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Primer-DNA Formation during Simian Virus 40 DNA Replication In Vitro

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Studies of simian virus 40 (SV40) DNA replication in vitro have identified a small (~30-nucleotide) RNA-DNA hybrid species termed primer-DNA. Initial experiments indicated that T antigen and the polymerase α-primase complex are required to form primer-DNA. Proliferating cell nuclear antigen, and presumably proliferating cell nuclear antigen-dependent polymerases, is not needed to form this species. Herein, we present an investigation of the stages at which primer-DNA functions during SV40 DNA replication in vitro. Hybridization studies indicate that primer-DNA is initially formed in the origin region and is subsequently synthesized in regions distal to the origin. At all time points, primer-DNA is synthesized from templates for lagging-strand DNA replication. These studies indicate that primer-DNA functions during both initiation and elongation stages of SV40 DNA synthesis. Results of additional experiments suggesting a precursor-product relationship between formation of primer-DNA and Okazaki fragments are presented.

With the exception of a single virally encoded protein termed T antigen (T-ag), replication of simian virus 40 (SV40) is dependent on simian or human host proteins (47). The dependence of SV40 replication on host proteins, and the presence of a single, well-defined origin of replication, has made SV40 a useful model system for studies of DNA replication in higher eukaryotes.

An in vitro SV40 replication system was developed by Li and Kelly (28), using simian extracts, and extended to human extracts by Stillman and Gluzman (44) and Wobbe et al. (54). This system has enabled the identity and function(s) of many of the host proteins required for SV40 DNA replication to be established (for reviews, see references 8, 22, and 43). It has also permitted an analysis of the mechanisms operating during various stages of the SV40 DNA replication to be studied in detail. We have used this system to study the T-ag-dependent initiation of SV40 DNA synthesis (4, 5).

The role played by T-ag during initiation of SV40 DNA replication has been reviewed recently (1a, 18). In summary, T-ag recognizes GAGGC binding sites within the SV40 origin, assembles as a double hexamer (30), and melts the early palindrome within the SV40 origin (2). In the presence of topoisomerase I and a single-stranded binding protein, such as the three-subunit complex present in humans (HSSB, also termed RFA or RPA) (8, 22, 43), the inherent helicase activity of T-ag (42) catalyzes further unwinding of the origin region (11, 12, 55). T-ag-dependent unwinding of the SV40 origin is a critical initiation event, since it allows additional replication factors to gain entry to the origin region. Less is known about subsequent steps during initiation of SV40 DNA replication.

We have conducted pulse-labeling experiments designed to more fully characterize postunwinding initiation events. We reported previously that SV40 DNA synthesis initiated on an unwound topological form termed form U_R (4). In addition, evidence that in the absence of nucleoside triphosphates other than ATP the complex of proteins that assembles at the SV40 origin (the preinitiation complex) remains within or close to the SV40 origin has been presented (5). In this system, upon addition of nucleoside triphosphates during pulse-labeling, DNA synthesis initiated outside the core origin on DNA strands that are templates for lagging-strand DNA synthesis (5). Moreover, we have reported that a small RNA-DNA hybrid species, termed primer-DNA, can be detected during SV40 DNA replication. Primer-DNA formed in crude extracts of HeLa cells was ~30 nucleotides (nt) long, was covalently linked to ~10-nt-long oligoribonucleotides, was T-ag dependent, and was suggested to be the product of the polymerase α-primase complex (pol α-primase complex) (5).

When primer-DNA was initially detected, we suggested that it might function during both initiation and elongation stages of SV40 lagging-strand DNA synthesis in vitro (5). The experiments described in this paper were conducted to test this prediction.

MATERIALS AND METHODS

Preparation of SV40 T-ag, HeLa cell crude extracts, and plasmid pSV01ΔEP DNA. SV40 T-ag was prepared by using a baculovirus expression vector containing the T-ag-encoding SV40 A gene (36) and isolated as previously described (54). T-ag was dialyzed against T-ag storage buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μg of leupeptin per ml, 0.1 μg of antipain per ml, and 10% glycerol) and frozen at −70°C. HeLa cell crude extracts, a gift from J. Hurwitz, were prepared as described elsewhere (54); extracts were dialyzed overnight against 4 liters of storage buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) prior to freezing at −70°C. Plasmid pSV01ΔEP, a 2,800-bp plasmid containing the SV40 *Eco*RII fragment cloned into the *Eco*RI site of pBR322, was isolated by standard techniques (40) and stored in TE buffer (10 mM Tris [pH 8.0]–1 mM EDTA). Note that sequences between the pBR322 *Eco*RV and *Pvu*II sites were deleted (ΔEP) during construction of this plasmid (see Fig. 3) (54).

Pulse-labeling reactions. Continuous pulse reactions (120

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μl) were performed essentially as described previously (5). Reaction mixtures contained 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 40 mM creatine phosphate (di-Tris salt [pH 7.7]), 2.8 μg of creatine phosphokinase, 1.5 μg of SV40 origin-containing pSV01ΔEP, 2 μg of T-ag, and 60 μl of HeLa cell extract (12.3 mg/ml). To reduce the T-ag-independent labeling of form II DNA, reaction mixtures were incubated at 37°C for 45 min prior to addition of T-ag (5). After the addition of T-ag, reaction mixtures were incubated at 37°C for 15 min prior to the addition of 8.3 μl of pulse mix (dATP, dGTP, and dTTP [final concentration of each, 100 μM]; CTP, GTP, and UTP [final concentration of each, 200 μM]; and [α-^{32}P]dCTP [final concentration, 3.0 μM; 228 cpm/fmol]) for various periods.

Late-labeling-pulse reactions were identical to the continuous pulse reactions except that the [α-^{32}P]dCTP label (final concentration, 3.00 μM; 276 cpm/fmol) was added only during the last 5 s of DNA synthesis. DNA synthesis was initiated by adding a mix containing dATP, dGTP, and dTTP (final concentration of each, 100 μM), dCTP (2.95 μM), and CTP, GTP, and UTP (final concentration of each, 200 μM) for various times.

Reactions were terminated by adding 12 μl of stop mixture (EDTA, *N*-lauroylsarcosine [pH 7.7], and proteinase K; final concentrations, 14 mM, 0.25 mg/ml, and 0.45 mg/ml, respectively). Aliquots (10 μl) were removed to monitor incorporated label. All reaction mixtures were further incubated for 30 min at 37°C, diluted to 0.2 ml with TE buffer, extracted with phenol-chloroform, and ethanol precipitated as described previously (5) and resuspended in various amounts of TE buffer (see figure legends).

Purification of nascent DNAs. Primer-DNA was separated from other nascent DNA products on denaturing 10% polyacrylamide gels containing 8 M urea; prior to electrophoresis, samples were boiled for 4 min in an equal volume of formamide loading buffer (40). Gels were electrophoresed at 1,000 V in 1× Tris-borate-EDTA buffer until the bromophenol blue dye had migrated 15 cm down a 20-cm gel. When used as a hybridization probe, primer-DNA was isolated from 10% acrylamide–urea gels following autoradiography. Regions of the gel containing primer-DNA were removed, crushed with a glass rod, and incubated overnight in 1.5 ml of elution buffer (0.1% sodium dodecyl sulfate [SDS], 0.5 M ammonium acetate, 10 mM magnesium acetate). Eluted primer-DNA was separated from acrylamide fragments by passing the samples through Millipore 0.45-μm-pore-size Millex-HV filters. Carrier tRNA (20 μg) was added to the samples, and the reaction mixtures were ethanol precipitated. Pellets containing purified primer-DNA were then resuspended in 100 μl of TE buffer.

Where indicated, RNA primers were removed from primer-DNA by incubating the samples in 0.3 N NaOH at 65°C for 45 min. Alkali-treated samples were neutralized with acetic acid, ethanol precipitated, washed with 80% ethanol, and dried.

Alkaline agarose gels (1.8%) were electrophoresed at 60 V for 15 h. Gels were dried on Whatman DE81 paper and then autoradiographed (40). Size markers used during alkaline gel electrophoresis consisted of a 123-bp DNA ladder (GIBCO-BRL) kinase labeled by standard methods (40).

Nascent DNA was purified from alkaline agarose gel fragments (see Fig. 5) by removal of the appropriate gel slices and electroelution (40). Ethanol precipitation was conducted in the presence of 20 μg of tRNA; pellets containing purified nascent DNA were resuspended in 100 μl of TE buffer.

Dot blot hybridization. The M13SV01 set of clones were affixed to Zeta probe filters with a Bio-Rad dot blot apparatus as described previously (5). As a negative control, 0.1- and 1-μg aliquots of M13mp19 DNA (M13) were applied to the Zeta probe filters. Also applied were 0.1- and 1.0-μg aliquots of plasmid pSV01ΔEP as positive controls. Prehybridization and hybridization solutions contained 50% formamide, 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% (wt/vol) SDS, 1 mM EDTA, and 50 μg of salmon sperm DNA per ml (hybridization buffer). Purified primer-DNA was boiled for 5 min prior to being added to 10 ml of hybridization buffer. Following hybridization for 12 to 14 h at 31°C, the filters were rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then washed (10 min per wash) at room temperature (~20°C) in 2× SSC–0.1% SDS, 1× SSC–0.1% SDS, and 0.5× SSC–0.1% SDS. The filters were air dried and exposed to Kodak X-Omat AR film. After development of the films, relevant sections of the Zeta probe filters were removed and the extent of primer-DNA hybridization was determined by liquid scintillation counting.

When nascent DNA fragments were used in hybridization experiments (see Fig. 6), the hybridization conditions were basically those described above; however, the hybridizations were conducted at 42°C. Moreover, the filters were rinsed in 2× SSC and then washed (15 min per wash) at room temperature in 2× SSC–0.1% SDS and 0.5× SSC–0.1% SDS, and the last wash was conducted at 65°C in 0.1× SSC–0.1% SDS.

Control experiments were conducted to establish the accuracy of the hybridization and wash conditions used to map primer-DNA. When Zeta probe filters containing the M13SV01 set of clones were hybridized to a kinase-labeled 20-nt oligonucleotide (5′CCTAACTCCGCCCATCCCGC3′) complementary to M13SV01 clone C_{Lag}, 98% of the bound probe hybridized to clone C_{Lag}. Thus, the hybridization and wash conditions used to map sequences complementary to primer-DNA are generally accurate. As a second control, we determined that nick-translated pSV01ΔEP (probe length, ~20 to 200 nt) hybridized with near-equal intensity (~10%) to all of the M13SV01 clones (data not shown). A slight variation (~2 to 4%) between the M13SV01 clones containing the largest and smallest inserts was observed. Thus, in general, there is no bias in the hybridization of nick-translated pSV01ΔEP DNA to any of the M13SV01 clones.

RESULTS

It was reported previously that primer-DNA can be detected in pulse reaction mixtures containing anti-proliferating cell nuclear antigen (anti-PCNA) serum at time points ranging between 5 s and 5 min of DNA synthesis (5). Since primer-DNA was shown to be covalently linked to RNA and was the only nascent DNA species detected in the presence of anti-PCNA serum, it was proposed that primer-DNA is the product of the pol α-primase complex (5).

To establish whether primer-DNA can be detected at various times after initiation of DNA synthesis in standard pulse reactions (i.e., in the absence of anti-PCNA serum), the experiments whose results are shown in Fig. 1 were conducted. Aliquots of reaction mixtures pulsed for various periods (10 to 270 s [4.5 min]) were displayed on a 10% polyacrylamide gel (Fig. 1). It is apparent from Fig. 1B, lane 3, that primer-DNA can be detected after 10 s of DNA synthesis (Fig. 1B shows a lighter exposure of lanes 1 to 6 in Fig. 1A). In addition, Fig. 1A (lanes 5 to 10) demonstrates

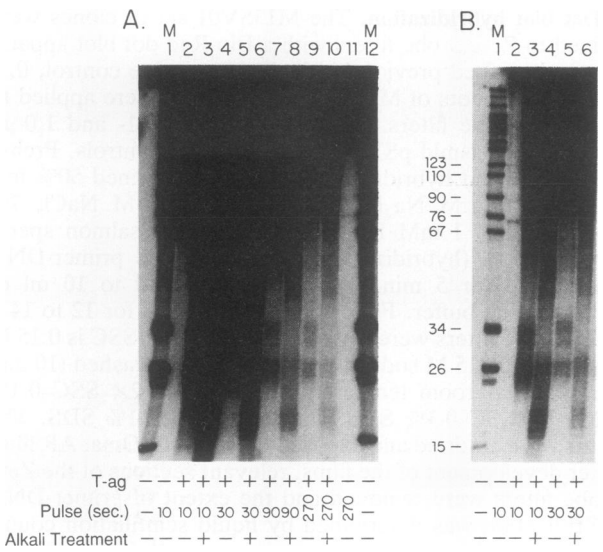

FIG. 1. Time course of primer-DNA formation. Reactions were pulse-labeled for the indicated times. Following ethanol precipitation, the pellets were resuspended in 20 μl of TE buffer and aliquots (12,000 cpm) were withdrawn. Where indicated, RNA primers were removed by alkali treatment (see Materials and Methods); after being resuspended in 10 μl of TE buffer, additional aliquots (12,000 cpm) were withdrawn. All samples were then boiled for 4 min in a 90% formamide loading buffer (40). The reaction mixtures were then applied to a denaturing 10% polyacrylamide gel that was run as described in Materials and Methods. Untreated primer-DNA contains two distributions centered around ~34 and ~24 nt. Lanes 2 and 11 demonstrate that primer-DNA formation is dependent on T-ag. Size markers (sizes given in nucleotides) used during gel electrophoresis (lanes 1 and 12) were from an *Msp*I digest of pBR322 (New England Biolabs) labeled with kinase by standard methods (40). Panel B presents a lighter exposure (1 day) of lanes 1 to 6 from the autoradiogram shown in panel A (4-day exposure).

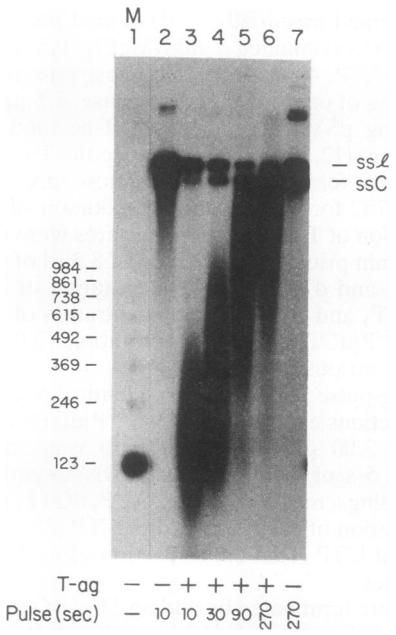

FIG. 2. Alkaline agarose gel analyses of the size of the nascent-DNA population formed during pulse-labeling. Aliquots (6,000 cpm) of the non-alkali-treated reaction mixtures used in Fig. 1 were applied to a 1.8% alkaline agarose gel that was processed as described in Materials and Methods. The pulse-labeling times and the positions of single-stranded linear (ssl) and single-stranded circular (ssC) forms of pSV01ΔEP are indicated. Size markers (lane 1; in nucleotides) were derived from a 123-bp DNA ladder (GIBCO-BRL).

that primer-DNA can be detected at all subsequent time points. Primer-DNA contains two major size distributions centered around ~34 and ~24 nt. Furthermore, primer-DNA formed during the indicated pulse times was reduced by ~10 nt by alkali treatment (Fig. 1A, lanes 4, 6, 8, and 10, and Fig. 1B, lanes 4 and 6); this demonstrates that at all time points, primer-DNA was covalently linked to RNA. Finally, the reaction mixtures displayed in lanes 2 and 11 of Fig. 1A confirm that primer-DNA formation is T-ag dependent.

The overall size distribution of the nascent DNA in the pulse reaction mixtures was established by loading aliquots on a 1.8% alkaline agarose gel (Fig. 2). Nascent DNA formed during a 10-s pulse (lane 3) had an average size distribution of ~175 nt. Nascent DNA formed during a 30-s pulse (lane 4) had an average size distribution of ~350 nt, while that formed during a 90-s pulse (lane 5) had an average size distribution of ~1,000 nt. Finally, lane 6 indicated that nascent DNA formed during a 270-s (4.5-min) pulse included full-length DNA products. Thus, primer-DNA (Fig. 1) was detected in reaction mixtures that contained molecules primarily undergoing initiation events as well as in reaction mixtures that contained molecules primarily in elongation stages of DNA synthesis (Fig. 2).

The experiments whose results are shown in Fig. 1 and 2 indicated that primer-DNA functions during both initiation and elongation of DNA replication. However, it could be argued that because of extensive unwinding of the substrate, primer-DNA present in reaction mixtures pulsed for brief periods (10 s) was synthesized from origin-distal regions. Moreover, primer-DNA present in reaction mixtures containing molecules primarily undergoing elongation events could be derived from origin-proximal regions; its presence could reflect delayed initiation events or, alternatively, the displacement of primer-DNA from parental DNA templates during initiation of synthesis. Therefore, it was necessary to demonstrate that primer-DNA is derived initially from the SV40 origin region and, at later times in the reaction, from origin-distal regions. A map of the plasmid used in these experiments, pSV01ΔEP, is presented in Fig. 3A.

Determining the template regions encoding primer-DNA and Okazaki fragments formed during initiation and elongation stages of DNA synthesis. Prior to initiation of SV40 DNA synthesis, a T-ag-dependent preinitiation complex is assembled at the SV40 origin. Previous studies indicated that the preinitiation complex requires approximately 10 min to assemble (17, 53). Consistent with these studies, reaction mixtures incubated with T-ag for less than 10 min do not support a pulse (data not presented). Additional studies indicated that once the preinitiation complex is assembled in HeLa cell crude extracts, it is maintained in the vicinity of the SV40 origin until the nucleotides other than ATP are introduced to the reaction mixture (5).

If primer-DNA is involved in initiation of SV40 DNA synthesis, then primer-DNA formed during a brief pulse (5 s) should hybridize to the SV40 origin region. Moreover, if primer-DNA also functions during elongation events, then primer-DNA isolated at later times should hybridize to regions distal to the SV40 origin. The experiments whose

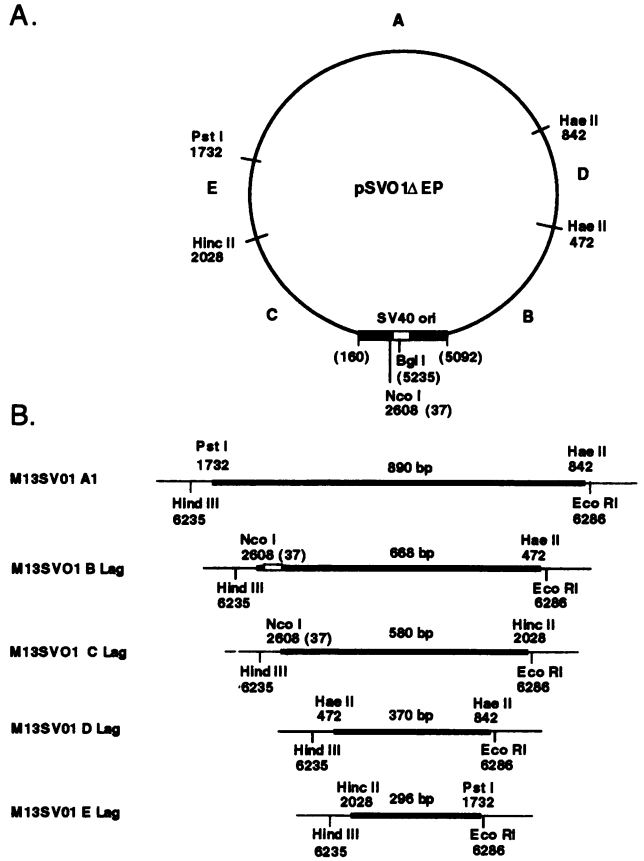

FIG. 3. Structure of pSV01ΔEP and the M13SV01 clones. (A) Map of plasmid pSV01ΔEP (5); the SV40 origin-containing 311-bp *Eco*RII fragment is depicted by the solid rectangle. The smaller open rectangle depicts the SV40 core origin. Numbers in parentheses represent SV40 nucleotides numbered according to Fiers (47); additional numbers represent nucleotide positions on plasmid pSV01ΔEP. Positions of the restriction endonuclease sites used to subclone the five pSV01ΔEP fragments (A to E) into M13mp19 to form the M13SV01 set of clones are indicated. (B) Clones containing both possible orientations of any given fragment were isolated; however, panel B presents maps of only those M13SV01 clones that contain DNA fragments complementary to nascent lagging-strand DNA (clones B lag to E lag; those in the opposite orientation are abbreviated B lead to E lead). Since bidirectional DNA replication terminates in pSV01ΔEP fragment A, single-stranded DNA from fragment A will contain segments complementary to both leading- and lagging-strand nascent DNA. Presented is the M13SV01 clone containing fragment A in an orientation termed A1. Plasmid pSV01ΔEP fragments A to E that were cloned into the *Sma*I site of M13mp19 (thick lines) are shown. Blunt-end ligation of the pSV01ΔEP fragments into the M13mp19 *Sma*I site resulted in the loss of the terminal restriction sites; this is symbolized by the absence of vertical bars at restriction endonuclease sites used to depict pSV01ΔEP fragment termini. Single-stranded DNA was isolated from M13 phage by standard protocols (40).

results are presented in Fig. 4 and 5 were conducted to test this hypothesis. However, we were concerned that primer-DNA isolated from reaction mixtures continuously pulse-labeled for longer times (>5 s) might not accurately reflect the location of the synthesis events taking place at the end of a reaction. For example, primer-DNA isolated from reaction mixtures pulse-labeled for more than 5 s might contain molecules that accumulated after being displaced from origin-proximal regions on the pSV01ΔEP template. Therefore, a late labeling pulse (see Materials and Methods) was used to form primer-DNA during the last 5 s of DNA synthesis.

Reaction mixtures were incubated in the presence of T-ag for 15 min prior to initiation of DNA synthesis. Nascent DNA, including primer-DNA, was formed during a 5-s pulse. Primer-DNA was also formed during a 5-s late labeling pulse following either 40 or 265 s of DNA synthesis. Aliquots of the reaction mixtures were separated on a 10% denaturing acrylamide gel (data not shown), and primer-DNA was isolated by standard methods (see Materials and Methods).

Purified primer-DNA was used as a probe in hybridization experiments with the M13SV01 set of clones (5) (Fig. 3B) affixed to Zeta probe filters. (Note that the origin-containing 311-bp SV40 *Eco*RII fragment [the solid rectangle in Fig. 3A] is subdivided into the B and C M13SV01 clones). Primer-DNA, formed during a 5-s pulse following a 15-min incubation in the presence of T-ag, hybridized primarily to origin-proximal M13SV01 clones C_{Lag} and B_{Lag} (Fig. 4A). However, purified primer-DNA, isolated from reaction mixtures pulsed for 5 s after either 40 or 265 s of synthesis, hybridized not only to M13SV01 clones C_{Lag} and B_{Lag} but also to origin-distal lagging-strand clones (Fig. 4B and C). Finally, it is apparent from Fig. 4A to C that at all stages of DNA synthesis, primer-DNA is derived exclusively from templates for lagging-strand DNA synthesis.

To quantitate the extent of primer-DNA hybridization to the M13SV01 clones after different times of DNA synthesis, DNA-containing regions of the Zeta probe filters were removed and the amount of radioactivity was determined. The sum of the labeled DNA hybridized to the M13SV01 clones minus the background hybridization to single-stranded M13 DNA was calculated and used to establish the percentage of hybridization to a given clone. A plot of these analyses is presented in Fig. 4D. The percentage of primer-DNA hybridized to the 1-μg aliquots of M13SV01 clones C_{Lag} and B_{Lag} is indicated by the upper solid line; hybridization to the 1-μg aliquots of M13SV01 clones D_{Lag}, E_{Lag}, A1, and A2 is indicated by the lower solid line. It is obvious from Fig. 4 that with increasing lengths of DNA synthesis, a greater percentage of primer-DNA is derived from regions distal to the SV40 origin.

Additional aliquots of the nascent DNA formed during a 5-s pulse following 0, 40, or 265 s of DNA synthesis were displayed on a 1.8% alkaline agarose gel (Fig. 5). This figure demonstrates that nascent DNA formed during a 5-s pulse was on average ~125 nt long (lane 2), while nascent DNA formed during a 5-s pulse following 40 s of synthesis was on average ~200 nt long (lane 3). Nascent DNA formed during a 5-s pulse following 265 s of synthesis contained two distributions centered at ~200 and ~1,000 nt. The DNA in the lower distribution was roughly the size of Okazaki fragments (~200 nt), while the upper distribution contained molecules that were nearly as long as full-length pSV01ΔEP (2,800 nt) (lane 4). The primer-DNA distribution is also indicated; primer-DNA was detected in lanes 3 and 4 upon longer exposures of the autoradiogram.

Regions of the alkaline agarose gel containing the nascent-DNA populations formed during a 5-s pulse (Fig. 5, lane 2) and during a 5-s pulse following 40 s of synthesis (Fig. 5, lane 3) and the two distributions formed during a 5-s pulse following 265 s of synthesis (Fig. 5, lane 4) were removed from the gel (see Materials and Methods), and the DNA was isolated by electroelution (40). The purified nascent-DNA populations were used in hybridization experiments with the

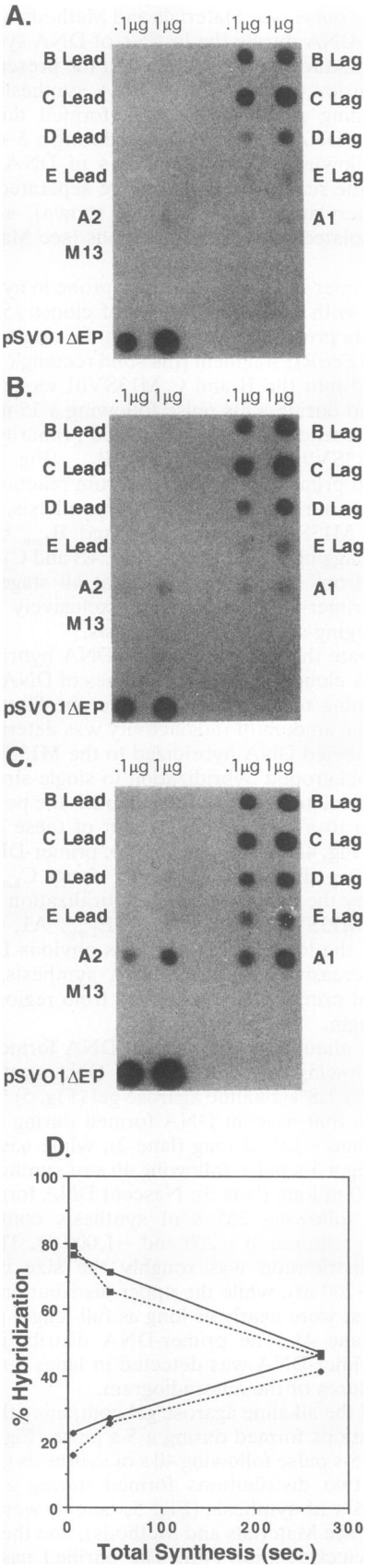

FIG. 4. Dot blot analyses of primer-DNA formed during a 5-s pulse following 0, 40, or 265 s of DNA synthesis (A to C, respectively). Purified primer-DNA (see below) was used in hybridization experiments with the M13SV01 set of clones affixed to Zeta probe filters. The reaction mixtures were incubated in the presence of T-ag for 15 min prior to initiation of DNA synthesis and subsequent pulse-labeling (late labeling pulse; see Materials and Methods). To obtain enough purified primer-DNA to use as a hybridization probe, the nascent DNA from 7 reaction mixtures formed during a 5-s pulse (202,490 cpm), from 13 reaction mixtures formed during a 5-s pulse following 40 s of synthesis (405,980 cpm), or from 14 reaction mixtures formed during a 5-s pulse following 265 s of synthesis (361,120 cpm) were pooled (~40,000-cpm aliquots were removed for alkaline agarose gel analyses; Fig. 5) and ethanol precipitated. The pellets were resuspended in 2.5 μl of TE buffer and an equal volume of formamide loading buffer (40); nascent-DNA samples were separated on a 10% polyacrylamide–urea gel as described in Materials and Methods (data not shown). Regions of the gel containing primer-DNA formed during a 5-s pulse or a 5-s pulse following 40 or 265 s of synthesis were removed (29,000, 10,075, and 9,183 cpm, respectively) and isolated as described in the text. Equal counts (~6,000 cpm) were used in the hybridization experiments. Hybridization and wash conditions are those described in Materials and Methods. For panels A to C, the sums of the radioactivity hybridized to the 1-μg aliquots of the M13SV01 clones were 132, 107, and 124 cpm, respectively. The extent of primer-DNA hybridization to the 1-μg aliquots of the origin-proximal C_{Lag} and B_{Lag} clones, as a function of total time of synthesis, is quantitated in panel D (solid upper line). Also presented is the extent of primer-DNA hybridization to the 1-μg aliquots of four origin-distal M13SV01 clones (D_{Lag}, E_{Lag}, A1, and A2; solid lower line) as a function of total time of synthesis. Nearly identical results were obtained when the extent of primer-DNA hybridization to the 0.1-μg aliquots was quantitated (data not shown). Moreover, virtually identical results were obtained when quantitation was performed with a Molecular Dynamics phosphoimager (data not shown).

M13SV01 clones (Fig. 6A to C). It is apparent that the nascent-DNA population formed after a 5-s pulse (Fig. 5, lane 2) hybridized predominantly to M13SV01 clones C_{Lag} and B_{Lag} (Fig. 6A). In contrast, the nascent-DNA population formed after a 5-s pulse following 40 s of synthesis (Fig. 5, lane 3) hybridized to M13SV01 clones C_{Lag}, B_{Lag}, and D_{Lag} and, to a lesser extent, to the other M13SV01 clones that are templates for lagging-strand synthesis events (Fig. 6B). The Okazaki-size-DNA distribution (nascent DNA below the 369-nt size marker) isolated from reaction mixtures pulsed for 5 s following 265 s of synthesis (Fig. 5, lane 4) hybridized to all of the M13SV01 lagging-strand clones (Fig. 6C). The upper part of the distribution shown in Fig. 5, lane 4, hybridized to both the leading- and the lagging-strand M13SV01 clones (Fig. 6D). Thus, at later time points, coordinated leading- and lagging-strand synthesis events are taking place in this system. Finally, the observation that the ~200-nt-long nascent DNA (Fig. 5, lane 3) hybridized to M13SV01 clones C_{Lag} and B_{Lag}, but not M13SV01 clones C_{Lead} and B_{Lead} (Fig. 6B), confirms that, in this system, initial synthesis events take place on lagging-strand templates outside the core origin. The same conclusion was drawn from previous studies (5).

The Okazaki fragment hybridization studies whose results are presented in Fig. 6A to C were quantitated by the same procedure used to quantitate the results shown in Fig. 4; the results of these analyses are presented in Fig. 4D. The percentage of nascent DNA hybridized to the 1-μg aliquots of M13SV01 clones C_{Lag} and B_{Lag} is indicated by the upper dotted line; hybridization to the 1-μg aliquots of M13SV01

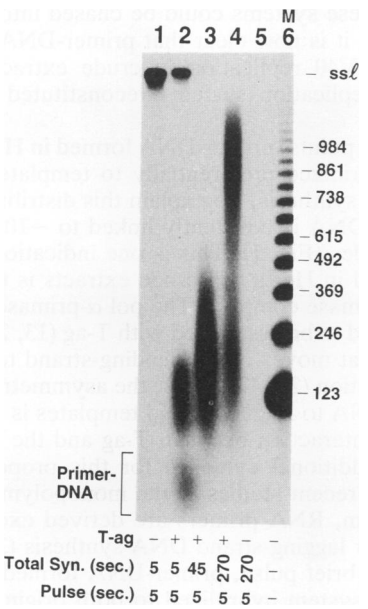

FIG. 5. Alkaline agarose gel analyses of the size of the nascent-DNA population formed during a 5-s pulse following synthesis for various periods. Aliquots (~40,000 cpm) of the pooled pulse reaction mixtures (see Fig. 4 legend) were applied to a 1.8% alkaline agarose gel that was electrophoresed and processed as described in Materials and Methods. The total time of DNA synthesis is indicated. Lanes 1 and 5 demonstrate that in the absence of T-ag, nascent DNA is not formed during pulse-labeling. The position of single-stranded linear (ssl) pSV01ΔEP is indicated. Size markers (lane 6) are identical to those described in the legend to Fig. 2. After autoradiography, regions of the gel containing the nascent-DNA population formed during a 5-s pulse (lane 2) or formed during a 5-s late labeling pulse following 40 s of synthesis (lane 3) were removed and the DNA was isolated (see Materials and Methods). Regions of the gel containing the lower Okazaki-size distribution (below the 369-nt size marker) and upper distribution (above the 492-nt size marker) formed during a 5-s late labeling pulse following 265 s of synthesis (lane 4) were also removed, and the DNA was subsequently isolated (see Materials and Methods). After ethanol precipitation, pellets containing purified nascent DNA (5,360 cpm isolated from lane 2, 12,950 cpm isolated from lane 3, 10,100 cpm isolated from the lower distribution in lane 4, and 10,750 cpm isolated from the upper distribution in lane 4) were resuspended in 100 μl of TE buffer.

clones D_{Lag}, E_{Lag}, A1, and A2 is indicated by the lower dotted line. It is clear from Fig. 6A to C and Fig. 4D that Okazaki-size nascent DNA is derived from templates for lagging-strand synthesis and that with increasing lengths of DNA synthesis, a greater percentage of Okazaki-size nascent DNA is derived from regions distal to the SV40 origin. Moreover, it is obvious from Fig. 4D that in a given reaction, primer-DNA and the Okazaki-size nascent-DNA population are derived from approximately the same template locations.

DISCUSSION

Upon addition of T-ag to an otherwise complete SV40 replication reaction mixture, a functional preinitiation complex forms at the SV40 origin after an incubation period of approximately 10 min (17, 53). Evidence indicating that once the preinitiation complex has assembled it is maintained in the vicinity of the origin region until initiation of DNA synthesis was previously presented (5). Indeed, when purified primer-DNA formed during a 5-s pulse following either 10- or 60-min incubation in the presence of T-ag was used in the hybridization experiments, the dot blot patterns were identical to that presented in Fig. 4A (11a). Collectively, these studies indicate that in this system DNA unwinding and replication events are coupled.

Once DNA synthesis is initiated, primer-DNA can be detected during various stages of DNA replication. Primer-DNA formed during initiation of synthesis (5-s pulse) hybridized to lagging-strand templates on either side of the SV40 origin (Fig. 4A). These studies suggest a role for primer-DNA during initiation of SV40 DNA synthesis in vitro. We previously suggested that primer-DNA, formed on lagging strands in the origin region, is used to prime leading-strand synthesis events (5). Consistent with this proposal, total nascent DNA formed during a 5-s pulse hybridized to origin-proximal templates for lagging-strand synthesis; after a longer labeling period, the nascent-DNA population also hybridized to templates for leading-strand synthesis (5).

As DNA synthesis proceeded out of the SV40 origin region, the percentage of primer-DNA derived from lagging-strand templates distal to the origin increased, while the percentage derived from the origin-proximal regions decreased (Fig. 4A to D). A similar change in the distribution of Okazaki-size DNA fragments was also observed (Fig. 6A to C and 4D). These experiments demonstrate that primer-DNA is a feature of lagging-strand synthesis events in regions distal to the SV40 origin. They also show that a correlation between the locations of primer-DNA and Okazaki fragments during lagging-strand synthesis events exists. Thus, there exists a possible precursor-product relationship between the formation of primer-DNA and Okazaki fragments. Finally, it is apparent from Fig. 4C and 6C that even after 4.5 min of DNA synthesis, a considerable percentage (~50%) of the primer-DNA and Okazaki fragments hybridized to origin-proximal lagging-strand templates. The percentage of primer-DNA and Okazaki fragments hybridizing to the origin-proximal fragments C_{Lag} and B_{Lag} continued to decrease with increased times of DNA synthesis (unpublished data). Nevertheless, it is apparent that synthesis events in the vicinity of the SV40 origin continued for several minutes after initiation of DNA synthesis. This may be due, in part, to the assembly and subsequent utilization of newly formed preinitiation complexes.

Okazaki fragments, but not primer-DNA molecules, were observed in previous in vitro studies of SV40 DNA synthesis in systems reconstituted with purified proteins (for examples, see references 23, 26, 38, 49, and 52). However, it is now clear that the concentrations of replication factors such as T-ag, HSSB, the pol α-primase complex, PCNA, and RFC (also termed activator I [8, 22, 43]) can have a profound effect on the size and composition of nascent DNA formed in these reactions (9, 15, 33, 49). Moreover, additional studies indicate that during the longer periods of synthesis employed in these studies (~15 to 60 min), the inherently nonprocessive pol α-primase complex can reinitiate DNA synthesis on the 3′ ends of newly formed DNA strands (9, 14, 49). Thus, the Okazaki-size DNA fragments reported in previous studies, particularly those employing only the pol α-primase complex, may reflect multiple dissociation-reassociation events between small nascent-DNA chains and pol α. Indeed, using a 1-min pulse, Eki et al. (15) have recently detected primer-DNA in both the monopolymerase system (T-ag, HSSB, topoisomerase I, and pol α-primase) and the dipolymerase systems (monopolymerase system plus pol δ, PCNA, and RFC [activator I]). Primer-DNA molecules

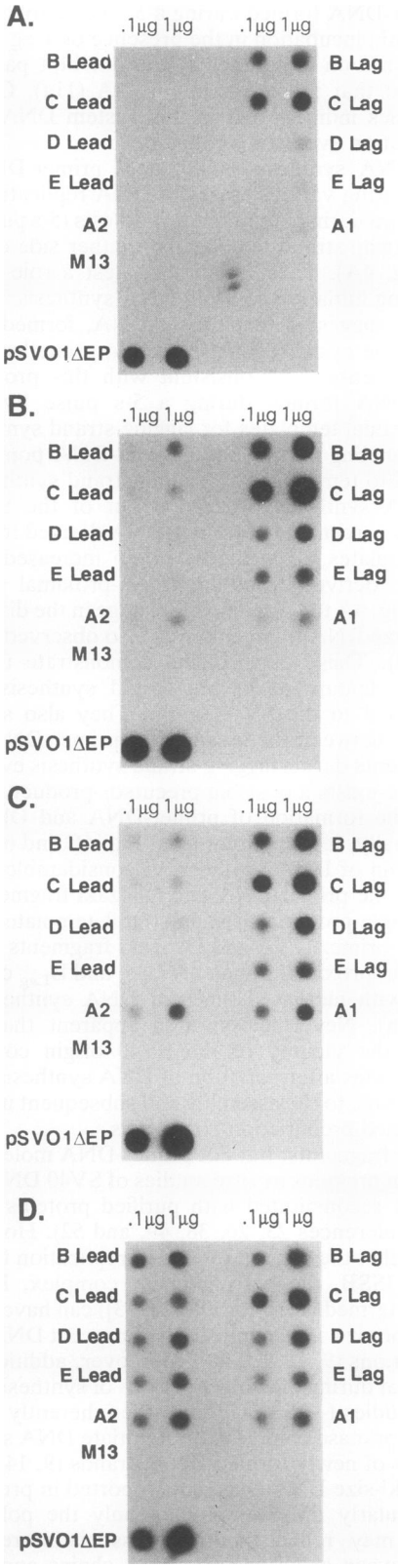

FIG. 6. Dot blot analyses of nascent DNA formed during a 5-s pulse following 0, 40, or 265 s of DNA synthesis. Purified Okazaki-size nascent DNA (purified from lanes 2 to 4 in Fig. 5) were used in hybridization experiments with the M13SV01 set of clones affixed to Zeta probe filters (A to C, respectively). Equal counts (~5,000 cpm) were used in the hybridization experiments. For panels A to C, the sums of the radioactivity hybridized to the 1-μg aliquots of the M13SV01 clones were 112, 298, and 240 cpm, respectively. The hybridization (~5,000 cpm) of the upper distribution of nascent DNA (Fig. 5, lane 4) to the M13SV01 set of clones is presented in panel D. The sum of the radioactivity hybridized to the 1-μg aliquots of the M13SV01 clones was 100 cpm. Hybridization and wash conditions were as described in the text (see Materials and Methods). The extent of hybridization of the Okazaki-size nascent-DNA populations to the 1-μg aliquots of the origin proximal C_{Lag} and B_{Lag} clones is quantitated in Fig. 4D (dotted upper line). The extent of hybridization to the four origin-distal M13SV01 clones (D_{Lag}, E_{Lag}, A1, and A2) is also presented (dotted lower line). Similar results were obtained when the 0.1-μg aliquots (panels A to C) were quantitated. Finally, nearly identical results were obtained when quantitation was performed with a Molecular Dynamics phosphoimager (data not shown).

formed in these systems could be chased into Okazaki-size DNA. Thus, it is now clear that primer-DNA is not only a feature of SV40 replication in crude extracts but also a feature of replication systems reconstituted with purified proteins.

At all time points, primer-DNA formed in HeLa cell crude extracts hybridized preferentially to templates for lagging-strand DNA synthesis. To explain this distribution, we note that primer-DNA is covalently linked to ~10-nt-long oligoribonucleotides (Fig. 1). This is one indication that primer-DNA formed in HeLa cell crude extracts is the product of the pol α-primase complex. The pol α-primase complex has been reported to be associated with T-ag (13, 20, 41); T-ag is a helicase that moves on the leading-strand template in the 3′-to-5′ direction (22, 42). Thus, the asymmetric distribution of primer-DNA to lagging-strand templates is likely a reflection of the interaction between T-ag and the pol α-primase complex. Additional evidence for this proposal has been provided by recent studies of the monopolymerase system; in this system, RNA primers are derived exclusively from templates for lagging-strand DNA synthesis (33). However, even after a brief pulse, primer-DNA formed in the monopolymerase system hybridized to both origin-proximal and origin-distal M13SV01 clones. The additional factor(s) required to cause stabilization of the preinitiation complex at the SV40 origin is currently unknown. We note that in the monopolymerase system, HSSB is absolutely required to form the ~10-nt-long RNA primers on duplex DNA containing the SV40 origin (31, 33). In contrast, related studies have shown that single-stranded DNA templates coated with HSSB inhibit the primase activity of the pol α-primase complex (9, 15, 49). Thus, synthesis of primer-DNA by the pol α-primase complex on newly exposed lagging-strand templates (single-stranded DNA) may be regulated, in part, by HSSB.

We previously demonstrated that in HeLa cell crude extracts, PCNA is necessary for the maturation of primer-DNA into larger DNA products (5). Okazaki-size DNA fragments were not detected in these experiments, indicating that once primer-DNA is formed in HeLa cell crude extracts, it is not a substrate for reinitiations by the pol α-primase complex. The failure of primer-DNA to mature into Okazaki-size DNA in the absence of PCNA, an essential SV40 replication factor (37), may reflect blockage of the 3′ OH group of primer-DNA by factors such as RFC (activator I) (15, 49) or perhaps poly(ADP-ribose) polymerase (14). We have now demonstrated that primer-DNA is a feature of both initiation and elongation events on lagging-strand templates.

Thus, a PCNA-dependent polymerase may be required to extend primer-DNA during initiation of leading-strand synthesis events as well as during maturation of Okazaki fragments. This implies that a PCNA-dependent polymerase may be involved in a polymerase switch (48, 49) with both origin-proximal and origin-distal primer-DNA molecules. Two PCNA-dependent polymerases, pol δ and pol ε, exist in HeLa and other eukaryotic cells (3, 25, 27, 32, 45; for a review, see reference 29). Both pol δ and pol ε can utilize activated 3′ OH ends of synthetic primers complexed with PCNA and RFC (activator I) (15, 27); thus, either polymerase may function during a polymerase switch. Studies of SV40 DNA replication in isolated nuclei also indicated that two DNA polymerases may be involved in the formation of Okazaki fragments (34, 35); also, an RNA-DNA species similar to primer-DNA has been observed in studies of replication of polyomavirus DNA in isolated nuclei (16). However, the exact roles played by pol δ and pol ε in these events are currently unclear.

The experiments presented here, and those reported previously (5), raise the possibility that during SV40 replication in HeLa cell crude extracts, the pol α-primase complex may be limited to the synthesis of primer-DNA. It is interesting that certain studies of the pol α-primase complex purified from higher eukaryotic cells have indicated that the processivity of this complex is very limited; the products were equal in size to that of primer-DNA (10, 19, 21, 50). Experiments addressing the fidelity of DNA replication also support the possibility that the pol α-primase complex may play a limited role during SV40 replication. In HeLa cell crude extracts, replication fidelity is similar for SV40 leading- and lagging-strand synthesis events (39). However, the pol α-primase complex purified from HeLa cells (7) and other higher eukaryotes (for reviews, see references 6, 24, and 51) generally does not contain an associated 3′-to-5′ exonuclease activity. Thus, if lagging strands are synthesized entirely by the pol α-primase complex, the fidelity of lagging-strand synthesis would be expected to be lower than that for leading-strand synthesis. pol δ and pol ε are both high-fidelity polymerases that contain 3′-to-5′ exonuclease activities (46; see references 1a, 6, 29, and 51 for reviews). Either of these polymerases could extend primer-DNA and synthesize lagging-strand DNA at an error rate similar to that for leading strands.

Maturation of Okazaki fragments is likely to require the removal of RNA primers by RNase H and a previously described 5′-to-3′ exonuclease purified from HeLa cells (23) and the subsequent ligation of the nascent-DNA strands. However, it is apparent that additional studies are required to more fully characterize primer-DNA and other lagging-strand synthesis events.

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