Initiation of simian virus 40 DNA replication *in vitro*: identification of RNA-primed nascent DNA chains

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ABSTRACT

Cell-free extracts of simian virus 40 (SV40)-infected CV-1 cells can initiate large tumor antigen dependent bidirectional replication in circular DNA molecules containing a functional SV40 origin of replication (*ori*). To determine whether or not DNA replication under these conditions involves RNA-primed DNA synthesis, replication was carried out in the presence of 5-mercuri-deoxycytidine triphosphate to label nascent DNA chains. Newly synthesized mercurated DNA was isolated by its affinity for thiol-agarose, and the 5'-ends of the isolated chains were radiolabeled to allow identification of RNA primers. At least 50% of the isolated chains contained 4 to 7 ribonucleotides covalently linked to their 5'-end; 80% of the oligoribonucleotides began with adenosine and 19% began with guanosine. About 60% of the nascent DNA chains annealed to the SV40 *ori* region, and about 80% of these chains were synthesized in the same direction as early mRNA. These results are consistent with the properties of SV40 DNA replication *in vivo* and support a model for initiation of SV40 DNA replication in which DNA primase initiates DNA synthesis on that strand of *ori* that encodes early mRNA.

INTRODUCTION

Simian virus 40 (SV40) provides a simple model of a single eukaryotic chromosomal replicon (see refs. 1 and 2 for review). The viral genome is a 5.2 kilobase circular double-stranded DNA molecule that replicates in the nucleus of its host as a chromosome with a histone composition and nucleosome structure similar to cellular chromatin. When the virus-encoded protein large tumor antigen (T-ag) and permissive cell factors interact with the *cis*-acting sequence genetically defined as the SV40 origin of replication (*ori*), replication is initiated bidirectionally at a unique site (the origin of bidirectional replication) within *ori*. Except for T-ag, all of the components required for the replication and assembly of SV40 chromosomes are provided by the host cell, and the events at replication forks in cellular chromosomes appear identical to those in SV40 and its close relative, polyoma virus (3, 4).

Short nascent DNA chains are repeatedly initiated on average once every 145 base pairs (bp). This process of discontinuous DNA synthesis occurs predominantly, if not exclusively, on the retrograde arm of replication forks where the direction of synthesis must be opposite to the direction of fork movement; synthesis on the forward arm is a relatively continuous process. A short oligoribonucleotide is synthesized first, serving as a primer for DNA synthesis. It is

then excised at the same rate at which completed Okazaki fragments are joined to the 5'-end of the continuously growing daughter strand. All steps in the synthesis of these RNA primed-DNA chains appear to be carried out by a multiprotein complex that includes DNA primase, DNA polymerase- α [or DNA polymerase- δ (5, 6)], C₁C₂ primer recognition proteins and other proteins relevant to DNA replication such as topoisomerase and exonuclease activities. Initiation sites for RNA-primed DNA synthesis are also found within the SV40 [and polyoma virus (3)] *ori* sequence on the strand that encodes early mRNA. These sites are indistinguishable from those found outside *ori* in terms of their frequency, sequence composition, confinement to retrograde arms of forks and average lengths of RNA primer, suggesting that initiation of DNA synthesis at *ori* occurs by the same mechanism used to initiate Okazaki fragments throughout the genome. Similar results have recently been obtained with polyoma virus-replicating DNA (4).

Soluble extracts from various permissive mammalian cells are capable of initiating replication in circular DNA molecules containing *ori* 6-12). Replication is bidirectional from *ori* and requires a functional T-ag, *ori*-core sequence, and DNA primase-DNA polymerase- α (11, 13) [or - δ (5,6)]. Although these results imply that DNA replication in these *in vitro* systems occurs in the same way that SV40 replicates *in vivo*, there has been no direct evidence of RNA-primed DNA synthesis. In fact, some data indicate that significant differences may exist. First, although DNA synthesis in these *in vitro* systems depends upon addition of deoxyribonucleoside triphosphates, with the exception of ATP, they do not depend on addition of ribonucleoside triphosphates, indicating that RNA synthesis is not a prerequisite of DNA synthesis. Secondly, although SV40-directed DNA replication *in vitro* is resistant to α -amanitin (6), it is inhibited by ribonuclease A (7, 14), suggesting a role for preformed RNA in the replication process. Finally, DNA primase-DNA polymerase- α purified from permissive monkey cells initiates synthesis at distinctly different sites and makes RNA primers significantly shorter when using SV40 DNA templates *in vitro* than observed on the same template sequences during SV40 DNA replication *in vivo* (15-17), indicating that additional proteins or even different enzymes are involved in DNA replication *in vivo*. Therefore, we employed a novel approach involving mercurated nucleotides in an effort to identify RNA-primed nascent DNA chains synthesized during SV40-directed DNA replication *in vitro*.

5-mercuri-deoxycytidine triphosphate (Hg-dCTP) has been used as a substrate for *in vitro* DNA replication in both bacterial (18-22) and mammalian cell systems (23) to facilitate the isolation and characterization of RNA-linked DNA under conditions where RNA primers are rapidly excised and low in abundance. Nascent Hg-labeled DNA chains are isolated by affinity chromatography on thiol-agarose and their 5'-ends analyzed for the presence of oligoribonucleotides. Application of this approach to a system that initiates SV40 DNA replication *in vitro* demonstrated the presence of RNA-primed nascent DNA chains in and around the *ori* region and revealed that the initial direction of DNA synthesis was the same as

that of early mRNA synthesis. The results are consistent with the model for initiation of SV40 DNA replication proposed by Hay and DePamphilis (24).

MATERIALS AND METHODS

***In Vitro* DNA Replication**

African Green monkey kidney cell line CV-1 was infected with SV40 wild-type 800 (25). From 36 to 38 hrs post infection, cytosol and nuclear extract was prepared as previously described (6, 11, 12). SV40-infected cells were washed with hypotonic buffer [20 mM HEPES (pH 7.8), 5 mM potassium acetate, 0.5 mM MgCl₂, and 0.5 mM dithiothreitol] and lysed in a Dounce homogenizer. Nuclei were sedimented by centrifugation at 1,200 x g for 5 min and then resuspended in hypotonic buffer supplemented with 500 mM potassium acetate to prepare a high-salt nuclear extract. SV40 chromosomes were removed from the high-salt nuclear extract by centrifugation at 300,000 x g for 1 hr. Cytoplasm (supernatant from nuclei sedimentation) was centrifuged at 100,000 x g for 1 hr to yield a cytosol fraction. The DNA substrate in these experiments was pSVori, a 2,643 bp plasmid constructed by replacing nucleotide 29 to 562 of plasmid vector pML-1 with a 206 bp segment of SV40 DNA containing *ori* (12).

DNA replication was carried out in a final volume of 125 μ L containing 30 mM HEPES (pH 7.8), 7 mM magnesium acetate, 1 mM EGTA, 4 mM ATP, 0.2 mM each of CTP, GTP, and UTP, 0.1 mM each of dATP and dGTP, 0.05 mM Hg-dCTP (Sigma), 100 μ Ci [³H]dTTP (26 Ci/mmol gives 0.03 mM dTTP), 0.5 mM dithiothreitol, 10 mM phosphoenol pyruvate, 3.75 μ g pyruvate kinase, 3% polyethylene glycol (14,000 Mr, Aldrich Chemical Co.), 0.25 μ g pSVori DNA, 50 μ L cytosol (7-12 mg protein/mL) and 25 μ L high salt nuclear extract (5-9 mg protein/mL). This mixture was incubated for 15 min at 30°C, unless otherwise indicated, before stopping the reaction by addition of 0.5 % sodium dodecyl sulfate and 15 mM EDTA. DNA was purified by treatment with proteinase K, extraction with organic solvents and precipitation with ethanol, as previously described (6, 11, 12).

Affinity Chromatography of Hg-DNA

DNA purified from the *in vitro* DNA replication reaction was dissolved in 50 μ L of water and chromatographed on Bio-Gel P4 (0.8 x 25 cm column) in 0.1 M triethyl ammonium bicarbonate (pH 7.5) to remove residual dithiothreitol. Use of siliconized columns and tubes was essential to prevent loss of Hg-DNA. DNA was recovered in the void volume, evaporated to dryness under vacuum, and then dissolved in 100 μ L 0.05 M Tris-HCl (pH 7.5), 0.1 M NaCl, and 1 mM EDTA. The DNA was denatured by heating for 5 min at 100°C, diluted to 1 mL with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM NaCl and then chromatographed on a 1 mL column of thiol-agarose (Affi-Gel 401, Bio-Rad), as previously described (20-22).

DNA Hybridization Assays

Single-stranded mSV01, mSV02, and M13mp8 DNA were treated with 0.3 N NaOH for 1

hr at 65°C, diluted with an equal volume of 2 M ammonium acetate, and then applied to MS1 Magna nylon filters in serial dilutions using a Schleicher and Schuell filtration manifold, as previously described (26). Pre-hybridization and hybridization were performed with [5'-³²P]Hg-DNA in a buffer containing 0.75 NaCl, 0.05 M NaH₂PO₄, 5 mM EDTA, 0.1% (v/v) Ficoll, 0.1% (v/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin, 1% sodium dodecyl sulfate, 200 µg/mL denatured salmon sperm DNA and 50% (v/v) formamide. After 24 hrs of hybridization at 37°C, the filters were washed as described (26). These conditions were optimal for preserving RNA in RNA-p-DNA chains and for maintaining Hg-DNA:DNA hybrids which have a lower melting point than DNA:DNA hybrids (27). Following preliminary autoradiography to locate samples, each spot was cut out of the filter and the amount of [5'-³²P]Hg-DNA measured by scintillation counting.

RESULTS

Labeling Nascent DNA with Hg-dCTP

The role of RNA primers during the initiation and continuation of SV40-directed DNA replication *in vitro* was explored using a cell-free system supplemented with 5-mercurideoxycytidine triphosphate (Hg-dCTP) and programmed with a circular plasmid (pSV_{ori}) containing the SV40 *ori* sequence. The original low-salt extract of SV40-infected CV-1 cells described by Su and DePamphilis (28, 29) that allowed SV40 replicating chromosomes to continue replication *in vitro* was modified to include a high-salt nuclear extract and polyethylene glycol (6). These modifications allowed initiation of multiple rounds of bidirectional replication from *ori* in SV40 chromosomes as well as in plasmid DNA. Replication depended on a functional SV40 T-ag and *ori*-sequence (6, 11, 12). Furthermore, promoter sequences that facilitate activation of *ori*-core *in vivo* [the 'GGGCGG'-repeats (1, 2)] were utilized in binding of initiation factor(s) specifically to SV40 *ori*-core (12). The products of replication included covalently-closed, superhelical DNA. Stillman (9) has shown that a high-salt nuclear extract is required for supercoiling of newly replicated DNA, which results from nucleosome assembly, although such extracts also reduce the amount of DNA synthesis observed. Therefore, several important characteristics of initiation of viral chromosome replication *in vivo* were reproduced under these *in vitro* conditions.

Substitution of Hg-dCTP for dCTP had no discernible effect on the characteristics of DNA replication that we have previously described for this system (6, 11). An average of 4.5 pmol [α -³²P]dCTP (18 pmols of all four dNTPs) was incorporated during the first hour of incubation using either dCTP or Hg-dCTP, and synthesis with either nucleotide was inhibited 50% in the presence of 3 µg/mL aphidicolin. Incorporation of [α -³²P]dNTPs was reduced at least 100-fold when plasmids in which SV40 *ori* was either absent or contained a 6 bp deletion at the BglI site were used, at least 100-fold when T-ag specific monoclonal antibody PAb419 was added, and at least 20-fold when linear DNA containing a functional *ori* was used. These results were

consistent with observations of other *in vitro* systems that initiate SV40 DNA replication (7-10). The products of replication with either dCTP or Hg-dCTP were essentially the same when fractionated by electrophoresis in agarose gels (data not shown). DNA synthesis with either dCTP or Hg-dCTP exhibited a delay of about 15 min; DNA synthesis during the first 15 min of incubation was only 6% of that observed after 2 hr. About 87% of the Hg-DNA synthesized during the initial 15 min of incubation could be recovered by affinity chromatography on thiol-agarose. Therefore, this material was further analyzed for the presence of 5'-terminal oligoribonucleotides.

RNA Primers on Nascent DNA Chains

The mercurated [^3H]DNA isolated above (Hg-DNA) was treated with bacterial alkaline phosphatase and then incubated with [$\gamma\text{-}^{32}\text{P}$]ATP in the presence of phage T4 polynucleotide kinase (24, 25) so that each polynucleotide chain carries a single phosphate and is radiolabeled at its 5'-end. Nonutilized [$\gamma\text{-}^{32}\text{P}$]ATP was removed by chromatography on Bio-Gel P4. Rechromatography of the [$5'\text{-}^{32}\text{P}$]DNA through Bio-Gel P4 confirmed that it eluted in the void

Figure 1. Fraction of [$5'\text{-}^{32}\text{P}$]HgDNA covalently attached to RNA primers. Panel A: [$5'\text{-}^{32}\text{P}$]HgDNA purified from the *in vitro* DNA replication reaction (see Materials and Methods) was subjected to gel filtration through Bio-Gel P4 (0.8 x 50 cm column) in 0.1 M triethylammonium bicarbonate (pH 7.5). Fractions (0.15 mL) were collected and analyzed for ^{32}P by scintillation counting. Panel B: An aliquot of the [$5'\text{-}^{32}\text{P}$]HgDNA analyzed in panel A was precipitated with ethanol, resuspended in 0.15 M NaOH, and incubated for 20 hrs at 37°C to hydrolyze RNA primers (24, 25). This material was subjected to gel filtration as in panel A.

volume (Fig. 1A). Since oligo(dT)18 also eluted in the void volume of this column, [5'-³²P]DNA chains were at least 18 residues in length. This material was incubated with alkali to hydrolyze RNA and then chromatographed on Bio-Gel P4 (Fig. 1B). At least 50% of the ³²P-label behaved as 3', 5'-ribonucleoside bisphosphates (20), revealing that at least 50% of the [3H]Hg-DNA carried one or more ribonucleotides at their 5'-ends.

The ³²P-labeled ribonucleosides generated by alkaline hydrolysis of isolated nascent DNA were chromatographed on poly(ethyleneimine) cellulose (20). About 80% of the radiolabel comigrated with adenosine 3', 5'-bisphosphate, 19% with guanosine 3', 5'-bisphosphate, and 1% with cytidine and uridine 3', 5'-bisphosphates (Fig. 2). Therefore, the putative 5'-oligoribonucleotides covalently linked to Hg-DNA began predominantly with adenosine and secondarily with guanosine.

To determine whether or not the oligoribonucleotides attached to the 5'-ends of nascent DNA chains were the size expected of RNA primers, [5'-³²P]Hg-DNA was digested exhaustively with the 3' to 5' exonuclease of bacteriophage T4 DNA polymerase (20, 24, 25). This enzyme specifically digests single-stranded DNA in RNA-primed DNA chains leaving a single deoxyribonucleotide attached to the 3'-end of the RNA moiety (15). The products were then fractionated by polyacrylamide gel electrophoresis (15, 20, 24, 25). Prior to digestion, [5'-³²P]DNA chains remained at the top of the gel (Fig. 3, lane A), whereas after digestion,

Figure 2. Identification of the 5'-ribonucleotide associated with RNA-p-[Hg]DNA. The [5'-³²P], 2',3'-ribonucleoside diphosphates released from [5'-³²P]HgDNA by alkaline hydrolysis and isolated by gel filtration (see Figure 1B) were fractionated by thin layer chromatography on PEI cellulose using 0.55 M ammonium sulfate as the solvent and a mixture of the four ribonucleoside 3', 5'-bisphosphates as standards. ³²P-nucleotides were detected by autoradiography and nucleotide standards by adsorption of short wave UV light. The autoradiogram was scanned using a soft laser scanning densitometer (Zeinek).

most of the ^{32}P -labeled material migrated with oligoribonucleotides 5 to 8 residues in length (Fig. 3, lane B). Digestion of this material with RNase T2 generated 5'-labeled mono- and dinucleotides (Fig. 3, lane C). Therefore, nascent DNA chains synthesized *in vitro* had 4 to 7 ribonucleotides covalently attached to their 5'-ends.

RNA-Primed Nascent DNA Chains Initiated In the *Ori*-Region

To determine whether or not any of these RNA-primed nascent DNA chains originated in the SV40 *ori*-region, Hg-DNA isolated after 15 minutes of incubation *in vitro* was hybridized with SV40 specific probes consisting of single-stranded M13 phage DNA containing either the SV40 *ori*-region DNA strand that encodes early mRNA (mSV01) or the strand that encodes late mRNA (mSV02). All 5'-ends of one aliquot of the isolated Hg-DNA were radiolabeled as

Figure 3. Size of RNA primers covalently attached to HgDNA. $[5'\text{-}^{32}\text{P}]\text{HgDNA}$ was subjected to electrophoresis in a 20% polyacrylamide gel containing 7 M urea (20) before (lane A) and after (lane B) digestion with the 3' to 5' exonuclease of phage T4 DNA polymerase or with both T4 DNA polymerase exonuclease and RNase T2 (lane C). The gel was calibrated by concurrent electrophoresis of a partial alkaline digest of $[5'\text{-}^{32}\text{P}]\text{poly(A)}$. Numbers indicate chain length in nucleotides.

TABLE 1. Hybridization of [5'-32P]Hg-DNA to the SV40 *Ori*-Region

Nascent	<u>Amount* of [5'-32PIHg-DNA Hybridized to:</u>			Ratio#
<u>DNA Structure</u>	<u>mSV01</u>	<u>mSV02</u>	<u>M13mp8</u>	<u>[mSV01/mSV02]</u>
RNA-p-DNA				
(15,000 cpm)	3460	844	60	4.3
(30,000 cpm)	4890	1522	105	3.4
RNA-p-DNA + DNA				
(15,000 cpm)	2254	1356	45	1.7
(30,000 cpm)	5070	2096	110	2.5

* All numbers are counts per minute.
Nonspecific DNA binding (M13mp8) was subtracted from the values obtained with mSV01 and mSV02 before calculating ratio. mSV01 and mSV02 are single-stranded circular DNA molecules obtained by inserting the 311 bp BstN1 SV40 DNA fragment containing *ori* into recombinant vector M13mp7 (24). mSV01 carries the early mRNA template strand of *ori*, and mSV02 carries the late mRNA template strand.

described above; about half the sample was [5'-32P]RNA-p-[Hg]DNA and half was [5'-32P]Hg-DNA. A second aliquot was first incubated in the presence of ATP and T4 polynucleotide kinase to insure that the 5'-ends of all polynucleotide chains were terminated by a phosphate. RNA primers were then removed by alkaline hydrolysis, and the resulting 5'-hydroxyl terminated DNA chains were radiolabeled using [γ -32P]ATP and T4 polynucleotide kinase (24, 25). Thus, all [5'-32P]DNA chains in the second aliquot represented those DNA chains whose 5'-ends used to be covalently attached to RNA (i.e. RNA-p-DNA chains). Two different amounts of 32P-labeled material was hybridized to each M13 probe to insure that the probe's hybridization capacity was not saturated. About 24% of all nascent DNA chains originated from the SV40 *ori*-region, (Table 1, RNA-p-DNA + DNA). About 20% of RNA-primed nascent DNA chains hybridized specifically to mSV01 and about 5% hybridized to mSV02 (Table 1, RNA-p-DNA).

Hybridization of Hg-DNA is comparatively inefficient relative to unsubstituted DNA due to the low melting point of Hg-DNA hybrids (27). Hybridization efficiency in these experiments was 40% as measured with a 5'-end labeled, Hg-substituted restriction fragment that was complementary to mSV01 (26). Therefore, about 60% of all 5'-end labeled nascent DNA contained a sequence complementary to the 209 bp SV40 *ori*-containing sequence present in pSVori, the template for *in vitro* DNA synthesis. About 50% of RNA-primed nascent DNA chains hybridized to mSV01 and about 13% hybridized to mSV02, revealing that about 80% were synthesized in the same direction as early mRNA and that as many as 63% of these chains

may have originated in the SV40 *ori*-region. The remaining 30-40% of RNA-primed nascent DNA chains presumably represented Okazaki fragments that were initiated at replication forks outside this *ori*-region as a result of bidirectional replication (6-11). If the *in vitro* metabolism of RNA-primed nascent DNA chains is representative of their *in vivo* metabolism, then most of the nascent DNA chains that did not contain RNA at their 5'-ends resulted from normal excision of RNA primers as Okazaki fragments are ligated to the ends of growing daughter strands (1, 2, 25). Alternatively, some nascent DNA chains may have been broken during their purification .

DISCUSSION

Our earlier attempts to detect RNA primers during replication of plasmid DNA containing the SV40 *ori*-region by incorporation of [α -³²P]dNTPs into newly replicated DNA were unsuccessful (unpublished data). Since pool sizes for rNTPs in mammalian cells exceed those of dNTPs by 500 to 1000-fold (30), dilution of radiolabeled rNTPs in the cell extracts may have prevented detection of RNA primer synthesis. In fact, a dependency of RNA-primed DNA synthesis on ribonucleotides has been difficult to demonstrate in subcellular systems where RNA-primed nascent DNA chains have been identified in a variety of ways (31-33). Therefore, we isolated newly synthesized DNA from the *in vitro* reaction by incorporation of an affinity label (Hg-dCTP) that allowed nascent DNA chains to be isolated from other DNA and RNA in the reaction by its affinity for thiol-agarose and then examined their 5'-ends for the presence of an oligoribonucleotide. This approach has been successfully used to detect RNA-primed DNA synthesis in other systems (18-23). The data revealed that at least 50% of the DNA chains synthesized during the initial "lag" period of pSVori DNA replication in an extract of SV40-infected CV-1 cells had 4 to 7 ribonucleotides covalently attached to their 5'-ends and that about 80% of these oligoribonucleotides began with adenosine and 19% with guanosine.

The results obtained with pSVori replication *in vitro* are remarkably similar to SV40 DNA synthesis *in vivo*. About 50% of the nascent DNA chains isolated from SV40 DNA replicating *in vivo* contain an average of 9 to 11 ribonucleotides attached to their 5'-ends; 61% of all oligoribonucleotides began with adenosine and 23% began with guanosine (25). Rapid degradation of RNA primers accounts for the fraction of oligoribonucleotides containing cytidine or uridine at their 5'-ends (25). When only (p)ppRNA-p-DNA chains were radiolabeled using vaccinia virus guanylttransferase, 80% of the RNA primers began with adenosine and 20% with guanosine; no initiation events were detected that began with either CTP or UTP (4).

The data on RNA-primed DNA synthesis during SV40 DNA replication *in vivo* and pSVori replication *in vitro* are consistent with the mechanism of action of purified DNA primase-DNA polymerase- α from permissive cells. The same regions of SV40 DNA analyzed *in vivo* have been used as single-stranded templates for purified DNA primase-DNA polymerase- α from CV-1 cells (15,16). This enzyme complex initiated RNA-primed DNA

synthesis 80% of the time with ATP and 20% of the time with GTP; initiation with either CTP or UTP was not detected. The typical length of RNA primers was 6 to 8 residues, although the length and base composition of individual RNA primers depended on the specific template initiation site chosen. A multiprotein complex containing DNA primase, DNA polymerase- α , C₁C₂ and other replication-related proteins initiated RNA-primed DNA synthesis on the same templates with similar characteristics except that RNA primers were typically 1 to 5 nucleotides long (17).

Part of the difference between the lengths of RNA primers synthesized under various conditions appears to result from the use of DNA size standards in the *in vivo* experiments and homo-oligomers of ribonucleotides as size standards in the *in vitro* experiments. Intact SV40 RNA primers synthesized *in vivo* typically contained 6 to 9 ribonucleotides when measured against oligo(rA) standards (4). Therefore, the typical RNA primer length synthesized during pSVori replication *in vitro* (4-7 bases) was characteristic of SV40 DNA replication *in vivo* (6-9 bases) and of CV-1 cell DNA primase-DNA polymerase- α DNA synthesis *in vitro* (6-8 bases). One important comparison remains. The initiation sites selected by purified DNA primase-DNA polymerase- α complexes *in vitro* were distinctly different from those selected by the cellular replication complex *in vivo* (15-17), suggesting that either additional proteins or different enzymes are required. Unfortunately, we were not able to map the initiation sites for RNA-primed nascent DNA chains synthesized on pSVori *in vitro* because restriction endonucleases failed to cut recognition sites containing Hg-dCMP (G. Banfalvi, S. Bhattacharya and N. Sarkar, manuscript in preparation). New methods for mapping these initiation sites are currently under development.

The data presented in this paper are consistent with the model proposed by Hay and DePamphilis (24, 25) for the initiation of SV40 DNA replication. In this model, a T-ag initiation complex separates the two strands of *ori* to allow DNA primase-DNA polymerase- α [or δ (5, 6)] to select one of several potential sites on the strand that encodes early mRNA to initiate RNA-primed DNA synthesis. From 60-70% of the RNA-primed nascent DNA chains synthesized during the first 15 minutes of incubation *in vitro* contained sequences complementary to the 209 bp *ori* region in pSVori. *Ori* itself consists of a 64 bp core sequence flanked on each side by an auxiliary sequence of 43-45 bp (1, 2). Since this comprises about 73% of the SV40 sequence present in pSVori, some RNA-primed DNA chains likely originated within *ori*. Moreover, 80% of these initiation events occurred on the template that also encodes early mRNA, suggesting that synthesis of the first DNA chain was initiated on that side of *ori* which encodes early mRNA. In fact, we have shown previously that the initial direction of DNA synthesis in SV40 chromosomes initiating replication under these *in vitro* conditions is the same as early mRNA synthesis (11) and that RNA-primed initiation events occur exclusively on the early mRNA template strand of *ori* during both SV40 (24) and polyoma virus (3) DNA replication *in vivo*. Further application of the techniques used in this study may allow precise

identification of the initiation site(s) for the first DNA chain synthesized during SV40 DNA replication.

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