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DNA Synthesis Generally Initiates outside the Simian Virus 40 Core Origin In Vitro

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The nucleotide positions at which DNA synthesis initiates in vitro, in the vicinity of the simian virus 40 origin, have been determined. Start sites for DNA synthesis are greatly suppressed over the simian virus 40 core origin. Relatively weak start sites are detected over the 21-bp repeats and T-antigen-binding site I; distal to these regions, stronger start sites are detected. Thus, studies using a model system for eukaryotic DNA replication indicate that DNA synthesis events initiate, in general, outside the core origin.

Multiple DNA-protein interactions govern many complex biological phenomena. Indeed, recombination, transcription, and replication events are all thought to be catalyzed by very precise nucleoprotein structures (1, 16). These specialized nucleoprotein structures confer the accuracy and regulation required by these diverse transactions.

An example of a complex phenomenon known to be mediated by precise protein-DNA interactions is the initiation of simian virus 40 (SV40) DNA synthesis. This process is dependent on the binding of the 82-kDa SV40 T antigen (T-ag) to the SV40 origin. Once bound to the origin, T-ag assembles as a double hexamer (30). Additional information regarding the interaction of T-ag with the SV40 origin has been reviewed (3, 20). Binding of T-ag (a DNA-dependent helicase [37]) to the SV40 origin initiates a series of additional protein assembly events that result in the unwinding of the SV40 origin (4, 5, 9, 15, 48) and ultimately in the formation of a poorly defined preinitiation complex. Initiation of SV40 DNA synthesis can be achieved with four purified proteins: T-ag, a topoisomerase that can remove positive supercoils, human single-stranded-DNA-binding protein (termed HSSB, RFA, or RPA [19, 46, 47]), and the polymerase α (pol α)-primase complex (27, 31, 33, 41, 43). However, it is likely that initiation of SV40 DNA synthesis normally depends on additional protein factors. Furthermore, studies with HeLa cell crude extracts indicate that once the preinitiation complex has assembled, it is maintained in the vicinity of the SV40 origin region until the addition of nucleotides (6).

The distribution of nascent DNA formed during a 5-s pulse in HeLa cell crude extracts, in the vicinity of the SV40 origin, was previously examined by blotting techniques; nascent DNA hybridized exclusively to templates for lagging-strand DNA synthesis (6, 12). Moreover, when the pulse reactions were conducted in the presence of anti-proliferating-cell nuclear antigen, DNA synthesis was limited to a small (~30-nucleotide [nt]) RNA-DNA hybrid species, termed primer RNA/DNA (6, 7, 12, 34). Primer-RNA/DNA has also been shown to hybridize exclusively to templates for lagging-strand DNA synthesis (6, 12). The available evidence indicates that primer-RNA/DNA is the product of the pol α-primase complex (6, 17, 34), the polymerase complex that initiates DNA synthesis. Therefore, to further characterize initiation events, primer-RNA/DNA has been used to determine the locations, in the vicinity of the SV40 origin, at which DNA synthesis initiated.

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MATERIALS AND METHODS

Commercial supplies of enzymes and reagents. The restriction endonuclease *Nco*I was obtained from United States Biochemicals, *Hin*dIII was obtained from Boehringer Mannheim Biochemicals, and *Ava*II and *Sph*I were obtained from Gibco BRL Life Technologies. Sequenase version 2.0 was obtained from United States Biochemicals. RNasin was obtained from Promega. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals.

Preparation of SV40 T-Ag, HeLa cell crude extracts, and DNA stocks. SV40 large T-ag, HeLa cell crude extracts, and plasmid pSV01ΔEP, a 2,796-bp plasmid with the SV40 origin containing an *Eco*RII fragment cloned into the *Eco*RI site of pBR322 (44), were isolated as described previously (6, 7, 44). Single-stranded DNA from M13 clones M13SV01ΔEP-Pst 1 (containing pSV01ΔEP DNA complementary to late mRNA) and M13SV01ΔEP-Pst 2 (containing pSV01ΔEP DNA complementary to early mRNA) (7) were purified by standard methods (35).

Labeling reactions. (i) Pulse reactions. Pulse reactions (120-μl mixtures) were performed exactly as described previously (6, 7); nascent DNA formed in 17 5-s pulse reactions was purified and subsequently pooled. Samples (~439,000 cpm; ~10 pmol) were precipitated with ethanol, washed with 80% ethanol, and dried. The pellets were resuspended in 10 μl of Tris-EDTA buffer plus an equal volume of formamide loading buffer (35). Samples were boiled for 4 min and loaded onto a 10% polyacrylamide gel containing 8 M urea. Primer-RNA/DNA was isolated (~35,600 cpm; ~0.8 pmol) by a previously described procedure (12) that is outlined below.

(ii) Continuous-labeling reactions. Continuous-labeling reactions were identical to the pulse reactions, except that the nucleotides in the "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; ~250 cpm/fmol]) were added at the same time as T-ag. After 15 min, 17 reactions were stopped and the mixtures were subsequently pooled. After ethanol precipitation, the samples were resuspended as described for pulse reactions, boiled for 4 min, and loaded onto a 10% polyacrylamide gel containing 8 M urea. Primer-RNA/DNA (~24 and 34 nt) was purified (~1.2 pmol) as described in detail previously (12); this procedure is summarized below.

After autoradiography, regions of the gel containing primer-RNA/DNA were removed and incubated overnight at 4°C in 1.5 ml of elution buffer (0.1% sodium dodecyl sulfate, 0.5 M ammonium acetate, 10 mM magnesium acetate). Primer-RNA/DNA was separated from acrylamide fragments by passing the samples through 0.45-μm-pore-size Millex-HV filters (Millipore Corp.). Carrier tRNA (20 μg) was added to the samples, and the reaction mixtures were precipitated with ethanol. Pellets containing purified primer-RNA/DNA were then resuspended in 40 μl of TE buffer.

Primer extension reactions. RNA primers were removed from one primer-RNA/DNA aliquot with alkali; phosphate residues were removed from a second aliquot with calf intestinal alkaline phosphatase (7). Treated primer-RNA/DNA molecules were hybridized to either M13SV01ΔEP-Pst 1 or M13SV01ΔEP-Pst 2; reaction conditions for the primer extension reactions (see Fig. 1) have been described previously (7). Restriction endonuclease digestions were conducted for 2 h in 40-μl reaction mixtures with 20 U of enzyme as recommended by the manufacturer; 40 U (1 μl) of RNasin was added to each reaction mixture to inhibit RNase activity.

Gel electrophoresis and PhosphorImager analysis. Sequencing reactions (36) were conducted with a kit supplied by United States Biochemicals (dITP labeling mix). Reactions were primed with a 21-nt oligonucleotide (5′TCATGAGCG

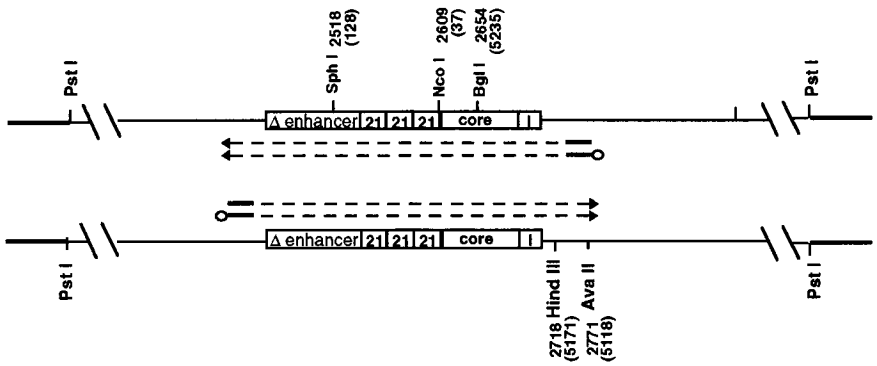

FIG. 1. Depiction of the SV40 origin region and the primer extension reactions used to map primer-RNA/DNA start sites. The relative positions of T-ag-binding site I (I), the SV40 core origin, the 21-bp repeats, and sequences from one of the SV40 enhancers (Δ enhancer) are indicated. Single-stranded M13 DNA is depicted by the thick line, and DNA derived from pSV01ΔEP is symbolized by the thinner line. Primer-DNA is represented by the small rectangle at the end of the dashed line; primer-RNA is depicted by the circle. Primer extension products, resulting from Sequenase 2-catalyzed elongation of primer-RNA/DNA molecules hybridized to either M13SV01ΔEP-Pst 1 (top line) or M13SV01ΔEP-Pst 2 (bottom line), are depicted by the dashed arrows. The primer extension products were cleaved at the indicated restriction endonuclease sites. Rectangles not associated with a circle represent primer-DNA molecules formed by alkali treatment; use of these molecules in primer extension reactions resulted in products either 8 or 9 nt shorter than nontreated samples. Nucleotide positions in plasmid pSV01ΔEP are indicated; numbers in parentheses correspond to the numbering system for SV40 nucleotides (40).

GATACATATTTG3′) (Oligos Etc. Inc.) hybridized to M13SV01ΔEP-Pst 2 DNA (7). Primer extension reaction products (~3,000 cpm per lane) and sequencing reaction products (~90,000 cpm of [^{35}S]dATP per lane) were loaded onto 8% polyacrylamide gels containing 8 M urea; the gels were electrophoresed and processed as described previously (7). The primer extension products were quantitated on a Molecular Dynamics PhosphorImager.

RESULTS

Figure 1 presents a map of the SV40 core origin and flanking regulatory sequences present in plasmid pSV01ΔEP (44); M13 clones M13SV01ΔEP-Pst 1 and M13SV01ΔEP-Pst 2 contain single-stranded DNA derived from this plasmid (see Materials and Methods). The SV40 core origin is both necessary and sufficient for initiation of replication (references 11, 29, and 39 and references therein). This figure also illustrates the use of primer extension techniques to map primer-RNA/DNA start sites. In an initial set of experiments, primer RNA-DNA molecules were formed during a 5-s pulse and primer extension reactions were conducted as described in Materials and Methods (7). Control reactions demonstrated that the non-restriction-endonuclease-treated primer extension products contained only high-molecular-weight products (data not shown; an example of the uncut products was reported previously [7]).

Figure 2A presents an example of studies conducted with primer-RNA/DNA and M13SV01ΔEP-Pst 2 (complementary to SV40 early mRNA). These experiments permitted mapping of initiation sites from the pSV01ΔEP *Hin*dIII site at position 2718 through the SV40 core origin, 21-bp repeats, and enhancer sequences. It is apparent from Fig. 2A (lanes 1 and 2) that primer-RNA/DNA start sites are, in general, absent over the core origin (quantitated in Fig. 3A; La and Lb are discussed below). Obvious start sites for primer-RNA/DNA (sites Lc to Li) were detected over the 21-bp repeats, although, compared with more-distal start sites, they were relatively weak (Fig. 2A, lanes 1 and 2; quantitated in Fig. 3A). Stronger primer-RNA/DNA start sites were present at template locations distal to the beginning of the SV40 enhancer. Mapping the distal (relatively strong) primer-RNA/DNA start sites was the subject of a previous report (7). Additional primer extension experiments, conducted with *Ava*II instead of *Hin*dIII (Fig. 1), demonstrated that primer-RNA/DNA start sites were not detected on the "lower strand" within T-ag-binding site I (data not presented). Finally, it is obvious that alkali treatment reduced the size of the primer extension products; with the exception of L1, alkali treatment reduced the primer extension products by either 9 nt (Ld to Li) or 8 nt (La to Lc) (Fig. 2A, lanes 1 and 2). Why L1 is particularly susceptible to alkali treatment is not understood.

Figure 2B presents the results of primer extension reactions conducted with primer-RNA/DNA and M13SV01ΔEP-Pst 1 (complementary to SV40 late mRNA); these experiments permit mapping of initiation sites from the pSV01ΔEP *Nco*I site at position 2609 through the core origin and T-ag-binding site I (Fig. 1). It is apparent from Fig. 2B (lanes 3 and 4) that on the "upper strand," primer-RNA/DNA start sites are not detected over the core origin. Upon longer exposure of the autoradiogram, start site Ex was detected within site I. Relatively strong primer-RNA/DNA start sites are detected at sites distal to T-ag-binding site I; the position of E1, the first strong start site (7), is indicated. Finally, additional primer extension experiments conducted with *Sph*I instead of *Nco*I (Fig. 1) demonstrated that start sites are not detected within the core origin near the *Nco*I site (data not presented).

A PhosphorImager was used to quantitate the data presented in Fig. 2A (lane 1) and B (lane 3). It is clear from the plot presented in Fig. 3A (a scan of Fig. 2A, lane 1) that start sites for primer-RNA/DNA molecules are, in general, not detected over the SV40 core origin. However, two very faint start sites (La and Lb) were noticed in this and several other PhosphorImager analyses of *Hin*dIII digests. Indeed, longer exposure of the autoradiogram in Fig. 2A revealed faint bands corresponding to start sites La and Lb. The PhosphorImager analyses presented in Fig. 3A do not suggest additional primer-RNA/DNA start sites over the core origin. If additional start sites exist, they are at least ~50% lower in intensity than La (data not presented). Quantification of primer-RNA/DNA start sites Lc to Li, detected over the 21-bp repeats, revealed that they were, on average, ~15% as strong as those at more-distal sites (the average of the seven flanking start sites [L1 to L7] [7]). Similarly, it is clear from Fig. 3B (a scan of Fig. 2B, lane 3) that on the upper strand, start sites for primer-RNA/DNA molecules are not detected over the SV40 core origin. A single primer-RNA/DNA start site (Ex) was detected within site I; however, this site was only ~17% as strong as E1, the neighboring strong start site. Finally, our analyses indicated that additional primer-RNA/DNA start sites over the core

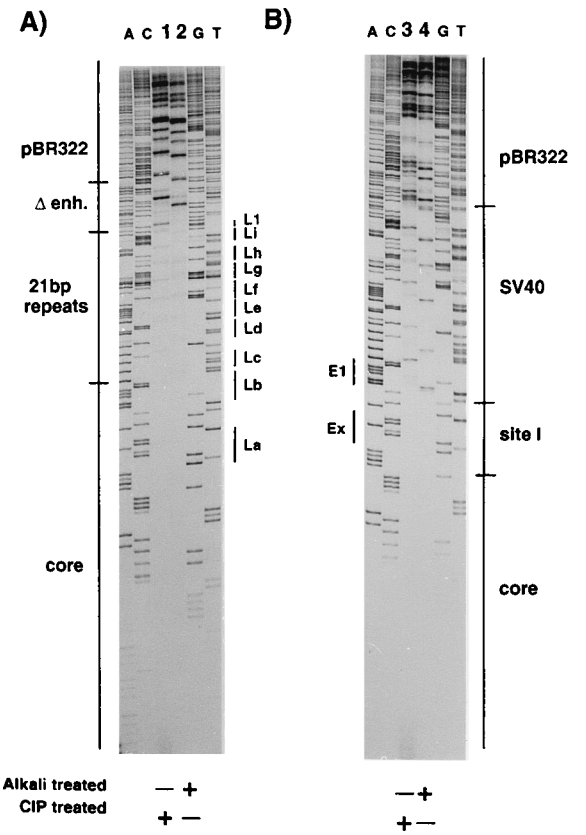

FIG. 2. Examples of primer extension reactions used to map primer-RNA/DNA start sites in the vicinity of the SV40 origin. (A) Aliquots of the primer extension products, formed with M13SV01ΔEP-Pst 2 (Fig. 1) and primer-RNA/DNA formed during a 5-s pulse (either CIP treated [lane 1] or treated with alkali [lane 2]) were restricted with *Hin*dIII (pSV01ΔEP site 2718). The digested samples were loaded alongside a sequencing ladder that served as a size marker (A, C, G, and T). To the left of the figure is a map of the pSV01ΔEP regions covered by the primer extension reaction. Also indicated are the positions of primer-RNA/DNA initiation sites La to Li (L stands for late; the tops of the vertical bars designate positions of bands in lane 1, and the bottoms designate positions of bands in lanes 2). The positions of La and Lb were suggested by additional experiments (see the text). (B) Aliquots of the primer extension products, formed with M13SV01ΔEP-Pst 1 (Fig. 1) and primer-RNA/DNA formed during a 5-s pulse (either CIP treated [lane 3] or treated with alkali [lane 4]), were restricted with *Nco*I (pSV01ΔEP site 2609). After digestion, the reactions were loaded alongside a sequencing ladder (A, C, G, and T). To the right of the figure is a map of the pSV01ΔEP regions covered by this primer extension reaction. The position of a faint primer-RNA/DNA initiation site within T-ag site I (Ex; E stands for early), detected on a longer exposure of this autoradiogram, is indicated.

origin on the upper strand, if they exist, are at least ~35% lower in intensity than Ex (data not presented).

We previously discussed the possibility that owing to greater than normal origin unwinding during a 15-min preincubation, SV40 initiation occurs at sites distal to the core origin in the pulse assay (6). Therefore, it could be argued that the distribution of primer-RNA/DNA start sites, relative to the SV40 origin (Fig. 2 and 3), is an artifact and that the start sites reflect only the locations of preinitiation complexes after a 15-min incubation. To eliminate this possibility, primer-RNA/DNA was isolated from continuous-labeling reactions (see Materials and Methods) (data not shown). In these experiments, the nucleotides in the pulse mix were added with T-ag and the reactions were stopped 15 min later. Thus, regardless of when a functional preinitiation complex was formed during the 15-min incubation period (~10 min [18, 45]), nucleotides were available for initiation of DNA synthesis. Obviously, primer-RNA/DNA molecules formed under these reaction conditions should reflect the initial position of the preinitiation complex.

Figure 4 presents the results of primer extension reactions conducted with primer-RNA/DNA isolated from continuous-labeling reactions. The experiments presented in Fig. 4A (lanes 1 and 2), conducted with M13SV01ΔEP-Pst 2, permit mapping of initiation sites from the pSV01ΔEP *Ava*II site at position 2771 through site I, the SV40 core origin, 21-bp repeats, and enhancer sequences (Fig. 1). It is apparent that characterization of primer-RNA/DNA molecules formed during continuous labeling permits the same conclusion to be drawn as for the pulse assays, i.e., that primer-RNA/DNA is generally not formed on the lower strand in the vicinity of the SV40 core origin. However, overexposure of the autoradiogram in Fig. 4A revealed very weak site, Lb, but not La. The experiments presented in Fig. 4B, conducted with M13SV01Δ EP-Pst 1, permitted mapping of initiation sites from the pSV01 ΔEP *Sph*I site at position 2518 through the SV40 core origin and site I (Fig. 1). It is apparent from Fig. 4B (lanes 3 and 4) that during a continuous-labeling reaction, primer-RNA/DNA molecules are not formed on the upper strand within the core origin.

The primer extension products shown in Fig. 2 and 4 and in eight additional experiments were run next to sequencing ladders that served as size markers; this permitted the origin-proximal primer-RNA/DNA start sites to be mapped. The locations of primer-RNA/DNA start sites, on lagging-strand templates on either side of the SV40 core origin, are presented in Fig. 5. Within the 21-bp repeats, the average distance between primer-RNA start sites was ~10 nt; a similar phasing of primer-RNA/DNA start sites was not observed at more-distal locations (7). Regarding the sequence composition of primer-RNA initiation sites in the vicinity of the SV40 origin, the nucleotides at the actual primer-RNA start sites (position 1) were always purines. This implies that pyrimidines will be situated at the 5′ end of primer-RNA/DNA molecules formed in this region. A second common feature of these sites are thymine residues situated immediately 5′ of the primer-RNA start sites (position 2). A thymine at this position was a feature of 98% of the previously characterized primer-RNA initiation sites (7). However, unlike most of the origin proximal faint primer-RNA/DNA start sites (La to Li and Ex), 72% of the previously characterized primer-RNA start sites were associated with an additional thymine at position 3 (7). Four consensus primer-RNA/DNA initiation sites (3′NTT5′ [7]) (Fig. 5; shown in boldface type), and several weak sites (3′NT5′) are present in the core origin but were not used. Therefore, a lack of potential start sites in this region does not explain the absence of initiation events over the core origin. Finally, it is not clear how many of the origin-proximal start sites (La to Li and Ex) are used on a given molecule of pSV01ΔEP (i.e., whether all of the start sites or only a subset are used).

DISCUSSION

We have determined that DNA synthesis events initiate in vitro in a very reproducible pattern relative to the SV40 origin. With the exception of the faint sites La and Lb, primer-RNA/DNA start sites were not detected within the SV40 core origin. However, since the signals from La and Lb sites were always very close to background (Fig. 3A), their significance is unclear. The molecular basis for the gap in primer-RNA/DNA initiation sites over the core origin has yet to be determined. It is interesting that previous studies of permeabilized cells indicated that the SV40 core origin and flanking GC boxes are part

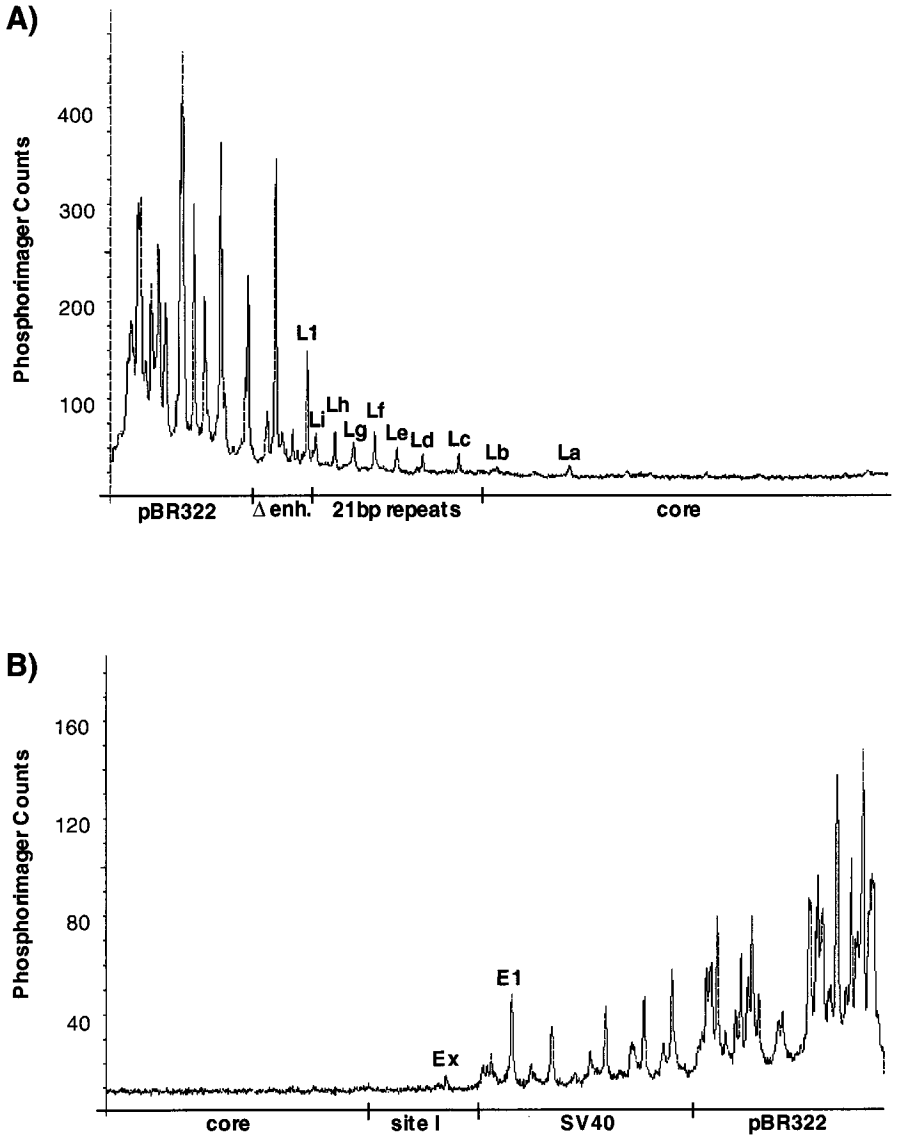

FIG. 3. PhosphorImager analyses of the primer extension reactions. (A) Results of a PhosphorImager scan of Fig. 2A (lane 1). Individual primer-RNA/DNA start sites, measured in PhosphorImager counts, are indicated. Below the scan is a map of the pSV01ΔEP regions covered by this primer extension reaction. (B) Results of a PhosphorImager scan of Fig. 2B (lane 3). Peaks corresponding to Ex and E1 are indicated. A map of the pSV01ΔEP regions covered by this primer extension reaction is presented below the scan.

of a nucleoprotein complex in vivo (49). The distribution of primer-RNA/DNA start sites may reflect the limits of a similar nucleoprotein structure in vitro. According to this hypothesis, a preinitiation complex, assembled at the SV40 origin, positions the pol α-primase complex to lagging-strand templates flanking the core origin. The paucity of primer-RNA/DNA initiation events over the SV40 origin could simply indicate that origin DNA bound by the preinitiation complex is not a substrate for the pol α-primase complex. The possibility that DNA synthesis events initiate outside genetically defined origins of replication has been suggested from studies with both prokaryotic (reviewed in references 6 and 38) and eukaryotic (6, 10, 25) replication systems.

Regarding the relatively low levels of primer-RNA/DNA initiation events over the 21-bp repeats and T-ag-binding site I, numerous studies have demonstrated that sequences flanking the core origin can stimulate SV40 replication in vivo (see references 11, 22, 26, and 29 and references therein). Exactly how these sequences facilitate DNA replication in vivo is not understood (see reference 13 for a review). Whether these sequences play a similar stimulatory role during initiation of SV40 DNA synthesis in vitro is controversial. For example, there are results suggesting that the 21-bp repeats and enhancer sequences do not have a significant effect on SV40 DNA replication in vitro (29, 39). However, it has also been reported that SV40 DNA replication in vitro depends on flanking sequences to the same extent as in vivo (22, 23). Others have noted stimulation of replication in vitro only by sequences within binding site I (29, 39). The relatively low level of primer-RNA/DNA initiation events over the 21-bp repeats and site I raises the possibility that under our experimental conditions, the flanking sequences—and proteins bound to these sequences—may play a role in the initiation of DNA synthesis in vitro.

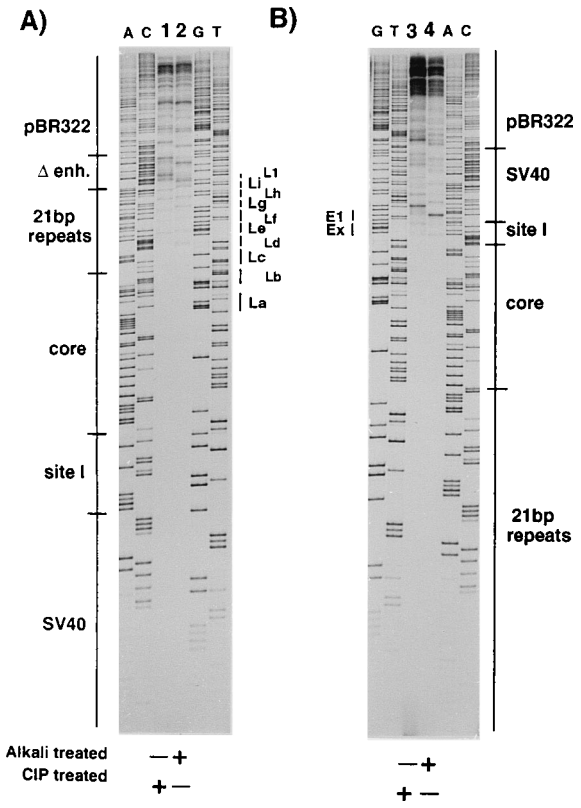

FIG. 4. Examples of primer extension reactions conducted with primer-RNA/DNA formed during continuous-labeling reactions. (A) Primer extension products, formed with M13SV01ΔEP-Pst 2 (Fig. 1) and primer-RNA/DNA present at the end of 15 min of synthesis (either CIP treated [lane 1] or treated with alkali [lane 2]), were restricted with *Ava*II (pSV01ΔEP site 2771). After digestion, the samples were loaded alongside a sequencing ladder (A, C, G, and T). A map of the pSV01ΔEP regions covered by this primer extension reaction is provided to the left of the figure. Also indicated are the relative positions of primer-RNA/DNA start sites La to Li (start site Lb was detected on a longer exposure of the autoradiogram). (B) Additional primer extension reactions, formed with M13SV01ΔEP-Pst 1 (Fig. 1) and primer-RNA/DNA present at the end of 15 min of synthesis (either CIP treated [lane 3] or treated with alkali [lane 4]), were restricted with *Sph*I (pSV01ΔEP site 2518). As in the previous examples, the reaction products were loaded alongside a sequencing ladder (A, C, G, and T). A map of the pSV01ΔEP regions covered by this primer extension reaction is provided to the right of the figure. Also provided are the locations of primer-RNA/DNA start sites Ex and E1.

The initiation sites mapped herein have both similarities to and significant differences from start sites mapped in vivo with Okazaki-sized DNA fragments (24). In both studies, start sites were not detected within the core origin on the strand encoding late mRNA (the upper strand; Fig. 1). Moreover, on the strand encoding early mRNA, the positions of primer-RNA/DNA start sites La to Li are similar to the locations of certain previously mapped initiation sites. However, the in vivo studies suggested that on the strand encoding early mRNA, many initiation sites, including the two strongest sites, were situated within the core origin. Strong primer-RNA/DNA start sites were not detected within the SV40 core origin in vitro (Fig. 2 to 4). Therefore, our studies do not suggest the existence of an origin of bidirectional replication (24) at the border between the core origin and T-ag site I (SV40 nt 5210 to 5211 [Fig. 5]). This conclusion is consistent with results of previous hybridization studies involving nascent DNA formed during a 5-s pulse (6, 12). Indeed, the finding that the initiation pattern is the same, whether one characterizes primer-RNA/DNA molecules formed in either continuous-labeling or pulse assays, confirms preliminary conclusions based on hybridization studies (6, 12), namely, that lagging-strand synthesis events initiate SV40 DNA replication and that primer-RNA/DNA molecules formed outside the SV40 core origin prime both leading-strand and lagging-strand synthesis events (6). Related experiments conducted initially with primate cell crude extracts (6, 12, 34) and subsequently with replication systems reconstituted with purified proteins (8, 17, 42) indicate that DNA primers formed by the pol α-primase complex are likely substrates for a polymerase switch (28, 41) with a proliferating-cell nuclear antigen-dependent polymerase. Analogous conclusions were drawn from disruption studies of the *POL2* gene in *Saccharomyces cerevisiae* (32).

Additional experiments are needed to determine whether factors such as chromatin structure explain the differences in the SV40 in vitro and in vivo initiation patterns. However, it is clear from the above discussion that primer-RNA/DNA molecules afford a distinct advantage for studies of the initiation of SV40 DNA synthesis; relative to full-length Okazaki fragments, they are the products of less-complicated synthesis and processing events (7).

Future experiments are required to establish the biological significance, if any, of the ~10-nt phasing of the primer-RNA/DNA start sites over the 21-bp repeats and the function(s) of the flanking sequences during initiation of DNA synthesis.

FIG. 5. Map of the nucleotide positions at which primer-RNA/DNA initiated in the vicinity of the SV40 origin in vitro. The locations of part of one of the SV40 enhancers (Δ enhancer), the 21-bp repeats (the origin-proximal repeat is imperfect [~21]), the core origin, and T-ag binding site I are indicated. Nucleotide positions are numbered as described in the legend to Fig. 1. Sequences encoding the 5′ termini of primer-RNA are indicated by boldface capital letters, and those encoding the 5′ termini of primer-DNA are indicated by boldface lowercase letters. The dashed arrows symbolize faint primer-RNA molecules mapped in this study; L1, L2, and E1 were previously mapped (7). Four consensus primer-RNA/DNA start sites in the core origin (3′NTT5′ [7]), which were not used to initiate DNA synthesis, are shown in boldface letters. Finally, Sp1-binding sites within the 21-bp repeats are underlined (21).

Additional studies are also required to determine whether the faint start sites over the core origin (La and Lb) are relevant to initiation. Nevertheless, in view of our studies, it is interesting that an ATP-dependent multiprotein complex (the origin recognition complex) is known to recognize yeast origins of replication; this complex extends over autonomously replicating sequences and functionally important flanking regions (2, 14). It will be intriguing to determine the extent to which initiation events have been conserved at these and related eukaryotic origins.

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