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Polyoma virus DNA replication is semi-discontinuous

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ABSTRACT

In marked contrast to simian virus 40 (SV40), polyoma virus (PyV) has been reported to replicate discontinuously on both arms of replication forks. In an effort to clarify the relationship between the mechanisms of DNA replication in these closely related viruses, the distribution of RNA-primed DNA chains at replication forks was examined concurrently in PyV and SV40 replicating DNA purified from virus-infected cells. About one third of PyV DNA chains contained 7 to 9 ribonucleotides covalently linked to their 5'-end. A similar fraction of DNA chains from replicating SV40 DNA contained an oligoribonucleotide that was 6 to 9 residues long and began with either (p)ppA or (p)ppG. Greater than 80% of PyV or SV40 RNA-primed DNA chains hybridized specifically to the retrograde template. Moreover, at least 95% of the RNA-primed DNA chains from either PyV or SV40 whose initiation sites could be mapped to unique nucleotide locations originated from the retrograde template. Therefore, PyV and SV40 DNA replication forks are essentially the same; DNA synthesis is discontinuous predominantly, if not exclusively, on the retrograde template.

INTRODUCTION

The antiparallel organization of DNA strands in double-stranded DNA, and the fact that all DNA polymerases synthesize only in the 5' to 3' direction requires that DNA synthesis on one arm of a replication fork (forward template) must be in the same direction as the fork opens while DNA synthesis on the other arm (retrograde template) must be in the direction opposite to fork movement. Therefore, to allow concomitant DNA synthesis on both sides of a replication fork, DNA synthesis on the retrograde template must be initiated repeatedly as the two parental template strands are unwound. This process, called discontinuous DNA synthesis, involves the repeated synthesis of comparatively short, nascent DNA chains referred to as Okazaki fragments. All DNA polymerases require a 3'-hydroxyl ribo- or deoxyribonucleotide to act as a primer on which to begin DNA synthesis. For Okazaki fragments, this requirement is provided by the 3'-hydroxyl terminus of an oligoribonucleotide 2 to 12 residues in length (RNA primer) that is synthesized by the enzyme DNA primase. In comparison, DNA synthesis on the forward template can be a continuous process; even if DNA polymerase dissociates from the template it can reinitiate on the 3'-hydroxyl end of the growing DNA strand. These concepts have been discussed in detail by Kornberg (1).

Mammalian DNA primase-DNA polymerase-α strongly favors initiating DNA synthesis on

the 3'-end of a preformed RNA or DNA primer over *de novo* synthesis of its own primer (2). Thus, repeated initiation of RNA-primed DNA synthesis on the forward template is neither required nor preferred. Never the less, evidence in support of discontinuous DNA synthesis on both sides of replication forks ("fully-discontinuous synthesis") as well as discontinuous synthesis only on the retrograde side ("semi-discontinuous synthesis") has been reported for eukaryotic systems (3). The importance of determining whether or not DNA synthesis is fully- or semi-discontinuous lies in the restrictions it places on the mechanism for DNA replication. If separation of DNA templates (i.e. DNA unwinding) is coupled to DNA synthesis, then replication forks will never proceed faster than DNA synthesis and extensive regions of single stranded forward DNA template will not be generated. Under these conditions, DNA replication will be semi-discontinuous. Conversely, if DNA unwinding proceeds faster than DNA synthesis, extensive amounts of single-stranded DNA template may be generated on both arms of replication forks, allowing Okazaki fragments to be initiated on the forward template in front of the growing daughter strand as well as on the retrograde template. However, if the enzyme complex responsible for the initiation of Okazaki fragments in eukaryotes behaves like the *E. coli* primasome and migrates along the DNA template in a 5' to 3' direction (4), it could operate only on the retrograde template where its direction of travel is toward the fork. On the forward template, where its direction of travel is away from the fork, the primasome would collide with DNA polymerase traveling in the opposite direction.

Polyoma virus (PyV) and simian virus 40 (SV40) replicating chromosomes offer relatively simple but appropriate models for cellular replication forks. With the exception of virally encoded large tumor antigen (T-Ag), which is required for initiation of viral DNA replication and may function as a helicase at replication forks, all steps in replication of viral DNA and its assembly into chromatin are carried out by the host cell (5, 6). The major difference between PyV and SV40 replication forks is that PyV DNA replication occurs only in mouse cells while SV40 DNA replication occurs only in certain monkey and human cells. Therefore, it was surprising that these two closely related papovaviruses appeared to differ fundamentally in their mechanism of DNA replication.

Most, if not all, Okazaki fragments in SV40 (RI)DNA originate from the retrograde template. When DNA was radiolabeled during synthesis in intact cells, at least 83% of Okazaki fragments annealed to the retrograde template while long nascent strands annealed equally well to both templates (7). Likewise, for Okazaki fragments synthesized *in vivo* and subsequently radiolabeled at their 5'-ends, 90% (8) and 96% (9) hybridized to the retrograde template. For DNA radiolabeled during synthesis in isolated nuclei, 95% of Okazaki fragments originated from the retrograde template (10, 11). Finally, 99% of the initiation sites for RNA-primed DNA synthesis were mapped to nucleotide sites on the retrograde template (9). The small number of initiation events that appeared to occur on the forward template could result from some viral chromosomes initiating replication at sites other than the genetically-defined origin of DNA replication (*ori*) (12)

or replicating unidirectionally (13). Thus, initiation of Okazaki fragments in SV40 appears to occur exclusively on retrograde templates.

Studies on the distribution of Okazaki fragments in PyV (RI)DNA have yielded conflicting results. If all PyV genomes replicate bidirectionally from a unique origin, then semi-discontinuous DNA synthesis will produce Okazaki fragments that are not self-complementary. However, the fraction of PyV Okazaki fragments that anneal to one-another has been reported as 70% (14-16), 52% (17), 30% (18-20) and 20% (19; short fragments only). The fraction of Okazaki fragments that annealed specifically to retrograde templates were 60% (16), 70% (15), 74% (17), and 80% (20). In addition, Narkhammer-Meuth et al. (16, 17) found that a larger fraction of Okazaki ffagments originating from the forward template contained RNA primers than from the retrograde template. Thus, in contrast to the consistent reports that SV40 DNA replication was semi-discontinuous, experiments with PyV DNA indicate that discontinuous DNA synthesis occur on both sides of replication forks.

We have attempted to resolve this paradox by applying the same techniques to PyV that were used to identify initiation sites for RNA-primed DNA synthesis in several regions of the SV40 genome (including the origin of replication), and to compare PyV (RI)DNA and SV40 (RI)DNA in the same experiment. The data revealed that the mode of replication in the two viruses was indistinguishable; both PyV and SV40 DNA replication is semi-discontinuous throughout the genome.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England Biolabs, T4 polynucleotide kinase and Sepharose CL-4B from Pharmacia, T4 DNA polymerase from P-L Biochemicals, nuclease P1 from Boehringer Mannheim, and radioactive nucleotides from New England Nuclear. Vaccinia virus guanylyltransferase was purified as described (21).

Preparation of PyV and SV40 Virus and Viral DNA

SV40 virus and SV40 DNA were prepared and analyzed as described (9). The following procedure yielded high titer, homogenous stocks of PyV. PyV strain A3 (22) was excised from a recombinant plasmid and then transfected onto secondary whole mouse embryo cultures. The resulting virus stock, which contained about 5 x 10^7 pfu/mL (hemmaglutin assay; 23), was plaque purified on mouse UC1B cells. Each plaque was dissolved in 2 mL of 137 mM NaCl, 5 mM KCl, 0.8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.0), overlayed onto a 100 mm tissue culture dish of 70-80% confluent baby mouse kidney (BMK) cells, and then incubated for 1 hr at 37°C before adding 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% horse serum, 0.03% penicillian and 0.01% streptomycin (DMEM+). When about 50% of the cells had lysed (ca. 6 days post-infection), cells were scraped from the dishes. The resulting lysate was

adjusted to pH 8.0 with 0.1 M $NaHCO_3$ and then frozen at -70°C and thawed at 30°C three times and then centrifuged at 2,500 x g for 5 min. 10 µL of the resulting supernatant was diluted 1:5000 with virus adsorption buffer [20 mM Tris (pH 6.5), 137 mM NaCl, 5 mM KCl, 100 mM $CaCl_2$, 50 mM $MgCl_2$, 2% horse serum, 0.03% penicillian G and 0.01% streptomycin] and 2 mL overlayed onto each of 25 150 mm tissue culture dishes of 70-80% confluent BMK cells. After 1 hr at 37°C, 20 mL of DMEM+ was added to each dish. Virus was harvested about 6 days post-infection as described above.

PyV DNA was prepared from virus-infected 3T6 cells. Optimal yields of viral DNA required careful control of the infection conditions. Cells, passaged at a 1:10 dilution in DMEM supplemented with 5% horse serum, routinely grew to confluency in 4 to 5 days and were not used after 20 passages. In preparation for infection, confluent dishes of 3T6 cells were passaged at a 1:12 to 1:14 dilution. Cells were infected with PyV at 72 hrs post-plating (ca. 70% confluency). PyV lysates were either used directly after adjusting the pH to 6.5 with 0.1 M $NaHPO_4$ or after dilution (1:2 -1:10) with virus adsorption buffer. PyV DNA was extracted at 26 hrs post-infection as described (24). On average, 3.2 µg of PyV covalently-closed, superhelical DNA [PyV (FI)DNA] and 0.33 µg of PyV replicating intermediate DNA [PyV (RI)DNA] were obtained per one 150 mm dish.

PyV viral DNA was tested for homogeneity by digesting purified PyV (FI)DNA with restriction endonucleases. Amplification of the Hpa II-5 fragment that contains the *ori*-region or the appearance of DNA fragments less than genome length following digestion at the single Bgl I site revealed the presence of mutant DNA. Only virus stocks in which mutant DNA was undetectable following Hpa II and Bgl I digestions were used in these experiments.

Radiolabeling Nascent DNA Chains Specifically At Their RNA-p-DNA Junctions

Nascent DNA chains in PyV or SV40 replicating DNA were radiolabeled uniquely at their RNA-p-DNA junction as previously described (9, 24, 25). This was accomplished by first phosphorylating the 5'-termini of all polynucleotide chains with non-radioactive ATP and then treating the sample with alkali to hydrolyze RNA and leave a 5'-hydroxyl end on those DNA chains that carried an RNA primer. The 5'-ends of this DNA was labeled with ^{32}P and fractionated by denaturing gel electrophoresis. [5'-^{32}P]DNA corresponding to 50-100, 100-200 or 200-300 nucleotides was excised from the gel and recovered by electroelution (24). Since each DNA chain was labeled only at its 5'-end, the amount of radiolabel was proportional to the number of nascent chains.

Preparation Of Hybrids Between Nascent Viral DNA And M13 Cloned Viral Sequences

DNA samples were hybridized to 5 µg of single-stranded M13 virion DNA containing a unique PyV or SV40 DNA sequence under conditions that allowed optimal specific annealing of the nascent chains to M13-PyV or M13-SV40 clones with minimal nonspecific aggregation (9; data not shown) [200 µL of 10 mM Tris-borate (pH 8.3), 0.1 mM EDTA and 75 mM NaCl (TBEN

buffer) at 65°C for at least 3 hrs]. DNA hybrids were separated from unhybridized DNA either by chromatography on Sepharose CL-4B (24 cm x 0.6 cm column) in TBEN buffer or by sedimentation through a 5-20% neutral sucrose gradient containing 75 mM NaCl (26). Gradients were centrifuged in a Beckman SW 60.1 rotor at 55,000 rpm for 170 min at 4°C. In both cases, 100 μL fractions were collected and counted in a Beckman LS 3801 scintillation counter until a 2% σ value of 5 or less was reached.

M13-PyV clones 9DB1 (complementary to early mRNA) and 8BD1 (complementary to late mRNA) contains 450 nucleotides of PyV A3 sequences from the Bcl I site (nucleotide 5046) to the Dde I site (nucleotide 200). This region includes the PyV enhancer(s), early and late promoters and *ori* (27). M13-PyV clones m662 (retrograde template, complementary to late mRNA) and m663 (forward template, complementary to early mRNA) contained the 333 nucleotide Ava II-6 fragment of Crawford small-plaque PyV sequence (nucleotides 1106 to 1439). The DNA sequence in this region is identical to PyV A3 DNA (28).

M13-SV40 clone mSV01 contains the 311 nucleotide BstN1 G-fragment from SV40 (22; nucleotides 5094 to 161). This region includes *ori* and the early gene promoter. M13-SV40 clones mSV07 (retrograde template, complementary to early mRNA) and mSV08 (forward template, complementary to late mRNA) contain the 360 nucleotide AccI to Pst I fragment of SV40 (9; nucleotides 1630 to 1990).

Identification of Initiation Sites for RNA-Primed DNA Synthesis

DNA hybrids isolated by gel exclusion chromatography were digested with a restriction endonuclease that cut at a single site within the cloned SV40 or PyV sequence, and the [5'-^{32}P]DNA chains released were fractionated by electrophoresis in a 22% polyacrylamide-8 M urea gel (9, 24). Each fragment corresponded to the position of the 5'-terminal deoxyribonucleotide at an RNA-p-DNA junction. The total amount of [5'-^{32}P]DNA fragments released was proportional to the number of RNA-primed DNA chains originating from that template. This approach has been used to map the nucleotide locations of initiation sites for RNA-primed DNA synthesis (9, 24, 29).

RESULTS

Identification of RNA-Primed DNA Chains

Nascent DNA chains in replicating DNA can be distinguished from damaged or degraded DNA that may exist in purified (RI)DNA preparations by the presence of a short oligoribonucleotide covalently attached to their 5'-ends. These RNA primers can be detected by radiolabeling the 5'-end of the RNA, digesting the DNA, and isolating the residual RNA. Therefore, to demonstrate the presence of these RNA-primed DNA chains in PyV replicating DNA, 5'-ends of polynucleotide chains in purified PyV (RI)DNA were treated first with alkaline phosphatase, radiolabeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase, and then fractionated by gel electrophoresis in order to isolate [5'-^{32}P]DNA 100-200 nucleotides long that would remain at the top of a sequencing gel. One fraction of this DNA was hybridized to M13-

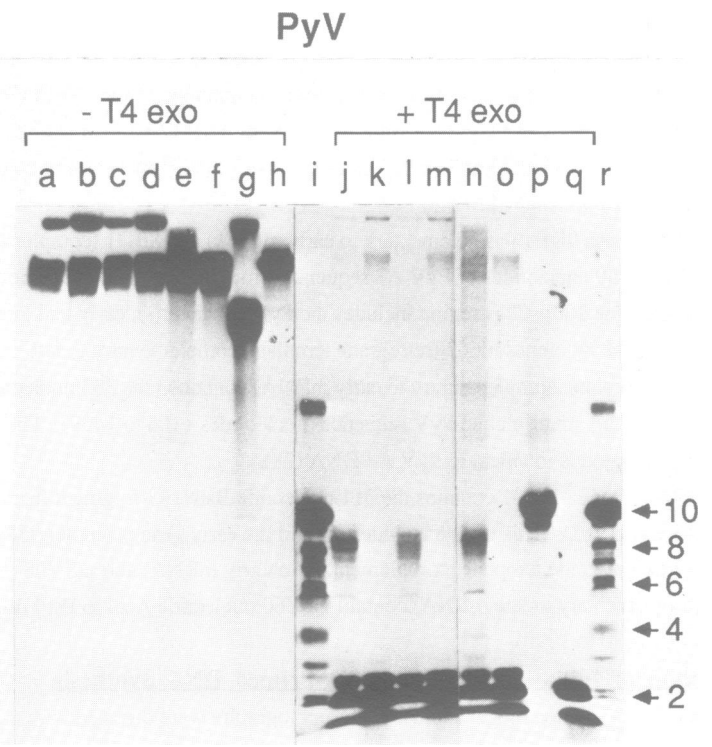

Figure 1. Identification of RNA Primers in PyV (RI)DNA. PyV (RI)DNA was radiolabeled and analyzed as described in the text. Polynucleotide chains were fractionated by electrophoresis in a 22% polyacrylamide-8 M urea gel, and 5'-^{32}P-labeled material was detected by autoradiography (8, 30). Lanes a-h are samples prior to digestion with T4 exo, and lanes j-q are samples after digestion with T4 exo. Lanes a and j: nascent DNA that hybridized to the PyV *ori*-region (M13-PyV clone 9DB1). Lanes b and k: sample run in lanes a and j but radiolabeled after incubation in alkali. Lanes c and l: nascent DNA that hybridized to a PyV sequence 1100 bp from *ori* (M13-PyV clone m662). Lanes d and m: sample run in lanes c and l but radiolabeled after incubation in alkali. Lanes e and n: total nascent PyV DNA. Lanes f and o: sample run in lanes e and n but radiolabeled after incubation in alkali. Lanes g and p: T4 exo digestion of [5'-^{32}P]rA_{10}d$A_{40\text{-}60}$. Lanes h and q: T4 exo digestion of a [5'-^{32}P]DNA restriction fragment. Lanes i and r: partial alkaline hydrolysis of [5'-^{32}P]rA_{10}.

PyV recombinant DNA clone containing the PyV*ori* -region, a second fraction was hybridized to M13-PyV clone m662 containing a PyV sequence 1100 base pairs from *ori*, and a third fraction was left unhybridized to represent total PyV nascent DNA. The DNA hybrids were isolated, and the three samples were either left untreated (Fig. 1, -T4 exo, lanes b, d and f, respectively) or incubated with the 3' to 5' exonuclease function of phage T4 DNA polymerase (Fig. 1, +T4 exo, lanes j, l and n, respectively) before fractionating the chains by gel electrophoresis (2, 9, 30).

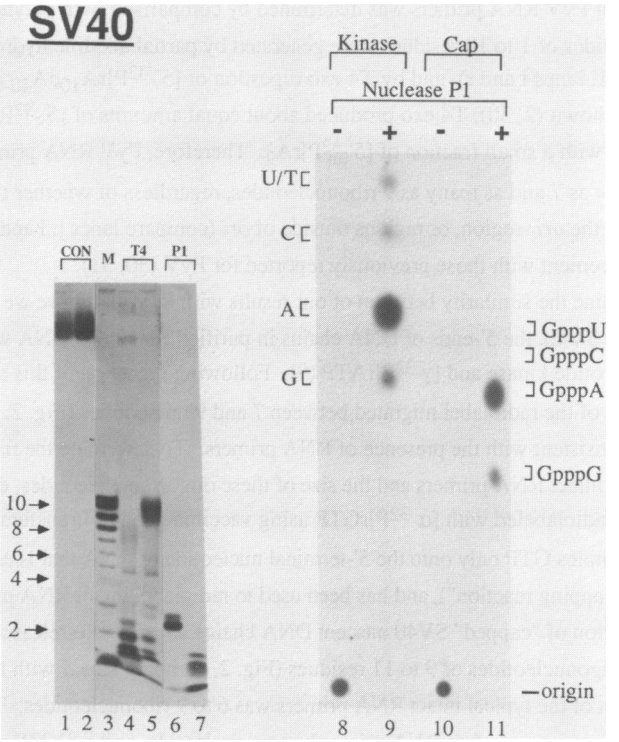

Figure 2. Identification of initiator RNA in SV40 (RI)DNA. SV40 (RI)DNA was radiolabeled and analyzed as described in the text. [5'-^{32}P]DNA chains 100-200 nucleotides long were either left untreated (CON; lanes 1 and 2) or digested with T4 exo (T4; lanes 4 and 5) or with nuclease P1 (P1; lanes 7 and 6). Lane 3 contains oligonucleotide size markers consisting of [5'-^{32}P]rA_{10}dA and partial alkaline hydrolysis of [5'-^{32}P]rA_{10}. Samples radiolabeled using T4 polynucleotide kinase and samles radiolabeled using capping enzyme were also chromatographed on PEI-cellulose before digestion with nuclease P1 (lanes 8 and 10, respectively) and after digestion with nuclease P1 (lanes 9 and 11, respectively) as described previously (29). Unlabeled rNMPs and "cap" standards were added to the appropriate samples and identified by UV light. Radioactive spots identified by autoradiography were excised and their radioactivities were quantitated by scintillation counting.

Under these conditions, T4 exo digests single-stranded DNA, but not RNA (2, 30). Exonuclease digestion released 33.2 ± 5.7% of the 5'-^{32}P-label as oligonucleotides that were predominantly 8-9 residues in length. In contrast, when PyV (RI)DNA was pretreated with alkali to remove RNA primers prior to radiolabeling, only mono- and di-nucleotides remained after digestion with T4 exo (Fig. 1, compare lanes b, d and f with k, m and o). Digestion of a [5'-^{32}P]DNA restriction fragment with T4 exo showed that mono- and di-nucleotides represented a limit digest of DNA molecules lacking RNA primers (Fig. 1, lane q). Therefore, the oligoribonucleotides represented RNA primers on about 33% of the DNA chains associated with replicating PyV DNA.

The size of PyV RNA primers was determined by comparison with polynucleotide standards. Polyribonucleotides of 1 to 10 residues were generated by partial alkaline hydrolysis of [5'-^{32}P]rA$_{10}$ (Fig. 1, lanes i and r), and by T4 exo digestion of [5'-^{32}P]rA$_{10}$dA$_{40\text{-}60}$ (Fig.1, lane p). As previously shown (2, 30), T4 exo produced about equal amounts of [5'-^{32}P]rA$_{10}$dA and [5'-^{32}P]rA$_{10}$ along with a small fraction of [5'-^{32}P]rA$_9$. Therefore, PyV RNA primers typically contained as few as 7 and as many as 9 ribonucleotides, regardless of whether the nascent chains originated from the *ori* -region, or regions outside of *ori* (compare lanes j, l and n). These results are in good agreement with those previously reported for PyV (30, 31).

To determine the similarity between of our results with PyV and those we had obtained previously with SV40, the 5'-ends of DNA chains in purified SV40 (RI)DNA were radiolabeled using polynucleotide kinase and [γ-^{32}P]rATP (9). Following digestion of this sample with T4 exo, about 30% of the radiolabel migrated between 7 and 9 nucleotides (Fig. 2, compare lane 4 with lane 1), consistent with the presence of RNA primers. To determine the fraction of DNA chains carrying intact RNA primers and the size of these oligoribonucleotides, denatured SV40 (RI)DNA was radiolabeled with [α-^{32}P]rGTP using vaccinia guanylyltransferase (2, 30). This enzyme incorporates GTP only onto the 5'-terminal nucleotide of RNA that is either a di- or triphosphate ("capping reaction"), and has been used to radiolabel intact RNA primers specifically (32-34). Digestion of "capped" SV40 nascent DNA chains with T4 exo released 74% of the radiolabel as oligonucleotides of 9 to 11 residues (Fig. 2, compare lane 5 with lane 2). Therefore, the actual length of the typical intact RNA primers was 6 to 9 ribonucleotides. The "cap" structure increases the apparent size of an RNA primer by two nucleotide, and a dNMP remains at the 3'-end of about half of the RNA chains (2, 30). The presence of the cap structure was confirmed by digesting the "capped" and "kinased" samples with nuclease P1 to release the 5'-terminal nucleotide (2, 30). A dinucleotide (G*pppN; *p symbolizes the ^{32}P-label) was released from the "capped" sample (Fig. 2, lane 6), while a mononucleotide (*pN) was the predominant form released form the "kinased" sample (Fig. 2, lane 7).

The 5'-terminal ribonucleotide was identified by subjecting a portion of the P1 digestion products to PEI-cellulose chromatography (Fig. 2, lanes 8-11). Prior to incubation with nuclease P1, [5'-^{32}P]RNA-p-DNA chains remained at the origin (lanes 8 and 10). Material that had been labeled using polynucleotide kinase and then digested by nuclease P1 released all four deoxyribonucleoside or ribonucleoside monophosphates (lane 9), 14% chromatographed with pG, 63% with pA, 12% with pC, and 12% with pU or pdT. In contrast, nuclease P1 released 71% of the radiolabel incorporated by guanylyltransferase as GpppA, and 29% as GpppG (lane 11). No radioactivity was detected in the region corresponding to GpppC or GpppU, suggesting that the presence of 5'-terminal U or C residues in the kinased material (lane 9) resulted from partially degraded RNA primers. Thus, intact SV40 RNA primers are 6 to 9 nucleotides in length and contain a 5'-terminal (p)pprA or (p)pprG residue in the ratio of 2.4 to 1. Unfortunately, a similar

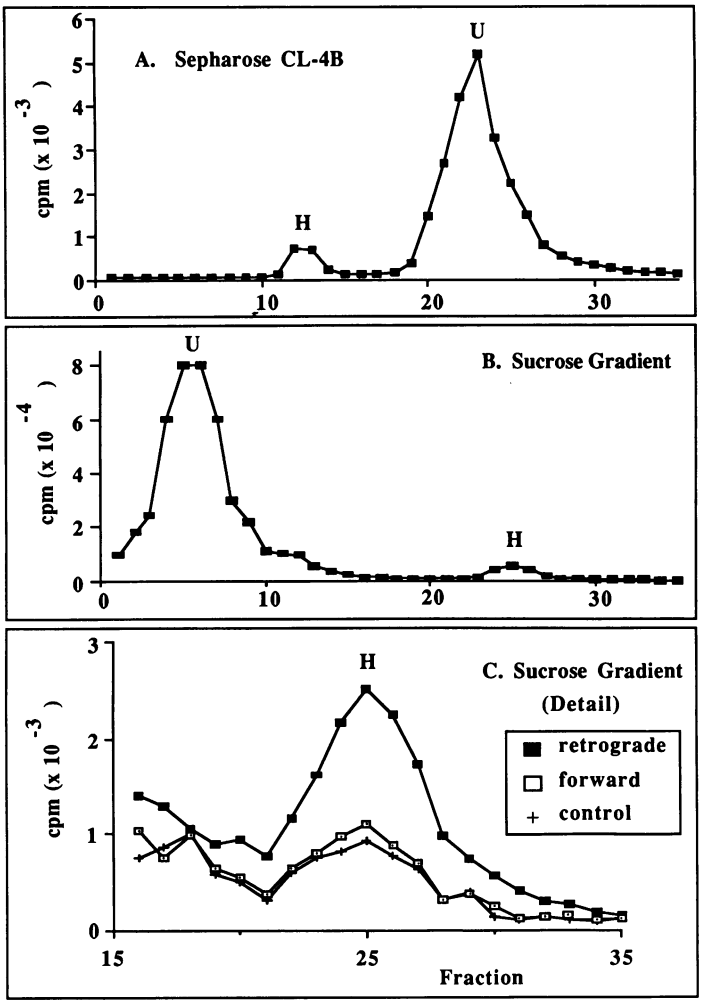

Figure 3. Separation of unhybridized PyV nascent DNA chains from their hybrids with M13-PyV clones. Purified PyV chains of selected sizes that had been radiolabeled with ^{32}P specifically at their RNA-p-DNA junctions, were hybridized to circular, single-stranded M13 virion DNA containing unique segments of PyV DNA that represent retrograde (m662) and forward (m663) template sequences. PyV:M13-PyV hybrids ("H") were then separated from unhybridized Okazaki fragments ("U") and radioactivity was quantitated as described in Materials and Methods. (A) 100-200 nucleotide long Okazaki fragments hybridized to m662 and separated by Sepharose CL-4B gel exclusion chromatography. (B) 100-200 nt long Okazaki fragments hybridized to m662 DNA and separated by neutral sucrose gradient sedimentation. The direction of centrifugation was from left to right. (C) 100-200 nucleotide long Okazaki fragments hybridized to m662 (retrograde), m663 (forward) or M13mp7 vector (control) DNA and separated by neutral sucrose gradient sedimentation. Only that portion of the gradient where hybrid DNA sedimented is shown, and the three gradient profiles are superimposed for the sake of comparison. The entire pool of hybridized [^{32}P]DNA (fractions 22-28 in panel C) was used to calculate the fraction of Okazaki fragments hybridized to each M13 DNA in Table 1.

analysis of PyV (RI)DNA was unsuccessful due to the small quantities of starting material available.

We previously reported a value of 9 to 11 nucleotides for the size of SV40 RNA primers synthesized in vivo based on comparison with deoxyribonucleotide standards of different sequence (9). The present values are based on comparison with poly(rA) standards. RNA primers synthesized by purified DNA primase-DNA polymerase-α from CV-1 monkey cells (the permissive host for SV40) are 6 to 8 nucleotides when compared with poly(rA) standards (30). Since electrophoretic mobility of polynucleotides in polyacrylamide gels depends on both nucleotide structure and polynucleotide sequence, comparison of RNA primer lengths must use common size standards.

Hybridization of Nascent DNA Chains to Forward and Retrograde Templates

PyV or SV40 replicating DNA was purified from virus-infected cells and radiolabeled specifically at their RNA-p-DNA junctions to distinguish these chains from mRNA, broken viral DNA or contaminating cellular DNA. [5'-^{32}P]DNA was fractionated into groups containing polynucleotide chains 50-100, 100-200, and 200-300 nucleotides long (9, 24). These size classes represented short, average, and long Okazaki fragments. Each group was hybridized to single-stranded M13 virion DNA containing cloned PyV or SV40 sequences that represented either forward or retrograde templates. Hybridization to M13mp7 DNA served as a control for nonspecific hybridization. DNA hybrids were separated from unhybridized Okazaki fragments either by Sepharose CL-4B exclusion column chromatography (Fig. 3A) or by neutral sucrose gradient sedimentation (Fig. 3B). Since each recombinant clone contained about 6% of the total PyV or SV40 genome, only about 6% of the total [5'-^{32}P]Okazaki fragments hybridized to the clones. The amount of material that hybridized to a clone containing a viral retrograde template was always greater than the amount that hybridized to the corresponding forward template. However, the fraction of Okazaki fragments that hybridized to forward templates was only slightly greater than the amount that hybridized to the M13 vector alone (Fig. 3C).

The amount of [5'-^{32}P]DNA that hybridized to each cloned DNA was measured by scintillation counting, and the fraction of RNA-primed DNA chains that were complementary to the retrograde template was calculated (Table 1). For the small and average length Okazaki fragments, DNA hybrids were isolated both by exclusion chromatography and by gradient sedimentation. The results from both techniques were similar and therefore were averaged together. The long Okazaki fragments could not be cleanly separated from their DNA hybrids by exclusion chromatography, so the experiment was carried out twice with sedimentation analysis. The results for SV40 were in good agreement with published results (8-10) and show that 83% to 90% of RNA-linked DNA chains were complementary to the retrograde template. Similarly, 80% to 92% of PyV DNA chains with RNA-p-DNA junctions were derived from the retrograde template.

Localization of RNA-Primed DNA Initiation Sites to Retrograde Templates

Presumably, the material that hybridized to the M13 vectors did so because of

Table 1. Hybridization of RNA-primed DNA Chains to Forward and Retrograde Templates

Virus	DNA Size (nucleotides)	DNA total cpm	Hybridization (%)		R/F Ratio	Retrogade Initiations (%)
PyV	50-100	130,000	R	3.8	5	84
			F	1.7		
			M13	1.2		
	100-200	70,000	R	3.6	11	92
			F	1.6		
			M13	1.4		
	200-300	170,000	R	2.5	4	80
			F	1.6		
			M13	1.3		
SV40	50-100	145,000	R	9.4	9	90
			F	2.5		
			M13	1.6		
	100-200	113,000	R	4.9	5	83
			F	2.1		
			M13	1.4		
	200-300	60,000	R	6.4	6	86
			F	3.2		
			M13	2.6		

For each size class, experiments with PyV and SV40 were conducted in parallel using the procedure described in figure 3. [5'-^{32}P]DNA chains radiolabeled at their RNA-p-DNA junctions were hybridized to M13 cloned sequences representing retrograde ("R", m662, mSV07) and forward ("F", m663, mSV08) templates, as well as to M13mp7 ("M13"). R/F ratios and the fractions annealed specifically to retrograde templates were determined after subtracting the values obtained for M13mp7 (8). Each value for "Hybridization (%)" represents the average of two experiments.

complementarity to the viral sequences. However, even under the stringent hybridization conditions employed, nonspecific hybridization of Okazaki fragments to M13 sequences has been documented (9). To demonstrate that the material which hybridized to the M13-viral clones originated specifically from sites within the viral sequences, initiation sites for RNA-primed nascent DNA chains were examined on both forward and retrograde template strands. PyV Okazaki fragments, 100 to 200 nucleotides in length, were radiolabeled specifically at their RNA-p-DNA junctions and then hybridized to either the retrograde or forward template of a PyV sequence 1106 to 1439 nucleotides from *ori*. Hybrids were isolated and cleaved at a unique restriction endonuclease site within the viral sequence, and the digestion products fractionated by gel electrophoresis (Fig.4, PyV). If an RNA-primed DNA chain had annealed correctly to this restriction site, and if its 5'-end was located less than 150 nucleotides upstream of this site, a [5'-

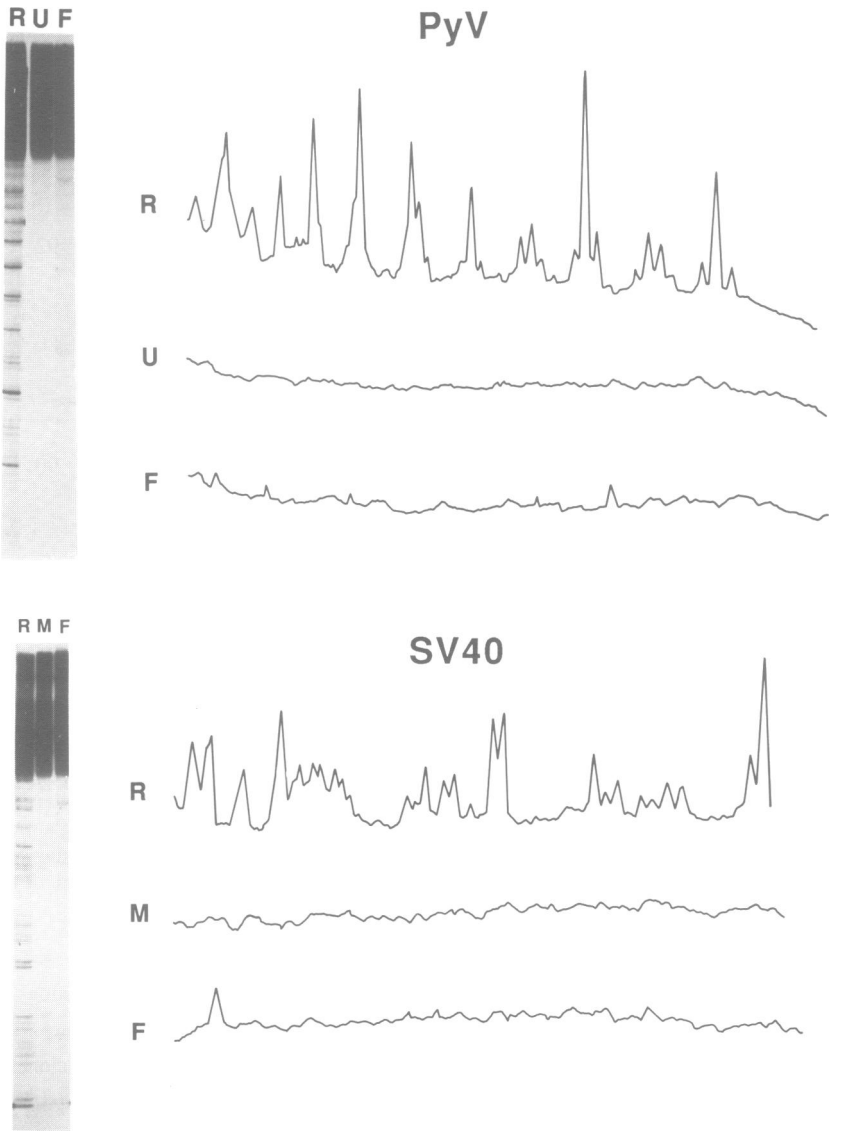

Figure 4. Localization of Okazaki fragments to retrograde templates. Nascent DNA chains from purified PyV (RI)DNA (PyV) or SV40 (RI)DNA were radiolabeled specifically at their RNA-p-DNA junctions. PyV [^{32}P]DNA was hybridized to M13-PyV clones m662 and m663 containing either the retrograde (R) or the forward template (F), respectively. DNA hybrids were then incubated with (R and F) or without (U) restriction endonuclease Nco I, which cleaves only once in the cloned PyV sequence (29). SV40 [^{32}P]DNA was hybridized to either M13mp7 (M) or M13-SV40 clones mSV07 and mSV08 containing either the retrograde (R) or the forward template (F),

respectively. Hybrid DNA was then digested with the restriction endonuclease EcoRI, which cleaves only once in the cloned SV40 sequence. The resulting digestion products from both PyV and SV40 experiments were fractionated by gel electrophoresis (9, 24, 29). Densitometer tracings were made from autoradiograms of identical exposure and care was taken to use exposures which were related linearly to the amount of radioactivity present.

32P]DNA fragment would migrate into the gel. This technique has been used to map the precise nucleotide initiation site for RNA-primed DNA chains in SV40 (9, 24) and PyV (29). The results from a similar experiment using a region of SV40 1628 to 1986 nucleotides from *ori* is presented for comparison (Fig. 4, SV40).

When DNA hybrids were incubated in the absence of restriction enzyme ("U") or when material that had hybridized to cloning vector M13mp7 DNA ("M") was digested, no [^{32}P]DNA bands were released that migrated into the gel. In contrast, when DNA hybrids with retrograde clones were cleaved, a series of [^{32}P]DNA bands were released ("R"). When DNA hybrids with clones containing forward templates were cleaved, only a few, very faint bands were detected ("F"). Quantitation of these data by densitometry showed that initiation of discontinuous DNA synthesis on the PyV retrograde template occurred 28-times more frequently than on the forward template. Hence, 97% of all PyV DNA chains with RNA-p-DNA linkages originated from the retograde arms of replication forks, as did 95% of all RNA-primed SV40 Okazaki fragments.

To demonstrate unequivocally that RNA-primed initiation sites originate specifically from retrograde templates and not from particular DNA sequences that may fortuitously represent retrograde templates, the distribution of RNA-primed DNA fragments in the *ori*-region was examined. Since these viruses replicate bidirectional from a unique *ori*-sequence referred to as the origin of bidirectional replication (5, 6), each DNA strand in the *ori* -region represents the forward template of one fork and the retrograde template of the opposite fork. Thus, on the same DNA strand, one side will exhibit sites where RNA-primed DNA synthesis was initiated (discontinuous DNA synthesis) and the other side will not (continuous DNA synthesis). Therefore, if PyV and SV40 indeed replicate semi-discontinuously, a sharp transition point marking the beginning of continuous DNA synthesis should be observed in their *ori* -regions (24, 29).

The experiment described in figure 4 was repeated using M13-PyV and M13-SV40 clones containing one strand of the viral *ori*-region. When DNA hybrids were incubated in the absence of a restriction endonuclease, no DNA fragments were observed following gel electrophoresis (Fig. 5, PyV, "-" lane). However, when DNA hybrids were digested at a unique restriction site within the PyV sequence, a series of [5'-^{32}P]DNA fragments were released that migrated near the top of the gel (Fig. 5, PyV, "+" lane). A similar result was obtained with SV40 (Fig. 5, SV40). Densitometer tracings of these gels revealed a distinct transition between the presence of RNA-p-DNA linkages to their complete absence. Hence, both PyV and SV40 DNA replication is semi-discontinuous.

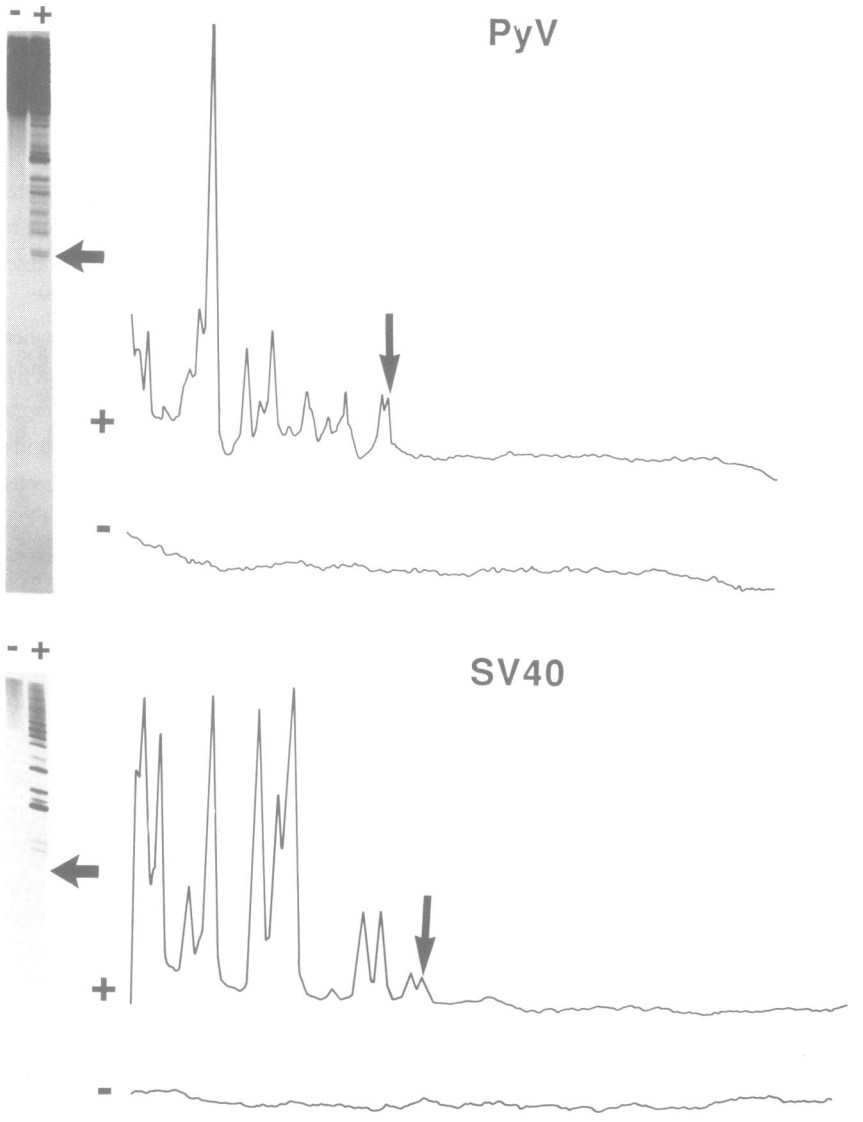

Figure 5. Transition from discontinuous to continuous DNA synthesis in the *ori*-region. RNA-p-DNA linkages in nascent PyV or SV40 DNA were labeled specifically, and [^{32}P]DNA was hybridized to single-stranded M13-PyV clone 8BD1 or M13-SV40 clone mSV01 DNA containing the *ori*-regions of their respective viruses. Hybrid DNA was either left untreated ("-" lane) or digested ("+" lane) with either Sty I (PyV) or Ava II (SV40). These restriction endonucleases cut the cloned viral sequence at a single site (22, 29). The resulting samples were then fractionated by gel electrophoresis. Densitometer tracings of autoradiograms were as described in Figure 4. Arrows indicate the transition from discontinuous to continuous DNA synthesis.

DISCUSSION

The data presented in this paper demonstrates that PyV DNA replication, like SV40 DNA replication, is discontinuous predominantly, if not exclusively, on the retrograde side of replication forks. About one third of the DNA chains in PyV (Fig. 1) and SV40 (Fig. 2) replicating DNA intermediates had an RNA primer covalently attached to their 5'-end that was 6 to 9 residues long. These viral DNA chains were radiolabeled on the 5'-deoxyribonucleotide that was covalently attached to an RNA primer. From 80% to 92% of PyV and SV40 RNA-primed DNA chains in the size range of Okazaki fragments hybridized specifically to the retrograde template (Fig. 3 and Table 1). Furthermore, when DNA hybrids between PyV or SV40 RNA-primed DNA chains and either the forward or retrograde template were cut at a unique restriction endonuclease site, 95% to 97% of the RNA-p-DNA junctions were located on the retrograde template (Fig. 4). This paucity of initiation events on the forward template was not an artifact of the sequence examined, because a clear transition was observed between the presence of RNA-primed DNA synthesis initiation sites and their absence in the *ori*-region of each virus (Fig. 5). The precise nucleotide locations of these transition sites have been mapped on both strands of the SV40 (24) and PyV (29) *ori*-regions, localizing the origin of bidirectional replication to the junction between *ori*-core and a strong T-Ag DNA binding site. Taken together, the data reveal that DNA replication at PyV replication forks is essentially the same as at SV40 replication forks; DNA synthesis is semi-discontinuous. This was also the conclusion of Franke and co-workers (18-20). The small number of initiation events localized to the forward strand can be explained if a fraction of the replicating intermediates proceeds unidirectionally instead of bidirectionally, or contains large deletions outside *ori*, or initiates DNA replication at alternative origins of replication. However, we cannot rule out the possibility that rapid excision of RNA primers and ligation of Okazaki fragments on the forward template masks the presence of fully-discontinuous DNA synthesis.

Under some conditions, significant amounts of PyV and SV40 can replicate unidirectionally, particularly with *in vitro* replication systems (12, 13, 36). Since up to half of the forward template in bidirectionally replicating molecules becomes a retrograde template in unidirectionally replicating molecules, contamination by unidirectionally replicating DNA will appear to generate Okazaki fragments from the forward template. Franke and Vogt (19) showed that deletion mutants of PyV that are still capable of DNA replication, which readily accumulate during preparation of virus stocks, initiate Okazaki fragments on what would have been the forward template in wild-type DNA, but, in a deletion mutant, is actually the retrograde template because bidirectional replication always terminates 180° from *ori* (5, 6). We also found that DNA deletions are readily packaged into virions in PyV-infected cells, and therefore our virus stocks were monitored closely to maintain homogeneous preparations of viral DNA that were nondefective (see Materials and Methods). Finally, SV40 is capable of initiating replication at alternative origins, albeit infrequently (12, 36). Since replication will terminate 180° away, this phenomena will also contribute to the appearance of Okazaki fragments on what is supposed to be the forward template.

Other problems may also contribute to the appearance that Okazaki fragments are initiated on forward templates. First, radiolabeling techniques that are not specific for RNA-primed DNA chains will also label any contaminating viral or cellular RNA and cellular DNA. Therefore, we radiolabeled specifically RNA-p-DNA junctions of polynucleotides associated with purified PyV and SV40 (RI)DNA. Second, preparation of single-stranded DNA templates by separation of strands of restriction fragments during gel electrophoresis can lead to contamination with the complementary strand or with DNA sequences from other parts of the viral genome. Small contaminations could generate significant error when the probe is present in excess over nascent DNA chains (20). Therefore, we used recombinant bacteriophage M13 clones to produce homogeneous preparations of single-stranded hybridization probes. Finally, it is conceivable that low concentrations of critical replication proteins in subcellular replication systems may allow initiation of Okazaki fragments on the forward template. In all but one of the previous studies (17), Okazaki fragments were radiolabeled as PyV (RI)DNA continued replication in isolated nuclei. Therefore, we chose PyV (RI)DNA that had been synthesized in intact cells as our starting material.

Narkhammer-Meuth et al. (17) also studied the distribution of Okazaki fragments in PyV (RI)DNA replicated *in vivo*, and found that 52% of pulse-labeled nascent DNA chains self-annealed and that 26% hybridized specifically to the forward template, suggesting discontinuous synthesis on both arms of replication forks. Remarkably, they also found that 53 - 86% of the radioactivity in 5'-end labeled polynucleotides was alkali-sensitive, and that a larger fraction of this radiolabel was liberated from material that hybridized to the forward template than to the retrograde template. This was interpreted to mean that a larger fraction of nascent DNA chains on the forward template carried RNA primers than on the retrograde template. However, the large amount of alkali-sensitive label and the fact that at least 50% of the radiolabel resided in pGp while a much smaller percentage was pAp suggest that a significant fraction of the material was contaminating viral or cellular RNA rather than RNA primers. Intact PyV RNA primers begin with ATP 2.4 times more frequently than GTP (32), similar to results with *Drosophila* (33), human (38) and SV40 RNA primers (9 and Fig. 2).

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