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Mapping of the three species of polyoma mRNA*

(restriction enzymes/hybridization)

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ABSTRACT **The polyoma mRNAs present in the cytoplasm of primary cultures of mouse kidney cells during lytic infection were characterized by sedimentation velocity analysis and by hybridization to polyoma DNA fragments generated by a specific endonuclease of *Hemophilus parainfluenzae* (*Hpa* II). Three distinct species of polyoma mRNAs were found and mapped on the viral genome: (*a*) early 19S polyoma mRNA, which is complementary to about 45% of the early strand and maps from approximately 78 (5′ end) to 23 (3′ end) map units; and (*b*) two related late mRNAs, a larger species (19 S), which is complementary to about 44% of the late strand and extends from approximately 70 (5′ end) to 26 (3′ end) map units, and a smaller species (16 S), which is complementary to about 31% and maps between 61 (5′ end) and 30 (3′ end) map units. Thus, the entire sequence of the late 16S mRNA is comprised within the late 19S mRNA, a finding which was also supported by two-dimensional chromatographic analysis of the RNase T_1 oligonucleotides of the two late mRNAs.**

Polyoma mRNA synthesized early in lytic infection (i.e., earlier than 12 hr after infection) sediments in sucrose density gradients as a homogeneous band with a sedimentation coefficient of 19 S and is designated early 19S polyoma mRNA (1). Polyoma mRNA synthesized late, when the rate of viral DNA synthesis has reached a maximum (i.e., later than 25 hr after infection), comprises two classes of RNA with sedimentation coefficients of 16 S and 19 S, which are designated late 16S and 19S polyoma mRNAs (2). As determined by exhaustive hybridization to polyoma DNA, early 19S mRNA (labeled from 8 to 11 hr after infection) accounts for about 0.03% and late mRNAs (labeled from 27 to 30 hr after infection) account for about 2% of the total radioactivity in cytoplasmic RNA. The sedimentation and hybridization patterns were closely similar for polyoma mRNAs isolated from polysomes (2) by chromatography on oligo(dT)-cellulose (3) or from total cytoplasm.

By using separated strands of polyoma DNA and of DNA fragments generated by an endonuclease of *Hemophilus parainfluenzae* (*Hpa* II), Kamen *et al.* (4) concluded that cytoplasmic polyoma-specific RNAs synthesized early and late in lytic infection are transcribed from opposite strands, with both transcripts starting near the origin of DNA replication in fragment 5 (5) and terminating in fragment 6 [see the physical map (6) in Fig. 3].

To identify the different species of polyoma mRNAs synthesized during lytic infection in primary cultures of mouse kidney cells and to map them on the viral genome we have combined sedimentation analysis of cytoplasmic RNA with hybridization to *Hpa* II fragments of polyoma DNA. Here, we present evidence for the existence of three distinct species of polyoma mRNAs, i.e., early 19S, late 16S, and late 19S mRNA, and show that the complete sequence of late 16S mRNA is comprised within late 19S mRNA.

Abbreviations: *Hpa* II, endonuclease from *Hemophilus parainfluenzae;* SSC, standard saline citrate (0.15 M sodium chloride–0.015 M sodium citrate, pH 7); x × SSC, concentration of the solution is x times that of SSC.

* Part of these results have been published in preliminary form [(1975) *Experientia* 31, 746 Abstr.] and were presented at the 9th Meeting of the European Tumor Virus Group in Mariehamn (Finland) in May 1975.

MATERIALS AND METHODS

Cultures of mouse kidney cells (1, 7) were infected 2 days after confluence with 0.4 ml of a suspension of wild type, plaque-purified polyoma virus containing 5×10^8 plaque-forming units (PFU)/ml.

Purified polyoma DNA I (8) was digested with *Hpa* II, and the fragments were separated by polyacrylamide gel electrophoresis (9). They were eluted from the gel electrophoretically into dialysis bags and concentrated by ethanol precipitation.

Polyoma DNA I was denatured and fixed on cellulose filters (30 mm) as described (10). *Hpa* II fragments were denatured in 0.2 M NaOH for 10 min at room temperature, neutralized with acetic acid, and fixed on filters by the same method. The 30-mm filters were cut into 25 square filters of 3.5 mm, each carrying 0.2 μg of polyoma DNA or 0.05–0.1 μg of an *Hpa* II fragment. These quantities assured DNA excess for hybridization. Using radioactive *Hpa* II fragments, we found that 90% of the input DNA of fragments 1, 2, and 4 were fixed on the filters, but only 65% of fragment 6.

Early RNA was labeled from 8 to 11 hr after infection with 500 μCi of [5-^{3}H]uridine (25 Ci/mmol, NEN Chemicals, Langen, Germany) in 1 ml of culture medium. Late RNA was labeled from 27 to 30 hr after infection with 250 μCi of [^{3}H]uridine in 1 ml of medium, or with 100 μCi of [^{32}P]orthophosphate (91 Ci/mg of P, Radiochemical Centre, Amersham, England) in 1 ml of phosphate-free medium.

Cytoplasmic RNA was extracted with phenol-choroform-isoamyl alcohol (2). Approximately 500 μg of RNA were layered onto a 10–25% (wt/vol) linear sucrose gradient in 0.05 M NaCl, 0.01 M Tris·HCl, 1 mM EDTA, pH 7.0, and centrifuged in a Spinco SW 40 rotor at 29,000 rpm for 16 hr at 7°. Fractions were collected from the bottom of the tube.

Hybridization of RNA to DNA filters was done either in 4 × SSC (SSC is 0.15 M NaCl, 0.015 M Na-citrate, pH 7) for 48 hr at 65° (10) followed by digestion with pancreatic RNase (EC 3.1.4.22; 20 μg/ml; Worthington Biochemical Corp.) or in 50% (vol/vol) formamide, 2 × SSC, for 60 hr at 37° (11). After hybridization in formamide, filters were washed once in SSC containing 0.5% sodium dodecyl sulfate, then incubated in the hybridization buffer for 30 min at 37°, followed by two washings in 0.1 × SSC. These filters were either used for elution of the hybridized RNA or they were assayed for radioactivity and then treated with RNase. Assay of the filters in toluene scintillation liquid followed by two washings with toluene and ether did not reduce the efficiency of the subsequent RNase treatment. Polyoma RNA is partially degraded during hybridization in 4 × SSC at 65°; although hybridization in formamide is somewhat less efficient (11), polyoma mRNAs

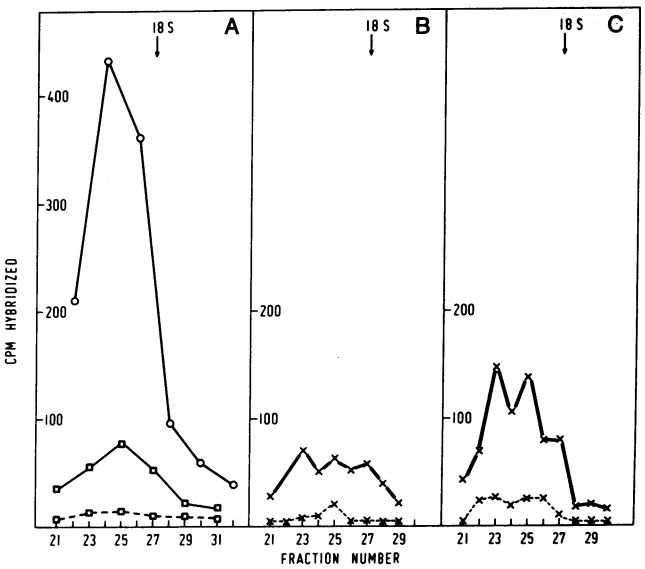

FIG. 1. Hybridization of early 19S mRNA containing fractions to individual *Hpa* II fragments of polyoma DNA. Early RNA was labeled, extracted, and sedimented through sucrose gradients; aliquots of the fractions were hybridized to total polyoma DNA to detect early 19S mRNA. (A) The bulk of the odd fractions containing 19S mRNA was hybridized in formamide to fragment 1 (late) (□). Radioactivity of the filters was measured before (solid line) and after (dashed line) RNase digestion. The bulk of the even fractions was hybridized to fragment 2 (early); radioactivity was determined without RNase digestion (○).

After the first hybridization, every fraction was hybridized to a filter with fragment 5 and to a filter with fragment 6. (B) Hybridization to fragment 5 without (solid line) and with (dashed line) RNase digestion. (C) Hybridization to fragment 6 without (solid line) and with (dashed line) RNase digestion.

remained intact, as judged by sedimentation in sucrose gradients.

Polyoma RNA was eluted from the filters that had been washed as described above by incubating twice in warmed 2 mM EDTA, pH 7.2, for 3 min at 85° (12). The first eluate contained 60–70% of the hybridized polyoma RNA and the second about 10%, whereas 10–20% of the radioactivity remained on the filter.

RESULTS

Early 19S mRNA. Early RNA was labeled with [^{3}H]uridine from 8 to 11 hr after infection, extracted, and sedimented as described in *Materials and Methods*. To localize polyoma mRNA, we hybridized an aliquot of each fraction to total polyoma DNA. The bulk of the odd fractions containing polyoma RNA was hybridized in formamide to filters with *Hpa* II fragment 1 (late) and that of the even fractions to filters with fragment 2 (early). More than 80% of polyoma RNA hybridized to fragment 2, forming a sharp band around 19 S (Fig. 1A). However, some hybridization was also observed with fragment 1; we do not know whether this was due to synthesis of very small amounts of late 19S mRNA or to some "anti-late" RNA hybridizing to the early strand of fragment 1. In a second hybridization every fraction was hybridized with a filter of fragment 5 and a filter of fragment 6. Fragment 5 (Fig. 1B) bound less RNA than fragment 6 (Fig. 1C), both before and after RNase treatment. The expected difference in hybridization between the odd fractions (which had not been hybridized to fragment 2) and the even fractions (which had been hybridized to fragment 2) was also more pronounced for fragment 6 than for fragment 5. Similar results were obtained with another preparation of early 19S mRNA that was hybridized in equal parts to filters with fragments 2, 5, and 6. The results (Table 1) show that before RNase treatment fragments 2 and 6 bound similar amounts of RNA, whereas fragment 5 bound only one-third of that amount. After RNase treatment fragment 6 again retained more RNA than did fragment 5. The RNase-resistant radioactivity on fragment 2 may seem quite low. It should be pointed out, however, that with late RNA hybridized to total polyoma DNA only 50% of the RNA retained before RNase treatment was RNase-resistant. This seems to be due to the fact that the fixed DNA is not freely available for ideal hybrid formation (unpublished observation).

Table 1. Hybridization of early 19S mRNA (labeled with [^{3}H]uridine from 8 to 11 hr after infection)

Hpa II fragment on filter	cpm hybridized* Before RNase	cpm hybridized* After RNase	% RNase-resistant
2	206†	43†	20.8
5	75	7	9.3
6	242	14	5.8
No DNA	66‡	6‡	

* Filters of the different fragments were hybridized in 50% formamide in separate tubes, each containing 800,000 cpm of RNA.
† Sum of cpm of two filters from which twice the value of the blank filter in the same tube was subtracted.
‡ Mean value of all blank filters.

To test whether early 19S mRNA is the contiguous transcript of the early region, we hybridized early 19S mRNA in formamide to either fragment 2 or 4. Hybridized RNA was eluted and aliquots of the eluates were rehybridized to fragments 2 and 4. The results (Table 2) show that both eluates rehybridized in the same proportion to the two fragments (the theoretical ratio of fragment 2 to fragment 4 is 1.6). This observation indicates that there is only one species of early 19S mRNA. The low proportion of rehybridization seems to be due to detachment of DNA from the filters during elution of RNA and to hybrid formation in solution (unpublished observation).

By sedimentation in dimethylsulfoxide-sucrose gradients, the molecular weight of early 19S mRNA was estimated to be around 700,000 (1), corresponding to about 2,000 nucleotides. From length measurements by electron microscopy, polyoma DNA I has been estimated to contain about 4,600 base pairs (Hirt, personal communication; and refs. 13 and 14). Based on this value, early 19S mRNA is the transcript of 44% of one strand. Our hybridization results suggest that early 19S mRNA is transcribed from fragments 4, 8, 7, and 2 and partially (about

Table 2. Rehybridization of early 19S mRNA eluted from fragments 2 and 4*

RNA eluted from fragment	cpm/tube	% cpm hybridized to fragment 2	% cpm hybridized to fragment 4	Ratio of fragment 2 to fragment 4
2	325	5.6	6.4	0.9
4	350	4.6	5.0	0.9

* Aliquots of early 19S mRNA were hybridized in formamide to filters with fragments 2 and 4. Hybridized RNA was eluted from the filters and each eluate was rehybridized in two equal parts to filters with fragments 2 and 4, respectively, in 4 × SSC followed by RNase digestion.

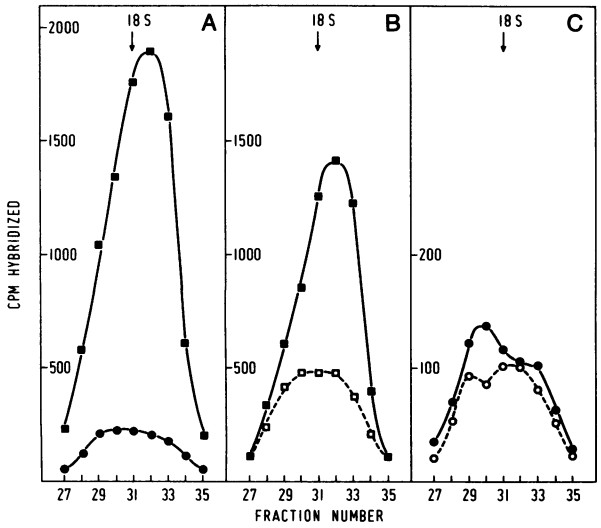

FIG. 2. Hybridization of late polyoma mRNA-containing fractions to individual *Hpa* II fragments. Late RNA was labeled, extracted, and sedimented through sucrose gradients; late polyoma mRNAs were detected by hybridizing aliquots of each fraction to total polyoma DNA. Other aliquots of the polyoma mRNA-containing fractions were then hybridized separately to *Hpa* II fragments 1, 2, 3, and 4 in 4 × SSC followed by RNase digestion. (A) Hybridization to the late fragments 1 and 3 (■) and to the early fragments 2 and 4 (●). (B) Hybridization to fragment 1 (■) and fragment 3 (□). (C) Hybridization to fragment 2 (●) and fragment 4 (○).

3 map units) from fragment 6, which comprise altogether 45% of the genome. By the methods used it was not possible to localize more precisely the beginning and end of transcription of early 19S mRNA.

Late mRNAs. Late RNA was labeled with [^{3}H]uridine from 27 to 30 hr after infection, extracted, and sedimented as described above. An aliquot of each fraction was first hybridized to total polyoma DNA. Other aliquots of the fractions containing polyoma RNA were then hybridized separately to *Hpa* II fragments 1, 2, 3, and 4 (in 4 × SSC followed by RNase treatment). Fig. 2A shows that polyoma RNA hybridized mostly to fragments 1 and 3; this result suggests that the two classes of late mRNA (2) are both transcribed from the late region. Fragment 1 bound more RNA than did fragment 3; the ratio of RNA bound to fragment 1 over RNA bound to fragment 3 increased from 19S to 16S RNA (Fig. 2B). Small amounts of RNA hybridizing to fragments 2 and 4 (early mRNA) were found in a rather broad peak (Fig. 2A) and hybridized in similar proportions to fragment 2 and 4 (Fig. 2C). Hybridization of polyoma RNA in the 16S region to fragments 2 and 4 may be due to increased turnover of early RNA late in infection (1).

To characterize the late mRNAs further, we pooled and analyzed the fractions of the sucrose gradient containing 19S and 16S RNA as follows.

Late 16S mRNA. The pooled fractions containing 16S RNA were hybridized in formamide to filters with fragments 1, 3, 5, and 6. The filters were washed and radioactivity was determined. They were treated with RNase and assayed for radioactivity again (Table 3). Before RNase treatment, fragments 1 and 3 bound similar amounts of RNA; fragments 5 and 6 bound 10–20% of that amount. After RNase treatment, fragment 1 retained 3.8 times more RNA than did fragment 3. More than 90% of the RNA was removed from fragment 5 or 6 by the RNase treatment. If one compares the RNase-resistant radioactivity on fragment 5 or 6 to that on fragment 1, it corresponds to a specific hybridization of about 0.5 map unit or 25 nucleotides for each of the two fragments. In three independent experiments the ratio of RNase-resistant radioactivity bound to fragment 1 over that bound to fragment 3 was determined seven times. The average value found was 3.3 ± 0.3.

Table 3. Hybridization of late 16S mRNA (labeled with [^{3}H]uridine from 27 to 30 hr after infection)

Hpa II fragment on filter	cpm hybridized* Before RNase	cpm hybridized* After RNase	% RNase-resistant	Ratio of fragment 1 to fragment 3
1	1575	672	42.7	3.8
3	1436	159	11.2	
5	171	12		
6	238	13		
No DNA	2	0		

* Each tube contained 40,000 cpm of RNA. For details, see footnotes of Table 1.

To test whether there is only one species of late 16S mRNA, we did the same type of experiment as described for early 19S mRNA. The results (Table 4) indicate that in the first hybridization both fragments 1 and 3 bound the same species of RNA, which, after elution, rehybridized to the two fragments in similar ratios as in the first hybridization.

From sedimentation in dimethylsulfoxide-sucrose gradients, the molecular weight of late 16S mRNA was estimated to be about 500,000 (2). This corresponds to approximately 1,400 nucleotides or to a transcript of 31% of the late strand. According to the hybridization results, this has to be divided between fragments 1 and 3 with a ratio of 3.3 to 1, yielding 24 map units on fragment 1 and 7 units on fragment 3. On the physical map shown in Fig. 3, late 16S mRNA is transcribed counterclockwise (4), the 5′ end being in fragment 3 near 61 units and the 3′ end in fragment 1 near 30 units. Taking into account the size estimate and the hybridization to fragments 1 and 3 of late 16S mRNA, it appears unlikely that this RNA is transcribed partially from fragment 5 or 6, as was suggested by the small proportion of hybridization observed with these two fragments (Table 3).

Late 19S mRNA. Hybridization in formamide of the pooled fractions containing 19S RNA to the *Hpa* II fragments 1, 2, 3, 5, and 6 showed that late 19S mRNA also hybridized predominantly to fragments 1 and 3 (Table 5). Before RNase digestion

Table 4. Rehybridization of late 16S mRNA eluted from fragment 1 or 3*

RNA eluted from fragment	Exp.	cpm/tube	% cpm hybridized to fragment 1	% cpm hybridized to fragment 3	Ratio of fragment 1 to fragment 3
1	(*i*)	800	26.1	9.2	2.8
	(*ii*)	500	29.4	12.6	2.3
	(*iii*)	2100	26.7	6.5	4.1
3	(*i*)	2150	30.3	9.7	3.1
	(*ii*)	600	22.0	19.2	1.1
	(*iii*)	1400	14.0	9.8	1.4

* Independent preparations of late 16S mRNA [(*i*) and (*ii*) labeled with [^{3}H]uridine, (*iii*) with [^{32}P]phosphate] were hybridized in formamide to fragments 1 and 3, eluted, and rehybridized in equal parts to fragments 1 and 3 in 4 × SSC followed by RNase digestion.

Table 5. Hybridization of late 19S mRNA (labeled with [^{3}H]uridine from 27 to 30 hr after infection)

Hpa II fragment on filter	cpm hybridized* Before RNase	cpm hybridized* After RNase	% RNase-resistant	Ratio of fragment 1 to fragment 3
1	1859	524	28.7	1.9
3	1650	250	15.2	
5	103	9		
6	328	24		
2	195	23		
No DNA	3	1		

* Each tube contained 49,000 cpm of RNA. For details, see footnotes of Table 1.

the two fragments bound similar amounts of RNA. Fragments 6 and 5 bound 5 and 15 times less RNA, respectively, than did fragment 1 or 3. After RNase treatment, fragment 1 retained 1.9 times more RNA than did fragment 3. RNase-resistant radioactivity bound to fragments 6 and 5, when compared to that on fragment 1, would correspond to a transcript of about 1.5 map units (about 70 nucleotides) on fragment 6 and about 0.5 map unit on fragment 5, assuming that all 19S mRNA molecules are identical. We cannot exclude, however, that this low degree of hybridization is the result of some size heterogeneity of a fraction of 19S mRNA molecules (2). As judged from the hybridization to fragment 2, both before and after RNase treatment, less than 10% of the 19S RNA was early 19S mRNA. The ratio of RNase-resistant RNA bound to fragment 1 over that bound to fragment 3 was determined four times in two independent experiments and gave an average value of 1.9 ± 0.1.

The rehybridization experiment shown in Table 6 suggests that there is only one species of late 19S mRNA. The molecular weight of 19S mRNA was estimated to be approximately

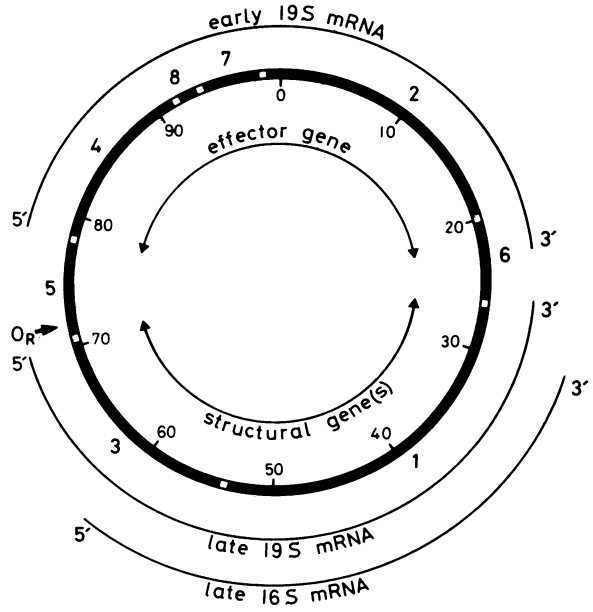

FIG. 3. Regions of transcription of the three polyoma mRNAs. The physical map of polyoma DNA (6) shows the *Hpa* II fragments numbered 1 to 8 on the outside and is divided into 100 map units (numbers inside) starting at the single site cleaved by the endonuclease *Eco* RI. O_R is the origin of bidirectional DNA replication (5). The direction of transcription for early and late RNAs has been determined by Kamen *et al.* (4).

Table 6. Rehybridization of late 19S mRNA eluted from fragments 1 and 3*

RNA eluted from fragment	cpm/tube	% cpm hybridized to fragment 1	% cpm hybridized to fragment 3	Ratio of fragment 1 to fragment 3
1	2940	15.0	7.5	2.0
3	2470	9.8	10.0	1.0

* Late 19S mRNA labeled with [^{32}P]phosphate. For details see footnote of Table 4.

700,000 (2), i.e., about 2,000 nucleotides or a transcript of 44% of the late strand. Fragments 1 and 3 account for 44% of the genome and give a slightly smaller ratio (1.7) than the experimental result. Taking into account the size estimate and the hybridization results (Tables 5 and 6), we assume that late 19S mRNA is complementary to the late strand between approximately 70 (5′ end) and 26 (3′ end) map units (see Fig. 3).

Chromatographic Analysis of Late 16S and 19S mRNA. The hybridization results obtained with the two late mRNAs suggested that the entire nucleotide sequence of late 16S mRNA is comprised within the late 19S mRNA. This has been confirmed by two-dimensional chromatographic analysis (15) of RNase T_1 (EC 3.1.4.8) digests of [^{32}P]phosphate-labeled late 19S and 16S mRNAs. The autoradiographs in Fig. 4 show very similar patterns. About 75 spots can be detected in the digest of the late 16S mRNA; all but one are also found in the digest of late 19S mRNA. On the other hand, the pattern obtained with late 19S mRNA shows nine spots that are either absent or much

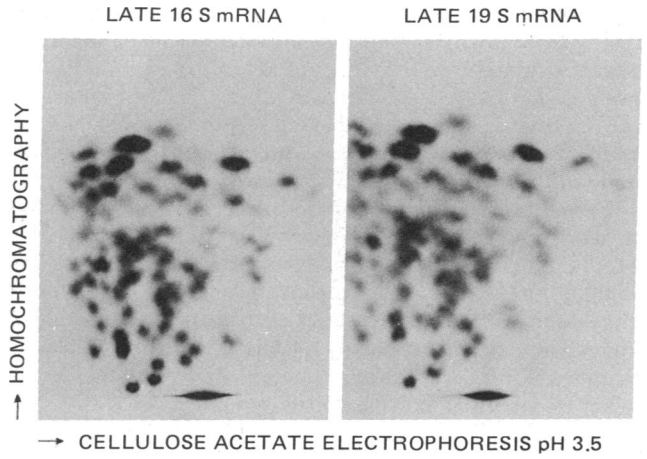

FIG. 4. Two-dimensional chromatograms of the RNase T_1 oligonucleotides of late 16S and 19S polyoma mRNAs. RNA was labeled from 27 to 30 hr after infection with 100 μCi of [^{32}P]orthophosphate in 1 ml of phosphate-free medium, extracted, and sedimented through sucrose gradients; polyoma mRNAs were localized by hybridizing aliquots of the fractions to total polyoma DNA and subdivided into 16S and 19S mRNAs. Each of these was hybridized in formamide to total polyoma DNA (5 μg on a 30-mm filter). The filters were washed, treated with RNase T_1 (Worthington Biochem. Corp.) at 10 units/ml for 30 min at room temperature, and washed again. RNA was eluted by incubating the filters twice with 0.5 ml of warmed 2 mM EDTA, pH 7.2, at 85° for 3 min each and precipitated after addition of 40 μg of purified yeast RNA. The dry pellet was digested with 5 μl of an RNase T_1 solution (Sankyo Co., Tokyo; 0.5 mg/ml) for 30 min at 37°. Chromatograms were obtained by cellulose acetate electrophoresis (pyridine acetate, 7 M urea, pH 3.5) in the first dimension and homochromatography on DEAE-cellulose thin layers with homomixture b at 60° in the second dimension (15).

weaker in the 16S mRNA digest. It is unlikely that the additional spots are due to the presence of early 19S mRNA because it amounts to less than 10% in the late 19S RNA preparations (Table 5).

DISCUSSION

In this paper we present evidence that three distinct species of polyoma mRNAs are synthesized during lytic infection in mouse kidney cells: an early 19S mRNA, a late 19S mRNA, and a late 16S mRNA. Their positions on the physical map of polyoma DNA are shown in Fig. 3.

Our results are based on hybridization of the two size classes (19S and 16S) of polyoma mRNAs to individual *Hpa* II fragments of polyoma DNA. To locate the 5′ and 3′ termini of the mRNAs we used the published estimates of the molecular weights of the mRNAs [700,000 for 19S RNA (1, 2) and 500,000 for 16S RNA (2)] and of polyoma DNA [3×10^6 molecular weight (13) or 4,600 base pairs (14)]. Assuming that the true molecular weights of the polyoma mRNAs are within ±10% of the published estimates, the indicated positions of the 5′ and 3′ ends have to be regarded as approximations with an accuracy of about ±3 map units. Therefore, our results do not exclude that the 3′ ends of the two late mRNAs coincide near 28 map units. Late polyoma mRNAs are synthesized in the nucleus as molecules equal to or larger than a full transcript of the late strand (4, 10, 16), and the two late mRNAs are most likely formed by exo- or endonucleolytic digestion followed by polyadenylation (3). Detailed analysis of the end-terminal sequences of the two late mRNAs might provide information on the mechanism and accuracy of processing mRNA precursors in mammalian cells.

Up to about 12 hr after infection early 19S mRNA is virtually the only virus-specific RNA found in the cytoplasm of cells undergoing lytic infection. Thereafter increasing amounts of the two late mRNAs appear in addition to early 19S mRNA, which is synthesized throughout lytic infection. Results to be presented elsewhere show that synthesis of late 19S and 16S mRNA begins before detectable viral DNA replication and also takes place under conditions where synthesis of both cellular and viral DNA is inhibited by 5-fluorodeoxyuridine.

Early 19S mRNA (or a very similar species) is also synthesized in abortive infection (1) and in polyoma virus-transformed cells (4, 16). It most likely directs synthesis of polyoma-specific tumor antigen, the hypothetical "pleiotropic effector" (1), whereas the late mRNAs direct synthesis of the capsid protein(s) (17). In lytic infection of the closely related simian virus 40, both the number of mRNAs and their function seem to be analogous to what we find with polyoma virus (18–20).

The experimental results presently available are compatible with the hypothesis that polyoma virus contains not more than three genes. Two of these most likely specify the viral capsid (21), whereas the other carries most of the information need for lytic infection and all the information for abortive infecti initiation, and maintenance of the transformed phenotype a probably also for induction of tumors in animals. Therefo we propose to designate the region of polyoma DNA fr which early 19S mRNA is transcribed as "(pleiotropic) effec gene" and the region of the viral genome that specifies the t late mRNAs as "structural gene(s)" (Fig. 3).

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