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Characterization of the 5'-Terminal Capped Structures of Late Simian Virus 40-Specific mRNA

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^{32}P-labeled, late simian virus 40-specific RNA was isolated from infected CV_1 cells and completely degraded with RNase T_2 and bacterial alkaline phosphatase. The RNase-resistant material was fractionated two dimensionally and further characterized with *Penicillium* nuclease and nucleotide pyrophosphatase. Two major 5' termini were identified in late simian virus 40 RNA, namely, 7-methyl Gppp 2',6-dimethyl ApUp and 7-methyl Gppp 2',6-dimethyl Ap 2'-methyl UpUp. Both 5' termini are present in unfractionated viral RNA as well as in the separated 16S and 19S species. As both caps differ only in secondary modification, it is possible that they are derived from the same site on the DNA. The relatively higher cap II content of the 16S mRNA may be related to its slower rate of turnover.

Blocked 5' termini are a general characteristic of eucaryotic cellular mRNA's (21, 24). Also, most of the eucaryotic viral mRNA's studied so far are blocked at their 5' end (24). The latter contain exclusively a purine nucleotide in the 5'-penultimate position, whereas cellular mRNA's may have either a purine or a pyrimidine. This penultimate nucleotide can be unmodified (cap O structure), but in many cases it appears to be methylated on the ribose, and in a few examples an additional base methylation also occurs in this position (cap I structure). Ribose methylation of the second nucleotide is often observed in the mRNA's from higher organisms and the corresponding viruses, thus leading to the formation of cap II structures. It is noteworthy that the methylation pattern of the caps increases as one moves up the evolutionary ladder.

Simian virus 40 (SV40) is a DNA-containing virus, which produces virus-specific RNA in the cytoplasm of lytically infected monkey kidney cells (3, 6, 17, 27, 31). Studies on the methylation pattern of SV40-specific RNA revealed 5'-terminal ends of the type ^{7m}GpppNm (13). Because of the general interest in SV40, we have characterized in more detail the 5' termini of the predominant cytoplasmic viral RNAs, and their structures are reported here.

MATERIALS AND METHODS

Preparation of ^{32}P-labeled late SV40 RNA. CV_1 monkey cells (Bio-Cult, Paisley, Scotland) were cultured in plastic petri dishes (Falcon Plastics, Oxnard, Calif.) and infected with 60 PFU of SV40 (strain 776) per cell. At 24 h after infection, the growing medium was changed to a low-phosphate medium (containing 10^{-5} M phosphate, i.e., 1% of the normal concentration), and 3 mCi of ^{32}P radioactivity (The Radiochemical Centre Ltd., Amersham, England) was added per 14-cm petri dish for a labeling period of 24 h. The medium was then removed, and the cells were washed a few times with phosphate-buffered saline and finally lysed with 1% Nonidet P-40. The nuclei were removed by centrifugation, and subsequently the lysate was subjected to several extractions with phenol-chloroform-isoamyl alcohol. The extracted RNA was precipitated at least twice with ethanol to minimize contamination with co-precipitating [^{32}P]phosphate, originating from the pool present in the cellular cytoplasm. Isolation of polyadenylic acid [poly(A)]-containing RNA on oligodeoxythymidylic acid-cellulose (P-L Biochemicals, Inc., Milwaukee, Wis.) was followed by selection of the SV40-specific sequences by hybridization on SV40 DNA linked to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) (9). The SV40 mRNA thus purified usually represented around 0.2% of the total cytoplasmic RNA fraction.

Preparative gel fractionation of ^{32}P-labeled RNA. In some cases the RNA preparation was fractionated by preparative electrophoresis on an agarose slab gel (160 by 120 by 3 mm). This step was carried out either before (see Fig. 4) or after the selection by hybridization. A solution of 1.4% agarose (Sigma Chemical Co., St. Louis, Mo.) in running buffer (0.02 M Tris-acetate plus 1 mM EDTA, pH 7.5) was used. The RNA samples were dissolved in running buffer, which had been mixed with 50% deionized formamide, heated for 1 min at 70°C, and loaded onto the gel. The electrophoresis was started immediately in the cold at 50 to 100 V for about 2 h. Next, the gel was dismantled, and the ^{32}P-labeled RNA was localized by autoradiography. The bands were cut out, and their radioactivity was estimated by Cerenkov counting. The material was recovered from squeezed gel bands by several extractions with small quantities (i.e.,

100 to 200 μl for a gel volume of 20 by 5 by 3 mm) of 0.5 M NaCl, containing 5 mM EDTA and 0.5% sodium dodecyl sulfate. The addition of a few drops of phenol may facilitate the release of RNA from the agarose pieces. Finally, the ^{32}P-labeled RNA was precipitated at −20°C together with carrier yeast RNA, by adding two parts of ethanol to the combined extracts. The recovery of the elution procedure amounts to 70% for RNA from the 28S region and reaches more than 80% for material of smaller S values.

Isolation of 5'-terminal structures. For degradation with RNase T_2, the pelleted RNA (i.e., ^{32}P-labeled viral or cellular mRNA and 40 μg of carrier yeast rRNA) was dissolved in a volatile buffer (0.1 M formic acid-pyridine, pH 4.5) and digested overnight at 37°C with 5 U of T_2 enzyme (Sigma Chemical Co.). Next, the hydrolysate was evaporated to dryness, and the residue was dissolved in 0.1 M ammonium hydrogen carbonate containing 0.5 U of bacterial alkaline phosphatase (Worthington Biochemicals Corp., Freehold, N.J.) for a further incubation of a few hours at 37°C. The reaction mixture was evaporated again and washed several times with distilled water to remove remaining traces of salt.

The separation procedure involved a classical electrophoresis on cellulose acetate in the first dimension (23), followed by ion-exchange chromatography on thin-layer plates in the second dimension. The T_2 hydrolysate, dissolved in a minimal volume of water, was applied as a thin line on prewetted cellulose acetate strips (Schleicher and Schüll GMBH, Dassel, West Germany) and subjected to electrophoresis at pH 3.5 in the presence of 5 M urea until the pink dye had migrated 20 to 25 cm. The material was transferred onto a thin-layer chromatographic plate of polyethyleneimine (PEI)-cellulose (Machery-Nagel and Co., Düren, West Germany) by the inversed blotting technique (26) and developed in the second dimension by ascending chromatography with a volatile buffer solution of 1 M (or 1.3 M) formic acid-pyridine (pH 4.3). Radioactive spots were visualized by autoradiography at −70°C, using preflashed X-ray films (RX Fuji X-Ray Film) (12) together with intensifying screens (CAWO, Schrobenhaussen, West Germany) (R. Laskey and A. Mills, FEBS Lett., in press). The nucleotide material can be recovered easily from thin-layer chromatography plates by the microelution technique described previously (28).

Analysis of capped termini. RNase T_2-resistant material was further degraded by *Penicillium* (P_1) nuclease (P-L Biochemicals, Inc.). The dry residue, after elution from the PEI plate, was redissolved in a volatile buffer (0.1 M formic acid-pyridine, pH 6), and 10 μg of P_1 enzyme was added. The reaction mixture was incubated for 30 min at 37°C and then evaporated to dryness. The material was thoroughly washed by the addition of a small volume of water followed by drying and then applied onto miniplates of PEI-cellulose (6.6 by 9.9 cm) for two-dimensional chromatography: the first dimension was developed with a 20% formic acid solution; in the second direction, a buffer system of 1 M formic acid-pyridine (pH 4.3) was used (27a).

Cap structures, resistant to P_1 hydrolysis, were analyzed by digestion with nucleotide pyrophosphatase (Sigma Chemical Co.). The dried material was washed with water to remove remaining traces of triethylamine carbonate. After it was redissolved in the appropriate enzyme solution (10 U of nucleotide pyrophosphatase per ml of 25 mM Tris-chloride–2 mM Mg^{2+}, pH 7.5), the pH was checked and eventually adjusted. The mixture was incubated for 30 min at 37°C, and the digest was transferred directly onto miniplates of PEI-cellulose (6.6 by 9.9 cm) for two-dimensional separation. A less concentrated solution (e.g., 0.5 M formic acid-pyridine, pH 4.3, in the second dimension) was used to obtain a good resolution between singly and doubly methylated nucleotides. Methylated nucleotides used as references were purchased from P-L Biochemicals, Inc.

RESULTS

RNase T_2 hydrolysis of total late SV40 RNA. ^{32}P-labeled late SV40 mRNA was prepared and completely degraded with RNase T_2 and bacterial alkaline phosphatase, as described above. A typical degradation pattern of SV40 RNA is shown in Fig. 1. Two spots are prominent, products 1 and 2, corresponding to two different 5′ termini in the late viral RNA. Inorganic [^{32}P]phosphate was run off the plate in the first dimension, and only some trailing radioactivity from this giant spot is seen. The dark spots at the top of the figure are cyclic mononucleotides; these intermediates are due to incomplete RNase T_2 hydrolysis and are resistant to the phosphatase.

The aforementioned products 1 and 2 contain the 7-methyl Gppp group, as will be shown below; sugar (2′) methylation of the following residue(s) renders these resistant to the action of RNase T_2 and generates the longer 5′-terminal oligonucleotides (1, 19, 32). That the caps are derived from "late" SV40 mRNA follows from a characterization of the cytoplasmic, poly(A)-containing, SV40-specific RNA by fingerprinting [G. Haegeman and W. Fiers, Nature (London), in press] and is in agreement with the well-known abundance of late RNA relative to "early" (3). Also, some minor spots may be observed in Fig. 1; they are probably not virus specified, but they are derived from a small contamination with cellular RNA. Indeed, in less purified preparations of SV40 RNA, the entire pattern of cellular cap structures was still visible, but as the viral RNA was more rigorously selected by stringent hybridization conditions, the two major spots became more and more prominent while the overall cellular contamination disappeared. The strongest contaminants correspond to the main cellular cap structures (e.g., products 3 and 4 in Fig. 5), but even in this example the two viral products 1 and 2 represent 65 to 70% of the total fraction of T_2-resistant material, whereas the latter spots amount to at most 20 to 25% of the entire cap population in

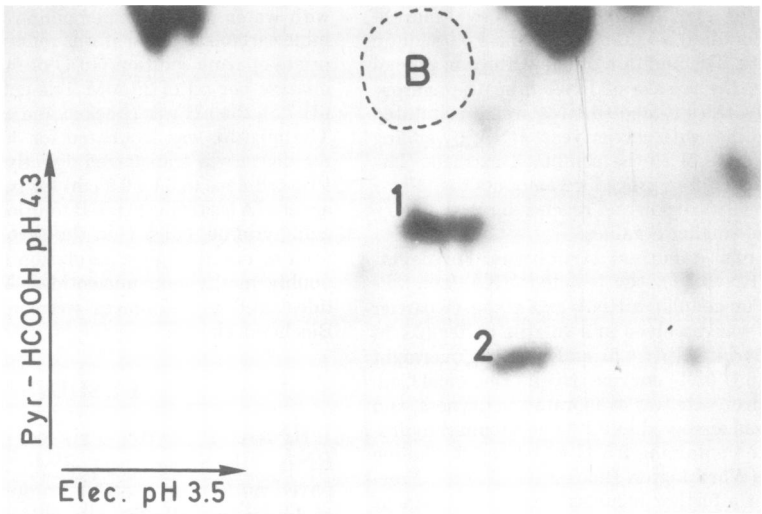

FIG. 1. *Two-dimensional map on PEI-cellulose of a combined RNase T_2 and phosphatase degradation of total late ^{32}P-labeled SV40 mRNA. The ion-exchange chromatography in the second dimension was carried out with 1.3 M formic acid-pyridine (pH 4.3). The dye marker, xylene cyanol FF, denoted by B, migrated 10.5 cm starting from the first-dimensional origin line.*

the case of a total cellular mRNA hydrolysate. Furthermore, the same RNA material, in which products 1 and 2 are the major cap spots, has been fingerprinted after RNase T_1 digestion and corresponds indeed to the viral RNA, as revealed by analysis of all of the T_1 oligonucleotides present and correlation with the known DNA sequence.

Furthermore, combined P_1 hydrolysis and phosphatase treatment of total cellular RNA yields two different cap structures, an A-cap and a G-cap, both of which are present in nearly equimolar amounts (unpublished data). On the other hand, similar analysis of the highly purified SV40 RNA showed almost exclusively an A-cap structure.

The molar yield of the 5′-terminal group was calculated from an analogous experiment, in which the radioactivity present in the P_1-resistant cap structure and representing exactly three phosphate residues was compared with the total radioactivity of the viral RNA. Assuming an average chain length of approximately 1,600 nucleotides for the mRNA molecules, the 5′-terminal structures appear in 0.5 molar ratio only. At present, we do not know whether this phenomenon is due to an inefficient capping mechanism or is the result of preferential losses of the 5′ terminus during the isolation procedure; however, T_1 oligonucleotides originating from the spliced 5′-terminal leader sequence appear in molar quantity (Haegeman and Fiers, in press).

Analysis of T_2-resistant cap structures. The T_2-resistant products 1 and 2 were eluted from the PEI plate and further characterized by digestion with P_1 nuclease (Fig. 2). Both patterns show a major component close to the blue dye and a lesser amount of radioactivity in the mononucleotide region; some undegraded product remains near the application point. The major compound, identical in both T_2-generated "caps," contains a P_1-resistant structure with the general formula $^{7m}G(5')ppp(5')X$. As mentioned above, complete P_1 hydrolysis of cellular RNA, followed by dephosphorylation, yields mainly two products derived from the 5′ termini, namely, 7-methyl GpppA and 7-methyl GpppG (our unpublished data). The position of these on the miniplates in the neighborhood of the blue dye was verified by several control experiments with ^{32}P-labeled material and with optical density (OD) references. In the case of the two SV40 products (Fig. 2), the 5′ terminus behaves as an A-cap (G-caps are more delayed in both dimensions), but a more refined analysis, as well as the determination of the degree of methylation, is described below.

Furthermore, enzymatic digestion of product 1 releases a mononucleotide, which behaves in this system as pU. Indeed, carrier mononucleotide pU, added to the reaction mixture as an internal OD marker, chromatographs in exactly the same position. In the case of product 2, two spots are visible in the mononucleotide region: the slowest one is again pU, whereas the faster-migrating spot might be the methylated analog (it chromatographs like pU in the first dimension, which separates mainly according to charge) (27a). It actually coincides with 2′-*O*-methyluridine-5′-phosphate, added as an OD

marker, but further confirmation was needed since one cannot unambiguously determine from the mobility pattern in this system whether the methylation occurs on the base or on the sugar moiety of the nucleotide. Therefore, the compound was rechromatographed on cellulose plates in a mixture of isopropanol–ammonia–0.1 M boric acid (7:1:2). Unmodified or base-methylated nucleoside-5'-phosphates are strongly delayed because of the complexing activity of boric acid with the *cis*-diol groups. The radioactivity recovered was not retained at the origin, but moved together with 2'-*O*-ribose-methylated pU, thus providing further proof for the identification (data not shown). This analysis leads to the partial structure ^{7m}GpppXmpU for product 1 and ^{7m}GpppXmpUmpU for product 2, where Xm indicates a ribose-methylated nucleotide, since the residue to the 3' end of Xm is not released by RNase T_2 digestion.

The identical P_1-resistant cap structures of the T_2 products 1 and 2 (Fig. 2) were reeluted from the PEI miniplate and independently analyzed by digestion with nucleotide pyrophosphatase (Fig. 3). The experiments were performed in duplicate for each product, applying in one 2'-*O*-methyladenosine-5'-phosphate (pAm) as an internal OD reference and in the other N^6,2'-*O*-dimethyladenosine-5'-phosphate (^{6m}pAm). Both sets of fractionations showed an identical pattern: inorganic phosphate (above the blue dye, but moving differently from undegraded P_1-resistant caps), ^{7m}pG (the fastest spot in both dimensions, also checked by an internal OD reference), and the pA residue, in both cases coinciding perfectly with the internal OD reference ^{6m}pAm and moving precisely one position faster than pAm (or ^{6m}pA), which in turn moves faster than pA. Although most of the capped termini studied so far contain a 2'-*O*-ribose-methylated purine nucleotide, some viral RNAs other than SV40 display a doubly methylated A-residue in this position (11, 15, 16, 20, 25); doubly methylated pG, however, has not been observed so far.

The structure of the two T_2-resistant products (1 and 2) is fully determined by these analyses. They are ^{7m}G(5')ppp(5')^{6m}AmpU and ^{7m}G-(5')ppp(5')^{6m}AmpUmpU, respectively, and will be further indicated by the terms cap I and cap II. It is quite possible that cap II is derived from cap I by a second methylation step, a phenomenon that already has been observed in the case of viral RNA (16, 20, 25) and appears rather general even in cellular mRNA populations (4, 5, 7, 19, 29, 32). However, as the late viral SV40 RNA contains at least two RNA species (27, 31), we cannot exclude a priori that one type of cap structure may be specifically derived from one RNA species. This was checked by identification of the caps in fractionated SV40 mRNA.

RNase T_2 hydrolysis of fractionated SV40 RNA species. Cytoplasmic, poly(A)-containing RNA was isolated from SV40-infected cells and fractionated on a 1.4% agarose gel. The fractionation pattern was divided into a set of bands, from which the RNA material was eluted and hybridized to SV40 DNA. A distribution profile of the SV40-specific sequences is shown in Fig. 4a. The majority was present in the 17S to 18S region and corresponds to the major, late RNA species, which sediments in sucrose gradients at 16S (30, 31). The 19S species is less prominent and represents about 25% of the 16S material.

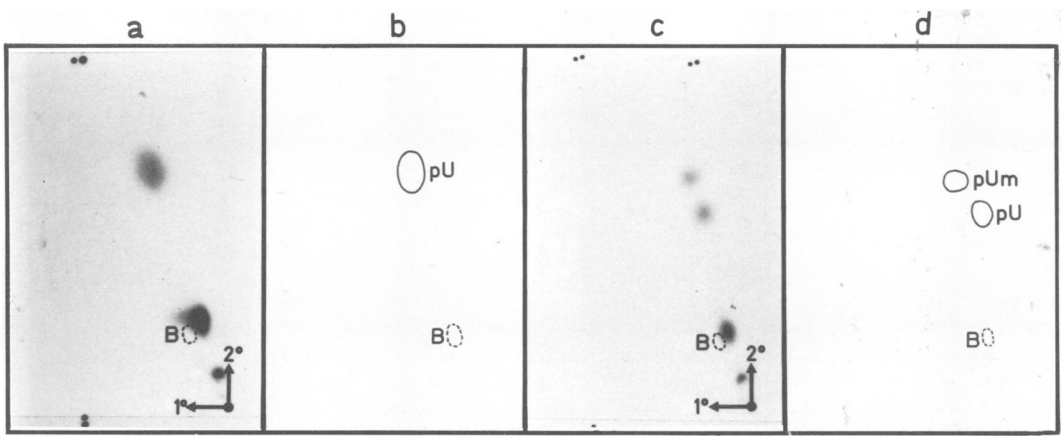

FIG. 2. *Two-dimensional chromatography on miniplates of PEI-cellulose of a P_1 digest of T_2 products 1 (a) and 2 (c) and the corresponding diagrams, respectively, (b) and (d), showing the position of relevant reference mononucleotides (revealed under UV light). The position of the blue dye is indicated by a dashed circle.*

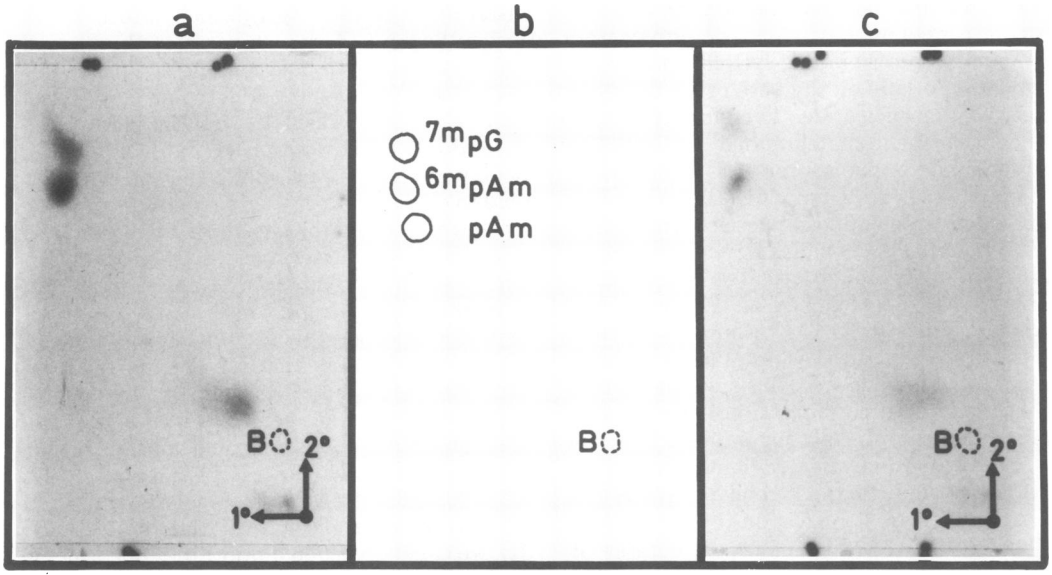

FIG. 3. *Nucleotide pyrophosphatase analysis of P_1-resistant cap structures, derived from products 1 and 2 (spots near the dye marker B in Fig. 2). (a) and (c) show the respective autoradiographs of a two-dimensional separation on PEI-cellulose. (b) denotes the position of methylated reference nucleotides, which were added as internal markers in different combinations and which were visualized under UV light. The two radioactive spots, in (a) as well as in (c), correspond precisely to the ^{7m}pG and ^{6m}pAm internal reference compounds, respectively. The monomethylated ^{6m}pA and (2')pAm move exactly the same in this system. B denotes the position of the blue dye. The spot that moves just ahead of B in both dimensions is inorganic phosphate.*

All of the SV40-specific fractions were subjected to hydrolysis by RNase T_2, followed by dephosphorylation, and subsequently fractionated in two dimensions on PEI-cellulose plates. Both cap I and cap II were present in all of the fractions analyzed, and they represented about 70% of the total amount of T_2-resistant products. As an example, the digestion patterns of the 18S and 19S SV40-specific material are compared in Fig. 5. There seems to be relatively more cap II in the material, which runs at 18S or faster, as compared with the 19S RNA. This is illustrated in Fig. 4b, in which the relative amount of cap II is plotted for the different size classes; these results, however, are based on counting very low amounts of radioactivities and must be considered tentative.

DISCUSSION

The 5′ termini of late poly(A)-containing SV40-specific RNA have been isolated and characterized in detail. It has been claimed that P_1 RNase hydrolysis of SV40 mRNA releases both ^{7m}G(5′)ppp 2′-*O*-methyladenosine and ^{7m}G-(5′)ppp 2′-*O*-methylguanosine (13). According to our data, however, no substantial amount of 2′-*O*-methylguanosine containing cap was present in SV40 mRNA; furthermore, we have shown that the modified adenosine residue is actually N^6,2′-*O*-dimethyladenosine. In fact, we conclude that only one major P_1 RNase cap is present in late SV40 mRNA, namely, ^{7m}G(5′)ppp(5′)^{6m}Am.

Analysis with RNase T_2 reveals two major types of cap structure, cap I and cap II, in cytoplasmic, late SV40 mRNA. Cap I was identified as ^{7m}G(5′)ppp(5′)^{6m}AmpU, and cap II was identified as ^{7m}G(5′)ppp(5′)^{6m}AmpUmpU. Both types of 5′ termini are also found among the wide variety of monkey cellular mRNA cap structures (our unpublished data). It is quite possible that the 5′ ends of both 16S and 19S late mRNA's are derived from the same site on the genome, but differ only in the degree of secondary modification. The molecular basis for such a hypothesis was not readily evident until very recently, as the 16S RNA is believed to be a processing product of the 19S molecule and shares common sequences with the 3′-terminal half of the 19S species (2, 10, 30). Recent results, however, indicate a common 5′-terminal leader sequence for the two late SV40 mRNA's, which become linked to transcripts from a nonadjacent region on the genome (14, 22; Haegeman and Fiers, in press; S. Lavi and Y. Groner, in press). Similar translocation of RNA segments has been shown in adenovirus mRNA, and in this system the sharing of a common leader sequence is even more obvious (3a, 8, 10a). Moreover, the localization of the SV40 cap structure in the *Hind* C fragment at position 0.72 or closer to the origin

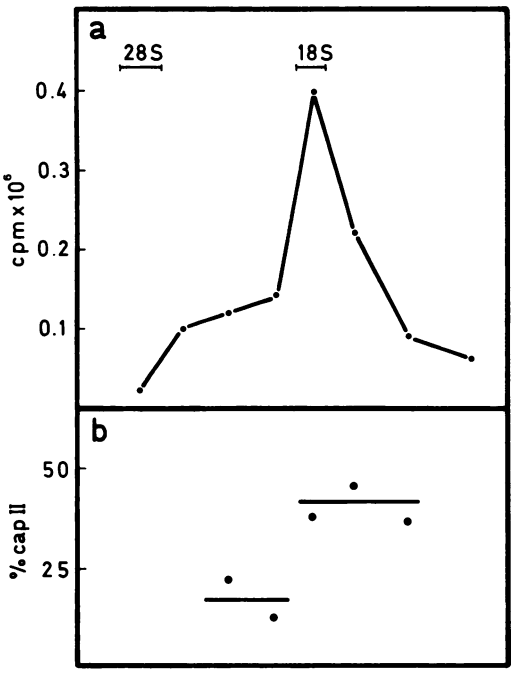

FIG. 4. *(a) Separation of the total cytoplasmic poly(A)-containing RNA by electrophoresis on a 1.4% agarose gel, followed by selection of the SV40-specific sequences by hybridization. The position of the rRNA markers is indicated. (b) The percent amount of cap II structure present in the total population (cap I plus cap II) is plotted against the corresponding gel fraction.*

of replication (Haegeman and Fiers, in press; Lavi and Groner, in press) suggests that the putatively common 5'-terminal structure is directly derived from the 5' end of the primary nuclear transcript. Indeed, a flow of the methylated cap structures from the heterogeneous nuclear RNA to the cytoplasmic mRNA population has been observed in L-cells (18).

The relatively higher percentage of cap II structure in the 16S mRNA may be related to a difference in half-life, as a slower turnover of the latter mRNA as compared with the 19S species has been reported (2). In the case of cellular mRNA's, it has in fact been found that the formation of cap II structures is correlated with the longevity of the mRNA molecules (18). Moreover, in the current view of "spliced" mRNA molecules, as is the case for late SV40 mRNA (see above), the relationship between the methylation of the cap structure and the turnover of the RNA may perhaps be more complex. Indeed, a higher representation of cap II in the 16S RNA population as compared with the 19S may be either directly related to the higher stability of the mRNA itself or formed by a longer exposure to methylating activity of the conserved 5'-terminal leader fragment, which functions in the 19S mRNA and, after cytoplasmic conversion (2), again in the 16S mRNA. Alternatively, the 5'-leader sequence containing the cap II structure could be preferentially used for transposition and linkage to the 16S coding portion.

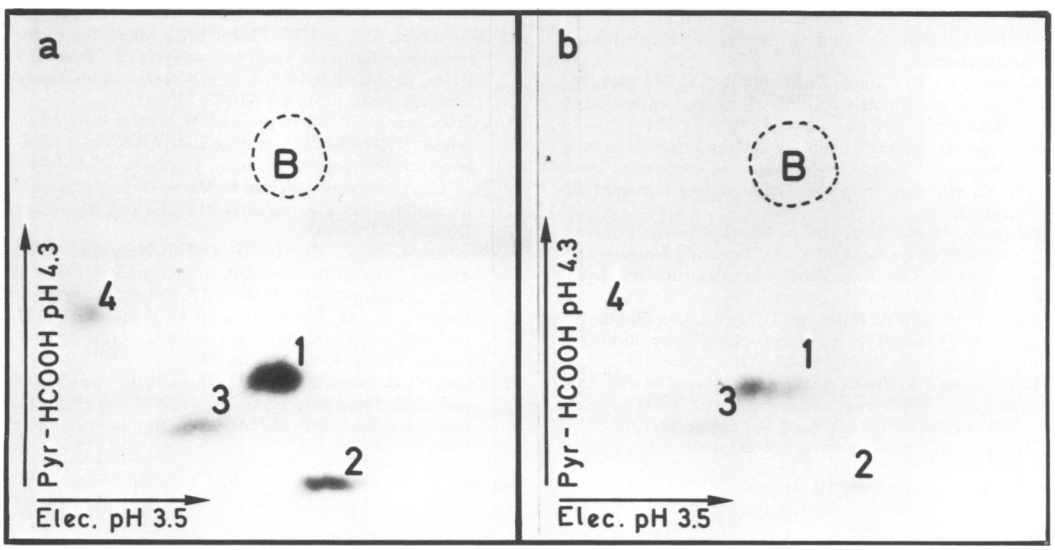

FIG. 5. *Two-dimensional map on PEI-cellulose of a combined RNase T_2 and phosphatase degradation of 18S (a) and 19S (b) SV40-specific RNA. The second dimension was developed with 1 M formic acid-pyridine (pH 4.3). The blue marker moved 7.5 cm in the second dimension.*

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