

cdc2 Phosphorylation of Threonine 124 Activates the Origin-Unwinding Functions of Simian Virus 40 T Antigen

DUNCAN McVEY,¹ SATYAJIT RAY,¹ YAKOV GLUZMAN,^{2†} LLOYD BERGER,³
ALAN G. WILDEMAN,³ DANIEL R. MARSHAK,² AND PETER TEGTMEYER^{1*}

Department of Microbiology, State University of New York, Stony Brook, New York 11794¹; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724²; and Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario N1G 2W1, Canada³

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Phosphorylation of simian virus 40 (SV40) T antigen on threonine 124 activates viral DNA replication in vivo and in vitro. We have manipulated the modification of T-antigen residue 124 both genetically and biochemically and have investigated individual replication functions of T antigen under conditions suitable for in vitro DNA replication. We find that the hexamer assembly, helicase, DNA polymerase α -binding, and transcriptional-autoregulation functions are independent of phosphorylation of threonine 124. In contrast, neither T antigen with an alanine mutation of threonine 124 made in human cells nor unphosphorylated T antigen made in *Escherichia coli* binds the SV40 replication origin as stably as phosphorylated wild-type T antigen does. Furthermore, modification of threonine 124 is essential for complete unwinding of the SV40 replication origin. We conclude that phosphorylation of threonine 124 enhances specific interactions of T antigen with SV40 origin DNA. Our findings do not exclude the possibility that phosphorylation of threonine 124 may affect additional undefined steps in DNA replication. We also show that DNase footprinting and KMnO₄ modification assays are not as stringent as immunoprecipitation and origin-dependent strand displacement assays for detecting defects in the origin-binding and -unwinding functions of T antigen. Differences in the assays may explain discrepancies in previous reports on the role of T-antigen phosphorylation in DNA binding.

The life cycle of simian virus 40 (SV40) is tightly controlled by large T antigen. T antigen down-regulates its own transcription, initiates viral DNA replication, unwinds DNA replication forks, and stimulates late viral transcription (15).

Down-regulation of early transcription and initiation of viral replication require the direct interaction of T antigen with viral regulatory sequences. T antigen binds two separate sites designated sites I and II (53). Each site consists of different arrangements of the recognition pentanucleotide 5'-GAGGC-3' (14). Site I lies to the early side of site II and contains two tandem pentanucleotides separated by an adenine tract (39). Site I is dispensable in vivo but is required for autoregulation of early transcription (7, 20, 38) and enhances DNA replication (3, 13). Site II is indispensable for virus reproduction and consists of four 5'-GAGGC-3' pentanucleotides arranged in a perfect palindrome in the center of a 64-bp core origin of replication (9, 11). Site II is flanked by additional origin domains: an inverted imperfect repeat on the early side and an adenine-and-thymine (AT)-rich segment on the late side (9, 10, 32). The AT segment is necessary for replication and has TATAA sequences for proper initiation of early transcription (35). To the late side of the origin of replication are promoter and enhancer elements for early and late transcription (15).

The initial stages of viral replication have been well defined. In the presence of ATP, T antigen binds to site II, assembles as two hexamers around the origin of replication, and distorts and unwinds origin DNA to form a replication bubble (5, 8, 25, 32, 33, 55). After release from site II by mechanisms that are not understood, T antigen acts as a helicase to extend the replication bubble in both directions

(48, 56). T antigen also interacts with cellular replication proteins to facilitate the initiation of viral DNA synthesis (16, 47).

T-antigen functions are positively and negatively regulated by phosphorylation (36). T antigen is phosphorylated on serine and threonine residues in two clusters at the amino and carboxy termini outside the DNA-binding domain (41). A number of studies have provided apparently contradictory evidence on the importance of phosphoserine residues in the binding of T antigen to origin DNA. Some studies demonstrated little change in origin binding after dephosphorylation of serines with alkaline phosphatase (2, 19, 44), while other studies showed that alkaline phosphatase treatment increased binding to origin DNA (30, 46). Recently, Virshup et al. showed that dephosphorylation of serines 120 and 123 by protein phosphatase 2A enhances DNA replication by decreasing the dissociation rate of T antigen from origin DNA (55). Therefore, phosphorylation of these serines negatively regulates DNA replication. McVey et al. (27) showed that phosphorylation of threonine at residue 124 with cdc2 kinase positively regulates DNA replication and site II binding in vitro. Furthermore, viruses harboring an alanine at residue 124 do not replicate in vivo (43).

In the present study, we manipulated threonine 124 genetically and biochemically to investigate further the role of this modification in a variety of replication functions. Previous studies of threonine 124 phosphorylation in DNA binding were done in the absence of ATP under conditions not suitable for DNA replication in vitro (27, 43). Since ATP is essential for the assembly of T-antigen hexamers and DNA unwinding, we used replication conditions for our studies. We find that hexamer assembly, helicase, DNA polymerase α -binding, and transcriptional-autoregulation functions are independent of phosphorylation of threonine 124. In contrast, phosphorylation augments site II binding and is crucial

* Corresponding author.

† Present address: Lederle Laboratory, Pearl River, NJ 10969.

for complete origin unwinding. By comparing the results of different DNA binding assays, we also provide an explanation for apparent discrepancies in previous studies of the role of phosphorylation in T-antigen binding to origin DNA.

MATERIALS AND METHODS

Purification of the T antigen. Wild-type and mutant T antigens were expressed in HeLa cells by using defective adenovirus vectors. The cDNA for mutant T antigen, with a substitution of an alanine for threonine 124, was a gift from Ellen Fanning. The gene was cloned into the same adenovirus vector used to express wild-type T antigen (30). Procedures for the overexpression and purification of T antigen have been described previously (28, 29, 45). In brief, defective adenoviruses expressing wild-type or mutant T antigens were used to infect HeLa cell suspension cultures in the presence of helper adenovirus. Cells were lysed 42 to 44 h after infection, and protein was purified by an immunoaffinity column (45). The protein was eluted with 20 mM triethylamine with 10% glycerol and neutralized with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.4). Following overnight dialysis at 0°C against buffer F (10% glycerol, 5 mM NaCl, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 10 mM HEPES-KOH [pH 7.4]), aliquots were stored at -70°C. Wild-type T antigen was also expressed in *Escherichia coli* as described by Mohr et al. (29) and purified as described above.

In vitro replication. Various amounts of T antigen were incubated with 0.3 µg of the pSV0-15 plasmid containing the SV40 core origin of replication (SV40 sequences 5209 to 30 in pUC118, kindly supplied by Bruce Stillman) and 18 µl of an S100 extract from 293 cells at 37°C for 2 h. The reactions were carried out in a final volume of 30 µl of replication buffer (30 mM HEPES [pH 7.5], 7 mM MgCl₂, 1 mM DTT, 40 mM creatine phosphate) containing 4 mM ATP; 0.2 mM GTP, UTP, and CTP; 0.1 mM dCTP, dGTP, and TTP; 0.025 mM dATP with [α-³²P]dATP (800 Ci/mmol; specific activity, 1,000 cpm/pmol); 20 µg of creatine phosphokinase per ml; and 2.5% glycerol. DNA synthesis was stopped by spotting the reactions onto DE81 paper (Whatman). The filters were washed three times with 0.5 mM Na₂PO₄ and twice with ethanol. The amount of DNA synthesis was quantitated by liquid scintillation counting.

Hexamer formation. T antigen was added to replication buffer in the presence or absence of 4 mM ATP and incubated in a final volume of 30 µl for 30 min at 37°C. The reaction products were analyzed immediately by nondenaturing 4 to 20% gradient polyacrylamide gel electrophoresis (PAGE) as described previously (33).

Helicase assay. Helicase substrates were prepared by elongating a ³²P-labeled primer on M13 single-stranded DNA in the presence of dideoxynucleotides as described by Stahl et al. (48). T antigen was added to unwinding buffer (30 mM HEPES [pH 7.5], 15 mM KH₂PO₄ [pH 7.5], 7 mM MgCl₂, 4 mM ATP, 0.05% Nodidet P-40, 1 mM DTT, 40 mM creatine phosphate, 0.1 mg of creatine phosphokinase per ml, 30 µg of *E. coli* single-stranded DNA-binding protein per ml) containing 10 ng of labeled substrate and incubated at 37°C for 30 min in a final volume of 30 µl. The reactions were stopped with 5 µl of 3.3% sodium dodecyl sulfate (SDS)-0.5 M EDTA, and the products were resolved by 6% PAGE.

Binding to DNA polymerase α. Wild-type and mutant threonine 124 T antigens (3 µg) were mixed with 50 µl of ³⁵S-labeled extract from SF9 cells infected with AcDHPα (a recombinant baculovirus overexpressing the 180 kDa sub-

unit of human DNA polymerase α [6]), 20 µl of a 1:1 slurry of PAb419 cross-linked to protein A-Sepharose, and 200 µl of phosphate-buffered saline. After incubation overnight at 4°C, the pellets were washed three times with 50 mM Tris-HCl (pH 8.0)-0.5 M LiCl-1 mM EDTA-10% glycerol. The pellets were visualized by SDS-6.5% PAGE and autoradiography.

Autoregulation of early transcription. In vitro runoff transcription was performed as described by Coulombe et al. (7). Whole-cell extracts for transcription reactions were prepared from HeLa S3 cells by the procedure of Manley et al. (24). Transcription reactions were carried out with 8 µl of extract in a final volume of 20 µl. The final concentrations of unlabeled ATP, GTP, and UTP were 0.5 mM, and the concentration of CTP was 2.5 µM. Radiolabeled CTP ([α-³²P]CTP; Amersham; >3,000 Ci/mmol) was added to a final concentration of 40 nM. All components, including purified T antigen (dialyzed against a mixture of 10 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 7.0], 1 mM DTT, 5 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride), were mixed on ice and incubated at 30°C for 60 min. Control reactions in the absence of T antigen included an equivalent amount of T-antigen dialysis buffer to standardize the salt concentration in all samples. DNA templates (pBEL1 and pBEL1.ori⁻) were digested with *Ava*II to generate runoff transcripts. Transcription products were recovered by SDS-phenol-chloroform extraction and precipitation with ethanol and analyzed by 6% PAGE.

Immunoprecipitation assay for DNA binding. We used the general immunoprecipitation procedure described by McKay (26) under two sets of conditions: the original conditions described by McKay (McKay conditions) and conditions that would support SV40 DNA replication in vitro (replication conditions). Plasmid DNA templates containing site I (pOS-1; nucleotides 5171 to 5228), site II (pSVodl3; nucleotides 5209 to 128), and the wild-type origin (pSVO+; nucleotides 5171 to 128) have been described previously (50, 52). Briefly, 50 ng of an equimolar mixture of these plasmids was cut with *Taq*I, ³²P end labeled with Klenow polymerase, and incubated with T antigen in 50 µl for 50 min. Five micrograms of PAb419 (21) was added, and the mixtures were incubated for 40 min and then incubated for 50 min with protein A-Sepharose beads. Under McKay binding conditions, reactions were done on ice in 10 mM HEPES-KOH (pH 7.4)-100 mM KCl-1 mM MgCl₂-5% glycerol-50 µg of bovine serum albumin (BSA) per ml. Ten microliters of protein A-Sepharose beads was added to the reaction in NET buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40) on ice. Under replication conditions, reaction components were assembled in replication buffer with 0.1 mg of BSA per ml and 4 mM ATP on ice and shifted to 37°C for 40 min. Ten microliters of protein A-Sepharose beads was added to the reaction mixture in replication buffer and left for 50 min at 37°C. Under both sets of conditions, immunocomplexes were pelleted and washed three times with 1 ml of 0°C NET buffer, resuspended in 1% SDS with 25 mM EDTA, and heated at 65°C for 15 min. The T-antigen-bound DNA fragments were resolved by 6% PAGE in a Tris-borate buffer. Gels were dried on DE81 paper and exposed to Kodak XAR film.

KMnO₄ and DNase I footprinting. KMnO₄ and DNase I footprinting were performed as described by Gralla (18) with minor modifications (32). T antigen in 15 µl of buffer F containing 300 ng of pOR 1 (13) was placed at 26°C for 1 min in the presence or absence of 4 mM ATP and 1 µl of cdc2

kinase. The 26°C incubation was continued for 30 min. An equal volume of 2× replication buffer containing 0.2 mg of BSA per ml and, when appropriate, 4 mM ATP was added, and the mixtures were shifted to 37°C for 30 min. DNAs were probed at 37°C with 30 mM KMnO₄ for 3 min or with 0.1 U of DNase I for 1 min. KMnO₄ reactions were stopped with 3 μl of β-mercaptoethanol, and DNase I reactions were stopped with 4 μl of 0.2 M EDTA. DNAs were extracted with phenol and were spun through Sephadex G-50 (Pharmacia) to suspend the DNA in distilled H₂O. Isolated DNAs were annealed to a primer that was 5' end labeled with [³²P]ATP. The primer was extended with 1 U of Klenow polymerase, and labeled products were separated on 8% polyacrylamide–8 M urea sequencing gels at 50°C.

Origin-dependent strand displacement assay. We used the strand displacement assay described by Virshup et al. (55) with minor modifications to determine the ability of T antigen to unwind linear, double-stranded DNA fragments in an origin-specific manner. pSV0-15 contains the core origin of replication (SV40 sequences 5209 to 30) cloned into pUC118. We constructed an isogenic, replication-defective plasmid (pSV0-17) with a deletion of three nucleotides in site II in the *Bgl*I site. The *Eco*RI-*Bam*HI origin-containing, 150-bp DNA fragment of the plasmids was purified and ³²P end labeled with Klenow polymerase. T antigen was incubated with 1 ng of the DNA fragment in the presence of 1 μg of salmon sperm DNA at 37°C for 30 min in 15 μl of unwinding buffer (30 mM HEPES [pH 7.5], 15 mM KH₂PO₄ [pH 7.5], 7 mM MgCl₂, 4 mM ATP, 0.05% Nonidet P-40, 1 mM DTT, 40 mM creatine phosphate, 0.1 mg of creatine phosphokinase per ml, 30 μg of *E. coli* single-stranded DNA-binding protein per ml). In some cases, T antigen was incubated with *cdc2* in kinase buffer (50 mM Tris-HCl [pH 8.0], 5 mM MgCl₂, 2 mM DTT, 2 mM EDTA, 10% glycerol, 120 μM ATP) containing 1 ng of labeled origin fragment and 50 ng of salmon sperm DNA in a final volume of 25 μl at 30°C for 20 min. These reaction mixtures were brought to 30 μl with 5 μl of 6× unwinding buffer and were incubated at 37°C for 30 min. An equal volume of stop buffer (2% SDS plus 50 mM EDTA containing 3 μg of proteinase K) was added and left for 5 min at 65°C. The reaction products were analyzed by 8% PAGE in 40 mM Tris acetate–1 mM EDTA. The gels were dried on DE81 paper and exposed to Kodak XAR film. Levels of radioactivity in the gel were directly quantitated by scanning with an AMBIS detector.

RESULTS

Experimental strategy. We manipulated threonine 124 genetically and, in some cases, biochemically to investigate the role of this modification in a variety of replication functions. Both wild-type T antigen and a mutant T antigen with an alanine substitution at residue 124 were produced in HeLa cells infected with an adenovirus expression vector. We compared the proteins by a variety of functional assays. Similar properties in a particular assay would indicate that phosphorylation of threonine 124 is not required for function. Loss of function by the mutant, however, could mean either that threonine 124 modification is directly required for function or that conformational changes indirectly distort other protein domains. To distinguish between these possibilities, full-length T antigen devoid of mammalian phosphorylation was produced in *E. coli*, and a phosphate at residue 124 was introduced in vitro by the *cdc2* kinase (27).

DNA replication in vitro. We compared the overall replication functions of wild-type and threonine 124 mutant T

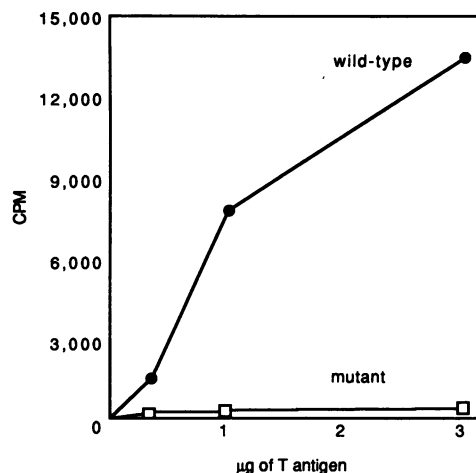


FIG. 1. DNA replication by wild-type and mutant T antigens in vitro. Wild-type and threonine 124 mutant T antigens were mixed with S100 cellular extracts and plasmids containing the SV40 origin of replication in replication buffer for 2 h at 37°C. Incorporation of radiolabeled precursors into DNA was quantitated by binding to DE81 paper and liquid scintillation counting.

antigens in vitro (Fig. 1). The T antigens were incubated with plasmid DNA containing the SV40 origin of replication and cellular extracts for 2 h at 37°C under conditions suitable for DNA replication (51). DNA replication was quantitated by measuring incorporation of radiolabel into DNA. Wild-type T antigen supported high levels of DNA replication, while T antigen with an alanine at position 124 produced 30-fold less DNA. These results are similar to those of McVey et al. (27), who showed that phosphorylation of unmodified T antigen on residue 124 by *cdc2* kinase strongly activated the DNA replication functions of T antigen in vitro. Therefore, our results further confirm that phosphorylation of T antigen at threonine 124 positively regulates one or more steps in DNA replication.

Hexamer formation. T antigen assembles as hexamers in solution or during binding to origin DNA (25, 33), and T-antigen mutants that do not assemble hexamers fail to replicate viral DNA (23, 37). We compared hexamer formation by wild-type and threonine 124 mutant T antigens in solution at 37°C with nondenaturing polyacrylamide gels (Fig. 2). In the absence of ATP, both purified T antigens consisted of a spectrum of oligomeric forms with a slight preference for monomeric and dimeric forms. Upon addition of ATP to 4 mM, both proteins readily formed hexamers and a small amount of double hexamers. Mutant T antigen did not manifest any abnormalities in either the quality or quantity of oligomerization as compared with the wild-type protein. These results show that phosphorylation of threonine 124 is not needed for hexamerization of T antigen and that the inability of threonine 124 mutant T antigen to replicate DNA is not the result of a defect in T-antigen oligomerization.

Helicase activity. One of T antigen's major functions in DNA replication is to unwind DNA at replication forks (48, 56). The helicase activity of T antigen is a complex function that requires a broad span of the protein, including the DNA-binding and ATPase domains (57). We compared the helicase activities of wild-type and mutant T antigens. The substrate for the helicase assay consisted of M13 single-stranded DNA on which a primer had been extended to

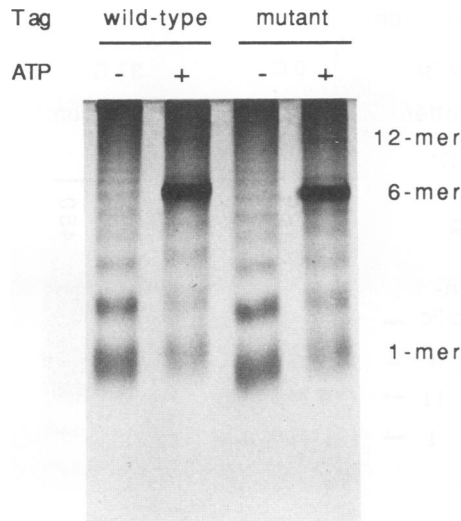


FIG. 2. Oligomerization of wild-type and threonine 124 mutant T antigens. Purified T antigens (T ag) (3 μ g) were incubated with or without ATP in modified replication buffer at 37°C for 30 min. The proteins were resolved in a nondenaturing gradient polyacrylamide gel (4 to 20%). Proteins were visualized by silver staining. T-antigen oligomers are identified on the right side of the figure.

various lengths up to several hundred bases. After incubation of T antigen with the substrate for 30 min under modified replication conditions, displacement of radiolabeled oligonucleotides was determined by gel electrophoresis (Fig. 3). The helicase activities of wild-type and mutant T antigens could not be distinguished at three different protein concentrations. In each case, both proteins unwound DNA segments of similar sizes and in similar quantities. We conclude that phosphorylation of threonine 124 is not necessary for the ATPase or helicase function of T antigen.

Binding to DNA polymerase α . T antigen binds to DNA polymerase α (16, 17, 47), which is thought to initiate the synthesis of the first segment of DNA at the SV40 replication origin (54). Presumably, these initial segments are then extended as continuous strands by DNA polymerase δ . Polymerase α also synthesizes retrograde, discontinuous strands at replication forks. It is logical to suppose that T-antigen binding to polymerase α may facilitate SV40 DNA replication. Therefore, we compared the binding of wild-type and mutant T antigens to the large subunit of DNA polymerase α . We mixed human polymerase, which had been overexpressed in insect cells by a baculovirus expression vector, with an excess of purified T antigen and immunoprecipitated T antigen with PAb419 to determine whether polymerase would coprecipitate. Figure 4 shows that wild-type and mutant T antigens bound small but similar amounts of polymerase. No polymerase was precipitated in the absence of T antigen. We conclude that phosphorylation of threonine 124 is not required for binding to the large subunit of polymerase α under replication conditions. We cannot exclude a defect in the interaction of the mutant protein with the intact tetrameric polymerase enzyme or with other cellular replication proteins.

Repression of early transcription. T-antigen binding to origin sites I and II is required for autoregulation of early transcription both in vivo and in vitro (7, 20, 38). The ability of wild-type and mutant threonine 124 T antigens to autoregulate early transcription was tested in vitro. The test

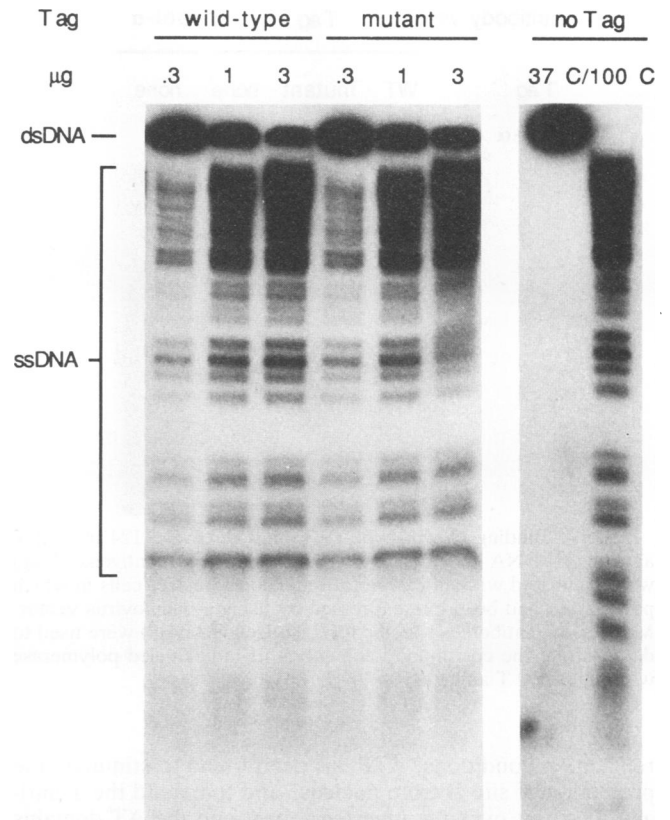


FIG. 3. Helicase activities of wild-type and threonine 124 mutant T antigens. Purified T antigens (T ag) were incubated in replication buffer containing partially double-stranded (ds) M13 DNA for 30 min at 37°C. Displaced single-stranded (ss) DNAs of various lengths were analyzed by gel electrophoresis as described in Materials and Methods. The double-stranded DNA substrate and boiled, single-stranded DNA are shown in the panel on the right.

plasmid (7) contains the entire regulatory region of SV40 and spans the early side of site I through the early promoter. DNA templates were digested with *Ava*II to generate runoff transcripts. This in vitro system allows the simultaneous measurement of both early and late transcription in the same assay. In the absence of T antigen, early transcripts are readily detected. The addition of either wild-type or mutant T antigen resulted in similar autorepression of early transcription (Fig. 5). This result suggests that the mutant T antigen binds well to the regulatory region of the SV40 early promoter. Additionally, both wild-type and mutant T antigens also repressed the promoter when one of the four pentanucleotides in site II was deleted (*ori*⁻). This result suggests either that optimal T-antigen binding to site II is not necessary for repression under these assay conditions or that T antigen may bind reasonably well to the replication-defective *ori*⁻ template.

DNA binding. The ability of wild-type and mutant T antigens to bind to sites I and II was tested by immunoprecipitation and DNase I footprinting assays. Previous studies on DNA binding by the threonine 124 mutant were done in 100 mM NaCl at 0°C in the absence of ATP under the immunoprecipitation conditions described by McKay (26). We compared DNA binding under those conditions with DNA binding in the absence of KCl at 37°C, conditions suitable for SV40 DNA replication in vitro (51). Under

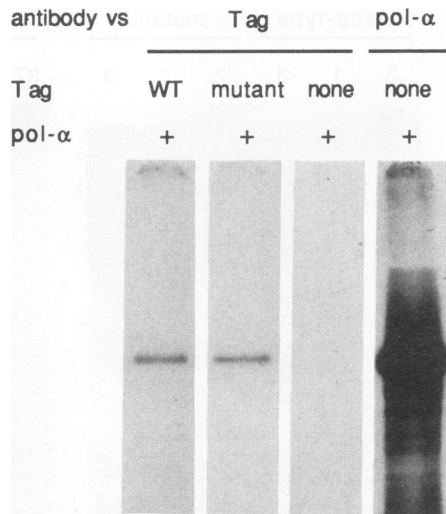


FIG. 4. Binding of wild-type (WT) and threonine 124 mutant T antigens to DNA polymerase α (pol- α). Purified T antigens (T ag) were incubated with an extract of radiolabeled insect cells in which polymerase had been overexpressed by using a baculovirus vector. Monoclonal antibodies specific for T antigen (PAb419) were used to demonstrate the coimmunoprecipitation of radiolabeled polymerase with unlabeled T antigen.

replication conditions, ATP has been found to stimulate the protection of site II from nuclease and to extend the T-antigen footprint over the inverted repeat and the AT domains (4, 12).

The ability of T antigen to interact with SV40 sites I and II

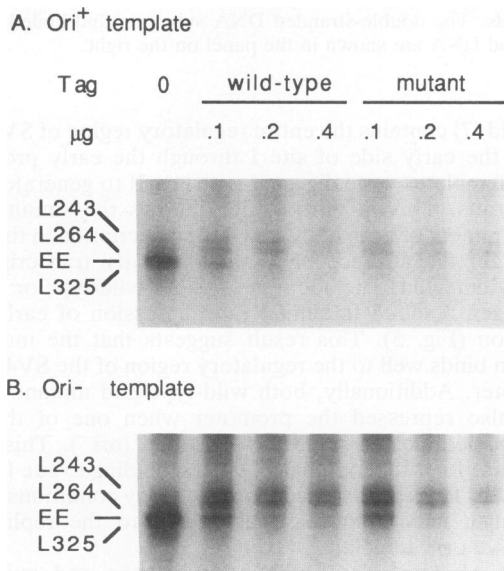


FIG. 5. Autoregulation of early transcription by wild-type and threonine 124 mutant T antigens (T ag). In vitro runoff transcription was performed as described in Materials and Methods. DNA templates with and without a functional origin of replication were digested with restriction endonuclease to generate runoff transcripts. Radiolabeled transcription products were recovered by SDS-phenol-chloroform extraction and precipitation with ethanol and analyzed on 6% polyacrylamide gels. The positions of early (EE) and late (L) transcripts are shown.

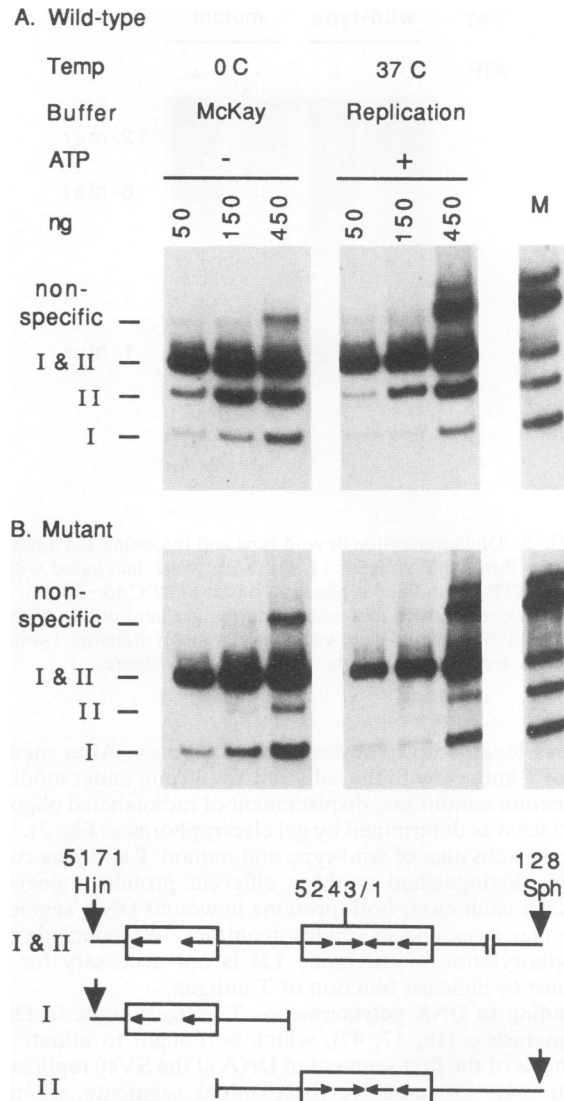


FIG. 6. Immunoprecipitation of T-antigen and bound-origin DNA fragments. Various amounts of purified wild-type (A) and threonine 124 mutant (B) T antigens were incubated with an equimolar mixture of end-labeled DNA fragments (M). After 60 min at the indicated temperature and buffer conditions, the protein-DNA complexes were immunoprecipitated, and the bound DNA was electrophoresed on a native 6% polyacrylamide gel. The positions of combined sites I and II, site II, and site I DNA fragments are indicated on the left, and the fragments are mapped at the bottom.

was measured first by the immunoprecipitation assay (Fig. 6). In this assay, T antigen was incubated with an equimolar mixture of three DNA fragments containing either site I, site II, or both sites to assess the relative affinity of each protein for these sites in one reaction mixture. The protein-DNA complexes were immunoprecipitated with a monoclonal antibody in McKay buffer, and the complexes were washed extensively at 0°C. The bound DNA fragments were then resolved on an acrylamide gel. In McKay immunoprecipitation buffer at 0°C, T antigen behaved as previously described. Wild-type (Fig. 6A) and mutant (Fig. 6B) T antigens bound to site I with similar affinities, while wild-type protein bound to site II at least 10-fold more efficiently than did the

threonine 124 mutant T antigen. In replication buffer at 37°C in the presence of ATP, both wild-type and mutant proteins bound origin DNAs somewhat less efficiently than under McKay immunoprecipitation conditions. Nevertheless, the patterns of binding to sites I and II were similar under immunoprecipitation and replication conditions. Wild-type and mutant T antigens bound the site I fragment with similar affinities, and the wild-type protein bound site II DNA at about 10-fold-lower protein concentrations than did the mutant protein. The mutant protein bound site II only at protein concentrations sufficient for binding to DNA fragments without origin sequences ("nonspecific DNA" in Fig. 6). Therefore, the immunoprecipitation assay demonstrates no significant specific binding of the threonine 124 mutant T antigen to an isolated site II under either immunoprecipitation or replication conditions. However, both wild-type and mutant T antigens bound tandem sites I and II better than they bound either individual site under all conditions tested. This result implies that mutant T antigen, like the wild-type protein, may bind to site II and thereby enhance binding to tandem sites I and II.

DNase I footprints generated by wild-type and threonine 124 mutant T antigens made in HeLa cells or by T antigen made in *E. coli* are shown in Fig. 7. All assays were done in replication buffer at 37°C. Similar concentrations of the three proteins protected site I from nuclease in the presence or absence of ATP, and, in contrast to results with the immunoprecipitation assay, wild-type T antigen consistently bound site I at protein concentrations lower than those at which it bound site II. As expected, ATP stimulated the binding of T antigen to site II. Surprisingly, mutant threonine 124 T antigen protected site II from nuclease almost as well as wild-type T antigen did. Furthermore, completely unmodified T antigen made in *E. coli* also bound site II, and phosphorylation of threonine 124 by cdc2 kinase induced only a modest increase in site II binding. When nuclease footprinting was performed on site II in the absence of site I, mutant T antigen bound site II almost as well as wild-type T antigen did (data not shown). We conclude that threonine 124 phosphorylation is not absolutely essential for site II-specific DNA recognition in either the presence or absence of site I.

A variety of differences in DNA binding were detected between the McKay and footprint assays. In the McKay assay, the wild-type protein bound to site II at protein concentrations lower than those at which it bound site I. In contrast, T antigen bound site I at protein concentrations lower than those at which it bound site II in the footprint assay. Wild-type T antigen bound site II at 10-fold-lower protein concentrations than did mutant T antigen in the McKay assay but at similar concentrations in the footprint assay. These differences in assays are real because the same protein preparations, buffers, and conditions were used in both assays and because the results were easily reproduced. It is apparent from these differences that the two assays measure different aspects of the interaction between T antigen and origin DNA.

Origin DNA distortion. To define further the steps in viral replication which are regulated by phosphorylation of threonine 124, two additional events were studied. These were the ability of T antigen to distort and to unwind the viral origin of replication (5, 8, 32, 55). Both events require ATP and occur prior to the initiation of DNA synthesis.

We measured origin distortion by using KMnO_4 modification under conditions identical to those used for DNase I footprinting, except that KMnO_4 was substituted for the

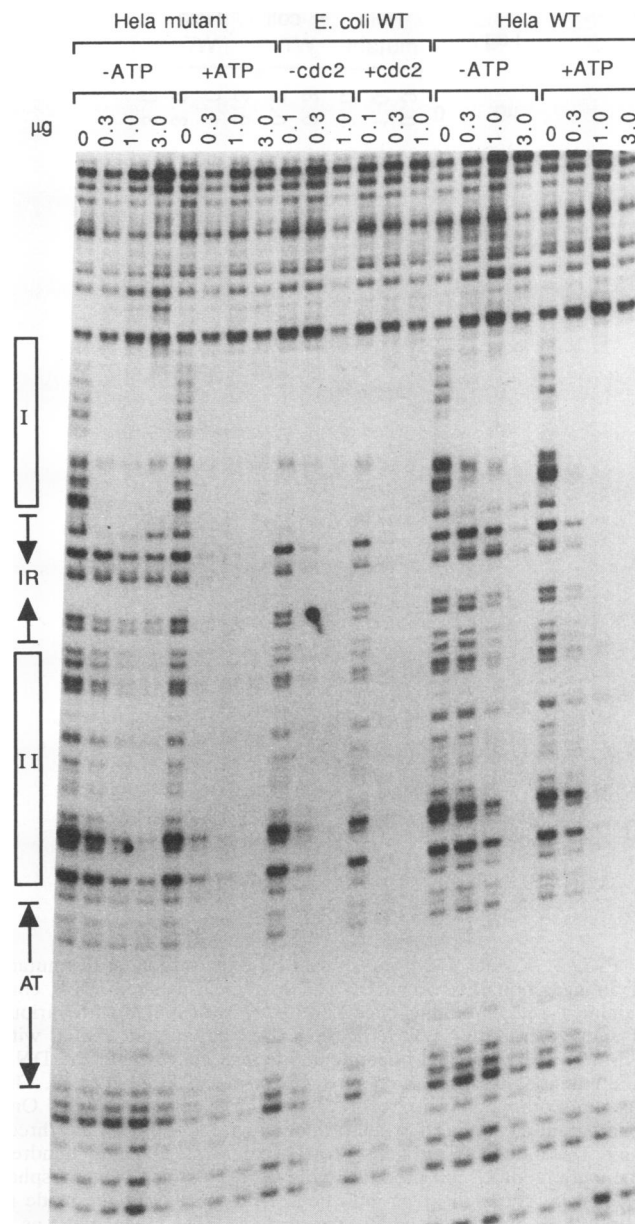


FIG. 7. DNase I footprints of T antigen bound to origin DNA. Supercoiled plasmid DNA, containing T-antigen-binding sites I and II, was incubated with various amounts of T antigen for 60 min at 37°C in replication buffer and digested with DNase I. A ^{32}P -labeled oligonucleotide was annealed to the DNA and extended with the Klenow fragment of DNA polymerase. The reaction products were resolved on a 6% acrylamide gel. HeLa-produced wild-type (WT) and mutant T antigens were tested in the presence or absence of 4 mM ATP. *E. coli*-produced T antigen was phosphorylated by cdc2 as indicated; all DNA binding assays with the bacterially produced T antigen were done in the presence of 4 mM ATP. Locations of site I, the inverted repeat domain (IR), site II, and the AT domain are shown.

nuclease (Fig. 8). KMnO_4 oxidizes the 5-6 double bond of the thymidine ring in single-stranded or distorted regions of double-stranded DNA. This chemical modification terminates a primer extension reaction by Klenow DNA polymerase, allowing mapping of the modified residues (5). The

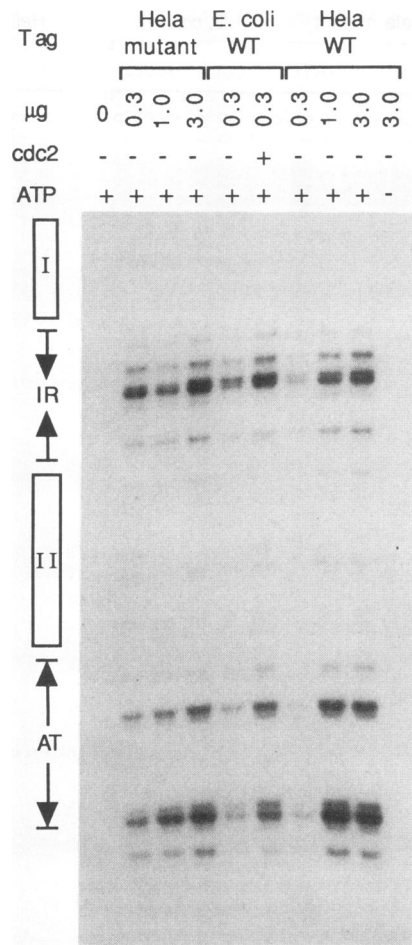
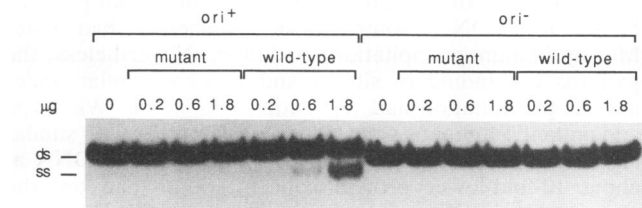


FIG. 8. KMnO_4 footprints of wild-type and threonine 124 mutant T antigens bound to origin DNA. Supercoiled plasmid DNA, containing T-antigen-binding sites I and II, was incubated with various T-antigen proteins at 37°C in replication buffer and treated with KMnO_4 . A ^{32}P -labeled oligonucleotide was annealed to the DNA and extended with the Klenow fragment of DNA polymerase. The reaction products were resolved on a 6% acrylamide gel. One microgram each of HeLa cell-produced wild-type (WT) and threonine 124 mutant T antigens (T ag) were tested. Three hundred monograms of *E. coli*-produced wild-type T antigen was phosphorylated by *cdc2* kinase as indicated. Wild-type T antigen made in HeLa cells was analyzed in the absence of ATP (-ATP) as a control. Locations of site I, the inverted repeat (IR) domain, site II, and the AT domain are shown.

distortions in the inverted repeat domain occur independently of site II, while those in the AT domain do not (32). Although distortion of the inverted repeat domain can occur in the absence of site II, site II stimulates this activity. Figure 8 shows the KMnO_4 modifications induced by modified wild-type T antigen, threonine 124 mutant T antigen, and fully unmodified T antigen made in *E. coli*. The activity was ATP dependent for each of the proteins, but this result was shown only for the wild-type protein made in HeLa cells. All three proteins deformed the origin at the same residues. Although *cdc2* phosphorylation of T antigen made in *E. coli* enhanced the DNA distortions induced by that protein to a small extent, threonine 124 mutant T antigen made in HeLa cells distorted DNA nearly as well as wild-type T antigen did. Therefore, distortion of the origin by T

A. Effect of mutation



B. Effect of *cdc2*

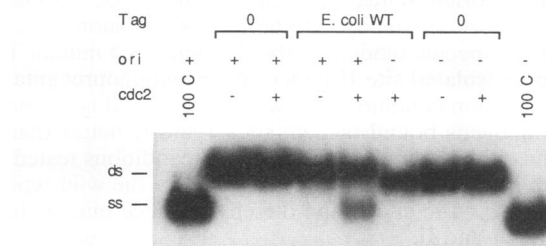


FIG. 9. Origin unwinding by wild-type and threonine 124 mutant T antigens. Wild-type and mutant T antigens made in HeLa cells, T antigen made in *E. coli*, single-stranded DNA-binding protein from *E. coli*, and end-labeled duplex DNA fragments were incubated at 37°C for 30 min in replication buffer. The DNA fragments contained either a functional origin of replication (ori^+) or a nonfunctional origin with a 3-bp deletion in site II (ori^-). The locations of the duplex (ds) and displaced (ss) DNAs are shown. (A) Comparison of the wild-type and mutant proteins. The amounts of protein used are shown. Reaction mixtures contained $1\ \mu\text{g}$ of salmon sperm DNA. (B) Comparison of wild-type (WT) T antigen (T ag) made in *E. coli* in the presence and absence of *cdc2* kinase. Reaction mixtures contained $0.6\ \mu\text{g}$ of T antigen and $50\ \text{ng}$ of competitor salmon sperm DNA. ori^+ and ori^- input DNAs were boiled (100 C) to identify the position of displaced DNA in the gel.

antigen can occur independently of phosphorylation of threonine 124 or other mammalian posttranslational modifications.

Origin unwinding. T-antigen-induced changes in the conformation of origin DNA are dependent on ATP but not on ATP cleavage (5). Complete unwinding of the origin region requires ATP hydrolysis. We investigated the effect of T antigen phosphorylation on origin-dependent unwinding by using an origin-dependent strand displacement assay (49, 55). In the assay, T antigen is incubated with linear duplex DNA containing the 64-bp core origin of replication (ori^+) or a mutant origin (ori^-) in modified replication buffer with ATP at 37°C . The mutant origin DNA is isogenic to ori^+ except for a 3-bp deletion in site II at the *Bgl*I site. Single-stranded DNA-binding protein is added to the assay mixture to stabilize the unwound DNA in a single-stranded conformation. The appearance of single-stranded DNA that is dependent both on T antigen and on a wild-type origin indicates origin unwinding. Origin unwinding by wild-type and threonine 124 mutant T antigens is shown in Fig. 9A. Mutant protein had no unwinding activity, while the wild-type protein had significant origin-dependent unwinding activity. These data strongly suggest that phosphorylation of threonine 124 is essential for origin-dependent unwinding.

To confirm that the inability of mutant T antigen to unwind the origin reflects a lack of phosphorylation on threonine 124 rather than a gross change in the protein conformation, we manipulated T antigen biochemically rather than genetically (Fig. 9B). We used purified *cdc2* kinase to phosphorylate

unmodified T antigen made in *E. coli* on threonine 124. In the absence of cdc2 kinase, unmodified wild-type T antigen did not unwind the ori⁺ DNA fragments. In the presence of cdc2 kinase, T antigen unwound approximately 30% of the ori⁺ DNA but less than 3% of ori⁻ DNA. Our results argue that phosphorylation of threonine 124 is the sole posttranslational modification required for origin-dependent unwinding by T antigen.

DISCUSSION

We investigated a variety of biochemical properties of SV40 T antigen to determine which are regulated by phosphorylation of threonine 124. First, we characterized a mutant T antigen with an alanine substitution at position 124. Scheidtmann et al. (40) have shown that this mutant T antigen has the same pattern of phosphorylation as wild-type T antigen except that residue 124 is not phosphorylated. Mutation of threonine 124 had no apparent effect on functions that are independent of T antigen's interaction with the core origin of DNA replication. These functions include ATP-dependent hexamer formation, ATPase-dependent helicase activity, binding to DNA polymerase α , binding to DNA site I, and autoregulation of early transcription *in vitro*. We think that it is unlikely that the alanine substitution at position 124 would overcome the need for phosphorylation of threonine 124 to perform these activities. In striking contrast, mutation of threonine 124 inhibited T-antigen functions that require a specific interaction with site II DNA in the core origin. These functions include binding to site II DNA and extended origin unwinding. Furthermore, we provide strong independent evidence that phosphorylation *per se* is required for these functions by showing that phosphorylation of unmodified wild-type protein by cdc2 kinase restores the origin-unwinding functions. When stringent assays are used, the magnitude of the origin-binding and -unwinding defects in the absence of threonine 124 phosphorylation is sufficient to explain the defect in DNA replication *in vitro*. We conclude that the primary function of phosphorylation of threonine 124 in DNA replication is to regulate specific interactions of T antigen with SV40 origin DNA.

Our findings extend published studies relating to the role of T-antigen phosphorylation in viral DNA replication. Schneider and Fanning (43) and Paucha et al. (34) showed that threonine 124 mutation interferes with binding of T antigen to site II and DNA replication *in vivo*. Mohr et al. (29) and McVey et al. (27) demonstrated that unmodified T antigen made in *E. coli* fails to bind site II and replicates little, if any, viral DNA *in vitro*. These DNA-binding studies were done in the absence of ATP and before key assays for T-antigen hexamer assembly and origin unwinding were developed. We have extended these studies to include a number of new assays performed in the presence of ATP under conditions suitable for DNA replication *in vitro*.

Previous conclusions relating to the role of threonine 124 phosphorylation in T-antigen binding to site II origin DNA need to be modified. Immunoprecipitation assays under McKay conditions and our present immunoprecipitation assays under replication conditions failed to detect any specific interactions of unmodified T antigen with site II in origin DNA (27, 43). In contrast, DNase I footprinting under replication conditions indicated that T antigen, either unmodified at threonine 124 or completely unmodified, binds to site II origin DNA in a pattern that resembles that of modified T antigen. Although the efficiency of binding may be slightly reduced in the footprints, threonine 124 phosphor-

ylation clearly is not an absolute prerequisite for site II recognition and binding. Furthermore, site II binding occurs at lower protein concentrations than site I binding in the immunoprecipitation assay, while the opposite is found in the footprinting assay under the same binding conditions. Therefore, the nuclease footprinting and immunoprecipitation assays must be measuring different DNA-binding parameters.

What are the differences in these DNA-binding parameters? In the immunoprecipitation assay, nucleoprotein complexes are precipitated at equilibrium and then are washed repeatedly over the course of up to 1 h to remove unbound DNA fragments. In contrast, in the footprint assay, undiluted complexes are treated with nuclease for 1 min under equilibrium conditions. Therefore, the immunoprecipitation assay would require more stable binding than the footprint assay and would be the more stringent assay, while the footprint assay would provide information for mapping of DNA-binding sites. Although beyond the scope of the present paper, a detailed analysis will be important to compare the kinetic parameters of the assays. Differences in results obtained by using the immunoprecipitation and nuclease protection assays may explain some of the conflicting conclusions on the role of phosphorylation in T-antigen binding to the origin of replication in a number of previous publications (2, 19, 27, 30, 42-44, 46, 55). Our findings emphasize that care must be taken when interpreting the results of a single DNA binding assay.

Optimal site II binding requires both protein-protein interactions and protein-DNA interactions. The fact that threonine 124 mutant T antigen forms hexamers well suggests that threonine 124 phosphorylation is required primarily for protein-DNA interactions. We have shown that, in addition to site II binding, T-antigen modification affects the unwinding of origin DNA. Both activities would require appropriate protein-DNA interactions. Under equilibrium binding conditions, the threonine 124 mutation enhances the origin distortion activity of T antigen only slightly. However, origin unwinding is completely dependent on phosphorylation of threonine 124. This result is not surprising since complete origin unwinding would require a number of different T-antigen interactions with DNA, including site II binding, stable origin distortion, and the conversion of T antigen from a site-specific DNA-binding protein to a mobile helicase.

Modifications of adjacent amino acids have opposite effects on DNA replication. Virshup et al. (55) showed that phosphorylation of serines 120 and 123 down-regulates viral DNA replication, while our present study and previous studies indicate that phosphorylation of threonine 124 up-regulates replication. Remarkably, these phosphorylation events appear to regulate similar steps in the initiation of DNA replication. The removal of phosphate from serines 120 and 123 with protein phosphatase 2A reduces the rate of T-antigen dissociation from the origin and increases the efficiency of origin unwinding. The addition of phosphate to threonine 124 with cdc2 kinase also increases the efficiency of origin unwinding by altering the interaction of T antigen with origin DNA. Furthermore, none of these modification events alter the helicase activity of T antigen. It has not been demonstrated at what point in the viral infection these phosphorylation events occur, but activation of phosphatase 2A and cdc2 kinase early in S phase would explain the observation that viral replication occurs during S phase of the cell cycle (31). Phosphorylation and dephosphorylation would provide a binary switch allowing the virus to sense when the cell was competent to support viral replication.

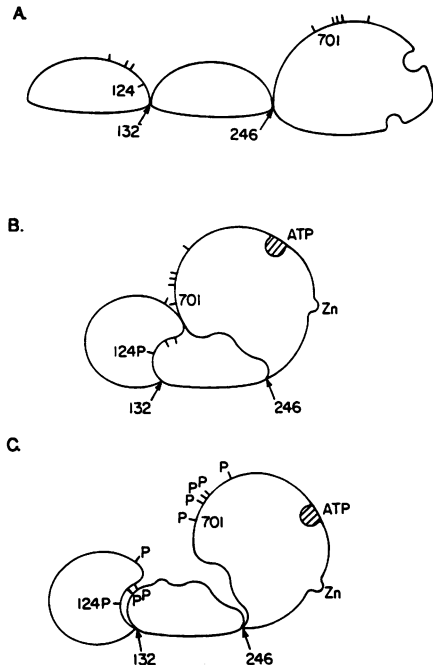


FIG. 10. Regulation of T-antigen interactions with the SV40 origin of replication by phosphorylation. Three domains of T antigen are shown, and the locations of phosphorylation sites are indicated by short lines. See Discussion for a description of the interactions among the three domains in DNA replication.

Figure 10 presents a model for the role of phosphorylation in SV40 DNA replication. In spite of the lack of direct data on the physical structure of T antigen, the protein can be represented as a three-domain structure (Fig. 10A). The central domain from amino acids 132 through 246 binds sites I and II (28, 52). Since both sites have some common recognition sequences, common protein residues are most likely responsible for specific binding. In Fig. 10B, ATP binding in the C-terminal domain induces changes in the tertiary structure of T antigen that lead to hexamer assembly and optimal DNA binding (25, 33). A functional zinc finger domain also contributes to hexamer assembly (23). Phosphorylation of threonine 124 in the N-terminal domain has no effect on hexamer assembly and is not required for DNA binding in the absence of the C-terminal domain (1, 28, 52). In the intact protein, however, phosphorylation of threonine 124 would lead to changes in the tertiary structure of T antigen that stabilize binding to site II DNA and increase the efficiency of origin unwinding. The ability of phosphorylation to cause large structural and enzymatic changes in other proteins has been well documented (22). Last, phosphorylation of serines 120 and 123 and possibly additional N- or C-terminal residues down-regulates site II binding (Fig. 10C). Whether this decrease comes about by a direct charge repulsion of the DNA by the phosphates or by a further conformation change in the protein is unknown. A better understanding of the regulation of the initiation of DNA replication will require definition of the interactions among these protein domains.

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