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Initiation of Simian Virus 40 DNA Replication In Vitro: Aphidicolin Causes Accumulation of Early-Replicating Intermediates and Allows Determination of the Initial Direction of DNA Synthesis

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Aphidicolin, a specific inhibitor of DNA polymerase α, provided a novel method for distinguishing between initiation of DNA synthesis at the simian virus 40 (SV40) origin of replication (*ori*) and continuation of replication beyond *ori*. In the presence of sufficient aphidicolin to inhibit total DNA synthesis by 50%, initiation of DNA replication in SV40 chromosomes or *ori*-containing plasmids continued in vitro, whereas DNA synthesis in the bulk of SV40 replicative intermediate DNA (RI) that had initiated replication in vivo was rapidly inhibited. This resulted in accumulation of early RI in which most nascent DNA was localized within a 600- to 700-base-pair region centered at *ori*. Accumulation of early RI was observed only under conditions that permitted initiation of SV40 *ori*-dependent, T-antigen-dependent DNA replication and only when aphidicolin was added to the in vitro system. Increasing aphidicolin concentrations revealed that DNA synthesis in the *ori* region was not completely resistant to aphidicolin but simply less sensitive than DNA synthesis at forks that were farther away. Since DNA synthesized in the presence of aphidicolin was concentrated in the 300 base pairs on the early gene side of *ori*, we conclude that the initial direction of DNA synthesis was the same as that of early mRNA synthesis, consistent with the model proposed by Hay and DePamphilis (Cell 28:767–779, 1982). The data were also consistent with initiation of the first DNA chains in *ori* by CV-1 cell DNA primase-DNA polymerase α. Synthesis of pppA/G$(pN)_{6-8}(pdN)_{21-23}$ chains on a single-stranded DNA template by a purified preparation of this enzyme was completely resistant to aphidicolin, and further incorporation of deoxynucleotide monophosphates was inhibited. Therefore, in the presence of aphidicolin, this enzyme could initiate RNA-primed DNA synthesis at *ori* first in the early gene direction and then in the late gene direction, but could not continue DNA synthesis for an extended distance.

Simian virus 40 (SV40) is a small (5.2 kilobases [kb]) DNA virus that replicates in the nuclei of permissive monkey or human cells as a circular chromosome whose nucleosome structure and histone composition are indistinguishable from that of its host (reviewed in reference 5 and in M. L. DePamphilis and M. K. Bradley, *in* N. P. Salzman, ed., *The Viruses: Polyoma Viruses*, in press). With the exception of initiation of viral DNA replication, all steps in the replication and assembly of viral chromosomes are carried out exclusively by cellular components. Initiation of SV40 DNA replication requires a *cis*-acting sequence that functions as the origin of replication (*ori*), SV40 large tumor antigen (T-ag), and one or more permissive cell factors. Bidirectional replication then begins at the junction between the strongest DNA-binding site for T-ag and the *ori* core. Analysis of initiation sites for RNA-primed DNA synthesis in SV40 replicating intermediates led to the hypothesis that initiation of DNA synthesis at *ori* involves the same mechanism used to synthesize Okazaki fragments at replication forks throughout the genome (11, 12), suggesting that DNA synthesis at *ori* is initiated by DNA primase-DNA polymerase α. These data also indicate that DNA synthesis at *ori* begins in the same direction as early mRNA synthesis.

The evidence is compelling that DNA polymerase α is solely responsible for DNA synthesis on both sides of SV40 replication forks. Aphidicolin, a specific inhibitor of α-polymerase (25), inhibits extension of long DNA chains and Okazaki fragments in replicating SV40 chromosomes (6, 14, 15, 37) as well as completion of Okazaki fragments (i.e., gap-filling) (14). In contrast, 2′,3′-dideoxythymidine triphosphate, under conditions that inhibit β- and γ-polymerases with little effect on α-polymerase, has no effect on any of these events (8, 15, 35, 37). Furthermore, only α-polymerase was able to reconstitute viral DNA replication in *N*-ethylmaleimide-inactivated nuclear extracts (15), and the extent of synthesis in viral chromosomes is proportional to the fraction of active α-polymerase (22, 37). Two questions that remain unanswered are: (i) is DNA primase-DNA polymerase α required for initiation of DNA replication, and (ii) what is the initial direction of DNA synthesis at *ori*?

Until recently, subcellular systems that supported SV40 DNA replication were limited to faithfully continuing the processes of DNA replication and chromatin assembly at active replication forks, separating the two sibling chromosomes when replication forks arrive at the termination region, and completing the final steps in the production of covalently closed, superhelical DNA monomers (form I DNA; reviewed in reference 5). With the availability of large quantities of pure T-ag and a sensitive assay for SV40 *ori*-dependent plasmid DNA replication, several laboratories have demonstrated that these initiation-deficient extracts are capable of T-ag-dependent, *ori*-specific initiation of bidirectional SV40 DNA replication (1, 17, 27, 39). Alternatively, in the absence of added T-ag or extracts from cells infected with a viral mutant that overproduces T-ag, initiation of SV40 DNA replication was achieved both in SV40 chromo-

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somes (present communication; R. S. Decker, M. Yamaguchi, M. K. Bradley, and M. L. DePamphilis, submitted for publication) and in plasmids containing SV40 *ori* (40) by addition of a hydrophilic polymer to extracts of wild-type SV40-infected permissive monkey cells. Using this system, we observed that initiation of DNA synthesis in the *ori* region of SV40 was much less sensitive to inhibition by aphidicolin than was DNA synthesis in replicative intermediates that had already initiated replication. This resulted in accumulation of DNA that was less than 10% replicated. Analysis of the distribution of nascent DNA with respect to *ori* revealed that most of these molecules had initiated replication in the direction of early mRNA synthesis, in agreement with the model of Hay and DePamphilis (11).

MATERIALS AND METHODS

Cells, virus, and plasmids. The African green monkey kidney cell line CV-1 was cultured in Dulbecco modified Eagle medium supplemented with 10% calf serum as previously described (12). CV-1 cells were infected at approximately 80% confluency with SV40 wt800 (12) at 40 to 50 PFU/cell and incubated at 37°C for 1 h. Fresh medium containing penicillin and streptomycin was then added to the cultures, and incubation was continued for 36 to 38 h. pSVori (2,643 base pairs [bp]) was made by replacing nucleotides 29 to 562 of plasmid vector pML-1 with a 206-bp segment of SV40 DNA containing the origin of replication (40).

Preparation of cellular fractions. (i) Cytosol. From 36 to 38 h after CV-1 cells were infected with SV40, viral DNA was radiolabeled for 1.5 h by replacing the medium in each 15-cm-diameter dish with 1 ml of isotonic buffer (20 mM Tris hydrochloride, pH 7.4, 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) containing 50 μCi of [^{3}H]thymidine (70 Ci/mmol; New England Nuclear Corp.) and 10% calf serum. This allowed all measurements of [α-^{32}P]deoxynucleotide triphosphate (dNTP) incorporation into total acid-insoluble products to be normalized to the amount of [^{3}H]DNA in the reaction. The following steps were carried out at 0 to 4°C. Cell monolayers were washed twice with isotonic buffer plus 250 mM sucrose and then once with hypotonic buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.8, 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol). Excess buffer was removed by stringent aspiration, and the cells were scraped free with a rubber policeman. Cell lysis was completed in a Dounce homogenizer (Wheaton) with three passes of pestle B. Nuclei were removed by centrifugation at 1,200 × *g* for 5 min and used to prepare the high-salt nuclear extract. This supernatant was centrifuged at 100,000 × *g* for 1 h in a Beckman type 50 Ti rotor, and the supernatant (cytosol) was stored at −70°C. Ten dishes of cells routinely yielded 2.5 ml of cytosol (7 to 12 μg of protein per μl [18]).

(ii) High-salt nuclear extract. Nuclei from 10 15-cm plates were suspended in 1.3 ml of hypotonic buffer plus 500 mM potassium acetate and extracted for 90 min on ice with occasional gentle vortexing to disperse nuclei aggregates. Nuclei were then removed by centrifugation at 8,000 × *g* for 10 min. SV40 nucleoprotein complexes (chromosomes) in these nuclear extracts were isolated by centrifugation at 300,000 × *g* for 1 h in a Beckman SV60 Ti rotor. The resulting supernatants (5 to 9 μg of protein per μl) were at least 99% free of ^{3}H-chromosomes and were stored at −70°C.

(iii) SV40 chromosomes. SV40 chromosomes containing [^{3}H]DNA constituted the pellet fraction from the high-salt nuclear extract preparation. This pellet was suspended in hypotonic buffer at 1/4 the original volume of nuclear extract for 1 h on ice with occasional gentle mixing by drawing into a pipette to disperse the pellet. The completeness of suspension was assessed by briefly centrifuging the mixture in an Eppendorf benchtop microfuge. Fully dispersed chromatin was stored at −70°C.

Initiation of DNA replication in SV40 chromosomes. In vitro replication was routinely carried out in 50-μl reaction volumes by combining 15 μl of cytosol and 8 μl of high-salt nuclear extract with 2 μl of SV40 chromosomes and then adding 6 μl of a mixture containing 140 mM HEPES, pH 7.8, 50 mM phosphoenolpyruvate, 17 mM ATP, 0.83 mM each CTP, GTP, UTP, dATP, and dGTP, and 83 μM each dCTP and dTTP, and 2 to 20 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dTTP (3,000 Ci/mmol; New England Nuclear), 0.8 μl of 3.75 M sucrose, 0.3 U of pyruvate kinase in 50% glycerol (Boehringer Mannheim Biochemicals), 2.5 μl (1 μg) of T-ag (where indicated), and 8 μl of a solution containing 125 mM HEPES, pH 7.8, 32 mM $MgCl_2$, 1.5 M ethylene glycol, 32% polyethylene glycol (14,000 M_r; Aldrich Chemical Co.), and 6 mM EGTA. Unlabeled nucleotides were purchased from P-L Biochemicals. Final reaction conditions were 45 mM HEPES, pH 7.8, 90 mM potassium acetate, 5 mM $MgCl_2$, 0.4 mM dithiothreitol, 1 mM EGTA, 60 mM sucrose, 240 mM ethylene glycol, 5% polyethylene glycol, 2 mM ATP, 100 μM each CTP, GTP, and UTP, 100 μM each dATP and dGTP, 10 μM each dCTP and dTTP, and 6 mM phosphoenolpyruvate. When aphidicolin (gift of the National Cancer Institute) was included, it was added from a stock solution of 2 mg/ml in dimethyl sulfoxide. The same volume of dimethyl sulfoxide alone had no effect on SV40 DNA replication. Reactions were incubated at 30°C for 1 h unless otherwise indicated. Reactions were terminated at 0°C by addition of one volume of 1% sodium dodecyl sulfate and 30 mM EDTA. Yeast tRNA (10 to 15 μg; Bethesda Research Laboratories) was added, and the mixture was digested with 200 U of proteinase K (Boehringer Mannheim Biochemicals) at 37°C for 1 h. Samples were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), once with chloroform-isoamyl alcohol (24:1), and twice with ether. Nucleic acids were precipitated at −70°C in 70% ethanol once in the presence of 300 mM sodium acetate and twice in the presence of 2 M ammonium acetate to concentrate the DNA and remove free ^{32}P-nucleotides.

Preparation of SV40 T-ag. Purified SV40 T-ag, kindly provided by Beth Weiner and Margaret Bradley, was prepared from Ad5SVRIII-infected human 293 cells or from SV40 cs1085-infected CV-1 cells by immunoaffinity chromatography with monoclonal antibody pAb419 (7, 24).

Restriction endonucleases. DNA was digested with restriction enzymes as suggested by Maniatis et al. (19). *Bst*NI was used under conditions of low ionic strength (10 mM Tris hydrochloride, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of gelatin per ml) at 60°C for 3 h under paraffin oil. Identical results were achieved under medium ionic strength conditions (as above plus 50 mM NaCl) and under conditions specified by the manufacturer. All other restriction enzymes were used under medium ionic strength conditions at 37°C for 3 h. All enzymes were purchased from New England Biolabs and used at a concentration of 4 U/μg of DNA.

Gel electrophoresis. SV40 DNA was purified from in vitro reactions and then fractionated by electrophoresis in 0.6% agarose gels in Tris-borate-EDTA (TBE) buffer (19). DNA restriction fragments were fractionated in a 6% polyacrylamide gel (29:1, acrylamide-bisacrylamide) in TBE buffer.

The gel was fixed in 10% acetic acid–25% methanol, dried, and analyzed by autoradiography. To discriminate between ^{3}H and ^{32}P, Kodak AR-5 film was used with a Cronex-Plus intensifying screen (E. I. du Pont de Nemours) and exposed at −70°C (16). Several exposures of each gel were scanned with a Joyce-Lobel MK-III microdensitometer to ensure that peak area was proportional to [α-^{32}P]DNA content and the results were corrected for individual fragment length. A standard curve was constructed by using SV40 form III [5′-^{32}P]DNA.

Isolation of early RI. When early replicative intermediate molecules (early RI) were analyzed by digestion with restriction endonucleases, the early RI were isolated from 0.6% low-melting-point agarose (Bethesda Research Laboratories) by first soaking the electrophoretic gels in ethidium bromide (0.5 μg/ml); the DNA was visualized under long-wavelength UV light, and the region between form I and form III SV40 DNA was excised. These agarose sections were frozen, crushed, and incubated in 2 ml of 10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA–100 mM NaCl–80 μg of yeast tRNA at 65°C for 30 min. Samples were then brought to 37°C, extracted three times with phenol (pH 8.0, 37°C), once with phenol-chloroform-isoamyl alcohol, and once with ether. DNA was precipitated in ethanol as above and suspended in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–20 mM NaCl, and this procedure was repeated twice. At least 70% of the DNA was recovered, as judged in a separate experiment with ^{32}P-labeled SV40 form III [5′-^{32}P]DNA.

RESULTS

Specific accumulation of SV40 early RI in the presence of aphidicolin. Conditions have been optimized in a cellular extract of SV40-infected CV-1 cells to promote initiation of SV40 chromosome replication in vitro (Materials and Methods). After 45 to 60 min of incubation, the major viral DNA product in this system was RI at various stages of replication (Fig. 1, lane 0). The genomic distribution of nascent DNA in this RI was consistent with bidirectional replication from *ori*, and further incubation in vitro converted these intermediate RI into RI that had completed at least 90% of their replication (RI*) as well as into covalently closed, superhelical, circular SV40 DNA monomers (form I) (Decker et al., submitted for publication). Most initiation events occurred during the first 20 to 40 min following an initial delay of 10 to 15 min (Fig. 2). Initiation of DNA replication depended on the presence of a high-salt nuclear extract from SV40-infected monkey cells. The major active component of this extract was SV40 T-ag, since purified T-ag could substitute for the high-salt nuclear extract, and addition of monoclonal antibodies directed against T-ag specifically inhibited initiation of DNA replication (R. Possenti, R. S. Decker, M. K. Bradley, and M. L. DePamphilis, submitted for publication). DNA replication also depended on the SV40 *ori* sequence, since plasmid DNA containing a functional SV40 *ori* replicated efficiently in these extracts, whereas plasmid DNA alone or carrying a defective SV40 *ori* or a functional

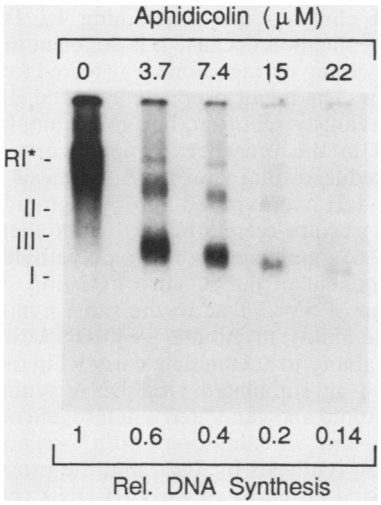

FIG. 1. SV40 DNA replication in vitro in the presence of increasing concentrations of aphidicolin. SV40 chromosomes were incubated for 45 min at 30°C with cytosol and high-salt nuclear extract from SV40-infected CV-1 cells supplemented with purified T-ag. Aphidicolin was added at the beginning of the reaction at the concentrations indicated (2.95 μM = 1 μg/ml). DNA was purified and fractionated by electrophoresis in 0.6% agarose, and [^{32}P]DNA was identified by autoradiography. Equal volumes of each reaction mixture were analyzed. The relative amounts of nucleotide incorporation are indicated at the bottom of each lane. The positions of SV40 form I, II, III, and RI* DNA, run in the same gel, are indicated.

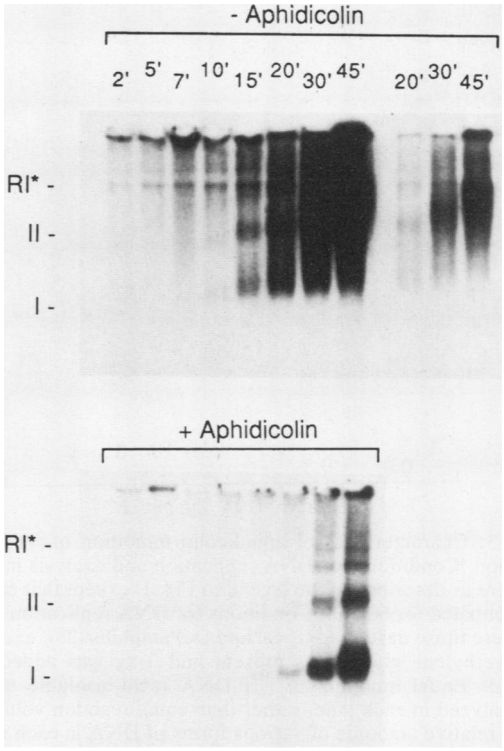

FIG. 2. Time course of SV40 DNA replication in the presence and absence of aphidicolin. Conditions for DNA replication and analysis were as described in the legend to Fig. 1. Individual reactions were stopped at the times indicated (in minutes) over each lane. The three lanes on the extreme right of the upper panel are shorter film exposures of the autoradiogram shown on the left. The same volume of sample was applied to each lane. Where indicated, DNA replication was carried out in the presence of 9 μM aphidicolin, which inhibited total DNA synthesis 70% at 45 min. To quantitate the amount of newly synthesized early RI, the gel was dried onto Whatman DE-81 paper and exposed to X-ray film, the region containing early RI DNA was excised and eluted in 1 ml of H_2O at 65°C, and the radioactivity was determined by liquid scintillation counting in Ultrafluor (National Diagnostics).

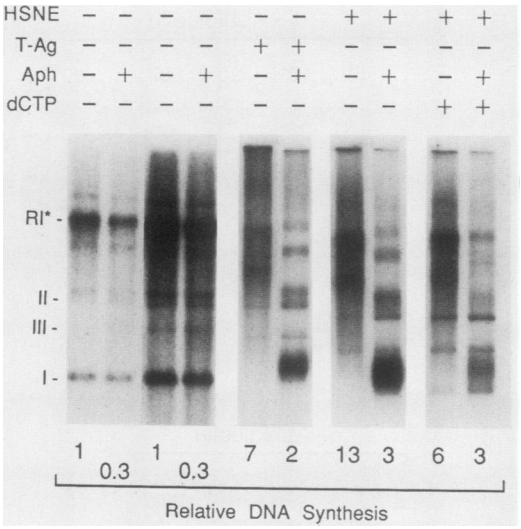

FIG. 3. Characteristics of aphidicolin inhibition of SV40 DNA replication. Conditions for DNA replication and analysis in lanes 7 to 10 were as described in the legend to Fig. 1, except that reactions were incubated for 60 min. Conditions for DNA replication in lanes 1 to 6 were those described by Su and DePamphilis (28), except that 5% polyethylene glycol was present and T-ag was added where indicated. Equal amounts of [^{32}P]DNA (acid-insoluble material) were analyzed in each lane, rather than equal reaction volumes, so that the relative amounts of various forms of DNA in each reaction could be compared directly. Dried gels were exposed to X-ray film for 8 h at −70°C (lanes 3 and 4 are the same as lanes 1 and 2 except that they were exposed for 24 h). The reactions in these experiments contained a slightly higher proportion of form II and III DNA than the experiments shown in Fig. 1 and 2, reflecting variation in cellular extracts and their levels of damage and repair enzymes. HSNE, High-salt nuclear extract; Aph, aphidicolin.

polyoma virus *ori* did not replicate (40). Finally, as described below, partial inhibition of SV40 DNA synthesis by aphidicolin, a specific inhibitor of DNA polymerase α (25), resulted in accumulation of SV40 replicating chromosomes with only their *ori* regions replicated.

Aphidicolin was added to the in vitro system containing SV40 chromosomes to assess its effect on initiation of SV40 DNA replication. As the concentration of aphidicolin was increased from 3.7 to 50 μM, SV40 DNA synthesis decreased from 60 to 5% of the uninhibited rate, with the concomitant accumulation of early SV40 RI DNA (Fig. 1); at 95% inhibition, no DNA products were detected (data not shown). At 40 to 60% inhibition, three distinct DNA products were recognized. The most prominent of these migrated between form I DNA and linear molecules one genome in length (form III), characteristic of intact SV40 RI at an early stage in their replication. A second, less prominent DNA species migrated slightly slower than topologically relaxed, circular monomers of SV40 DNA (form II), corresponding to early RI molecules whose parental DNA strands were topologically relaxed by endonuclease or topoisomerase activity. The DNA product in the lowest amount migrated as RI* that presumably arose from the 1 to 2% replicating viral chromosomes present in SV40 chromosomes isolated at the peak of viral DNA replication in vivo, most of which are more than 70% replicated (30, 31, 33). The electrophoretic mobility in agarose gels of SV40 RI, forms I, II, and III, and other SV40 DNA species has been rigorously characterized (29, 31, 38).

The prominent appearance of early RI in the presence of aphidicolin suggested that one or more events in the initiation of SV40 DNA replication were significantly more resistant to inhibition by this drug than was continued replication of RI DNA. This hypothesis was confirmed by the experiments described below. First, the products of SV40 DNA replication were analyzed as a function of incubation time in the presence and absence of aphidicolin. In the absence of aphidicolin, a lag period of 10 min was followed by the rapid onset of DNA synthesis, with the transient appearance of both intact and topologically relaxed early RI (Fig. 2). At 20 min, the amount of [^{32}P]DNA extracted from the early RI region (form I to form III) of the control reaction reached its highest level, 21% of the total [^{32}P]DNA present. By 45 min, the bulk of the newly synthesized DNA was in the form of middle to late RI. In the presence of 9 μM aphidicolin, DNA synthesis was detected after 15 min of incubation in the form of early RI that accumulated but failed to elongate significantly (Fig. 2). After 20 min in the presence of aphidicolin, 27% of the total [^{32}P]DNA was present as early RI, and by 45 min this value had increased to 45%. In contrast, the fraction of early RI present after 45 min in the control reaction accounted for only 5% of the viral DNA synthesized. Thus, aphidicolin caused the accumulation of early RI to more than twice the maximum level observed in its absence, consistent with continued initiation of SV40 DNA replication in the absence of significant elongation.

Parameters that affect the action of aphidicolin on SV40 DNA replication. If the accumulation of early RI observed in the presence of aphidicolin depends on de novo initiation of SV40 DNA replication in vitro, then a subcellular system capable only of elongating RI and terminating replication should not accumulate early RI in the presence of aphidicolin. The soluble system described by Su and DePamphilis (28) uses the same hypotonic extract from virus-infected CV-1 cells and the same SV40 nucleoprotein complexes used in the present system to initiate DNA replication, and it supports continued replication of endogenous SV40 chromosomes containing RI DNA but not initiation of replication because it is deficient in T-ag. Therefore, the hypotonic extract alone was tested for its response to aphidicolin. The major products included RI* and form I DNA, as previously reported, but no accumulation of early RI occurred in the presence of aphidicolin even though aphidicolin reduced total viral DNA synthesis by 70% (Fig. 3, lanes 1 and 2; overexposed autoradiograms, lanes 3 and 4). Identical results were obtained with these hypotonic extracts when supplemented with polyethylene glycol, a normal component of our in vitro initiation system. However, addition of SV40 T-ag to the same hypotonic extract conferred the ability to initiate SV40 DNA replication and restored the ability to accumulate early RI in the presence of aphidicolin. T-ag stimulated viral DNA synthesis a maximum of sevenfold and generated a heterogeneous population of RI (Fig. 3, lane 5). Addition of both T-ag and aphidicolin reduced DNA synthesis by 70%, and the products of replication consisted primarily of early RI DNA (Fig. 3, lane 6). A high-salt nuclear extract could substitute for the purified T-ag (Fig. 3, lanes 7 and 8), increasing total DNA synthesis 13-fold over the hypotonic extract alone. This extract presumably provided T-ag as well as other factors required for viral DNA replication. Therefore, early RI accumulated in the presence of aphidicolin only when initiation of viral DNA replication occurred.

Accumulation of early RI DNA in the presence of aphidicolin appeared to result from the action of this drug on DNA polymerase α. Inhibition of DNA polymerase α by aphidicolin involves competition with dCTP (20). Addition

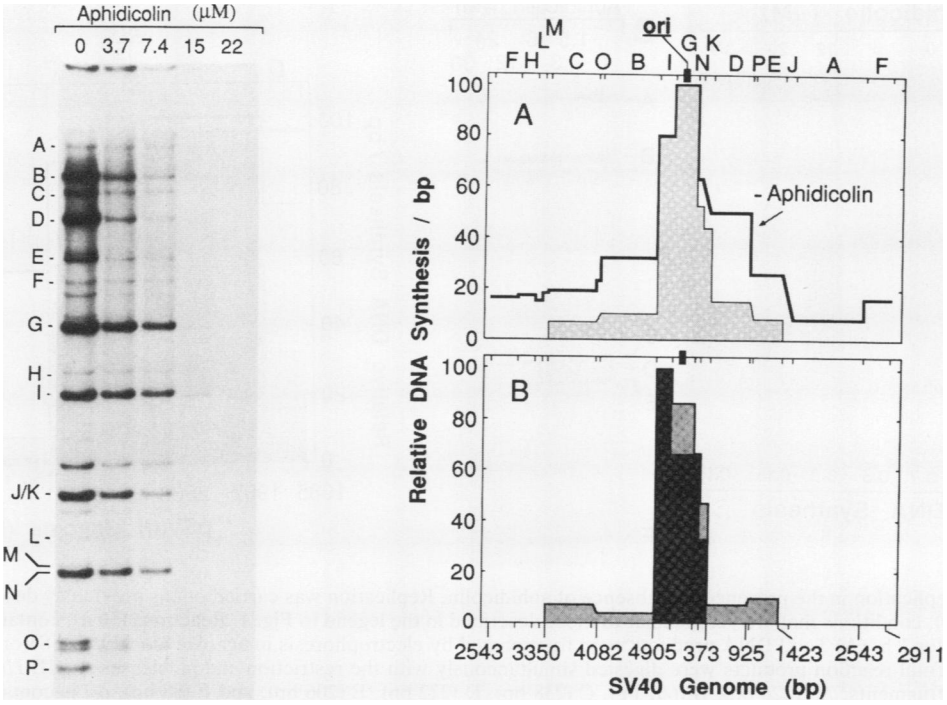

FIG. 4. Genomic distribution of nascent DNA in SV40 DNA replicated in the presence and absence of aphidicolin. (Left) DNA replication and analysis conditions are as described in the legend to Fig. 1. In vitro reactions are the same as those shown in Fig. 1. One-tenth of the total DNA product was digested with *Bst*NI, which digests SV40 wt800 form I DNA into 16 fragments 54 to 993 bp in length. Samples were fractionated by electrophoresis in a 6% polyacrylamide gel, and the gel was dried and subjected to autoradiography. Several exposures were developed, and the relative radioactivity in each DNA fragment was quantitated by densitometry and divided by the number of base pairs per restriction fragment. Relative DNA synthesis was calculated by defining the fragment with the highest specific radioactivity as 100% and then calculating the specific radioactivity of all other fragments relative to the 100% fragment. SV40 form I DNA digested with *Bst*NI was fractionated in parallel to provide standards for comparison; the positions of the standard fragments are indicated by letters. SV40 wt800 contains 5,256 bp (12); numbering proceeds in the direction of the SV40 late genes. (Right) (A) Symbols: 0 μM aphidicolin (solid line); 3.7 μM aphidicolin (shaded area). (B) Symbols; 7.4 μM aphidicolin (shaded area); 15 μM aphidicolin (solid area). All DNA fragments containing less than 2% of the radiolabel in fragments G and I were considered nonspecific DNA repair background and not included in the analysis. *ori* is located in fragment G. Fragments J (126 bp) and K (127 bp) migrated as a single band and were therefore ignored in the analysis of the sample incubated in the absence of aphidicolin. When aphidicolin was present, all of the label in the J/K band was assumed to be in the K fragment, because fragment E, which is adjacent to fragment K, was poorly labeled.

of 600 μM dCTP to SV40 chromosomes replicating in the presence of the high-salt nuclear extract reduced net incorporation of [α-^{32}P]dNTP more than twofold as a result of diluting the specific radioactivity of [α-^{32}P]dCTP, but it did not affect the type of DNA products synthesized (Fig. 3, lane 9). Addition of aphidicolin to this reaction was markedly less effective in causing the accumulation of early RI than when it was added under normal reaction conditions (Fig. 3, lane 10). Thus, dCTP interfered with the action of aphidicolin, as expected if aphidicolin was inhibiting DNA polymerase α.

In contrast to aphidicolin, 2′,3′-dideoxythymidine triphosphate (ddTTP), a nucleotide analog that inhibits DNA polymerases β and γ without inhibiting DNA polymerase α (8), had no effect on SV40 DNA replication at ratios of 4:1 and 20:1 over dTTP (data not shown). These results are in agreement with previous reports on the lack of inhibition by ddTTP of SV40 replication in isolated nuclei and nuclear extracts that do not support initiation of replication (8, 15) and are consistent with a requirement for DNA polymerase α in both the initiation and continuation of SV40 DNA replication.

DNA synthesis in the presence of aphidicolin restricted to regions in and around *ori*. The identity of the early RI DNA that accumulated in the presence of aphidicolin was confirmed by analysis of the genomic distribution of nascent, radiolabeled DNA in these molecules. The samples shown in Fig. 1 were also digested with the *Bst*NI restriction endonuclease, and the amount of [^{32}P]DNA in each fragment was determined as a measure of relative DNA synthesis. In the absence of aphidicolin, the most intensely labeled regions of DNA were in and around the *ori* region (fragments G, I, and N), and the amount of radiolabel per base pair decreased in both directions from *ori* to the termination region (fragments A, F, and H; Fig. 4, lane 0 and panel A). However, the mobility of the same RI DNA in agarose gels (Fig. 1, lane 0) appears to indicate more extensive replication. Together, these data suggest that RI becomes topologically relaxed during incubation in vitro, limiting the fractionation range during gel electrophoresis from the position of form II (0% replication) to RI* (85 to 95% replication) (32, 33). As replication continued in vitro for up to 3 h, the amount of DNA synthesis in the termination region increased in the manner expected for bidirectional replication of SV40 (data not shown). Addition of 3.7 μM aphidicolin inhibited DNA synthesis 40% coincident with early RI accumulation (Fig. 1), and restriction enzyme analysis of the total DNA products revealed that DNA synthesis in regions distal to both the early and late gene borders of *ori* had decreased relative to that in the *ori* region (fragments G and I in Fig. 4, lane 3.7 and panel A [shaded region]). Further inhibition of DNA

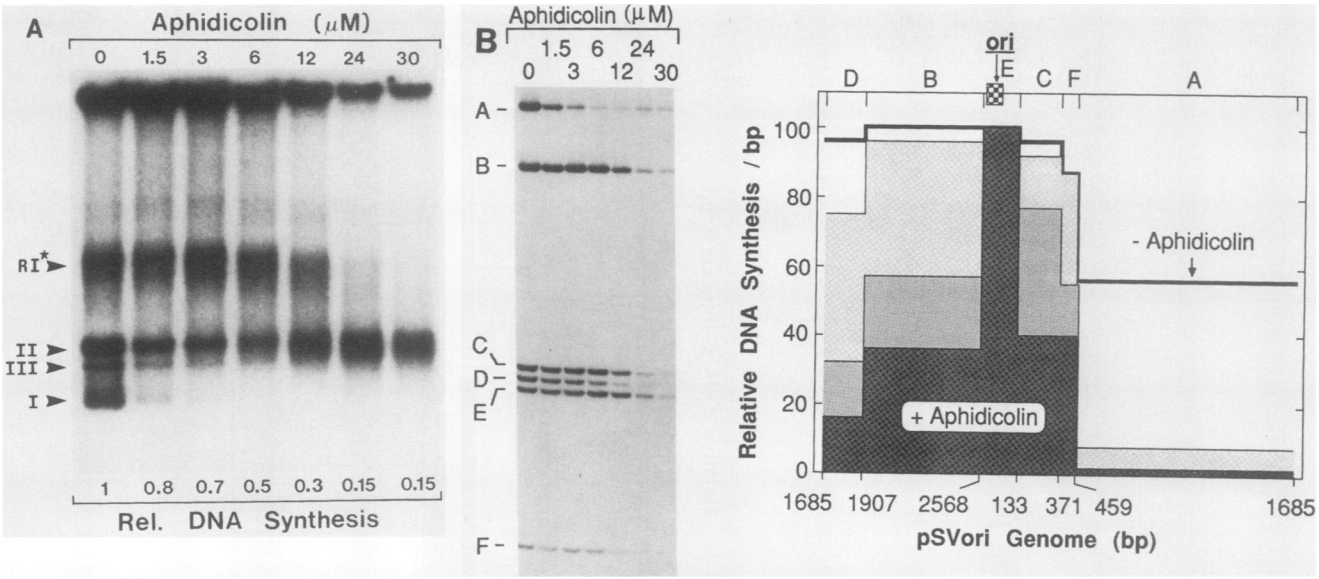

FIG. 5. pSVori replication in the presence and absence of aphidicolin. Replication was carried out as previously described by Yamaguchi and DePamphilis (40), conditions that are very similar to those described in the legend to Fig. 1. Reactions (50 μl) contained 130 ng of pSVori and were incubated for 1 h. (A) Total DNA products were fractionated by electrophoresis in agarose along with pSVori forms I, II, III, and RI* DNA (38). (B) Total reaction products were digested simultaneously with the restriction endonucleases *Ava*II, *Hin*dII, and *Sph*I. This generated six DNA fragments: A (1226 bp), B (661 bp), C (238 bp), D (222 bp), E (206 bp), and F (88 bp). *ori* is contained within fragment E. Restriction fragments were separated by electrophoresis in 6% polyacrylamide. Results were quantitated as described in the legend to Fig. 4. Diagram: 0 μM aphidicolin (solid line), and 3 μM (lightest shading), 12 μM (medium shading), and 30 μM (darkest shading) aphidicolin.

synthesis with 7.4 and 15 μM aphidicolin confined synthesis almost exclusively to the G, I, and N fragments. All of the DNA fragments in the 22 μM aphidicolin sample were equally faint, consistent with a low level of nonspecific DNA damage and repair that obscured any replication from *ori*. In addition, replication forks that remained in fragments G, I, and N would prevent normal migration of these DNA fragments during gel electrophoresis (4, 26). These results were confirmed by extracting DNA from the early RI region of each gel as described below and then digesting it with a restriction endonuclease (data not shown).

In some instances, depending on the particular restriction digest conditions used, additional DNA fragments were observed that were not present in digests of purified SV40 form I DNA. The DNA fragment immediately above the J/K doublet (Fig. 4, lane 0) was observed in extracts of mock-infected CV-1 cells and therefore was ignored. Minor DNA fragments were sometimes observed immediately below fragments F and O that resulted from secondary cleavage sites when high concentrations of *Bst*NI were used (*Bst*NI* sites [9]). These fragments constituted less than 3% of the total radiolabeled DNA fragments used in our calculations and therefore did not affect our conclusions.

Effects of aphidicolin on replication of plasmid DNA containing the SV40 *ori* sequence. To determine whether the relative insensitivity of initiation of SV40 DNA replication to aphidicolin was due to the nucleoprotein structure of the chromosome substrate, a purified plasmid DNA (pSVori) containing the 206-bp *Hin*dIII-*Sph*I fragment of SV40 wt800, which includes the *ori* sequence, was incubated under in vitro conditions that allowed *ori*-dependent, T-ag-dependent initiation of DNA replication (40). These conditions were similar to the ones used to initiate replication in SV40 chromosomes and resulted in bidirectional replication from *ori*. DNA synthesis was linear for up to 3 h (Decker et al., submitted for publication).

The effects of aphidicolin on replication of pSVori were analogous to its effects on replication of SV40 chromosomes. At 6 μM aphidicolin, replication of both plasmid DNA (Fig. 5A) and chromosomal DNA (Fig. 1) was inhibited 50%. pSVori replication was inhibited 85% by the addition of 30 μM aphidicolin, consistent with published reports of similar experiments in other laboratories (17, 27, 39). Low concentrations of aphidicolin completely inhibited production of form I DNA (Fig. 5A). As the concentration of aphidicolin was increased, the fraction of newly synthesized DNA in RI decreased while the fraction in the region of the gel containing form II DNA increased. These data are consistent with the accumulation of early RI containing topologically relaxed parental strands. Plasmid DNA may be more sensitive to endonucleases in the cell extract than DNA in viral chromosomes, as suggested by the relatively high proportion of form II product in the control sample (Fig. 5A, lane 0). Accumulation of early plasmid RI in the presence of aphidicolin indicates that plasmid replication in vitro was similar to replication of SV40 chromosomes. The high-molecular-weight products at the top of the gel are quantitatively converted to form III by cleavage with a single-site endonuclease (40) and may consist of multiple interlocked catenates, the result of inefficient separation of sibling DNA molecules (38). In addition, nonspecific DNA aggregation can occur that traps a random population of molecules (32). Problems with completion of circular plasmid DNA replication in vitro have been observed in other systems as well (27, 39).

pSVori DNA that replicated in the presence of aphidicolin contained most of its newly synthesized DNA in the *ori* region. After 1 h of incubation in the absence of drug, all regions of the genome had replicated to a similar extent except for the termination region (fragment A, Fig. 5B). However, in the presence of increasing amounts of aphidicolin, DNA synthesis was increasingly confined to the

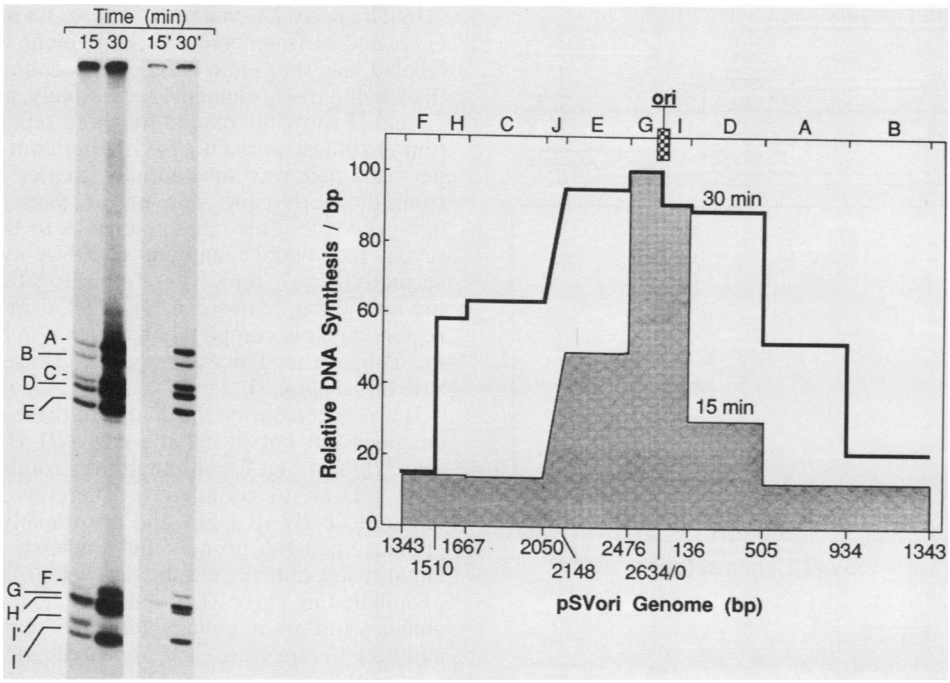

FIG. 6. Replication of pSVori in the absence of aphidicolin. DNA replication conditions were described as in the legend to Fig. 5, except that incubation was terminated after either 15 or 30 min, as indicated above the lanes. Total nucleotide incorporation was 15,000 cpm at 15 min and 84,000 cpm at 30 min. The nonlinear relationship resulted from a 10- to 15-min delay prior to extensive replication, as observed in Fig. 2. Total DNA was digested with the restriction endonucleases *Bgl*I, *Dde*I, *Sph*I, and *Xmn*I to produce DNA fragments of 429 bp (A), 409 bp (B), 383 bp (C), 369 bp (D), 328 bp (E), 167 bp (F), 158 bp (G), 157 bp (H), 140 bp (I'), and 136 bp (I). Fragment I' resulted from failure of *Bgl*I, which recognizes only a single site (2643/0) in pSVori, to cleave 100% of the DNA. Radiolabel in fragments I' and I was therefore combined. Since the *Bgl*I site is only four nucleotides from the *Dde*I site, cleavage by *Dde*I may have inhibited subsequent cleavage at the *Bgl*I site, as previously observed for *Hin*fI and *Pst*I enzymes (2). The 6% polyacrylamide gel was exposed for 40 h (lanes 15 and 30) or for 5 h (lanes 15' and 30'). Fragment J (98 bp) was lost at the anode. After DNA replication had proceeded for 1 h, all DNA fragments were nearly uniformly labeled (data not shown). Diagram: solid line, 30-min incubation; shaded area, 15-min incubation.

ori region, in particular fragment E (Fig. 5B, lane 30), confirming the identity of the plasmid DNA that accumulated in the region of form II as early RI molecules. Thus, the effects of aphidicolin inhibition on plasmid replication closely paralleled the effects described during chromosome replication and suggest equivalent mechanisms of replication in vitro.

Early-gene side of *ori* replicated first in vitro. Newly replicated SV40 DNA appeared first on the early-gene side of the *ori* region in SV40 chromosomes that initiated replication in vitro and then on the late-gene side. As observed with SV40 chromosomes, pSVori replication exhibited a 10- to 15-min initial lag period. Thus, after 15 min of incubation in the presence of [α-^{32}P]dCTP and [α-^{32}P]dTTP, DNA synthesis was confined mainly to the *ori* region and flanking sequences (Fig. 6). Furthermore, the relative amount of DNA synthesis per base pair was significantly and reproducibly greater on the early-gene side of *ori* (fragments G and E) than on the late-gene side (fragments I and D). This asymmetry was no longer discernible after 30 min of incubation.

Similar results were observed with SV40 chromosomes. Early mRNA synthesis began within *ori* (fragment G, Fig. 4) and proceeded in the opposite direction (fragment I, Fig. 4) to that used in numbering the genome (5). A bias in the direction of DNA replication towards the early genes was evident in the absence of aphidicolin, but it was markedly pronounced in the presence of aphidicolin (Fig. 4). At aphidicolin concentrations sufficient to inhibit replication by 80% (15 μM), the specific radioactivity (counts per minute per base pair) in *Bst*NI fragment I was routinely 1.5-fold greater than in fragments G and N and 2-fold more than in fragment K (Fig. 4). Therefore, regardless of whether purified pSVori form I DNA or isolated SV40 chromosomes were added to the cell extract, a greater fraction of DNA replication forks that were initiated in vitro moved towards the early-gene side of *ori* than towards the late-gene side, particularly in the presence of aphidicolin.

The asymmetric distribution of replication forks in early RI DNA was confirmed and refined by analysis of the genomic distribution of nascent DNA in an isolated population of early RI. SV40 chromosomes were incubated for 45 min in the presence of 15 μM aphidicolin, conditions that allow initiation of replication and subsequent accumulation of early RI [^{32}P]DNA. This DNA was extracted from agarose gels and digested with restriction enzymes, and the relative amount of DNA synthesis that occurred in each restriction fragment was quantitated as described for the data in Fig. 4. Replication forks must traverse the entire length of a restriction fragment for that fragment to be identified by gel electrophoresis, because restriction fragments containing replication forks migrate more slowly than those that are completely replicated, and the rate of migration varies with the number of forks and extent of replication (4, 26). This complication means that the amount of a [^{32}P]DNA fragment will be inversely proportional to its length as well as to its distance from the origin of DNA synthesis. Ideally, one would like to cut the origin region into a series of equal segments to compare the extent of the replication in each

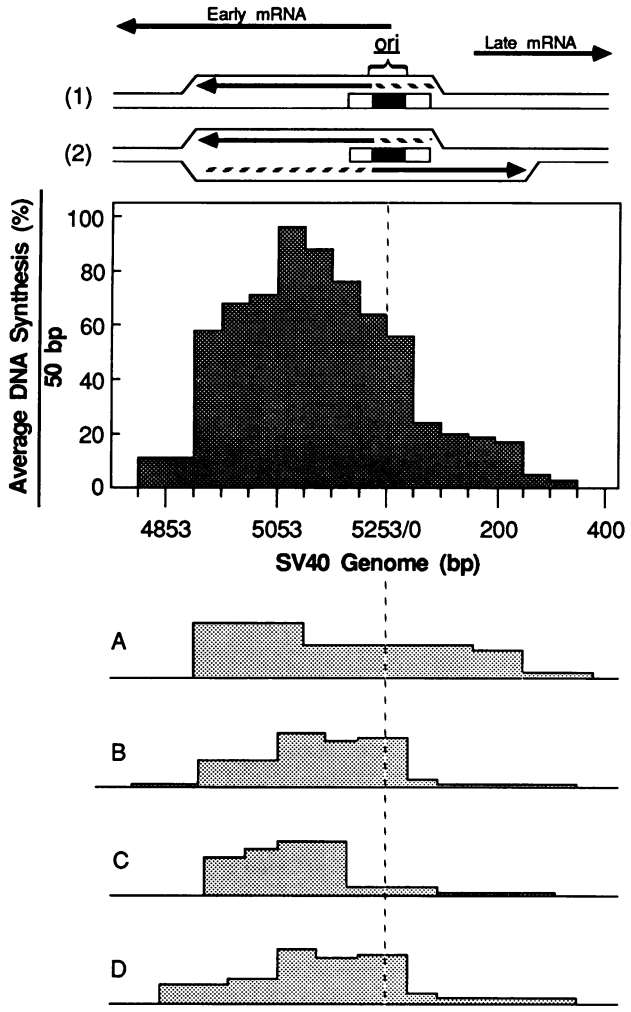

FIG. 7. Initial direction of DNA replication after initiation of replication at *ori*. SV40 chromosome replication was initiated in the presence of 15 μM aphidicolin and incubated for 45 min, and the replicated DNA was fractionated in low-melting-point agarose as described in the legend to Fig. 1. Early RI DNA was isolated and digested with one of four sets of restriction endonucleases: (A) *Bst*NI; (B) *Fok*I, *Hin*fI, *Nco*I, and *Stu*I; (C) *Fok*I, *Hin*dIII, and *Rsa*I; or (D) *Ava*II, *Fok*I, *Nco*I, and *Stu*I. The restriction products were electrophoresed in 15% polyacrylamide, the DNA fragments were detected by autoradiography, and the results were quantitated by densitometry as described in the legend to Fig. 4. Individual DNA fragments are indicated as bars in histograms A to D. The data of histograms A to D were combined by calculating the average relative synthesis per 50 bp, and the result was plotted as a single histogram (center panel). The simplest interpretation of these data is diagrammed at the top: 1, DNA synthesis (solid arrows) is initiated in the same direction as early mRNA synthesis at one of several possible sites (broken line) within the origin of replication (*ori* core, solid box; auxiliary origin regions, open boxes); 2, when sufficient template is available on the opposite (retrograde) strand, DNA synthesis is then initiated in the direction of late mRNA synthesis at one of many possible sites.

segment. Since this was not possible, we digested the same preparation of early RI DNA with a variety of different restriction enzymes and pooled the results.

*Bst*NI digestion of isolated early RI (Fig. 7, panel A) gave the result previously described for the *Bst*NI digestion of total viral DNA radiolabeled under the same conditions (Fig. 4B). The early RI contained 98% of its nascent DNA in the G, I, and N fragments; the I fragment was most intensely labeled, and the G and N fragments contained 58 and 55% of the label in the I fragment, respectively. Similarly, panels B, C, and D show the results for three separate sets of restriction enzymes. In each case, the amount of DNA synthesis per base pair was substantially greater in DNA fragments from the early-gene side of *ori* than in those from the late-gene side. The data in panels A to D were combined to reveal the average amount of DNA synthesis per 50-bp segment (Fig. 7, top). These data, together with those from the restriction analysis of total DNA, indicate that the first region which is completely replicated in SV40 encompasses the 63-bp *ori* sequence and about 300 bases on the early-gene side of *ori* (Fig. 7).

It was noted during the course of these studies that several enzymes did not digest the early RI DNA to completion (e.g., *Bgl*I in Fig. 6), although they routinely digested SV40 form I DNA to completion. Therefore, the problem was unique to early RI DNA and presumably resulted from the presence of RNA primers and single-stranded gaps remaining at restriction sites in the *ori* region. Even in the absence of aphidicolin, early RI contain sufficient RNA-primed DNA chains to allow mapping of their 5′ ends (11). Sequential addition of enzymes and repeated addition of the same enzyme alleviated this problem in part, although *Bgl*I and *Stu*I consistently failed to complete digestion. However, the genomic position of the undigested fragment was identified and therefore included in calculating the data presented in Fig. 7.

RNA-dependent DNA synthesis by DNA primase-DNA polymerase α in the presence of aphidicolin. DNA primase from mice, humans, and yeast is resistant to inhibition by aphidicolin (10, 21, 34, 36), suggesting that the increased resistance to aphidicolin of DNA synthesis at SV40 *ori* may be due to initiation of RNA-primed DNA synthesis by CV-1 cell DNA primase-DNA polymerase α. Therefore, we examined the effect of aphidicolin on purified DNA primase-DNA polymerase α by using M13mp7 single-stranded circular DNA. This template represents a more natural substrate for DNA polymerization than single-stranded homopolymers such as poly(dT) or random copolymers such as poly(dI,dT) used in previous studies. To circumvent interference by DNA primase-independent DNA synthesis that can occur with natural single-stranded DNA templates (41), only DNA chains with an intact RNA primer were radiolabeled at their 5′ ends. This was accomplished by incubating the purified reaction products with [α-^{32}P]GTP in the presence of guanylyltransferase, which "caps" the ends of polynucleotide chains bearing a 5′-terminal ribonucleoside di- or triphosphate with GDP. A portion of the products were treated with T4 DNA polymerase-associated 3′-to-5′ exonuclease under conditions that degraded single-stranded DNA but not RNA, leaving G$\overset{*}{p}$pp(rN)$_n$-dN (41). Thus, the amount of radioactivity observed was proportional to the number rather than the length of nascent DNA chains. Nascent DNA chains before and after treatment with exonuclease were then fractionated by gel electrophoresis (Fig. 8).

In the absence of aphidicolin, DNA primase-DNA polymerase α synthesized polynucleotide chains greater than 60 nucleotides in length, most of which were too long to enter the gel (Fig. 8, lane a). These chains contained 5′-oligoribonucleotides seven to nine residues long (Fig. 8, lane d; the cap structure contributed two residues to the oligonucleotide's mobility and the 3′-dNMP contributed one residue [41]). Addition of 60 μM aphidicolin to this reaction had no

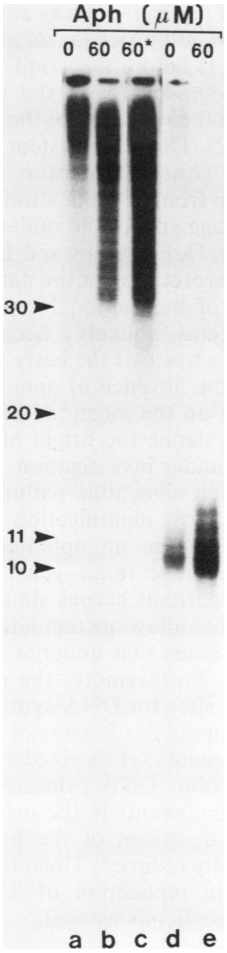

FIG. 8. Effects of aphidicolin (Aph) on synthesis by purified DNA primase-DNA polymerase α. DNA primase-DNA polymerase α was purified from CV-1 cells as described by Yamaguchi et al. (41). DNA synthesis on single-stranded, circular M13mp7 DNA was carried out at 30°C for 20 min in the presence of 100 μM each [^{3}H]dATP, [^{3}H]dCTP, [^{3}H]dGTP, and [^{3}H]dTTP at a concentration of 0.4 μCi/nmol. (p)pprN(rN)$_n$(dN)$_m$ products were specifically radiolabeled at their 5′ ends with [α-^{32}P]GTP with vaccina virus guanylyltransferase. A portion of the radiolabeled material was digested with T4 DNA polymerase under conditions in which the associated 3′-to-5′ exonuclease activity removed all but a single dNMP from the 3′ ends of RNA chains (41). Equal amounts of radiolabel from each sample were electrophoresed in 22% polyacrylamide in the presence of 8 M urea and exposed to X-ray film with an intensifying screen. Unfortunately, part of the sample used for lane d was lost. Polynucleotide lengths (indicated on the left) were derived from a continuous series of pA(Ap)$_n$ size standards fractionated on the same gel. Lane a, Products synthesized in the absence of aphidicolin; lane b, products synthesized in the presence of 60 μM aphidicolin; lane c, an overexposed autoradiogram of lane b; lane d, a portion of the sample in lane a digested with exonuclease; lane e, a portion of the sample in lane b digested with exonuclease. The material which remained at the top of lane d has also been observed in the absence of DNA primase-DNA polymerase α (42) and is presumed to be [^{32}P]GMP bound to guanylyltransferase (23). The anode of electrophoresis is on the bottom.

discernible effect on the lengths of RNA primers (lane e), although it did reduce the frequency of initiation events about 22%, as judged by a reduction in the incorporation of [α-^{32}P]GTP. The primary effect of aphidicolin was to reduce the rate of DNA chain elongation after 21 to 23 deoxyribonucleotides were added onto the 3′ end of an RNA primer. Incorporation of approximately the first 30 nucleotides was completely resistant to inhibition by aphidicolin, after which DNA synthesis came under the inhibitory influence of this drug and the length distribution of DNA products was markedly decreased (Fig. 8, lanes b and c). This concentration of aphidicolin was sufficient to reduce total incorporation of [^{3}H]dNTP on the M13 DNA template by 73%. Inhibition was even more pronounced (85%) with DNase I-activated calf thymus DNA (3) as template; most of the synthesis was initiated on the 3′ ends of DNA chains. Therefore, DNA primase-DNA polymerase α synthesizes RNA-primed DNA chains in at least two stages; synthesis of the RNA primer and subsequent oligodeoxynucleotide synthesis (21- to 23-mer), which is completely resistant to aphidicolin, while extended elongation of nascent DNA chains is sensitive to aphidicolin.

DISCUSSION

Aphidicolin, a specific inhibitor of DNA polymerase α (25), provided a novel method for distinguishing between initiation of replication at SV40 *ori* and continuation of DNA replication beyond *ori*. Initiation of DNA replication took place in SV40 chromosomes in vitro despite the presence of sufficient aphidicolin to inhibit DNA synthesis in replicating SV40 chromosomes that had already initiated replication in vivo. This resulted in the in vitro accumulation of early RI in which most nascent DNA was localized within a 600- to 700-bp region centered at *ori*. DNA synthesis in the *ori* region was not resistant to aphidicolin, but simply less sensitive to the drug than DNA synthesis at forks farther away from *ori*. The amount of DNA synthesis in the *ori* region decreased in proportion to the concentration of aphidicolin until it was undetectable (Fig. 4, autoradiogram, fragment G), but the residual DNA synthesis was always localized in the *ori* region (Fig. 4, histograms). Since this phenomenon was observed with purified plasmid DNA containing the SV40 *ori* sequence as well as viral chromosomes, it does not appear to involve substrate structure. These data are consistent with the observation by Dinter-Gottlieb and Kaufmann (6) that aphidicolin does not prevent reinitiation of SV40 DNA replication in vivo when *ts*A virus-infected CV-1 cells were shifted from the restrictive to the permissive temperature. Since aphidicolin does inhibit SV40 DNA replication in vivo, the resulting RI contained newly synthesized DNA localized in the *ori* region. Therefore, both in vitro and in vivo, DNA synthesis in the SV40 *ori* region is less sensitive to aphidicolin than DNA synthesis throughout the remainder of the genome.

The effects of aphidicolin on SV40 DNA replication are similar to its effects on CV-1 cell DNA primase-DNA polymerase α, suggesting that this enzyme is used to initiate DNA synthesis at *ori*. Insensitivity to aphidicolin appears to be a general property of eucaryotic DNA primases, whereas DNA polymerase α activity on preprimed templates is very sensitive to aphidicolin (10, 21, 34, 36). We found that drug concentrations sufficient to inhibit 85 to 95% of the SV40 DNA synthesis observed in vitro reduced the frequency of RNA primer initiation by purified CV-1 cell DNA primase-DNA polymerase α on a single-stranded DNA template by only 22%. However, once initiation occurred, incorporation of approximately the first 30 nucleotides was completely resistant to aphidicolin, at which point the probability of extending DNA chains decreased rapidly. This phenomenon was even more pronounced in isolated nuclei from SV40-

infected CV-1 cells. Recently, residual SV40 DNA synthesis in isolated nuclei incubated with high concentrations of aphidicolin was observed to consist of RNA-primed DNA chains less than 40 residues long that represented aborted synthesis of Okazaki fragments, and long DNA chains that represented addition of a few nucleotides to the 3′ ends of the growing daughter strands (G. Dinter-Gottlieb, T. Nethanel, and G. Kaufmann, personal communication). Using SV40 chromosomes as the substrate, we observed a similar accumulation of nascent chains 20 to 70 nucleotides long in early RI radiolabeled in vitro in the presence of 45 μM aphidicolin (data not shown). Since the 22 to 28 residues incorporated by CV-1 cell DNA primase-DNA polymerase α per initiation event (41) corresponds closely to the number of nucleotides whose synthesis was completely resistant to aphidicolin, DNA primase may synthesize the entire $pppA/G(pN)_{6-8}(pdN)_{21-23}$ moiety before DNA polymerase α takes over. In fact, purified DNA primase has been shown to synthesize chains 20 to 60 nucleotides long in the presence of very low concentrations of dNTPs that are mixed polymers of oligoribo- and oligodeoxyribonucleotides (13).

Although the inability of aphidicolin to prevent DNA synthesis at *ori* is qualitatively consistent with initiation of DNA synthesis by DNA primase-DNA polymerase α, quantitative differences may arise from proteins associated with either the enzyme or the DNA template. For example, the initiation sites selected by purified CV-1 or HeLa cell DNA primase-DNA polymerase α on natural single-stranded DNA templates were distinctly different than those selected in vivo (42; J. K. Viswanatha, M. Yamaguchi, M. L. DePamphilis, and E. Baril, submitted for publication). A more complex form of this enzyme increased selection of initiation sites that used ATP instead of GTP and made shorter RNA primers but still did not mimic the behavior of DNA primase-DNA polymerase α in vivo (Viswanatha et al., submitted for publication). Therefore, SV40 chromosomes may provide a more valid template than plasmid DNA for investigating the mechanism of initiation of DNA synthesis in vitro.

The effects of aphidicolin are consistent with the model proposed by Hay and DePamphilis (11) for initiation of SV40 DNA replication. A preinitiation complex binds to the initiation factor-binding site (40), opens the *ori* region to create a replication bubble, and thus allows DNA primase-DNA polymerase α to select, by some stochastic process, one of several possible template initiation sites within this initiation zone. Since the initiation sites for RNA-primed DNA synthesis in *ori* are indistinguishable from those found outside *ori* in terms of their frequency, sequence composition, confinement to the retrograde sides of forks, and average length of RNA primers, it was postulated that the mechanism used to initiate synthesis of the first DNA chain in the *ori* region is the same as that used to initiate synthesis of Okazaki fragments throughout the genome (12). Furthermore, since all of the RNA-primed DNA synthesis initiation sites within *ori* were located on the strand that encodes early mRNA, the first DNA chain synthesized was expected to extend in the direction of the early genes. In fact, the first DNA sequences completely synthesized in vitro included *ori* and the region about 300 bases in the direction of early mRNA synthesis (Fig. 4 and 7). Since bidirectional replication originates at the junction of T-ag-binding site I and the *ori* core (nucleotides 5223 to 5226 in SV40 wt800; shown in Fig. 7), initiation of the first RNA primer must occur somewhere upstream of this point. It is this nascent RNA-primed DNA chain that becomes the continuously synthesized forward arm of replication forks advancing towards the early-gene side of *ori* and proceeds an average of 250 ± 150 bp (Fig. 7, diagram 1) before a second "initiation zone" is created on the retrograde arm of the fork to allow DNA synthesis to begin in the direction of the late mRNA synthesis (Fig. 7, diagram 2). This is consistent with the hypothesis that the average center-to-center distance between nucleosomal cores in front of replication forks (220 ± 72 bp) determines the average size of the initiation zone (reviewed in reference 5 and in DePamphilis and Bradley, in press).

An alternative interpretation of the data is that aphidicolin changes the origin of bidirectional replication to position 5053–5083. This seems unlikely, because a bias in the direction of synthesis towards the early-gene side of *ori* was detectable even in the absence of aphidicolin (Fig. 6). The effect of aphidicolin on the locations of 5′ ends of nascent DNA chains, which define the origin of bidirectional replication, is currently under investigation.

The ability to inhibit elongation without markedly impairing initiation could allow identification of *ori* sequences in other systems that utilize an aphidicolin-sensitive DNA primase-DNA polymerase (e.g., yeast, mammalian cells, polyoma virus, and perhaps herpes simplex virus), if conditions are available that allow accumulation of early replicating intermediates. Genes that undergo amplification would be good candidates. Furthermore, the nucleotide locations of the first initiation sites for DNA synthesis within *ori* may be identified by mapping the 5′ ends of the most abundant, short nascent DNA chains synthesized on the template in the presence of aphidicolin. DNA primase activity should be restricted to the initial events in the *ori* region; initiation of Okazaki fragments upstream of the first initiation events should be substantially reduced. Thus, the techniques developed to explore the replication of SV40 DNA may be applicable to other replicons as well.

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