Dde*I. This fragment was further end labeled to high specific activity (12) with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [$\alpha\text{-}^{32}\text{P}$]TTP and subsequently cleaved with *Hin*FI at nt 5075. This yielded a 411-bp fragment which was resolved on a 6% polyacrylamide gel and electroeluted. Various amounts of both wild-type and mutant T Ags were incubated for 15 min at 33°C in a buffer containing 40 mM creatine phosphate di-Tris salt (pH 7.7), 4 mM ATP, 7 mM MgCl_2 , 0.5 mM dithiothreitol, 10 μ g of bovine serum albumin, 0.1 μ g of plasmid pAT153, and 5 fmol of ^{32}P end-labeled ori DNA fragment. DNase I (10 ng) diluted in 20 mM CaCl_2 was then added for 2 min at 33°C. Reactions were terminated by adding 50 μ l of DNase I stop solution (2 M ammonium acetate, 0.2% SDS, 100 mM EDTA, 100 μ g of salmon sperm DNA per ml). DNA was purified from reaction mixtures by phenol-chloroform extraction followed by ethanol precipitation, then resuspended in deionized formamide, and analyzed on 8% polyacrylamide-8 M urea gels followed by autoradiography.

RESULTS

T187A but not T278A can initiate Py ori DNA replication in vivo. Py T Ag was shown to possess two NLSs surrounding Lys 192 and Lys 282 (44). Both are in the immediate vicinity of prospective phosphorylation sites, a feature that is common to many NLSs, and both are potential CDK sites (Fig. 1). To further investigate whether either of these can serve as a potential phosphorylation site which may be involved in T Ag replication function, we constructed mutant T Ag genes in which threonines at residues 187 and 278 were changed to alanine residues singly, generating T187A and T278A, or doubly, generating the DM (T187A T278A). Mouse 3T6 cells were transfected with DNAs expressing wild-type and mutant Py T Ags under the control of the viral regulatory region that includes the early promoter and replication origin. As a negative control, pBE102, which contains the Py replication origin but lacks T Ag-expressing sequences, was transfected alone into 3T6 cells. Viral DNA replication was assessed by Southern blot

FIG. 2. Wild-type and T187A but not T278A Py T Ag can support ori DNA replication in vivo. (A) cDNAs (100 ng) encoding wild-type (lanes 8 and 9) and mutant (T187A [lanes 2 and 3], T278A [lanes 4 and 5], or DM [lanes 6 and 7]) Py T Ag were transfected into 3T6 cells, and low-molecular-weight DNA was extracted 48 h later and analyzed as described in Materials and Methods. pBE102 Py origin-containing DNA (100 ng) was also transfected into cells (lanes 10 and 11) without Py T Ag. Lanes 1 and 12 contain Py cDNA cleaved with *Bam*HI. (B) Wild-type (lanes 8 and 9)- and mutant (T187A [lanes 2 and 3], T278A [lanes 4 and 5], and DM [lanes 6 and 7]) Py T Ag-encoding plasmids or pBE102 DNA (lanes 10 and 11) was cotransfected into 3T6 cells along with wild-type Py T Ag DNA (100 ng; lanes 2 to 7) or with no added DNA (lanes 8 to 11) and analyzed as for panel A. (C) Plasmids (10 μ g) containing cDNAs encoding wild-type and mutant T Ags were cloned into the vector pCDNA3 under the CMV promoter and transfected into 3T6 cells. Cell extracts were prepared 48 h after transfection and immunoprecipitated with Py T Ag antibodies PAb F5 and 820 coupled to Sepharose beads. Immunoprecipitates were analyzed by Western blotting using as probes a mixture of PAb F4, F5, and 820. Lane 1, T187A Py T Ag; lane 2, T278A Py T Ag; lane 3, wild-type Py T Ag; lane 4, pBE102 (negative control); lane 5, immunopurified wild-type Py T Ag.

analysis of *Dpn*I-digested low-molecular-weight DNA extracted from transfected cells (Fig. 2). We first established that maximal amounts of viral DNA replication occurred at 48 h after transfection with 100 ng of DNA (data not shown). While the wild-type and T187A proteins yielded comparable levels of *Dpn*I-resistant DNA product (Fig. 2A; compare lanes 2 and 3 with lanes 8 and 9, respectively), the mutant T278A (lanes 4 and 5) and the DM (lanes 6 and 7) failed to show detectable levels of replication products. These data are consistent with the previously published observation that a mutant Py large T Ag lacking sequences between bp 962 and 1338 bp and therefore lacking T187 was both capable of initiating DNA replication and able to complete a viral infection cycle in rapidly growing mouse 3T6 cells (27). When cDNAs expressing wild-type and mutant T Ags were cotransfected, viral DNA replication products were detected in each case, suggesting that the wild-type function is dominant over the mutant function in vivo (Fig. 2B). The plasmids used for these experiments yielded only marginally detectable levels of Py T Ags (data not shown). To ensure that all Py T Ag variants were similarly stable in cells, cDNAs encoding wild-type and mutant T Ags were moved into a vector containing the strong CMV promoter and transfected into 3T6 cells. The levels of Py T Ags detected in transfected cells were similar, showing that the wild-type and mutant T Ags were comparably expressed and accumulated to similar steady-state levels in these cells (Fig. 2C). In a separate experiment in which these constructs were also cotransfected into 3T6 cells along with pBE102, which, as mentioned above, contains the Py replication origin but does not express T Ag, essentially similar results were obtained, showing that the wild type and T187A but not T278A and DM were capable of mediating replication of pBE102 (data not shown). We therefore conclude that T278 is a critical residue for the replication function of Py T Ag in vivo.

FIG. 3. T278A mutant Py T Ag is defective in mediating ori DNA replication in vitro. (A) Py T Ags were immunopurified from Sf9 cells infected with recombinant baculoviruses expressing wild-type (WT) and T187A and T278A mutant Py T Ags. Py ori DNA replication reaction mixtures containing various amounts of the different T Ags were incubated for 3 h, and then replication products were analyzed by acid precipitation. (B) Ori DNA replication reaction mixtures containing 100 ng of Py T Ag were immunoprecipitated with PAb F5 coupled to Sepharose, resolved by SDS-PAGE, and detected by Western blotting using PAb 820 as a probe. Lane 1, T187A Py T Ag; lane 2, T278A Py T Ag; lane 3, wild-type Py T Ag; lane 4, no protein. LT, large T.

T278A and the DM cannot initiate Py ori DNA replication in vitro. To further characterize the mutant Py T Ags, baculoviruses expressing them were generated and T Ags were immunopurified as described in Materials and Methods. The abilities of the wild-type and mutant T Ags to support DNA replication in vitro in murine FM3A cell extracts were tested over a range of concentrations. Confirming and extending results obtained with transfection assays, T278A was unable to mediate DNA replication in vitro, while T187A was essentially comparable to wild-type T Ag (Fig. 3). Although in the experiment shown in Fig. 3 the T187A mutant T Ag appeared to be more active than the wild-type form at lower concentrations, this was not consistently observed, and our data indicate that the two proteins are similar in mediating ori DNA replication in vitro. By contrast, several different preparations of T278A were each completely defective over all concentrations tested. By subjecting the replication mixtures at the end of the 3-h incubation period to immunoprecipitation with antibody F5, which recognizes Py T Ag, followed by Western blot analysis (Fig. 3B), it was demonstrated that the wild-type and mutant T Ags were comparably stable throughout the replication reaction (Fig. 3B). Thus, mutation of T278 but not T187 leads to an ori DNA replication-defective T Ag in vivo and in vitro.

T278A Py T Ag is defective in origin unwinding and binding. The defect in the T278A mutant T Ag could affect any of its several biochemical functions that are important for initiating DNA replication. Wild-type Py T Ag is capable of unwinding duplex DNA fragments containing the replication origin. To examine the DNA-unwinding activity of Py T Ag in vitro, a 120-bp double-stranded DNA fragment which spans the replication origin was excised from the plasmid pBE102 (*Pvu*II-*S*tyI, nt 5267 to 90), end labeled, and used as a substrate in origin-unwinding reactions. While the wild-type and T187A

FIG. 4. DNase I footprint analysis of wild-type and mutant Py T Ags on Py ori DNA. Increasing quantities of wild-type (WT) or DM Py T Ag (0.1, 0.4, 0.8 and 1.2 μ g) or T278A T Ag (0.4, 0.8, and 1.2 μ g) were added to DNA binding mixtures. Binding, DNase I treatment, purification, and PAGE of DNA fragments were as described in Materials and Methods. Lane 1 shows DNase I cleavage in absence of added protein. Structural features of the ori DNA fragment (core origin containing the late-side A/T region, PEN site, and part of the A site) and the three T Ag binding sites (A, B, and C) are indicated at the left.

mutant forms of Py T Ag were able to unwind double-stranded DNA with comparable efficiencies, the T278A mutant protein was impaired in its ability to do so (data not shown).

Ori DNA unwinding by T Ag involves ori DNA binding, ATP hydrolysis, and DNA helicase activities. The abilities of wild-type and mutant Py T Ags to bind to the ori DNA were compared in a DNase I footprinting assay performed under replication conditions, i.e., in the presence of ATP and Mg^{2+} (Fig. 4). The wild-type protein in the presence of ATP showed its characteristic pattern of protection of the core origin (A/T,

FIG. 5. Wild-type Py T Ag is phosphorylated more efficiently by CDK2-cyclin A and CDC2-cyclin B than by CDK2-cyclin E. (A) Py T Ag (100 ng; top) or pRB (300 ng; bottom) was incubated with increasing amounts of CDK2-cyclin A (lanes 2 to 4), CDC2-cyclin B (lanes 5 to 7), and CDK2-cyclin E (lanes 8 to 10). Lane 1 contained a reaction mixture that lacked kinase. Mixtures were separated on an SDS-8% polyacrylamide gel, silver stained, and autoradiographed. WT Py LT, wild-type Py large T. (B) The silver-stained gel shown as an autoradiograph in panel A. Note the difference in mobility between phosphorylated wild-type Py T Ag (WT LT-P) and wild-type Py T Ag (WT LT).

PEN [pentanucleotide-containing origin palindrome], and A sites) and adjacent B and C sites (28). By contrast, both T278A and the DM Py T Ags showed defective and altered protection of the Py T Ag regulatory region. While at the highest concentration they protected the A, B, and C sites, and also showed the characteristic DNase I hypercutting on the late side of the origin, they displayed a nearly complete absence of protection of the A/T and PEN regions within the core origin. This defect in interactions with the core origin is likely to be the primary reason for the failure of T278A to support ori DNA synthesis.

Wild-type Py T Ag can be phosphorylated by S and G₂/M but not G₁/S CDKs. Our experiments showed that T278 but not T187 is critical for the replication function of Py T Ag. Since both are potential CDK phosphorylation sites, we wished to characterize the ability of Py T Ag to serve as a substrate for this class of protein kinases. Three cyclin-CDK complexes, cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDC2, which were immunopurified from baculovirus-infected insect cells, were tested for the ability to transfer ^{32}P from [γ - ^{32}P]ATP to Py T Ag (Fig. 5A, upper panel). Their activities were normalized by using purified pRB (lower panel). When comparable activities of these kinase complexes were used to phosphorylate wild-type Py T Ag, marked differences were observed: whereas CDK2-cyclin A and CDC2-cyclin B phosphorylated T Ag significantly, CDK2-cyclin E did so very poorly. Phosphorylation of Py T Ag by the cyclin A and cyclin B complexes was efficient since it caused the protein to exhibit a lower mobility on silver-stained gels (Fig. 5B), an observation consistent with the fact that many phosphorylated polypeptides, including Py T Ag itself (2, 20), display altered and usually lower electrophoretic mobility than do their unphosphorylated or underphosphorylated counterparts. While we did not test a complex consisting of a D-type cyclin paired with CDK4, it is predicted that such a complex would be defective in phosphorylating T Ag, based on the extremely restricted substrate specificity of

FIG. 6. Cyclin B-cdc2 can phosphorylate wild-type and mutant T187A Py T Ags more efficiently than T278A Py T Ag. Increasing amounts of cyclin B-CDC2 complex were added to wild-type (WT; lanes 2 to 4), T187A (lanes 6 to 8), and T278A (lanes 10 to 12) T Ag reaction mixtures. Mixtures in lanes 1, 5, and 9 did not contain protein kinase. The mixtures were separated on an SDS-8% polyacrylamide gel, silver stained, and exposed to autoradiography. The lower panel is a separate silver-stained gel showing the proteins that were used in the assay. LT, large T.

cyclin D-CDK complexes (22). Thus, Py T Ag is a markedly better substrate for S-phase (cyclin A-CDK2) and G₂/M-phase (cyclin B-cdc2) complexes than a G₁/S-phase (cyclin E-CDK2) complex. Since Py T Ag is a substrate for CDKs *in vitro*, this suggests that the T Ag's enhanced state of phosphorylation by the S-phase CDK possibly contributes to its efficiency in replication functions.

Reduced phosphorylation of T278A mutant large T Ag by the cdc2-cyclin B kinase complex. We next examined the ability of cyclin B-cdc2 to phosphorylate wild-type and mutant Py T Ags. Proteins were incubated with the protein kinase under conditions similar to those used for Fig. 5, followed by SDS-PAGE and autoradiography (Fig. 6). Both wild-type and T187A Py T Ags were capable of being phosphorylated by the CDK complex (upper panel). Note that in this experiment, the phosphorylated proteins did not exhibit significantly reduced electrophoretic mobility. Whether this was due to less efficient phosphorylation or less well resolving gel conditions in this experiment is not known. Unfortunately, despite repeated attempts, we were unable to prepare immunopurified T187A protein that was free of a contaminating protein kinase activity. Nevertheless, when the background level of this activity was subtracted (using data from phosphorimaging), it appeared that T187A was actually a somewhat better substrate (1.5- to 2-fold more phosphate was incorporated) for phosphorylation by the cyclin B-cdc2 kinase complex than was wild-type Py T Ag. By contrast, T278A was poorly phosphorylated under these conditions; data from phosphorimaging revealed that 18-fold more phosphate was incorporated into wild-type than T278A at an intermediate level of added protein kinase, and 3-fold more phosphate was incorporated at the highest level of enzyme used. These results suggest that T278 is an important target for phosphorylation by CDKs. In further experiments, it was observed that the wild type and T187A could be phosphorylated to similar extents (in comparison to each other) over a range of concentrations of unlabeled ATP (50 to 1,000 μ M; data not shown). In comparison, T278A was a poor substrate in the presence of 400 μ M ATP, with only about 9 to 10% of the wild-type level of phosphorylation seen; in the presence of 1,000 μ M ATP, we did not detect any phosphorylation of this mutant (data not shown). Since there was a small but significant amount of phosphorylation of the T278A mutant, however, our results suggest that there is one (or more) additional CDK sites on Py T Ag. Supporting this assumption is the result of an earlier experiment performed in our laboratory in which we observed that two bacterially produced Py T Ag peptides spanning residues 60 to 240 and 24 to 521, respectively, were

both phosphorylated *in vitro* by cdc2-cyclin B purified from HeLa cells (40a). In the context of the full-length protein, our data indicate that T278 is the predominant acceptor of phosphate from CDKs.

We also tested whether phosphorylation of Py T Ag by cyclin B-cdc2 affected its ability to mediate Py ori DNA replication *in vitro*. T Ags were preincubated with or without cdc2-cyclin B for 30 min on ice, prior to addition to replication mixtures. As expected, T278A and the DM were inactive in replication when they were subjected to preincubation with the kinase. Additionally, although the wild type and T187A could be efficiently phosphorylated in presence of 400 μ M ATP in our *in vitro* kinase assay, these preincubated T Ags exhibited greatly reduced replication function (data not shown). McVey et al. (31) similarly observed that SV40 T Ag which had been prephosphorylated with cdc2-cyclin B displayed greatly reduced ori DNA replication function. Those authors suggested that ATP induces oligomerization of T Ag which, in the absence of DNA, is incapable of assuming the correct nucleoprotein-ori DNA complex and thus is prevented from initiating DNA replication. Wang and Prives (52) similarly showed that ATP induces Py T Ag to form hexamers in the absence of DNA in a manner similar to what has been observed with SV40. Indeed, we found that the reduction in replication activity could be overcome by including the origin-containing plasmid DNA (pBE102) in the preincubation mixture (data not shown). Even under these conditions, however, no dramatic or reproducible stimulation of replication was observed with either wild-type or T187A protein compared with reactions that did not contain exogenously supplied CDK (data not shown). Since we have found that the FM3A cell extract contains a protein kinase(s) capable of efficiently phosphorylating Py T Ag (data not shown), such activity may well maintain phosphorylation of the crucial site(s) on the protein necessary to render it active. On a related note, the fact that Py T Ag can be phosphorylated in FM3A replication extracts may explain an apparent discrepancy in our results: our data show that Py T Ag needs to be phosphorylated by CDKs at T278, yet the wild-type baculovirus-expressed T Ag is an excellent substrate for CDK phosphorylation, suggesting that it is in a relatively underphosphorylated state. If Py T Ag becomes phosphorylated at T278 in FM3A extracts, this would explain any inconsistency in our experimental results. We are currently working on discerning what sites on Py T Ag are phosphorylated in FM3A cell extracts.

DISCUSSION

Previous work from this laboratory established that the replication function of Py T Ag is regulated by phosphorylation (54). We analyzed the effects of removal of phosphates from Py T Ag with calf intestinal alkaline phosphatase on its different replication activities *in vitro*. Our results indicated the presence of both repressing and stimulatory phosphate residues on T Ag. These data suggested that there are protein kinases that can regulate the function of Py T Ag. Here we present evidence that CDKs are very likely to be critical to Py T Ag function. Specifically, we have shown that Py T Ag is a substrate for S- and G₂-phase-specific CDKs at a site, T278, that is critical for its replication function.

Our observations provide evidence not only that the Py and SV40 T Ags are functionally similar as viral ori DNA replication initiators but that their functions are regulated by CDK phosphorylation analogous fashions. Detailed analyses of the impact of the CDK site at T124 of SV40 T Ag have elucidated the mode by which this protein is regulated by phosphorylation. Moarefi et al. (35) and McVey et al. (32) showed that

mutant (T124A) SV40 T Ag is incapable of mediating SV40 ori DNA replication *in vitro*, despite the fact that it displays ATP-dependent hexamerization, DNA helicase activity, binding to site I DNA adjacent to the core origin, and melting of sites within the origin to extents similar to those of wild-type T Ag. The most striking difference between the two T Ags, however, was seen with origin unwinding assays. Moarefi et al. (35) showed that T124A was completely defective in unwinding closed circular DNA, and McVey et al. (32) observed that the mutant T Ag was inefficient in unwinding a duplex origin-containing DNA fragment. In another study, it was reported that fragments of SV40 T Ag spanning the origin binding domain are stimulated to bind to the origin after phosphorylation by cdc2 kinase (33). Our data comparing wild-type and T278A mutant Py T Ags were essentially similar. The T278A mutant is incapable of unwinding Py ori DNA, with defective interactions within the origin but not with the sites adjacent to the core. Like SV40 T Ag, Py T Ag induces specific structural distortions within the Py core origin (1, 26). The defect in the interactions of mutant T278A with the core origin suggests that it might also be defective in its ability to unwind ori DNA. However, since the corresponding mutation in SV40 did not result in defective origin-unwinding function, this remains an open question.

We found that Py T Ag is phosphorylated by cyclin A-CDK2 and cyclin B-CDC2 but not cyclin E-CDK2. This is not the first reported example of differential abilities of G₁- and S-phase cyclin complexes to phosphorylate proteins. Dynlacht et al. (8) and Wang and Prives (55) showed that E2F and p53, respectively, are phosphorylated well by S- and G₂-phase but not G₁-phase complexes. Differences within and adjacent to the canonical (S/T)PX(R/K) site are likely to contribute to the differential activities of cyclin E-CDK2 and cyclin A-CDK2 to phosphorylate different substrates (18, 19).

Our results are consistent with previous observations suggesting changes in the phosphorylation state of Py T Ag *in vivo*. Py T Ag was shown to be in an underphosphorylated state early in the infection of growth-arrested cells; as infection proceeds, the protein becomes detectably more phosphorylated, as evidenced by reduced electrophoretic mobility (2, 20). While it is not known whether this results from phosphorylation of Py T Ag by CDKs, our data support this likelihood. First, phosphorylation by CDKs results in slowed electrophoretic mobility of Py T Ag protein *in vitro*; second, our results suggest that S- and G₂-phase CDK complexes are uniquely able to phosphorylate Py T Ag. The implication of the latter is that a resting host cell would be unlikely to contain CDKs capable of phosphorylating T Ag, and only after the infected cell was induced to enter the active cycling mode would the appropriate CDKs be available to phosphorylate T Ag.

In vitro studies suggest that once ori DNA is unwound, both SV40 and Py T Ags form initiation complexes with DNA polymerase α and replication protein A (RP-A) from their respective primate and murine host cells (47). Interestingly, in addition to T Ag, DNA polymerase α (34) and RP-A (7, 40) are potentially substrates of CDKs. However, it is possible that CDK phosphorylation is not required to activate the functions of RP-A and DNA polymerase α . Indeed, it has been shown that the RP-A CDK sites are not essential for its function *in vitro* (25).

Our result that the late G₁ CDK complex (cyclin E-CDK2) is ineffective in phosphorylating Py T Ag is thus intriguing: given the importance of the CDK site on Py T Ag at T278, these data suggest that the cell must enter a state where kinases capable of phosphorylating this site on T Ag are present. This, in turn, suggests the existence of a cellular regulatory

mechanism that coordinates the phosphorylation status of the T Ag presumably in a cell cycle-dependent manner so that regardless of the time of infection of the host cell, viral DNA replication commences only when cellular DNA replicates in S phase. SV40 viral DNA replication begins when the host cell enters the S phase regardless of the stage of the cell cycle at which the cell was infected (39). Also, S-phase cell extracts were found to be 10-fold more efficient than G₁-phase cell extracts in supporting SV40 DNA replication *in vitro* (45). Since Py DNA replication, like that of SV40, is likely to require entry into S phase, one question that arises is, how do these viruses ensure that they will encounter a cycling host cell, or if they do not, then how do they induce the host which is in a resting mode to initiate DNA synthesis? Py T Ag has been shown to bind to pRB (9), harboring a specific sequence which by deletion analysis has been demonstrated to be important for its interaction with pRB (24). It could be speculated that in the resting host cell, Py T Ag inactivates pRB, thus allowing the cell to progress into S phase. This would then allow for the appropriate CDK(s) to accumulate and regulate the phosphorylation status of T Ag, leading to the initiation of viral DNA replication. Gjørup et al. (15) in an elegant study provided evidence that the N terminus of Py T Ag is necessary for the replication function of the Py T Ag C terminus only in resting cells. They reported, further, that the N terminus actually induces increased phosphorylation of the C terminus, a result that is entirely consistent with our data. Furthermore, their data showed that a truncated Py T Ag starting at residue 264 supported replication while one starting at 280 did not. Thus, it would appear that the region between these residues is essential for initiating DNA replication. It should be noted, however, that they also provided evidence that T Ag itself can stimulate cell cycle progression without interacting with pRB. However T Ag stimulates cell cycle progression, our results and those of Gjørup et al. show that the phosphorylation status of the protein is paramount for its ability to effect DNA replication.

Our data for T187 were more difficult to interpret. Although this site contains some features common to CDK consensus sites (49), it is a rather imperfect version of such sites. Clearly mutation of this site is neutral for DNA replication *in vitro* and *in vivo*. Furthermore, our results suggest that this site is only weakly phosphorylated by the CDKs tested. Nevertheless, T187 is likely to be phosphorylated on Py T Ag (3, 17). Perhaps it is a substrate for mitogen-activated protein kinase or proline-directed cellular protein kinases. Its location in the vicinity of an NLS is intriguing, given the frequency with which such signals are near phosphorylation sites and the positive role that such phosphorylation frequently plays in nuclear transport. Additional experiments testing the effect of this mutation in resting cells, or in other functions of Py T Ag such as transcriptional regulation or cellular immortalization assays, may provide further insight into this residue. Given the multifunctional nature of T Ag, it is conceivable that it may be differentially phosphorylated in order to carry out those activities. Although these issues are unresolved, our results strongly suggest the importance of CDKs in Py ori DNA replication. Since CDKs were recently shown to be essential for the initiation of mammalian DNA replication *in vitro* (23), studies with viral T Ags may provide a paradigm for regulation of initiators of cellular DNA replication.

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