CHARACTERIZATION OF THE RNA INITIATING THE DISCONTINUOUS SYNTHESIS OF POLYOMA DNA

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SUMMARY

The discontinuous synthesis of polyoma DNA in infected isolated nuclei from 3T6 cells is initiated with RNA. From experiments in which this RNA was labelled from [^{3}H] or β-[^{32}P] labelled ribonucleoside triphosphates we conclude that the 5′ end of initiating RNA starts with ATP or GTP, but not with CTP or UTP. When the RNA was released from progeny DNA strands by digestion with pancreatic DNAse and characterized by gel electrophoresis its position on the gel corresponded approximately to that of a decanucleotide. While initiating RNA was quite homogenous in size it had no unique nucleotide sequence.

INTRODUCTION

A function of RNA as an initiator for DNA synthesis was suggested from experiments with microorganisms (1, 2). A similar involvement of RNA was proposed for the replication of the DNA of polyoma, a mammalian virus (3). With isolated infected nuclei from 3T6 cells we found that the growth of DNA progeny strands was discontinuous and occurred by multiple initiation events. RNA appeared to participate in the initiations, since ribonucleotides were covalently attached to the 5′ end of both short and long growing DNA strands. At the RNA-DNA link all four ribo- and deoxyribonucleotides were recovered (4), suggesting that the RNA was not a unique molecular species. This lack of base specificity at the 3′ end of the RNA differed from the situation in E. coli where the sequence p(rPy)p(dC)p was found at the RNA-DNA link (5). In this communication we describe studies of the 5′ end of the RNA which initiates polyoma DNA.

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METHODS

Infection of cells, preparation of nuclei and general conditions of incubations were described earlier (4, 6). In the two experiments described here nuclei (6 mg of total DNA) were incubated for 5 min at 25° in a final volume of 4 ml. In the first experiment progeny strands were labelled with [^{3}H]GTP (12 μM, 8,100 cpm/pmole) + α-[^{32}P]dCTP (11 μM, 2,800 cpm/pmole), in the second experiment with β-[^{32}P]GTP (16 μM, 23,000 cpm/pmole) + [^{3}H]UTP (15 μM, 11,000 cpm/pmole) + [^{14}C]dATP (10 μM, 270 cpm/pmole).

For the purification of progeny strands Hirt supernatants (7) (36 ml) were digested for 2 hrs at 37° with proteinase K (0.2 mg/ml), treated with phenol and passed through a column of Biogel P100 (4 x 30 cm) in 0.25 M NaCl - 0.05 M Tris, pH 7.6, - 0.01 M EDTA (= buffer A). The material in the void volume was precipitated with isopropanol (4), centrifuged, dissolved in 2 ml of buffer A and chromatographed on a 2 x 200 cm column of Sepharose 4B. The material from the void volume was again precipitated with isopropanol, dissolved in 4 ml of 0.02 M Tris, pH 7.2, - 0.01 M EDTA (buffer B) and centrifuged to equilibrium in a propidium iodide-CsCl gradient (4) to separate the replicative intermediates from supercoiled DNA and RNA. Fractions containing the replicative intermediates were combined, propidium iodide was extracted (8), the material was precipitated with isopropanol after addition of 0.4 mg of carrier calf thymus DNA, and finally dissolved in 1-2 ml of 0.05 M Tris, pH 7.2. Progeny strands were released from the replicative intermediates by heating at 100° for 3 min followed by quick cooling.

Separation of progeny strands according to size and analysis of pppGp†. In experiment 1 the released single-stranded progeny strands were chromatographed in buffer B on a column of Sepharose 4B (4). Fractions were combined in four different pools as indicated in Fig. 1, and precipitated with isopropanol. Small aliquots from each pool were used to determine s-values in

† pppGp = guanosine 5′-triphosphate, 2′(3′) monophosphate.

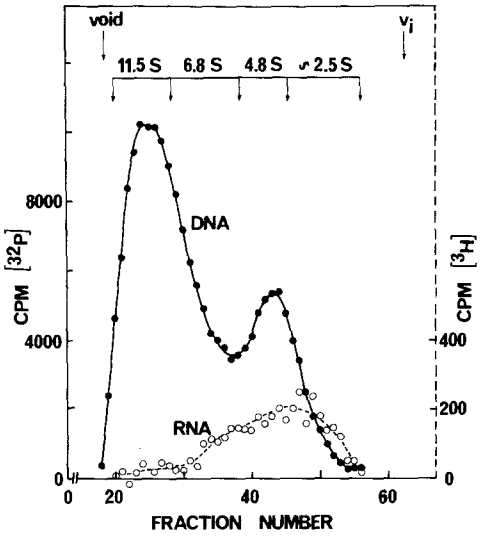

Fig. 1. Separation on Sepharose 4B (4) of progeny strands into four size classes. Fractions were combined in four pools as indicated and the average sedimentation coefficients of the [^{32}P] labelled progeny strands present in each pool were determined by sedimentation in alkaline sucrose gradients (4). The materials were then used for determinations summarized in Table 1.

alkaline sucrose gradients. For the analysis of pppGp the remaining material was incubated in 1 ml of 0.3 M KOH at 37° for 16-18 hrs and then neutralized to pH 7-8 by addition of a slurry of Dowex-50 (H^+). After centrifugation, about 1 μmole each of GDP and guanosine 5′-tetraphosphate were added as UV markers and the solution was chromatographed on a 5 ml column of DEAE-Sephadex with a linear gradient (0.1 M to 1.0 M, 50 ml each) of triethanolamine-HCO_3. pppGp was recovered immediately after guanosine 5′-tetraphosphate, approximately 80 ml from the start of the chromatogram.

Polyacrylamide electrophoresis of initiating RNA. In experiment 2 the released progeny strands were incubated at 37° for 1 hr with pancreatic DNAse (0.1 mg/ml, free from RNAse) in 0.02 M $MgCl_2$ and 0.015 M $CaCl_2$. The reaction was stopped by addition of solid urea (final conc. 7 M) and the material was electrophoresed in 7 M urea on 12% polyacrylamide columns for 4 hrs at 20 V/cm with bromphenol blue as an internal marker (9). The mobilities of $tRNA^{Phe}$ (76 nucleotides), aniline cleaved $tRNA^{Phe}$ (36 and 39

nucleotides, respectively) and a T1 RNAse digest of $tRNA^{Phe}$, containing i. al. a dodeca- and an octanucleotide (9), were determined in parallel runs. The radioactive gels were sliced and counted after incubation of each slice at 37° in 0.3 ml of 0.3 M NaOH for 16 hrs. Without quench corrections the over all recovery of radioactivity was close to 100% for $[^{32}P]$, about 80% for $[^{14}C]$ and about 65% for $[^{3}H]$.

RESULTS AND DISCUSSION

Presence of pppGp in progeny strands of different length. In experiment 1 the initiating RNA was labelled with $[^{3}H]$GTP while DNA was labelled with α-$[^{32}P]$dCTP. After alkaline hydrolysis the distribution of $[^{3}H]$ between pppGp and GMP measured the amount of 5′ ends of RNA, newly synthesized from GTP, relative to the total amount of RNA synthesized. We determined this parameter for the replicative intermediates at different stages of purification and also for progeny strands of different length obtained from the final preparation of replicative intermediates (Table 1). During purification a considerable enrichement of pppGp occurred, as demonstrated by the decrease in the GMP/pppGp ratio. In the Hirt extract most pppGp was present in contaminating RNA. After propidium iodide centrifugation essentially all labelled pppGp was present in the initiating RNA (see experiment 2 below). However, at this stage the preparation of replicative intermediates still contained some labelled GMP from contaminating RNA. The presence of pppGp in all four classes of progeny strands demonstrates that initiating RNA could start with GTP at all stages of replication. The low GMP/pppGp ratios indicate that the initiating RNA was quite short.

Length of initiating RNA. In experiment 2, the 5′ ends were labelled with β-$[^{32}P]$GTP, $[^{3}H]$UTP was used as an internal RNA label and DNA was labelled with $[^{14}C]$dATP. In the purified replicative intermediates all $[^{32}P]$ was recovered as pppGp after alkaline hydrolysis. The initiating RNA was released from its attachment to progeny DNA by digestion with pancreatic DNAse and analyzed by gel electrophoresis. Fig. 2 shows that after

Table 1. pppGp in replicative intermediates at different stages of purification and in different size classes of progeny strands.

	DNA	RNA		
	$[^{32}P]$dCMP (pmole)	$[^{3}H]$GMP (pmole)	$[^{3}H]$pppGp (pmole)	$\frac{GMP}{pppGp}$
Hirt extract	190	730	4.4	166
Sepharose 4B	135	14	0.35	40
Propidium iodide	105	1.3	0.18	7
Progeny strands: 11.5 s	40	0.09	0.025	3.5
6.8 s	17	0.13	0.024	5
4.8 s	13	0.18	0.016	11
2.5 s	7	0.22	0.031	7

Replicative intermediates were purified as described. After centrifugation in a propidium iodide-CsCl gradient progeny strands were released and separated into four different size classes as described in Fig. 1. Each fraction was analysed for $[^{32}P]$ (DNA synthesis), total $[^{3}H]$ (RNA synthesis) and $[^{3}H]$ in pppGp.

enzyme digestion two $[^{3}H]$RNA peaks were found: one at the top of the 12% gel and another one which migrated slightly behind the dye marker. All $[^{32}P]$ added to the gel was recovered in the second peak. In the control (no DNAse treatment) the second peak was absent. Most of the $[^{14}C]$ and $[^{32}P]$ did not enter the spacer gel and was lost. We propose that the second RNA peak, observed after treatment with DNAse, represents initiating RNA. Its position on the gel corresponded to that of a decanucleotide, but the

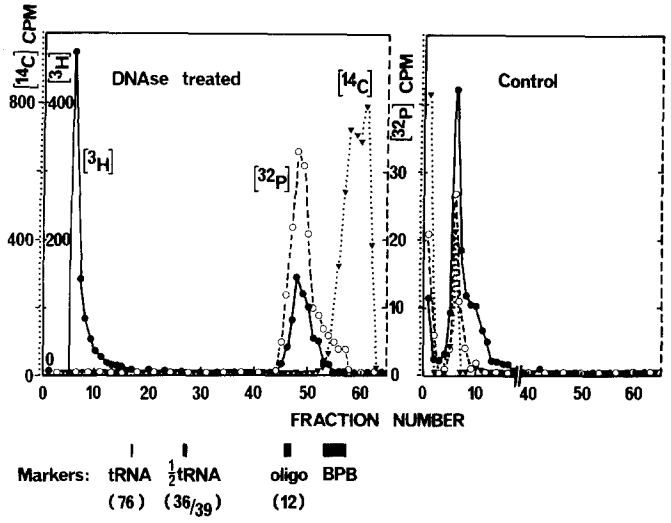

Fig. 2. Polyacrylamide gel electrophoresis in 7 M urea (9) of initiating RNA. RNA was labelled internally from [^{3}H]UTP and at its 5′ end from β-[^{32}P]GTP, DNA was labelled from [^{14}C]dATP. Progeny strands from purified replicative intermediates were digested with pancreatic DNAse and the digest was electrophoresed on 12% polyacrylamide. Fractions 1 - 6 are from the spacer gel (5% polyacrylamide). $tRNA^{Phe}$, aniline cleaved $tRNA^{Phe}$ and a T1 RNAse digest of $tRNA^{Phe}$ were used as markers in parallel runs. Bromphenol blue (BPB) was used as an internal marker in each run.

presence of the 5′-terminal triphosphate and the possibility that the RNA still might contain a few deoxynucleotides are complicating factors. From the [^{3}H]/[^{32}P] ratio one can calculate that about 9 UMP were present for each pppGp.

In separate experiments we did not recover labelled tetraphosphates after alkaline hydrolysis of replicative intermediates synthesized from [^{3}H]UTP or [^{3}H]CTP. However, [^{32}P]-labelled adenosine tetraphosphate was recovered in good yield in similar experiments in which β-[^{32}P]ATP and [^{3}H]GTP were used simultaneously. In these experiments which will be described elsewhere we found that the ratio between ATP and GTP at the 5′ end of the initiating RNA was 2.5 - 3 showing a preference for ATP as the starting nucleotide. Similar to the results described in Fig. 2, DNAse treatment gave rise to a well defined [^{32}P] and [^{3}H]-labelled RNA species with the properties of a decanucleotide.

From our data it appears that the RNA initiating polyoma DNA during discontinuous synthesis of progeny strands is quite homogenous with respect to size. However, the molecules did not have a unique nucleotide sequence. At the 5′ end the RNA started with either ATP or GTP and at the 3′ end all four ribonucleotides were present (4). Additional evidence for heterogeneity came from unpublished experiments in which the RNA, labelled with [^{32}P] at the 5′ end, was degraded with ribonuclease A.

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