EVIDENCE FOR RNA-LINKED NASCENT STRANDS IN POLYOMA VIRUS DNA REPLICATION[1]

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SUMMARY

Replicating polyoma virus DNA, pulse-labeled with ^{3}H-thymidine, was isolated from infected mouse embryo cells by velocity sedimentation in neutral sucrose and purified by benzoylated-naphthoylated DEAE-cellulose chromatography. Nascent strands, prepared by heat denaturation of purified replicative intermediate, banded at a slightly higher buoyant density in neutral cesium sulfate gradients than single strands derived from superhelical viral DNA. Treatment of nascent strands with a mixture of ribonucleases 1A and T1 shifted their buoyant density to that of single strands derived from superhelical viral DNA. These results indicate that an oligoribonucleotide component is covalently associated with replicating polyoma DNA strands.

INTRODUCTION

Recent evidence from this laboratory (Kowalski and Cheevers, unpublished) has indicated the involvement of DNA-dependent RNA synthesis in the control of DNA replication in py[2]-infected cells. In these studies, the rate of decay of ^{3}H-TdR incorporation into viral DNA was measured after inhibition of DNA-dependent transcription by actinomycin D. The rate of inhibition of new rounds of viral DNA replication in the absence of RNA synthesis follows a first order exponential decay, corresponding to a half-life of 1 - 1.5 hr for the RNA requirement(s). In contrast, residual labeling of py RI in the presence of actinomycin decays with a half-life of more than 3 hr. This result suggested that the formation of relatively stable RNA is required for the progression of replicating molecules into mature superhelical DNA. Since the maturation of py RI is not dependent upon protein synthesis (Yu and Cheevers, unpublished), it was reasoned that the DNA-dependent transcript probably is not messenger RNA.

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[2]Abbreviations: Py, polyoma virus; RI, replicative intermediate DNA; RNase, ribonuclease; BND-cellulose, benzoylated-naphthoylated DEAE cellulose; TdR, thymidine; SDS, sodium dodecyl sulfate; SSC, standard saline citrate (1); Tris-EDTA, 0.01M Tris-HCl, pH 8.1 - 0.001M EDTA (2).

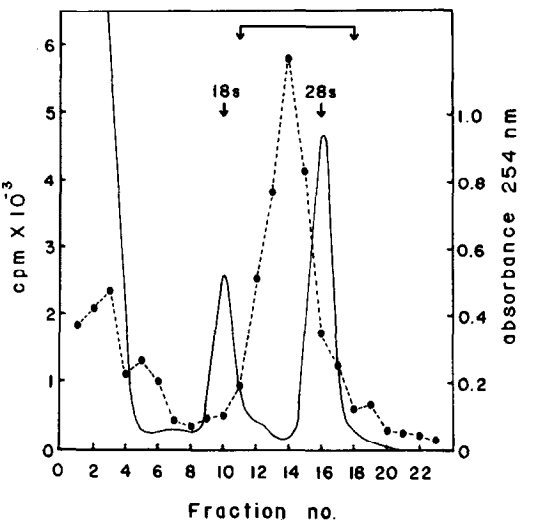

Figure 1. Primary isolation of ^{3}H-labeled py RI. Py-infected cultures were pulse-labeled for 4 min at 30 hr post-infection. The total DNA was extracted with 1% SDS and sedimented in a neutral SDS-sucrose gradient as previously described (5).

Sugino et al. (3) and Sugino and Okazaki (4) have recently reported on the involvement of RNA in the discontinuous replication of Escherichia coli DNA. In view of this work, we postulated that our results indicated the existence of a DNA-dependent oligoribonucleotide covalently associated with nascent strands in replicating py DNA. Preliminary evidence supporting this hypothesis is reported.

MATERIALS AND METHODS

Confluent secondary cultures of Swiss mouse embryo cells infected with py (strain TSP-1) at an input multiplicity of 150 plaque-forming-units/cell were used. Cell cultivation procedures and methods for virus infection have been described (5,6).

Py RI synthesized in py-infected cells was pulse-labeled at 37°C with (methyl-^{3}H)TdR (52-60 c/mmole) at a final concentration of 100 μc/ml in McCoy 5A medium (6) without serum. Form I py DNA (5), used as marker in sedimentation and buoyant density analyses, was labeled with (2-^{14}C)TdR (59 mc/mmole) at a concentration of 0.1 μc/ml. Incorporation in all experiments was in terms of 5% trichloroacetic acid-insoluble radioactivity.

The procedure for primary fractionation of viral and cellular DNA by velocity sedimentation in neutral SDS-sucrose gradients has been described (5).

BND-cellulose chromatography was carried out as described by Sedat et al. (2) and Kiger and Sinsheimer (7). SERVA BND-cellulose was purchased from Gallard-Schlesinger, Carle Place, N.Y.

Sedimentation analysis in alkaline sucrose gradients was done as follows: DNA was dissolved in 0.01 x SSC and mixed with an equal volume of 1N NaOH-0.001M EDTA-0.1% sodium N-lauroyl sarcosine. The denatured DNA was then layered onto an alkaline sucrose gradient (15-30% (w/w) in 0.5M NaCl-0.25N NaOH-0.001M EDTA-0.1% sodium N-lauroyl sarcosine) and centrifuged at 26000 rpm for 12 hr at 23°C using a Spinco SW 27 rotor.

For equilibrium centrifugation in Cs_2SO_4 gradients, py RI was dissolved in 0.01 x SSC and denatured by heating at 100°C for 10 min, followed by rapid cooling in ice. Marker ^{14}C py DNA I was heated at 100°C in 0.01 x SSC for 20-30 min. Prolonged heating in dilute salt causes strand scissions in form I py DNA, which is normally resistant to heat denaturation, yeilding denaturable form II molecules and linear fragments (8). Denatured py RI and marker py DNA were mixed in a polyallomer tube with 2 ml of a solution of saturated Cs_2SO_4 in SSC, and the volume was adjusted to 4 ml with SSC. The solution was overlaid with liquid paraffin and centrifuged at 36000 rpm for 48-60 hr at 15°C using a Spinco SW 50L rotor.

RESULTS AND CONCLUSIONS

Purification of py RI

Figure 1 shows a primary molecular weight fractionation by velocity sedimentation in neutral SDS-sucrose of DNA pulse-labeled with ^{3}H-TdR in py-infected cells. About 75% of the DNA is comprised of high-molecular-weight cellular DNA, which sediments at +70s (5) into the bottom of the tube (not shown). The remaining low-molecular-weight DNA is resolved into (i) material sedimenting at 10s or less, consisting primarily of newly-initiated cellular DNA (5), (ii) a prominent peak of 25s DNA, consisting of py RI (5,9), and (iii) an underlying heterogeneous DNA component, sedimenting throughout the gradient. Component (iii) (2-5% of the total) is cellular in nature, arising by fragmentation of high-molecular-weight cellular DNA during lysis of the cells (Kowalski and Cheevers, unpublished).

The crude py RI isolated by sedimentation in neutral sucrose (indicated by the bar in Figure 1) was further purified by BND-cellulose chromatography. The purpose of this step was to remove cellular DNA fragments and any form I py DNA (20s in neutral solution) which may have been completed during the pulse-labeling interval.

Table 1 shows the chromatographic properties on BND-cellulose of ^{14}C-labeled cellular DNA fragments and ^{3}H-labeled py RI. 86% of native cellular DNA was eluted in 0.65M NaCl; upon heat denaturation, 98% of this DNA was eluted in 1M NaCl-2% caffeine. Two points may be made from these results: (i) Single-stranded DNA is essentially quantitatively eluted from BND-cellulose in 2%

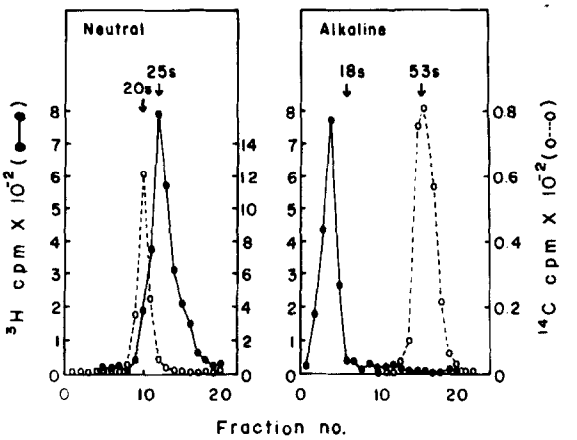

Figure 2. Neutral and alkaline sedimentation analysis of ^{3}H-labeled py RI eluted from BND-cellulose. 25s DNA, eluted from BND-cellulose in 1M NaCl + 2% caffeine (Table 1), was concentrated by ethanol precipitation and dissolved in 0.1M NaCl-0.001M EDTA-0.01M Tris-HCl, pH 7.4-0.5% SDS. DNA was then mixed with marker py DNA I (20s in neutral solution) and sedimented in a neutral SDS-sucrose gradient (5). Alternatively, the DNA mixture was denatured in alkaline solution and analyzed by velocity sedimentation in an alkaline sucrose gradient as described in Materials and Methods.

caffeine, confirming the properties of this column described by Kiger and Sinsheimer (7). (ii) Fragmentation products of cellular DNA contain approximately 13% single-stranded or partially single-stranded molecules. 18% of the ^{3}H py RI preparation obtained from the experiment in Figure 1 was eluted in NaCl and the remaining 82% in caffeine.

The sedimentation properties in neutral and alkaline sucrose gradients of caffeine-eluted DNA are shown in Figure 2. At neutral pH, the DNA sedimented as relatively homogeneous 25s material; 95% of the alkaline denaturation products of this DNA sedimented at less than 18s. These data indicate (i) that py RI is eluted from BND-cellulose in 2% caffeine (9-11) and (ii) that any labeled superhelical py DNA and the majority of fragmented cellular DNA are eluted in NaCl. Sedimentation analysis of the NaCl-eluted DNA in Table 1 confirmed these results. This material consisted of a small amount of form I viral DNA and heterogeneous fragments of cellular DNA; no evidence for the presence of 25s DNA was apparent. It is concluded that under our conditions, py RI preparations of approximately 95% purity are obtained.

Equilibrium centrifugation of purified denatured py RI in neutral Cs_2SO_4

The buoyant density distribution of purified ^{3}H-labeled nascent strands of py RI in neutral Cs_2SO_4, relative to ^{14}C-labeled heat-denatured py DNA I, is shown in Figure 3 (left panel). The py RI bands at a slightly heavier density than single-stranded py DNA I. Figure 3 (right panel) shows that treatment of denatured py RI with a mixture of RNase 1A and RNase T1 shifts it buoyant density

Table 1. BND-cellulose chromatography of cellular and viral DNA

Column fraction	Percent of DNA recovered		
	^{14}C-cellular DNA		^{3}H-25s DNA
	Native	Denatured	
0.65M NaCl	86	1	12
1M NaCl	1	1	6
1M NaCl + 2% caffeine	13	98	82

25s DNA, indicated by the bar in Figure 1, was concentrated by ethanol precipitation (5) and dissolved in 0.3M NaCl (Tris-EDTA). Cellular DNA of uninfected cells was extracted and sedimented in neutral sucrose as described previously (5). Material sedimenting between 15s and 60s was concentrated by ethanol precipitation and dissolved in 0.0015M NaCl (Tris-EDTA). Half of this DNA was heat denatured, and the NaCl concentration of native and denatured DNA was made 0.3M. DNA was bound to BND-cellulose (2 ml, packed volume), previously washed with 100 ml of 0.3M NaCl (Tris-EDTA), in a 5 cm x 15 cm glass column. DNA was eluted by successive passage through the column of 20 ml of 0.65M NaCl (Tris-EDTA), 1M NaCl (Tris-EDTA), and 1M NaCl (Tris-EDTA) + 2% caffeine. Aliquots of each fraction were assayed for trichloroacetic acid-insoluble radioactivity.

distribution to that of single-stranded py DNA I. This experiment indicates that a single-stranded oligoribonucleotide is covalently linked to replicating py DNA strands *in vivo*. Py RI labeled for a longer period (90 sec) did not show a buoyant density shift toward the heavier side of marker DNA. This result is consistent with the suggestion of Sugino *et al.* (3) that RNA associated with discontinuously replicating DNA fragments is removed during the assimilation of these fragments into longer units of replication.

In summary, the experiments presented in this report indicate that RNA is covalently linked to the daughter strands of py RI purified by BND-cellulose chromatography. While this work was in progress, Magnusson *et al.* (12) reported that isolated nuclei of py-infected mouse 3T6 cells elongate the daughter strands of viral DNA *in vitro*, via the formation of 4-5s DNA fragments linked at their 5' end to RNA. It remains to be determined if the oligoribonucleotide segment indicated in the present work is analogous to that observed by these workers.

As indicated in the Introduction, we have obtained evidence implicating a non-messenger DNA-dependent transcription event involved in the assimilation of daughter strands of replicating py DNA. This evidence, together with the present results, suggest the existence of a DNA-dependent transcript directly involved in the replication of py DNA.

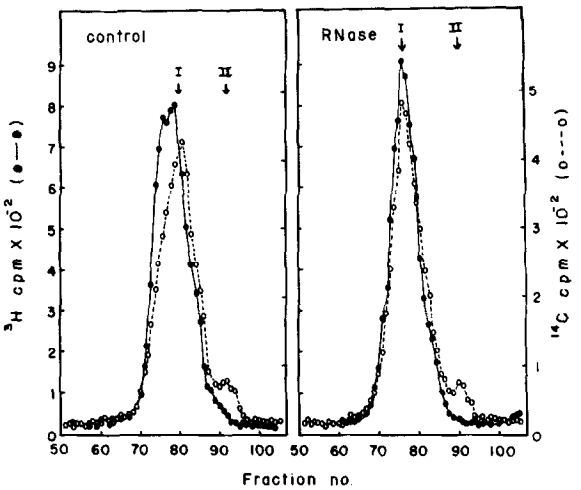

Figure 3. Cesium sulfate equilibrium centrifugation of ^{3}H-labeled nascent strands of BND-cellulose-purified py RI. Py-infected cells were pulse-labeled for 20 sec at 30 hr post-infection. Py RI was extracted and purified by velocity sedimentation in neutral sucrose followed by chromatography of 25s DNA on BND-cellulose. Material eluted from BND-cellulose in 1M NaCl + 2% caffeine was concentrated by ethanol precipitation and dissolved in 0.01 x SSC. Nascent DNA strands, prepared by heat-denaturation of BND-cellulose purified py RI, was mixed with heat-denatured marker py DNA I and centrifuged to equilibrium in a neutral cesium sulfate gradient as described in Materials and Methods. Alternatively, denatured py RI was made 2x in SSC and incubated for 1 hr at 37°C with a mixture of RNase 1A and RNase T1 (final concentrations of 50μg/ml and 10μg/ml, respectively). RNase-treated py RI nascent strands were then mixed with marker denatured py DNA I and centrifuged in cesium sulfate. Single-stranded marker py DNA and residual double-stranded superhelical py DNA are indicated in the buoyant density profiles as I and II, respectively.

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