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Mechanisms of Simian Virus 40 T-Antigen Activation by Phosphorylation of Threonine 124

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Previous studies have shown that phosphorylation of simian virus 40 (SV40) T antigen at threonine 124 enhances the binding of T antigen to the SV40 core origin of replication and the unwinding of the core origin DNA via hexamer-hexamer interactions. Here, we report that threonine 124 phosphorylation enhances the interaction of T-antigen amino acids 1 to 259 and 89 to 259 with the core origin of replication. Phosphorylation, therefore, activates the minimal DNA binding domain of T antigen even in the absence of domains required for hexamer formation. Activation is mediated by only one of three DNA binding elements in the minimal DNA binding domain of T antigen. This element, including amino acids 167, 215, and 219, enhances binding to the unique arrangement of four pentanucleotides in the core origin but not to other pentanucleotide arrangements found in ancillary regions of the SV40 origin of replication. Interestingly, the same four pentanucleotides in the core origin are necessary and sufficient for phosphorylation-enhanced DNA binding. Further, we show that phosphorylation of threonine 124 promotes the assembly of high-order complexes of the minimal DNA binding domain of T antigen with core origin DNA. We propose that phosphorylation induces conformational shifts in the minimal DNA binding domain of T antigen and thereby enhances interactions among T-antigen subunits oriented by core origin pentanucleotides. Similar subunit interactions would enhance both assembly of full-length T antigen into binary hexamer complexes and origin unwinding.

Simian virus 40 (SV40) T antigen initiates DNA unwinding and replication via elaborate interactions with the viral origin of replication. T antigen binds two adjacent sites in the SV40 origin (42). Both sites are composed of repeats of the pentanucleotide 5′-GAGGC-3′, but the organization of the two sites differs. Site I, which has an ancillary role in replication, consists of two pentanucleotides in the same orientation separated by a short adenine tract (33). Site II consists of four pentanucleotides that form a perfect palindrome in the center of the minimal or core origin of replication. This pentanucleotide palindrome (PEN) is flanked by an inverted repeat (IR) on the early side of PEN and an adenine-thymine (AT)-rich region on the late side of PEN (27). Footprint analyses demonstrate that the core origin can be divided into functional early and late halves through the middle of PEN (29). T-antigen hexamers bind to isolated half origins (18, 29) and deform their DNA (2, 7, 9, 44). Only in an intact origin, however, do double hexamers of T antigen progressively unwind origin DNA bidirectionally (28).

Many functions of T antigen have been mapped to specific regions of the protein (10). Particularly well characterized is the minimal DNA binding domain which has been mapped to amino acids 131 through 259 (1, 23, 30, 37, 41). About 50 amino acids from the DNA binding domain, a single zinc finger motif extends from amino acids 302 to 320 (13, 16). This region is required for the formation of T-antigen hexamers in the presence of ATP (17). Minimal domains for the assembly of hexamers and double hexamers have not been mapped. The ATP binding and ATPase domains map within amino acids 418 to 627 (3, 6). The helicase and origin-unwinding activities of T antigen require the minimal DNA binding, zinc finger, and ATPase domains (46, 47).

T antigen is regulated both negatively and positively by phosphorylation (10, 11, 31). Phosphorylation of serine residues 120 and 123 inhibits viral replication (4, 5, 34, 43), whereas phosphorylation of threonine 124 (Thr-124) enhances replication (21). None of these phosphorylation sites are in the minimal DNA binding domain of T antigen between amino acids 131 and 259 (1, 23, 30, 37, 41). Mechanisms by which phosphorylation of Thr-124 enhances T-antigen activity are not completely understood. Several studies have implicated Thr-124 phosphorylation in binding to the core origin, in the assembly of T-antigen double hexamers on core origin DNA, and in origin unwinding (5, 22, 24, 43). Thr-124 modification is not required for the assembly of single hexamers (22, 24, 32). Here, we identify regions of T antigen and the core origin of replication that are involved in the modulation of T-antigen function by Thr-124 phosphorylation. Further, we show that these regions contribute to interactions among T-antigen subunits bound to origin DNA.

MATERIALS AND METHODS

Expression vectors. pT7f1A-259.0, a derivative of pT7f1A-259 (41), expresses T-antigen amino acids 1 to 259 in bacteria as a nonfusion protein. Mutations were made in this vector as described by Kunkel (15). Mutated plasmids expressed the following amino acid substitutions in the T-antigen segment spanning amino acids 1 to 259 (T1-259): an S-to-T mutation at position 152 (152 S-T), 159 F-Y, 167 K-R, 205 V-L, 207 A-G, 215 L-V, and 219 S-T. pT7f1A-82-259, derived from pT7f1A-259.0 by site-directed mutagenesis, encodes wild-type T-antigen residues 82 to 259. The adenovirus vector for the expression of full-length wild-type p53 was previously described (26). The same vector was used to express cDNA for T antigen with a substitution of an alanine for Thr-124, a generous gift from Ellen Fanning (35). All mutations were confirmed by sequence analysis.

Purification of T antigen. Segments of wild-type and mutant T antigen were expressed in *Escherichia coli* cells as described by Mohr et al. (25). Full-length wild-type and mutant T antigens were expressed in HeLa cells by using defective adenovirus vectors as described by Mohr et al. (26). After extraction, wild-type and mutant T antigens were purified by immunoaffinity chromatography (36) by using PAb419 or PAb416 (12). The proteins were 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 on

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ice against 10 mM HEPES–KOH (pH 7.4)–5 mM NaCl–0.1 mM EDTA–1 mM dithiothreitol–10% glycerol, aliquots were stored at −70°C.

Phosphorylation of truncated proteins. Purified proteins were incubated at 30°C for 20 min in 50 mM Tris (pH 8.0)–10 mM $MgCl_2$–2 mM dithiothreitol–5% glycerol–2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid]–100 μM ATP in the presence or in the absence of purified cdc2 kinase as previously described (21).

Immunoprecipitation assay for DNA binding. Labeled DNAs were derived from plasmids either by digestion with restriction enzymes or by PCR. Plasmids containing site I (pOS-1, SV40 nucleotides 5171 to 5228), the core origin (pSVOdl3, SV40 nucleotides 5209 to 128), or the wild-type origin (pSVO+, SV40 nucleotides 5171 to 128) have been described previously (40, 41). Fifty nanograms of an equimolar mixture of these plasmids cut with *Taq*I and end labeled with Klenow polymerase was used in immunoprecipitation assays. DNA fragments were also made by PCR with a single set of primers from plasmids pOR1 (SV40 core origin, nucleotides 5209 to 42), pEH (early half, nucleotides 5209 to 5243), pLH (late half, nucleotides 5243 to 42), pIR/PEN (IRs and pentanucleotides, nucleotides 5209 to 11), pPEN/AT (pentanucleotides and AT region, nucleotides 5230 to 42), pIR/AT (IRs and AT domains, nucleotides 5209 to 30 and 11 to 42, respectively), pIR (IRs, nucleotides 5209 to 5230), pPEN (pentanucleotides, nucleotides 5230 to 11), and pAT (AT region, nucleotides 11 to 42) which have been described previously (8, 29). As a negative control, we replaced the origin sequences of pOR1 with a *Hae*III fragment from lambda phage which was cloned into the *Sma*I site of pOR1 to create pOR-lambda. DNA fragments generated by PCR with labeled oligonucleotides represented origin sequences between the *Hin*dIII and *Nco*I sites of the plasmids. DNA fragments were purified by gel electrophoresis. Twenty thousand counts per minute of each fragment was used in each reaction.

We used the immunoprecipitation procedure described by McKay (20) with modifications. Briefly, T antigen was incubated with ^{32}P-labeled DNA in 50 μl of 10 mM HEPES–KOH (pH 7.4)–100 mM KCl–1 mM $MgCl_2$–5% glycerol–50 μg of bovine serum albumin per ml (DNA binding buffer) for 50 min on ice. Hybridoma supernatant, containing monoclonal antibody PAb416 or PAb419, was added to 10 μl of protein A-Sepharose beads in NET buffer (50 mM Tris · HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40) and rocked at 4°C for 50 min. The beads were suspended in DNA binding buffer before addition to the T antigen-DNA complexes. The reaction mixtures were incubated for an additional 50 min with occasional mixing before being pelleted, washed three times with 1 ml of NET buffer at 0°C, suspended in 1% sodium dodecyl sulfate with 25 mM EDTA, and heated at 65°C for 15 min. The T-antigen-bound DNA fragments were resolved by 6% polyacrylamide gel electrophoresis. The gels were dried on DE81 paper and exposed to Kodak XAR film.

Gel shift assay. Core origin DNA (nucleotides 5209 to 42) was prepared by digestion of pOR1 with *Hin*dIII and *Nco*I and end labeled with Klenow polymerase. T-antigen segment T1-259 was purified and, in some cases, phosphorylated with cdc2 kinase as described above. Labeled core origin DNA (1 ng) and unlabeled poly(dA)-poly(dT) (300 ng) were incubated with T1-259 (1 μg) in 25 μl of DNA binding buffer for 50 min on ice. Under these conditions, competition experiments indicate that binding by T antigen is specific for core origin DNA. In some cases, reaction mixtures were incubated for 20 min on ice with glutaraldehyde at a final concentration of 0.1%. The reaction products were resolved in a 6% polyacrylamide gel containing 10% glycerol and 50 μg of BSA per ml at room temperature. The gels were dried on DE81 paper and exposed to X-ray film.

RESULTS

Thr-124 phosphorylation activates the minimal DNA binding domain of T antigen. Previous studies of the role of T-antigen phosphorylation in DNA binding used the full-length protein. We first asked whether Thr-124 together with the minimal DNA binding domain of T antigen (amino acids 131 to 259) is sufficient to activate DNA binding in the absence of domains for hexamerization, ATPase, and helicase functions. For this purpose, we overexpressed T-antigen amino acids 82 to 259 (T82-259) in bacteria and purified the segment for DNA binding studies in vitro. We used a stringent immunoprecipitation assay to quantitate site-specific binding of segment T82-259 to origin DNA in the absence and in the presence of phosphorylation by cdc2 kinase. In this assay, T antigen was mixed with an equimolar mixture of end-labeled DNA fragments containing nonspecific plasmid DNA, both site I and the core origin, core origin alone, and site I alone. DNA bound to T antigen was immunoprecipitated, washed extensively, and visualized by gel electrophoresis and autoradiography. We found that ATP had no effect on the binding of segment T89-259 to origin DNA (data not shown). Because this T-antigen segment has no ATP binding domain, this result is not surprising.

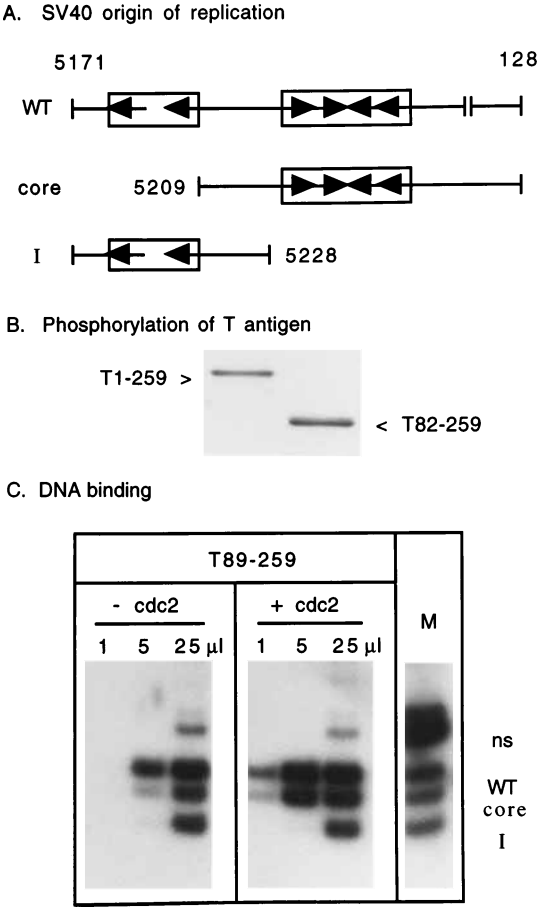

FIG. 1. Binding of T-antigen amino acids 82 to 259 (segment T82-259) to DNA in the absence and in the presence of phosphorylation by cdc2 kinase. (A) Map of the SV40 origin of replication. Arrows indicate pentanucleotide repeats. Numbers indicate sequence positions in the SV40 genome. WT, wild type. I, site I DNA. (B) Phosphorylation assay. T-antigen segments in bacterial extracts were immunoprecipitated with PAb416 on protein A-Sepharose beads and washed extensively. After incubation with cdc2 kinase and [^{32}P]ATP, phosphorylation of T1-259 and T82-259 was demonstrated by gel electrophoresis and autoradiography. (C) DNA binding assay. After T82-259 was purified by immunoprecipitation and incubated in the absence or in the presence of cdc2 kinase, the immunocomplexes were incubated with an equimolar mixture of ^{32}P-labeled DNA fragments. T antigen-DNA complexes were washed and analyzed by gel electrophoresis. Input DNA is shown in lane M. The positions of nonspecific DNA (ns), wild-type origin DNA with sites I and II (WT), core origin DNA with site II (core), and site I DNA (I) are indicated.

Figure 1A reviews the arrangement of pentanucleotide repeats in the SV40 origin of replication, and Fig. 1B confirms that T82-259 was phosphorylated by cdc2 kinase in this study. Figure 1C shows that phosphorylation of segment T82-259 enhanced binding to core origin DNA. Increasing concentrations of either unphosphorylated or phosphorylated T-antigen segments bound intact origin, core origin, site I, and nonspecific DNAs, in that order of preference. Phosphorylated segment T82-259 bound core origin DNA fivefold more efficiently than did unphosphorylated T82-259. Given that only 20 to 30% of T antigen was phosphorylated by cdc2 kinase (data not shown), the observed increase in core origin DNA binding is quite significant. Because purified cdc2 kinase phosphorylates only Thr-124 in T antigen (21) and because ATP is essential for the reaction, we conclude that enhanced binding to core origin

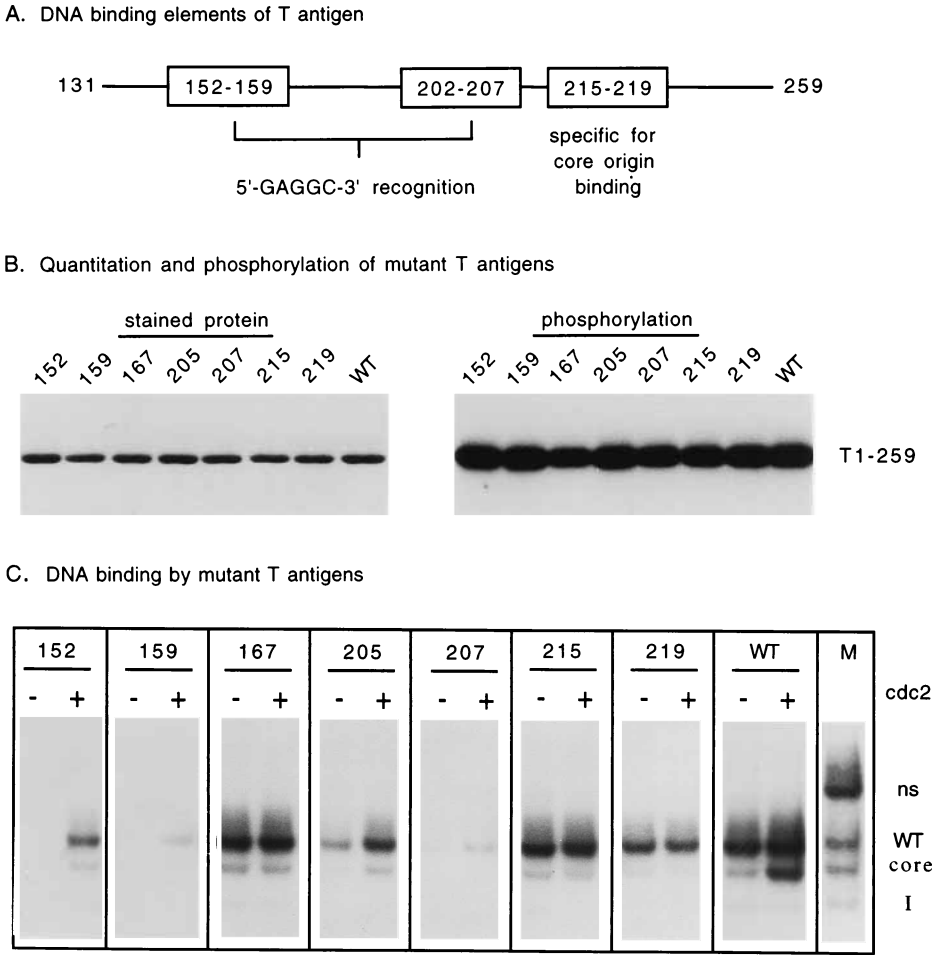

FIG. 2. DNA binding by wild-type (WT) and mutant T-antigen segments consisting of amino acids 1 to 259 (T1-259). (A) DNA binding elements of T antigen. The minimal DNA binding domain of T antigen has three critical DNA binding elements with functions defined by Simmons et al. (38). Numbers indicate amino acid positions. (B) Quantitation and phosphorylation of mutant T antigens. Mutant T-antigen segments (3 μg) were purified from *E. coli* cells by immunoaffinity chromatography. After ^{32}P phosphorylation with cdc2 kinase, T-antigen segments were stained with Coomassie blue or autoradiographed. Numbers indicate positions of amino acid changes. (C) DNA binding by mutant T-antigen segments. WT and mutant T-antigen segments T1-259 were incubated with a mixture of end-labeled DNA fragments as described for Fig. 1. T antigen-DNA complexes were immunoprecipitated with PAb419 on protein A-Sepharose beads, washed, and analyzed by gel electrophoresis. Input DNAs are shown in lane M. The positions of DNAs are as described in the legend to Fig. 1.

DNA reflects phosphorylation of Thr-124 rather than some other modification of T antigen. In contrast to its effect on core origin binding, phosphorylation had no effect on the binding of T82-259 to site I DNA or to nonspecific DNA. These results are similar to those previously reported for full-length T antigen (21, 22). We conclude that phosphorylated Thr-124 interacts with the minimal DNA binding domain of T antigen to enhance binding to core origin DNA. Further, our results indicate that enhanced binding does not require the ATP binding, hexamer assembly, or ATPase domains of T antigen.

Phosphorylated Thr-124 requires amino acids 167, 215, and 219 for activation of T antigen. Simmons et al. (38) scanned the DNA binding domain of T antigen with mutations and identified three regions that are crucial for site-specific DNA binding (Fig. 2A). Amino acids 152 to 159 and 202 to 207 are required for binding to either site I or core origin DNA. Because the pentanucleotide 5′-GAGGC-3′ is the only feature that is common to these sites, Simmons et al. proposed that these regions of T antigen may interact with the pentanucleotide. In contrast, amino acids 167, 215, and 219 contribute to optimal binding to core origin DNA but not to site I. We asked whether these regions might contribute to the enhancement of core origin DNA binding via phosphorylation of Thr-124. For this purpose, we introduced the mutations characterized by Simmons et al. (38) into a T-antigen segment consisting of amino acids 1 to 259 (T1-259). We overexpressed the mutant segments in bacteria and purified the segments for DNA binding studies in the absence and in the presence of phosphorylation by cdc2 kinase. Figure 2B shows the purity of the mutant T-antigen segments and confirms that each of the segments was phosphorylated by cdc2 kinase with similar efficiency.

Figure 2C compares the DNA binding efficiencies of the mutant T1-259 segments, using an immunoprecipitation assay. T-antigen segments with substitutions of amino acids 152, 159, 205, and 207 bound DNAs containing core origin DNA much less efficiently than the wild-type T1-259 did. Nevertheless, phosphorylation of these mutant segments by cdc2 kinase activated binding to origin DNA. In contrast, T-antigen segments with substitutions of amino acids 167, 215, and 219 bound core origin DNA to a greater extent than the other mutant segments did, but these mutant segments were not activated by phosphorylation. These findings argue that phosphorylated

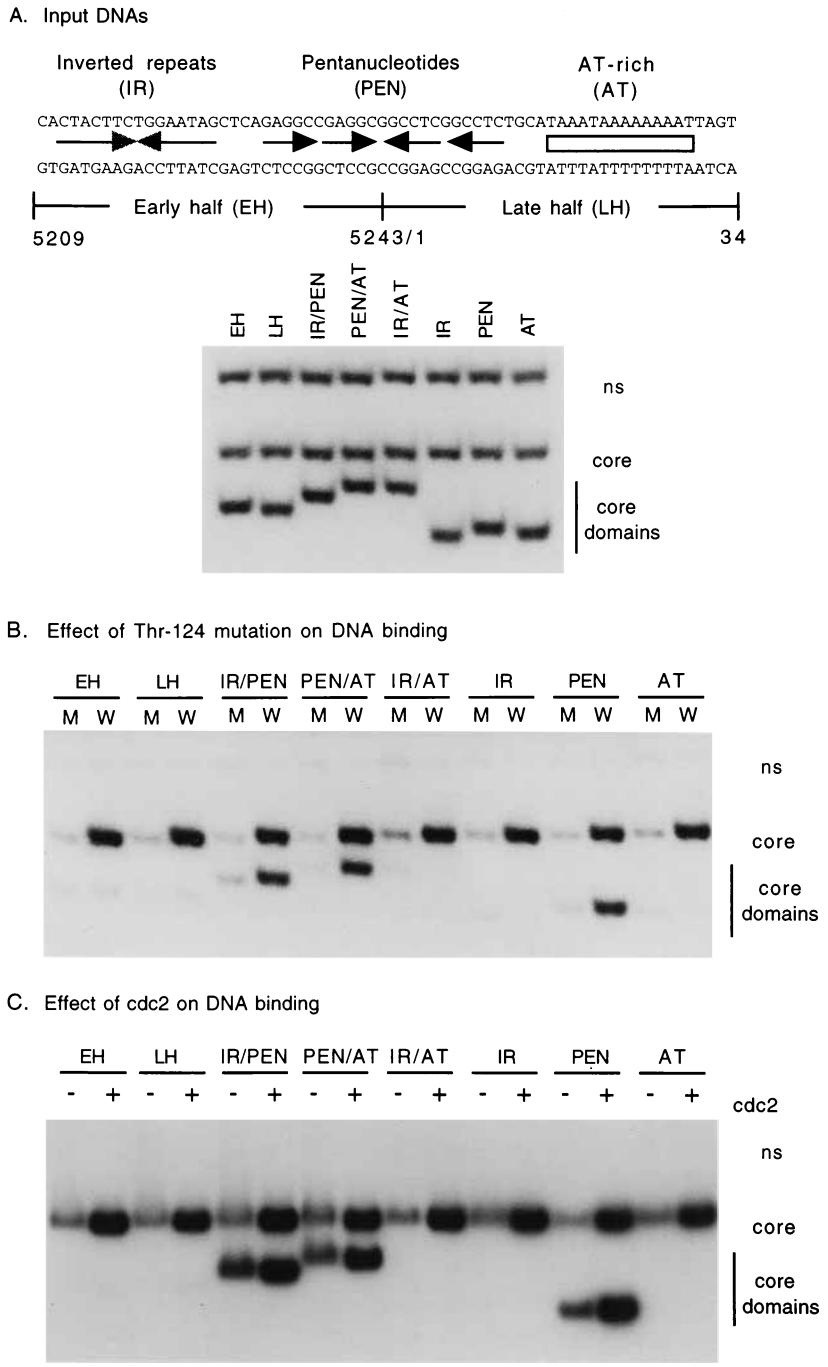

FIG. 3. Domains of the core origin of replication that are sufficient for enhanced binding by T antigen phosphorylated at Thr-124. (A) Input DNAs. The core origin consists of an IR domain, a PEN domain, and an AT-rich domain. Division of PEN at its center yields early-half and late-half origins. Numbers indicate sequence positions in the SV40 genome. Nonspecific DNA (ns), intact core origin DNA (core), and isolated domains of the core origin (core domains) were prepared by PCR. (B) Immunoprecipitation assay for DNA binding by full-length, mutant T antigen (M) with an alanine substitution for Thr-124 or by wild-type T antigen (W). Both proteins were made in HeLa cells and purified by immunoaffinity chromatography. Domains of core DNA are identified above the lanes (EH and LH, early-half and late-half origins, respectively); the locations of nonspecific DNA (ns), core, and core domains are indicated. (C) Immunoprecipitation assay demonstrating the effect of phosphorylation by cdc2 kinase on DNA binding. T-antigen segment T1-259 was made in bacteria and was phosphorylated with cdc2 kinase. Abbreviations are as described for panel B.

Thr-124 modulates core origin DNA binding via amino acids 167, 215, and 219.

The PEN domain of the core origin is sufficient for enhanced DNA binding by T antigen phosphorylated at Thr-124. To determine which domains within core origin DNA are required for enhanced binding by phosphorylated T antigen, we used a set of plasmids encoding well-characterized subregions of the core origin (27, 29). These DNA domains were amplified by PCR with radiolabeled primers. The names of the DNA segments indicate the core domains that they contain (Fig. 3A).

The DNAs were mixed with intact core origin and nonspecific DNAs which served as internal positive and negative DNA binding controls, respectively, for immunoprecipitation assays. Because the assays were done under conditions of limiting protein, the intact core origins compete with the mutant origins for T-antigen binding and indicate relative binding affinities. Equimolar mixtures of the DNAs are shown in Fig. 3A.

Figure 3B compares the binding of full-length, wild-type T antigen (lanes W) with the binding of mutant T antigen with an alanine substitution for Thr-124 (lanes M). Both proteins were purified from HeLa cells fully competent for phosphorylation of all available sites in T antigen. Wild-type T antigen bound the core origin of replication far more efficiently than did the Thr-124 mutant T antigen. Furthermore, wild-type T antigen bound all origins that contain PEN (IR/PEN, PEN/AT, and PEN), and binding was more efficient than that of mutant T antigen. In the presence of competitor core origin DNA, neither half of the PEN domain (early or late) was sufficient for efficient binding. Interestingly, the presence of threonine at position 124 strongly enhanced binding to the isolated PEN domain. This finding argues that the PEN domain alone is sufficient to demonstrate enhanced binding by T antigen phosphorylated at Thr-124. To confirm this conclusion, we did similar experiments with unphosphorylated T1-259 made in bacterial cells (Fig. 3C). This wild-type segment bound the same core origin domains that the full-length T antigen did. Furthermore, phosphorylation of T1-259 by cdc2 kinase stimulated binding of the PEN domain in isolation from other core origin DNA domains. We conclude that the PEN domain is necessary and sufficient to demonstrate enhanced binding by T antigen phosphorylated at Thr-124.

Phosphorylation of Thr-124 promotes the assembly of high-order complexes of T antigen with core origin DNA. Phosphorylation of Thr-124 might enhance binding of T1-259 to core origin DNA by two general mechanisms: enhanced protein-DNA interactions and enhanced protein-protein interactions. That DNA binding is activated by the palindromic arrangement of four pentanucleotides in the PEN domain of the core origin, but not by site I DNA, argues that Thr-124 phosphorylation enhances protein-protein interactions rather than protein-DNA interactions. In the absence of an ATP binding domain, however, segment T1-259 would not be expected to form hexamers; we have confirmed this expectation experimentally (data not shown).

We asked whether phosphorylation of Thr-124 might enhance assembly of high-order complexes of T1-259 on core origin DNA even in the absence of hexamer formation. For Fig. 4, we used a gel retardation assay to compare complexes formed by core origin DNA and T1-259 without and with phosphorylation. T1-259 was prepared in bacteria, purified by immunoaffinity chromatography, and phosphorylated by cdc2 kinase. In the absence of phosphorylation, T1-259 bound the core origin as a single distinct species. After phosphorylation, the same segment bound core origin DNA as a significantly larger complex. Cross-linking with glutaraldehyde did not stabilize DNA binding by either unphosphorylated or phosphorylated T82-259. We conclude that phosphorylation of Thr-124 enhances the formation of high-order complexes of T1-259 with core origin DNA. Although qualitative differences in the binding of T1-259 to core origin DNA in the absence and in the presence of phosphorylation are striking, little difference in the efficiency of overall DNA binding is apparent in the gel shift assay. We previously showed that the immunoprecipitation assay, which requires extensive washing of protein-DNA complexes before analysis, is far more stringent than equilibrium assays like footprinting or gel retardation assays (22).

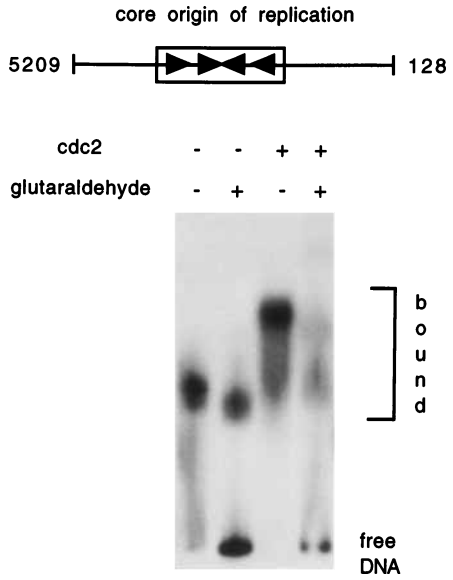

FIG. 4. Analysis of protein-DNA complexes formed by T-antigen segment T1-259 without and with phosphorylation. T1-259 was made in bacteria, purified by immunoaffinity chromatography, and phosphorylated with cdc2 kinase. Unphosphorylated or phosphorylated T1-259 was bound to core origin DNA. Complexes, without or with fixation by glutaraldehyde, were identified by gel shift analysis. The positions of bound and free DNAs are indicated.

DISCUSSION

The minimal DNA binding domain of T antigen is sufficient for enhancement of core origin DNA binding via phosphorylation of Thr-124. This result indicates that ATP binding and hydrolysis, hexamer assembly, and the formation of double hexamers are not required for this enhanced core origin binding. In addition, there is considerable evidence that Thr-124 phosphorylation stabilizes the assembly of double hexamers on core origin DNA and promotes unwinding of core DNA (5, 22, 24, 43). Moarefi et al. (24) have presented evidence that the enhancement of DNA binding by phosphorylation of Thr-124 may not be of sufficient magnitude to explain completely the enhanced unwinding of origin DNA. Nevertheless, it is clear that the stable binding of T antigen to origin DNA would contribute significantly to subsequent DNA unwinding.

Phosphates are known to interact with charged residues to induce conformational changes in proteins (14). We propose that Thr-124 phosphorylation induces conformational changes in the minimal DNA binding domain of T antigen. Amino acids 167, 215, and 219 promote the binding of T antigen to core origin DNA after phosphorylation of Thr-124. Because these amino acids, like phosphorylated Thr-124, contribute to optimal binding to core origin DNA but not to site I DNA (39), they are likely to operate through a common mechanism with Thr-124. Most probably, phosphorylated Thr-124 interacts with charged amino acids in the minimal DNA binding domain to initiate a change in conformation; Lys-167 could contribute to such interactions. Additional areas in the minimal DNA binding domain, like that represented by amino acids 215 to 219, could participate in the conformational shift by encoding structural signals. In addition, amino acids 215 to 219 might contribute directly to intermolecular interactions among subunits of T antigen. Our present data, therefore, provide an explanation for the role of amino acids 167, 215, and 219 in DNA binding and replication (38). These amino acids are

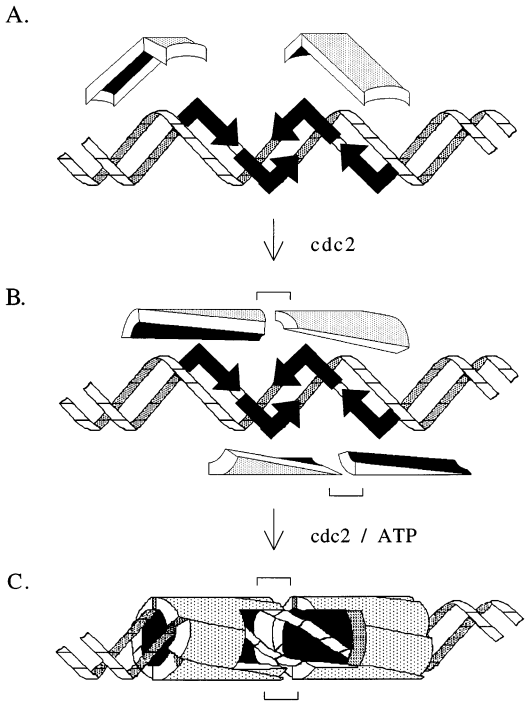

FIG. 5. Steps in the initiation of SV40 DNA replication that are enhanced by phosphorylation of Thr-124. Black arrows on the DNA indicate pentanucleotide repeats. Brackets indicate protein-protein interactions. See Discussion for a description.

required for structural changes in T antigen in response to Thr-124 phosphorylation.

Conformational changes in the minimal DNA binding domain could lead to a variety of related changes in T-antigen function. A model, consistent with published findings and our present findings, is shown in Fig. 5. Figure 5A shows possible interactions of the minimal DNA binding domain of T antigen with pentanucleotide repeats of core origin DNA in the absence of Thr-124 phosphorylation. The direction of pentanucleotides orients the minimal DNA binding domains. Because T antigen does not bind single pentanucleotides well (45), we have shown the binding of two T-antigen subunits to two pentanucleotide repeats. These interactions would result in the single complex of unphosphorylated T1-259 with core origin DNA demonstrated by the gel shift assay (Fig. 4). Figure 5B shows the effects of Thr-124 phosphorylation by cdc2 kinase. Because phosphorylation of Thr-124 does not enhance binding to site I, these changes are not likely to affect the interaction of T antigen with individual pentanucleotides per se. Instead, conformational shifts induced by phosphorylation would foster interactions among T-antigen subunits, but only when the subunits are properly spaced and oriented by core origin pentanucleotides. Presumably, in the absence of ATP binding and hexamerization (29), phosphorylation would enhance the binding of four subunits of T antigen to four pentanucleotide repeats as shown by Mastrangelo et al. (19). These enhanced protein-protein interactions, indicated in Fig. 5B (see the legend), would promote assembly of the larger complexes of phosphorylated T1-259 with core origin DNA demonstrated by the gel shift assay (Fig. 4). Phosphorylation of Thr-124, therefore, would operate at the earliest stages of DNA replication by enhancing contacts among minimal DNA binding domains of T antigen. Figure 5C shows the assembly of full-length T-antigen on origin DNA. The assembly of hexamers requires ATP binding (18, 29). The protein-protein interactions that contribute to core origin binding by the minimal DNA binding domain of T antigen after Thr-124 phosphorylation could also enhance hexamer-hexamer interactions and origin unwinding by a binary complex of T-antigen hexamers (36). Conformational changes in T antigen after Thr-124 phosphorylation could contribute to DNA unwinding by additional mechanisms.

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