Mechanism of Activation of Simian Virus 40 DNA Replication by Protein Phosphatase 2A

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The catalytic subunit of protein phosphatase 2A (PP2A_c) stimulates the initiation of replication of simian virus 40 DNA *in vitro* by dephosphorylating T antigen at specific phosphoserine residues (K. H. Scheidtmann, D. M. Virshup, and T. J. Kelly, *J. Virol.* 65:2098-2101, 1991). To better define the biochemical mechanism responsible for this stimulation, we investigated the effect of PP2A_c on the interaction of T antigen with wild-type and mutant origins of replication. Analysis of the binding of T antigen to the wild-type origin as a function of protein concentration revealed that binding occurs in two relatively discrete steps: the assembly of a T-antigen hexamer on one half-site of the origin, followed by the assembly of the second hexamer on the other half-site. The major effect of PP2A_c was to stimulate binding of the second hexamer, so that the binding reaction became much more cooperative. This observation suggests that dephosphorylation of T antigen by PP2A_c primarily affects interactions between the two hexamers bound to the origin. Pretreatment with PP2A_c increased the ability of the bound T antigen to unwind the origin of replication but had no effect on the intrinsic helicase activity of the protein. Thus, dephosphorylation of PP2A_c appears to increase the efficiency of the initial opening of the origin by T antigen. An insertion mutation at the dyad axis in the simian virus 40 origin, which altered the structural relationship of the two halves of the origin, abolished the effect of the phosphatase on the cooperativity of binding and completely prevented origin unwinding. These findings suggest that the ability of T antigen to open the viral origin of DNA replication is critically dependent on the appropriate functional interactions between T-antigen hexamers and that these interactions are regulated by the phosphorylation state of the viral initiator protein.

The initiation of chromosomal DNA replication is likely to be a vital control point in the maintenance of normal cell proliferation. Significant insights into the mechanisms and controls involved in mammalian DNA synthesis have derived from analysis of a model system, the papovavirus simian virus 40 (SV40) (5, 55). SV40 DNA synthesis is initiated at a specific viral origin of replication and requires only a single virus-encoded protein, the SV40 large T antigen. The remaining proteins required for the replication of the viral genome are supplied by the host cell and are presumably involved in cellular DNA synthesis as well. In infected cells, the initiation of SV40 DNA replication appears to be critically dependent upon events that occur in the late G₁ or early S phase of the cell cycle, suggesting that viral DNA replication may be subject to the normal mechanisms which control cellular entry into the S phase (39, 46). The development and biochemical characterization of a cell-free system for SV40 DNA replication have resulted in the purification of a number of cellular proteins required for the reconstitution of efficient DNA replication *in vitro*. A detailed understanding of how the activities of these cellular enzymes are controlled during the initiation of viral DNA replication may aid our understanding of how cellular DNA replication is regulated as well.

Detailed mutational analysis has demonstrated that the SV40 origin of replication (*ori*) contains a 64-bp sequence which is necessary and sufficient for initiation of replication *in vivo* and *in vitro*. This minimal origin of replication is flanked by auxiliary sequences which stimulate replication

severalfold *in vivo* but are not absolutely required. The 64-bp minimal origin contains four copies of a pentameric T-antigen recognition sequence (GAGGC), with a central dyad axis of symmetry. On the early side of the pentamers is a 15-bp imperfect inverted repeat, and on the late side is a sequence of 17 A/T base pairs. The A/T-rich region includes a required sequence of eight adenines, which induce a significant bend in the DNA structure (10, 11, 16, 17, 24, 31).

The earliest steps in the initiation of SV40 DNA replication, recognition of the origin and local unwinding of the duplex, require T antigen and the cellular replication protein RP-A (8, 20, 62-64). In the presence of ATP, T-antigen monomers assemble cooperatively on the 64-bp minimal origin, forming an oligomeric DNA-protein complex (3, 9, 13). Quantitative electron microscopy and cross-linking studies suggest that this complex consists of two hexamers of T antigen in close proximity (32, 42). The binding of T antigen induces structural alterations in the origin, leading to local destabilization of the helix (4, 41, 45). The helicase activity of the bound T antigen (54) then catalyzes unwinding of the DNA. RP-A facilitates this phase of the initiation reaction by stabilizing the unwound strands, and other single-stranded DNA-binding proteins, such as *Escherichia coli* SSB, can provide the same function. The unwound complex serves as an efficient template for priming and DNA synthesis by the eukaryotic DNA polymerase α (26, 36, 37, 56, 60, 61, 65).

We have previously shown that the catalytic subunit of protein phosphatase 2A (PP2A_c) stimulates the initial stages of the SV40 DNA replication reaction *in vitro* by dephosphorylating T antigen at specific phosphoserine residues (51, 57, 58). The present studies were undertaken to better define

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the biochemical mechanism by which PP2A_c-mediated dephosphorylation of T antigen stimulates the initiation of SV40 DNA replication. Analysis of the binding of T antigen to the viral origin as a function of protein concentration revealed that binding occurs in two relatively discrete steps: the binding of a T-antigen hexamer on one half-site of the origin, followed by the binding of the second hexamer on the other half-site. The major effect of PP2A_c was to stimulate binding of the second hexamer, so that the apparent cooperativity of the binding reaction increased markedly. This finding suggests that dephosphorylation of T antigen by PP2A_c primarily affects the interactions between hexamer units bound to the origin. Although pretreatment with the phosphatase increased the ability of the bound T antigen to unwind the origin of replication, the intrinsic helicase activity of the protein was unaffected. Thus, dephosphorylation by PP2A_c appears to enhance the initial opening of the origin by T antigen. An insertion mutation in the SV40 origin which altered the structural relationship of the two halves of the origin abolished the effect of the phosphatase on the cooperativity of binding and completely prevented origin unwinding. These findings suggest that the ability of T antigen to open the viral origin of DNA replication is critically dependent on the appropriate functional interactions between T-antigen hexamers and that these interactions are regulated by the phosphorylation state of the viral initiator protein.

MATERIALS AND METHODS

To generate short DNA fragments containing the minimal origin of SV40 DNA replication, restriction sites were engineered immediately adjacent to the boundaries of the minimal origin by amplification of the origin from pUC.HSO (64) by polymerase chain reaction (PCR). The primers used, 1 (5'-GGCTCGAGTGCCTACTTCTGGAATAGC-3') and 2 (5'-CCAAGCTTACGTAATTTTATTTATGTC-3'), contain *Xho*I and *Apa*LI restriction sites on the early side and *Hind*III and *Sna*BI sites on the late side of the SV40 origin of replication. The resultant PCR products were digested with *Xho*I and *Hind*III and ligated to pBluescript KS(-) (Stratagene) digested with the same enzymes. Mutant origins were similarly amplified from pOR.1174 and pOR.105 (31). The resultant plasmids were designated pDV.XH, pDV.1174, and pDV.105, respectively, and are hereafter referred to by number only. pDV.XH thus contains 64 bp of SV40 sequence, corresponding to nucleotides 5211 to 32, encompassing the minimal origin of replication. Additional origin mutations were constructed by PCR mutagenesis (25). The sequence of all clones was confirmed by dideoxy sequencing.

SV40 large T antigen was immunoaffinity purified from recombinant-adenovirus-infected HeLa cells on monoclonal antibody 419 coupled to Affigel-10 (Bio-Rad) as previously described (65). PP2A_c was purified from HeLa extracts (58) and beef heart by a modification of previously published methods (14, 35a).

DNA mobility shift assays. Origin-containing fragments were obtained by digesting the pDV plasmids with *Apa*LI and *Sna*BI restriction endonucleases. This gave an origin-containing fragment of 67 bp. A nonspecific 70-bp fragment was obtained by digesting pUC19 with *Apa*LI and *Hae*II. After enzyme digestions, the fragments were separated by agarose gel electrophoresis and visualized with ethidium bromide, and the desired fragments were electrophoresed onto NA45 paper (Schleicher & Schuell). The DNA fragments were eluted from the paper with 300 μ l of 50 mM

arginine base and 1 M sodium chloride, extracted with phenol-chloroform, precipitated with ammonium acetate and ethanol in the presence of 20 μ g of glycogen, and resuspended in 10 mM Tris-HCl-1 mM EDTA, pH 7.2. The fragments were labeled with [α -³²P]dCTP with the Klenow fragment of DNA polymerase I, and ³²P incorporation was quantitated by thin-layer chromatography of an aliquot of the labeling reaction mix on 0.1-mm cellulose-polyethyleneimine sheets.

DNA gel retardation assay mixes contained 3 fmol (0.3 nM) of ³²P-labeled DNA fragment in 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.8)-7 mM MgCl₂-40 mM creatine phosphate-1 mM dithiothreitol (DTT), with 4 mM adenylyl-imidodiphosphate (AMP-PNP; Boehringer Mannheim). Binding reaction mixes were assembled on ice, and reactions were initiated by shifting the tubes to 37°C and then adding T antigen to give a final volume of 10 μ l. After a 30-min incubation, the protein-DNA complexes were cross-linked by the addition of glutaraldehyde to a final concentration of 0.5%, incubated for a minimum of 5 min at 37°C, and then analyzed by electrophoresis through a 0.5% agarose-2.4% acrylamide vertical slab gel in 89 mM Tris-borate-2 mM EDTA (TBE) for 45 min at 300 V constant voltage. The gels were then dried on DE81 paper (Whatman) and autoradiographed on XAR film at -70°C with Cronex intensifying screens.

The time course of dissociation of T-antigen double hexamers from the origin was assessed by incubating 1.2 μ g of T antigen with 45 fmol of ³²P-labeled origin fragment, with or without 1.5 μ g of PP2A_c, in a 15-fold-increased reaction volume. After a 30-min binding period at 37°C, 9.75 μ g of unlabeled pSori64 (containing 64 copies of the SV40 origin of replication) was added to each reaction mix. Then, 10- μ l aliquots were removed from the cocktail at the indicated times and added to tubes containing 2 μ l of 3% glutaraldehyde. Competition assays were performed by incubating 80 ng of T antigen with 3 fmol of ³²P-labeled wild-type or pDV.1'C origin fragment, without or with 100 ng of PP2A_c, in the presence of the indicated amount of sheared calf thymus DNA (average length, 500 to 1,000 bp). Following electrophoresis and autoradiography, the amount of DNA in the double hexamer form was quantitated by laser densitometry on an LKB Ultrascan.

To obtain a quantitative estimate of the stimulation of cooperative binding by PP2A_c, the ratio K_2/K_1 of the affinities of T antigen for unoccupied and singly shifted DNA was determined, where K_1 is the apparent association constant for the reaction $T + D \leftrightarrow TD$ (where T is the T antigen hexamer, D is free DNA, and TD is the single hexamer-origin complex) and K_2 is the affinity constant for the reaction $TD + T \leftrightarrow TDT$ (where TDT is the double hexamer-DNA complex). Thus,

$$K_2/K_1 = \frac{4(TDT)(D)}{(TD)^2}$$

The equation $T + D \leftrightarrow TD$ is a simplification of the two possible initial binding reactions, namely, the association of a T-antigen hexamer with either the early or the late side of the origin of replication. The statistical factor of 4 is based upon the simplifying assumption that T-antigen hexamers bind equally well to the early and late sides of the origin. This assumption is supported by the results of the exonuclease assay shown in Fig. 2B (see Results). The effect of PP2A_c was expressed in terms of a cooperativity index (see

Fig. 8), which was calculated as the K_2/K_1 ratios in the presence and absence of PP2A_c.

DNase I and exonuclease III assays. Uniformly labeled 168-bp fragments containing the minimal origin of replication and flanking pBluescript sequence were amplified from pDV.XH by PCR with the T3 and SK primers (Stratagene) in the presence of 25 μ M [α -³²P]dCTP. The labeled PCR product was purified by electroelution from 8% polyacrylamide gels and used in gel retardation assays as described above. The DNA bands were visualized by autoradiography of the wet gel, excised individually, and electroeluted in 0.1 $\times$ TBE. Equal quantities of DNA and DNA-protein complexes were exhaustively digested with 1 U of DNase I for 20 min at 37°C in a final volume of 90 μ l (15). Reactions were stopped by the addition of proteinase K to 0.25 mg/ml and sodium dodecyl sulfate (SDS) to 0.25%, and the reaction mixes were incubated for an additional 2 h at 37°C. The size of the DNA region protected by T-antigen binding was determined by electrophoresis on a nondenaturing 12% polyacrylamide gel.

Exonuclease III assays were performed by the method of Shalloway et al. (53). A 300-bp *Xba*I-*Ase*I fragment from pDV.XH (containing the 64-bp minimal origin and flanking pBluescript sequence) was 5'-end labeled on the late (*Xba*I) side, incubated with T antigen in a standard gel retardation assay, and then electrophoresed as described above. The DNA bands were visualized by autoradiography of the wet gel, excised individually, and electroeluted in 0.25 $\times$ TBE. Equal quantities of DNA were digested in 200 μ l (final volume) of 30 mM HEPES (pH 7.8)–5 mM MgCl₂–2 mM DTT by 70 U of exonuclease III (Boehringer Mannheim) for 15 min at 37°C. Reactions were stopped by adjusting the reaction mix to 10 mM EDTA, 0.05% SDS, and 0.1 mg of proteinase K per ml and further incubated overnight at 37°C. Samples were then extracted with phenol-chloroform, ethanol precipitated, and analyzed on a 6% polyacrylamide sequencing gel.

Helicase and unwinding reactions. Unwinding and helicase reactions were performed as described previously (54, 58) with the following modifications. The substrate for origin unwinding was pDV.XH or the indicated mutants, digested with *Asp* 718 and *Pvu*II and labeled with the Klenow fragment of DNA polymerase I and [α -³²P]dCTP. This gave a 279-bp labeled fragment containing the minimal origin of replication and a 220-bp labeled vector fragment. The unwinding buffer contained 30 mM HEPES (pH 7.8), 7 mM MgCl₂, 4 mM ATP, 40 mM creatine phosphate, 15 mM potassium phosphate, 100 μ g of bovine serum albumin per ml, and 2 mM DTT. The indicated amount of T antigen was incubated in reaction buffer without or with 200 ng PP2A_c for 15 min, after which 200 ng of *E. coli* SSB (United States Biochemical), 100 ng of sheared calf thymus DNA, and 0.5 fmol of labeled template DNA were added to give a final volume of 10 μ l. After an additional 30 min of incubation at 37°C, the reactions were stopped, and the reaction mixes were analyzed as described before (58).

The nonspecific helicase substrate was prepared by annealing 20 pmol of 21M13 primer to 1 μ g of M13mp18 and extending from the primer with the Klenow fragment of DNA polymerase I in the presence of dATP, dGTP, and [α -³²P]dCTP (54). The labeled DNA was purified with Glassmilk (Bio 101) to give a 25-base labeled primer annealed to M13. The helicase buffer contained 30 mM HEPES (pH 7.8), 7 mM MgCl₂, 4 mM ATP, 100 μ g of bovine serum albumin per ml, and 2 mM DTT. The indicated quantities of T antigen were preincubated without or with 200 ng of PP2A_c

FIG. 1. SV40 large T antigen binds to the minimal origin of replication in two distinct steps. Increasing quantities of T antigen were incubated under replication conditions in the presence of AMP-PNP with 3 fmol of a 67-bp fragment from pDV.XH containing the minimal origin of SV40 DNA replication. After 30 min at 37°C, the protein-DNA complex was cross-linked in the presence of 0.5% glutaraldehyde for 5 min and then analyzed by acrylamide-agarose gel electrophoresis. T-antigen concentrations (in nanograms); lane 1, 0; lane 2, 20; lane 3, 40; lane 4, 50; lane 5, 60; lane 6, 80; lane 7, 100.

in helicase buffer for 15 min at 37°C. After the addition of 1 ng of helicase substrate, the reaction mixes were incubated for an additional 30 min at 37°C. Reactions were stopped by the addition of 5 μ l of 1% SDS–100 mM EDTA–40% glycerol, and the reaction mixes were electrophoresed on 0.75-mm-thick 12% acrylamide gels in 1 $\times$ TBE at 200 V for 30 min.

RESULTS

Two-stage assembly of T-antigen oligomers on the SV40 origin of replication. In initial studies, we characterized the interaction of T antigen with the viral origin in the absence of PP2A_c. For this purpose, increasing concentrations of T antigen were incubated with a 67-bp radioactive DNA fragment containing the minimal origin of DNA replication or with a control 70-bp nonspecific DNA fragment. At the end of the incubation period, DNA-protein complexes were stabilized by chemical cross-linking and analyzed by gel electrophoresis (Fig. 1). Unexpectedly, we observed two distinct protein-DNA complexes which were retarded in mobility relative to the free DNA. The faster-migrating of the two complexes was the predominant retarded species observed at low T-antigen concentrations. As the T-antigen concentration was increased, the amount of this species first increased and then decreased. Simultaneously, a second, more slowly migrating form appeared which became the predominant retarded species at high T-antigen concentrations. At a T-antigen monomer concentration of 22 nM (Fig. 1, lane 2) approximately 15% of the input DNA was retarded in mobility; of this, over 80% was in the faster-migrating form. When 50% of the input DNA was retarded in mobility (55 nM T antigen; Fig. 1, lane 4), 30% was in the faster-migrating form and 70% was in the more slowly migrating form. At the highest T-antigen concentration tested (110 nM), 83% of the input DNA fragment was in the more slowly migrating form.

Previous studies have established that T antigen readily forms hexamers in solution and assembles on the origin as a double hexamer which is centered approximately on the dyad axis (32, 42). It seemed likely that the two DNA-protein complexes that we observed represented origins with one

FIG. 2. (A) DNase I protection of *ori* by single and double hexamer complexes. A standard gel retardation assay was performed on a uniformly labeled 168-bp fragment containing the 64-bp minimal origin of replication flanked by vector sequence. Unshifted (un) and singly and doubly shifted (1 and 2, respectively) complexes were excised, purified, and incubated with (+) or without (-) DNase I as described in the text. Samples were then analyzed by electrophoresis on a nondenaturing 12% polyacrylamide gel. The singly shifted species contains a protected band of approximately 47 bp, while the doubly shifted species contains a protected band of approximately 80 bp and a somewhat smaller band of about 65 bp, large enough to cover the entire minimal origin of replication. Lane M, size markers. (B) T-antigen hexamer binding site on singly and doubly shifted origin fragment determined by exonuclease III digestion. (Left) A 300-bp fragment containing the 64-bp minimal origin and flanking vector sequence was 5'-end labeled on the late side,

and two bound hexamers, respectively. To verify this possibility, we carried out two kinds of nuclease protection experiments. In the first experiment, the lengths of the DNA segments protected by T antigen in the two complexes were determined. T antigen was incubated with a uniformly labeled 168-bp *ori* fragment generated by PCR amplification. The two complexes were isolated by electrophoresis and exhaustively digested with DNase I. After removal of the cross-linked protein by digestion with proteinase K, the sizes of the protected fragments were measured by electrophoresis on a 10% polyacrylamide gel (Fig. 2A). The faster-migrating complex yielded a protected fragment with an average size of about 47 bp, while the more slowly migrating complex yielded protected fragments of about 80 bp, with some less prominent fragments of about 65 bp. A protected segment of 80 bp at high T-antigen concentrations has been reported previously (3, 13) and is in good agreement with the electron microscopic measurements of the amount of DNA associated with the double hexamer (32). The finding that the protected segment in the faster-migrating form is about half this length is consistent with the hypothesis that this species represents a single hexamer bound to the origin.

In a second experiment, T antigen was incubated with an origin fragment which had been labeled at the 5' terminus on the late side of the origin (Fig. 2B). The two DNA-protein complexes and the uncomplexed DNA were excised from a polyacrylamide gel as before and digested with exonuclease III. Following deproteinization, the lengths of the labeled DNA strands were determined by electrophoresis on a sequencing gel (Fig. 2B). As expected, no strong exonuclease stops were observed in the case of the free DNA. The faster-migrating complex exhibited T-antigen-dependent exonuclease stops of roughly equal intensities near the dyad axis and near the 3' edge of the origin. By contrast, the more slowly migrating complex showed a single prominent exonuclease stop at the 3' edge of the origin. Similar results were obtained with a 279-bp fragment labeled at the 5' terminus on the opposite side of the origin (data not shown). Taken together with the DNase I protection data, these findings indicate that the faster-migrating complex consists of molecules with a single T-antigen hexamer bound on either the early or the late side of the dyad. While it is possible that the slower-mobility complex could be due to a change in conformation of the protein-DNA complex, e.g., DNA unwinding, rather than an increase in protein bound to the DNA, this is unlikely, since the amount of DNA protected from DNase I doubled in the slower complex. The more slowly migrating complex most likely consists of molecules with two hexamers bound in close proximity on

incubated with T antigen in a standard band shift assay, and then separated by acrylamide-agarose gel electrophoresis as described in the text. The unshifted and singly and doubly shifted cross-linked complexes were isolated and incubated with exonuclease III as described in the text. Lanes: M, size markers; D, mixture of individual digestions of the 300-bp fragment with the indicated restriction enzymes; 1, unshifted fragment; 2, singly shifted fragment; 3, doubly shifted fragment. (Right) Schematic representation of SV40 minimal origin, with restriction sites and T-antigen-dependent exonuclease III stops indicated. The singly shifted fragment has stops of roughly equal intensity immediately adjacent to the early palindrome (open arrow) and in the pentamers (solid arrow), while the majority of the stops in the doubly shifted fragment occur adjacent to the early palindrome. Similar results were obtained with a 279-bp fragment 5'-end labeled on the early side (not shown).

FIG. 3. Requirements for formation of double hexamer. A gel retardation assay was performed as described in the text with the omission of the indicated components: - nucleotide, AMP-PNP omitted; + ATP, 4 mM ATP substituted for AMP-PNP; + PP2A_c, 100 ng of PP2A_c added; - ori, 30 fmol of a 90-bp nonspecific duplex DNA fragment was substituted for the SV40 *ori* fragment.

either side of the dyad. These results are consistent with those of Parsons et al. (42), who showed that T-antigen hexamers can assemble on isolated half-origins. Parsons et al. also reported that T antigen shows some preference for binding to the early versus the late half-origin. Although it is difficult to obtain precise quantitation from nuclease protection experiments, the similar intensities of the two exonuclease stops in digests of the complex containing a single hexamer suggest that any difference in occupancy of the two half-origins was relatively small in our experiments. Our protection data are also consistent with the electron microscopic observation of both single and double hexamers bound to origin DNA (32).

Requirements for assembly of double hexamers on the SV40 origin of replication. The requirements for double hexamer assembly were investigated by omitting various components of the binding reaction mixture (Fig. 3). No retarded complexes were observed in the absence of T antigen or when a DNA fragment lacking the SV40 origin was substituted for the *ori* fragment. Assembly of double hexamers was absolutely dependent upon the presence of Mg²⁺ and ATP or an ATP analog, as expected (3, 13). Both ATP and the nonhydrolyzable analog AMP-PNP supported the formation of double hexamers, consistent with previous observations that such analogs support DNase I protection (3, 4). However, at subsaturating T-antigen concentrations, significantly more protein-DNA complexes were observed in the presence of the nonhydrolyzable analog AMP-PNP. The use of the nonhydrolyzable analog in these experiments has the considerable advantage of separating the binding step from the

FIG. 4. Stimulation of doubly shifted complex by PP2A_c. Gel retardation assays were performed in the absence and presence of 100 ng of PP2A_c. T-antigen per 10-μl reaction mixture (in nanograms): lane 1, 0; lane 2, 20; lane 3, 40; lane 4, 50; lane 5, 60; lane 6, 80; lane 7, 100.

subsequent unwinding step, which requires ATP hydrolysis (8, 54, 64). When the nucleotide or Mg²⁺ was omitted, only a small fraction of the *ori* fragments formed DNA-protein complexes, and the mobility of the retarded species was greater than that of double hexamers. In the absence of the cross-linking agent glutaraldehyde, the DNA-protein complexes displayed a reduced mobility, and single and double hexamers were not clearly resolved. Moreover, the complexes appeared to be unstable, as evidenced by the smear of radioactivity extending to the position of free DNA. The increase in mobility of complexes treated with a cross-linker may be due to the generation of a more compact structure or to an increase in net negative charge from reaction of the agent with free amino groups. The increase in mobility, as well as the ability to resolve the single and double hexamer species, was not dependent upon the particular cross-linking agent used, as identical results were obtained with Lomant's reagent, dithiobis(succinimidylpropionate) (data not shown).

PP2A_c increases the cooperativity of double hexamer assembly. PP2A_c dephosphorylates T antigen at specific serine residues (primarily Ser-120, Ser-123, and Ser-677), thereby enhancing the ability of the protein to initiate SV40 DNA replication (51, 57, 58). In preliminary experiments, we noticed that incubation of T antigen with PP2A_c stimulated the formation of double hexamers on the origin (Fig. 3, lane 7). To analyze this effect further, T-antigen titrations were performed in the presence and absence of PP2A_c (Fig. 4). In both titrations, most of the origin fragment was driven into double hexamer complexes at high T-antigen concentrations. In addition, at corresponding points in the two titrations, the fraction of the input *ori* fragment complexed with T antigen differed by less than a factor of 2. However, at subsaturating T-antigen concentrations, the amount of the double hexamer complex was dramatically increased relative to that of the single hexamer complex by incubation with PP2A_c. In the presence of the phosphatase, the double hexamer species was evident even at the lowest concentration tested (+PP2A_c; Fig. 4, lane 2). Moreover, at intermediate T-antigen concentrations, at which less than half of the origin fragment was complexed with T antigen (Fig. 4, lanes 3 and 4), the double hexamer complex was the predominant form in the presence of PP2A_c, while the single hexamer complex was the predominant form in the absence of PP2A_c.

FIG. 5. (A) Effect of PP2A_c on stability of the double hexamer complex. The rate of dissociation of double hexamer complexes preformed in the presence (○) and absence (●) of PP2A_c was measured after the addition of a 1,000-fold excess of unlabeled *ori*-containing DNA. (B) Effect of PP2A_c on formation of the doubly shifted complex in the presence of competitor DNA. Formation of the doubly shifted form was measured in the presence of increasing amounts of competitor (sheared calf thymus DNA). T antigen (80 ng) was incubated with 3 fmol of labeled *ori* fragment derived from the wild-type (○, ●) or mutant pDV.1stC (□, ■) plasmid, with (○, □) or without (●, ■) 100 ng of PP2A_c, in the presence of the indicated amount of sheared calf thymus DNA.

Thus, the major effect of PP2A_c was to increase the apparent cooperativity of double hexamer assembly.

The data in Fig. 4 were quantitated by densitometry and analyzed in terms of a simple two-step process involving the sequential assembly of two T-antigen hexamers on the origin. This analysis indicated that in the presence of PP2A_c, the efficiency of formation of a second hexamer on an origin already occupied by the first hexamer increased at least 10-fold relative to the formation of the first hexamer on an unoccupied origin. The simplest interpretation of these observations is that the dephosphorylation of T antigen by PP2A_c primarily affects interactions between hexamer units bound to the origin. The increase in apparent cooperativity is explained by a net increase in favorable hexamer-hexamer interactions.

If dephosphorylation of T antigen by PP2A_c promotes favorable hexamer-hexamer interactions at the origin (or reduces unfavorable interactions), it would be expected that treatment with the enzyme would stabilize the double hexamer against dissociation. In the experiment shown in Fig. 5A, T antigen-*ori* complexes preformed in the presence and absence of PP2A_c were challenged with a 1,000-fold molar excess of T-antigen binding sites, and the fraction of double hexamers remaining after various incubation times was determined. Double hexamer complexes formed in the absence of PP2A_c dissociated relatively quickly, with a half-time of about 2 min. The half-time of disappearance of double hexamers formed in the presence of PP2A_c was approximately 18 min. The obvious biphasic kinetics of disappearance of the complexes formed from T antigen that had not been incubated with phosphatase is probably due to the fact that preparations of T antigen are quite heterogeneous in extent of phosphorylation (50). The slow rate of dissociation of a small fraction of the double hexamers can

be explained simply by the presence of some underphosphorylated T antigen in the preparation.

The stabilization of double hexamers by treatment with PP2A_c was also observed in equilibrium competition experiments with nonspecific DNA. In these experiments, radioactive *ori* fragments (3 fmol) were incubated with T antigen in the presence of increasing concentrations of sheared calf thymus DNA, and the amount of labeled double hexamer complex was determined. Incubation with PP2A_c significantly increased the resistance of double hexamers to competition with nonspecific DNA (Fig. 5B). Double hexamer-*ori* complexes formed on mutant *ori* fragments (pDV.1stC; see below) were not stable, irrespective of the presence of PP2A_c.

PP2A_c stimulates origin unwinding and DNA replication but does not stimulate the helicase activity of T antigen. We have previously reported that treatment of T antigen with PP2A_c increased the ability of the protein to unwind duplex DNA molecules containing the viral origin of replication (57, 58). This finding was verified in the experiment shown in Fig. 6A. Increasing amounts of T antigen were incubated in the presence or absence of PP2A_c and then added to unwinding-reaction mixtures containing *E. coli* SSB, ATP, and a double-stranded *ori* fragment. PP2A_c greatly stimulated the unwinding of the *ori* fragment. The unwinding reaction was absolutely dependent upon T antigen and was specific for *ori*-containing DNA. In preliminary experiments, the presence or absence of site I adjacent to the minimal origin was found to have no effect on the ability of PP2A_c to stimulate origin unwinding. Okadaic acid, which inhibits the phosphatase activity of PP2A_c, completely blocked stimulation of the origin-unwinding activity of T antigen (data not shown). In parallel reactions, the same T-antigen preparations were tested for helicase activity. The substrate for these assays

FIG. 6. Origin unwinding but not nonspecific DNA helicase activity of T antigen is stimulated by PP2A_c. The indicated amount of T antigen was preincubated for 15 min at 37°C in helicase buffer in the absence or presence of 200 ng of PP2A_c. (Top) Origin-containing substrate (1 ng) was added, with *E. coli* SSB and 100 ng of sheared calf thymus DNA, and incubated under unwinding conditions (see text) for an additional 30 min. (Bottom) Helicase substrate (1 ng) was added and incubated under helicase conditions (see text) for an additional 30 min. ori, DNA fragment containing the minimal origin of replication; ns, nonspecific DNA fragment.

consisted of a radioactive oligonucleotide annealed to M13mp18 single-stranded DNA. PP2A_c did not stimulate, and may have slightly inhibited, the helicase activity of T antigen. This result extends previous studies indicating that nonspecific alkaline phosphatase does not affect the ATPase or helicase activity of T antigen (22, 35).

Conceptually, the unwinding of duplex DNA containing the SV40 origin can be divided into two sequential steps: the initial opening of the duplex in the immediate vicinity of the origin, which presumably involves some rearrangement of the bound T-antigen hexamers, and the extension of the initial opening by the T-antigen helicase, resulting in complete unwinding of the duplex. Our data suggest that dephosphorylation of T antigen by PP2A_c is important in the initial opening of the duplex. One reasonable hypothesis, consistent with all of our data, is that dephosphorylation permits interactions between bound hexamers that are critical for duplex opening.

Figure 7 shows the effects of PP2A_c on the complete DNA replication reaction in a partially reconstituted system. In the presence of PP2A_c, DNA synthesis increased sharply at 200 to 300 ng of T antigen and then leveled off. In the absence of PP2A_c, DNA synthesis increased only slightly at the same T-antigen concentration and reached only about one-fifth of the level observed in the presence of PP2A_c, even at very high T-antigen concentrations. The fact that addition of untreated T antigen to a high concentration did

not increase the extent of replication confirms the finding that the relative inactivity of the phosphorylated form of the protein is not simply a result of reduced affinity for the origin sequence. This conclusion is in fundamental agreement with our binding studies, which showed a less than twofold difference in occupancy of the origin in the presence and absence of PP2A_c at all T-antigen concentrations tested. Thus, in the absence of PP2A_c, double hexamer complexes can clearly assemble at the origin, but because of their state of phosphorylation, such complexes are not competent to initiate unwinding.

Interaction of T antigen with mutant origins of replication. To further characterize the mechanism of activation of T antigen by dephosphorylation, we examined the effect of PP2A_c on the interaction of T antigen with several mutant origins of replication. Mutant *ori* fragments were incubated with a subsaturating quantity of T antigen in the presence or absence of PP2A_c and then analyzed by gel retardation assays as before. In each case, the relative amounts of free DNA, single hexamers, and double hexamers were quantified by densitometry. The increase in cooperativity resulting from incubation with PP2A_c was calculated by a simple two-step binding model (Fig. 8 and Materials and Methods). Using the experimentally determined values for the concentrations of the two complexes and the free DNA, we calculated the ratio of the apparent dissociation constants for a T-antigen hexamer bound to an origin half-site when the

FIG. 7. Effect of PP2A_c on SV40 DNA replication. Increasing amounts of T antigen were added to a reconstituted in vitro replication reaction mix in the presence (○) or absence (●) of 200 ng of PP2A_c.

other half-site was either unoccupied or occupied by a second hexamer. The cooperativity index presented in Fig. 8 represents the factor by which this ratio increased upon dephosphorylation of T antigen by PP2A_c. In addition to binding studies, we also assayed each mutant origin for its ability to support DNA replication and origin-dependent unwinding in vitro. Both of these activities were measured under standard conditions (65) in the presence of PP2A_c.

The mutant origins that we studied fell into several classes. Three of them (mutants 11, 105, and 5222) behaved similarly to the wild-type SV40 origin in their response to incubation with PP2A_c, exhibiting significant increases in the cooperative assembly of double hexamers. All of these mutants supported DNA replication and origin-dependent unwinding with efficiencies that were comparable to those of the wild type. Mutants 11 and 105 contained insertions that altered the spacing between the central core of pentamer repeats and the early palindrome or A/T-rich region, respectively. Thus, the precise spatial relationship between the repeats and these adjacent elements is apparently not critical for DNA replication. Mutant 5222 contains a single base substitution in the early palindrome. The relatively minor effect of this mutation confirms previous observations (7, 10). The spacing mutant 105 has been reported previously to support low levels of replication in vivo and in vitro in the complete origin (31) but to be inactive in vivo in the core origin of replication (10).

Two mutants (1174 and 5241) readily formed single hexamer complexes but were defective in the assembly of double hexamers even in the presence of PP2A_c. One of these mutants (1174) had a single base deletion in a central pentamer (31), and the other (5241) had a base substitution in the same pentamer (12). These mutations would be expected to reduce the efficiency of hexamer formation on the half-origin to the early side of the dyad. Although we have not directly examined this point by nuclease protection, we assume that the single hexamers that were observed with

these mutant origins were assembled on the half-origin on the late side of the dyad. Since both mutants showed severe defects in DNA replication and origin unwinding, it is apparent that a single hexamer bound to the origin is not sufficient to mediate these activities. This finding is in agreement with the results of Parsons et al., which demonstrated that isolated half-origins did not support DNA replication in vitro (42). It should be noted that at higher T-antigen concentrations, we observed double hexamer assembly with these mutants (data not shown), but PP2A_c still had no effect on the cooperativity of binding. This finding strongly suggests that one effect of mutations in the central pentamers is to perturb the normal interactions between T-antigen hexamers bound to the origin.

Assembly of double hexamers on a mutant origin with a single base substitution in an outer pentamer (mutant 8) was stimulated by PP2A_c but to a much lesser extent than in the case of the wild-type origin. Additionally, the double hexamers formed showed higher mobilities than other double hexamers, suggesting that mutation of an outer pentamer allowed fewer T-antigen molecules to bind to the origin. This mutant also displayed low but detectable replication and unwinding activities. Mutant 26, which contained an A to G transition in the A/T-rich region, also exhibited intermediate levels of replication, unwinding, and T-antigen binding (Fig. 8) (7).

To directly assess the role of hexamer-hexamer interactions in T-antigen function, we constructed a mutant origin containing a single base pair insertion at the dyad axis (mutant 1). The effect of this mutation is to rotate the two halves of the origin approximately 36° with respect to each other without altering the sequence of the recognition pentamers. As expected, since both half-origins were intact, T antigen assembled into both single and double hexamers on the mutant origin. However, assembly of double hexamers was noncooperative and completely unaffected by PP2A_c, consistent with the hypothesis that the insertion disrupted

FIG. 8. Effect of *ori* mutations on SV40 DNA replication, unwinding, and cooperative two-step binding. (A) Sequence of mutations in the SV40 minimal origin of replication. The solid arrowhead indicates the numbering system from the center of the origin; open wedges show restriction sites used to generate origin fragments for gel retardation assays. (A, *Apa*LI; S, *Sna*BI). Mutated and deleted bases are marked with asterisks and open triangles, respectively, and the pentameric T-antigen binding sites are underlined. DNA replication and unwinding reactions were performed as described in the text. The cooperativity index is the K_2/K_1 ratio for the binding reactions performed in the absence and presence of PP2A_c. (B) Gel retardation assays were performed with 30 ng of T antigen and 3 fmol of origin-containing fragment from the indicated wild-type (WT) or mutant plasmids, in the absence and presence of 100 ng of PP2A_c.

hexamer-hexamer interactions. In contrast to the finding with wild-type origins, incubation with PP2A_c did not increase the resistance of the double hexamers formed on mutant 1 to competition with nonspecific DNA (Fig. 5B). Significantly, the mutant origin containing the single base pair insertion was profoundly defective in the DNA replication and origin-unwinding assays (6, 12). These findings strongly support the conclusion that specific hexamer-hex-

amer interactions are critically important for T-antigen function in the initiation of DNA replication, presumably during the initial opening of the duplex.

DISCUSSION

Ability of T antigen to open the viral origin is regulated by phosphorylation. The SV40 T antigen assembles on the viral

FIG. 9. Activation of DNA replication by PP2A_c. T-antigen hexamers bind in two discrete steps to the SV40 origin of replication. Double hexamers containing inhibitory phosphoserine residues have a rapid off-rate and cannot unwind the origin region. Removal of specific phosphoryl groups from T antigen permits essential functional interactions between bound hexamers. These protein-protein interactions may drive a conformational change in the T antigen-DNA complex, resulting in initial opening of the duplex DNA.

origin to form a double hexameric nucleoprotein structure with a molecular weight of about 1,000,000 (32). This large structure functions to unwind the origin and coordinate the subsequent initiation of DNA synthesis. A number of studies, including ours, have demonstrated that the activity of T antigen in initiation of DNA replication is modulated by protein phosphorylation. During fractionation of the SV40 cell-free replication system, we purified a protein factor, subsequently identified as PP2A_c, which significantly stimulated SV40 DNA synthesis in reconstitution assays by dephosphorylating T antigen at specific phosphoserine residues (51, 57, 58). Two-dimensional phosphopeptide analysis revealed that the phosphates at Ser-120 and Ser-123, which lie on the amino-terminal side of the DNA-binding domain of T antigen, were removed most rapidly by PP2A_c. However, phosphates at other sites, particularly Ser-677 and perhaps Ser-679, were also removed at a slower rate. We have previously shown that dephosphorylation of T antigen by PP2A_c stimulates the early stages of SV40 DNA replication and reduces the time lag normally observed prior to the onset of DNA synthesis (58).

Using gel mobility shift assays, we have demonstrated that binding of T antigen to the SV40 origin occurs in two relatively discrete steps: the assembly of a T-antigen hexamer on one half-site of the origin, followed by the assembly of the second hexamer on the other half-site. The major effect of PP2A_c was to increase the apparent cooperativity of the binding reaction. Incubation of T antigen with PP2A_c did not markedly alter the concentration of T antigen required for binding of the first hexamer. However, the apparent dissociation constant for the second hexamer decreased more than 10-fold. The simplest explanation of our data is that the two T-antigen hexamers bound to the origin interact with each other and that dephosphorylation of T antigen causes a change in these interactions. We suggest that removal of phosphates from Ser-120 and Ser-123 permits favorable contacts between the hexamers which are otherwise prevented and that the resulting decrease in the free energy of the double hexamer complex is reflected in increased cooperativity of binding. As illustrated in Fig. 9, we

further suggest that the favorable protein-protein interactions made possible by dephosphorylation drive a change in the conformation of the double hexamer complex which results in the initial opening of the duplex. The open duplex then serves as a substrate for the intrinsic helicase activity of T antigen, which catalyzes more extensive unwinding of the origin region. Consistent with this suggestion, dephosphorylation by PP2A_c significantly stimulated unwinding of the duplex DNA molecules containing the viral origin but did not affect the helicase activity of the protein.

Analysis of the interaction of T antigen with various mutant origins has provided additional support for this model. In one particularly informative case, we studied the properties of a mutant with a single nucleotide insertion at the dyad axis of the core origin. This mutation would be expected to alter the structural relationship between the two bound hexamers but not to alter the contacts of T antigen with its pentameric recognition sequences. Double hexamers of T antigen were assembled on this mutant origin, but the binding reaction was noncooperative and unaffected by PP2A_c. Moreover, the mutant was completely defective in origin unwinding and DNA replication *in vitro*. These findings indicate that simple occupancy of the SV40 origin by T antigen, while necessary for initiation of DNA synthesis, is not sufficient. The ability of T antigen to unwind the viral origin of DNA replication is critically dependent on the appropriate functional interactions between T-antigen hexamers, and this interaction is regulated by the phosphorylation state of the viral initiator protein.

Positive and negative regulation of T-antigen activity by phosphorylation. The regulation of the replication activities of T antigen by protein phosphorylation appears to be quite complex (see reference 44 for a review). T antigen contains two clusters of phosphoserine and phosphothreonine residues, one near the amino terminus (Ser-106, Ser-112, Ser-120, Ser-123, and Thr-124) and one near the carboxyl terminus (Ser-639, Ser-677, Ser-679, and Thr-701) (48, 49). As indicated above, phosphorylation of Ser-120 and Ser-123 in the N-terminal cluster inhibits DNA replication by reducing the efficiency with which T antigen opens the duplex at the viral origin. Removal of these phosphates probably explains, at least in part, the previously observed stimulatory effect of nonspecific phosphatases. For example, treatment of T antigen with calf intestinal alkaline phosphatase, which dephosphorylates all of the phosphoserine residues in T antigen but not the phosphothreonine residues, increased the stability of T antigen bound to the SV40 origin and also stimulated DNA replication *in vitro* (22, 35). It has also been demonstrated that okadaic acid, a relatively specific inhibitor of protein phosphatases 1 and 2A, inhibits SV40 DNA replication *in vitro* by up to 65% (28). The inhibitory effect was not observed if the T antigen was first dephosphorylated by treatment with alkaline phosphatase.

At present, there is no evidence that phosphorylation of serine residues in the carboxyl-terminal cluster of T antigen has any direct effect, either positive or negative, on the ability of the protein to support initiation of SV40 DNA replication. Indeed, the last 38 amino acids of T antigen can be deleted without significantly affecting viral DNA replication (43). However, there is evidence that the amino- and carboxyl-terminal clusters may be in close proximity in the native T-antigen molecule, so that phosphorylation of residues in the carboxyl-terminal cluster can affect the phosphorylation state of residues in the amino-terminal cluster. Thus, phosphorylation of Ser-120 and Ser-123 is drastically reduced *in vivo* by substitution of the serine residue at 677

with an alanine residue (48). On the basis of this observation, it has been suggested that phosphorylation of Ser-677 may indirectly stimulate phosphorylation at Ser-120 and Ser-123 by inducing structural changes in the amino-terminal domain. Since Ser-677 is a preferred site of dephosphorylation by PP2A_c (51), it follows that the phosphatase may reduce the level of phosphorylation at Ser-120 and Ser-123 by both direct and indirect effects.

Analysis of T antigen produced in *E. coli* has demonstrated that completely unphosphorylated T antigen is inactive in SV40 DNA replication in vitro (34). However, full activity can be restored by phosphorylation at Thr-124 by the cell cycle-regulated p34^{cdc2} protein kinase (33). This finding is consistent with genetic studies indicating that T-antigen molecules with a Thr-to-Ala substitution at residue 124 are defective in DNA replication in vivo and in vitro (52; unpublished data). It has been suggested that phosphorylation at Thr-124 is required for stable binding of T antigen to the origin (1, 27, 33, 50, 52). Thus, depending on the residue affected, phosphorylation of T antigen can have either positive (Thr-124) or negative (Ser-120 and Ser-123) effects on DNA replication. It is possible that additional effects due to phosphorylation at other residues will be uncovered by more detailed analysis in the future. In any case, the final level of activity of the protein in initiation will be determined by the balance of positive and negative effects, and these, in turn, may be influenced by a variety of environmental factors.

There is evidence that the serine phosphates in T antigen turn over during the viral multiplication cycle (47). Thus, the level of phosphorylation of a given residue depends upon the relative activities of cellular protein kinases and protein phosphatases. Little is currently known about the nature of the kinases responsible for phosphorylation of serines in either the amino- or carboxyl-terminal cluster. Serines 123 and 679 can be phosphorylated in vitro by casein kinase I (23). A recently described protein kinase which is activated by double-stranded DNA (DNA-PK) efficiently phosphorylates Ser-677 in vitro (29). Since phosphorylation of this residue may be a prerequisite for phosphorylation at the inhibitory residues Ser-120 and Ser-123 (see above), the DNA-PK could indirectly affect initiation of DNA replication. Kinases that phosphorylate other serine residues have not been identified. The phosphate at Thr-124 of T antigen does not appear to turn over significantly during SV40 infection, at least in the usual cell culture systems (47).

Role of T-antigen phosphorylation in the viral multiplication cycle. T antigen performs multiple roles in the SV40 multiplication cycle. In addition to its crucial function in the initiation of viral DNA replication, the protein is required for the repression of early transcription and the activation of late transcription. T antigen also appears to modulate the growth state of the host so that the intracellular environment is conducive to replication of the viral genome. This function is probably accomplished, at least in part, by interactions with the cellular proteins Rb and p53 (see reference 30 for a recent review). One possible role for phosphorylation of T antigen is to regulate the different activities of the protein so that each is expressed at the proper level and at the proper time during the viral multiplication cycle. Indeed, there is considerable evidence that phosphorylation of certain residues can have differential effects on the various activities of T antigen. Phosphorylation of Thr-124 is required for the replication function of T antigen but is not required for binding to the site (I) where T antigen functions as a transcriptional repressor (33, 34, 52). On the other hand, mutants with substitutions at residue 677 that cannot be phosphorylated are

defective in binding to site I but support DNA replication with normal efficiency (52).

Genetic and biochemical studies are consistent with the hypothesis that T-antigen phosphorylation plays a critical role in the SV40 multiplication cycle. Viruses with substitution mutations at Ser-120, Ser-123, and Thr-124 are nonviable (52). As expected, mutants with substitutions at residue 124 fail to synthesize viral DNA. A mutant with a substitution of alanine for serine at residue 123 synthesizes SV40 DNA but at a low level and with altered kinetics, suggesting some derangement in the normal progression of the infectious cycle (52). Biochemical studies have shown that SV40 small t antigen forms specific complexes with protein phosphatase 2A (40, 66). Although the role of complex formation in virus replication is not yet clear, it has been demonstrated that, under certain circumstances, small t antigen inhibits the activity of the phosphatase (66). Such inhibition could, in principle, affect the phosphorylation state, and hence the activity, of large T antigen.

SV40 as a model for control of cellular DNA replication. The control of SV40 DNA replication by phosphorylation of the viral initiator protein T antigen may have some features in common with the control of cellular DNA replication in the cell cycle. There is a large body of evidence suggesting that cellular DNA replication and mitosis are regulated by cascades of protein phosphorylation. Both genetic and biochemical data indicate that cellular DNA replication is dependent upon the activity of a protein kinase(s) containing the key cell cycle regulator p34^{cdc2} (2, 19, 21, 38). As noted above, the replication activity of T antigen is absolutely dependent upon phosphorylation by such a kinase (33). Physiological studies with synchronized cell populations indicate that SV40 DNA replication cannot occur until the host cell has passed through the boundary between the G₁ and S phases of the cell cycle, suggesting that viral DNA replication may be subject to the normal mechanisms which control cellular entry into the S phase (39). Moreover, extracts prepared from primate cells in the G₁ phase are much less efficient in supporting SV40 DNA synthesis in vitro than are extracts from S-phase cells (46). Significantly, the replication of SV40 DNA in G₁ extracts is markedly stimulated by incubation with PP2A_c or a partially purified fraction containing p34^{cdc2}, consistent with a critical role for protein phosphorylation in activating DNA synthesis at the S phase (46, 57). We suspect that cellular initiator proteins may be activated in much the same way as SV40 T antigen. It has been observed that RP-A, a cellular protein required for initiation of SV40 DNA replication, is phosphorylated in a cell cycle-dependent fashion; however, no significant differences in the biochemical activities of the phosphorylated and dephosphorylated forms of the protein have yet been demonstrated (18). It seems likely that the critical initiator protein, the cellular counterpart(s) of T antigen, remains to be discovered.

It has been noted previously that the regulation of SV40 DNA replication differs from that of cellular DNA replication in one significant feature—the viral genome is reduplicated numerous times during a relatively short period of time, whereas cellular DNA replication occurs once and only once during each cell cycle. Thus, although we have observed negative regulation of the replication activity of T antigen by phosphorylation of specific serine residues, this regulatory mechanism clearly does not operate to prevent reinitiation of DNA viral replication on individual templates. However, the detailed analysis of the regulation of SV40 DNA replication by protein phosphorylation has suggested

possible ways in which positive and negative control mechanisms, similar to those that operate upon T antigen, might be combined to achieve replication of a genome once and only once per cell cycle. We have previously described a general model for the control of cellular DNA replication which is based upon this analysis (59).

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REFERENCES

- Baumann, E. A. 1985. DNA-binding properties of phosphorylated and dephosphorylated D2-T antigen, a simian virus 40 T antigen related protein. *Eur. J. Biochem.* **147**:495-501.
- Blow, J. J., and P. Nurse. 1990. A cdc2-like protein is involved in the initiation of DNA replication in *Xenopus* egg extracts. *Cell* **62**:855-862.
- Borowiec, J. A., and J. Hurwitz. 1988. ATP stimulates the binding of simian virus 40 (SV40) large tumor antigen to the SV40 origin of replication. *Proc. Natl. Acad. Sci. USA* **85**:64-68.
- Borowiec, J. A., and J. Hurwitz. 1988. Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. *EMBO J.* **7**:3149-3158.
- Challberg, M. D., and T. J. Kelly. 1989. Animal virus DNA replication. *Annu. Rev. Biochem.* **58**:671-717.
- Cohen, G. L., P. L. Wright, A. L. DeLucia, B. A. Lewton, M. E. Anderson, and P. Tegtmeyer. 1984. Critical spatial requirement within the origin of simian virus 40 DNA replication. *J. Virol.* **51**:91-96.
- Dean, F. B., J. A. Borowiec, Y. Ishimi, S. Deb, P. Tegtmeyer, and J. Hurwitz. 1987. Simian virus 40 large tumor antigen requires three core replication origin domains for DNA unwinding and replication in vitro. *Proc. Natl. Acad. Sci. USA* **84**:8267-8271.
- Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz. 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. *Proc. Natl. Acad. Sci. USA* **84**:16-20.
- Dean, F. B., M. Dodson, H. Echols, and J. Hurwitz. 1987. ATP-dependent formation of a specialized nucleoprotein structure by simian virus 40 (SV40) large tumor antigen at the SV40 replication origin. *Proc. Natl. Acad. Sci. USA* **84**:8981-8985.
- Deb, S., A. L. DeLucia, C. P. Baur, A. Koff, and P. Tegtmeyer. 1986. Domain structure of the simian virus 40 core origin of replication. *Mol. Cell. Biol.* **6**:1663-70.
- Deb, S., A. L. DeLucia, A. Koff, S. Tsui, and P. Tegtmeyer. 1986. The adenine-thymine domain of the simian virus 40 core origin directs DNA bending and coordinately regulates DNA replication. *Mol. Cell. Biol.* **6**:4578-4584.
- Deb, S., S. Tsui, A. Koff, A. L. DeLucia, R. Parsons, and P. Tegtmeyer. 1987. The T antigen binding domain of the simian virus 40 core origin of replication. *J. Virol.* **61**:2143-2149.
- Deb, S. P., and P. Tegtmeyer. 1987. ATP enhances the binding of simian virus 40 large T antigen to the origin of replication. *J. Virol.* **61**:3649-3654.
- DeGuzman, A., and E. Y. C. Lee. 1988. Preparation of low-molecular-weight forms of rabbit muscle protein phosphatase. *Methods Enzymol.* **159**:356-368.
- DeLucia, A., B. Lewton, R. Tjian, and P. Tegtmeyer. 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of pentanucleotide interaction sites at the origin of replication. *J. Virol.* **46**:143-150.
- DeLucia, A. L., S. Deb, K. Partin, and P. Tegtmeyer. 1986. Functional interactions of the simian virus 40 core origin of replication with flanking regulatory sequences. *J. Virol.* **57**:138-144.
- DePamphilis, M. L. 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. *Cell* **52**:635-638.
- Din, S., S. J. Brill, M. P. Fairman, and B. Stillman. 1990. Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. *Genes Dev.* **4**:968-977.
- D'Urso, G., R. L. Marraccino, D. R. Marshak, and J. M. Roberts. 1990. Cell cycle control of DNA replication by a homologue from human cells of the p34cdc2 protein kinase. *Science* **250**:786-791.
- Fairman, M. P., and B. Stillman. 1988. Cellular factors required for multiple stages of SV40 DNA replication in vitro. *EMBO J.* **7**:1211-1218.
- Fang, F., and J. W. Newport. 1991. Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* **66**:731-742.
- Grässer, F. A., K. 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.
- Grässer, F. A., K. H. Scheidtmann, P. T. Tuazon, J. A. Traugh, and G. Walter. 1988. In vitro phosphorylation of SV40 large T antigen. *Virology* **165**:13-22.
- Guo, Z.-S., C. Gutierrez, U. Heini, J. M. Sogo, and M. L. Depamphilis. 1989. Origin auxiliary sequences can facilitate initiation of simian virus 40 DNA replication in vitro as they do in vivo. *Mol. Cell. Biol.* **9**:3593-3602.
- Higuchi, R. 1989. Using PCR to engineer DNA, p. 61-70. *In* H. A. Erlich (ed.), *PCR technology*. Stockton Press, New York.
- Ishimi, Y., A. Claude, P. Bullock, and J. Hurwitz. 1988. Complete enzymatic synthesis of DNA containing the SV40 origin of replication. *J. Biol. Chem.* **263**:19723-19733.
- Klausing, K., K. H. Scheidtmann, E. A. Baumann, and R. Knippers. 1988. Effects of in vitro dephosphorylation on DNA-binding and DNA helicase activities of simian virus 40 large tumor antigen. *J. Virol.* **62**:1258-1265.
- Lawson, R., P. Cohen, and D. P. Lane. 1990. SV40 large T antigen-dependent DNA replication is activated by protein phosphatase 2A in vitro. *J. Virol.* **64**:2380-2383.
- Lees-Miller, S. P., Y. R. Chen, and C. W. Anderson. 1990. Human cells contain a DNA-activated protein kinase that phosphorylates simian virus 40 T antigen, mouse p53, and the human Ku autoantigen. *Mol. Cell. Biol.* **10**:6472-6481.
- Levine, A. J. 1990. Tumor suppressor genes. *Bioessays* **12**:60-66.
- Li, J. J., K. W. C. Peden, R. A. F. Dixon, and T. J. Kelly. 1986. Functional organization of the simian virus 40 origin of DNA replication. *Mol. Cell. Biol.* **6**:1117-1128.
- Mastrangelo, I. A., P. V. Hough, J. S. Wall, M. Dodson, F. B. Dean, and J. Hurwitz. 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. *Nature (London)* **338**:658-662.
- 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.
- Mohr, I. J., Y. Gluzman, M. P. Fairman, M. Strauss, D. McVey, B. Stillman, and R. D. Gerard. 1989. Production of simian virus 40 large tumor antigen in bacteria: altered DNA binding specificity and DNA replication activity of underphosphorylated large tumor antigen. *Proc. Natl. Acad. Sci. USA* **86**:6479-6483.
- Mohr, I. J., B. Stillman, and Y. Gluzman. 1987. Regulation of SV40 DNA replication by phosphorylation of T antigen. *EMBO J.* **6**:153-160.
- Mumby, M. (University of Texas Southwestern, Dallas, Tex.). Personal communication.
- Murakami, Y., T. Eki, and J. Hurwitz. 1992. Studies on the initiation of simian virus 40 replication in vitro: RNA primer synthesis and its elongation. *Proc. Natl. Acad. Sci. USA* **89**:952-956.
- Murakami, Y., C. R. Wobbe, L. Weissbach, F. B. Dean, and J.

- Hurwitz. 1986. Role of DNA polymerase α and DNA primase in simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. USA* **83**:2869–2873.
38. Nurse, P. 1991. The Florey Lecture, 1990: How is the cell division cycle regulated? *Phil. Trans. R. Soc. London (Biol.)* **332**:271–276.
39. Pages, J., S. Manteuil, D. Stehelin, M. Fiszman, M. Marx, and M. Girard. 1973. Relationship between replication of simian virus 40 DNA and specific events of the host cell cycle. *J. Virol.* **12**:99–107.
40. Pallas, D. C., L. K. Shahrik, B. L. Martin, S. Jaspers, T. B. Miller, D. L. Brautigan, and T. M. Roberts. 1990. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. *Cell* **60**:167–176.
41. Parsons, R., M. E. Anderson, and P. Tegtmeier. 1990. Three domains in the simian virus 40 core origin orchestrate the binding, melting, and DNA helicase activities of T antigen. *J. Virol.* **64**:509–518.
42. Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeier. 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the replication origin. *J. Virol.* **65**:2798–2806.
43. Pipas, J. M., K. W. C. Peden, and D. Nathans. 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T antigen gene. *Mol. Cell. Biol.* **3**:203–213.
44. Prives, C. 1990. The replication functions of SV40 T antigen are regulated by phosphorylation. *Cell* **61**:735–738.
45. Roberts, J. M. 1989. Simian virus 40 (SV40) large tumor antigen causes stepwise changes in SV40 origin structure during initiation of DNA replication. *Proc. Natl. Acad. Sci. USA* **86**:3939–3943.
46. Roberts, J. M., and G. D'Urso. 1988. An origin unwinding activity regulates initiation of DNA replication during the mammalian cell cycle. *Science* **241**:1486–1489.
47. Scheidtmann, K. H. 1986. Phosphorylation of simian virus 40 large T antigen: cytoplasmic and nuclear phosphorylation sites differ in their metabolic stability. *Virology* **150**:85–95.
48. Scheidtmann, K. H., M. Buck, J. Schneider, D. Kalderon, E. Fanning, and A. E. Smith. 1991. Biochemical characterization of phosphorylation site mutants of simian virus 40 large T antigen: evidence for interaction between amino- and carboxy-terminal domains. *J. Virol.* **65**:1479–1490.
49. Scheidtmann, K. H., B. Echle, and G. Walter. 1982. Simian virus 40 large T antigen is phosphorylated at multiple sites clustered in two separate regions. *J. Virol.* **44**:116–133.
50. Scheidtmann, K. H., M. Hardung, B. Echle, and G. Walter. 1984. DNA-binding activity of simian virus 40 large T antigen correlates with a distinct phosphorylation state. *J. Virol.* **50**:1–12.
51. 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 the DNA-binding activity. *J. Virol.* **65**:2098–2101.
52. Schneider, J., and E. Fanning. 1988. Mutations in the phosphorylation sites of simian virus 40 (SV40) T antigen alter its origin DNA-binding specificity for site I or II and affect SV40 DNA replication activity. *J. Virol.* **62**:1598–1605.
53. Shalloway, D., T. Kleinberger, and D. M. Livingston. 1980. Mapping of SV40 DNA replication origin region binding sites for the SV40 T antigen by protection against exonuclease III digestion. *Cell* **20**:411–422.
54. Stahl, H., P. Droge, and R. Knippers. 1986. DNA helicase activity of SV40 large tumor antigen. *EMBO J.* **5**:1939–1944.
55. Stillman, B. 1989. Initiation of eukaryotic DNA replication in vitro. *Annu. Rev. Cell Biol.* **5**:197–245.
56. Tsurimoto, T., T. Melendy, and B. Stillman. 1990. Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. *Nature (London)* **346**:534–539.
57. 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.
58. Virshup, D. M., and T. J. Kelly. 1989. Purification of replication protein C, a cellular protein involved in the initial stages of SV40 DNA replication in vitro. *Proc. Natl. Acad. Sci. USA* **86**:3584–3588.
59. Virshup, D. M., M. S. Wold, D. H. Weinberg, M. G. Kauffman, and T. J. Kelly. 1990. Cellular proteins involved in SV40 DNA replication in vitro, p. 303–314. *In* C. C. Richardson and I. R. Lehman (ed.), *Molecular mechanisms in DNA replication and recombination*, vol. 127. Alan R. Liss, Inc., New York.
60. Weinberg, D. H., K. L. Collins, P. Simancek, A. Russo, M. S. Wold, D. M. Virshup, and T. J. Kelly. 1990. Reconstitution of simian virus 40 DNA replication with purified proteins. *Proc. Natl. Acad. Sci. USA* **87**:8692–8696.
61. Weinberg, D. H., and T. J. Kelly. 1989. Requirement for two DNA polymerases in the replication of simian virus 40 DNA in vitro. *Proc. Natl. Acad. Sci. USA* **86**:9742–9746.
62. Wobbe, C. R., L. Weissbach, J. A. Borowiec, F. B. Dean, Y. Murakami, P. Bullock, and J. Hurwitz. 1987. Replication of simian virus 40 origin-containing DNA in vitro with purified proteins. *Proc. Natl. Acad. Sci. USA* **84**:1834–1838.
63. Wold, M. S., and T. J. Kelly. 1988. Purification and characterization of replication protein A, a cellular protein required for in vitro replication of simian virus 40 DNA. *Proc. Natl. Acad. Sci. USA* **85**:2523–2527.
64. Wold, M. S., J. J. Li, and T. J. Kelly. 1987. Initiation of simian virus 40 DNA replication in vitro: large-tumor-antigen- and origin-dependent unwinding of the template. *Proc. Natl. Acad. Sci. USA* **84**:3643–3647.
65. Wold, M. S., D. W. Weinberg, D. M. Virshup, J. J. Li, and T. J. Kelly. 1989. Identification of cellular proteins required for SV40 DNA replication. *J. Biol. Chem.* **264**:2801–2809.
66. Yang, S. I., R. L. Lickteig, R. Estes, K. Rundell, G. Walter, and M. C. Mumby. 1991. Control of protein phosphatase 2A by simian virus 40 small-t antigen. *Mol. Cell. Biol.* **11**:1988–1995.