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# Both Strands of Polyoma DNA Are Replicated Discontinuously with Ribonucleotide Primers In Vivo

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Nascent polyoma DNA molecules were isolated after pulse-labeling of infected murine 3T6 cells with [$^3$H]thymidine. The extent of digestion of these DNA molecules by spleen exonuclease was increased by exposure to alkali or RNase, suggesting that ribonucleotides were present at or near the 5′ termini of the newly synthesized pieces of DNA. Intermediates shorter than 300 nucleotides were hybridized to the separated strands of restriction enzyme fragments of the polyoma genome: 2.5 to 3-fold more radioactivity was found in the strand whose synthesis is necessarily discontinuous (the lagging strand) than in the strand whose synthesis is potentially continuous (the leading strand). Separation of the strands of [5′-$^{32}$P]DNA molecules showed that the excess [$^3$H]thymidine in lagging-strand molecules was not simply the result of an increased number of molecules. Therefore, assuming equivalent efficiencies of labeling, lagging-strand pieces must be slightly longer than those with leading-strand polarity. The presence of ribonucleotides on the 5′ termini of molecules with both leading- and lagging-strand polarity was demonstrated by (i) release of $^{32}$P-ribonucleoside diphosphates upon alkaline hydrolysis of [5′-$^{32}$P]DNA separated according to replication polarity and (ii) the change in the degree of self-annealing of nascent molecules upon preferential degradation of DNA molecules possessing initiator RNA moieties by spleen exonuclease. We conclude that replication of polyoma DNA in vivo occurs discontinuously on both sides of the growing fork, using RNA as the major priming mechanism.

During replication of polyoma and simian virus 40 DNA, radioactivity is incorporated into short molecules of both replication polarities (3, 6, 8, 24). Most of these molecules are derived from the strand whose synthesis is necessarily discontinuous (the lagging strand). Short molecules derived from the strand whose synthesis is potentially continuous (the leading strand) have been assumed to be the products of repair or radiolysis (8, 24). In the preceding paper (20), evidence was presented that these molecules are indeed intermediates of discontinuous DNA synthesis because they contain initiator RNA (iRNA). It was concluded that polyoma DNA is synthesized discontinuously on both strands during in vitro replication.

The in vitro replication system used consisted of nuclei isolated from infected 3T6 cells; it mimicked in vivo replication according to several criteria (16, 26, 29). For example, Kowalski and Denhardt (11) have shown that the intact 3T6 host cell of polyoma uses ribonucleotides to prime DNA synthesis. Nevertheless, it is not certain to what extent events in isolated nuclei accurately reflect DNA synthesis in unbroken cells. Excessive reinitiation of synthesis in subcellular systems could cause an accumulation of iRNA-containing molecules on the strand whose synthesis is normally continuous in vivo. To extend our knowledge of polyoma DNA replication to the in vivo situation, we have investigated the extent of use of iRNA during viral DNA synthesis in the intact cell by exploiting the fact that nascent DNA molecules bearing ribonucleotides become substrates for spleen exonuclease after treatment with alkali or RNase (11, 12, 18). Herein we demonstrate the presence of iRNA moieties on both leading- and lagging-strand nascent DNA molecules. Our data are consistent with discontinuous RNA-primed synthesis of both progeny strands at the growing fork during replication in vivo of polyoma DNA.

## MATERIALS AND METHODS

Most of the procedures used in cell culture, the preparation of DNA, and the hybridization experiments with the nascent molecules were as described in the preceding paper (20).

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**Enzymes and reagents.** [$^3$H]thymidine (50 to 60 Ci/mmol), [$\gamma$-$^{32}$P]ATP, and [$^{32}$P]phosphate were purchased from New England Nuclear Corp. or synthesized by published procedures (5). Polyoma cRNA synthesized by *Escherichia coli* RNA polymerase was kindly provided by R. Kamen. The separated strands of restriction enzymes *Eco*R1-*Hha*I fragment 2 of the polyoma genome were generous gifts from G. Magnusson. Pancreatic DNase I and staphylococcal nuclease were obtained from Calbiochem. RNases A and $T_1$ were from Worthington Biochemicals Corp. *E. coli* alkaline phophatase purified according to Weiss et al. (28) was kindly provided by Neil Miyamoto. When assayed on 5′-$^{32}$P-phosphorylated DNA, it removed more than 90% of the label. T4 polynucleotide kinase was from P-L Biochemicals. Phosphorylation was complete under the conditions used as judged by quantitative labeling with [$\gamma$-$^{32}$P]ATP and resistance to spleen exonuclease of 5′-hydroxyl DNA of defined length after phosphorylation. Spleen phosphodiesterase was prepared from hog spleen by Neil Miyamoto by the method of Bernardi and Bernardi (1). It was assayed according to Razzell and Khorana (25). By this assay, the final preparation had a specific activity of 7.5 nmol of synthetic substrate hydrolyzed per h per mg of protein. It was assayed on several nucleic acid substrates, including freshly 5′-phosphorylated 18S rRNA and fragments generated by staphylococcal nuclease or DNase I treatment of 3T6 DNA. The enzyme preparation possessed a low level of endoribonuclease activity which cochromatographed with the 5′-hydroxyl-specific exonuclease activity on Sephadex G-75 and on concanavalin A-Sepharose. The two activities also had identical temperature inactivation curves.

**Labeling of DNA in vivo.** The cells were labeled directly in the culture dish at 26 to 28 h postinfection. To hasten the equilibration of the cellular pool with [$^3$H]thymidine during the very short pulse, the cells were preincubated for 5 min on ice in 10 ml of the medium containing 2.5 mCi of [$^3$H]thymidine (50 to 60 mCi/mmol) (24). The ice-cold medium was then replaced with 10 ml of equivalent medium prewarmed to 37°C, and the petri dish was floated on a 37°C water bath for about 30 s. The cells were quickly washed with cold Tris-buffered saline and lysed with 2 ml of 0.01 M Tris-hydrochloride (pH 7.6)–0.01 M EDTA–0.7% sodium dodecyl sulfate for 10 to 15 min at room temperature.

**Purification of the nascent molecules.** A purification procedure similar to that described (20) was used with a few modifications. The early Bio-Gel P-30 step and the CsCl equilibrium centrifugation in the presence of propidium diiodide were omitted. To minimize losses in the nitrocellulose chromatography, the column was prewashed with 100 to 200 $\mu$g of denatured carrier DNA, and the sample was eluted with low-salt buffer without sodium dodecyl sulfate so that the DNA could be precipitated with ethanol directly. After the size fractionation on Ultrogel AcA 34, three size classes were pooled: molecules less than 300, those 300 to 600, and those more than 600 nucleotides long. The average distribution of [$^3$H]thymidine in the size classes was 18, 15, and 65%, respectively. By mass, the amount of DNA in the shortest size class was 1 to 2% of the total labeled polyoma DNA. Typically 25 ng of DNA shorter than 300 nucleotides was recovered from 10 15-cm culture dishes. The overall recovery was 35 to 40%. When the material was to be 5′-end labeled with polynucleotide kinase, the carrier DNA was omitted from the purification. The short molecules were then isolated from the denatured preparation on a neutral sucrose gradient (5 to 20% sucrose, 0.01 M Tris-hydrochloride, pH 7.6, and 0.3 M NaCl) centrifuged in an SW50.1 rotor at 50,000 rpm for 3 h at 4°C.

**Analysis of the polarity of the nascent molecules.** Nascent molecules, labeled in vivo with [$^3$H]-thymidine or in vitro with $^{32}$P by using polynucleotide kinase were hybridized to a 10- to 20-fold sequence excess of the separated strands of *Hpa*II fragment 1 and *Eco*RI-*Hha*I fragment 2 (see Fig. 3) which had been tailed at the 3′ end with polydeoxycytidylate [poly(dC)] by using terminal transferase. The preparation of the separated strands of polyoma DNA restriction fragments tailed with poly(dC) and controls for cross-contamination are described in the accompanying paper (20). The hybridizations were in 0.5 to 1 M NaCl–0.015 M Tris-hydrochloride (pH 7.6)–5 mM EDTA at 65°C for 10 to 20 $\times$ $C_0t_{1/2}$. Polyinosinic acid [Poly(I)]-Sephadex chromatography was carried out as described earlier (20), except that when the samples were to be tested for pNp release, 90% formamide–0.01 M Tris-hydrochloride (pH 7.6) was used as the eluent instead of $NH_3$. The eluate was diluted with 1 volume of water, adjusted to 1 M NaCl, and precipitated with ethanol.

**Sedimentation in alkaline sucrose gradients.** Samples were applied on top of a linear gradient of 5 to 20% sucrose in 0.3 M NaOH–0.7 M NaCl–0.001 M EDTA. The gradients were centrifuged at 4°C either in an SW56 rotor at 50,000 rpm for 3.5 h or in an SW40 rotor at 36,000 rpm for about 20 h. Fractions were collected from the bottom or the top, respectively, and analyzed for radioactivity.

**Spleen exonuclease hydrolysis.** DNA was first reacted with polynucleotide kinase (10 to 20 U/ml; 60 min at 25°C) and a 500- to 1,000-fold molar excess of ATP over the number of 5′ ends in the sample. It was then subjected to one of the following three treatments: (i) alkaline hydrolysis (0.4 M NaOH, 16 h, 37°C); (ii) RNase digestion (RNase A at 20 $\mu$g/ml plus RNase $T_1$ at 20 U/ml heated for 10 min at 80°C to inactivate DNase) for 30 min at 37°C; or (iii) dephosphorylation with alkaline phosphatase (1 U/ml for 30 min at 65°C). Digestion with spleen exonuclease was performed with 30 $\mu$g of the enzyme per ml in 0.15 M $CH_3COONa$ (pH 5.5)–0.01 M EDTA–0.05 M $Na_2SO_4$ with DNA at 30 to 500 $\mu$g/ml. Incubation was for 60 min at 45°C. The proportion of acid-soluble radioactivity in the sample was determined by precipitation and centrifugation in 0.4 M $HClO_4$ together with 200 $\mu$g of carrier DNA per ml and comparison with an equivalent sample that had been heated at 80°C for 30 min after acid precipitation. The sensitivity of phosphorylated and dephosphorylated DNA to this enzyme is discussed in more detail elsewhere (11).

**5′-End labeling and assay for 5′-terminal ribonucleotides.** Purified nascent polyoma DNA less than 300 nucleotides long was dephosphorylated with alkaline phosphatase and reacted with polynucleotide kinase (20 U/ml) and [$\gamma$-$^{32}$P]ATP (8,400 Ci/mmol; 2.5

μM). The reaction was stopped with 20 mM EDTA and a 100-fold excess of unlabeled ATP. After addition of carrier DNA and precipitation with ethanol, the remaining [γ-$^{32}$P]ATP was removed by chromatography on nitrocellulose. The 5'-$^{32}$P-labeled nascent DNA was then hybridized to the separated strands of polyoma DNA restriction enzyme fragments (see above). $^{32}$P-labeled material eluting from poly(I)-Sephadex with formamide was concentrated by alcohol precipitation and subjected to alkaline hydrolysis (0.4 M NaOH, 16 h, 37°C). The samples were neutralized and spotted on polyethyleneimine (PEI)-cellulose thin layers and chromatographed as previously described (18).

## RESULTS

**Strand polarity of nascent short molecules.** Polyoma DNA was pulse-labeled in vivo for about 30 s with [$^3$H]thymidine and purified as described in Materials and Methods. From the final size fractionation of the material by chromatography on Ultrogel, three size classes of nascent DNA molecules were isolated: less than 300, 300 to 600, and more than 600 nucleotides long (Fig. 1a). Sedimentation in alkaline sucrose gradients revealed that the class of molecules 300 to 600 nucleotides long also contained some molecules over 1,000 nucleotides in length (Fig. 1b). This contamination was likely due to the retardation of some of the longer DNA strands on the agarose column. The labeled molecules in the largest size class ranged from about 600 nucleotides to full-length progeny strands as judged by the sedimentation profile in alkaline sucrose gradients (data not shown).

The isolated nascent DNA was 90 to 100% polyoma DNA as judged by its ability to hybridize to an excess of polyoma form I DNA. Figure 2 shows the kinetics of a typical self-annealing experiment with the shortest molecules. Table 1 shows examples of the maximal amount of duplex DNA formed during reassociation of the three size classes of molecules. The degree of self-complementarity of the DNA decreased as the length of the DNA decreased. The annealing of the longest molecules does not truly represent their complementarity because of the presence of an excess of unlabeled non-nascent parental molecules in this size class. The shortest molecules reannealed to 52 ± 6% (average of 10 experiments), suggesting a greater degree of asymmetry between the two strands in the replication fork than in vitro, where the value was 72 ± 4% (20).

The strand bias of these nascent molecules was determined by hybridization to separated strands of *Hpa*II fragment 1 as described in the preceding paper (20). As expected, a greater fraction of label in the shortest chains annealed to the template for lagging-strand synthesis, 1E (Table 1). As pointed out in the preceding paper (20) and illustrated in Fig. 3, leading-strand molecules initiated at the origin and complementary to *Hpa*II fragment 1L will not be present among

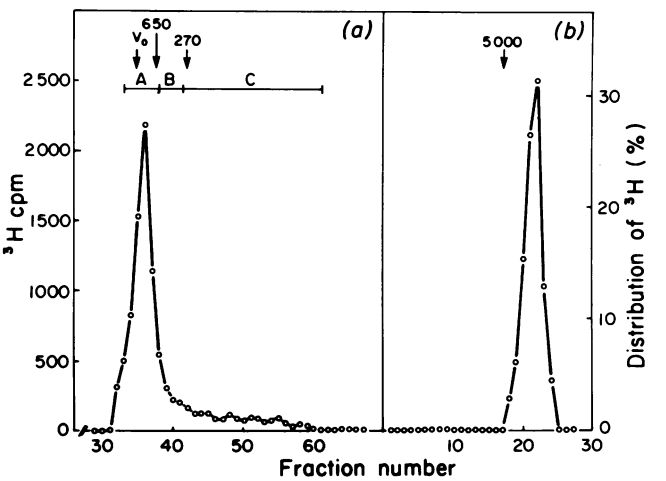


FIG. 1. *(a) Chromatography on Ultrogel AcA34. Polyoma DNA pulse-labeled with [$^3$H]thymidine and purified as described in Materials and Methods was denatured and applied to a column (1.7 by 95 cm) previously calibrated with denatured $^3$H-labeled restriction enzyme HpaII fragments 4 and 7 (about 650 and 270 nucleotides long). The column was eluted with 0.05 M Tris-hydrochloride (pH 7.6)-0.001 M EDTA, and fractions of 2.0 to 2.5 ml were collected and assayed for radioactivity. The material that was pooled is indicated with bars. (b) Sedimentation velocity centrifugation in alkaline sucrose of pool B from the Ultrogel filtration. A 5 to 20% sucrose gradient was centrifuged in an SW50.1 rotor at 50,000 rpm for 3.5 h at 4°C. The position of a marker of 5,000 nucleotides (denatured form II DNA) is indicated. Total radioactivity was 3,000 cpm.*

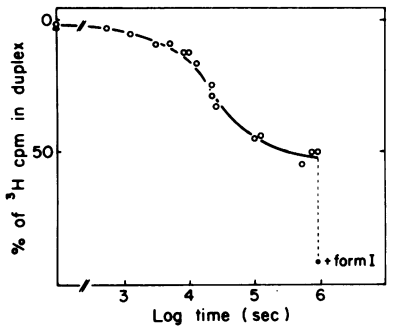


FIG. 2. *Self-annealing of short nascent polyoma DNA molecules. Portions of nascent ³H-labeled polyoma DNA molecules shorter than 300 nucleotides were denatured at 100°C for 3 min and self-annealed at 65 C in 1 M NaCl-0.015 M Tris-hydrochloride (pH 7.6)-0.005 M EDTA. The products were analyzed on hydroxyapatite columns at 65°C. One portion of the material was incubated with an excess of denatured sonicated polyoma form I DNA under the same conditions. Each point represents between 500 and 1,500 cpm.*

TABLE 1. *Asymmetry of nascent polyoma DNA molecules synthesized in vivo*[a]

| DNA size (nucleotides) | % Self-annealing | % Hybridization to: | |
|---|---|---|---|
| | | *Hpa*II-1E | *Hpa*II-1L |
| <300 | 55 | 13.8 | 5.7 |
| | 46 | 12.0 | 3.5 |
| 300–600 | 85 | 13.3 | 15.3 |
| | 75 | 8.2 | 12.0 |
| >600 | 97 | 12.5 | 11.4 |
| | 95 | 11.1 | 11.4 |

[a] Purified (as described in Materials and Methods) nascent polyoma DNA was treated as follows. (i) Self-annealed at 50 to 250 ng/ml for at least 50 × $C_0t_{1/2}$ to ensure that the plateau of the reaction had been reached. The $C_0t_{1/2}$ was corrected for the length of the molecules; $C_0t_{1/2}$ for polyoma DNA of about 350 nucleotides was determined to be about $3 \times 10^{-3}$ mol·s per liter in 0.12 M $NaPO_4^{2-}$ buffer. The product was analyzed on hydroxyapatite at 65°C as described. Input radioactivity: 500 to 1,500 cpm. (ii) Hybridized to a 20-fold excess (6 to 12 ng) of poly(dC)-extended separated strands of *Hpa*II fragment 1 (27% of the viral genome; Fig. 3) in 50 to 100 μl (final volume) at 20 × $C_0t_{1/2}$. $C_0t_{1/2}$ was adjusted for the size of the fragment 1 and for the sequence excess of one of the strands over the complementary sequences in the nascent DNA. Nascent sequences complementary to the respective strands were isolated by poly(I)-Sephadex chromatography. Input radioactivity: 1,000 to 3,000 cpm.

the isolated small Okazaki pieces because the fragment is about 1,000 nucleotides from the origin. The asymmetry between the two strands varied somewhat from experiment to experiment. Similar variations were obtained whether the [³H]deoxythymidine used for labeling was of very high or low specific activity. Furthermore, there was no correlation between the strand asymmetry and the time elapsed from labeling to hybridization. Thus, we conclude that radiolysis caused by ³H decay did not generate the short molecules on the leading strand.

The distribution of label between the strands agreed quantitatively with the asymmetry observed in the self-annealing data. Thus, the preparation of short nascent DNA did not contain significant amounts of non-nascent unlabeled DNA. In accord with the observations of Hunter et al. (6) and Perlman and Huberman (24), a larger proportion of intermediate-sized molecules showed leading-strand polarity. Because of the presence of a proportion of radioactivity in very long molecules in this class (see Fig. 1), the radioactivity observed on the leading strand was most likely contributed by molecules initiated at the origin rather than by intermediates in repair processes.

**Presence of iRNA on nascent molecules of polyoma DNA.** Spleen exonuclease digestion has been used in several previous studies to detect putative RNA primers (11, 12, 17, 18). This enzyme degrades molecules with a free 5'-hydroxyl group. Nascent molecules were phosphorylated at their 5' termini with polynucleotide kinase and treated with alkali or RNase to expose new 5'-hydroxyl groups on DNA molecules having 5'-terminal or internal ribonucleotides. For quantification of the fraction of mol-

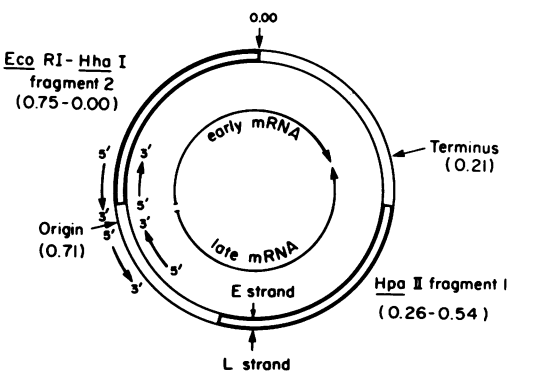


FIG. 3. *Polyoma genome. The restriction enzyme fragments that were used were generated with either HpaII or EcoRI-HhaI. Their relation to the origin and terminus of replication is shown. Also indicated are the replication polarities of the progeny strands and the polarities of both the major mature late message transcribed from the E strand counterclockwise from the region 0.68 to 0.25 and the early message transcribed from the L strand clockwise from the region 0.72 to 0.25 (7).*

ecules possessing iRNA, the maximal digestion of 5'-hydroxyl-terminated molecules was determined after dephosphorylation of all 5' ends with bacterial alkaline phosphatase. The advantage of using this approach to demonstrate iRNA molecules is that even extensively degraded RNA primers can be detected.

The background digestion of phosphorylated non-nascent DNA with or without alkali or RNase treatment was 5% (Table 2). As observed earlier (11), the nascent phosphorylated DNA was hydrolyzed to a greater degree than the control DNA, even in the absence of alkali or RNase treatment. This is apparently because the spleen exonuclease contains an endoribonuclease activity that we have been unable to separate from the exonucleolytic activity (see Materials and Methods). This activity attacks the RNA primer internally and creates a substrate for the exonuclease. Thus, to obtain the true increase in sensitivity to spleen exonuclease resulting from alkali or RNase treatment of the nascent material, the observed values should only be corrected for the digestion of authentic non-nascent DNA as measured with uniformly labeled DNA. All size classes of nascent DNA showed an increased sensitivity to spleen exonuclease after exposure to alkaline conditions or RNase, with the largest increment occurring in the shortest molecules. The values after alkali and RNase treatment corresponded well, suggesting that the increased sensitivity was truly due to the removal of ribonucleotides.

TABLE 2. *Percentage of digestion by spleen exonuclease of nascent DNA after exposure to alkali, RNase, or phosphatase*[a]

| DNA size (nucleotides) | Radioactivity released (%) | | | |
|---|---|---|---|---|
| | Control | Alkali | RNase | Phosphatase |
| Nascent | | | | |
| <300 | 36 | 45 | 47 | 51 |
| 300–600 | 17 | 32 | 32 | 29 |
| >600 | 22 | 30 | 30 | 25 |
| Non-nascent | | | | |
| <300 | 4.5 | 3.0 | 4.5 | 75 |
| 300–600 | 2.0 | 1.5 | 3.5 | 33 |
| >600 | 3.0 | 4.0 | 4.5 | 25 |

[a] Three size classes of purified [$^3$H]thymidine pulse-labeled polyoma DNA were combined with uniformly $^{32}$P-labeled 3T6 or φX174 DNA fractionated into similar size classes after exposure to DNase I. The 5' termini were phosphorylated with polynucleotide kinase and unlabeled ATP, and the preparation was divided into four parts. One portion received no further treatment, one was exposed to RNase, another was exposed to alkali, and the fourth was treated with phosphatase. All samples were then treated with spleen exonuclease, and the acid-soluble radioactivity released was determined. Total radioactivity analyzed for $^3$H and $^{32}$P ranged from 100 to 7,500 cpm. The average of values from three to four independent experiments are presented.

The maximal digestion by spleen exonuclease after phosphatase treatment of uniformly labeled DNA decreased with increasing size of the molecules, and the nascent DNA showed the same pattern. The shortest nascent molecules were not degraded to the same extent as the control, probably because of slight differences in average molecular weight of the DNA in the two preparations (the nascent DNA was a bit longer on average). Also the longer intermediates were less well digested than the control DNA, probably because the $^3$H pulse-label here was more concentrated nearer the 3' terminus. Comparison of the extent of digestion after removal of RNA primers with the extent of digestion after phosphatase treatment indicated that up to 80% of the shortest molecules possessed iRNA. Also, a substantial proportion of the longer molecules contained RNA primers.

**Distribution of iRNA on leading and lagging strands.** The proportion of iRNA on the leading and lagging strands during in vivo replication of polyoma DNA was determined in the following way. Purified nascent DNA molecules were dephosphorylated, and their 5' termini were quantitatively labeled by using polynucleotide kinase and [γ-$^{32}$P]ATP. The [$^{32}$P]DNA was hybridized to separated strands of *Hpa*II fragment 1 and *Eco*RI-*Hha*I fragment 2 (see Fig. 3). As described in the previous paper (20), the separated strands of the restriction enzyme fragments had been tailed at their 3' termini with poly(dC) to allow isolation of hybrids on poly(I)-Sephadex columns. A portion of the strand-separated material was treated with alkali, and the $^{32}$P-labeled molecules that were liberated by this treatment were identified as 2'(3'), 5'-ribonucleoside diphosphates (pNp) by PEI chromatography. Since this approach measures all 5' termini, the total number of molecules with leading- and lagging-strand polarity can be estimated after the poly(I)-Sephadex chromatography. The asymmetry in hybridization of the [$^{32}$P]DNA (1.3-fold; data not shown) to the separated strands was less than for the [$^3$H]DNA (2.5-fold; see Table 1). This was probably because the $^{32}$P label represents a number average of the molecules in the two classes, whereas the $^3$H label represents more of a mass average. Thus, the results are consistent with a somewhat longer average length of the short nascent DNA molecules from the lagging strand as compared with molecules from the leading strand; this was also the conclusion drawn for the in vitro system (20).

Table 3 shows the percentage of the total 5'-end label that was released as pNp from poly(I)-Sephadex-purified DNA. A substantial release of pNP from both the leading and lagging strands was observed after alkaline hydrolysis. The diphosphates released preferentially were pAp and pGp (Fig. 4). Reichard and co-workers have shown that iRNA is initiated with either ATP or GTP (26). Thus, our results suggested that the preparation of nascent DNA contained a high proportion of intact iRNA molecules on strands of both polarities. The leading-strand molecules, measured by hybridization to *Hpa*II fragment 1L and *Eco*RI-*Hha*I fragment 2E, released slightly more pNp (about 1.3-fold) in both

TABLE 3. *Percentage of total radioactivity released as [$^{32}$P]pNp by alkali after strand separation of nascent molecules less than 300 nucleotides long*[a]

| Restriction enzyme fragment | % pNp |
|---|---|
| *Hpa*II-1E | 67 |
| *Hpa*II-1L | 86 |
| *Eco*RI-*Hha*I-2E | 67 |
| *Eco*RI-*Hha*I-2L | 53 |

[a] Purified nascent polyoma DNA molecules less than 300 nucleotides in length were 5'-phosphorylated with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. A portion of the preparation was divided into four parts. Each part was hybridized to an excess of one of four poly(dC)-extended separated strands of restriction enzyme fragments of the polyoma genome: *Hpa*II-1E and -1L and *Eco*RI-*Hha*I-2E and -2L. The nascent DNA molecules complementary to the respective strands were isolated on poly(I)-Sephadex columns. Each strand-specific portion was divided into two parts. One received no further treatment; the other was hydrolyzed with 0.4 M NaOH at 37°C overnight. The release of [$^{32}$P]pNp was determined by chromatography on PEI-cellulose thin-layer plates (Fig. 4) and is expressed as the percentage of the $^{32}$P radioactivity hybridizing to the restriction fragment strand that migrated as pNp upon alkaline hydrolysis. The values given have been corrected for background radioactivity migrating similarly to pNp in samples not subjected to alkaline hydrolysis (10 to 30%). They are also corrected for background originating from 5'-end-labeled material that did not hybridize to poly(dC)-extended restriction fragment strand but was nevertheless retained on poly(I)-Sephadex. This was determined by analyzing a portion of the material exactly as described above, but omitting hybridization to the restriction fragment strand. This material represented less than 10% of the radioactivity applied to the poly(I)-Sephadex and about 50% of the radioactivity eluted by formamide. The source of this material is considered in the discussion. A control for RNA contamination consisting of 5'-$^{32}$P-non-nascent cellular DNA did not release any detectable pNp after alkali treatment, even though the DNA was less extensively purified than the viral DNA. Input radioactivity averaged 450 cpm of $^{32}$P per sample chromatographed on PEI-cellulose.

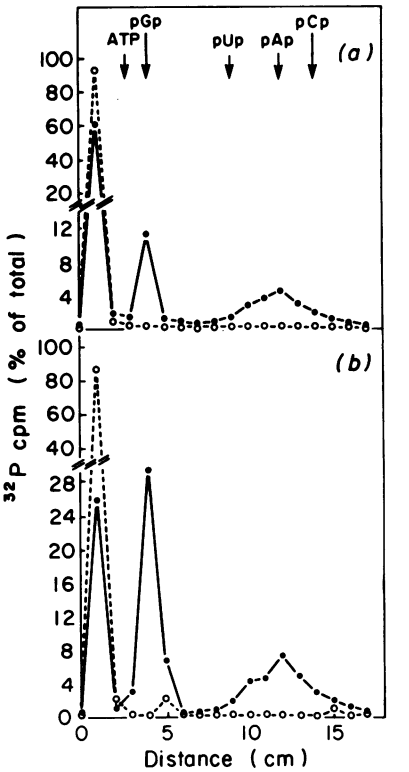


FIG. 4. *Purified nascent polyoma DNA molecules less than 300 nucleotides in length were labeled at the 5' termini with $^{32}$P. (a) A portion of the labeled DNA was divided into two, and one half was subjected to alkaline hydrolysis. Both halves were then chromatographed on PEI-cellulose thin-layer plates. Symbols: ●, Alkaline hydrolysate; ○, control. (b) A second portion of the DNA was hybridized to poly(dC)-extended HpaII fragment 1L and chromatographed on poly(I)-Sephadex. The column-bound DNA was eluted with formamide, and the eluate was divided into two portions and treated as in (a). The thin-layer plates were cut into 1-cm strips for scintillation counting. One strip below that containing the origin was counted to ensure recovery of all radioactivity applied. Input radioactivity was $10^5$ cpm in (a) and $1.2 \times 10^3$ cpm in (b). The positions of authentic $^{32}$P-labeled pNp (18) are indicated.*

pairs of separated strands. Since this is a number average of the molecules, the data indicate that the number of leading-strand nascent DNA molecules that possess iRNA is larger than the number of lagging-strand molecules bearing iRNA.

**Effect of spleen exonuclease digestion on self-annealing of iRNA-containing nascent polyoma DNA.** Another approach for ascertaining the presence of iRNA on nascent molecules from the leading strand uses the preferential digestion of DNA molecules containing ri-

bonucleotides by spleen exonuclease after removal of the RNA. Given the amount of material available, the most feasible method to measure the asymmetry of the remaining molecules was the extent of self-annealing. As shown in the preceding paper (20), the self-annealing of the DNA correlated well with the degree of asymmetry determined by hybridization to separated strands. The interpretation of this experiment relies on the fact (shown above in Table 1) that there is an excess of radioactivity in the DNA molecules with the polarity of the lagging strand in the shortest size class. If only these lagging-strand molecules possess iRNA, the fraction of labeled DNA that self-annealed after various degrees of digestion of the alkali-treated DNA by spleen exonuclease should increase as the excess lagging-strand DNA is degraded. Only if a large fraction of these molecules possess iRNA will the situation occur that leading-strand pieces are unable to find complements, therefore leading to a decrease in self-annealing.

On the other hand, if a substantial fraction of the molecules from the leading strand also contain iRNA, then the extent of self-annealing will either remain unchanged or decrease, depending on the relative amounts of leading and lagging strands possessing ribonucleotides and their relative size. The conclusions drawn from the actual experimental data depend on the evaluation of models for the behavior of the DNA given various assumptions. Knowing the proportion of $^3H$ pulse-label in strands of each polarity and the overall extent of hydrolysis by spleen exonuclease before and after removal of iRNA moieties, one can calculate whether the results are compatible with the absence of RNA-terminated leading-strand DNA pieces.

The nascent DNA was phosphorylated at its 5′ terminus with polynucleotide kinase, treated with alkali or RNase, and precipitated with ethanol. One half was digested with spleen exonuclease, and the other half was incubated under the same conditions but without the enzyme. After purification on nitrocellulose and ethanol precipitation, the DNA was self-annealed for at least 50 × $C_0t_{1/2}$ to ensure completion of the reaction.

Table 4 presents the results of the experiments, including a control consisting of uniformly $^{32}P$-labeled φX174 replicative form II DNA. The first self-annealing value of each pair was determined before the material was subjected to any treatment and served as an additional control. After spleen exonuclease digestion, a substantial decrease in self-annealing was observed, especially among the smaller molecules. The change in the larger size classes was generally less pronounced except for the RNase-

TABLE 4. *Percentage of self-annealing of DNA molecules before and after digestion by spleen exonuclease*[a]

| DNA | Size (nucleotides) | Treatment | Hydrolysis by spleen exonuclease (%) | Self-annealing (%) | |
|---|---|---|---|---|---|
| | | | | Before digestion | After digestion |
| $^3H$-labeled polyoma DNA | <300 | Alkali | 25 | 47; 47 | 30 |
| | | RNase | 56 | 62; 64 | 35 |
| | 300–600 | Alkali | 23 | 82; 84 | 87 |
| | | RNase | 41 | 80; 87 | 57 |
| | >600 | Alkali | 21 | 95; 95 | 95 |
| | | RNase | 31 | 90; 87 | 81 |
| $^{32}P$-labeled φX174 DNA | <300 | RNase | 5 | 100 | 94 |
| | 300–600 | RNase | 5 | 100 | 92 |
| | >600 | RNase | 5 | 100 | 100 |

[a] Polyoma DNA pulse-labeled with [$^3H$]thymidine was purified, mixed in the RNase experiment (only) with uniformly $^{32}P$-labeled φX174 replicative form DNA of the same size, and incubated with polynucleotide kinase to ensure phosphorylation of all 5′ termini. The samples were then treated with RNase or alkali to expose 5′-hydroxyl groups wherever ribonucleotides existed. After ethanol precipitation, one half of the sample was digested with spleen exonuclease, and the other half was incubated under the same conditions but without the enzyme. The percentage of acid-soluble radioactivity (500 to 3,000 total cpm) released was determined. After chromatography on nitrocellulose and precipitation with ethanol, the DNA preparations were self-annealed for at least 50 × $C_0t_{1/2}$. The double-stranded DNA content was analyzed on hydroxyapatite columns. Total radioactivity after spleen exonuclease digestion ranged from 7,500 to 45,000 cpm for both $^3H$ and $^{32}P$. Experiments with alkali-treated and RNase-treated molecules represent different DNA preparations. Sample calculation: Self-annealing of molecules of <300 nucleotides before RNase and spleen exonuclease digestion is 63% (above). This corresponds to 30% leading-strand and 70% lagging-strand molecules, assuming similar size and specific activity on both sides of the fork. After RNase digestion, spleen exonuclease digests 56% of these molecules. If only the lagging strands possess RNA, then in order to get 56% digestion overall, 80% of the lagging strand DNA would have to be digested. This would leave lagging- to leading-strand molecules in the ratio of 14 to 30. The expected self-annealing would then be 2(14)/44, or 64%. This was not observed. Alternatively, consider the data of Table 3, where 53 and 67% of lagging and leading strands, respectively, were shown to possess 5′-ribonucleotides. Digestion of these molecules by RNase and then spleen exonuclease would result in digestion of (0.53 × 0.70) + (0.67 × 0.30), or 57%, of the radioactivity. This is what is observed above. The product of this digestion would consist of lagging- and leading-strand molecules in the ratio of 33 to 10, which would self-anneal to the extent of 47%. The self-annealing is thus expected to decrease, as was observed. These calculations can be made in various ways depending on what assumptions are made. In no case, however, is it possible to account for the data with the assumption that only the lagging-strand molecules possess 5′-terminal ribonucleotides.

treated intermediate-sized molecules.

A complication in evaluating the results is that, as shown in Table 2, spleen exonuclease did not completely hydrolyze even short molecules. The incomplete hydrolysis by the exonuclease especially affects the results obtained with the longer size classes of nascent DNA. In this case, the interpretation is further complicated by the possible concentration of the pulse-label at the 3′ termini of these molecules. However, considering only the shortest newly synthesized strands, the most straightforward interpretation of the data (Table 4, legend) is that molecules on both strands were made sensitive to the exonuclease by alkali or RNase and therefore contained iRNA.

## DISCUSSION

In the preceding paper (20), evidence was presented supporting a totally discontinuous process for the replication of polyoma DNA in isolated nuclei. We undertook the experiments described in this paper to determine whether a similar process could be detected in vivo, since results obtained in an in vitro system unfortunately cannot with certainty be extrapolated to the environment of the intact cell. We have shown that a majority of the short nascent viral DNA molecules possess 5′-terminal ribonucleotides, which we assume were used to prime DNA synthesis. These iRNA-containing DNA molecules are synthesized on both strands of the replication fork, indicating that polyoma DNA is replicated by a totally discontinuous mode in vivo. Nascent molecules with leading-strand polarity more frequently possessed RNA primers and appeared to be shorter than the nascent molecules with lagging-strand polarity. A substantial, but smaller, fraction of longer intermediates also possessed RNA primers, suggesting the same precursor-product relationship of iRNA-primed Okazaki pieces and longer progeny molecules demonstrated during in vitro replication (2, 20).

The quantification of iRNA was based on the amount of $^{32}$P-labeled pNp released after hydrolysis of the separated strands of 5′-end-labeled nascent DNA. Poly(I)-Sephadex was used to isolate the nascent polyoma DNA complementary to the separated strands of *Hpa*II fragment 1 and *Hha*I fragment 2 [tailed with poly(dC)]. A small amount of nonspecifically bound DNA [probably DNA containing stretches of poly(dC)] eluted together with the strand-specific molecules, but controls (Table 3) showed that this background material did not completely account for the pNp's released. It is unlikely that the nonspecific material was free RNA contributing to the pNp release for the following reasons. (i) The amount of label incorporated from [$\gamma$-$^{32}$P]ATP at the 5′ termini was in agreement with the amount expected from calculations based on the known amount of DNA and its average length. (ii) The preparations were chromatographed twice on nitrocellulose, which efficiently removes RNA not linked to DNA. (iii) 5′-$^{32}$P-labeled phosphorylated cellular non-nascent DNA, less extensively purified than the viral DNA, did not release any pNp upon alkaline hydrolysis (Table 3). (iv) As a consequence of the purification procedure of the nascent DNA, any free RNA molecules that contaminated the short DNA molecules would have had to be degradation products; it seems unlikely that this would produce molecules preferentially terminated at the 5′ end with AMP or GTP. (v) It is improbable that any polyoma-specific mRNA associated with the replicative intermediates was responsible for the pNp release from the leading strands. This is because less than 1% of the extracted mRNA is late polyoma mRNA (30), and only one strand in each pair of separated strands used for analysis is transcribed at the time of isolation of the nascent DNA, yet we observed alkali-labile material hybridizing to both strands.

The short DNA molecules bearing iRNA were not confined to the origin of replication, since one of the probes used in the analysis was considerably removed from the origin (see Fig. 3). Also, similar extents of strand bias were determined from the self-annealing of the total population of nascent DNA molecules and from the fraction of molecules complementary to the separated strands of the specific part of the viral genome encompassed by *Hpa*II fragment 1 (see Fig. 3).

Okazaki pieces could arise from the leading strand through a unidirectional mode of replication of the genome or initiation at secondary origins during replication of defective genomes. To account for the high proportion of molecules we detected on this strand, a majority of the viral molecules would have to have replicated unidirectionally or used other origins. That this could be the case in our system is highly unlikely. First, almost all polyoma DNA molecules have been shown to replicate bidirectionally from one defined origin (15). Second, our virus stock was prepared from a doubly plaque-purified virus, and the cleavage pattern generated by digestion with *Hpa*II of polyoma form I DNA synthesized during infection with this stock was normal.

Many experiments have been performed in an effort to distinguish semidiscontinuous from totally discontinuous synthesis of papovavirus DNA with varying results. The strand asym-

metry of the shortest nascent molecules has varied, depending on the replication conditions, the method of analysis, and the virus-host system. One point of universal agreement is that molecules of leading-strand polarity represent less than 50% of the total (3, 6, 8, 20, 24). Our own results indicate that about 35% of the short molecules synthesized in vitro (20) and about 25% of those made in vivo (Table 1) are of leading-strand polarity. There appears to be a quantitative difference between polyoma virus and simian virus 40, since a lower fraction of molecules of leading-strand polarity is consistently found during simian virus 40 replication (8, 24) as compared with polyoma virus replication (3, 6, 20). The observed variations may be due to different concentrations of factors necessary for iRNA removal, gap filling, and ligation in the two systems.

Arbitrary dismissal of short leading-strand molecules as products of degradation (e.g., radiolysis) or of repair synthesis is not justified (21). On the contrary, our results (20; Tables 3 and 4) indicate that a large proportion of these molecules possess iRNA and therefore are the products of discontinuous DNA synthesis. The presence of iRNA on short intermediates was not found during simian virus 40 replication in vitro (9), perhaps because a faster rate of assimilation of these intermediates in this system (discussed above) made them harder to detect.

Polyoma virus utilizes host cell enzymes for its replication (10, 19), and hence the viral genome is in many respects analogous to a mammalian replicon. In view of our data, one may infer that previous research showing that most of the radioactivity incorporated into mammalian DNA in a short pulse is in short intermediates indicates a totally discontinuous mode of eucaryotic replication (4, 27). In procaryotes, also there is strong evidence that the potentially continuously synthesized strands of phages P2 (13, 23) and $\phi$X174 (14, 17) are synthesized discontinuously via short intermediates, at least some of which bear 5'-ribonucleotides. These data from representative procaryotes and eucaryotes indicate that polyribonucleotides may serve as primers of DNA synthesis even when a 3'-OH DNA terminus is available, as is the case on the leading strand. Such a mechanism would permit multiple initiation events to occur simultaneously on the leading strand, thereby maximizing the rate of DNA synthesis in vivo.

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## LITERATURE CITED

1. **Bernardi, A., and G. Bernardi.** 1968. Studies on acid hydrolases. IV. Isolation and characterization of spleen exonuclease. Biochim. Biophys. Acta **155**:360–370.
2. **Eliasson, R., and P. Reichard.** 1978. Replication of polyoma DNA in isolated nuclei. Synthesis and distribution of initiator RNA. J. Biol. Chem. **253**:7469–7475.
3. **Flory, P. J., Jr.** 1977. Strandedness of newly synthesized short pieces of polyoma DNA from isolated nuclei. Nucleic Acids Res. **4**:1449–1463.
4. **Gautschi, J. R., and J. M. Clarkson.** 1975. Discontinuous DNA replication in mouse P-185 cells. Eur. J. Biochem. **50**:403–412.
5. **Glynn, J. M., and J. B. Chappell.** 1964. A simple method for preparation of $^{32}$P-labelled adenosine triphosphate of high specific activity. Biochem. J. **90**:147–149.
6. **Hunter, T., B. Francke, and L. Bacheler.** 1977. In vitro polyoma DNA synthesis: asymmetry of short DNA chains. Cell **12**:1021–1028.
7. **Kamen, R., J. Sedat, and E. Ziff.** 1976. Orientation of the complementary strands of polyoma DNA with respect to the DNA physical map. J. Virol. **17**:212–218.
8. **Kaufmann, G., R. Bar-Shavit, and M. L. DePamphilis.** 1978. Okazaki pieces grow opposite to the replication fork direction during simian virus 40 DNA replication. Nucleic Acids Res. **5**:2535–2545.
9. **Kaufmann, G., and G. Dinter-Gottlieb.** 1980. Initiator RNA of Okazaki pieces in replicating SV 40 DNA. J. Supramol. Struct. **4**(Suppl.):360.
10. **Kit, S., D. R. Dubbs, and P. M. Frearson.** 1966. Enzymes of nucleic acid metabolism in cells infected with polyoma virus. Cancer Res. **26**:638–646.
11. **Kowalski, J., and D. T. Denhardt.** 1979. Ribonucleotides in DNA newly synthesized in 3T6 cells in vivo. Nature (London) **281**:704–706.
12. **Kurosawa, Y., T. Ogawa, S. Hirose, T. Okazaki, and R. Okazaki.** 1975. Mechanism of DNA chain growth. XV. RNA-linked nascent DNA pieces in *Escherichia coli* strains assayed with spleen exonuclease. J. Mol. Biol. **96**:653–664.
13. **Kurosawa, Y., and R. Okazaki.** 1975. Mechanism of DNA chain growth. XIII. Evidence for discontinuous replication of both strands of P2 phage DNA. J. Mol. Biol. **94**:229–241.
14. **Machida, Y., T. Okazaki, and R. Okazaki.** 1977. Discontinuous replication of replicative form DNA from bacteriophage $\phi$X174. Proc. Natl. Acad. Sci. U.S.A. **74**: 2776–2779.
15. **Magnusson, G., and M.-G. Nilsson.** 1979. Replication of polyoma DNA in isolated nuclei: analysis of replication fork movement. J. Virol. **32**:386–393.
16. **Magnusson, G., E.-L. Winnacker, R. Eliasson, and P. Reichard.** 1972. Replication of polyoma DNA in isolated nuclei. II. Evidence for semiconservative replication. J. Mol. Biol. **72**:539–552.
17. **Matthes, M., and D. T. Denhardt.** 1980. The mechanism of replication of $\phi$X174 DNA. XVI. Evidence that the $\phi$X174 viral strand is synthesized discontinuously. J. Mol. Biol. **136**:45–63.
18. **Miyamoto, C., and D. T. Denhardt.** 1977. Evidence for the presence of ribonucleotides at the 5' termini of some DNA molecules isolated from *Escherichia coli polAex2*. J. Mol. Biol. **116**:681–707.
19. **Närkhammar, M., and G. Magnusson.** 1976. DNA polymerase activities induced by polyoma virus infection of 3T3 mouse fibroblasts. J. Virol. **18**:1–6.
20. **Närkhammar-Meuth, M., R. Eliasson, and G. Magnusson.** 1981. Discontinuous synthesis of both strands at the growing fork during polyoma DNA replication in vitro. J. Virol. **39**:11–20.

21. **Nilsson, S., P. Reichard, and L. Skoog.** 1980. Deoxyuridine triphosphate pools after polyoma virus infection. J. Biol. Chem. **255**:9552–9555.
22. **Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, R. Kainuma, A. Sugino, and N. Iwatsuki.** 1968. In vivo mechanism of chain growth. Cold Spring Harbor Symp. Quant. Biol. **33**:129–143.
23. **Okazaki, T., Y. Kurosawa, T. Ogawa, T. Seki, K. Shinozaki, S. Hirose, A. Fujijama, Y. Kohara, Y. Machida, F. Tamanoi, and T. Hozumi.** 1978. Structure and metabolism of the RNA primer in the discontinuous replication of prokaryotic DNA. Cold Spring Harbor Symp. Quant. Biol. **43**:203–219.
24. **Perlman, D., and J. A. Huberman.** 1977. Asymmetric Okazaki piece synthesis during replication of simian virus 40 DNA in vivo. Cell **12**:1029–1043.
25. **Razzell, W. E., and H. G. Khorana.** 1961. Studies on polynucleotides. X. Enzymatic degradation, some properties and mode of action of spleen phosphodiesterase. J. Biol. Chem. **236**:1144–1149.
26. **Reichard, P., R. Eliasson, and G. Söderman.** 1974. Initiator RNA in discontinuous polyoma DNA synthesis. Proc. Natl. Acad. Sci. U.S.A. **71**:4901–4905.
27. **Tseng, B. Y., and M. Goulian.** 1975. DNA synthesis in human lymphocytes: intermediates in DNA synthesis, in vitro and in vivo. J. Mol. Biol. **99**:317–337.
28. **Weiss, B., T. R. Live, and C. C. Richardson.** 1968. Enzymatic breakage and joining of deoxyribonucleic acid. V. End group labeling and analysis of deoxyribonucleic acid containing single strand breaks. J. Biol. Chem. **243**:4530–4542.
29. **Winnacker, E.-L., G. Magnusson, and P. Reichard.** 1972. Replication of polyoma DNA in isolated nuclei. I. Characterization of the system from mouse fibroblast 3T6 cells. J. Mol. Biol. **72**:523–537.
30. **Zeev, L., R. Kamen, and H. Manor.** 1979. Topography of polyoma virus-specific giant nuclear RNA molecules containing poly(A) sequences. Virology **93**:445–457.