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Initiator RNA in Discontinuous Polyoma DNA Synthesis*

(isolated nuclei/Okazaki fragments/RNA–DNA link)

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ABSTRACT **During replication of polyoma DNA in isolated nuclei, RNA was found attached to the 5′ ends of growing progeny strands. This RNA starts with either ATP or GTP and can be labeled at its 5′ end with ^{32}P from β-labeled nucleotides. Digestion of progeny strands with pancreatic DNase released ^{32}P-labeled RNA that, on gel electrophoresis, gave a distinct peak in the position expected for a decanucleotide. We believe that this short RNA is involved in the initiation of the discontinuous synthesis of DNA and propose the name "initiator RNA" for it. The covalent linkage of initiator RNA to 5′ ends of growing DNA chains was substantiated by the finding that ^{32}P was transferred to ribonucleotides by alkaline hydrolysis of purified initiator RNA obtained by DNase digestion of polyoma progeny strands synthesized from [α-^{32}P]dTTP. While initiator RNA was quite homogeneous in size, it had no unique base sequence since digestion with pancreatic RNase of initiator RNA labeled at its 5′ end with ^{32}P released a variety of different [^{32}P]oligonucleotides. The switch from RNA to DNA synthesis during strand elongation may thus depend on the size of initiator RNA rather than on a specific base sequence.**

Elongation of progeny strands of the DNA of polyoma or simian virus 40 is discontinuous (1, 2) and requires multiple initiation events. From studies with isolated nuclei from polyoma-infected 3T6 cells we concluded that RNA participates in the initiations (1, 3), as was previously suggested for microbial systems (4, 5). Alkaline hydrolysis of purified progeny strands synthesized from any of the four α-^{32}P-labeled deoxynucleoside triphosphates transferred isotope to all four ribonucleotides, demonstrating that RNA was attached to the 5′ ends of growing DNA chains and that the RNA·DNA link was not unique (3). The 5′ ends of RNA labeled with [^{3}H]-GTP or [β-^{32}P]GTP could be recovered as guanosine tetraphosphate (6). Furthermore, digestion of DNA progeny strands with pancreatic DNase released an RNA species that on polyacrylamide gels had the size of approximately a decanucleotide. We suggested that this might be the RNA that initiates the discontinuous synthesis of polyoma DNA (6).

In the present communication we demonstrate that this "decanucleotide," for which we propose the name "initiator RNA (iRNA)," is covalently linked to the 5′ ends of DNA progeny strands. It has no unique base sequence but appears to be quite homogeneous in size.

MATERIALS AND METHODS

All enzymes were obtained from Worthington Biochemical Corp. Pancreatic DNase I was treated with bentonite (7) and was free from RNase activity; pancreatic RNase, heated at 95° for 5 min before use, was free from DNase; snake venom diesterase was free from monoesterase activity (8).

^{3}H-Labeled ribonucleoside triphosphates and [^{14}C]dATP were obtained from New England Nuclear Corp. β-^{32}P-Labeled ATP and GTP were made by exchange of $^{32}P_i$ with the corresponding diphosphates (9) and subsequent phosphorylation of the diphosphates (10). The sources and preparation of other nucleotides and reagents, as well as the infection of cells and preparation and incubation of nuclei, were described earlier (11, 12). All incubations of nuclei were at 25° for 5 min in a final volume of 4–6 ml with nuclei containing 6–8 mg of DNA.

Radioactivity was measured by liquid scintillation counting, applying the proper corrections for overlap of isotopes. The composition of iRNA was calculated from the radioactivities of the isolated products, and the specific activities of the precursors were corrected for the dilution by the pools of ribonucleoside triphosphates present in the nuclei. These pools were measured in separate experiments by isotope dilution and found to be 4 nmol of UTP and 3 nmol of GTP per mg of nuclear DNA.

5′-Terminal ATP or GTP was determined by quantitation of pppAp and pppGp formed after alkaline hydrolysis of RNA and is here illustrated by an experiment in which RNA was labeled from both [β-^{32}P]ATP and [^{3}H]GTP. Fig. 1 shows the separation on DEAE–Sephadex of the labeled products formed by alkaline hydrolysis of the crude desalted RNA fraction. The positions of the three major ^{3}H peaks correspond to water, Gp, and pppGp. Alkaline hydrolysis caused approximately 30% of the ^{3}H to exchange with water, and for our calculations it was assumed that this loss of isotope equally affected Gp and pppGp. The ^{32}P pattern in Fig. 1 showed two major peaks, the first corresponding to P_i, the second to pppAp. With purified progeny strands labeled from either [β-^{32}P]ATP or GTP, essentially all ^{32}P was recovered as tetraphosphate. The identity of the tetraphosphate peak was established by enzymatic digestion of pppGp with alkaline phosphatase or snake venom diesterase, which liberated P_i or PP_i (14).

RESULTS

Purification of Progeny Strands Containing iRNA. Initiator RNA is attached covalently to the 5′ end of growing progeny strands of polyoma DNA. During incubation of nuclei, iRNA was labeled internally from [^{3}H]ribonucleoside triphosphates while the 5′ ends were labeled from [β-^{32}P]GTP or ATP. However, most of the isotope was incorporated into other RNA.

The fact that iRNA forms part of the structure of the replicative intermediates of polyoma DNA proved useful for its

Abbreviations: pppAp and pppGp, the 5′-triphosphates, 2(3′)-monophosphates of adenosine and guanosine, respectively; iRNA, initiator RNA.

* This paper is no. IV in a series "Replication of Polyoma DNA in Isolated Nuclei." The preceding paper is ref. 3.

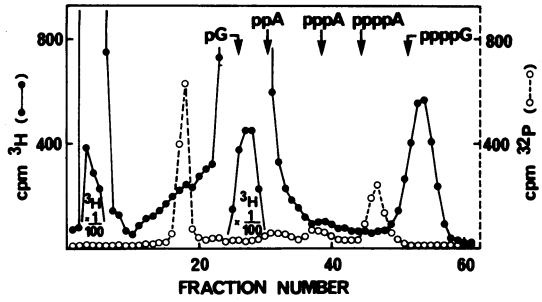

FIG. 1. Identification of pppGp and pppAp by chromatography on DEAE-Sephadex. Nuclei were incubated for 5 min at 25° with [β-^{32}P]ATP (0.33 mM, 14,000 cpm/pmol), [^{3}H]GTP (25 μM, 6700 cpm/pmol), and [^{14}C]dATP (11 μM, 270 cpm/pmol). Other conditions were as described earlier (3, 11). Hirt supernatants (13) were prepared, treated with proteinase K and phenol, and passed through a Biogel column (6). An aliquot of the material from the void volume (50 pmol of ^{3}H and 0.35 pmol of ^{32}P) was made 0.3 M with KOH, incubated at 37° for 18 hr, neutralized to pH 7–8 with Dowex-50-H$^+$, and chromatographed at 4° on a 5-ml column of DEAE-Sephadex with a linear gradient of triethylamine-HCO_3 (0.1–1.0 M, pH 8, 50 ml each) after addition of ultraviolet markers. Fractions (1.7-ml) were first analyzed for the ultraviolet markers and then for radioactivity after addition of 15 ml of Instagel. The total recovery was about 65% for ^{3}H and 80% for ^{32}P.

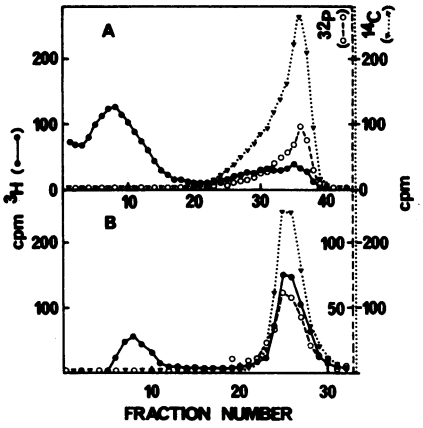

FIG. 2. (Panel A) Purification of replicative intermediates by centrifugation in a CsCl–propidium diiodide gradient (12). Polyoma DNA was labeled from [β-^{32}P]GTP (11 μM, 68,000 cpm/pmol), [^{3}H]CTP (20 μM, 5300 cpm/pmol), and [^{14}C]dATP (10 μM, 270 cpm/pmol) and purified through the Sepharose 4B step (6). The material was then centrifuged in a Spinco type 65 rotor in six tubes at 35,000 rpm for 60 hr at 15°. Fractions were collected, and 0.03 ml of each was analyzed for radioactivity after addition of 0.5 ml of water and 5 ml of Instagel. Fractions 23–39 were combined, propidium diiodide was extracted, the material was precipitated with isopropanol, dissolved in 2 ml of 50 mM Tris·HCl (pH 7.2), and used for further experiments. (Panel B) Cs_2SO_4 centrifugation of progeny strands. Replicative intermediates (0.15 ml of the pooled material from panel A) were heated at 100° for 5 min after addition of 2.5 ml of water, cooled quickly, and centrifuged to equilibrium (48 hr, 35,000 rpm at 20°, SW50 rotor) in a neutral Cs_2SO_4 gradient (12). Fractions (about 0.1 ml) were analyzed for radioactivity as described for panel A.

purification. Replicative intermediates were purified first by chromatography on Sepharose followed by centrifugation in a CsCl–propidium diiodide gradient (6, 12). Fig. 2A illustrates the dye centrifugation step from an experiment in which iRNA was labeled internally from [^{3}H]CTP and at its 5′ end from [β-^{32}P]GTP, while DNA was labeled from [^{14}C]dATP. Most of the ^{32}P, but only a minor part of the ^{3}H, banded together with DNA. The pooled DNA fractions were then heat-denatured to liberate progeny strands from the replicative intermediates and centrifuged to equilibrium in a neutral Cs_2SO_4 gradient (Fig. 2B). All ^{32}P and more than 80% of the ^{3}H label now banded together with DNA. Such results are usually considered to indicate a covalent linkage of RNA to DNA. In the next section we will provide evidence, however, that in this experiment all ^{32}P but only about one-third of the ^{3}H label in effect was covalently attached to DNA. Artifactual binding in isopycnic Cs_2SO_4 gradients of RNA to DNA after heat denaturation was recently also reported by Probst *et al.* (15).

When these heat-denatured progeny strands were hybridized to filters containing polyoma DNA, 40% of ^{32}P, 35% of ^{14}C, and 37% of ^{3}H were bound to the filters after 48 hr (as compared to 43% of authentic polyoma DNA).

Characterization of iRNA after DNase Digestion of Progeny Strands. Heat-denatured material after the dye centrifugation step (see Fig. 2A) was digested with an excess of DNase (6), and the products of the reaction were separated by electrophoresis on 12% polyacrylamide in 7 M urea (16, 6). ^{3}H was recovered in two peaks, one at the top of the gel, the second (about one-third of the total radioactivity) at approximately the position of a decanucleotide (Fig. 3). All ^{32}P appeared in the second peak. Material extracted from this peak could be again subjected to electrophoresis and appear in good yield in the same position (insert of Fig. 3). This material represents iRNA. Its ^{32}P/^{3}H ratio indicates the presence of eight molecules of CMP for each 5′-terminal GTP. In a similar experiment we earlier found nine molecules of UMP per molecule of GTP (6).

The stoichiometries described above suggested that GTP was not the only nucleotide starting the synthesis of iRNA. We, therefore, incubated nuclei with both [β-^{32}P]ATP and [^{3}H]GTP in order to compare these two nucleotides as initiators. Progeny strands were purified and hydrolyzed with alkali. The amount of [^{32}P]pppAp relative to [^{3}H]pppGp was determined. The molar ratios between the two tetraphosphates was 2.4, showing a preference for ATP as the starting nucleotide.

We then digested progeny strands from this experiment with DNase and separated the products by gel electrophoresis (Fig. 4). Again a radioactive peak containing both ^{32}P and ^{3}H was found in the position expected for iRNA, and again all ^{32}P was recovered in this peak. In this case the 5′ ends were labeled with both ^{32}P (from ATP) and ^{3}H (from GTP), while the internal label originated exclusively from [^{3}H]GTP. From the ^{3}H/^{32}P ratio of the peak fractions the presence of 1.8 moles of ^{3}H per mole of ^{32}P was calculated. Since all ^{32}P was present as pppAp, the data suggest that iRNA contained 1.4 moles of GMP (+0.4 mole of pppGp) per mole of pppAp.

Initiator RNA is Covalently Attached to DNA. It seemed likely that iRNA released from progeny strands by digestion with DNase might contain one or several deoxyribonucleotides at its 3′ end. This would allow ^{32}P-transfer experiments with purified iRNA, similar to those carried out earlier with com-

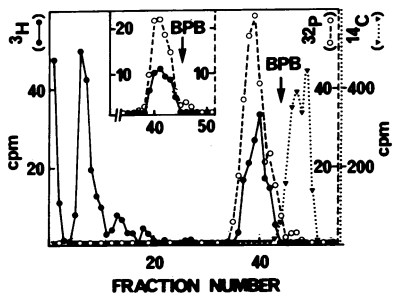

FIG. 3. Polyacrylamide gel electrophoresis of DNase-treated progeny strands. Pooled material from Fig. 2, panel A (0.4 ml) was heated at 100° for 5 min, treated with DNase (6), and subjected to electrophoresis on six separate 12% polyacrylamide columns (16, 6). One column was sliced and analyzed for radioactivity. iRNA (fractions 37–42) was extracted from the remaining columns with water, again subjected to electrophoresis (insert), or used for the experiment described in Fig. 7. Bromphenol blue (BPB) was used in this and all other electrophoretic experiments as an internal marker and all mobilities (R_B values) were determined relative to this marker. In parallel runs the following R_B values were determined for standards: dodecanucleotide, 0.77–0.81; octanucleotide, 0.91; heptanucleotide carrying a 5′-terminal triphosphate, 1.04. The first two oligonucleotides were obtained by T1 RNase digestion of yeast $tRNA^{Phe}$ (17); the heptanucleotide was a generous gift from Dr. H. Schaller. The R_B values are in good agreement with published data (16). In different experiments, iRNA had R_B values between 0.85 and 0.88.

plete progeny strands (3). For this purpose infected nuclei were incubated with [^{3}H]GTP and [α-^{32}P]dTTP in order to label RNA with ^{3}H and DNA with ^{32}P. A small fraction of the ^{32}P should be located at the RNA·DNA link. During the electrophoretic separation of the products formed by DNase treatment of progeny strands, about 0.8% of the ^{32}P appeared together with ^{3}H in the position of iRNA (Fig. 5). On reelectrophoresis (insert to Fig. 5), ^{32}P split into two peaks, the first of which coincided with the ^{3}H peak and was in the position of iRNA. This material was treated with alkali to hydrolyze iRNA and to transfer ^{32}P to any ribonucleotide linked in diester linkage to the 5′ position of dTMP (3). The relative transfers to the four ribonucleotides are summarized in Table 1, which also shows that the corresponding values from a parallel alkaline hydrolysis of intact progeny strands were very similar. The total transfer of ^{32}P, as defined earlier (3), was 0.23% for intact progeny strands and 0.15% for iRNA. The latter value was calculated on the basis of the total amount of ^{32}P applied to the gels used for the preparation of iRNA and is not corrected for any incomplete recovery, e.g., during extraction of the gels. It is thus a minimum value. The

TABLE 1. *Ribonucleotide distribution at RNA·DNA link with [α-^{32}P]dTTP as substrate*

	Total transfer* (%)	% ^{32}P in Cp	Ap	Gp	Up
Initiator RNA	0.15	28	33	12	26
Intact progeny strands	0.23	22	31	14	32

* Total transfer (3) = $\frac{\Sigma \text{ cpm in ribonucleotides}}{\text{cpm in DNA}}$

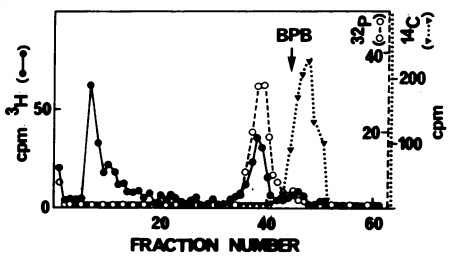

FIG. 4. Electrophoresis of DNase-treated progeny strands labeled from [β-^{32}P]ATP, [^{3}H]GTP, and [^{14}C]dATP. The conditions for the incubation of nuclei are described in the legend of Fig. 1. Progeny strands were purified and treated with DNase. The split products were separated by gel electrophoresis (6).

data strongly suggest that iRNA was responsible for the ^{32}P transfer observed with intact progeny strands and that iRNA, therefore, was the RNA species linked to the 5′ ends of growing polyoma DNA chains.

Only 31% of the ^{32}P of iRNA was recovered as ribonucleotides after alkaline hydrolysis. The rest was present as short oligonucleotides, suggesting that iRNA as isolated contained on the average about three deoxynucleotides at its 3′ end. The iRNA that had been again subjected to electrophoresis (see Fig. 5) contained a total of 1.2 moles of ^{32}P per mole of ^{3}H, as calculated from its ^{32}P/^{3}H ratio, corresponding to the presence of 0.38 mole of [^{32}P]dTMP at the 3′ end for each mole of guanosine nucleotide (GMP + pppGp).

Initiator RNA Has No Unique Nucleotide Sequence. Exhaustive digestion with pancreatic RNase of iRNA labeled at its 5′ end with [β-^{32}P]ATP or [β-^{32}P]GTP should generate only one labeled oligonucleotide, provided iRNA has a unique base sequence but a series of different ^{32}P-labeled products if iRNA consists of a mixture of different molecules.

We treated progeny strands containing iRNA labeled from either [β-^{32}P]GTP or ATP with two different amounts of RNase and separated the products in 7 M urea on columns of DEAE–cellulose (18). Fig. 6 shows that the [^{32}P]oligonucleotide patterns in three experiments were quite similar irrespective of the nature of label at the 5′ end or the amount of

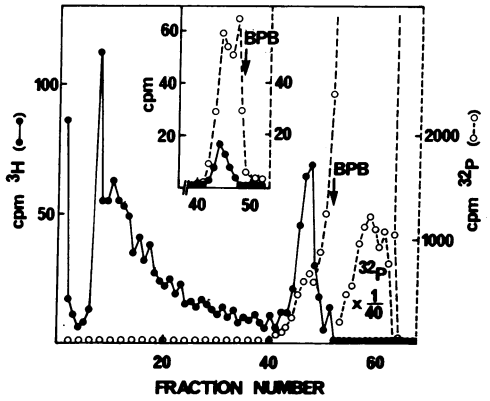

FIG. 5. Electrophoresis of DNase-treated progeny strands labeled from [α-^{32}P]dTTP (11 μM, 42,000 cpm/pmol) and [^{3}H]-GTP (6900 cpm/pmol). A total of 480,000 cpm of ^{32}P and 1200 cpm of ^{3}H was applied to one gel. Material corresponding to fractions 43–48 was extracted from four separate gels, again subjected to electrophoresis (insert), and used for the ^{32}P-transfer experiments summarized in Table 1.

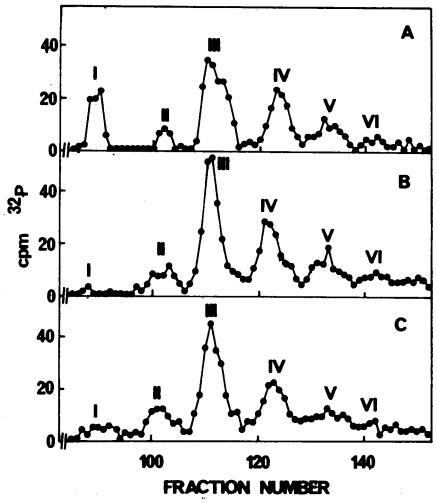

FIG. 6. Chromatography on DEAE–cellulose of [^{32}P]oligonucleotides from RNase digests of progeny strands. Replicative intermediates, labeled from [β-^{32}P]ATP (panel A) or [β-^{32}P]GTP (panels B and C) were heated (100° for 5 min) and then digested with RNase (0.05 mg/ml in panels A and B, 0.25 mg/ml in panel C) for 1 hr at 37° in 50 mM Tris·HCl (pH 7.4), 10 mM EDTA. After addition of ultraviolet markers, the solutions were made 7 M with respect to urea and chromatographed on columns (0.3 × 75-cm) of DEAE–cellulose in 7 M urea with linear gradients from 0–0.3 M NaCl (250 ml each). Fractions (2.3 ml) were analyzed as described in the legend of Fig. 1. Total recovery was 70–80%. The positions of ultraviolet markers are given in Fig. 7.

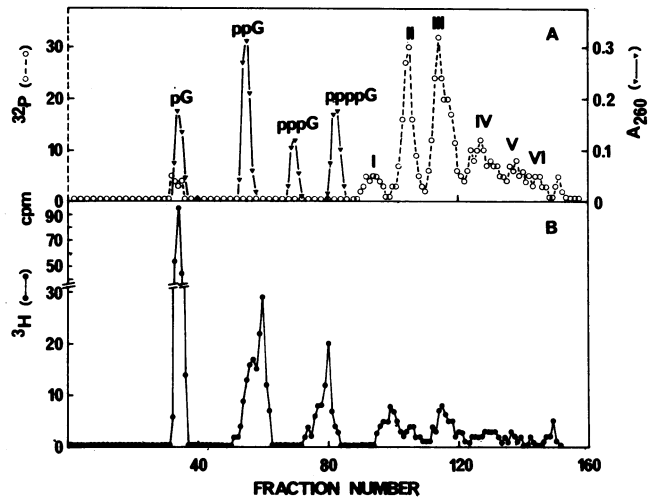

FIG. 7. DEAE–cellulose chromatography of ^{32}P- and ^{3}H-labeled oligonucleotides from an RNase digest of purified iRNA. iRNA, labeled from [β-^{32}P]GTP and [^{3}H]CTP, was purified by gel electrophoresis, as shown in Fig. 3. After treatment with RNase (0.15 mg/ml), oligonucleotides were separated on DEAE–cellulose, as described in the legend of Fig. 6. Panel A gives the positions of ultraviolet markers and the ^{32}P pattern; panel B gives the ^{3}H pattern.

RNase used. In all cases, at least six different peaks (labeled I–VI) can be discerned. Peak I corresponds to the position of pppAp or pppGp, while peak III is in the position expected for a dinucleotide carrying a triphosphate at its 5′ end (e.g., pppApPyp). Peaks IV–VI would then be the corresponding tri- to pentanucleotides. Peak II is probably a dinucleotide that has lost the 5′-terminal γ-phosphate (e.g., ppApPyp).

In a final experiment, purified iRNA labeled from [β-^{32}P]-GTP and [^{3}H]CTP was digested with RNase and chromatographed on DEAE–cellulose. Since in this experiment other RNA had been removed by gel electrophoresis, both the ^{32}P and ^{3}H values reflect the oligonucleotide patterns of iRNA and are given in Fig. 7. The ^{32}P pattern (Fig. 7A) shows the presence of oligonucleotides in positions corresponding to those of peaks I–VI in Fig. 6. There is a striking increase in the amount of isotope in peak II. We believe that this reflects dephosphorylation of the γ-phosphate at the 5′ end during the extended purification procedure. This conclusion is supported by the finding that after alkaline hydrolysis of purified iRNA about 30% of the ^{32}P during DEAE–cellulose chromatography was recovered in a peak corresponding to ppGp. The ^{3}H pattern (Fig. 7B) shows the presence of at least five different peaks (from CMP to pentanucleotides).

DISCUSSION

Two classes of radioactive RNA species were found tightly bound to replicative intermediates purified from polyoma-infected nuclei that had been incubated with labeled ribonucleoside triphosphates. The two classes differed widely in molecular weight and could be separated by gel electrophoresis after digestion with DNase. Our results strongly suggest that the small RNA, with the size of approximately a decanucleotide, functions as initiator in the discontinuous synthesis of Okazaki fragments (iRNA) while the large RNA may represent tightly bound messenger RNA (19). The following arguments support the proposed function of iRNA: (*i*) iRNA is covalently linked to the 5′ ends of progeny strands, as shown by the ^{32}P transfer from [α-^{32}P]dTTP. The quantitative aspects of this experiment indicate that iRNA was essentially the only RNA bound to progeny strands. (*ii*) Earlier pulse-chase experiments (3) demonstrated the transient nature of the RNA·DNA link. (*iii*) The 5′ ends of iRNA start with ATP or GTP, suggesting initiation by a transcriptional process.

The presence of a triphosphate at the 5′ end increases the electrophoretic mobility of iRNA. On the other hand, our data indicate that iRNA after DNase treatment still contained about three deoxynucleotides at its 3′ end. These two effects should about cancel each other (16), and from gel electrophoresis we, therefore, estimate that iRNA is close to 10 ribonucleotides long.

The double isotope technique used permits certain calcualtions of the composition of iRNA which also bear on its size. From the ^{32}P/^{3}H ratios of different experiments we can calculate the stoichiometry between 5′ ends and internal nucleotides. For these calculations we use the measured ATP/GTP ratio of 2.4 at the 5′ end. The results then indicate the presence of 2.4 molecules of CMP (Fig. 3), 1.0 molecule of internal GMP (Fig. 4), and 2.6 molecules of UMP (6) for each 5′ end. For technical reasons similar results are not available for internal AMP. The uncertainties in these values are large because they were calculated by combining data from different incubations. Nonetheless they support the conclusion that iRNA is about 10 ribonucleotides long.

Our results also support the conclusion that essentially all iRNA contained a labeled 5′ end and thus consisted of complete molecules. We demonstrated earlier that a majority of progeny strands contained RNA·DNA links (3) and that all size classes of progeny strands contained initiating GTP (6).

The combined data suggest that in isolated nuclei removal of iRNA is a rate-limiting step during chain elongation.

iRNA has no unique base sequence in spite of the homogeneity in size, since RNase treatment of molecules labeled at their 5′ ends with ^{32}P did not result in the release of unique labeled oligonucleotides. While this result implies that transcription of iRNA can start at many points, it does not necessarily suggest a complete random process and does not exclude that initiations of iRNA may occur at a limited number of preferred sites.

From our results we would like to propose that the size of intermediates rather than base sequences may play a role as "signals" for the discontinuous mechanism of polyoma DNA synthesis. This seems particularly striking for the relation between the switch from RNA to DNA synthesis and the discrete size of iRNA, but may also apply to the process that determines the initiation of iRNA and, thereby, of Okazaki fragments. We need, however, better information of the size distribution of completed Okazaki fragments.

Sugino and Okazaki proposed that in *Escherichia coli* the RNA·DNA junction has the unique base sequence... prPyprAprPypdCp... (20). With the limited data available, it is of course not possible to refer the differences between the results obtained by Okazaki's group and our results to differences between prokaryotic and eukaryotic systems. There is, however, good reason to believe that the elongation of polyoma DNA is carried out by the replication machinery of the mouse cell and that the results obtained for polyoma DNA, therefore, may be of more general significance for our understanding of how mammalian DNA is replicated.

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