MOLECULAR AND CELLULAR BIOLOGY, Apr. 1990, p. 1719–1728
0270-7306/90/041719-10$02.00/0

Vol. 10, No. 4

Simian Virus 40 Origin Auxiliary Sequences Weakly Facilitate T-Antigen Binding but Strongly Facilitate DNA Unwinding

CRISANTO GUTIERREZ,[1]† ZONG-SHENG GUO,[1] JAMES ROBERTS,[2] AND MELVIN L. DEPAMPHILIS[1]*

Department of Cell and Developmental Biology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110,[1] and Department of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104[2]

Received 22 September 1989/Accepted 15 December 1989

The complete simian virus 40 (SV40) origin of DNA replication (*ori*) consists of a required core sequence flanked by two auxiliary sequences that together increase the rate of DNA replication in monkey cells about 25-fold. Using an extract of SV40-infected monkey cells that reproduced the effects of *ori*-auxiliary sequences on DNA replication, we examined the ability of *ori*-auxiliary sequences to facilitate binding of replication factors and to promote DNA unwinding. Although the replicationally active form of T antigen in these extracts had a strong affinity for *ori*-core, it had only a weak but specific affinity for *ori*-auxiliary sequences. Deletion of *ori*-auxiliary sequences reduced the affinity of *ori*-core for active T antigen by only 1.6-fold, consistent with the fact that saturating concentrations of T antigen in the cell extract did not reduce the stimulatory role of *ori*-auxiliary sequences in replication. In contrast, deletion of *ori*-auxiliary sequences reduced the efficiency of *ori*-specific, T-antigen-dependent DNA unwinding in cell extracts at least 15-fold. With only purified T antigen in the presence of topoisomerase I to unwind purified DNA, *ori*-auxiliary sequences strongly facilitated T-antigen-dependent DNA conformational changes consistent with melting the first 50 base pairs. Under these conditions, *ori*-auxiliary sequences had little effect on the binding of T antigen to DNA. Therefore, a primary role of *ori*-auxiliary sequences in DNA replication is to facilitate T-antigen-dependent DNA unwinding after the T-antigen preinitiation complex is bound to *ori*-core.

The genetically defined simian virus 40 (SV40) origin of DNA replication (*ori*) consists of three components: *ori*-core, *aux-1* and *aux-2* (Fig. 1). *ori*-core is the minimal *cis*-acting sequence required for replication under all conditions. Bidirectional DNA replication originates at a specific locus within *ori*-core when *ori* interacts with SV40 large tumor antigen (T-ag) in the presence of permissive cell factors (reviewed in references 17 and 18). *aux-1* and *aux-2* are noninterchangeable sequences that flank *ori*-core and facilitate its activity in vivo (2, 15, 24, 27, 32, 34). In the absence of these *ori*-auxiliary sequences, *ori*-core activity is reduced up to 100-fold, depending on experimental conditions and the method used to measure DNA replication (24).

Although the effects of SV40 *ori*-auxiliary components on SV40 DNA replication in vivo have been characterized extensively, the role of these sequences in initiating replication remains speculative. One possibility is that the SV40 early gene promoter, which encompasses *aux-2* and part of *ori*-core (Fig. 1), is needed to transcriptionally activate *ori*-core. However, this is ruled out by the fact that initiation of SV40 DNA replication is resistant to α-amanitin (14, 33). A second hypothesis is that proteins that bind to promoters and enhancers modify the chromatin structure of *ori*-core, making it more accessible to the T-ag preinitiation complex. However, stimulation of SV40 replication by *ori*-auxiliary components did not depend on the presence of nucleosomes (24). These considerations directed our attention to two other mechanisms that can explain the role of *ori*-auxiliary components.

Proteins that bind to transcriptional elements in SV40 (e.g., T-ag, Sp1, and the AP series [reviewed in references 30 and 38]) may also stabilize the binding of SV40 replication factors to *ori* (e.g., T-ag, DNA primase-DNA polymerase α, topoisomerases, and several stimulatory proteins [reviewed in references 11, 31, and 48]). For example, AP-2 binds to T-ag as well as to the SV40 enhancer and *aux-2* sequences (39). *aux-2* is part of the binding site for one or more SV40-specific factors that stimulates replication (55), and *ori* mutations in the A+T-rich motif of *ori*-core can be suppressed by mutations in *aux-2* (22). Alternatively, proteins recognizing these transcriptional elements may increase the activity of replication factors that bind to *ori*-core rather than their affinity for *ori*. For example, auxiliary components could promote localized DNA strand separation. Either mechanism would account for the fact that auxiliary components must be in close proximity to SV40 *ori*-core (8, 29, 32).

One obstacle to elucidating the role of SV40 *ori*-auxiliary sequences has been the inability to reproduce their effects in vitro either with cell extracts or with purified replication components (11, 34, 49). This problem was recently overcome by using extracts from SV40-infected CV-1 cells containing relatively high salt concentrations and an assay for replication that measured only completed DNA products (24), suggesting that the function of *ori*-auxiliary components depended upon the balance of cellular and viral replication factors and physiological conditions normally present during viral replication in vivo. Under these conditions, which reproduced all of the known characteristics of viral chromosome replication in vivo, *ori*-auxiliary sequences stimulated replication in vitro to the same extent that they stimulated replication in vivo (24). In the present study, we used these in vitro replication conditions to determine whether the primary role of SV40 *ori*-auxiliary sequences was to facilitate the binding of replication factors to *ori* or to stimulate a subsequent step in replication.

* Corresponding author.
† Present address: Centro de Biologia Molecular, Facultad de Ciencias, Cantoblanco, 28049 Madrid, Spain.

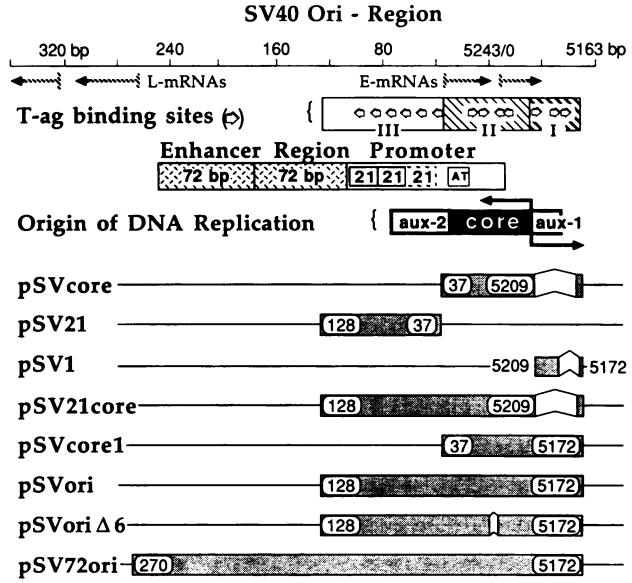

FIG. 1. SV40 *ori*- region and structures of plasmids carrying SV40 *ori* components. Restriction sites used in the construction of plasmids and nucleotide map positions for SV40 777 are indicated. The terminal SV40 nucleotide in each plasmid is numbered. The complete origin of DNA replication consists of *ori*-core (accurately defined by single-base-pair deletions as nucleotides 31 to 5211 [12]) and two auxiliary components, *aux-1* (5210 to between 5193 and 5164) and *aux-2* (32 to about 72), whose minimum sequences are defined by deletions described in Fig. 7 of reference 18. The origin of bidirectional DNA replication (↹, 5210 to 5211) is defined by the transition from discontinuous to continuous DNA synthesis on each template strand (25). The enhancer region consists of two 72-bp repeats. The key elements in the promoter region consist of two perfect 21-bp repeats, one imperfect repeat, an A+T-rich region that contains the TATA box, and six G+C-rich sequences that contain the 5′-(G>T)(A>G)GGC-3′ recognition sequence for T-ag (⇨) (16, 52) overlapping the 5′-GGGCGG-3′ recognition sequence for Sp1 (30). Under conditions that support DNA replication (ATP, 37°C), T-ag binds primarily to regions I and II (3, 13). T-ag has also been shown to bind to region III (see Fig. 3 and 4 of reference 18). Plasmids were constructed from pML-1 and SV40 DNA as described in Materials and Methods.

MATERIALS AND METHODS

Plasmids. SV40 DNA fragments were cloned into pML-1 (2,969 base pairs [bp]), which is pBR322 minus sequences that poison DNA replication in mammalian cells (35). Plasmids pSV72ori, pSVori, pSV21core, pSVcore1 and pSVcore have been described previously (15, 24, 55). pSV21 was made by deleting the *Nco*I-*Hin*dIII fragment from pSVori. To construct pSV-1, SV40 sequences between *Hin*dIII and *Sph*I sites of pSVori were replaced by a synthetic oligonucleotide 17-bp sequence corresponding to T-ag-binding site I (nucleotides 5209 to 5191 [43]) flanked by the sequence complementary to a *Hin*dIII cleavage site on the 5′ side and a unique *Nco*I-*Sph*I site on the 3′ side. pPyVori contains the polyomavirus origin of DNA replication (α-β-core; nucleotides 5039 to 90 [53]). Superhelical plasmid DNA was prepared from transformed *dam*$^+$ *Escherichia coli* DH5 or AG1 (Stratagene, Inc.) by lysing cells in alkali and sodium dodecyl sulfate and then purifying the DNA by sedimentation to equilibrium in CsCl as described previously (36).

DNA replication in vitro. Extracts from SV40-infected CV-1 monkey kidney cells and SV40 T-ag were prepared, and replication assays were carried out at 37°C for 2 h as previously described (24) with minor modifications. After DNA was treated with proteinase K and extracted with phenol, the aqueous phase was passed through a Sephadex G-50 spin column to remove unincorporated ^{32}P-deoxynucleoside triphosphates and the DNA was precipitated in 70% ethanol–2.5 M ammonium acetate for at least 1 h at −70°C. Purified DNA was digested simultaneously with *Dpn*I to eliminate all plasmid [^{32}P]DNA that had not replicated completely (pSVori contains 14 *Dpn*I sites) and with *Eco*RI to cut the single *Eco*RI site and convert all pSVori that completed replication into linear molecules of unit length. DNA was then fractionated by electrophoresis in 0.8% agarose gel, the appropriate *Dpn*I-resistant [^{32}P]DNA band was excised and placed in Aquasol (Du Pont Co.), and radioactivity was measured in a liquid scintillation counter. More than 90% of the pSVori [^{32}P]DNA appeared as completely replicated, *Dpn*I-resistant DNA.

DNA unwinding assay. Detection of DNA unwinding in cell extracts was carried as previously described (7), with minor modifications. Reaction mixtures (30 μl) containing 30 mM creatine phosphate (di-Tris salt [pH 7.7]), 7 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM ATP, 33 μg of creatine kinase per ml, 3 μg of bovine serum albumin per ml, 200 to 400 ng of relaxed plasmid DNA, 5 U of calf thymus topoisomerase I (Bethesda Research Laboratories, Inc.), HeLa cell cytosol (100 to 150 μg of protein), and 0.3 to 0.5 μg of immunoaffinity-purified T-ag were incubated at 37°C for 60 min. Reactions were terminated with 0.8% sodium dodecyl sulfate and 20 mM EDTA. After digestion with proteinase K (10 μg) at 37°C for 30 min, DNA was extracted with phenol, precipitated with ethanol, fractionated by electrophoresis in 1.2% agarose gels, and then stained with ethidium bromide. Portions of interest were transferred to Zeta-Probe membranes (Bio-Rad Laboratories), and plasmid DNA was detected by hybridization with pSVori [^{32}P]DNA. After autoradiography, reaction products were quantitated by excising the portions of the blot containing the substrate and the product (unwound DNA located close to the position of form I DNA) and measuring the radioactivity by scintillation counting. Background radioactivity present in the equivalent position of a reaction lacking T-ag was subtracted in every case.

Relaxed DNA substrate was prepared by incubating superhelical plasmid DNA for 2 h at 37°C with calf thymus topoisomerase I in 50 mM Tris hydrochloride (pH 7.5)–50 mM KCl–10 mM $MgCl_2$–0.5 mM dithiothreitol–0.1 mM EDTA–30 μg of bovine serum albumin per ml. DNA was extracted with phenol, precipitated with ethanol, and suspended in 10 mM Tris (pH 7.6)–1 mM EDTA. HeLa cell cytosol was prepared as described for CV-1 cells (24).

The initial steps of DNA unwinding were measured as described previously (41). SV40 *ori*-containing DNA fragments 500 to 650 bp long were prepared by digesting the plasmids in Fig. 1 at unique restriction sites as follows: *Eco*RI and *Hin*dIII (pSV72ori), *Ssp*I and *Sal*I (pSVori, pSVoriΔ6), *Hin*dIII and *Eag*I (pSVcore1, pSV21core), *Eag*I and *Eco*RI (pSVcore), and *Hin*dIII and *Sal*I (pML-1). The origin DNA fragments were purified by gel electrophoresis, and their ends were filled in with *E. coli* polymerase I (Klenow fragment) in the presence of [α-^{32}P]dCTP, [α-^{32}P]dTTP, dGTP, and dATP. Substrate minicircles were formed by ligation under dilute conditions with T4 DNA ligase and purified from polyacrylamide gels. Unwinding assays contained 0.5 ng of circular [^{32}P]DNA, 750 ng of immunoaffinity-purified T-ag, 5 U of calf thymus topoisomerase I, 2 mM ATP, 8 mM $MgCl_2$, 40 mM *N*-2-hydroxyeth-

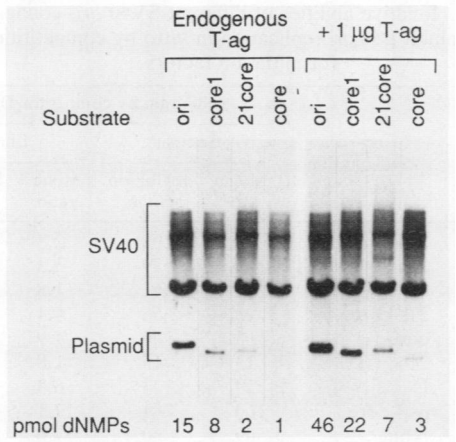

FIG. 2. DNA replication of SV40 *ori*-containing plasmids in vitro. Newly replicated plasmid DNA was radiolabeled with ^{32}P-deoxynucleoside triphosphates in an extract of SV40-infected CV-1 cells under standard conditions (see Materials and Methods). Purified DNA replication products were digested with *Eco*RI and *Dpn*I, fractionated by gel electrophoresis, and detected by autoradiography. The positions of linear plasmid DNA and SV40 DNA are indicated. The amount of deoxynucleoside monophosphates (dNMPs) (picomoles) incorporated into plasmid DNA in reactions containing 100 ng of plasmid DNA incubated for 1.5 h at 37°C is indicated at the bottom.

ylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), 50 mM NaCl, 0.5 mM dithiothreitol and 100 ng of mouse 3T3 cell DNA in a final volume of 25 μl. Reactions were carried out at 37°C for 1 h. DNA was purified, and the reaction products were fractionated by electrophoresis through a 3.5% polyacrylamide gel at 7 V/cm for 12 h. Gels were dried, and the reaction products were visualized by autoradiography.

RESULTS

***ori*-auxiliary sequences stimulate SV40 DNA replication in vitro.** The ratio of replication rates for the complete origin (*ori*, pSVori in Fig. 1) relative to the minimal origin (*ori*-core, pSVcore in Fig. 1) varied from 10- to 30-fold, depending on the conditions used. The maximum ratio was observed with saturating levels of T-ag, the optimum ratio of DNA substrate to cell extract, and extract prepared from either SV40-infected CV-1 cells or uninfected HeLa cells (24). The ratio of *ori* to *ori*-core replication rates observed in vitro was also strongly affected by ionic strength and the method used to measure DNA replication (24), the major reasons why investigators in other laboratories observed little if any effect of *ori*-auxiliary sequences in vitro (34, 49).

The absolute rates of DNA replication, but not the relative rates of *ori* to *ori*-core replication, were strongly affected by the T-ag concentration, the cell type from which the extract was prepared, and the method of extract preparation (24). Although replication rates were varied from 10 to 200 pmol of deoxynucleoside monophosphate incorporated in 1.5 h, the ratio of *ori* to *ori*-core was fixed by the amount of DNA substrate used. For example, when the endogenous T-ag concentration present in extracts from SV40-infected CV-1 cells was used, the rate of *ori*-core replication was stimulated 10- to 15-fold by the addition of *ori*-auxiliary sequences (Fig. 2). Supplementing endogenous T-ag with enough purified T-ag to give the maximum rate of DNA replication increased the rate of plasmid replication three- to fivefold, but the ratio of *ori* to *ori*-core replication remained unchanged (Fig. 2).

The ratio of *ori* to *ori*-core varied with the amount of DNA substrate because the rate of *ori* replication was much more sensitive to the ratio of DNA substrate to cell extract than was the rate of *ori*-core replication (24). At high ratios of pSVori to cell extract, replication was reduced to the level of pSVcore. This phenomenon revealed that the activity of *ori*-auxiliary sequences required *ori* to interact either with two or more different initiation factors (e.g., T-ag and some transcription factors such as Sp1 or AP-2) or with a molar excess of a single initiation factor (e.g., T-ag). The simplest explanation would be that *ori*-auxiliary sequences facilitated the binding of one or more initiation factors to *ori*-core. Since increasing the concentration of T-ag until the rate of plasmid replication no longer changed did not overcome the need for *ori*-auxiliary sequences in DNA replication (Fig. 2) (24), it did not appear that *ori*-auxiliary sequences served to increase the affinity of T-ag for *ori*. Therefore, we measured the relative affinity of SV40 *ori* components for whatever viral and cellular initiation factors may bind to *ori* under conditions in which *ori*-auxiliary sequences are active in DNA replication.

***ori*-auxiliary sequences have a small effect on the binding of initiation factors to *ori*.** The effect of various *ori* components on the relative affinity of some unidentified limiting initiation factor(s) for *ori* was measured by incubating pSVori with increasing amounts of a second plasmid (competitor DNA) containing various *ori* components (Fig. 1) in extracts of SV40-infected CV-1 cells that initiate replication in vitro. If *ori*-auxiliary sequences facilitate the binding of one or more factors required to initiate SV40 DNA replication, deletion of these sequences from the competitor plasmid should reduce its ability to inhibit pSVori replication by removing initiation factors, as previously demonstrated by Yamaguchi and DePamphilis (55).

The amount of pSVori DNA substrate used in these titration experiments was chosen to give a strong replication signal that was sensitive to inhibition by further addition of DNA containing the SV40 *ori*. This effect was reproducible in different cell extracts. The results revealed an exponential relationship between the fraction of pSVori replication and the amount of competitor DNA added (Fig. 3). This permitted the calculation of the competitor/pSVori molar ratio that gave 50% inhibition, which provided a convenient method to compare the relative affinities of different *ori* configurations and individual *ori* components for initiation factors (Table 1).

The sensitivity of this assay was demonstrated by doubling the amount of pSVori DNA (adding pSVori as competitor), which reduced the rate of pSVori replication by 50%. Competition was specific for the SV40 initiation factor(s) because an 80-fold molar excess of circular pPyVori or a 250-fold molar excess of circular pML-1 competitor DNA was required to inhibit pSVori replication by 50% (Table 1), consistent with previously published results (55). Moreover, the replication factor removed by competitor DNA was bound specifically to the SV40 *ori* sequence rather than nonspecifically to replication forks, because the ability of competitor DNA to inhibit replication was not proportional to its ability to replicate (Fig. 2). For example, linear pSVori and circular pSVcore replicated only 5 and 10% as well as circular pSVori, respectively, but were equivalent to circular pSVori as competitor DNAs in their ability to inhibit pSVori replication (Table 1).

Of the three SV40 *ori* components (Fig. 1), only *ori*-core (pSVcore) competed strongly for SV40-specific initiation factor(s). The 21-bp repeats (pSV21) and T-ag-binding region

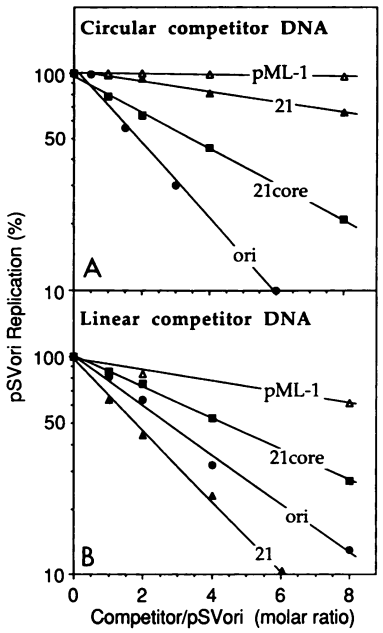

FIG. 3. Competitive inhibition of SV40 DNA replication by SV40 *ori* components. Circular competitor DNA and 100 ng of pSVori (A) or linear competitor DNA and 100 ng of pSVori (B) were combined at the indicated ratio and incubated in the standard replication assay, and the amount of pSVori DNA replication was analyzed (see Materials and Methods). Linear competitor DNA was obtained by digesting superhelical plasmid DNA with *Eco*RI. When linear pSVori was added as the competitor DNA, replication products were digested with *Sal*I instead of *Eco*RI to separate competitor DNA from the pSVori substrate. pSVori contains one *Sal*I cleavage site. Results were expressed as the percentage of pSVori replication observed in the absence of competitor DNA and plotted as a function of the molar ratio of competitor DNA to pSVori DNA. The best fit to a straight line was determined by computer to be exponential, with a regression coefficient of ≥0.99. pSVori replication in the presence of linear pSV21 was 7.5% at a molar ratio of 8. Representative experiments with circular (Form I) and linear (Form III) pML-1, pSV21core, pSVori, and pSV21 (Fig. 1) competitor DNA are shown. Results for these and other plasmids are summarized in Table 1.

I (pSV1) were four- to ninefold less effective than *ori*-core (Table 1). Addition of either of these auxiliary sequences to *ori*-core (pSV21core and pSVcore1) had no significant effect, although addition of both sequences (pSVori) increased the affinity of *ori*-core for this initiation factor by 1.6-fold. The importance of *ori*-core in binding initiation factor was confirmed with pSVoriΔ6, which contained a 6-bp deletion at the center of *ori*-core that eliminated its ability to replicate and significantly reduced its ability to bind initiation factor.

The role of DNA conformation in binding SV40-specific replication factors was also considered. Circular superhelical DNA was equivalent to circular DNA topologically relaxed by topoisomerase I in its ability to compete for initiation factors (data not shown), perhaps because superhelical DNA was rapidly converted into relaxed DNA when added to cell extracts. Linear and circular DNAs containing the *ori*-core component were also equivalent as competitor DNAs (Fig. 3; Table 1), revealing that the failure of linear DNA to replicate efficiently is not reflected in its ability to bind replication factor(s). However, binding of replication factors to DNA that did not contain *ori*-core was strongly affected by DNA topology: pSV1, pSV21, pPyVori, and

TABLE 1. Relative abilities of different SV40 *ori*- configurations to inhibit pSVori replication in vitro by competition for initiation factors

Plasmid[a]	*ori* configuration	Inhibition by competitor DNA:			
		Circular		Linear	
		Molar ratio[b]	Inhibition activity[c]	Molar ratio	Inhibition activity
pSVori	21s-core-I	1.9	100	2.3	100
pSVoriΔ6	21s-co(Δ6)re-I	4.9	39	3.3	70
pSVcore	core	3.1	61	1.8	128
pSV21core	21s-core	3.4	56	4.4	52
pSVcore1	core-I	3.4	56	4.9	47
pSV21	21s	13	15	1.8	128
pSV1	I	29	7	6.3	36
pPyVori	α-β-core	160	1	7.1	32
pML-1	None	509	0.4	11.6	20

[a] Plasmids are described in Fig. 1.
[b] Molar ratio is the ratio of competitor DNA to pSVori DNA that gave 50% inhibition of pSVori replication as determined from two to four independent experiments such as the one shown in Fig. 3. The standard error of the mean varied from 3.5 to 8.8% of the value shown.
[c] Inhibition activity was calculated from the molar ratio data by defining pSVori as 100% inhibition activity.

pML-1 were 5-, 7-, 22-, and 44-fold, respectively, more effective at inhibiting SV40 DNA replication as linear rather than circular molecules. To test the possibility that replication factors were binding to the ends of linear molecules, we digested pSVori DNA with a variety of restriction enzymes to produce ends with different structures and to generate different numbers of ends per mole of DNA. The extent of inhibition was dependent only on the competitor/substrate molar ratio and was independent of the number of ends present, the structure of the ends, and the position of *ori* relative to the ends (data not shown). Thus, linear DNA had a greater nonspecific affinity for replication factors than circular DNA did.

T-ag is the limiting DNA-binding initiation factor in SV40-infected cellular extracts. The monophasic titration curves represented in Fig. 3 indicated that a single binding component was being removed from the replication assay by addition of competitor DNA, although this component might consist of a multifactor complex. To identify this component, we inhibited pSVori replication by addition of competitor DNA and then restored its activity by addition of cellular or viral components. Thus, sufficient circular pSVori or pSVcore competitor DNA was added to an in vitro reaction mixture to reduce pSVori replication by 90%. Cytosol or high-salt nuclear extract (see Materials and Methods) from uninfected CV-1 cells failed to restore replication activity, whereas high-salt nuclear extract from SV40-infected CV-1 cells restored full activity (data not shown). This cellular fraction, which was rich in SV40 T-ag, could be replaced completely by immunoaffinity-purified SV40 T-ag (Fig. 4A). Therefore, the replicationally active form of SV40 T-ag appears to be the DNA-binding protein in lowest concentration in extracts of SV40-infected cells.

Linear forms of pSVori and pSVcore were as effective as their circular forms in depleting initiation factors in extract (Table 1). Again, immunoaffinity-purified T-ag restored activity (Fig. 4A). The linear form of pSV21, which was as effective a competitor as either circular or linear forms of pSVcore, also was found to remove T-ag from the replication assay (Fig. 4A). Therefore, the replicationally active form of T-ag can bind to *aux-2* as well as to *ori*-core,

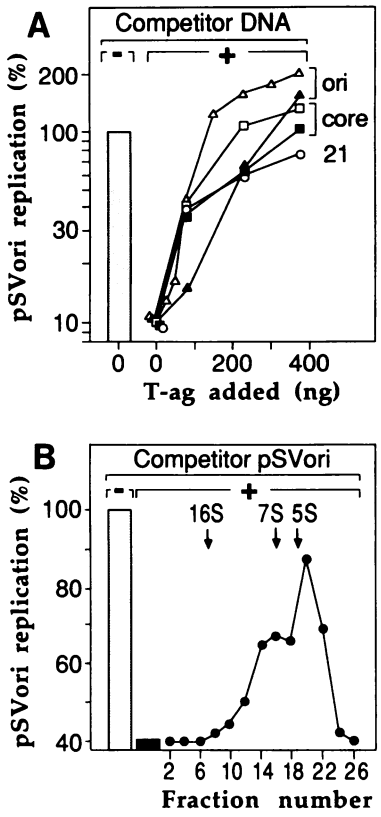

FIG. 4. Reconstitution of pSVori replication with immunoaffinity-purified T-ag in cell extracts that were inactivated by competitor DNA. (A) Extracts from SV40-infected CV-1 cells were incubated with the indicated amount of immunopurified SV40 T-ag, 100 ng of pSVori DNA (substrate), and the amount of pSVori, pSVcore, or pSV21 superhelical (solid symbols) or linear (open symbols) competitor DNA that was required to reduce pSVori replication to 10% (Fig. 2; Table 1). pSVori replication was then determined as described in the legend to Fig. 2. (B) T-ag monomer restores pSVori replication in cell extracts depleted of initiation factor by excess superhelical pSVori competitor DNA. Purified T-ag (20 μg) was sedimented through a continuous 5 to 40% glycerol gradient in 50 mM HEPES (pH 7.8)–1 mM dithiothreitol–100 mM potassium acetate–50 μg of phenylmethylsulfonylfluoride per ml in a Beckman SW60 rotor for 14 h at 52,000 rpm (4°C). Sedimentation markers (serum albumin, 4.3S, 66 kilodaltons; alcohol dehydrogenase, 7.6S, 150 kilodaltons; apoferritin, 17.6S, 443 kilodaltons) were fractionated simultaneously in a separate gradient. Fractions were collected from the bottom of the gradient, and 40-μl samples were tested for their ability to reconstitute pSVori replication in the presence of sufficient pSVori competitor DNA to reduce replication to 40% of control levels. pSVori replication was determined as described in the legend to Fig. 2.

although its affinity for *aux-2* is strongly dependent on the DNA topology.

SV40 large T-ag is known to occur in several oligomeric states (5) and to form specific complexes with cellular proteins such as DNA polymerase α (46), oncogene product p53 (6, 21), and transcription factor AP-2 (39). Thus, it was possible that the activity that restored replication in depleted cell extracts was a cell factor that copurified as a complex with T-ag. Such a complex should sediment more rapidly than T-ag monomer (39). Therefore, a sample of immunoaffinity-purified T-ag was fractionated by sedimentation through a glycerol gradient, and individual fractions were tested for their ability to restore SV40 replication in extracts that had been partially inhibited by the addition of pSVori DNA.

Although T-ag was distributed throughout the gradient, only T-ag present in the T-ag monomer and T-ag dimer regions of the gradient was able to reconstitute SV40 DNA replication in vitro (Fig. 4B). These were also the only fractions of T-ag that supported SV40 DNA replication in extracts of uninfected CV-1 or HeLa cells (data not shown). Therefore, the replicationally active form of T-ag itself is the SV40 *ori* DNA-binding protein that is rate limiting in virus-infected CV-1 cells.

***ori*-auxiliary sequences facilitate T-ag-dependent, *ori*-specific DNA unwinding.** The small effect of *ori*-auxiliary sequences on binding of the replicationally active form of T-ag to *ori* relative to their effect on DNA replication suggested that the primary role of *ori*-auxiliary sequences occurred after T-ag was bound to *ori*. After the formation of a complex, the next step in SV40 replication uses T-ag ATP-dependent helicase activity (47) to unwind the SV40 *ori* region (10, 20, 47, 54) and provide a single-stranded template for the enzymatic machinery that initiates DNA replication. Therefore, the effect of *ori*-auxiliary sequences on the ability of T-ag to unwind pSVori DNA was assayed under conditions in which *ori*-auxiliary sequences stimulated the replication of pSVori DNA in vitro.

pSVori DNA, topologically relaxed by treatment with calf thymus topoisomerase I, was incubated at 37°C with T-ag, topoisomerase I, and ATP in the presence of a low-salt extract from HeLa cells (7). When all four deoxynucleoside triphosphates were also added to HeLa cytosol supplemented with T-ag, replication in pSVori was up to 30-fold more efficient than in pSVcore (24). Plasmid DNA was then purified, and topoisomers were fractionated by gel electrophoresis and detected by blotting-hybridization with pSVori [^{32}P]DNA. Unwound DNA appeared in the form of several topoisomers migrating slightly more slowly than form I DNA (Fig. 5A). The fraction of DNA substrate that was unwound was quantified as the ratio of rapidly migrating [^{32}P]DNA to total [^{32}P]DNA in each gel lane.

The formation of unwound DNA was a time-dependent process (Fig. 5A and B) that required the presence of T-ag (Fig. 5A and C), cytosol extract (Fig. 5A), and a functional SV40 origin of replication (Fig. 5A to C). DNA unwinding did not result from DNA replication, because addition of [^{32}P]dCTP alone produced no detectable [^{32}P]DNA and addition of 50 μM aphidicolin had no effect on the unwinding reaction. Furthermore, the products of DNA unwinding were completely sensitive to *Dpn*I digestion. Under optimal conditions derived from the data in Figure 5, the extent of pSVori unwinding was at least 15-fold greater than that of pSVcore (Fig. 5A). pSVoriΔ6, which was inactive in DNA replication assays, was also inactive in DNA-unwinding assays. These data demonstrated a role for the *ori*-auxiliary sequences in facilitating T-ag-dependent unwinding of DNA containing a functional SV40 *ori* region.

***ori*-auxiliary sequences stimulate the initial step in *ori* unwinding.** The effects of *ori*-auxiliary sequences on the initial steps in SV40 T-ag-dependent, *ori*-dependent DNA unwinding were examined by incubating small covalently closed DNA circles containing one or more SV40 *ori* components with saturating amounts of T-ag, topoisomerase I, and ATP. Under these conditions, small changes in the topological linking number result in large changes in DNA conformation, which are readily observed by gel electrophoresis (41). The actual rate of migration depends on what fraction of the change in linking number is represented by changes in helical

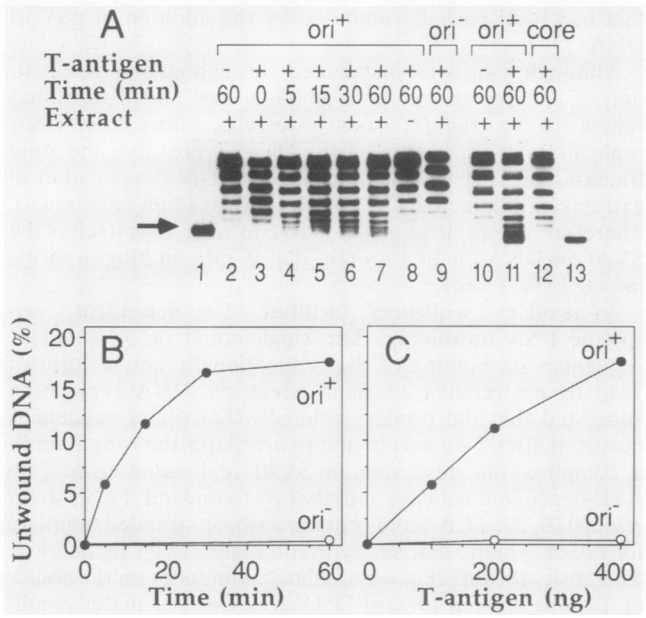

FIG. 5. Auxiliary sequences stimulate SV40 *ori*-specific, T-ag-dependent unwinding of DNA in cell extracts. (A) Topologically relaxed pSVori (ori$^+$), pSVoriΔ6 (ori$^-$), or pSVcore (core) DNA was incubated with T-ag, topoisomerase I, ATP, an ATP-regenerating system, and HeLa cell extract for the times indicated, and topoisomers were fractionated by gel electrophoresis as described in Materials and Methods. Superhelical pSVori (form I DNA) was used as a standard (lanes 1 and 13). The arrow slightly above this marker indicates the position of unwound DNA products. The distribution of topoisomers observed in the absence of T-ag (lanes 2 and 10), cell extract (lane 8), or a functional origin (lane 9) are also shown. Lanes 2 to 9 and lanes 10 to 12 were taken from two independent experiments. (B and C) The fraction of DNA unwound in these and similar experiments were quantified as described in Materials and Methods and presented as a function of time (panel B) or T-ag concentration (panel C).

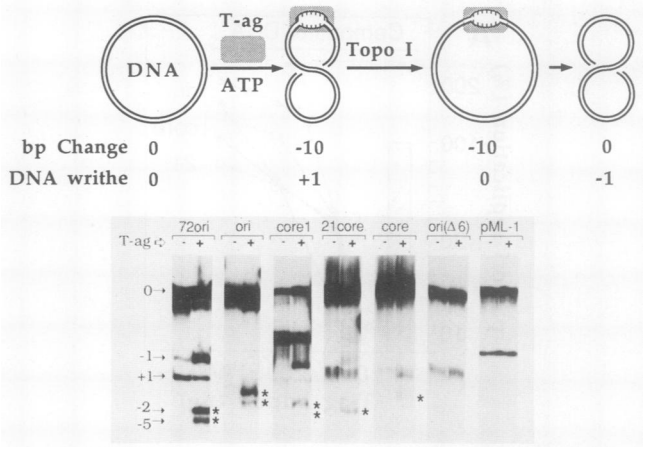

FIG. 6. Auxiliary sequences stimulate *ori*-specific, T-ag-dependent unwinding of SV40 *ori* by using purified T-ag and topoisomerase (Topo I). At the top is an illustration of how each 10 bp of DNA unwinding in a covalently closed DNA molecule can be detected as a change in DNA twist. Below this diagram are gels of the small DNA circles. The plasmids listed in Fig. 1 were used to prepare small [^{32}P]DNA circles (41) containing the SV40 *ori*-components indicated ([72ori, 571 bp; ori, 512 bp; core1, 629 bp; 21core, 552 bp; core, 516 bp; ori(Δ6), 506 bp; pML-1, 622 bp]). These DNA substrates were incubated with saturating amounts of purified T-ag and topoisomerase I in the presence of ATP (see Materials and Methods). Topological DNA isomers with linking differences of +1, 0, −1, −2 and −5 were fractionated by gel electrophoresis. The positions of topoisomers −2 and −5 for all functional *ori*-containing circles are indicated with asterisks. Each lane received 2,000 cpm of [^{32}P]DNA. Autoradiograms of the gels were aligned with relaxed DNA (0) bands in the register. Variations in DNA migration patterns resulted from the different sizes and sequence compositions of DNA substrates. The linking differences of the reaction products were determined both by comparison with standards generated by gyrase treatment of the template DNA and by examination of isolated products with topoisomerase I as previously described (41). The −1 topoisomer for large ori is just beneath the relaxed template DNA and is clearly visible in a lighter exposure.

twist and what fraction is represented by spatial writhe (9), which can account for the fact that small DNA circles slightly different in size can have the same linking number but different mobilities during gel electrophoresis. However, the fraction of DNA unwound by T-ag depended solely on the DNA sequence composition of the SV40 origin and not on plasmid size (Fig. 6).

In the absence of T-ag, topoisomerase I alone generates an equilibrium distribution of topoisomers that depends precisely upon the integral number of helical turns in the substrate minicircle (28, 45). With circles of the size used in this experiment (506 to 629 bp), this distribution contained primarily one or two topoisomers (the 0 and +1 topoisomers; Fig. 6, 72ori). A minicircle containing the 72-bp repeats in their normal position relative to *ori* (72ori) was included in these studies since the −1 and +1 topoisomers of the minicircle containing *ori* alone (Fig. 6, ori) were clearly distinguished from the 0 topoisomer only in autoradiogram exposures too brief to see the −2 and −5 topoisomers.

Incubation of the complete SV40 origin with T-ag, topoisomerase I, and ATP yielded three specific reaction products: the −1, −2, and −5 topoisomers (41) (Fig. 6, 72ori, ori). No T-ag-dependent products were obtained with minicircles lacking *ori* (Fig. 6, pML-1) or containing a nonfunctional *ori* [Fig. 6, ori(Δ6)]. These data are consistent with unwinding of 10, 20, and 50 bp in the *ori* region (Fig. 6, top). Alternatively, changes in the linking number could result from coiling of DNA around T-ag multimers, much as DNA is coiled around histone octamers.

The efficiency and extent of T-ag-dependent unwinding of *ori* were strongly dependent on the presence of *ori*-auxiliary sequences. Deletion of *aux-2* (Fig. 6, core1) reduced the efficiency of unwinding about 4-fold, deletion of *aux-1* (Fig. 6, 21core) reduced it about 5-fold, and deletion of both auxiliary elements (Fig. 6, core) reduced it more than 10-fold. This is readily apparent by inspecting the yield of −2 and −5 topoisomers (the unwound products most easily identified) relative to the remaining DNA forms. The enhancement of T-ag-dependent, *ori*-dependent DNA unwinding by *aux-1* and *aux-2* is consistent with their effects on DNA unwinding in HeLa cytosol (Fig. 5) and with their enhancement of SV40 DNA replication both in vivo and in vitro (Fig. 2) (24), suggesting that *aux-1* and *aux-2* facilitate *ori*-core replication activity by promoting T-ag-dependent unwinding of *ori*-core. *ori*-auxiliary sequences also affected the extent to which T-ag could unwind the *ori* region, since progressive deletion of *ori*-auxiliary components led to a progressive decrease of the −5, −2, and finally, −1 topoisomers. The subtle effect of SV40 enhancers on the −5 topoisomer (Fig. 6, compare 72ori with ori) was not detected in DNA replication studies (24).

We considered the possibility that *ori*-auxiliary sequences stimulated the binding of purified T-ag to purified DNA, in

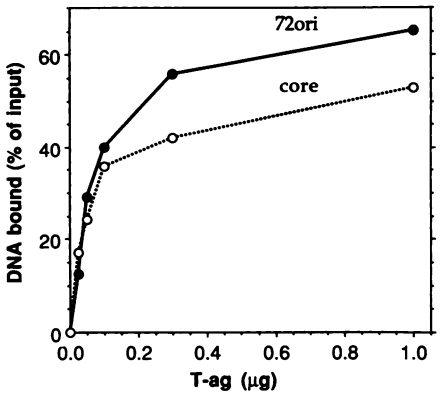

FIG. 7. Effect of auxiliary sequences on equilibrium binding of purified T-ag to purified circular DNA containing SV40 *ori*-components. [^{32}P]DNA minicircles (0.5 ng) containing either the complete origin (72ori) or the *ori*-core (core) were incubated at 37°C for 30 min with increasing amounts of immunoaffinity-purified T-ag under conditions identical to those used for the unwinding assay (see Materials and Methods). The extent of binding was measured by vacuum filtration through Millipore 0.45-μm-pore-size HA nitrocellulose filters, followed by washing with 10 ml of 20 mM HEPES-KOH (pH 7.4)–50 mM NaCl–1.5 mM $MgCl_2$. Under these conditions, control DNA fragments containing no T-ag binding sites were not retained on the filter.

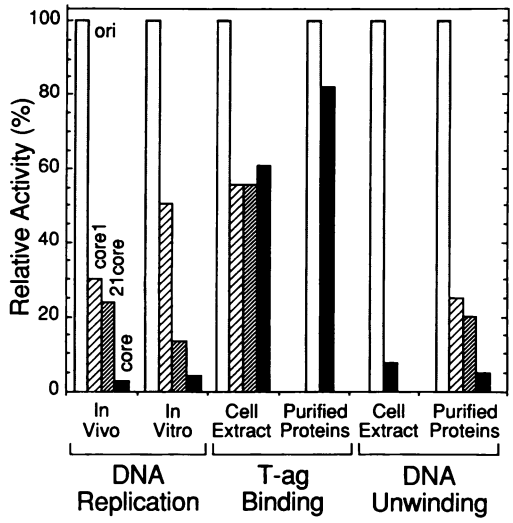

FIG. 8. Summary of experimental results. The relative activities of plasmids containing the indicated *ori* configuration are compared in six different assays. The rates of DNA replication following transfection of induced CMT-3 cells (DNA Replication in vivo) were taken from reference 24. The rates of DNA replication in extracts of SV40-infected CV-1 cells with or without the addition of saturating amounts of T-ag (DNA replication in vitro), inhibition of pSVori DNA replication in extracts from SV40-infected CV-1 cells resulting from competition for binding of the replicationally active form of T-ag (T-ag binding in cell extract), binding of purified T-ag to minicircle DNA (T-ag binding with purified proteins), unwinding of plasmid DNA in cell extracts (DNA unwinding in cell extract), and initiation of unwinding of minicircle DNA by T-ag (DNA unwinding with purified proteins) were taken from Fig. 2, Table 1, and Fig. 7, 5, and 6, respectively.

contrast to their effects on T-ag binding in cell extracts, and thus indirectly stimulated DNA unwinding. However, the affinity of [^{32}P]DNA minicircles for nitrocellulose filters in the presence of increasing amounts of purified T-ag revealed that binding of T-ag to *ori*-core at equilibrium was increased only slightly by its association with *aux-1* and *aux-2* (Fig. 7). Thus, the primary effect of *aux-1* and *aux-2* on SV40 DNA replication is mediated by their enhancement of T-ag-dependent unwinding of the replication origin, rather than by an effect on binding of T-ag to the replication origin.

DISCUSSION

***ori*-auxiliary sequences facilitate T-ag-dependent DNA unwinding rather than binding of the T-ag preinitiation complex.** The object of this study was to determine whether the role of SV40 *ori*-auxiliary components was to facilitate the binding of some viral or cellular replication factor to *ori* or to facilitate some step in the initiation of replication that occurs after the binding of initiation proteins. To this end, we took advantage of the recent discovery by Guo et al. (29) that the effects of *ori*-auxiliary sequences on the relative rates of SV40 *ori*-dependent DNA replication in monkey cells expressing T-ag (Fig. 8, DNA replication in vivo) could be reproduced by incubating the same DNA molecules in an extract of SV40-infected monkey cells (Fig. 8, DNA replication in vitro) or in HeLa cell cytosol supplemented with T-ag. These results were independent of the T-ag concentration in vivo or in vitro, suggesting that auxiliary sequences were not required for binding the T-ag preinitiation complex to *ori*. This hypothesis was confirmed in the present study by competitive titration of the DNA-binding factor or factors present in the lowest concentration in cell extracts (Fig. 3), which turned out to be SV40 T-ag monomer (Fig. 4). *ori* was only 1.6-fold more effective than *ori*-core alone in competing for that form of T-ag required to initiate SV40 DNA replication (Table 1; Fig. 8, T-ag binding in cell extract), whereas under the same conditions *ori* was 25-fold more effective than *ori*-core in replicating DNA (24). By measuring the ability of various *ori* components to compete for replication factors rather than measuring the binding of purified T-ag to DNA, only the relative affinities of these sequences for the replicationally active form of T-ag were observed; their affinity for the total T-ag population was not observed. The replicationally active form of T-ag probably represents a small fraction of the total T-ag in virus-infected cells that consists of newly synthesized, underphosphorylated T-ag monomers (23, 40, 44, 50). Thus, the affinity of active T-ag for *ori* was only slightly increased by the presence of auxiliary sequences, and this increase could not account for the much stronger effect of these auxiliary sequences on SV40 DNA replication. If cellular proteins are also involved in forming the complex that binds to *ori*, they must be present in excess over T-ag monomer in our cell extracts.

The affinity observed for T-ag binding to DNA regions I, II, and III (Fig. 1) depends on experimental conditions of pH, ionic strength, temperature, and cofactors (reviewed in reference 18). For example, in the absence of ATP, purified T-ag binds to region I more strongly than to region II or III, whereas in the presence of 2 to 4 mM ATP at 37°C, binding to region II is increased dramatically, such that the affinities for regions I and II are similar (3, 13). In addition, binding is also affected by particular posttranslational modifications such as phosphorylation (40, 44) and perhaps by association with cellular proteins (6, 21, 39, 46). Since the replicationally active form of T-ag binds strongly to region II (as defined by *ori*-core) and weakly to region I (Table 1), other T-ag molecules in the total population must account for the strong binding to region I observed in either the presence or absence of ATP (3, 13). *ori*-auxiliary sequences had little

effect on binding of the total T-ag population to *ori* when the ratio of T-ag to DNA was high (Fig. 7) (3), consistent with independent binding to two regions (regions I and II) with similar affinity for T-ag. Borowiec and Hurwitz (3) did observe an effect of *ori*-auxiliary sequences on total T-ag binding at low ratios, but interpretation of this observation is complicated because they used linear DNA fragments in which binding of the replicationally active fraction of T-ag to the 21-bp repeats and to region I was significantly increased (Table 1).

Since the effect of *ori*-auxiliary sequences on binding of the T-ag complex could not account for their effect on DNA replication, the role of *ori*-auxiliary sequences must be to facilitate one or more steps in replication that occur after T-ag binding. The activity of *ori*-auxiliary sequences cannot be explained as a requirement for transcription through *ori*-core because replication in vitro is insensitive to α-amanitin (14, 33). Nor does their activity require the presence of nucleosomes, because there is no correlation between the number of nucleosomes present and the effect of *ori*-auxiliary sequences (24). In fact, *ori*-auxiliary sequences can stimulate replication 30-fold in cytosol supplemented with T-ag (24), conditions under which no nucleosomes are assembled on the DNA (C. Gruss, C. Gutierrez, W. Burhans, T. Koller, M. DePamphilis, and J. Sogo, submitted for publication). However, there was a strong correlation between the ability of *ori*-auxiliary sequences to facilitate T-ag-dependent DNA unwinding of an *ori*-containing plasmid in cell extracts and their ability to stimulate the replication of the same plasmid (Fig. 8). Moreover, the same was true for the ability of purified T-ag and topoisomerase I to initiate DNA unwinding at *ori* in minicircle DNA under conditions where *ori*-auxiliary sequences had little effect on binding of purified T-ag to *ori*-core (Fig. 8). Therefore, one function of *ori*-auxiliary sequences is to promote the initial steps in DNA unwinding after the T-ag preinitiation complex binds to *ori*-core. Umek and Kowalski (52a) have suggested an analogous role for the readily melted 3′-auxiliary component of yeast autonomously replicating sequences.

How do *ori*-auxiliary sequences facilitate replication? All of the *cis*-acting DNA sequence information required to initiate DNA replication in SV40 (and its close relative, polyomavirus) is contained within *ori*-core; the flanking auxiliary sequences appear simply to increase the efficiency of initiation without altering its mechanism (19). We suggest that a primary function of SV40 *ori*-auxiliary sequences is to prevent unwound replication intermediates from dissociating before DNA primase-DNA polymerase α can initiate DNA synthesis. Strong T-ag binding to *ori*-core is eliminated as unwinding proceeds, because T-ag binds weakly and nonspecifically to single-stranded *ori* DNA (1). Thus, T-ag literally destroys the very DNA-binding site required for it to initiate DNA replication. Under these conditions, continued DNA unwinding may be facilitated through weak but specific binding of T-ag to *aux-1* and *aux-2*. In this way, *ori*-auxiliary sequences would facilitate the initiation of DNA synthesis by shifting the steady-state population of DNA molecules in favor of the transient unwound intermediates.

The replicationally active form of T-ag binds strongly to *ori*-core (T-ag binding region II) and weakly to *ori*-auxiliary sequences (T-ag binding regions I and III) and then begins unwinding DNA at the origin of bidirectional replication (Fig. 9). The −1 topoisomer produced by T-ag in Fig. 6 corresponds to the single-stranded DNA bubble observed by Borowiec and Hurwitz (4) at nucleotides 5210 to 5217 when T-ag binds to *ori* in linear DNA; both structures require ATP

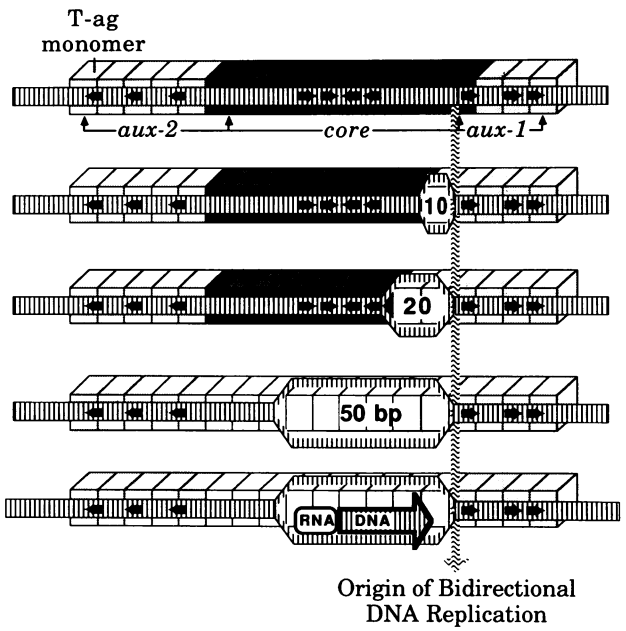

FIG. 9. A model that can account for the effects of *ori*-auxiliary sequences in T-ag-dependent DNA unwinding at *ori*-core is presented in the Discussion. Based on electron microscopy of T-ag binding to *ori*-core in the presence of ATP (37), 12 T-ag monomers are centered on the origin (nucleotide 5243/0) and extended over 89 ± 5 bp. T-ag monomers that bind strongly and specifically to *ori*-core (dark boxes) protect 80 to 84 bp from DNase I digestion (3, 13). Additional T-ag monomers (light boxes) that bind weakly but specifically to *aux-1* and *aux-2* (Table 1) were then added to accommodate the complete *ori*. Melting of *ori*-core results in single-stranded DNA that binds T-ag (open boxes) weakly and nonspecifically at physiological ionic strength (1) and eventually disrupts the four pentanucleotide recognition sequences (bold arrows) required for strong, specific T-ag binding to *ori*-core.

but not ATP hydrolysis (41). One boundary of this bubble corresponds precisely to the transition from discontinuous to continuous DNA synthesis that marks the origin of bidirectional replication (nucleotides 5210 to 5211 [25]). Topoisomers −2 and −5 could represent extensions of this bubble into *ori*-core to create the first initiation zone for DNA synthesis (25). Initiation of RNA-primed DNA synthesis occurs within *ori* only on the DNA template used for early mRNA synthesis and predominantly at sites between nucleotides 5239 and 30 (25, 51). The characteristics of these initiation sites are the same as those for Okazaki fragments throughout the genome (26), and therefore synthesis presumably uses the same proteins, including DNA primase-DNA polymerase α (19). Initiation of the first nascent DNA chain at one of these sites would account for the observation that newly synthesized DNA appears first on the early-gene side of *ori* (14).

T-ag must interact with *ori*-auxiliary sequences for them to facilitate replication. This was revealed by the fact that when the concentration of DNA substrate was low, *ori*-auxiliary sequences stimulated replication, but when the substrate DNA concentration was high, the replication rate was reduced to that observed in the absence of *ori*-auxiliary sequences (24). Therefore, either two or more different initiation factors or multiple copies of the same initiation factor must bind to *ori* for maximum replication activity. Since the replicationally active form of T-ag was the limiting *ori*-specific DNA-binding protein in these cell extracts (Fig.

3 and 4) (24), multiple T-ag monomers must bind to the same *ori* before *aux-1* and *aux-2* can facilitate *ori*-core activity. Some of these T-ag monomers must associate with *ori*-auxiliary sequences, because deletion of these sequences in the presence of excess T-ag had the same effect as reducing the pool of available T-ag by increasing the ratio of *ori* to cell extract; in both cases, the replication rate was reduced dramatically.

Although the affinity of the 21-bp repeats or T-ag-binding region I for T-ag was 5- to 10-fold lower than observed with *ori*-core, it was 14- to 30-fold higher than for the plasmid DNA sequence (Table 1). Therefore, *ori*-auxiliary sequences have a weak but specific affinity for the replicationally active form of T-ag. This could explain why mutations in the AT motif of *ori*-core that prevent replication can be suppressed by alterations in *aux-2* (22). Furthermore, the affinity of active T-ag for *aux-2* was increased sevenfold by conversion of the competitor DNA from a circular to a linear form (Table 1), suggesting that changes in DNA conformation that occur during *ori* unwinding could increase their affinity for T-ag. The importance of weak binding during T-ag-dependent DNA unwinding would not have been observed when measuring binding and dissociation rates of the total T-ag population with *ori*, because only a small fraction of T-ag–DNA complexes participated in the unwinding process under these assay conditions.

Although stimulation of T-ag-dependent DNA unwinding by *ori*-auxiliary sequences did not require any additional proteins other than topoisomerase I (Fig. 6), it is possible that other cellular proteins further facilitate this reaction. The ratio of *ori* to *ori*-core activity in extracts of SV40-infected CV-1 cells or uninfected HeLa cells was at least 10 times higher than in extracts of uninfected confluent monolayers of CV-1 cells (24). This may reflect the existence of an S-phase-specific factor that stimulates T-ag-dependent unwinding of *ori* (42). Enhancer-binding proteins may also participate. The SV40 enhancer region, which does not have T-ag-binding sites, can partially substitute for the 21-bp repeats in activating *ori*-core (8). Perhaps enhancer-binding proteins such as AP-2, which can also bind SV40 T-ag (39), can mediate the interaction between T-ag and *ori*-auxiliary sequences. This could explain how a variety of enhancers function as strong *ori*-auxiliary sequences in polyomavirus, in which T-ag binds weakly to *ori*-core. It would be analogous to the way in which NF1 facilitates the initiation of DNA replication by the adenovirus DNA polymerase-terminal protein complex (17).

The role of *ori*-auxiliary sequences in T-ag-dependent DNA unwinding must be coupled to the formation of replication forks. Although SV40 *ori*-auxiliary sequences can stimulate the formation of completely replicated DNA molecules 25-fold, they stimulate total DNA synthesis only 2- to 3-fold (24). Therefore, replication forks formed at *ori*-core are less likely to complete replication than are forks formed at a complete origin. These data suggest that *ori*-auxiliary components appear to facilitate the transition from a sequence-dependent T-ag preinitiation complex that initiates DNA unwinding to a sequence-independent T-ag postinitiation complex that unwinds DNA at replication forks. This hypothesis is currently under investigation.

ACKNOWLEDGMENTS

We are indebted to Peter Tegtmeyer for pOR-1, Linda Malkas and Earl Baril for AdSVR284 virus and advice on the purification of T-antigen, Bill Burhans for useful discussions, and Sharon Perry for assistance in preparation of the manuscript.

LITERATURE CITED

1. **Auborn, K. J., R. B. Markowitz, E. Wang, Y. T. Yu, and C. Prives.** 1988. Simian virus 40 (SV40) T antigen binds specifically to double-stranded DNA but not to single-stranded DNA or DNA-RNA hybrids containing the SV40 regulatory sequences. J. Virol. **62**:2204–2208.
2. **Bergsma, D. J., D. M. Olive, S. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. USA **79**:381–385.
3. **Borowiec, J. A., and J. Hurwitz.** 1988. ATP stimulates the binding of simian virus 40 large tumor antigen to the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **85**:64–68.
4. **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.
5. **Bradley, M. K., J. Hudson, M. S. Villaneuva, and D. Livingston.** 1984. Specific *in vitro* adenylation of the simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA **81**:6574–6578.
6. **Braithwaite, A. W., H.-W. Sturzbechter, C. Addison, C. Palmer, K. Rudge, and J. R. Jenkins.** 1987. Mouse p^{53} inhibits SV40 origin-dependent DNA replication. Nature (London) **329**:458–460.
7. **Bullock, P. A., Y. S. Seo, and J. Hurwitz.** 1989. Initiation of simian virus 40 DNA replication *in vitro*: pulse-chase experiments identify the first labeled species as topologically unwound. Proc. Natl. Acad. Sci. USA **86**:3944–3948.
8. **Chandrasekharappa, S. C., and K. N. Subramanian.** 1987. Effects of positions and orientation of the 72-base-pair-repeat transcriptional enhancer on replication from the simian virus 40 core origin. J. Virol. **61**:2973–2980.
9. **Crick, F. H. C.** 1976. Linking numbers and nucleosomes. Proc. Natl. Acad. Sci. USA **73**:2639–2643.
10. **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.
11. **Dean, F. B., M. Dodson, J. A. Borowiec, Y. Ishimi, G. S. Goetz, P. Bullock, S. W. Matson, H. Echols, and J. Hurwitz.** 1988. SV40 origin-dependent DNA unwinding and nucleoprotein complex formation by SV40 T antigen. Cancer Cells **6**:113–122.
12. **Deb, S., A. DeLucia, C.-P. Baur, A. Koff, 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**:1663–1670.
13. **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.
14. **Decker, R. S., M. Yamaguchi, R. Possenti, and M. L. DePamphilis.** 1986. Initiation of SV40 DNA replication in vitro: aphidicolin causes accumulation of early-replicating intermediates and allows determination of the initial direction of DNA synthesis. Mol. Cell. Biol. **6**:3815–3825.
15. **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.
16. **DeLucia, A. L., B. A. Lewton, R. Tjian, and P. Tegtmeyer.** 1983. Topology of SV40 A protein-DNA complexes: arrangement of pentanucleotide interaction sites at the origin of replication. J. Virol. **46**:143–150.
17. **DePamphilis, M. L.** 1988. Transcriptional elements as components of origins of eukaryotic DNA replication. Cell **52**:635–638.
18. **DePamphilis, M. L., and M. K. Bradley.** 1986. Replication of simian virus 40 and polyoma virus chromosomes, p. 99–246. *In* N. P. Salzman (ed.), The papovaviridae, vol. 1. Plenum Publishing Corp., New York.
19. **DePamphilis, M. L., E. Martinez-Salas, D. Y. Cupo, E. A. Hendrickson, C. E. Fritze, W. R. Folk, and U. Heine.** 1988. Initiation of polyomavirus and SV40 DNA replication, and the requirements for DNA replication during mammalian development. Cancer Cells **6**:165–175.

20. **Dodson, M., F. B. Dean, P. Bullock, H. Echols, and J. Hurwitz.** 1987. Unwinding of duplex DNA from the SV40 origin of replication by T antigen. Science **238**:964–967.
21. **Gannon, J. V., and D. P. Lane.** 1987. p^{53} and DNA polymerase α compete for binding to SV40 T antigen. Nature (London) **329**:456–458.
22. **Gerard, R., and Y. Gluzman.** 1986. Functional analysis of the role of the A+T-rich region and upstream flanking sequences in simian virus 40 DNA replication. Mol. Cell. Biol. **6**:4570–4577.
23. **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.
24. **Guo, Z.-S., C. Gutierrez, U. Heine, 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.
25. **Hay, R. T., and M. L. DePamphilis.** 1982. Initiation of simian virus 40 DNA replication in vivo: location and structure of 5′-ends of DNA synthesized in the *Ori* region. Cell **28**:767–779.
26. **Hay, R. T., E. A. Hendrickson, and M. L. DePamphilis.** 1984. Sequence specificity for the initiation of RNA primed-SV40 DNA synthesis *in vivo*. J. Mol. Biol. **175**:131–157.
27. **Hertz, G. Z., and J. Mertz.** 1986. Bidirectional promoter elements of simian virus 40 are required for efficient replication of the viral DNA. Mol. Cell. Biol. **6**:3513–3522.
28. **Horowitz, D. S., and J. C. Wang.** 1984. Torsional rigidity of DNA and length dependence of the free energy of DNA supercoiling. J. Mol. Biol. **173**:75–91.
29. **Innis, J. W., and W. A. Scott.** 1984. DNA replication and chromatin structure of simian virus insertion mutants. Mol. Cell. Biol. **4**:1499–1507.
30. **Jones, N. C., P. W. J. Rigby, and E. B. Ziff.** 1988. *Trans*-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
31. **Kelly, T. J.** 1988. SV40 DNA replication. J. Biol. Chem. **263**:17889–17892.
32. **Lee-Chen, G.-J., and M. Woodworth-Gutai.** 1986. Simian virus 40 DNA replication: functional organization of regulatory elements. Mol. Cell. Biol. **6**:3086–3093.
33. **Li, J. J., and T. Kelly.** 1984. Simian virus 40 DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **81**:6973–6977.
34. **Li, J. J., K. W. C. Peden, R. A. F. Dixon, and T. Kelly.** 1986. Simian virus 40 DNA replication: functional organization of regulatory elements. Mol. Cell. Biol. **6**:1117–1128.
35. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Mastrangelo, I., 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.
38. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46**:795–805.
39. **Mitchell, P., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription *in vitro*: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
40. **Mohr, I. J., B. Stillman, and Y. Gluzman.** 1987. Regulation of SV40 DNA replication by phosphorylation of T antigen. EMBO J. **6**:153–160.
41. **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.
42. **Roberts, J. M., and G. D'Urso.** 1988. An origin unwinding activity regulates initiation of DNA replication during mammalian cell cycle. Science **241**:1486–1489.
43. **Ryder, K., E. Vakalopoulo, R. Mertz, I. Mastrangelo, P. Hough, P. Tegtmeyer, and E. Fanning.** 1985. Seventeen base pairs of region I encode a novel tripartite binding signal for SV40 T antigen. Cell **42**:539–548.
44. **Schirmbeck, R., and W. Deppert.** 1988. Analysis of mechanisms controlling the interactions of SV40 large T antigen with the SV40 ORI region. Virology **165**:527–538.
45. **Shore, D., and R. Baldwin.** 1983. Energetics of DNA twisting. J. Mol. Biol. **170**:983–1007.
46. **Smale, S. T., and R. Tjian.** 1986. T-antigen DNA polymerase complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. **6**:4077–4087.
47. **Stahl, H., P. Dröge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5**:1939–1944.
48. **Stillman, B.** 1988. Initiation of eukaryotic DNA replication *in vitro*. BioEssays **9**:56–60.
49. **Stillman, B., R. D. Gerard, R. A. Guggenheimer, and Y. Gluzman.** 1985. T antigen and template requirements for SV40 DNA replication in vitro. EMBO J. **4**:2933–2939.
50. **Tack, L. C., J. H. Wright, and E. G. Gurney.** 1986. Free and viral chromosome-bound simian virus 40 T antigen: changes in reactivity of specific antigenic determinants during lytic infection. J. Virol. **58**:635–646.
51. **Taljanidisz, J., R. S. Decker, Z.-S. Guo, M. L. DePamphilis, and N. Sarkar.** 1987. Initiation of SV40 DNA replication in vitro: identification of RNA-primed nascent DNA chains. Nucleic Acids Res. **15**:7877–7888.
52. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
52a. **Umek, R. M., and D. Kowalski.** 1988. The ease of DNA unwinding as a determinant of initiation at yeast replication origins. Cell **52**:559–567.
53. **Wirak, D. O., L. E. Chalifour, P. M. Wassarman, W. J. Muller, J. A. Hassell, and M. L. DePamphilis.** 1985. Sequence dependent DNA replication in preimplantation mouse embryos. Mol. Cell. Biol. **5**:2924–2935.
54. **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.
55. **Yamaguchi, M., and M. L. DePamphilis.** 1986. DNA binding site for a factor(s) required to initiate simian virus 40 DNA replication. Proc. Natl. Acad. Sci. USA **83**:1646–1650.