JOURNAL OF VIROLOGY, Sept. 1992, p. 5248–5255
0022-538X/92/095248-08$02.00/0

Vol. 66, No. 9

Inhibition of Structural Changes in the Simian Virus 40 Core Origin of Replication by Mutation of Essential Origin Sequences

JAMES A. BOROWIEC

Department of Biochemistry, New York University Medical Center, New York, New York 10016

Received 15 May 1992/Accepted 16 June 1992

Mutation of the simian virus 40 (SV40) origin of replication (*ori*) has revealed the presence of three critical domains needed for DNA replication. The outer two domains, the AT tract and early palindrome element (EP), colocalize with DNA regions that become structurally altered in the presence of the SV40 large tumor antigen (T antigen) and ATP. Mutations within each domain were examined for their effect on the distortion of *ori* DNA by T antigen, as assayed by the sensitivity of DNA to $KMnO_4$ oxidation. We have found that mutations in the AT tract that inhibit SV40 DNA replication also inhibit the distortion of the AT tract. Similarly, mutations in the EP inhibited the generation of structural changes in this element by T antigen. Although AT-tract mutations or mutations on the late side of *ori* affected structural changes only in the AT tract, certain EP mutations or mutations on the early side of *ori* also inhibited AT-tract distortion. Mutation of the flanking regions did not significantly affect either the affinity of T antigen for *ori* or the rate of binding to *ori*. We conclude from these results that the primary function of the flanking *ori* domains is to undergo structural changes required during the initiation of SV40 DNA replication. Moreover, our results suggest that the efficiency of replication initiation is significantly affected by the degree to which the flanking elements undergo a structural transition.

The initiation of DNA replication from internal sites on chromosomes presents a challenging obstacle for the cellular replication machinery. Sufficient lengths of double-stranded DNA must be rendered single stranded to serve as templates for DNA polymerases to synthesize the nascent strands. The manner by which replication factors interact with the DNA helix to convert it to single-stranded DNA remains poorly understood. The use of relatively simple model systems for eukaryotic DNA replication has made this problem tractable. Examination of simian virus 40 (SV40) indicates that the presynthetic changes in DNA structure take place in an elaborate series of steps (2).

An essential first step in SV40 DNA replication is the binding of the SV40 large tumor antigen (T antigen) to the viral origin of replication (*ori*). At elevated temperatures (37°C), stable binding of T antigen to core sequences within *ori* requires ATP and results in the formation of a multimeric complex containing up to 12 monomers of protein (4, 14, 24). This ATP-dependent complex is notable for its bifurcated structure with each half containing a hexamer of T antigen (10, 24, 28). In the presence of human single-stranded DNA-binding protein (human SSB, RP-A, RF-A [19, 35, 36]) or certain heterologous SSB proteins, the intrinsic DNA helicase activity of T antigen can catalyze the large-scale unwinding of DNA molecules from *ori* (8, 22, 37). Mechanistic parallels to these events were observed in prokaryotic model systems such as *oriC* of *Escherichia coli* and *oriλ* of bacteriophage λ (1, 17).

The nucleation of DNA melting in the SV40 system appears to take place within the ATP-dependent complex. Chemical probing of the *ori* DNA within this complex reveals two regions of altered DNA structure. In one arm of an imperfect inverted repeat, termed the early palindrome element (EP) or inverted repeat, approximately 8 bp of DNA are denatured. On the opposite flank of the *ori* situated toward the late gene side of SV40, nearly 20 bp of an adenine-thymine (AT) tract undergo a conformational distortion while remaining primarily double stranded (3, 5, 26, 28). These observed structural changes appear to lead, at least in part, to topological untwisting of the *ori*-containing DNA (11, 29).

That each of these conformational changes in *ori* structure is a critical step during the initiation of SV40 DNA replication is suggested by high-resolution genetic mapping of *ori*. Tegtmeyer and colleagues have described three discrete elements within the 65-bp core *ori* that are essential for SV40 DNA replication in vivo or in vitro (7, 12, 13, 15). Two of these functional elements closely overlap the two conformationally flexible regions in the EP and AT-tract regions. The third central region, containing four GAGGC elements in a perfect mirror repeat, acts as a recognition element for T-antigen binding. Point mutations in any of these three elements can reduce DNA replication activity by 2 orders of magnitude in vivo (12, 13, 15).

The relationship between the induction of conformational changes within *ori* and the initiation of replication was tested by examining the effect of replication-defective mutations on the distortion of the two flanking regions of *ori* by T antigen. Single-base-pair mutations in critical late side elements significantly inhibited the generation of structural changes within the AT tract while having only minor effects on structural changes in the opposite flanking region. Conversely, while all early side mutants had significantly lower levels of structural changes in the EP, two of these mutants were also defective in the generation of conformational changes in the AT tract. These results provide direct evidence that the importance of the two flanking regions lies in

their ability to undergo conformational changes required during the initiation of SV40 DNA replication.

MATERIALS AND METHODS

Preparation of protein and DNA reagents. T antigen was purified from Sf9 insect cells infected with recombinant baculovirus vEV55SVT (25) or Ac941SVT. Ac941SVT, a kind gift of Monika Lusky, Cornell University Medical College, New York, N.Y., was prepared by initially cloning a T-antigen cDNA into the transfer vector pVL941 (23). Ac941SVT, although producing T antigen identical in all respects to that isolated from vEV55SVT, yields three to five times more T antigen per liter of infected Sf9 cells. Preparation of infected-cell lysates and immunoaffinity purification of T antigen were done by the method of Borowiec et al. (3). The DNA constructs used in these experiments were a kind gift of Peter Tegtmeyer, State University of New York, Stony Brook. The nomenclature used by Tegtmeyer and colleagues in reference 7 is maintained in this paper, with the additional change that each point mutation is termed *bs* (for base). For example, the *ori* mutated at position 5212 is termed *bs*5212. Each DNA was prepared from DH5α cells (21) by alkali lysis and CsCl banding (30).

$KMnO_4$ footprinting of DNA. Standard reaction mixtures (30 μl) contained 40 mM creatine phosphate (di-Tris salt [pH 7.8]), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 μg of the appropriate plasmid *ori* DNA, 4 mM ATP, 25 μg of creatine phosphokinase per ml, and various quantities of T antigen. Reactions were incubated at 37°C for 60 min before modification with $KMnO_4$. Although T antigen can form a stable complex with *ori* DNA after shorter times (e.g., see Fig. 7), the induction of structural changes that are detectable by $KMnO_4$ requires incubation times in excess of 30 min (3). $KMnO_4$ was added from a freshly prepared stock solution (200 mM) to give a final concentration of 20 mM, and the reaction was incubated for an additional 4 min. Primer extension and gel electrophoresis conditions were essentially as described by Borowiec and Hurwitz (5).

The level of $KMnO_4$ oxidation in each region was quantitated by densitometric scanning of autoradiographs with an LKB Ultrascan XL laser densitometer. Autoradiographs with various exposure times were used to ensure that the band intensity was within the linear range of film sensitivity. The extent of $KMnO_4$ oxidation of the early palindrome was determined by quantitating the intensity of the region corresponding to top-strand thymine residues 5217 and 5218. Although the thymine at *bs*5214 produces a $KMnO_4$ signal, this signal constitutes only approximately 15% of the signal derived from the major reactive nucleotides at positions 5217 and 5218 and can be easily distinguished. For the AT tract, bands corresponding to thymine residues 16, 20, 29, and 30 of the top strand were used. The overall intensity of each region was separately normalized by comparison with four control thymine residues located outside the SV40 core *ori*. The mutant origins were always probed in parallel with the wild-type (wt) *ori*, which served as an internal control. The maximum normalized value for the early palindrome and the AT tract of wt *ori* for each titration was set at 1.0. The fraction of maximum oxidation for the mutant origins was then calculated for each datum point.

Gel mobility shift analysis of T antigen-*ori* complexes. The ^{32}P-end-labeled *ori* DNA was prepared by first digesting the appropriate mutant plasmid DNA with *Eco*RI and *Hin*dIII to release the *ori*-containing DNA fragment (approximately 100 bp in length). After treatment with calf intestinal alkaline phosphatase (US Biochemical), the DNA was end labeled in the presence of [γ-^{32}P]ATP and polynucleotide kinase, and the labeled *ori* DNA was purified after electrophoresis through an 8% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1). Each gel shift assay used approximately 50 fmol of DNA (at 1×10^6 to 2×10^6 cpm/pmol). For gel mobility shift assays, standard reaction mixtures (20 μl) contained 40 mM creatine phosphate (di-Tris salt [pH 7.8]), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 25 μg of creatine phosphokinase per ml, *ori* DNA, and various quantities of T antigen. Standard binding reactions were incubated at 37°C for 60 min and then cross-linked by the addition of glutaraldehyde to 0.1% and incubation for 15 min at 37°C. Kinetic measurements used shorter binding times (see Fig. 8), followed by the identical cross-linking procedure. The reaction mixtures were then loaded onto a 4% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) and electrophoresed in the presence of 25 mM Tris, 190 mM glycine, and 1 mM EDTA (pH 8.5). Gel slices corresponding to the shifted T-antigen–DNA complex were removed, and the radioactivity was determined by scintillation counting.

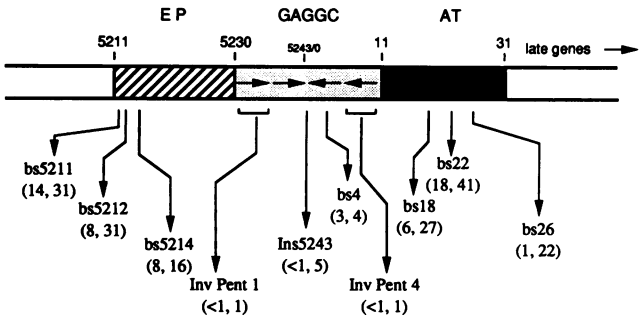

FIG. 1. Mutations in the SV40 core *ori* that were tested for their effect on the distortion of *ori* DNA by T antigen. The general location of each mutant is shown. *bs*5211, *bs*5212, and *bs*5214 are located in the EP; Inv Pent 1, *Ins*5243, *bs*4, and Inv Pent 4 are located in the central GAGGC element (GAGGC); and *bs*18, *bs*22, and *bs*26 are located in the AT tract. The numbers below each mutation indicate the replication efficiency relative to wt *ori* (in percentages). The first number indicates the activity in vivo, while the second number indicates the activity in vitro, obtained by using crude cytoplasmic extracts of HeLa cells (data from reference 7).

RESULTS

The binding of T antigen to the SV40 *ori*, in the presence of ATP, results in the induction of significant structural changes at two flanking regions within *ori*. Each of these deformable DNA elements resides in regions found previously to be critical for *ori* function. We therefore wished to understand the relationship between the structural deformation of these elements by T antigen and the importance of these elements for DNA replication. We used a series of mutations located within *ori* that were initially constructed by Tegtmeyer and colleagues (7, 12, 13, 15). The mutations were changes of one to three base pairs or single-base-pair insertions and were located within one of the three critical regions of *ori*, i.e., the flanking EP or AT-tract regions or the central GAGGC element (Fig. 1). Each mutant *ori* was present as a core *ori* lacking the flanking T-antigen-binding site I and SV40 transcriptional control elements.

The induction of structural changes in each mutant *ori*, contained in plasmid DNA, was probed by using $KMnO_4$ oxidation. While $KMnO_4$ reacts poorly with DNA in the

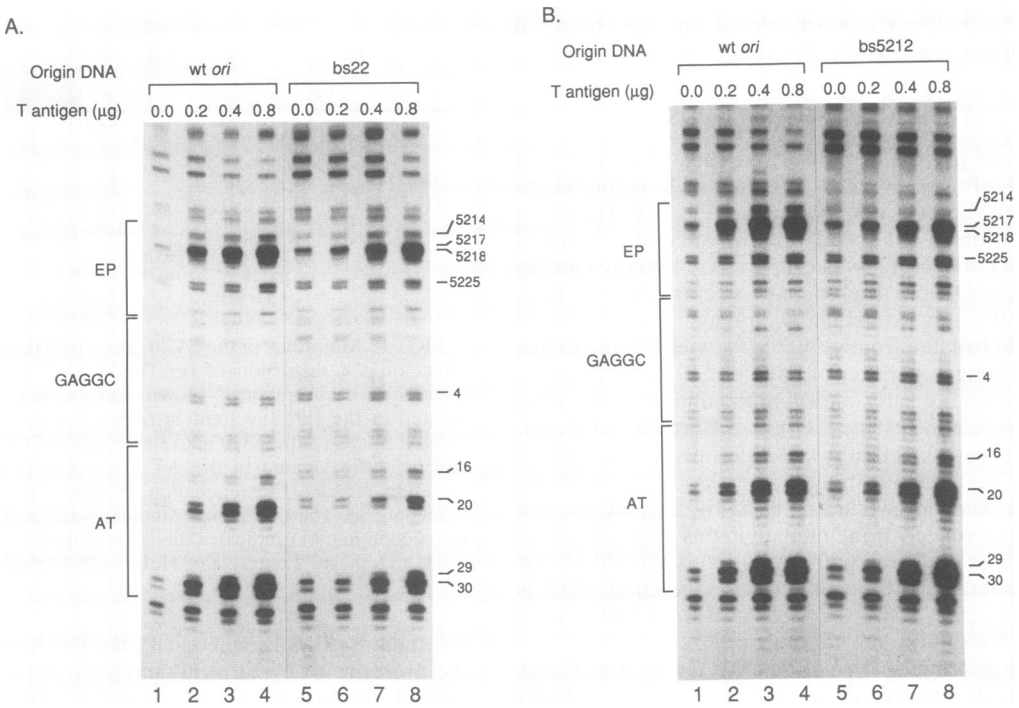

FIG. 2. Effect of T-antigen levels on the induction of structural changes in the wild-type *ori* and in *ori* DNA molecules mutated in the AT tract or EP. A representative autoradiograph showing the changes in DNA reactivity to $KMnO_4$ as a function of T antigen is shown. To wt *ori* (A and B; lanes 1 to 4), *bs*22 (A; lanes 5 to 8), or *bs*5212 (B; lanes 5 to 8) was added 0.0 μg (lanes 1 and 5), 0.2 μg (lanes 2 and 6), 0.4 μg (lanes 3 and 7), or 0.8 μg (lanes 4 and 8) of T antigen. After incubation for 60 min, the DNA was modified with 20 mM $KMnO_4$. The oxidation reaction was then quenched and the sites of oxidation were determined as described in Materials and Methods. Various sequence positions are indicated on the right side of each panel. The locations of the EP, central GAGGC, and AT tract are shown on the left side of each panel.

B-form, DNA that is melted or apparently bent sharply or significantly untwisted (of altered helical pitch) becomes hyperreactive to $KMnO_4$ oxidation, predominantly at thymine residues (5, 6). These residues can be precisely located by first extending a ^{32}P-labeled DNA primer across the region of interest with the Klenow fragment of DNA polymerase I. Because the oxidized nucleotides can stall the polymerase, analysis of the extension products by denaturing gel electrophoresis and autoradiography indicates the sites and relative extent of $KMnO_4$ oxidation. With a single exception (*bs*5214; see Materials and Methods), none of the mutations affected thymines producing the $KMnO_4$ oxidation signal.

The induction of structural changes in the wt *ori* DNA was first compared with an *ori* DNA mutated in the AT-tract element at nucleotide 22 (*bs*22). The *bs*22 mutation was found previously to inhibit SV40 DNA replication to approximately 40% of wt levels when tested in vitro with crude extracts, and under 20% of wt levels when examined in vivo (7, 13, 15). These DNA molecules were incubated with increasing levels of T antigen, and the DNA was then modified with $KMnO_4$. A representative autoradiograph of the primer extension products compares the $KMnO_4$ oxidation patterns for wt *ori* and *bs*22 (Fig. 2A). As the amount of T antigen added to wt *ori* was increased from 0.0 to 0.8 μg, the level of $KMnO_4$ oxidation also increased at two regions within *ori* (Fig. 2A, lanes 1 to 4). These regions, as indicated on the left side of the figure, correspond to the EP and AT tract of *ori*. Titration of similar amounts of T antigen to *bs*22 induced identical nucleotides within this mutant to become hypersensitive to $KMnO_4$ oxidation (Fig. 2A, lanes 5 to 8). Visual comparison of the amount of $KMnO_4$ oxidation in these two *ori* DNA molecules indicated that modification of the AT tract of *bs*22 was significantly decreased relative to wt *ori*. Although oxidation of the EP element of *bs*22 appeared slightly less than that of wt *ori*, quantitative analysis of the data found no significant difference (see Fig. 3B). Thus, in this qualitative comparison, a single base pair mutation of the AT tract inhibited the generation of structural changes in the AT tract by T antigen.

A similar experiment was performed comparing the $KMnO_4$ oxidation patterns of wt *ori* and an *ori* DNA mutated in the EP at nucleotide 5212 (*bs*5212; Fig. 2B). This point mutation inhibits SV40 replication to levels that are 31% in vitro and 8% in vivo of wt levels (7, 12, 15). The EP mutation significantly decreased the extent of $KMnO_4$ oxidation in this region when intermediate levels of T antigen were used (0.2 and 0.4 μg; compare lanes 2 and 3 with lanes 6 and 7 in Fig. 2B). No observable effects of the *bs*5212 mutation were noted on modification of the AT tract. Thus, mutations in the EP or AT tract that are inhibitory to SV40 DNA replication also inhibit distortion of the mutated region by T antigen.

We performed a comprehensive analysis of the effect of *ori* mutation on the distortion of *ori* structure by T antigen. Each mutation tested was found previously to significantly reduce SV40 replication both in vivo and in vitro (Fig. 1). Each *ori* mutant was incubated with various amounts of T antigen, in the presence of ATP, and the DNA was then probed with $KMnO_4$. The extent of $KMnO_4$ oxidation for the AT-tract and EP regions in each *ori* were separately quantitated by densitometric scanning of the autoradiographs. The levels of modification in these two *ori* elements were sepa-

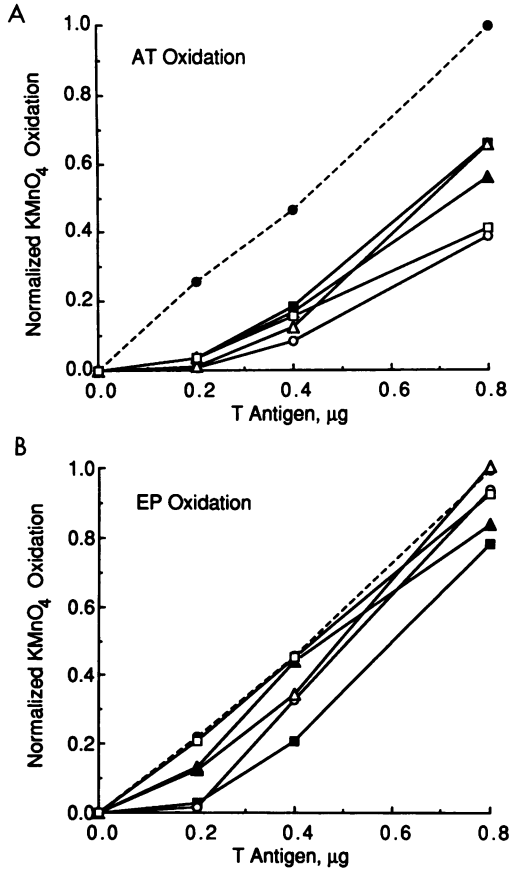

FIG. 3. Effect of T-antigen levels on $KMnO_4$ oxidation in the EP and AT tract of *ori* DNA molecules mutated on the late side (nucleotides 1 to 31). Quantitation of oxidation in the AT tract (A) and EP (B) is shown. The mutant *ori* DNAs tested were wt *ori* (●), *bs*18 (■), *bs*22 (▲), *bs*26 (○), *bs*4 (△), and Inv Pent 4 (□). Various amounts of T antigen were incubated with the wt or mutant *ori* plasmid, and the sites of oxidation were visualized as described in the legend to Fig. 2. The levels of $KMnO_4$ oxidation for the EP and AT tract were individually determined for each *ori* by densitometric analysis of the autoradiograph. The data for each element were normalized with respect to the maximum level of oxidation determined for wt *ori* (at 0.8 μg of T antigen) by comparison with control bands located outside *ori*.

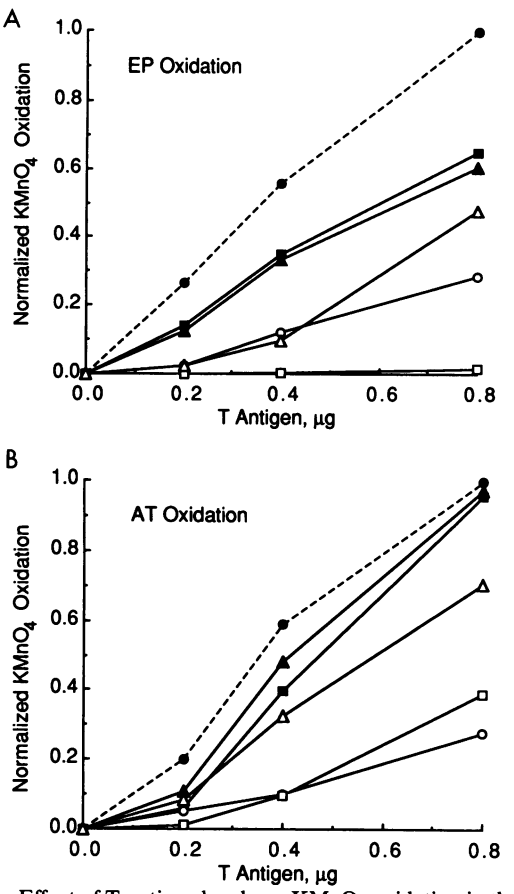

FIG. 4. Effect of T-antigen levels on $KMnO_4$ oxidation in the EP and AT tract of *ori* DNA molecules mutated on the early side (nucleotides 5211 to 5243). Quantitation of oxidation in the EP (A) and AT tract (B) is shown. The mutant *ori* DNAs tested were wt *ori* (●), *bs*5211 (■), *bs*5212 (▲), *bs*5214 (○), *Ins*5243 (△), and Inv Pent 1 (□). Quantitation of oxidation was as described in the legend to Fig. 3.

rately normalized by comparison with control bands located outside the *ori* region. The normalized levels of $KMnO_4$ oxidation in the AT-tract and EP regions were then plotted as a function of added T antigen for each mutant *ori*. The data were grouped according to whether the mutation was located on the late side (nucleotides 1 to 26; Fig. 3) or the early side (nucleotides 5211 to 5243; Fig. 4) of *ori*. Control experiments examining *ori* mutations that did not significantly affect SV40 DNA replication were also performed. These mutations (at nucleotides 5222, 5228, and 12) did not significantly affect the distortion of *ori* structure (data not shown).

Five late-side mutations were examined, and each had severe effects on the distortion of the AT tract by T antigen (Fig. 3A). Two were located in the central GAGGC region (*bs*4 and Inv Pent 4), and the other three were located within the AT tract (*bs*18, *bs*22, and *bs*26). The Inv Pent 4 mutation is an inversion of the most rightward (late) GAGGC sequence. At moderate levels of T antigen (0.4 μg), each mutation reduced $KMnO_4$ oxidation of the AT tract to levels 18 to 40% of that of wt *ori*. High levels of T antigen (0.8 μg) did not overcome the effect of the mutation, as $KMnO_4$ modification within the AT tract remained 39 to 66% of wt levels. In contrast, these identical mutations had less significant effects on the generation of structural changes within the EP (Fig. 3B). In the presence of 0.4 μg of T antigen, four of the five mutations reduced $KMnO_4$ modification of the EP to levels 72 to 99% of wt values. At 0.8 μg of T antigen, oxidation within the EP of any of these mutants did not fall below 79% of the wt level. Therefore, late-side mutations that are deleterious to SV40 DNA replication significantly inhibited AT-tract distortion by T antigen while having only minor effects on distortion of the EP.

Five early-side *ori* mutants were tested for their ability to undergo T-antigen-mediated changes in DNA structure. Three of these mutations were located in the EP (*bs*5211, *bs*5212, and *bs*5214). The fourth mutation was an inversion of the most leftward (early) GAGGC sequence (Inv Pent 1), and the last was a single-base insertion between the middle two GAGGC pentamers (*Ins*5243). The *Ins*5243 mutation, because of its position in the center of *ori*, would presumably separate the two hexameric lobes of T antigen by 0.34 nm and rotate one lobe with respect to the other by approxi-

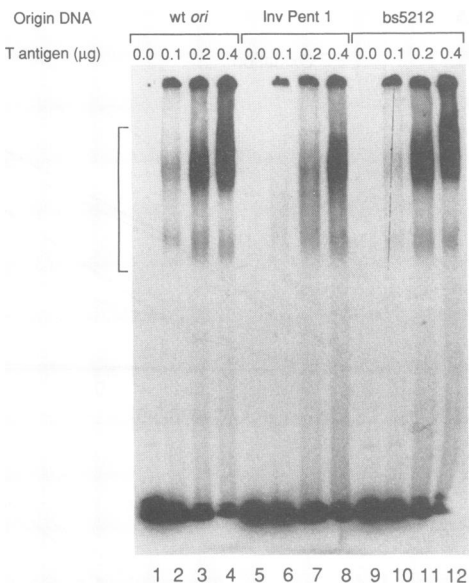

FIG. 5. Effect of *ori* mutation on the formation of the ATP-dependent complex by T antigen. Various amounts of T antigen, as indicated, were incubated with ^{32}P-end-labeled DNA fragments containing either wt *ori* (lanes 1 to 4), Inv Pent 1 (lanes 5 to 8), or *bs*5212 (lanes 9 to 12), in the presence of ATP. After 60 min, the T-antigen–DNA complexes were cross-linked by the addition of glutaraldehyde. The complexes were then separated from free *ori* DNA by electrophoresis through a nondenaturing gel, and the gel was dried and autoradiographed. The T-antigen–DNA complex is indicated by a bracket on the left side of the gel.

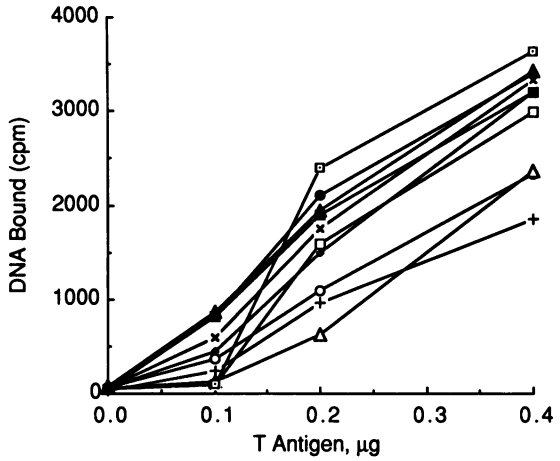

FIG. 6. Quantitation of the effect of *ori* mutation on the formation of the ATP-dependent complex by T antigen. Increasing levels of T antigen were incubated with ^{32}P-end-labeled DNA fragments containing wt or a mutant *ori*. The T-antigen–*ori* complexes were then cross-linked by using glutaraldehyde and separated from free *ori* DNA by nondenaturing gel electrophoresis as shown in Fig. 5. The portions of the gel corresponding to the T-antigen–*ori* complexes, after detection by autoradiography, were then removed, and the radioactivity present was determined by scintillation counting. The *ori* DNA constructs tested were wt *ori* (●), *bs*5212 (■), *bs*5214 (▲), Inv Pent 1 (○), *Ins*5243 (□), *bs*4 (△), Inv Pent 4 (+), *bs*18 (⊡), *bs*22 (×), and *bs*26 (◆).

mately 34°. Each mutation significantly inhibited the induction of structural changes within the EP (Fig. 4A). The mutations located in the EP region reduced the modification of this region 28 to 65% compared with the wt *ori* at high levels of T antigen (0.8 μg). The Inv Pent 1 mutation had the greatest effect on the generation of structural changes, with the extent of $KMnO_4$ modification reduced to less than 2% of wt levels.

Early-side mutations had differential effects on the induction of structural changes in the AT tract (Fig. 4B). At the highest level of T antigen (0.8 μg), AT-tract distortion was significantly inhibited for two mutants, *bs*5214 and Inv Pent 1, to 28 and 39%, respectively, of wt levels. The Inv Pent 1 mutation is particularly informative since the corresponding mutation on the late side, Inv Pent 4, had insignificant effects on $KMnO_4$ modification of the EP. Two other early-side mutations (*bs*5211 and *bs*5212) had only minor effects on $KMnO_4$ modification of the AT tract. The last mutant tested, *Ins*5243, had moderately reduced levels of AT-tract oxidation compared with the wt *ori*. We conclude that although late-side mutations have little effect on structural changes in the EP, certain early-side mutations can affect the generation of structural changes in both the EP and AT tract.

The inhibitory effect of *ori* mutation on *ori* distortion could be either an indirect result of the mutation decreasing T-antigen binding or a direct effect of the mutation decreasing the intrinsic ability of the flanking *ori* elements to undergo a conformational change. To distinguish between these two possibilities, we used a gel shift assay to examine the binding of T antigen to a ^{32}P-labeled DNA fragment containing either a wt or mutant *ori*. Increasing levels of T antigen were added to the *ori* DNA molecules, and the T-antigen–DNA complexes were then cross-linked with glutaraldehyde. The T-antigen–DNA complexes were separated by nondenaturing gel electrophoresis and detected by autoradiography.

The binding of T antigen to wt *ori*, Inv Pent 1, and *bs*5212 was examined first (Fig. 5). The addition of a small amount of T antigen (0.1 μg) to wt *ori* led to the production of two distinct complexes (lane 2). Comparison with the work of Parsons et al. (28) indicates that the faster and slower complexes are *ori* molecules bound by a hexamer or double hexamer of T antigen, respectively. Increasing the level of T antigen to 0.4 μg increased the amount of DNA bound by a double hexamer of T antigen (lane 4). Qualitative inspection of the autoradiograph shows that the Inv Pent 1 mutation, as expected, decreased the ability of T antigen to bind to the *ori* (lanes 5 to 8), whereas the *bs*5212 mutation had little observable effect (lanes 9 to 12).

Similar conditions were used to examine the binding of T antigen to the mutant *ori* DNAs. The binding of T antigen to each DNA was quantified by determining the sum of radioactivity present in both bands of the complex (Fig. 6). Certain mutations located in the central GAGGC region significantly reduced the binding of T antigen. At 0.4 μg of T antigen, the Inv Pent 1, *bs*4, and Inv Pent 4 mutations each decreased the amount of T-antigen–*ori* complex to approximately two-thirds of the wt level. On the other hand, T-antigen binding to DNA molecules containing mutations in the AT tract or EP (*bs*5212, *bs*5214, *bs*18, *bs*22, and *bs*26) or to the *Ins*5243 mutant was not significantly different from binding to wt *ori*, and any small differences were within the normal variation of this assay. Thus, mutations in the EP and AT tract did not inhibit the induction of the conformational changes in these regions by preventing T-antigen binding to *ori*. Our results suggest instead that these mutations directly affect the ability of the AT-tract and EP regions to undergo structural changes mediated by T antigen.

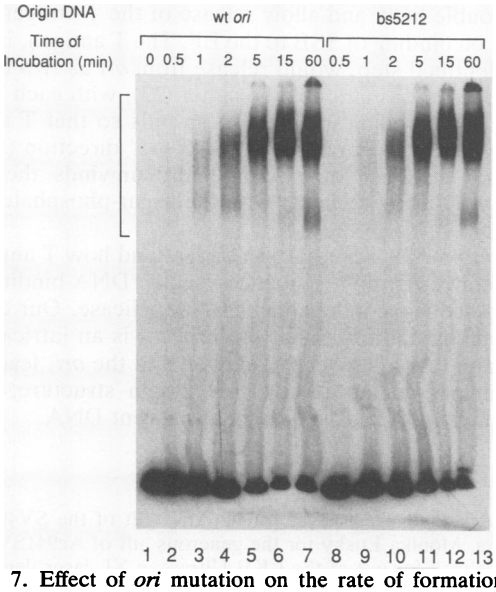

FIG. 7. Effect of *ori* mutation on the rate of formation of the ATP-dependent complex. T antigen (0.2 μg) was incubated with ^{32}P-end-labeled DNA fragments containing either wt *ori* (lanes 1 to 7) or *bs*5212 (lanes 8 to 13). After various intervals (as indicated), the T-antigen–DNA complexes were cross-linked by the addition of glutaraldehyde. The complexes were then separated from free *ori* DNA by electrophoresis through a nondenaturing gel, and the gel was dried and autoradiographed. The T-antigen–DNA complex is indicated by a bracket on the left side of the gel.

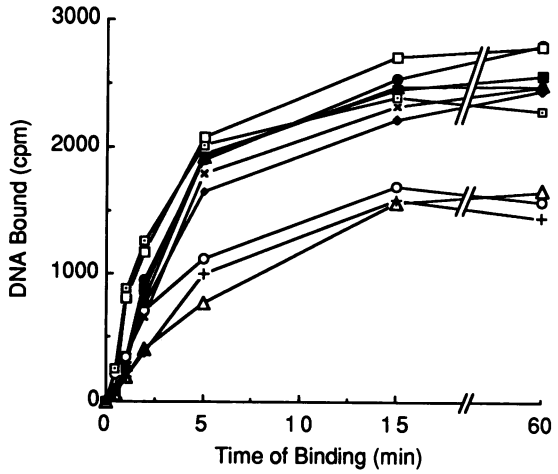

FIG. 8. Quantitation of the effect of *ori* mutation on the kinetics of formation of the ATP-dependent T-antigen–*ori* complex. T antigen (0.2 μg) was incubated with wt *ori* or a mutant *ori* for various times. After cross-linking of the T-antigen–DNA complex, the complex was separated from free *ori* DNA by electrophoresis through a nondenaturing gel (as shown in Fig. 7). The portions of the gel corresponding to the T-antigen–*ori* complexes, after detection by autoradiography, were removed, and the radioactivity present was determined by scintillation counting. The *ori* DNA constructs tested were wt *ori* (●), *bs*5212 (■), *bs*5214 (▲), Inv Pent 1 (○), *Ins*5243 (□), *bs*4 (△), Inv Pent 4 (+), *bs*18 (⊡), *bs*22 (×), and *bs*26 (◆).

ori mutation may in turn affect the rate of T-antigen binding to *ori*. To examine this possibility, we incubated T antigen (0.2 μg) for various times with ^{32}P-end-labeled DNA fragments containing a mutant or wt *ori*. The T-antigen–*ori* complexes were cross-linked with glutaraldehyde, and the relative amount of complex formed was examined by a gel shift assay. A time course of T-antigen binding to the wt *ori* and the EP mutant *bs*5212 indicated that the rates of binding to the wt *ori* (lanes 1 to 7) and *bs*5212 (lanes 8 to 13) were very similar (Fig. 7). The amount of T antigen bound to these and other mutant origins at each time point was quantitated by determining the amount of radioactivity in gel slices corresponding to both bands of the T-antigen–DNA complex. T antigen bound all mutant *ori* DNA molecules at rates similar to that for binding to wt *ori* DNA (Fig. 8). Although the overall amount of T-antigen binding to DNA fragments containing mutations in the GAGGC element of *ori* (*bs*4, Inv Pent 1, and Inv Pent 4) was reduced, the rate of T-antigen binding to these fragments was similar to that for fragments containing wt *ori*.

DISCUSSION

The ATP-dependent binding of T antigen to the SV40 origin of replication results in the structural distortion of two flanking regions of *ori*. These two regions overlap two *ori* elements required for origin activity. We have examined the effect of replication-defective *ori* mutations on the distortion of *ori* DNA by T antigen. Mutation of critical nucleotides in either the EP or the AT tract decreased the ability of T antigen to induce structural changes within that region. These flanking mutations have no significant effect on either the amount of T antigen bound or the rate of T-antigen binding to *ori*. We therefore conclude that the importance of the two flanking regions lies in their ability to undergo structural changes required during the initiation of SV40 DNA replication.

Of the 10 mutations tested, 6 were located in the flanking sequence elements. We found similar qualitative effects of the mutations on the generation of structural changes in the mutated region and on SV40 DNA replication. The *bs*5214 and *bs*26 mutations had the greatest effect on SV40 DNA replication in vitro and were also the most inhibitory to the generation of structural changes in the EP and AT tract, respectively. These results suggest that the efficiency of replication initiation is significantly affected by the degree to which the flanking elements undergo a structural transition.

Mutation of the central GAGGC element inhibited the ability of T antigen to bind to *ori*. This result is consistent with various studies demonstrating close contacts of T antigen with 5′-GAGGC-3′ sequences in both the core *ori* and the adjacent site I (5, 16, 32) and agrees with the results of Parsons et al. (26). Somewhat surprisingly, mutation of the core sequences inhibited only the level of T antigen binding to the *ori* but did not significantly inhibit the overall rate of T-antigen–*ori* complex formation. In other words, normalization of each set of binding data to the maximum amount of DNA bound indicated that the binding of the mutant *ori* DNA was never less than 68% of the wt *ori* level at each time point tested. Although the GAGGC elements are presumably required to properly position T antigen on *ori*, the rate-limiting step of binding, under our reaction conditions, does not appear to be the recognition of these sequences. We have not performed extensive kinetic analysis of the binding of T antigen to wt *ori*, but it would seem plausible that a "slow" step occurs after the initial interaction of T antigen with the DNA. Dean et al. (9) have shown that preformed hexamers of T antigen cannot properly bind to *ori* and that therefore T-antigen hexamers are probably formed on the DNA by sequential addition of monomers (see

also reference 28). A possible interpretation of our data is that the formation of the hexameric T antigen on *ori* after the initial GAGGC recognition is the rate-limiting step in the formation of the ATP-dependent complex. The use of other assays (e.g., dimethyl sulfate footprinting) may allow detection of intermediate complexes during the initial stages of binding.

Why does mutation of the flanking regions inhibit their distortion by T antigen? The minimal effect of such mutations on T-antigen binding demonstrates that the reduced structural distortion is not a consequence of poor T-antigen binding to the origin. However, the work by Parsons et al. indicates that T antigen can weakly recognize the EP region in the absence of the central GAGGC and AT-tract regions (26). Thus, one possibility is that T antigen still binds with wt efficiency to the mutant templates because of the intact GAGGC elements, yet forms weaker contacts with the mutated flanks. T antigen would therefore be unable to form contacts sufficiently stable to fully distort the flanking sequence. An alternative model is that each flanking element is predisposed to undergo a structural transition and that mutation lessens their ability to become distorted. In this model, T antigen can interact with the flanking regions but these contacts are inefficiently converted into an observable structural change. These two models are not mutually exclusive, and our data cannot yet distinguish between them.

Our data bear upon the interaction of T-antigen molecules bound to *ori* in the presence of ATP. Mutation of either the EP or the AT tract affected primarily the generation of structural changes within the mutant element while having less significant effects on the opposite flanking region. Thus, these data support the hypothesis that the ATP-dependent complex is composed of two somewhat independent domains, each containing a hexamer of T antigen (3, 28). It is important to note that certain early side mutations (*bs*5214, Inv Pent 1) significantly inhibited conformational changes within the AT tract. Similarly, the *Ins*5243 mutation, a single-base-pair insertion between the early and late halves of *ori*, reduced the level of structural changes in both flanks of *ori*. Our data therefore suggest that the two hexamers do have significant interactions and that the hexamer bound to the early half of *ori* appears to be dominant to the T antigen bound to the late half. This hypothesis has also been independently suggested by Parsons and Tegtmeyer (27), who examined the spacing requirements between conserved elements in *ori*.

The events occurring prior to the synthesis of nascent DNA during the initiation of SV40 DNA replication have been the focus of significant effort (for a review, see reference 2). These studies suggest that T antigen, binding as monomeric units to *ori*, forms a hexamer over each half of *ori* (24, 28). In the early half of *ori*, the EP acts as the nucleation site for DNA melting within the *ori* (5, 26). Both human SSB protein (RF-A, RP-A) and some noncognate SSB proteins can allow the unwinding of *ori*-containing DNA molecules by T antigen (8, 22, 37). Because specific interactions between T antigen and certain nonhuman SSB proteins (e.g., *E. coli* SSB) seem unlikely, the major role for SSB is presumably to bind to the single-stranded DNA within the EP and thus further destabilize the double-stranded structure of *ori*. We note, however, that T antigen can specifically interact with human SSB (18) and that these contacts may facilitate this event. The AT tract, relative to the EP, does not appear to be greatly melted within the ATP-dependent complex (5, 26). The structural changes within the AT tract would both further decrease the stability of the double helix and allow release of the T-antigen DNA helicase on binding of SSB to the EP. The T antigen, in an as yet unidentified step, would release from *ori* as two hexamers, or possibly as a double hexamer (33), with each T-antigen hexamer binding to opposite strands so that T antigen can move outward from *ori* in the 3′→5′ direction (20, 31, 34). Each hexamer then individually unwinds the DNA primarily by using contacts with the sugar-phosphate backbone (31).

Further work is necessary to understand how T antigen is able to convert from a sequence-specific DNA-binding protein to a sequence-independent DNA helicase. Our current understanding indicates that the process is an intricate and subtle interaction between T antigen and the *ori*, leading to the complete denaturation of the origin structure, which allows the subsequent synthesis of nascent DNA.

ACKNOWLEDGMENTS

We thank Peter Tegtmeyer for his kind gift of the SV40 origin constructs, Monika Lusky for the generous gift of Ac941SVT, and Warren Jelinek for use of the LKB Ultrascan XL laser densitometer. We also thank Frank Dean and Jerry Hurwitz for critical comments on the manuscript and Dhruba SenGupta, Len Blackwell, and Tom Gillette for helpful discussions. We appreciate the technical assistance provided by Richard Tomko and Max Kallet.

Research was supported by NIH grant AI29963, the Pew Biomedical Scholars Program (T88-00457-063), and Kaplan Cancer Center Developmental Funding and Cancer Center Support core grant (from NCI P30CA16087).

REFERENCES

1. **Baker, T. A., K. Sekimizu, B. E. Funnell, and A. Kornberg.** 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the Escherichia coli chromosome. Cell **45**:53–64.
2. **Borowiec, J. A., F. B. Dean, P. A. Bullock, and J. Hurwitz.** 1990. Binding and unwinding—how T antigen engages the SV40 origin of DNA replication. Cell **60**:181–184.
3. **Borowiec, J. A., F. B. Dean, and J. Hurwitz.** 1991. Differential induction of structural changes in the simian virus 40 origin of replication by T antigen. J. Virol. **65**:1228–1235.
4. **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.
5. **Borowiec, J. A., and J. Hurwitz.** 1988. Localized melting and structural changes in the SV40 origin of DNA replication induced by T antigen. EMBO J. **7**:3149–3158.
6. **Borowiec, J. A., L. Zhang, S. Sasse-Dwight, and J. D. Gralla.** 1987. DNA supercoiling promotes formation of a bent repression loop in *lac* DNA. J. Mol. Biol. **196**:101–111.
7. **Dean, F., J. 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.
8. **Dean, F., 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.
9. **Dean, F. B., J. A. Borowiec, T. Eki, and J. Hurwitz.** 1992. The simian virus 40 T antigen double hexamer assembles around the DNA at the replication origin. J. Biol. Chem. **267**:14129–14137.
10. **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.
11. **Dean, F. B., and J. Hurwitz.** 1991. Simian virus 40 large T antigen untwists DNA at the origin of DNA replication. J. Biol. Chem. **266**:5062–5071.
12. **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**:1663–1670.

13. **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.
14. **Deb, S., 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.
15. **Deb, S., S. Tsui, A. Koff, A. 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.
16. **DeLucia, A. L., B. A. 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.
17. **Dodson, M., H. Echols, S. Wickner, C. Alfano, K. Mensa-Wilmot, B. Gomes, J. LeBowitz, J. D. Roberts, and R. McMacken.** 1986. Specialized nucleoprotein structures at the origin of replication of bacteriophage λ: localized unwinding of duplex DNA by a six-protein reaction. Proc. Natl. Acad. Sci. USA **83**:7638–7642.
18. **Dornreiter, I., L. F. Erdile, I. U. Gilbert, D. von Winkler, T. J. Kelly, and E. Fanning.** 1992. Interaction of DNA polymerase alpha-primase with cellular replication protein A and SV40 T antigen. EMBO J. **11**:769–776.
19. **Fairman, M. P., and B. Stillman.** 1988. Cellular factors required for multiple stages of SV40 DNA replication *in vitro*. EMBO J. **7**:1211–1218.
20. **Goetz, G. S., F. B. Dean, J. Hurwitz, and S. W. Matson.** 1988. The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. J. Biol. Chem. **263**:383–392.
21. **Hanahan, D.** 1983. Studies on the transformation of Escherichia coli with plasmids. J. Mol. Biol. **166**:557–580.
22. **Kenny, M. K., S.-H. Lee, and J. Hurwitz.** 1989. Multiple functions of human single-stranded-DNA binding protein in simian virus 40 DNA replication: single-strand stabilization and stimulation of DNA polymerases α and δ. Proc. Natl. Acad. Sci. USA **86**:9757–9761.
23. **Luckow, V. A., and M. D. Summers.** 1989. High level expression of nonfused foreign genes with Autographa californica nuclear polyhedrosis virus expression vectors. Virology **170**:31–39.
24. **Mastrangelo, I. A., P. V. C. 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.
25. **O'Reilly, D. R., and L. K. Miller.** 1988. Expression and complex formation of simian virus 40 large T antigen and mouse p53 in insect cells. J. Virol. **62**:3109–3119.
26. **Parsons, R., M. E. Anderson, and P. Tegtmeyer.** 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.
27. **Parsons, R., and P. Tegtmeyer.** 1992. Spacing is crucial for coordination of domain functions within the simian virus 40 core origin of replication. J. Virol. **66**:1933–1942.
28. **Parsons, R. E., J. E. Stenger, S. Ray, R. Welker, M. E. Anderson, and P. Tegtmeyer.** 1991. Cooperative assembly of simian virus 40 T-antigen hexamers on functional halves of the replication origin. J. Virol. **65**:2798–2806.
29. **Roberts, J.** 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.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **SenGupta, D., and J. A. Borowiec.** 1992. Strand-specific recognition of a synthetic DNA replication fork by the SV40 large tumor antigen. Science **256**:1656–1661.
32. **Tjian, R.** 1979. Protein-DNA interactions at the origin of simian virus 40 DNA replication. Cold Spring Harbor Symp. Quant. Biol. **43**:655–661.
33. **Wessel, R., J. Schweizer, and H. Stahl.** 1992. Simian virus 40 T-antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. J. Virol. **66**:804–815.
34. **Wiekowski, M., M. W. Schwarz, and H. Stahl.** 1988. Simian virus 40 large T antigen DNA helicase. J. Biol. Chem. **263**:436–442.
35. **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.
36. **Wold, M. S., and T. 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.
37. **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.