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Orientation of the Complementary Strands of Polyoma Virus DNA with Respect to the DNA Physical Map

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The chemical polarities of the two strands of polyoma virus DNA with respect to the DNA physical map have been determined by hybridization of restriction endonuclease fragments specifically labeled with [^{125}I]dCMP at their 3′ termini to asymmetric polyoma complementary RNA (the product of in vitro transcription of viral DNA by *Escherichia coli* RNA polymerase). The orientations of the polyoma-specific stable RNA transcripts present in the cytoplasm of productively infected mouse cells have been deduced from this result: the 5′ ends of the early and late viral transcripts map very near the origin of viral DNA replication.

The RNA transcribed from polyoma virus DNA during productive infection of mouse cells has recently been characterized (15). At late times after infection, two classes of viral transcripts were found in the cytoplasm. The minor class is transcribed from approximately one-half of one strand of polyoma DNA, designated the E strand, whereas the major class is transcribed from about one-half of the complementary, or L strand, of the DNA. Only the E-strand transcripts were found in the cytoplasm during the early phase of viral infection prior to the onset of polyoma DNA replication. The availability of a physical map of polyoma DNA (12) made it possible to determine which regions of the DNA molecule are templates for the two classes of viral transcripts. The E-strand transcripts derive from a continuous region comprising about 50% of the DNA, whereas the L-strand transcripts are from the other half of the molecule (see Fig. 1). We describe here an experiment which determines the chemical polarity of the E and L strands of polyoma DNA with respect to the physical map. This knowledge enables us to assign the 5′ to 3′ orientation of stable viral transcripts. A preliminary account of the experiment presented here has already appeared (15).

MATERIALS AND METHODS

Cells, virus, and viral DNA. The A2 strain of large-plaque polyoma virus (12) was generously provided by B. Gifffin. Virus stocks were prepared by infecting monolayers of secondary whole mouse embryo cells at 0.05 PFU/cell and incubating the cultures for 10 to 14 days at 37 C until advanced cytopathic effects were obtained. Superhelical polyoma DNA was prepared from subconfluent monolayers of 3T6 cells (22) 48 to 55 h after infection (20 PFU/cell) by sodium dodecyl sulfate (SDS) selective extraction (13) followed by ethidium bromide-CsCl equilibrium centrifugation (17) and velocity sedimentation through sucrose gradients. DNA prepared by this procedure was free from defective molecules as judged by its appropriate cutting pattern (11, 12) when digested with each of the three restriction endonucleases HpaII, HhaI, and EcoRI.

Synthesis of asymmetric polyoma cRNA Complementary RNA (cRNA) is defined as the RNA synthesized in vitro by *Escherichia coli* RNA polymerase and was prepared as follows. Superhelical polyoma DNA was transcribed in vitro using a modification of the conditions previously described (15). A typical reaction mixture contained: 40 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 400 mM KCl, 1 mM each ATP, CTP, GTP, and UTP, 50 μg of polyoma DNA and 60 μg of *E. coli* RNA polymerase holoenzyme in a final volume of 2.5 ml. The KCl was added last. Under these conditions, incorporation at 39.5 C was linear for more than 200 min; a 20-fold net synthesis of cRNA was obtained after 12 h of incubation. The reaction was terminated by addition of SDS and EDTA to final concentrations of 0.1% and 20 mM, respectively, followed by 3 volumes of 4 M LiCl. After 24 h at 0 C, the precipitated RNA was recovered by centrifugation at 10,000 rpm in the HB-4 rotor of a Sorvall RC-2 centrifuge and redissolved in 2 ml of 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$. The contaminating template DNA was hydrolyzed by incubation at 37 C for 30 min with 20 μg of repurified (14) RNase-free DNase (Worthington) per ml. After phenol extraction in the presence of 0.1% SDS,

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the cRNA was precipitated with ethanol from 0.1 M sodium acetate (pH 5.3). The RNA was then self-annealed at 68 C for 1 h in 1 ml of TSE buffer (50 mM Tris-hydrochloride, pH 7.5–0.1 M NaCl–1 mM EDTA), and double-stranded material was removed by chromatography on a 5-ml CF-11 (Whatman) cellulose-ethanol column (9). Approximately 80% of the cRNA was recovered in the single-stranded peak of the column.

Preparation of [^{125}I]dCTP. [^{125}I]dCTP was prepared by a modification of the Commerford reaction (4). An aliquot (6.25 nmol) of dCTP (P. L. Biochemicals) in 25 μl of a buffer containing 0.120 M sodium acetate (pH 4.6) and 0.001 M $TiCl_3$ (dissolved immediately before use) was added to 25 μl of carrier-free Na^{125}I (Amersham IMS 30; 100 mCi/ml) and incubated at 37 C for 4 h, at which time 100 μl of a solution containing 0.1 M triethylammonium bicarbonate (pH 9.5), 0.01 M EDTA, and 0.14 M 2-mercaptoethanol was added. After a further 30 min at 37 C, the [^{125}I]dCTP was purified by chromatography on Sephadex G-10 in distilled water, evacuated to dryness, redissolved in 100 μl of water followed by 100 μl of ethanol, and stored at −20 C. Of the added ^{125}I, 64% was recovered in the final product, which thus had a specific activity of approximately 260 Ci/mmol.

Terminal labeling of polyoma DNA fragments. Polyoma form I DNA (10 μg) was digested with restriction endonuclease HpaII (12) in 50 μl of 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol. After the addition of 20 μl of a buffer containing 0.1 M Tris-acetate (pH 8.0), 0.014 M $MgCl_2$, and 0.01 M dithiothreitol, the digested DNA was transferred to a siliconized glass test tube in which 1.5 nmol of [^{125}I]dCTP had been dried. The 3′ OH termini of the DNA fragments were labeled by incubation at 12 C for 10 h with approximately 13 μg of T4 DNA polymerase. The reaction was stopped by the addition of EDTA (20 mM final concentration) and 2 μl of diethylpyrocarbonate (Baycovin) followed by incubation for 10 min at 37 C. Unreacted [^{125}I]dCTP was removed by Sephadex G-25 chromatography in 0.1 mM EDTA. Of the terminally labeled DNA, 80% was subsequently digested with restriction endonuclease EcoRI in 500 μl of 10 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$–100 mM NaCl–1 mM dithiothreitol. After concentration by ethanol precipitation, the HpaII/EcoRI digested DNA, as well as the remaining 20% of the original HpaII digest, was fractionated in parallel on a 3.5% acrylamide–0.5% agarose gel (20 by 40 cm) (12). All labeled fragments (localized by autoradiography) were cut out from the gel and counted in a Nuclear Enterprises gamma counter at suboptimal window settings to achieve relative quantitation within the linear range of the machine. The fragments of interest (HpaII-2 and the two EcoRI subfragments of HpaII-2, designated 2A and 2B for the larger and smaller, respectively) were eluted from the gel by electrophoresis onto an 0.5-ml pad of benzoylated, naphthoylated DEAE-cellulose (Boehringer), recovered from the resin by washing with a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, 1 M NaCl, and 1% caffeine, and then concentrated by ethanol precipitation. The final yield was approximately 5×10^5 counts/min per pmol of 3′ end when counted at optimal settings in the gamma counter.

Hybridization of terminally labeled DNA with asymmetric polyoma cRNA. Terminally labeled DNA fragments were diluted to the equivalent of approximately 0.25 μg of full-length polyoma DNA per ml in 0.8 ml of 0.1 mM EDTA and heated to 100 C for 3 min and then chilled on ice; 0.4-ml aliquots with or without the addition of 13 μg of polyoma cRNA were then mixed with 0.4 ml of 2× hybridization buffer (2 M NaCl–0.1 M Tris-hydrochloride, pH 7.5–native calf thymus DNA [20 μg/ml]–0.2% SDS–0.02 mM EDTA), and the DNA was reannealed at 70 C. At various times, 50-μl aliquots were removed and diluted with 1 ml of 0.14 M sodium phosphate buffer (pH 6.8) containing 0.1% SDS. The fraction of the DNA reannealed was determined by batchwise hydroxylapatite chromatography (3): 2 drops of a 20% (wt/vol) suspension of hydroxylapatite (Bio-Rad, DNA grade HTP) was added to the diluted DNA samples. After 3 min of incubation at 68 C with frequent agitation, the HTP was removed by low-speed centrifugation (2,200 × *g*) for 5 min. The supernatant, containing single-stranded DNA, and the hydroxylapatite pellets, containing absorbed double-stranded DNA, were counted in a Nuclear Enterprises gamma counter.

Enzymes. *E. coli* RNA polymerase was the generous gift of W. Mangel. Restriction endonucleases EcoRI and HpaII were prepared by published methods (R. N. Yoshimori, Ph.D. thesis, University of California, San Francisco, 1971; 20). T4 DNA polymerase was purified from *E. coli* infected with T4 amber N82 (R. Kamen, unpublished procedure) to approximately 80% purity as judged by SDS-polyacrylamide gel electrophoresis. The preparation used was free of detectable double-strand-specific endonuclease (assayed by incubation with polyoma form I DNA) and contaminating exonuclease (assayed by incubation with restriction endonuclease fragments of polyoma DNA in the presence of dCTP to inhibit the 3′ exonuclease activity of the polymerase).

RESULTS

Experimental design. The protocol used for determination of the chemical orientation of the complementary strands of polyoma DNA is schematically shown in Fig. 1. We have chosen an approach similar in principle to that used by Sambrook et al. (19) for simian virus 40 DNA. It depends on the observation (15) that, under appropriate in vitro conditions, *E. coli* RNA polymerase preferentially transcribes all of one strand of polyoma form I DNA. It is thus possible to prepare large amounts of in vitro transcript ("cRNA") complementary to a single DNA strand. The polyoma DNA strand complementary to the cRNA is called the L strand, whereas that with the same sense as cRNA is called the E strand (15).

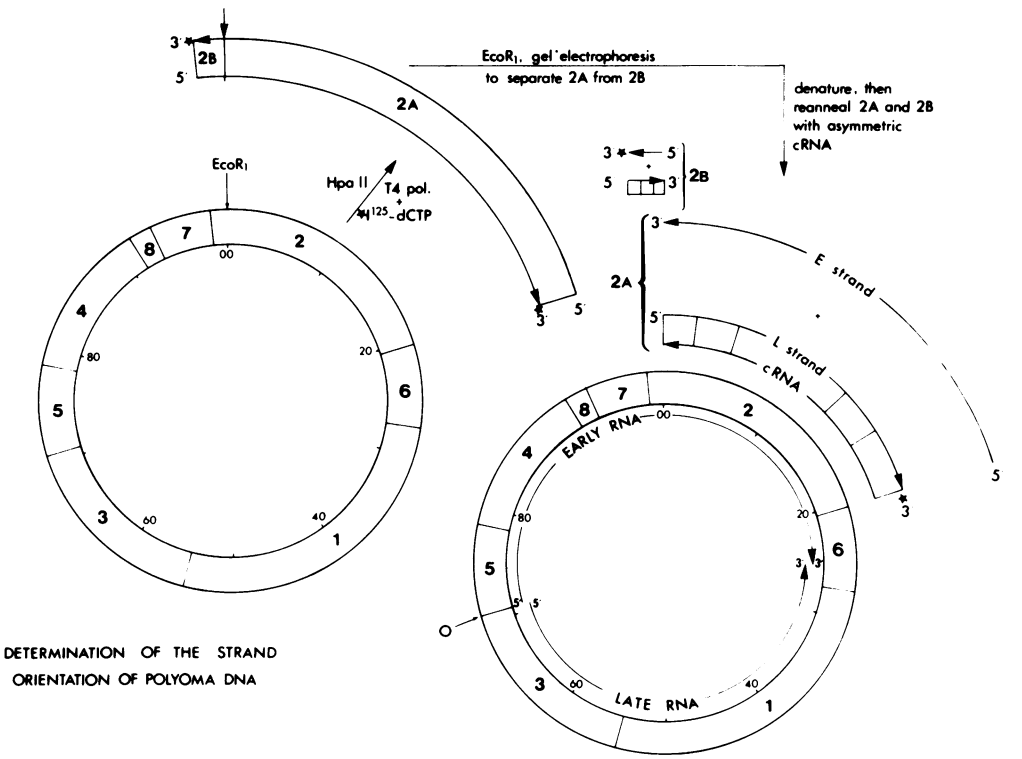

FIG. 1. *Protocol for the determination of the chemical orientation of the complementary strands of polyoma DNA. The left circle is the physical map of polyoma DNA, showing the positions of the eight HpaII fragments (numbered 1 to 8) relative to the unique site of cleavage by EcoRI (12). The regions of early and late transcription (15) are indicated by the innermost lines on the right circle.*

Polyoma DNA was digested with restriction endonuclease HpaII to generate eight specific fragments whose relative positions on the circular DNA are known (12). The 3′ OH termini of the fragments were radioactively labeled prior to redigestion with restriction enzyme EcoRI. Only HpaII fragment 2 (HpaII-2) is cut by EcoRI (12), generating two fragments, 2A and 2B, which are 20% and 1.5% of the total DNA, respectively (Fig. 1). Denaturation and reannealing of fragments 2A and 2B in the presence of asymmetric cRNA were then used to determine which subfragment contained the 3′-terminal label of the L strand of HpaII-2.

Terminal labeling of restriction fragments. Restriction fragments of polyoma DNA were labeled at their 3′ ends by incubation with T4 DNA polymerase in the presence of high-specific-activity [^{125}I]dCTP. Englund (8) has shown that T4 polymerase can exchange the 3′-terminal nucleotide of a double-stranded DNA through a combination of its 3′ to 5′ exonuclease and 5′ to 3′ polymerase activities. In the particular situation of DNA fragments generated by HpaII, the terminal sequence is . . .GGCp (10). Thus, when supplied with [^{125}I]dCTP, the polymerase can exchange the 3′-terminal C as well as add a further [^{125}I]dCMP complementary to the nonpaired G.

TABLE 1. *Quantitation of [^{125}I]CMP terminal labeling of polyoma DNA restriction fragments*

DNA fragment	Counts/min ($\times 10^{-5}$)	Relative radioactivity
HpaII-1	1.6	0.94
HpaII/EcoRI-2A	1.0	0.59
HpaII-3	1.7	1.00
HpaII-4	1.7	1.00
HpaII-5	1.3	0.76
HpaII-6	1.9	1.1
HpaII-7	1.4	0.82
HpaII-8	2.3	1.35
HpaII/EcoRI-2B	0.8	0.47

Figure 2 (slot B) shows the gel pattern of HpaII-digested polyoma DNA after terminal iodination. As anticipated for terminally labeled material, the intensity of the eight frag-

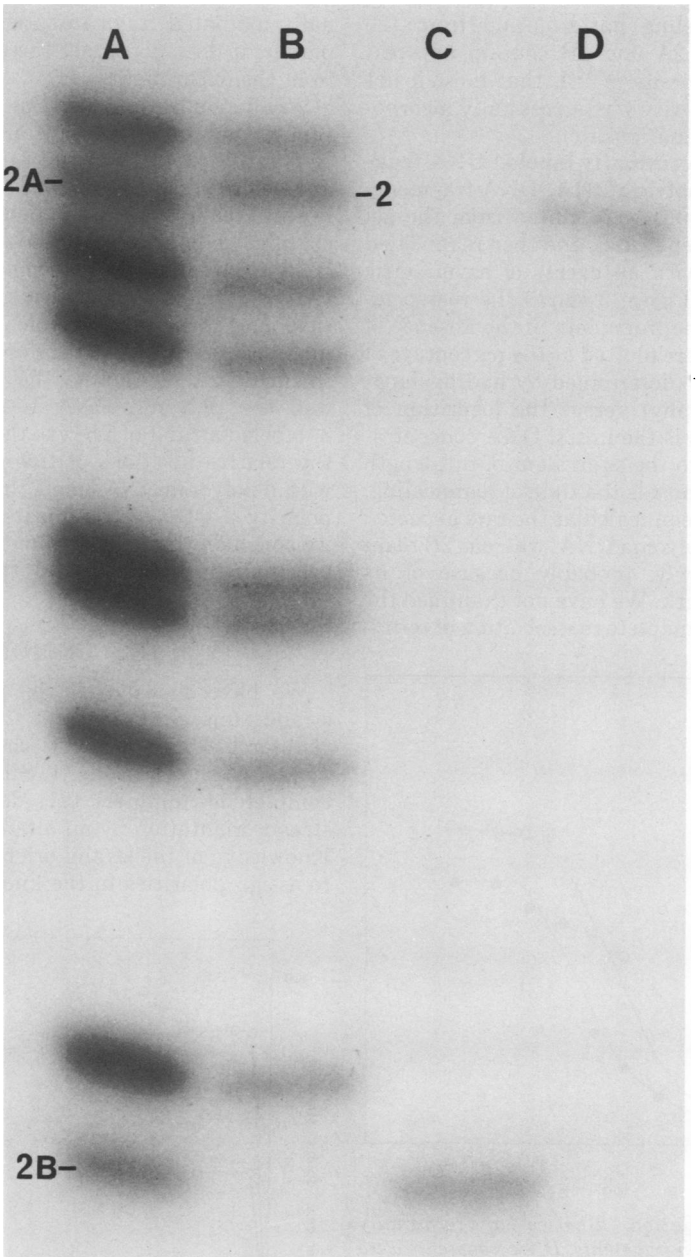

FIG. 2. *Gel electrophoresis of 3'-terminally iodinated polyoma DNA fragments. Electrophoresis conditions are described in Materials and Methods. (A) Terminally labeled HpaII fragments after subsequent digestion with EcoRI; (B) terminally labeled HpaII fragments; (C) purified fragment 2B; (D) purified fragment 2A.*

ments, which ranges from 27% to 1.8% of the total DNA length, is independent of their molecular weight. Figure 2 (slot A) shows the gel pattern of the same labeled DNA fragments after redigestion with EcoRI. A single fragment, HpaII-2, was cut, generating fragments 2A and 2B. As shown in Table 1, the HpaII fragments contain similar quantities of radioactivity (which we estimate to be 2 to 4 dCMP's per molecule), whereas 2A and 2B contain one-half this quantity of ^{125}I. The smallest HpaII fragment, HpaII-8, contains somewhat more label than the others (1.3 times more than HpaII-3).

We conclude from the chain-length-

independent labeling pattern, and from the observation that 2A and 2B contain approximately equal amounts of ^{125}I, that most if not all of the radioactivity was successfully incorporated in the terminal position.

Annealing of terminally labeled DNA fragments to asymmetric cRNA. DNA fragments HpaII-2, 2A and 2B were eluted from the gel shown in Fig. 2, denatured, and then reannealed either alone or with an excess of asymmetric polyoma cRNA. Figure 3 shows the reassociation kinetics of the fragments in the absence of cRNA. The data are plotted as the percentage of DNA reannealed (determined by hydroxylapatite chromatography) versus the logarithm of the C_0t, where C_0 is the initial DNA concentration normalized to the equivalent of full-length polyoma DNA, and t is the time of reannealing. HpaII-2 and 2A reannealed at the rate expected for fragmented polyoma DNA, whereas 2B reannealed more slowly, probably because of its shorter chain length. We have not examined the reason for the incomplete reassociation of terminally iodinated fragments; as will become apparent, it does not affect the conclusion drawn from the experiment.

When reannealed with an excess of cRNA (Fig. 4), approximately 50% of the radioactivity in HpaII-2 rapidly entered DNA-RNA hybrid, and no further annealing was obtained at longer times. This is the result expected since HpaII-2 contains radioactivity at the end of both of its DNA strands. In contrast, most of the radioactivity in 2A entered hybrid, whereas none of that in 2B became double stranded in the presence of cRNA. Therefore, the strand of fragment 2A containing the 3'-terminal label was the polyoma DNA L strand, and the 3'-labeled strand of 2B was the E strand. From the relative positions of fragments 2A and 2B within polyoma DNA, using the convention that polarity of a DNA strand is its 5' to 3' direction, we conclude that the polyoma DNA L strand is oriented clockwise around the physical map (Fig. 1).

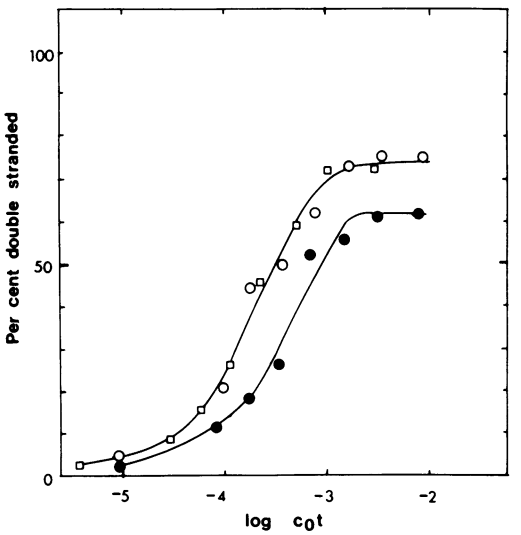

FIG. 3. *Reassociation kinetics of terminally iodinated restriction fragments. DNA fragments were denatured and reannealed, and the percentage of DNA reassociated was determined by hydroxylapatite chromatography as described in Materials and Methods. The data are plotted as a function of the logarithm of the product of the initial DNA concentration (C_0) and the time (t) of reannealing, in units of $m \cdot l^{-1} \cdot s$. C_0 was calculated from the estimated specific activity of the ^{125}I-labeled restriction fragments and was normalized to the equivalent concentration of full-length polyoma DNA by dividing the concentration of fragment used by the fractional length of the fragment. Symbols: □, HpaII-2; ○, 2A; ●, 2B.*

DISCUSSION

We have shown that the polyoma DNA L strand runs clockwise and the E strand runs counterclockwise around the physical map viewed as shown in Fig. 1. Since this work was completed, Summers (21) deduced the same strand orientation by an alternative procedure. Knowledge of the strand orientation enables us to assign polarities to the known transcripts of

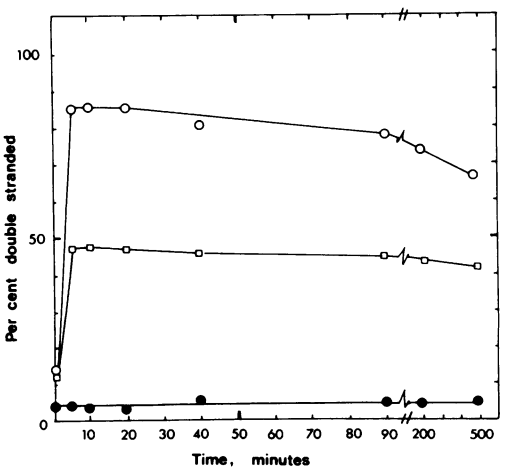

FIG. 4. *Annealing of terminally iodinated restriction fragments with asymmetric cRNA. Conditions of the hybridization are described in Materials and Methods. The fraction of radioactive DNA annealed to cRNA was measured by hydroxylapatite chromatography and is plotted as a function of reannealing time. Symbols: □, HpaII-2; ○, 2A; ●, 2B.*

polyoma DNA. Polyoma cRNA, by definition, has the same orientation as the E strand, and since *E. coli* RNA polymerase synthesizes RNA in the 5′ to 3′ direction, the enzyme must move counterclockwise around the circular template during in vitro transcription. More interesting are the implications of the strand orientation for in vivo transcription. During lytic infection of permissive mouse cells, the appearance of virus-specific RNA in the cytoplasm occurs in early and late phases. At early times, prior to the onset of viral DNA replication, RNA sequences complementary to slightly more than 50% of the E strand are found in the cytoplasm; these sequences are transcribed from a continuous region extending clockwise from about 71 to 25 units on the physical map (Fig. 1) (15) and are conventionally called "early" viral RNA. After the initiation of viral DNA replication, far larger amounts of RNA complementary to the L strand of the DNA are also found in the cytoplasm. These "late" RNA sequences hybridize to the DNA region between 25 and 71 map units (15). More recent experiments (A. E. Smith, S. T. Bayley, W. F. Mangel, H. Shure, T. Wheeler, and R. I. Kamen, FEBS Symposium, Proceeding of the 10th FEBS Meeting, Paris, 1975, in press) have shown that the early RNA sequences are contained in a single 20-21*S* poly(A)-terminated mRNA molecule, whereas the late RNA sequences comprise two molecular species, a 19*S* molecule complementary to the entire late region of the genome and a smaller 16*S* molecule containing 50 to 60% of the 19*S*, lacking sequences complementary to the L strand of HpaII-3. Because the early 20-21*S* RNA and the late 19*S* RNA are transcribed from opposite DNA strands, they must be synthesized in opposite directions. From the DNA strand orientation determined in this report, we can conclude that the 5′ ends of these early and late mRNA's must be near the HpaII-5/HpaII-3 juncture, whereas their 3′ ends lie in HpaII-6.

The localization of the 5′ ends of polyoma mRNA's near the HpaII-5/HpaII-3 juncture (71 map units) suggests an interesting control possibility. The origin of the bidirectional replication of polyoma DNA is at 71 ± 2% map units (5, 12). Since the switch from early to late phases of viral gene expression is coupled to DNA replication, it is tempting to suggest that a single region of the DNA contains sequences controlling the initiation of both DNA and RNA synthesis. The proximity of the origin of DNA replication and the possible promotors for RNA synthesis would allow both events to be controlled by a single DNA binding protein. The genetic organization of the monkey papova virus, simian virus 40, is strikingly similar to that described here for polyoma virus (7, 16). In the case of simian virus 40, it is likely that the viral T antigen is involved in the control of both DNA and RNA synthesis (4) and, moreover, that T antigen preferentially binds to viral DNA in the region of the origin of viral DNA replication (18).

As attractive as this hypothesis may appear as an explanation for coordinate control of macromolecular synthesis, it is at present limited by the fact that we cannot unambiguously identify the position of the 5′ ends of the stable viral mRNA's with the location of the start of viral transcription. Aloni and Aloni and Locker initially showed that all of the DNA of both simian virus 40 and polyoma is transcribed into RNA in infected cells (1, 2). Nuclear polyoma RNA is known to comprise total transcripts of both L and E strands (15); we have more recently shown that the polyoma L-strand nuclear transcript is very large, containing a majority of molecules longer than once around the DNA circle (R. Kamen, manuscript in preparation). It is thus likely that mRNA molecules are generated from larger nuclear RNA precursors by processing. The ultimate 5′ ends need not correspond to those of the nascent RNAs. A more complete understanding of the control of viral gene expression thus requires further experiments determining the map position of the 5′ ends of nuclear transcripts.

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