Strand-Specific Transcription of Polyoma Virus DNA Early in Productive Infection and in Transformed Cells

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The DNA strand origin of nuclear and cytoplasmic polyoma-specific RNA in productively infected mouse cells and in a line of polyoma-transformed hamster cells was determined by hybridization of unlabeled RNA with radioactively labeled separated strands of polyoma DNA. Early in the productive cycle (10 h postinfection) nuclear viral RNA is complementary to only about 40% of the E strand of viral DNA. No RNA complementary to the L strand was detected even when the RNA was first self-annealed to enrich for possible minor species. Early cytoplasmic RNA is complementary to the same 40% of the E strand. Thus, only that part of the polyoma genome which codes for early viral messenger RNA appears to be transcribed. Late in infection, nuclear viral RNA is complementary to most or all of the L strand and to at least 60% of the E strand. Late cytoplasmic viral RNA hybridizes to 40 to 45% of the E strand and 50 to 55% of the L strand. The transformed cell nuclear viral RNA is complementary to 60% of the E strand, whereas cytoplasmic RNA is complementary to 40% of the E strand and comprises the same polyoma-specific sequences as are found in RNA early in productive infection. No L strand transcripts could be detected. Thus, in the transformed cells and late in productive infection, viral RNA sequences in the cytoplasm are a specific subset of those in the nucleus.

During the late phase of the infectious cycle of polyoma virus or simian virus 40 extensive regions of the viral genomes are transcribed symmetrically (2, 3, 13, 14). Thus, both strands in a given region of DNA serve as template for the synthesis of RNA, and the RNA newly synthesized by this mechanism is self-complementary. In contrast, stable viral RNA species which accumulate in the cytoplasm of the infected cells, and which include the viral messenger RNA, are not self-complementary (13–15, 20). These findings led to the suggestion (2) that a control of the expression of viral genes may operate at a post-transcriptional level by the selection, from the symmetrical nuclear transcripts, of the specific viral RNAs which function as cytoplasmic messengers.

To test how general this proposed control mechanism may be, we studied the DNA strand origin of polyoma virus-specific RNA isolated from (i) lytically infected cells early in the infectious cycle, i.e., before the viral DNA is replicated (24) or integrated extensively (4, 11; Türler, personal communication) into cellular DNA; (ii) a line of baby hamster kidney cells

transformed by polyoma. Using the separated strands of radioactively labeled polyoma DNA to detect virus-specific RNA by hybridization, we found no evidence that the polyoma-specific RNA either early in lytic infection or in the transformed cells is synthesized symmetrically. With the clear proviso that viral RNA with a very short life would not be detected in this assay, we tentatively conclude that the synthesis of polyoma messenger RNA can be regulated at the level of transcription. Symmetrical polyoma-specific RNA was detected at later times in the lytic cycle, even when the replication of viral DNA was inhibited by 5-fluorodeoxyuridine.

MATERIALS AND METHODS

Plaque-purified polyoma virus was grown on primary baby mouse kidney (MK) cell cultures (27). Superhelical polyoma DNA labeled with [³H]thymidine was prepared according to Germond et al. (10). Py BHK₁ is a line of baby hamster kidney cells transformed by polyoma, containing on average four polyoma genomes per cell and a relatively high proportion of polyoma-specific RNA (Maxwell, submitted for publication).

Isolation of RNA. MK cells infected with polyoma virus (10 to 20 PFU/cell) were washed with cold

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isotonic buffer (0.25 M sucrose, 0.025 M NaCl, 0.005 M MgCl$_2$, 0.01 M triethanolamine, pH 7.4) and lysed in the same buffer containing 1% Nonidet P-40 (8). The lysate was centrifuged at 12,000 × g for 20 min, and RNA in the supernatant ("cytoplasmic extract") was extracted at room temperature with 1% (wt/vol) sodium dodecyl sulfate-phenol:chloroform:isoamylalcohol in the ratio of 50:50:1 as previously described (8, 17). The nuclear pellet was washed by suspension in phosphate-buffered saline and centrifugation. Nuclear RNA was extracted as previously described (1, 22) with hot (65 C) phenol-1% sodium dodecyl sulfate in 0.01 M sodium acetate, pH 5.1. RNA extracts were precipitated twice with ethanol. RNA preparations were incubated at 37 C for 30 min with 50 µg of deoxyribonuclease I (ribonuclease free, Worthington) per ml, reextracted with hot phenol, precipitated twice with ethanol, and further purified by precipitation with 2 M LiCl overnight at 0 C. Py BHK$_1$ cell RNA was isolated as described (Maxwell, submitted for publication).

Separation of the complementary strands of polyoma DNA. The method followed closely that of Sambrook et al. (19). Superhelical DNA from nonplaque-purified stocks of our strain of polyoma virus was found (J. Lewis and R. Portmann, personal communication) to be transcribed asymmetrically in vitro by RNA polymerase of *Escherichia coli* (Kamen et al. [13] have shown that DNA from plaque-purified polyoma virus is transcribed asymmetrically only at elevated salt concentrations). The RNA synthesis mixture (1 ml), containing 0.05 M Tris(pH 7.9), 0.08 M NaCl, 0.04 M KCl, 0.01 M MgCl$_2$, 0.2 mM EDTA, 2% glycerol, 1 mM dithiothreitol, 5 mM each of the four ribonucleoside triphosphates, 100 µg of polyoma DNA I, and 50 µg of RNA polymerase (gift of R. Portmann), was incubated for 1 h at 37 C. After phenol extraction and digestion with deoxyribonuclease, RNA was annealed in 0.37 M NaCl, 0.037 M sodium citrate at 68 C for 1 h, then chromatographed on Whatman CF11 cellulose (9). (We use the word "anneal" to mean: incubate under conditions which favor the formation of base pairing in nucleic acids.) Most (85 to 90%) of the RNA eluted as single-stranded RNA. Five micrograms of singly nicked (5) polyoma DNA (from plaque-purified stocks) labeled with [³H]thymidine (specific activity 3.5 × 10⁵ counts/min per µg of DNA) was denatured at 100 C, then incubated with a 10-fold excess of in vitro synthesized single-stranded RNA at 60 C for 20 min in a solution (1 ml) containing 0.01 M Tris-hydrochloride (pH 7.5), 0.001 M EDTA, 0.045 M NaCl. DNA-RNA hybrids, containing about half the added DNA, were separated from single-stranded DNA by chromatography on hydroxyapatite (Bio-Rad HTP) at 60 C. RNA was removed from each fraction with 0.7 M NaOH at 37 C for 12 h. After neutralization with HCl, each DNA preparation was annealed for 30 h at 68 C in 0.6 M NaCl, and single-stranded DNA (70 to 85% of the total) was reisolated using hydroxyapatite. The DNA which did not hybridize with in vitro RNA was one of the strands of polyoma DNA (termed the E strand, since it is complementary to early viral RNA). The DNA which did hybridize was the other strand (L strand).

E and L strand DNA, when tested immediately after the first hydroxyapatite separation, were of full polyoma genome length as judged by co-sedimentation with singly nicked polyoma DNA in alkaline sucrose gradients. After E strand or L strand DNA was annealed alone under the conditions given below for hybridization, about 5% of the DNA was resistant to digestion by single-strand-specific S$_1$ nuclease (23). When annealed together the two strands became more than 70% resistant to S$_1$.

Hybridizations. Hybridizations were done in a solution (0.125 ml) containing 0.02 M Tris-hydrochloride (pH 7.5 at room temperature), 0.001 M EDTA, 1 M NaCl, 5 µg of denatured calf thymus DNA, about 1 ng (350 counts/min) of E or L strand DNA, and the indicated amount of RNA. The mixtures were incubated at 70 C for 60 h (about 40 × C$_0$t$_{1/2}$ for the reassociation of 1 ng of a strand of polyoma DNA with 1 ng of the complementary strand). After dilution to a final volume of 1 ml containing 0.05 M sodium acetate (pH 4.5), 0.001 M ZnSO$_4$, and addition of 2 U of S$_1$ nuclease per 10 µg of total nucleic acid present (1 U of S$_1$ nuclease degrades 10 µg of single-stranded DNA in 10 min at 37 C), the reaction was incubated at 37 C for 30 min. After the addition of 10 µg of calf thymus DNA carrier, trichloroacetic acid-insoluble material was collected on Whatman GF/B filters and washed, and the radioactivity was counted during 20-min periods. The total radioactivity in E or L strand in each experiment was determined by incubating duplicate mixtures with 10 µg of yeast RNA and omitting the S$_1$ nuclease digestion. The amount of E or L strand rendered resistant to S$_1$ is given as a percentage of this total, without deduction of the background (2 to 8% of the input) of S$_1$ nuclease-resistant radioactivity seen after annealing without RNA or with RNA from uninfected MK cells. In this assay the proportion of input DNA protected by saturating amounts of RNA gives a measure of the fraction of that DNA strand complementary to the RNA being tested. If a small amount of RNA complementary to the major virus-specific RNA is present, only the more abundant RNA may be detected by this method; RNA-RNA association could compete with RNA-DNA hybridization and prevent the minor RNA species from hybridizing with the DNA probe. To detect low concentrations of an RNA complementary to the major species, the following procedure (see references 2 and 13) was used.

Isolation of self-complementary RNAs. RNA at a concentration of 5 to 7 mg/ml in 0.01 M Tris-hydrochloride (pH 7.4), 0.001 M EDTA, 1 M NaCl was annealed at 70 C for 24 h. The solution was diluted 10-fold with 0.01 M Tris-hydrochloride (pH 7.5), the concentration of NaCl was adjusted to 0.3 M, and single-stranded RNA was digested with 100 µg of pancreatic ribonuclease per ml at 37 C for 1 h. After treatment with Pronase at 100 µg/ml, 37 C for 1 h, and two extractions with phenol/chloroform (1:1), the ribonuclease-resistant RNA was recovered by precipitation with 50 µg of yeast RNA carrier per ml and 2 volumes of ethanol. By this means most of the major viral RNA species, having no complement, was degraded, whereas any RNA complementary to it would be relatively enriched. The presence and nature of

polyoma-specific sequences in the ribonuclease-resistant RNA was determined by hybridization of the denatured (100 C, 4 min) RNA with labeled E and L strand DNA as above.

RESULTS

Early lytic polyoma-specific RNA. Cytoplasmic RNA was isolated from MK cells 10 h after infection in the presence of 6×10^{-5} M 5-fluorodeoxyuridine and annealed in increasing amounts with E strand or L strand DNA. At saturation about 40% of the E strand entered hybrids (Fig. 1A). No hybridization to the L strand was seen. RNA extracted from unin-
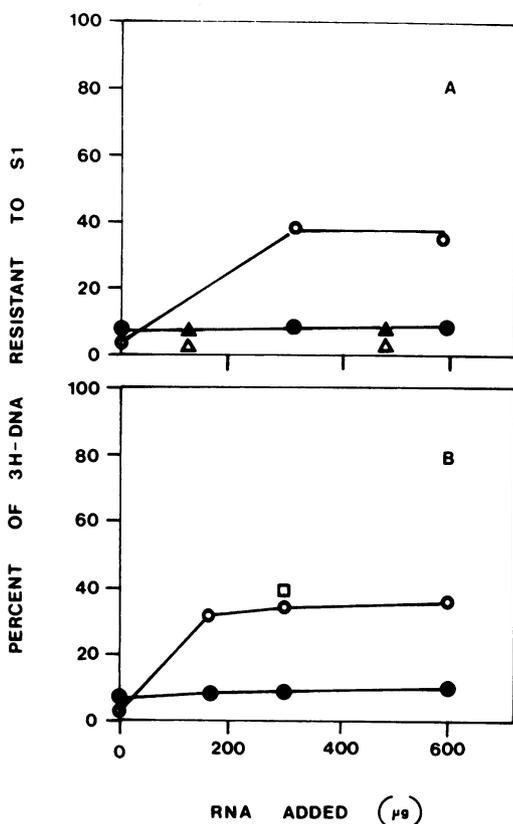
FIG. 1. *Hybridization of early lytic polyoma-specific RNA with the separated strands of polyoma DNA. RNA was isolated from (A) cytoplasm or (B) nuclei 10 h after infection in the presence of 6×10^{-5} M 5-fluorodeoxyuridine and annealed in increasing amounts with labeled E strand (empty symbols) or L strand (filled symbols) DNA. The percentage of DNA in hybrids was measured by resistance to digestion by S_1 nuclease. Symbols: infected cell RNA (O and ●), uninfected whole MK cell RNA (△ and ▲). The point (□) shows the hybridization of a mixture containing 300 μg of nuclear RNA and 300 μg of cytoplasmic RNA with E strand DNA.*

fected MK cells showed no hybridization with either polyoma DNA strand. Nuclear RNA isolated at the same time also formed hybrids with a maximum of 40% of the E strand and did not hybridize significantly to the L strand (Fig. 1B). When this early nuclear RNA was self-annealed (see above) and the ribonuclease-resistant fraction was denatured and tested for the presence of polyoma-specific sequences, none was detected (Table 1). Cytoplasmic and nuclear RNA extracted 10 h postinfection were mixed and annealed together with E strand DNA; no additive effect on the proportion of E strand in hybrids was seen (Fig. 1A and B). Therefore, the polyoma-specific RNA detected in nuclei early after infection comprises the same sequences as the E-strand transcripts which accumulate in the infected cell cytoplasm.

Late lytic polyoma-specific RNA. RNA extracted from the cytoplasm of MK cells 30 h after infection hybridized with a maximum of 40 to 45% of the E strand and about 55% of the L strand (Fig. 2A). In contrast nuclear RNA isolated at this time hybridized to only a low level, about 25%, with the E strand, whereas nearly 100% of the L strand entered hybrids (Fig. 2B). Late nuclear RNA should contain at least the transcript of 45% of the E strand seen in cytoplasmic RNA, since polyoma-specific RNA is synthesized in the nucleus. Late nuclear RNA therefore seemed to contain self-complementary polyoma-specific sequences. The data could not be explained by contamination of nuclear RNA preparations by viral DNA. Self-complementary viral RNA was demonstrated

TABLE 1. *Hybridization of self-annealed polyoma-specific RNAs with the separated strands of polyoma DNA*

Source of nuclear RNA	RNA concentration during annealing (mg/ml)	RNA in hybridization (μg)[a]	DNA resistant to S_1 (%)	
			E strand	L strand
No RNA			2–5	3–8
Late lytic	5	70	59	57
FU lytic[b]	5	125	42	38
Early (10 h) lytic	5	300	6	3
Early (10 h) lytic	7	200	7	5
Transformed	6	160	7	6
Transformed	6	200	4	8

[a] The amounts of RNA in each hybridization mixture are given as the equivalent amount of nuclear RNA before annealing and digestion by ribonuclease.

[b] FU, 15 h postinfection in the presence of 6×10^{-5} M 5-fluorodeoxyuridine.
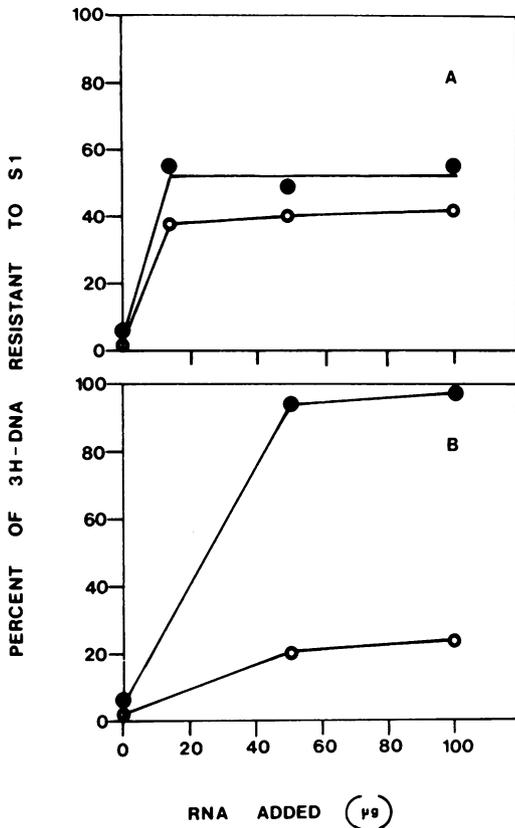
FIG. 2. *Hybridization of late lytic polyoma-specific RNA with the strands of polyoma DNA. RNA isolated 30 h after infection from (A) cytoplasm or (B) nuclei was annealed with E strand (O) or L strand (●) DNA, and the amount of DNA rendered resistant to S_1 nuclease digestion was determined.*

directly by self-annealing late nuclear RNA, isolating the double-stranded RNA, and measuring its ability after denaturation to hybridize to the E and L strands (Table 1). The double-stranded RNA was found to contain polyoma-specific sequences representing 55 to 60% of the viral genome.

Polyoma-specific RNA synthesized 15 h postinfection in the presence of 5-fluorodeoxyuridine. Fluorodeoxyuridine has previously been employed as an inhibitor of the replication of polyoma virus or simian virus 40 DNA and of the synthesis of late viral messenger RNA and proteins (12, 16, 18, 19). Figure 3A shows the hybridization of cytoplasmic RNA isolated 15 h after infection of MK cells in the presence of 5-fluorodeoxyuridine, with the strands of polyoma DNA. Forty-three percent of the E strand, but very little of the L strand, formed hybrids. Nuclear RNA extracted under the same conditions hybridized with at least

85% of the L strand and with about 20% of the E strand (Fig. 3B). Qualitatively the nuclear RNA closely resembled late nuclear RNA (cf. Fig. 2B); it contained self-complementary polyoma-specific sequences (Table 1). Synthesis of L strand transcripts therefore can occur in the absence of extensive viral DNA replication, although a small amount of DNA replication cannot be excluded. It is noteworthy that under these conditions the L strand transcripts seem to be largely confined to the nucleus.

Viral RNA in cells transformed by polyoma. Cells transformed by polyoma virus or by simian virus 40 contain viral DNA which is believed (7, 21) to be integrated covalently into the cellular genome. The transforming viral genomes are transcribed (6). To test for the presence of symmetrically transcribed polyoma-specific RNA in transformed cells, RNA from the nuclear and cytoplasmic fractions of Py BHK$_1$ cells was hybridized with polyoma E and L strand DNA (Fig. 4). Saturating amounts of Py BHK$_1$ cytoplasmic RNA hybridized with
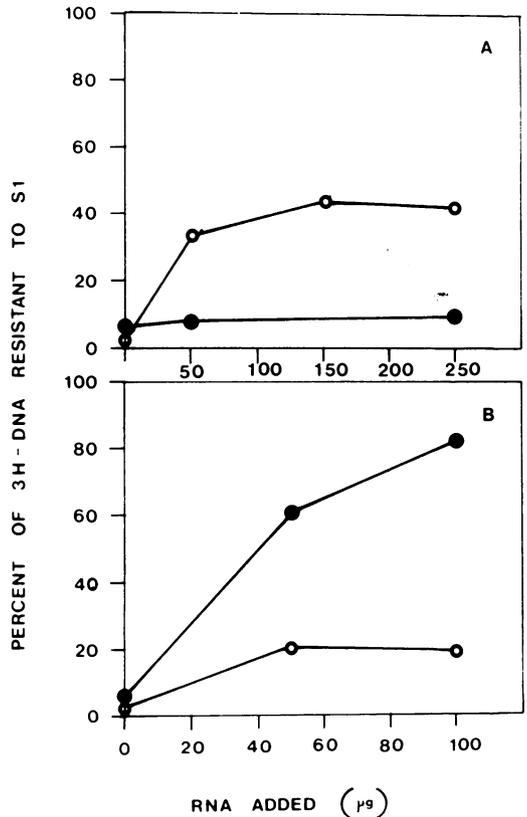
FIG. 3. *Hybridization of (A) cytoplasmic and (B) nuclear RNA isolated 15 h after productive infection in the presence of 6×10^{-5} M 5-fluorodeoxyuridine, with polyoma E strand (O) or L strand (●) DNA.*
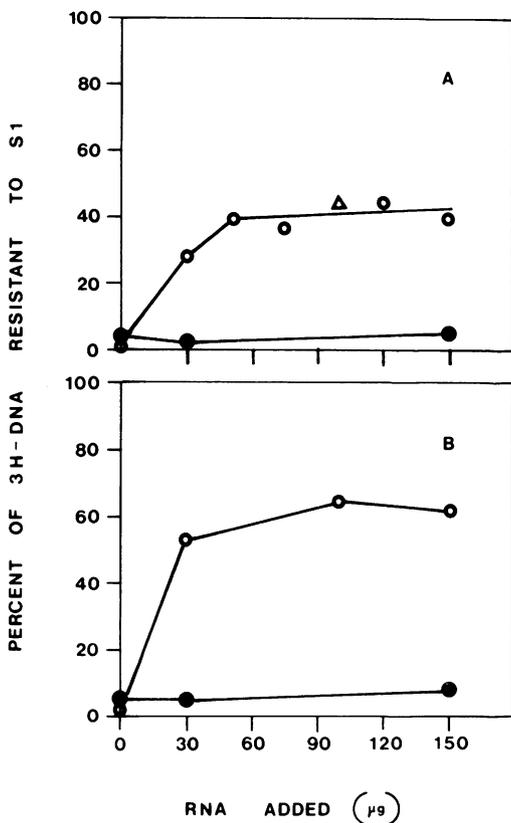
FIG. 4. *Polyoma-specific RNA in the transformed cell line Py BHK₁. RNA isolated from (A) cytoplasm or (B) nuclei was annealed with the separated strands of polyoma DNA. The amount of E strand (O) or L strand (●) in hybrids was measured as in Fig. 1. The point (△) shows the hybridization of a mixture of 100 µg of Py BHK₁ cytoplasmic RNA together with 100 µg of cytoplasmic RNA isolated 15 h after productive infection in the presence of 5-fluorodeoxyuridine (see Fig. 3a) with E strand DNA.*

about 40% of the E strand. No hybridization with the L strand was detected. The viral RNAs from transformed and early lytic cytoplasm represent essentially the same region of the E strand, since no additive effect was seen when the two RNAs (100 µg of each) were hybridized together with E strand DNA. Nuclear RNA from Py BHK₁ hybridized with substantially more of E strand DNA than did cytoplasmic RNA, with a maximum of more than 60% of the E strand entering hybrids. The transformed cell nuclear RNA appeared to contain no L strand transcripts. Since this RNA contained extensive E strand transcript, a small amount of RNA complementary to the L strand may have escaped detection due to RNA-RNA base pairing. However when the ribonuclease-resistant frac-

tion of self-annealed Py BHK₁ nuclear RNA was denatured and hybridized with E and L strand, no hybridization to either DNA was detected (Table 1).

DISCUSSION

Our experiments show that early in the infectious cycle (10 h postinfection), nuclear polyoma-specific RNA is complementary to only about 40% of the E strand of viral DNA. No RNA complementary to the L strand could be detected. Early cytoplasmic RNA is complementary to the same 40% of the E strand. These findings suggest that, early in infection, only that part of the polyoma genome which codes for early messenger RNA is transcribed.

In contrast, late nuclear polyoma-specific RNA contains transcripts of most or all of the L strand, as well as a lower concentration of RNA complementary to at least 60% of the E strand. Thus, as Aloni and Locker (3) and Kamen et al. (13) have already shown, late nuclear RNA contains self-complementary viral sequences. Late cytoplasmic RNA, on the other hand, contains sequences complementary to a maximum of 45% of the E strand and 55% of the L strand. Therefore, late in infection, the appearance of viral RNA in the cytoplasm cannot be regulated solely at the level of RNA synthesis. Regulation seems also to occur by specific selection or transport of RNA from nucleus to cytoplasm.

RNA from a line of polyoma-transformed BHK cells, like early lytic RNA, contains transcripts of only the E strand. However, more of this strand is represented in nuclear RNA (60 to 65%) than in cytoplasmic RNA (40 to 45%), suggesting that also in transformed cells posttranscriptional selection mechanisms are present. A similar conclusion was drawn from Maxwell's (submitted for publication) studies of the radioactive labeling and sedimentation properties of polyoma-specific RNA in Py BHK₁ cells. However, there is no proof that large viral RNAs, either in transformed cells or late in productive infection, are precursors of smaller cytoplasmic viral RNA.

Our conclusion that nuclear RNA isolated 10 h after lytic infection or from transformed cells contains no detectable self-complementary viral sequences is based on two types of experiment. Firstly, saturation hybridization with these RNAs protected only a specific fraction of the viral E strand from digestion by S₁ nuclease. Secondly, when each RNA was self-annealed and the resulting ribonuclease-resistant RNA tested for content of polyoma-specific se-

quences, none was found. Together, these results suggest that the early lytic and transformed cell nuclear RNAs contain neither "anti-early" nor true late transcripts of the L strand. Clearly the hybridization methods used could fail to detect L strand transcripts if present at very low concentration. However, the finding of large amounts of anti-sense L strand transcripts in nuclear RNA 15 h or more after infection seems to suggest that once synthesized such RNA is relatively stable in the nucleus. Knowing the concentration of nuclear RNA required to give detectable hybridization with the polyoma DNA strands (Fig. 1 and 4), we estimate that a complementary RNA species amounting to respectively 10% (early lytic) or 5% (transformed) of the major nuclear viral RNA would have been detected.

Inhibition of viral DNA replication by 5-fluorodeoxyuridine did not prevent the synthesis of late polyoma-specific RNA, a conclusion also suggested by the sedimentation studies of Weil et al. (25). Kamen et al. (13) found that nuclei of cytosine arabinoside-treated cells 20 h after infection with polyoma contain abundant transcripts of most of the E strand as well as less abundant L strand transcripts. The viral RNA accumulating in the presence of cytosine arabinoside or 5-fluorodeoxyuridine thus clearly differs from true early (10 h postinfection) RNA. Therefore, the presence of these inhibitors of DNA synthesis late in infection may alter viral transcription (cytosine arabinoside), or may not completely block the onset of the late pattern of transcription (5'-fluorodeoxyuridine).

It is noteworthy that the sedimentation properties of polyoma-specific RNA from lytically infected cells are consistent with our interpretation of the hybridization experiments reported here. The bulk of viral RNA labeled early in infection, and isolated from either whole cells or cytoplasm, sediments in the region of $19S$ (26). The estimated molecular weight of this $19S$ RNA (approximately 7×10^5) is close to the expected value for a transcript of 40 to 45% of one strand of polyoma DNA (0.4 to 0.45 $\times$ 1.5 $\times$ 10^6 = 6×10^5 to 7×10^5). Late in infection most of the newly synthesized viral RNA sediments at a rate greater than $26S$ (corresponding to a length equal to or greater than the polyoma genome, reference 1), whereas stable cytoplasmic viral RNA sediments at 16–19S (8). Therefore, control of both the size and the sequence composition of late (but not early) cytoplasmic polyoma-specific RNA seems to involve specific cleavage, degradation, or transport mechanisms.

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