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The Replication Functions of Polyomavirus Large Tumor Antigen Are Regulated by Phosphorylation

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Polyomavirus (Py) large T antigen (T Ag) contains two clusters of phosphorylation sites within the amino-terminal half of the protein. To characterize possible regulatory effects of phosphorylation on viral DNA replication, Py T Ag was treated with calf intestinal alkaline phosphatase (CIAP). Incubation of the protein with a range of phosphatase concentrations caused progressive loss of phosphate without affecting its stability. Treatment with smaller quantities of CIAP stimulated the ability of the viral protein to mediate replication of constructs containing the viral replication origin, while higher concentrations of CIAP caused a marked diminution of this replication function. Several biochemical activities of Py T Ag were examined after CIAP treatment. Py T Ag DNA unwinding and nonspecific DNA binding were only slightly affected by dephosphorylation. However, as determined by DNase I footprinting experiments, treatment with smaller amounts of CIAP stimulated specific binding to the Py replication origin by Py T Ag, while treatment with larger amounts of CIAP caused marked inhibition of origin-specific binding by the viral protein. Phosphotryptic maps of Py T Ag before or after treatment with CIAP revealed changes in individual phosphopeptides that were uniquely associated with either the stimulation or the inhibition of replication. Our data therefore suggest that Py T Ag is regulated by both repressing and activating phosphates.

The large tumor antigen (T Ag) of polyomavirus (Py) is a 90- to 100-kDa nuclear protein which has multiple functions in the viral life cycle. Py T Ag is involved in the initiation of viral DNA replication in vivo (10) and in vitro (23) and the regulation of early transcription from the viral genome (5). The role of Py T Ag in DNA replication involves a number of biochemical properties, including its specific interaction with the viral origin of replication (6, 7, 19, 32), as well as its duplex double-stranded DNA fragment unwinding (38), DNA helicase (29, 38), and ATPase (12, 29) activities. Some of these properties of Py T Ag have been mapped to distinct structural domains (7, 24, 32). However, it is of interest to determine if and how the various functions of Py T Ag are regulated. One possibility is that posttranslational modifications play a role. Py T Ag is a phosphoprotein (27) that exists in multiple forms as detected by differences in electrophoretic mobility (17). The more slowly migrating forms represent phosphorylated molecules of Py T Ag which are converted to faster migrating species upon treatment with alkaline phosphatase (1). By analogy with the well studied large T Ag encoded by simian virus 40 (SV40), phosphorylation may regulate the replication activity of Py T Ag.

The replication functions of the highly homologous SV40 T Ag are both negatively and positively regulated by phosphorylation (for reviews, see references 9 and 25 and references therein). Treatment of SV40 T Ag with either calf intestinal alkaline phosphatase (CIAP) (13, 18, 22, 30) or the catalytic subunit of phosphatase 2A ($PP2A_c$) (28) stimulated its ability to mediate SV40 origin-dependent DNA replication in vitro. Both enzymes specifically removed phosphates from serine residues. However, phosphorylation by cdc2 kinase of a single amino acid, Thr-124, was sufficient to confer high levels of DNA replication activity, similar to those observed with eukaryotically expressed protein, to bacterially produced inert SV40 T Ag (21).

A comparison of the phosphorylation sites on Py and SV40 T Ags shows both similarities and differences (Fig. 1). Py T Ag, like SV40 T Ag, contains two clustered sites of phosphorylation (2, 14). However, unlike SV40, both clusters are located within the amino-terminal half of the polypeptide. Alignment of the two proteins, so that they exhibit the highest degree of sequence and functional homology, revealed that one cluster of sites, located adjacent to the nuclear localization signal and origin binding domain, is in a region that is highly conserved between the two T Ags. Within SV40 this region encompasses the phosphorylated threonine residue (Thr-124) which is essential for SV40 T Ag function in viral DNA replication. Strikingly, the second cluster of phosphorylated residues in Py and SV40 T Ag are each located within regions unique to the respective proteins. This suggests that altering the phosphorylation state of Py T Ag may affect its replication function in a manner different from that observed with SV40 T Ag. We report here the effects of the removal of phosphates from Py T Ag with CIAP on its different replication functions in vitro.

MATERIALS AND METHODS

Removal of phosphates from Py T Ag with CIAP. Py and SV40 T Ag were prepared from insect Sf-27 cells infected with recombinant baculoviruses, vEV55PyT and vEV55SVT, respectively, as previously described (37, 38). ^{32}P-labeled protein was purified from extracts derived from infected Sf-27 cells which were labeled for 4 h with 0.5 mCi of [^{32}P]P_i in 3 ml of phosphate-free TC-100 medium (Specialty Media) per 150-mm-diameter dish. Purified Py or SV40 T Ag was incubated with CIAP (Boehringer Mannheim) at 33°C for 5 to 20 min in either 1× CIP buffer (10 mM Tris-HCl [pH 8.5], 1 mM $MgCl_2$, 1 mM $ZnCl_2$) or 1× dephosphorylation buffer (50 mM Tris-HCl [pH 8.5], 0.1 mM EDTA) as indicated. The pretreated T Ag was then added directly to reaction mixtures and analyzed in biochemical assays described below.

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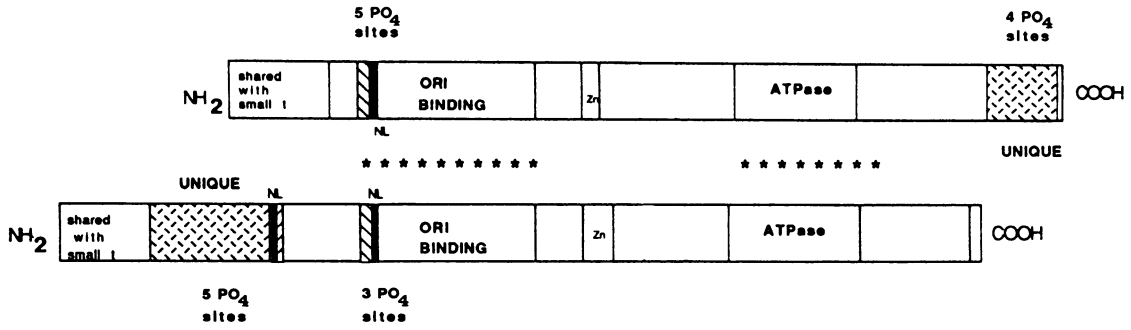


FIG. 1. Comparison of the large T Ags of Py (785 residues; lower diagram) and SV40 (708 residues; upper diagram). In the schematic diagrams, the two viral proteins have been aligned so that they exhibit the highest degree of sequence homology. The regions of greatest similarity are indicated by the asterisks. The domains identified to be important for origin DNA binding and ATPase activity are also indicated. The position of the nuclear localization signals (NL) and phosphorylation sites (PO_4 sites) in each protein are shown. The region containing the putative zinc finger is designated.

In vitro ori-DNA replication. Py and SV40 replication reactions (50 μl) as previously described (37) were carried out in mouse FM3A cell and human HeLa cell extracts, respectively. Standard reaction mixtures (50 μl) contained 40 mM creatine phosphate (pH 7.7; di-Tris salt); 7 mM $MgCl_2$; 0.5 mM dithiothreitol; 4 mM ATP; 200 μM each CTP, UTP, and GTP; 100 μM each dATP, dGTP, and dCTP; 20 μM [α-^{32}P]dTTP (2×10^4 cpm/pmol); and 100 μg of creatine kinase per ml. The Py ori-DNA plasmid pPyCAT (34) and Py T Ag, which had been preincubated in the presence or absence of CIAP, were incubated at 33°C. SV40 DNA replication reactions, which required the SV40 ori-DNA plasmid pSVS (15) and CIAP-treated or untreated SV40 T Ag, were carried out at 37°C. After a 3-h incubation at the appropriate temperature, the amount of DNA synthesis was determined by acid precipitation. Alternatively, progeny DNA was purified from the reaction mixtures, linearized with *Bam*HI in the presence or absence of *Dpn*I, a restriction enzyme which selectively digests the fully methylated unreplicated input DNA. The samples were then separated on 0.8% agarose gels, and the replication products were visualized by autoradiography.

Duplex double-stranded DNA unwinding assay. The DNA substrate used in unwinding reactions consisted of a 97-bp Py core replication origin fragment (nucleotide 5265 to 64) derived from a *Sal*I-*Xho*I digestion of pΔ76-4 (26). The fragment was 3′-end-labeled and filled in by using the Klenow fragment of DNA polymerase I in the presence of [^{32}P]TTP and the remaining unlabeled nucleotides. Unwinding reactions, which were carried out under conditions previously described (36, 38), contained 4 mM ATP, 7 mM $MgCl_2$, 0.3 μg of *Escherichia coli* single-stranded binding protein (purchased from Boehringer Mannheim), 2 ng of the labeled DNA fragment, and Py T Ag that had been pretreated with increasing amounts of CIAP. After a 30-min incubation at 33°C, sodium dodecyl sulfate (SDS) proteinase K, and EDTA were added to a final concentration of 1%, 1 mg/ml, and 25 mM, respectively. The mixtures were incubated an additional 30 min at 37°C, followed by 65°C for 5 min. The DNA was resolved on 8% polyacrylamide gels and visualized by autoradiography.

Nitrocellulose filter-binding assay. Binding of Py T Ag to filters was essentially as previously described (19). Untreated and CIAP-treated Py T Ag was added to binding reaction mixtures containing 40 mM creatine phosphate (pH 7.7; di-Tris salt), 0.5 mM dithiothreitol, 4 mM ATP, 7 mM $MgCl_2$, 0.2 μg of nonspecific DNA (pAT153) per ml, and 0.3 ng of a 3′-^{32}P-end-labeled 97-bp Py core replication origin fragment used in DNA unwinding reactions. Binding was allowed to proceed for 15 min at 33°C. Reaction mixtures were then passed through 0.45-μm-pore-size nitrocellulose filters presoaked in wash buffer (20 mM $NaPO_4$ [pH 7.0], 0.1 mM EDTA, 0.1 mM dithiothreitol, and the filters were washed three times with the same buffer. The amount of DNA bound was determined by counting in liquid scintillation.

DNase I protection assay. The DNA fragment used for DNase I footprinting was prepared as follows. A 2,056-bp *Dde*I fragment derived from p373.A2 (33) was gel purified and 3′-end-labeled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP, [α-^{32}P]dGTP, and [α-^{32}P]TTP. The isolated labeled fragment was digested with *Hin*fI, generating a 411-bp fragment labeled at the *Dde*I site (nucleotide 187). This fragment was purified by electroelution from 6% polyacrylamide and used in subsequent experiments. Conditions for the binding of Py T Ag to the above-described origin fragment (1.5 to 3.0 ng) were identical to those used in filter-binding experiments. After a 15-min incubation at 33°C, 15 μl of a 40- to 100-μg/ml DNase I solution diluted in 20 mM $CaCl_2$ was added to binding reaction mixtures. DNase I digestion was continued at 33°C for 2 min and was terminated with the addition of 50 μl of DNase I stop mix (2 M ammonium acetate, 100 mM EDTA, 0.2% sodium dodecyl sulfate, 100 μg of sheared salmon sperm DNA per ml). The DNA was then purified from the reaction mixtures by phenol-chloroform extraction followed by ethanol precipitation. Samples were resuspended in 90% deionized formamide sample buffer in 1× Tris-borate-EDTA and analyzed on 8% polyacrylamide–8 M urea sequencing gels and by autoradiography.

Tryptic peptide maps. ^{32}P-labeled immunopurified Py T Ag was incubated with 0, 2, or 12 U of CIAP at 33°C for 20 min. Aliquots of Py T Ag samples were tested in Py ori-DNA replication reactions prior to tryptic peptide mapping procedures, which were essentially as described previously (4, 14, 20). Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and then electrotransferred to nitrocellulose filter paper, which was stained with Ponceau-S stain to identify the Py T Ag and then exposed to film. The labeled Py T Ag bands were then excised from the membrane and soaked in 0.5% polyvinylpyrrolidone (M_r of 360,000; Sigma) in 100 mM acetic acid for 30 min at 37°C. The membrane slices were then washed five times in distilled water and then twice in freshly prepared ammonium bicarbonate (pH 7.8) prior to incubation in buffer containing 10 μg of tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin for 2 h in 200 μl of 50 mM NH_4HCO_3 (pH 7.8) at 37°C, followed by incubation with a second aliquot of TPCK-treated trypsin for an addi-

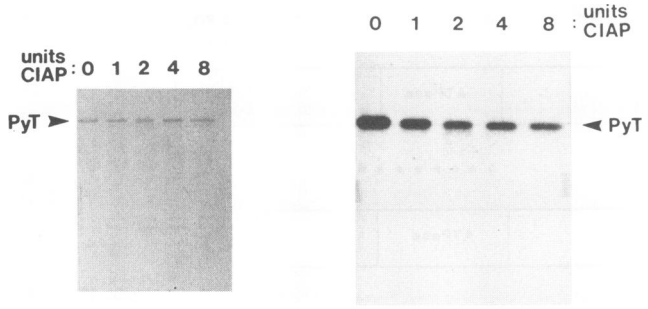


FIG. 2. Dephosphorylation of Py T Ag by CIAP. (A) Purified in vivo ^{32}P-labeled Py T Ag (1 μg) was incubated with the indicated amount of CIAP in 1× CIP buffer for 30 min at 33°C. The CIAP-treated proteins were separated on SDS–10% polyacrylamide gels and visualized by silver staining. (B) The same gel was air dried between cellophane sheets and autoradiographed.

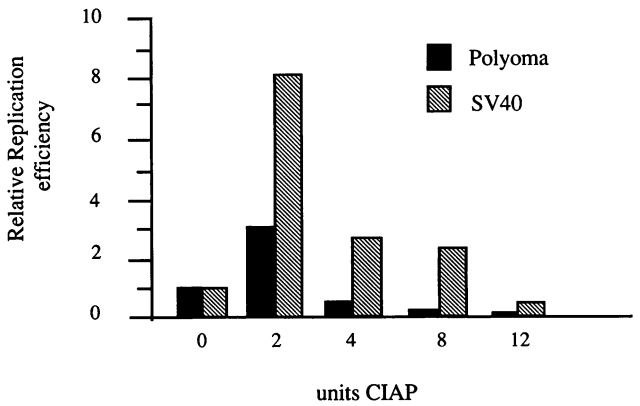


FIG. 3. CIAP differentially affects the ability of Py and SV40 T Ag to mediate ori-DNA replication in their respective in vitro systems. Py T Ag (1.5 μg) was preincubated in 1× CIP buffer with the indicated units of CIAP for 20 min at 33°C. The pretreated protein (0.75 μg) was then added to replication reaction mixtures containing a Py ori-DNA plasmid and mouse FM3A cell extract. After a 3-h incubation at 33°C, 5-μl aliquots were acid precipitated to determine the level of DNA synthesis, and the relative replication efficiency was calculated and the number of picamoles incorporated in the absence of CIAP was assigned a value of one and used to calculate the relative replication efficiency of the different reaction mixtures. The number of picamoles incorporated in the absence of CIAP was 27. SV40 T Ag (1 μg) was treated as described for Py T Ag, and half the mixture was added to replication reaction mixtures containing an SV40 ori-DNA plasmid and human HeLa cell extract. Following 3 h at 37°C, the amount of acid-insoluble material in 5-μl aliquots was determined and quantitated as described for Py. A total of 23 pmol of synthesis material was mediated by SV40 T Ag in the absence of CIAP.

tional 12 to 16 h. Then, 300 μl of H_2O was added, the sample was centrifuged, and the liquid was transferred to a fresh tube and evaporated to dryness in a SpeedVac centrifuge. The dried peptides were then oxidized in 50 μl of performic acid for 1 h at 4°C. One milliliter of H_2O was then added, and the samples were again evaporated to dryness prior to being dissolved in 6 μl of pH 1.9 buffer (4) and stored at −20°C until further use. Comparable amounts of radioactively labeled material from the different samples were then subject to two dimensional electrophoresis and chromatographic separation as follows. Phosphopeptides were first separated on cellulose thin-layer chromatographic plates by using the Hunter Thin-Layer Electrophoresis Apparatus (C.B.S. Scientific) for 50 min in pH 1.9 buffer and 0.25% pyridine. Plates were then subjected to ascending chromatography in buffer containing isobutyric acid-pyridine-acetic acid-butanol-H_2O (65:5:3:2:29 [vol/vol]) for 6 to 8 h and then dried and exposed to X-ray film. Autoradiograms were then analyzed by scanning densitometry with a Bioimage Visage 210 to quantify some of the peptides.

RESULTS

CIAP removes the majority of phosphates from Py T Ag. To identify a relationship between the phosphorylation state and the replication activities of Py T Ag, we chose to examine the effects of phosphatase treatment on the viral protein. Using Py T Ag purified from ^{32}P-labeled insect cells, we first compared $PP2A_c$ from two noncommercial sources, as well as commercially available potato acid phosphatase and CIAP. Over the range of enzyme concentrations tested, neither preparation of $PP2A_c$ removed more than 10% of the phosphate label from Py T Ag and such treated protein displayed virtually no changes in any of its replication functions (data not shown). For these reasons, $PP2A_c$ was not used for further experiments. While potato acid phosphatase removed a far greater proportion of the labeled phosphate from Py T Ag, there was also considerable degradation of the viral protein under these conditions, rendering it unsuitable for further study (data not shown). By contrast, when Py T Ag was incubated with increasing quantities of CIAP we observed both a marked loss of phosphate and little effects, if any, on its stability. The results of a typical experiment are shown in Fig. 2. When ^{32}P-labeled Py T Ag was incubated for 30 min at 33°C followed by SDS-PAGE and silver staining, we observed that there was no change in the overall level of protein after CIAP treatment (Fig. 2A, left panel). An autoradiogram of the same gel revealed that proportionally greater quantities of phosphates were removed with increasing levels of enzyme such that 1, 2, 4, and 8 U removed approximately 50, 60, 75, and 80%, respectively, of the labeled phosphates from Py T Ag, as estimated from phosphoimaging densitometry (Fig. 2B, right panel). Although when various batches of CIAP and labeled T antigen were similarly tested essentially similar results were obtained, there was some variation in the amount of CIAP required to remove similar proportions of labeled phosphate. However, in general, treatment with up to 12 U of CIAP removed between 60 and 80% of the ^{32}P label but did not cause detectable reduction in the amount of total immunoreactive Py T Ag.

CIAP treatment differentially affects Py and SV40 T Ags. CIAP treatment of SV40 T Ag was shown to stimulate its ability to mediate SV40 ori-DNA replication in vitro (14, 22). It was therefore of interest to compare the relative effects of CIAP treatment on the ability of both T antigens to mediate ori-DNA replication in their respective replication systems. Preparations of Py and SV40 T Ag were chosen for their similar levels of replication activity in mouse and human cell extracts, respectively. The effects of preincubating Py and SV40 T Ags with CIAP on their ability to support Py or SV40 ori-DNA synthesis in murine or human cell extracts, respectively, were examined (Fig. 3). The amount of DNA synthesis after 3 h at 33°C was determined by acid precipitation. Since, as mentioned above, we observed some variation among the activities of different batches of CIAP utilized, the results shown are a composite of a large number of experiments with Py T Ag and with SV40 T Ag (20 and 6 experiments, respectively). Two general observations were made reproduc-

ibly. (i) With either T Ag, at lower levels of CIAP, there was a marked stimulation of replication while at higher levels of phosphatase replication was reduced, and (ii) the relative sensitivity of both proteins differed: Py T Ag was consistently both stimulated to a lesser degree and inhibited more dramatically by CIAP treatment than was SV40 T Ag. Indeed, with some preparations, Py T Ag was inhibited at all CIAP concentrations tested. Since the results obtained with the SV40 system confirmed previously published studies, in which CIAP dramatically enhanced SV40 T Ag-dependent DNA replication, this supported the validity of the somewhat contrasting effects of dephosphorylation on Py T Ag. Treatment of SV40 T Ag with greater quantities of CIAP reduced the fold increase. However, at any given CIAP concentration, the relative replication efficiency of the SV40 ori-DNA reaction, compared with that in the absence of phosphatase treatment, was greater than that detected with Py ori-DNA. We observed that SV40 T Ag preparations that had maximal overall replication activity similar to that of Py T Ag in cell extracts (i.e., ~10 to 30 pmol of nucleotide incorporated into DNA products) were strongly stimulated by CIAP treatment. By contrast, preparations of SV40 T Ag that were more active (i.e., 70 to 100 pmol of nucleotide incorporated maximally) were stimulated to lesser extents (data not shown). It was previously established that the optimal temperatures of the SV40 and Py ori-DNA replication reactions are 37 and 33°C, respectively. However, by performing CIAP treatment experiments with both T Ags at both temperatures, we ascertained that a difference in temperature was not the reason for the difference between the responses of the two T antigens to dephosphorylation (data not shown). Thus, Py and SV40 T Ags display disparate responses to the removal of phosphates by CIAP.

Controls were used to support the likelihood that the effects observed with Py T Ag were the direct result of phosphatase treatment of the viral protein. First, CIAP that had been boiled prior to addition to reaction mixtures failed to cause any inhibitory effect (Fig. 4A). Second, in parallel experiments, identical quantities of Py T Ag and CIAP, which had undergone no preincubation, were also examined in replication reactions (Fig. 4B). It should be noted that the level of synthesis mediated by Py T Ag that had been preincubated in the absence of CIAP was approximately 50% of that of similar quantities of Py T Ag that were added directly to the reaction mixtures. When CIAP was thus added directly to Py T Ag containing replication reactions, a greater number of units of enzyme were required before even a slight (25%) decrease was detected. As the enzyme remained present throughout the 3-h incubation period, these experiments do not rule out the possibility that CIAP exerted additional effects on one or more cellular factors involved in the replication of Py ori-DNA. However, the fact that preincubation of Py T Ag with CIAP led to a markedly greater reduction in Py ori-DNA synthesis suggests strongly that the removal of phosphates from Py T Ag directly affected its replication function.

The double-stranded DNA fragment unwinding activity of Py T Ag is unaltered by CIAP. To further understand how CIAP treatment might affect Py T Ag function, the biochemical properties of the viral protein involved in Py DNA synthesis were analyzed. Py T Ag DNA helicase and unwinding activities are involved in the initiation of DNA replication, i.e., the formation of the open DNA complex. The ability of CIAP-treated Py T Ag to unwind a double-stranded DNA fragment encompassing the Py origin of replication was examined in the presence of ATP and single-stranded DNA-binding protein (Fig. 5). The preincubation of Py T Ag with increasing amounts of CIAP had no affect on the amount of single-

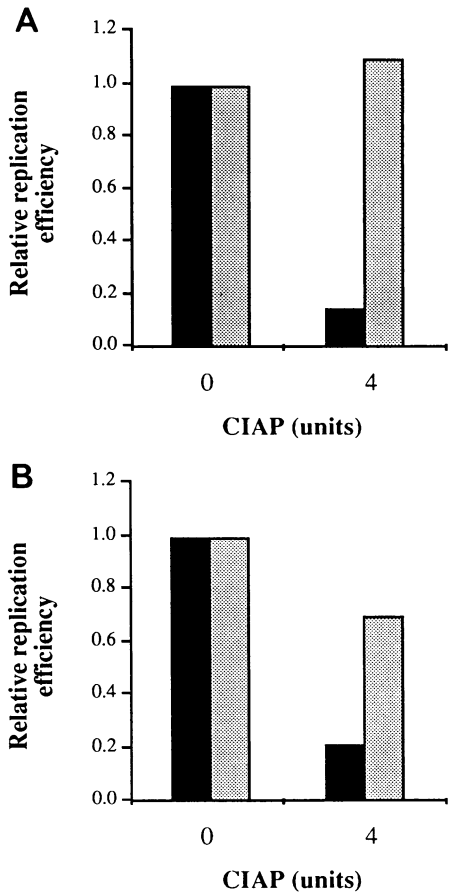


FIG. 4. CIAP inhibits Py DNA replication by removing phosphates from Py T Ag. (A) Py T Ag (0.8 μg) was preincubated with CIAP (■) or heat-inactivated (boiled) CIAP (□) for 10 min at 33°C in 1× dephosphorylation buffer. The proteins were then added to replication reaction mixtures containing a Py ori-DNA plasmid and mouse FM3A cell extract. The level of synthesis was measured after 3 h at 33°C by acid precipitation. A total of 59.6 pmol of incorporation was detected by acid precipitation in the absence of CIAP. (B) Purified Py T Ag (0.6 μg) and 4 U of CIAP were preincubated for 20 min at 33°C in 1× dephosphorylation buffer and added to replication reaction mixtures (■). In parallel, equivalent amounts of Py T Ag and CIAP were added directly to reaction mixtures (□). All reaction mixtures were incubated for 3 h at 33°C and analyzed as described for panel A. Py T Ag supported 13.2 pmol of incorporation when preincubated in the absence of CIAP and 26.5 pmol when added directly to reaction mixtures which lacked CIAP.

stranded DNA generated by the viral protein. The unwinding of double-stranded DNA fragments by Py T Ag has been shown to occur in a relatively sequence-independent manner, i.e., in the absence of the viral replication origin (38). Under these conditions, the unwinding activity of Py T Ag exhibits sequence requirements which differ from those determined for Py ori-DNA replication and may not be entirely representative of the events which precede chain elongation. Furthermore, using a DNA helicase assay in which the ATP-dependent removal of complementary DNA fragments from single-stranded M13 DNA by Py T Ag was measured, we also observed that preincubation of Py T Ag with a range of quantities of CIAP had little significant effect on such fragment displacement activity (data not shown).

CIAP treatment of Py T Ag reduces its binding to the

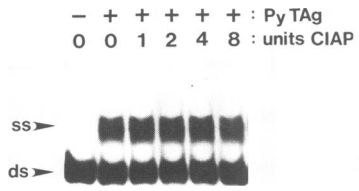


FIG. 5. Dephosphorylation of Py T Ag with CIAP does not inhibit double-stranded DNA fragment unwinding. Py T Ag (0.3 μg) was pretreated with increasing amounts of CIAP in 1× dephosphorylation buffer, as indicated above each lane. After 20 min at 33°C, the pretreated protein was added to DNA unwinding reaction mixtures containing 2 ng of a ^{32}P-labeled 97-bp Py ori-DNA fragment. The positions of single-stranded (ss) and double-stranded (ds) DNA are indicated.

palindrome within the replication origin. For the initiation of Py DNA synthesis, prior to melting of the DNA duplex, the binding of Py T Ag to specific sequences within the replication origin is required. To determine whether CIAP treatment affected Py T Ag DNA binding, we first used nitrocellulose filter retention assays. When increasing concentrations of Py T Ag, which had been preincubated in the presence or absence of CIAP, were added to binding reaction mixtures containing a fragment encompassing the Py core origin of replication, no difference in DNA binding was detected between the treated and untreated proteins (Fig. 6A). To confirm that the large amount of CIAP used in these experiments was inhibitory to Py ori-DNA replication, aliquots of the CIAP-treated and untreated Py T Ags were added to replication reaction mixtures. As seen in Fig. 6B, no *Dpn*I-resistant progeny DNA was detected with Py T Ag that had been preincubated with CIAP, while reaction mixtures that contained the untreated viral protein generated significant amounts of replication products.

We have observed that Py T Ag possesses a relatively high affinity for nonspecific DNA (38). The fragments retained in nitrocellulose filter-binding reactions most likely reflect both specific and nonspecific interactions of Py T Ag with the DNA. For this reason we turned to the method of DNase I protection to examine in more detail the specific binding of Py T Ag to Py ori-DNA. Footprinting experiments were carried out under conditions that simulated the replication reaction, i.e., with ATP and $MgCl_2$ in mixtures containing Py T Ag and a 3′-end-labeled DNA fragment containing the entire Py regulatory region (nucleotide 5075 to 187). Since the conditions of the experiments were established such that there was maximal protection by Py T Ag in the absence of CIAP it was not possible in this case to identify any stimulation of DNA binding with smaller amounts of phosphatase. We observed that treatment of Py T Ag with increasing quantities of CIAP markedly reduced the amount of protection detected over T Ag-binding sites within core origin (ori) (Fig. 7A). It is of interest that the DNase I hypercutting region on the late side of the origin (indicated by the open arrow) that we have previously described (19) was unaffected at all concentrations of CIAP, even under conditions when protection over the origin region was completely eliminated. This suggested that while the specific interaction of Py T Ag with sequences within the origin was strongly blocked after CIAP treatment, the ability of the protein to interact with neighboring regions was less affected by dephosphorylation.

The reduction in binding to the origin palindrome was also examined by using a range of concentrations of Py T Ag that had been treated with a quantity of CIAP which had completely inhibited protection of the origin by that preparation of Py T Ag

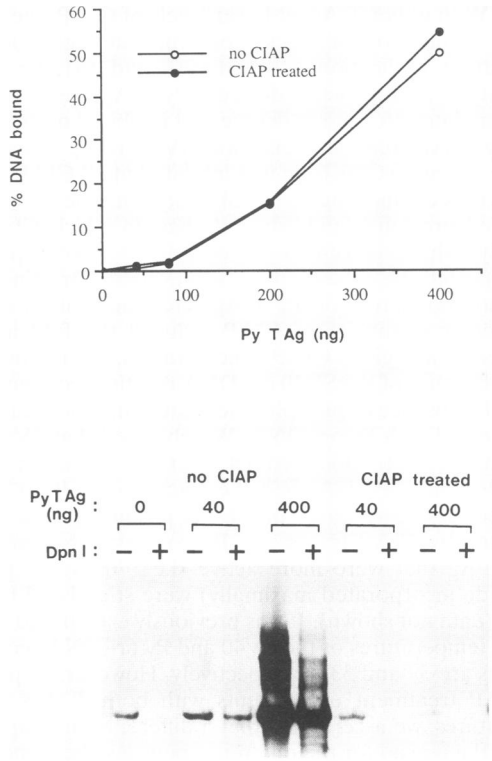


FIG. 6. Nitrocellulose filter-binding reactions do not reveal an effect of CIAP on the DNA-binding activity of Py T Ag. (A) Py T Ag (1.5 μg) was preincubated with 0 (no CIAP) or 25 U (CIAP treated) of CIAP for 20 min at 33°C in 50 μl of 1× CIP buffer. Increasing quantities of the untreated and CIAP-treated proteins were added to binding reaction mixtures containing the ^{32}P-end-labeled Py core origin DNA fragment as described in the legend to Fig. 5. Reaction mixtures were incubated for 15 min at 33°C and passed through nitrocellulose filters. The amount of input DNA retained on the filter was determined by liquid scintillation counting. (B) Py T Ag, as indicated above each lane, that was preincubated in the presence or absence of CIAP, as described for panel A, was added to Py DNA replication reaction mixtures and incubated for 3 h at 33°C. Progeny DNA was then purified and linearized with *Bam*I restriction enzyme digestion in the absence (–) or presence (+) of Dpn I. The products were separated on a 0.8% agarose gel and visualized by autoradiography.

(Fig. 7B). The preparation of Py T Ag used in these experiments was also less active in Py DNA replication reactions such that more viral protein was required to obtain detectable levels of DNA binding. In addition, more DNA was added to these reaction mixtures compared with the amount used for the experiment for which the results are shown in Fig. 7A. However, in this experiment there was a significantly greater reduction in Py T Ag binding to the core origin region (particularly to the central palindrome) than to the other T Ag-binding sites. The facts that protection of the origin palindrome was relatively the most affected by CIAP treatment and that Py T Ag has a strong nonspecific DNA-binding activity may explain our inability to detect an effect of CIAP on the origin-binding function of Py T Ag by nitrocellulose filter binding. We conclude that the extensive removal of phosphates by CIAP alters the replication function of Py T Ag by reducing its binding to specific sequences within the core origin of replication.

That CIAP is capable of reducing the protection of the Py origin by Py T Ag provides a mechanistic explanation by which phosphatase at higher concentrations inhibits Py ori-DNA

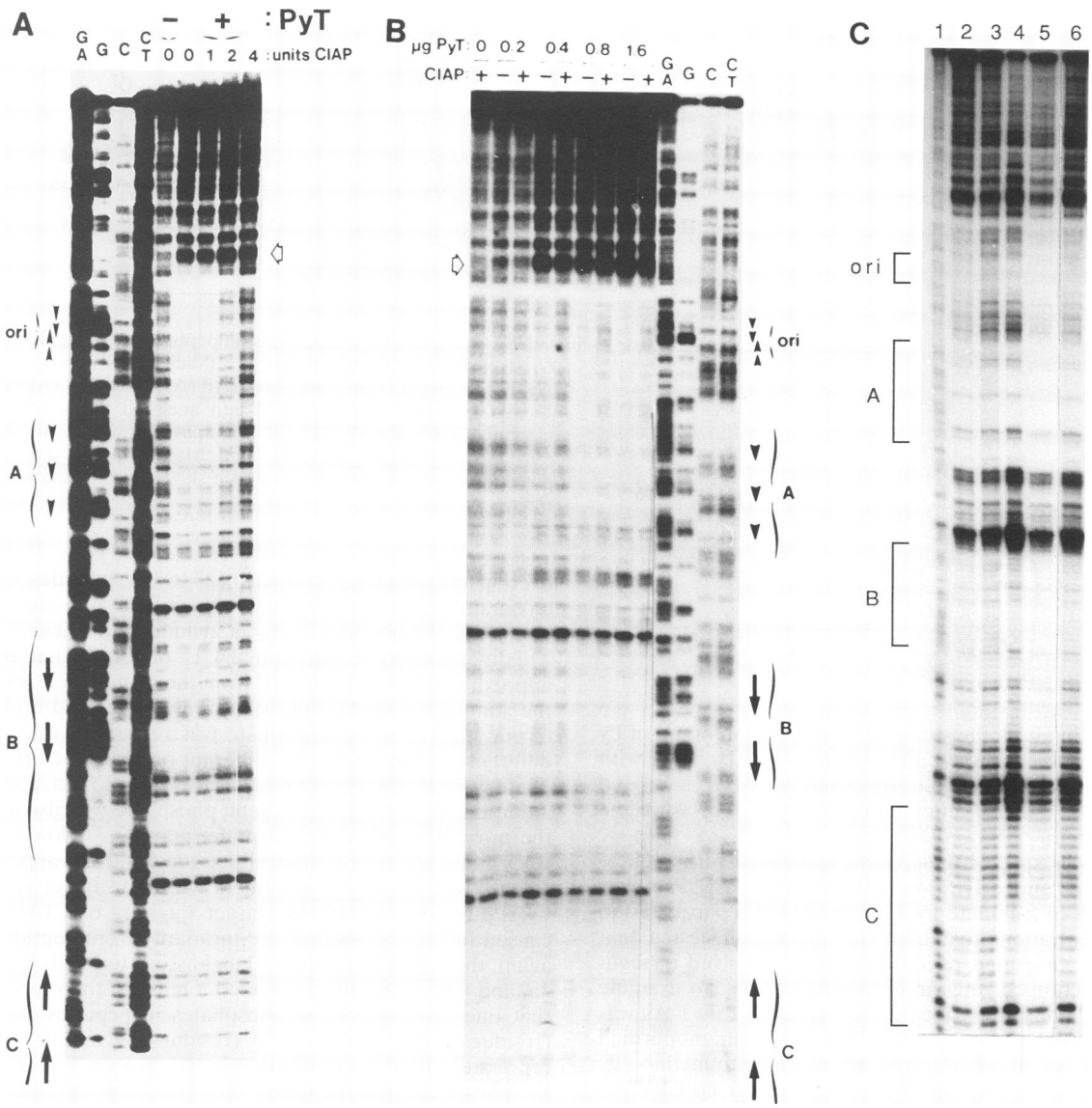
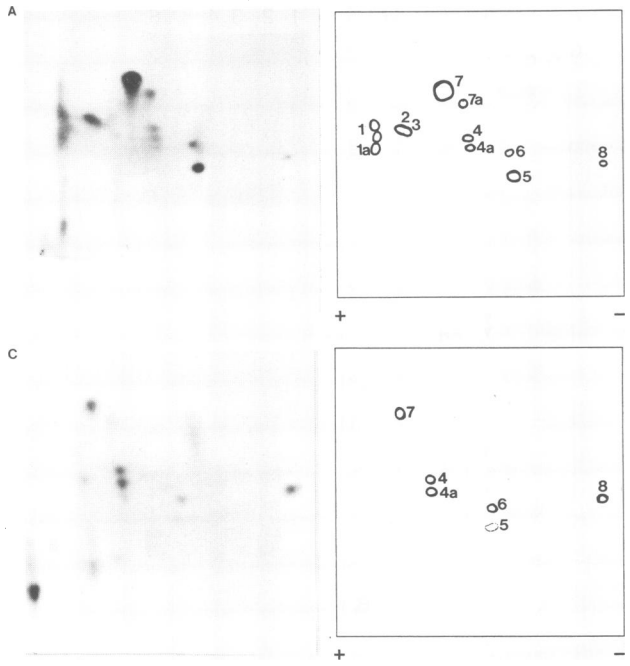


FIG. 7. Treatment of Py T Ag with CIAP inhibits the protection of T Ag-binding sites within the origin palindrome. (A) Py T Ag (0.25 μg) was preincubated with increasing units of CIAP in 1× dephosphorylation buffer. After 5 min at 33°C, the proteins were added to binding reaction mixtures containing 1.5 ng of the Py origin fragment 3′-end-labeled at position 187. Reaction mixtures were incubated an additional 15 min at 33°C and digested with DNase I. The products were purified, separated, and detected by autoradiography. Py T Ag-binding sites are indicated by braces. The arrows designate the location of pentanucleotide consensus sequences within each site. The lanes on the left, designated A+G, A, C, and C+T, contain Maxam-Gilbert sequencing reactions. The open arrow indicates the region of DNase I hypercutting induced by Py T Ag. (B) Reaction conditions were as described for panel A except that the indicated amount of Py T Ag was preincubated with 0 (−) or 4 (+) U of CIAP and added to binding reaction mixtures containing 3 ng of the Py ori-DNA fragment. The samples were digested with DNase I and analyzed as described for panel A. (C) Reaction conditions were as described for panel A except that a smaller quantity of Py T Ag (0.1 μg) was preincubated for 10 min in CIAP buffer with either 0 (lane 3), 0.8 (lane 4), 1.6 (lane 5), or 4.8 (lane 6) U of CIAP prior to binding to the same Py ori-DNA fragment as shown in panels A and B. Lane 2 shows the protection of the DNA fragment by Py T Ag that was not preincubated in CIAP buffer, and lane 1 shows DNase I cleavage of the fragment in the absence of added protein.

replication. It was interesting to examine the possibility that the stimulation of Py ori-DNA replication observed with treatment of Py T Ag with lesser quantities may, in an analogous fashion, be related to an increase in ori-DNA binding as determined by DNase I footprinting (Fig. 7C). To test this, binding conditions were adjusted such that Py T Ag preincubated in the absence of CIAP displayed only partial protection of the same Py origin-containing fragment that was used in the experiment shown in Fig. 7A and B. Increasing quantities of CIAP were then preincubated with Py T Ag prior to binding to the origin fragment. In this case, treatment of Py T Ag with 1.6 U of CIAP actually caused a significant increase in protection of the origin palindrome and adjacent Py T Ag-binding sites A, B, and C. However, after treatment with a higher concentration of CIAP, the amount of protection of this region was decreased, consistent with what we had observed under conditions when we started with more extensive occupancy of the Py T Ag-binding sites. Taken together, our data



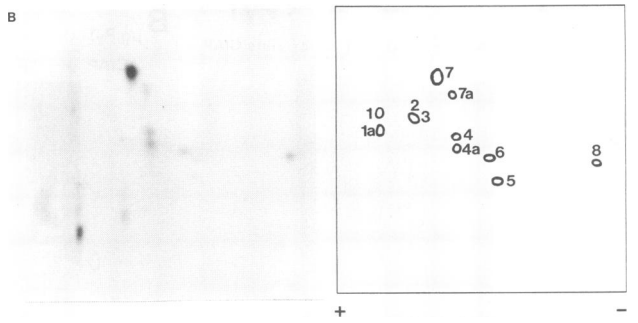


FIG. 8. Py T Ag tryptic phosphopeptides respond differently to CIAP treatment. Tryptic digests of ^{32}P-labeled immunopurified Py T Ag that had been treated with no CIAP (A), 2.4 U of CIAP (B), or 12 U of CIAP (C) were prepared and analyzed by two-dimensional electrophoresis-ascending chromatography followed by autoradiography as described in Materials and Methods. The corresponding positions of the peptides (1 to 8) are shown in the diagrams on the right.

thus indicate that both the stimulation and the inhibition of Py ori-DNA synthesis observed after treatment of Py T Ag with lesser and greater quantities, respectively, of CIAP is related directly to the specific interaction of Py T Ag with the replication origin.

CIAP removes specific phosphates associated with replication repression and activation. The ability of CIAP at low concentrations to stimulate Py T Ag-mediated replication and at high concentrations to inhibit this process suggested that different phosphorylated residues might be involved in regulating the functions of the viral phosphoprotein. To examine this possibility phosphotryptic peptide analysis of Py T Ag was performed (Fig. 8). ^{32}P-labeled Py T Ag was immunopurified from insect cells and, when tested for its ability to mediate Py ori-DNA replication after incubation with either no CIAP or lesser or greater amounts of the phosphatase, yielded results essentially similar to those that we had observed with unlabeled T Ag. The preparation of T Ag that had been used to generate the maps shown, when preincubated without CIAP, supported the incorporation of 23.8 pmol of [^{3}H]TdR into acid-precipitable DNA, while treatment with 2.4 and 12 U of CIAP led to incorporation of 44.5 and 4.2 pmol, respectively. Thus, the stimulation and inhibition at lower and higher phosphatase concentrations, respectively, was reproduced. Further, no change in the amount of immunoreactive Py T Ag was detected with the higher quantity of CIAP used (data not shown). We found that trypsinized Py T Ag synthesized in insect cells yielded a pattern of phosphopeptides that was indeed qualitatively similar to those reported by Hassauer et al. (14), who had mapped the peptides from ^{32}P-Py T Ag isolated from infected and transformed rodent cells. The similarity of the overall pattern of phosphopeptides led us to adopt their numbering system. In some cases the relative intensity of the labeled peptides was different and at least one additional minor phosphopeptide (designated peptide 8) that was not described by that group was detected. However, assuming that our peptides do indeed correspond to those described by Hassauer et al., then peptides 1, 1a, 2, and 3 overlap and contain amino acids 123 to 127; peptides 4, 4a, and 6 contain amino acids 170 to 191, peptide 5 spans amino acids 89 to 110, and peptides 7 and 7a include amino acids 71 to 88. Treatment of Py T Ag with 2.4 U of CIAP that removed approximately 60% of the ^{32}P from the protein and resulted in the stimulation of its replication function yielded a phosphotryptic map in which phosphate was removed with markedly different efficiency from the different peptides such that phosphate was selectively removed from peptides 1, 1a, 2, 3, and 5. Additionally, with this treatment, phosphate was also lost from the most strongly labeled peptide, peptide 7. However, peptides 4, 4a, and 6 or the novel peptide 8 did not appear to be affected significantly by treatment with the smaller amount of CIAP. Treatment with the larger quantity of CIAP (12 U) caused further removal of ^{32}P, primarily from peptide 7, but, again, the relative amount of label associated with peptides 4, 4a, and 6 was virtually unaffected. Our data therefore suggest that one or more of the phosphates in peptides 1, 1a, 2, 3 (residues 133 to 167), and 5 (residues 89 to 110) normally repress the Py T Ag replication function. Removal of some of the phosphate from peptide 7 may also be involved in repression, although in several additional experiments that were performed the label associated with this peptide appeared to be relatively more stable than was seen in this experiment. By contrast, the loss of phosphate that was most clearly associated with reduced replication function when the larger amount of CIAP was used occurred with peptide 7 (residues 71 to 88).

DISCUSSION

Our experiments have shown that the replication function of Py T Ag is both negatively and positively regulated by phosphorylation. In this respect it shares features with its more well-studied primate homolog from SV40. However, the two T Ags respond in somewhat different manners to dephosphorylation by CIAP. We found that, as is the case for SV40, low concentrations of CIAP stimulated the replication functions of Py T Ag. However, higher levels of CIAP markedly inhibited the replication function of Py T Ag and tended to have a much less profound effect on SV40 T Ag. Thus our results indicate that there are both similarities and differences between two viral proteins that share extensive structural and functional homology. The responses of the two proteins to dephosphorylation may be related to the fact that both Py and SV40 T Ags

each contain tight clusters of phosphorylated residues directly adjacent to their respective origin-binding domains. In the case of SV40, the presence of a single phosphate at Thr-124, which can be added by cdc2 kinase but cannot be removed by CIAP, was sufficient to confer on inert bacterially produced SV40 T Ag at high levels of replication activity (21). While similar experiments with Py T Ag have not been reported, it might therefore be predicted that the presence of a phosphorylated residue(s) in the analogous region of Py T Ag is also necessary for its function in viral DNA synthesis. Indeed, we have observed that two regions of Py T Ag encompassed by amino acids 63 to 240, and 240 to 521, respectively, contain cdc2 kinase phosphorylation sites (36a). If such a phosphate(s), as is the case with SV40, is solely required to confer replication competence on Py T Ag, then this phosphate differs markedly from its SV40 counterpart in its accessibility to CIAP. However, another possibility remains as well and is consistent with the fact that the two T Ags each possess additional phosphate clusters within their unique regions. The more NH_2-proximal phosphate cluster of Py T Ag, of which no comparable region exists in SV40, may contain at least one phosphate whose presence is necessary for replication and which can be removed by CIAP. Our tryptic maps suggest that, interestingly, the phosphorylated residue(s) in peptide 7 that is most distal to the origin binding domain plays a positive role in DNA binding.

We confirmed that dephosphorylation of SV40 T Ag stimulated greatly subsequent SV40 ori-DNA replication in permissive cell extracts. While our experiments show that Py T Ag is also negatively regulated by phosphorylation, it is worth noting that at no concentration of CIAP tested did we observe a stimulation of Py T Ag replication function of greater than threefold. Furthermore, using $PP2A_c$, which was also shown to serve as a stimulatory SV40 replication factor by removing phosphate from SV40 amino acids Ser-120 and Ser-123 (28, 35), we have detected no significant increase (or decrease) in Py T Ag replication function in vitro (data not shown).

Our sources of Py and SV40 T Ag were insect cells infected with the appropriate recombinant baculovirus. The SV40 T Ag produced in these cells was reported to be hypophosphorylated compared with the viral T Ag expressed in SV40-infected monkey cells (16). Additionally, Höss et al. (16) reported that SV40 T Ag purified from insect cells is overphosphorylated on Thr-124 relative to the SV40 T Ag isolated from mammalian COS cells and, correspondingly, binds better to the SV40 origin. However, we observed that the replication activity of matched preparations of SV40 T Ag produced either in insect cells or in mammalian cells responded to CIAP treatment similarly (data not shown). This may suggest that the state of the infected cells, whether of insect or mammalian origin, may influence the phosphorylation of T Ag. Whether Py T Ag produced in insect cells is differentially phosphorylated compared with its mammalian counterpart has yet to be determined. Nevertheless, it should be again mentioned that the extent of both the stimulatory and inhibitory effects of CIAP varied somewhat with different preparations of Py T Ag isolated from insect cells (data not shown). The differential response of Py T Ag preparations to CIAP could reflect differences in their initial phosphorylation states.

The development of in vitro DNA replication systems has been extremely beneficial to our understanding of the essential process of DNA synthesis. Studies, which have used SV40 as a model system, have identified an ordered series of events which lead to the initiation of viral DNA replication (reviewed in reference 3). Prior to the incorporation of the deoxynucleoside triphosphates, a nucleoprotein complex consisting of SV40 T Ag hexamers is assembled at the viral replication origin and induces a localized melting of the duplex within this region. The DNA helicase activity of SV40 T Ag can further unwind the replication origin, generating the primary replication bubble. Because of the high degree of sequence homology between the two viral proteins it is likely that the function of Py T Ag in DNA replication is similar to that of SV40 T Ag. As was reported to be the case for SV40 (14, 18, 22), the DNA helicase activity of Py T Ag was unaffected by extensive CIAP treatment (data not shown). ATP induces the assembly of Py T Ag into hexamers (39). However, our experiments suggest that neither Py T Ag nor SV40 T Ag hexamer formation is substantially affected by CIAP treatment (unpublished data). SV40 T Ag was shown in several studies to interact with DNA polymerase-α primase (8, 11, 31). We have similarly determined an association of Py T Ag with murine DNA polymerase-α primase (22a). Whether this interaction is influenced by the phosphorylation state of Py T Ag remains to be determined.

Examination of the effects of CIAP treatment on several biochemical properties of Py T Ag revealed that the primary defect of dephosphorylation was a reduced affinity of Py T Ag specifically for the origin palindrome as evidenced by DNase I footprinting experiments. Our inability to detect an effect on origin binding by using a nitrocellulose filter-binding assay suggests that the nonspecific DNA-binding activity of Py T Ag is unaffected by CIAP. This is consistent with the observation that the removal of phosphates by CIAP did not reduce the ability of the viral protein to unwind double-stranded DNA fragments, an activity of Py T Ag which exhibits little sequence specificity. It is noteworthy that not only was the inhibition of ori-DNA replication caused by CIAP treatment linked directly to reduction in ori-DNA binding by Py T Ag, but also the increased DNA synthesis seen after removal of the repressing phosphate(s) was reflected in increased binding to the origin. This suggests that both the positive and negative regulation of the replication function of Py T Ag by phosphorylation is due to a direct effect on the protein-DNA interactions that are the initial stage of ori-DNA synthesis. Further clarification of the roles of the different phosphorylated residues of Py T Ag in ori-DNA synthesis will require additional combined biochemical and genetic analysis.

Do our results bear any relationship to the state of phosphorylation of Py T Ag in permissive cells? In lytic viral infection of murine cells, it was determined that the growth state of the cells affected the phosphorylation of Py T Ag (1). Early in the infection of growth-arrested cells, Py T Ag was found to be underphosphorylated compared with the T Ag found in rapidly proliferating cells. The differences were mapped to two distinct phosphopeptides which became more abundant as the infection progressed. Furthermore, studies with mutant Py, which produce a temperature-sensitive Py T Ag defective in DNA replication, have shown that at the nonpermissive temperature, Py T Ag is underphosphorylated (1). The less highly phosphorylated forms were also detected under conditions when viral DNA replication did not occur at high levels, such as in the infection of nonpermissive rat cells. The conversion of Py T Ag to the more highly phosphorylated state appears to be correlated with both viral and cellular DNA replication. These results suggest that the infection of quiescent cells induces cellular growth, producing an environment suitable for viral DNA replication and activating a kinase(s) capable of phosphorylating Py T Ag. Therefore, an increased state of phosphorylation may be important for the function of Py T Ag in viral DNA synthesis. Our experiments thus may provide a mechanistic basis for changes in the phosphorylation state of Py T Ag during the viral lytic cycle.

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The first two authors contributed equally to this work.

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REFERENCES

1. **Bockus, B. J., and B. Schaffhausen.** 1987. Phosphorylation of polyomavirus large T antigen: effects of viral mutations and cell growth state. J. Virol. **61:**1147–1154.
2. **Bockus, B. J., and B. Schaffhausen.** 1987. Localization of the phosphorylations of polyomavirus large T antigen. J. Virol. **61:** 1155–1163.
3. **Borowiec, J. A., F. Dean, P. A. Bullock, and J. Hurwitz.** 1990. Binding and unwinding: how SV40 T antigen engages the origin of replication. Cell **60:**181–184.
4. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:** 110–149.
5. **Cogen, B.** 1978. Virus-specific early RNA in 3T6 cells infected by a tsa mutant of polyoma virus. Virology **85:**222–230.
6. **Cowie, A., and R. Kamen.** 1984. Multiple binding sites for polyomavirus large T antigen within regulatory sequences of polyomavirus DNA. J. Virol. **52:**750–760.
7. **Cowie, A., J. deVilliers, and R. Kamen.** 1986. Immortalization of rat embryo fibroblasts by mutant polyomavirus large T antigens deficient in DNA binding. Mol. Cell. Biol. **6:**4344–4352.
8. **Dornreiter, I., A. Hoss, A. K. Arthur, and E. Fanning.** 1990. SV40 T antigen binds directly to the large subunit of purified DNA polymerase alpha. EMBO J. **9:**3329–3336.
9. **Fanning, E., and R. Knippers.** 1992. Structure and function of simian virus 40 large tumor antigen. Annu. Rev. Biochem. **61:**55–85.
10. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology **55:**127–135.
11. **Gannon, J. V., and D. P. Lane.** 1987. p53 and DNA polymerase α compete for binding to SV40 T antigen. Nature (London) **329:** 456–458.
12. **Gaudray, P., P. Clertant, and F. Cuzin.** 1980. ATP phosphohydrase ATPase activity of a polyomavirus T antigen. Eur. J. Biochem. **109:**553–558.
13. **Grässer, F. A., K. H. Mann, and G. Walter.** 1987. Removal of serine phosphates from simian virus 40 large T antigen increases its ability to stimulate DNA replication in vitro but has no effect on ATPase and DNA binding. J. Virol. **61:**3373–3380.
14. **Hassauer, M., K. H. Scheidtmann, and G. Walter.** 1986. Mapping of phosphorylation sites in polyomavirus large T antigen. J. Virol. **58:**805–816.
15. **Hertz, G. Z., and J. Mertz.** 1986. Bidirectional promoter elements of simian virus 40 are required for efficient replication of the viral DNA. Mol. Cell. Biol. **6:**3513–3522.
16. **Höss, A., I. Moarefi, K.-H. Scheidtmann, L. J. Cisek, J. L. Corden, I. Dornreiter, A. K. Arthur, and E. Fanning.** 1990. Altered phosphorylation pattern of simian virus 40 T antigen expressed in insect cells by using a baculovirus vector. J. Virol. **64:**4799–4807.
17. **Hutchinson, M., T. Hunter, and W. Eckhart.** 1978. Characterization of T antigens in polyoma-infected and transformed cells. Cell **17:**65–77.
18. **Klausing, K., K.-H. Scheidtmann, E. A. Baumann, and R. Knippers.** 1988. Effects of in vitro dephosphorylation of DNA-binding and DNA helicase activities of simian virus 40 large T antigen. J. Virol. **62:**1258–1265.
19. **Lorimer, H. E., E. H. Wang, and C. Prives.** 1991. The DNA-binding properties of polyomavirus large T antigen are altered by ATP and other nucleotides. J. Virol. **65:**687–699.
20. **Luo, K., T. R. Hurley, and B. M. Sefton.** 1991. Cyanogen bromide cleaveage and proteolytic peptide mapping of protein immobilized to membranes. Methods Enzymol. **201:**149–152.
21. **McVey, D., L. Brizuela, I. Mohr, D. R. Marshak, Y. Gluzman, and D. Beach.** 1989. Phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. Nature (London) **341:**503–507.
22. **Mohr, I. J., B. Stillman, and Y. Gluzman.** 1987. Regulation of SV40 DNA replication by phosphorylation of T antigen. EMBO J. **6:**153–160.

22a. **Moses, K., and C. Prives.** Unpublished data.

23. **Murakami, Y., T. Eki, M.-A. Tamada, C. Prives, and J. Hurwitz.** 1986. Species-specific in vitro synthesis of DNA containing the polyomavirus origin of replication. Proc. Natl. Acad. Sci. USA **83:**6347–6351.
24. **Nilsson, S. V., and G. Magnusson.** 1984. Activities of polyomavirus large-T-antigen proteins expressed by mutant genes. J. Virol. **51:**768–775.
25. **Prives, C.** 1990. The replication functions of SV40 T antigen are regulated by phosphorylation. Cell **61:**735–738.
26. **Prives, C., Y. Murakami, F. G. Kern, W. R. Folk, C. Basilico, and J. Hurwitz.** 1987. DNA sequence requirements for replication of polyomavirus DNA in vivo and in vitro. Mol. Cell. Biol. **7:**3694–3704.
27. **Schaffhausen, B., J. Silver, and T. Benjamin.** 1978. Tumor antigens in cells productively infected by wild-type polyoma virus and mutant NG-18. Proc. Natl. Acad. Sci. USA **75:**79–83.
28. **Scheidtmann, K. H., D. M. Virshup, and T. J. Kelly.** 1991. Protein phosphatase 2A dephosphorylates simian virus 40 large T antigen specifically at residues involved in regulation of DNA binding activity. J. Virol. **64:**2098–2101.
29. **Seki, M., T. Enomoto, T. Eki, A. Miyahima, Y. Murakami, R. Hanaoka, and M. Ui.** 1990. DNA helicase and nucleoside 5′ triphosphatase activities of polyoma virus large tumor antigen. Biochemistry **29:**1003–1009.
30. **Simmons, D. T., W. Chou, and K. Rodgers.** 1986. Phosphorylation downregulates the DNA-binding activity of simian virus 40 T antigen. J. Virol. **60:**888–894.
31. **Smale, S. T., and R. Tjian.** 1986. T-antigen-DNA polymerase α complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. **6:**4077–4087.
32. **Tang, W.-T., and W. R. Folk.** 1989. Asp-286 → Asn-286 in polyomavirus large T antigen relaxes the specificity of binding to the polyomavirus origin. J. Virol. **63:**242–249.
33. **Tyndall, C., G. LaMantia, C. M. Thacker, J. Favaloro, and R. Kamen.** 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequence is required in *cis* for both early gene expression and viral DNA replication. Nucleic Acids Res. **9:**6231–6250.
34. **Veldman, G. M., S. Lupton, and R. Kamen.** 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. **5:**649–658.
35. **Virshup, D. M., M. G. Kauffman, and T. J. Kelly.** 1989. Activation of SV40 DNA replication in vitro by cellular protein phosphatase 2A. EMBO J. **8:**3891–3898.
36. **Virshup, D. M., and T. J. Kelly.** 1989. Purification of replication protein C, a cellular protein involved in the initial stages of simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA **86:**3584–3588.

36a. **Wang, E., D. Pearlstein, J. Manfredi, H. Lorimer, and C. Prives.** Unpublished data.

37. **Wang, E. H., P. N. Friedman, and C. Prives.** 1989. The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. Cell **57:**379–392.
38. **Wang, E. H., and C. Prives.** 1991. DNA helicase and duplex DNA fragment unwinding activities of polyoma and SV40 large T antigen display similarities and differences. J. Biol. Chem. **266:** 12668–12675.
39. **Wang, E. H., and C. Prives.** 1991. ATP induces the assembly of polyoma large tumor antigen into hexamers. Virology **184:**399–403.