Volume 5 Number 7 July 1978 Nucleic Acids Research

Localization of the 5' terminus of late SV40 mRNA

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Received 24 April 1978

ABSTRACT

A cap-containing oligonucleotide has been isolated from a T_1 ribonuclease hydrolysate of total Simian Virus 40-specific late RNA and its structure has been determined as $^{7m}G(5')ppp(5')^{m}AmpU(m)pUp(Up,Cp)ApGp$. This oligonucleotide constitutes the major 5' terminus of both late mRNA species, 16S and 19S. Assuming that viral mRNA "caps" are derived from a 5'-terminal triphosphate or diphosphate, these results mean that late transcription is (mainly) initiated at nucleotide L 308 (numbering system of Simian Virus 40 DNA, cf. Fiers et al. (1978) Nature, 273, 113-120). The cap is followed by a contiguous leader sequence which is at least 194 nucleotides long.

INTRODUCTION

Simian Virus 40 (SV40) RNA is capped (1), and the cap structures of the viral late mRNAs have been characterized in detail (2,3). At least 70 % of the SV40 late, cytoplasmic mRNAs start with either 7-methyl-guanosine-5'-triphosphoryl-5'-6,2'-dimethyl-adenylyl-uridylic acid or 7-methyl-guanosine-5'-triphosphoryl-5'-6,2'-dimethyladenylyl-2'-methyl uridylyl-uridylic acid, the latter being relatively enhanced in the 16S mRNA species (2). Furthermore, the SV40 late mRNAs are spliced, consisting of a 5'-terminal untranslated leader segment of about 150-200 nucleotides joined covalently to the coding portion of the RNAs (4-9). In the case of 16S RNA, the approximate localization of the leader sequence on the genome has been established by direct structural analysis (8,9), but no information was obtained as to which site on the genome actually corresponded to the capped 5' end of the leader sequence, nor had a capped T_1 oligonucleotide been detected in the ribonuclease T_1 fingerprints of the

entire 16S RNA molecule or the isolated leader segment.

In the present study we report the isolation of a cap-containing oligonucleotide from T_1 digests of SV40-specific, cytoplasmic late mRNA. This information plus supplementary data on T_1 oligonucleotides present in the mRNA digest in correlation with the primary nucleotide sequence of the SV40 genome allowed us to localize the 5' terminus of the untranslated leader sequence at nucleotide L 308 (numbering system as in ref. 10).

MATERIALS AND METHODS

Highly ^{32}P-labeled SV40 late mRNA was isolated from SV40-infected CV-1 monkey cells and purified from the cytoplasmic extract by oligo(dT)-cellulose selection and hybridization to SV40 DNA-Sepharose as described previously (2). The viral RNA was dissolved in a small volume (10 λ) of 0.02 M Tris-Cl, 2 mM EDTA, pH 7.4, and hydrolysed for 30 min at 37°C with T_1 ribonuclease (Sankyo Co. Ltd). Subsequently the reaction mixture was diluted with an excess (e.g., 200 λ) of 0.6 M KCl, 0.05 M morpholine-HCl buffer, pH 8.5, and loaded directly onto an oligo (dT)-cellulose column which had been equilibrated with the same buffer. This step eliminated the poly(A)-containing material which could interfere with the ensuing isolation procedure. The effluent of the column, containing the non-bound oligonucleotide material, was collected and diluted with ethanol to a final concentration of 20 %. In this way the resulting composition corresponded to the composition of the starting solution used for isolation of sugar derivatives containing a 2',3' cisdiol group by chromatography on acetylated dihydroxyboryl (DBAE)-cellulose (11,12). The oligonucleotide material was loaded onto a pre-equilibrated column (0.7 x 0.2 cm) of acetylated DBAE-cellulose (Collaborative Research, Inc.) at 4°C and the column was washed with approximately 10 column-volumes of starting buffer (i.e., 0.6 M KCl, 0.05 M morpholine-HCl buffer, pH 8.5, 20 % ethanol). A T_1 hydrolysate of 400 µg of carrier yeast ribosomal RNA diluted with 0.5 ml starting buffer was then loaded onto the DBAE-cellulose column and the column was washed again with another 10 column-volumes of starting solution. The material still bound was then eluted with starting buffer containing also

0.1 M sorbitol, and finally the column was stripped with 0.2 M NaCl, 0.05 M sodium acetate buffer, pH 5.0. The peak fractions of each elution step were collected, diluted with water to a final salt concentration of 0.02 M and desalted on a column (0.7 x 1.0 cm) of DEAE-cellulose (Whatman DE-52). The oligonucleotide material retained was recovered by elution with 1 - 1.5 ml of 1 M triethylammonium carbonate, pH 10, and the eluate was evaporated to dryness. The residue was redissolved in a few λ of water and fractionated two-dimensionally by homochromatography on PEI (polyethyleneimine)-cellulose plates (Machery-Nagel & Co.) (13).

Specific products were recovered from the thin-layer plate by a micro-elution technique (13) and further analyzed by digestion with pancreatic ribonuclease A (14). Ribonuclease-A-resistant products were reeluted and further degraded with Penicillium nuclease P_1 (2).

RESULTS

A typical elution profile of the DBAE-cellulose column is shown in Fig. 1. Fingerprint analysis revealed that the huge peak of non-bound material contains all the products present in a typical T_1 map of total SV40 late mRNA. Ap-rich oligonucleotides, however, are preferentially retained by the cellulose and therefore appear in submolar quantity (data not shown). The irregular pattern of the material eluted by the T_1 hydrolysate of carrier yeast RNA represents material that attaches rather non-specifically to the column resin. The next peak, eluted by the sorbitol solution, contained the capped oligonucleotide(s) - as will be demonstrated below - in addition to a relatively high background of material of random origin. The final wash again consisted of non-specifically bound oligonucleotides but did not contain any capped product, as checked by further analysis of the eluate with P_1 nuclease and bacterial alkaline phosphatase as described previously (2).

The peak eluted with the sorbitol-containing buffer was collected, desalted, and fingerprinted as described in Materials and Methods. An autoradiograph of the two-dimensional fractionation is shown in Fig. 2 together with a regular fingerprint of

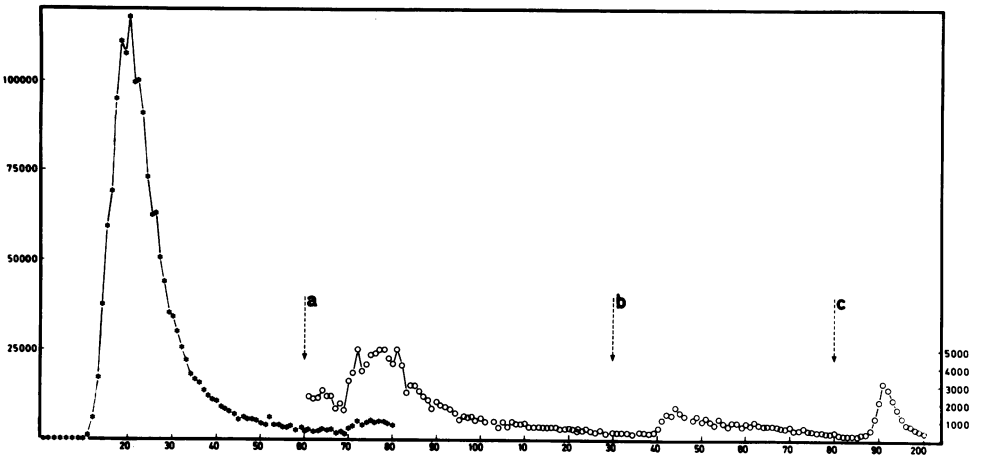

Figure 1 : Elution profile of the DBAE-cellulose chromatography. (^{32}P)-labeled, SV40-specific, cytoplasmic mRNA was digested with ribonuclease T_1, and after dilution with "starting buffer" (0.6 M KCl, 0.05 M morpholine-HCl buffer, pH 8.5, 20 % ethanol), loaded onto a DBAE-cellulose column. The first peak to the left corresponds to non-bound material that runs through the column (the radioactivity corresponds to the left ordinate). The more expanded scale at the right applies to the remainder of the elution pattern. The successive elutions are indicated by arrows : (a) T_1 hydrolysate of carrier yeast RNA diluted with starting buffer. (b) "starting solution" containing in addition 0.1 M sorbitol, and (c) 0.2 M NaCl, 0.05 M sodium acetate (pH 5).

total SV40 late mRNA. As the 19S mRNA is only a minor species, all the major spots present in the latter fingerprint are derived from the 16S mRNA (8). Although the normal, 16S mRNA type T_1 pattern is recognizable in the sorbitol peak from the DBAE-cellulose chromatography, a discrete set of new products can readily be seen. All the spots were further characterized by double digestion with pancreatic ribonuclease and at least 6 contained the previously characterized 5' cap structure (vide infra, Fig. 4). No structural difference between these spots was evident from the ribonuclease-A analysis. Hence, the resolution between this variety of spots (at least 6 were resolved) is presumably due to the procedures used and does not necessarily indicate different oligonucleotides. A similar occurrence of doubled spots in the first (i.e., the electrophoretic) dimension has recently also been described for the capped 5' terminus of adenovirus-2 late mRNAs isolated by chromatography on DBAE-cellulose (12,15). The authors suggested that the spot doubling

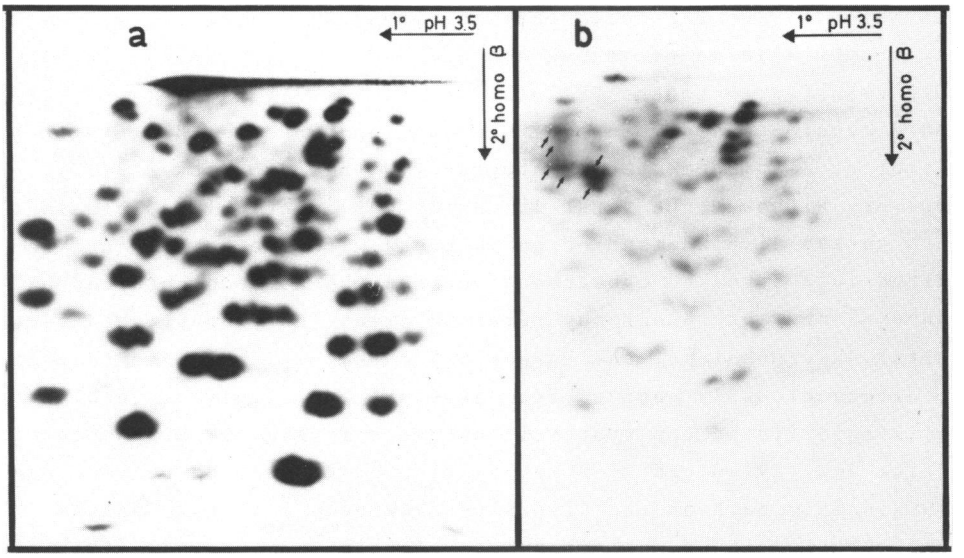

Figure 2 : Two-dimensional fractionation on PEI-cellulose thin-layer plates (20 x 20 cm) according to the procedure of Volckaert et al. (13) of : (a) a T_1 hydrolysate of cytoplasmic SV40 late mRNA. The analysis of such a fingerprint has been described in a previous publication (8). As the 19S RNA represents at most 20 % of the viral late RNA, the 19S-specific oligonucleotides are hardly visible; and (b) material derived from a similar T_1 hydrolysate as in (a), but which was retained on a DBAE-cellulose column and was eluted with the sorbitol-containing buffer. Spots indicated by an arrow were shown upon enzymic analysis to contain the cap structure (5 of them were rather well-defined spots, while the 6th had a streaked appearance).

might perhaps be caused by a different degree of methylation at the N^6 position of the adenosine residue of the cap. However, this explanation is not applicable to our observations because only the 6,2'-doubly-methylated adenosine was found to be part of the SV40 cap structure in our preparations (2). Moreover, singly- or doubly-methylated adenosine residues are not expected to give any resolution in the corresponding cap structures upon electrophoretic fractionation at pH 3.5 (3,16-18). Also, the apparent heterogeneity in the second (i.e., chromatographic) dimension is primarily not due to a difference in composition, as shown by analysis with nucleases, but is largely caused by an artifact which arises in the separation of polyphosphate groups by homochromatography on PEI cellulose. Indeed, nuclease P_1-generated cap structures of cellular mRNA were separated two-

dimensionally on PEI cellulose: the first fractionation was by electrophoresis on cellulose acetate and the separated components were transferred to the PEI plate by reversed blotting; the second dimension was developed either by ion-exchange chromatography (2) or by displacement chromatography with homomixture β (13). However, although the first dimension was identically clean in both cases, displacement chromatography with the homomixture resulted in a streaked appearance whereas the pattern obtained by ion-exchange chromatography remained clean (our unpublished results). Nevertheless, the larger and more complex cap-containing T_1 oligonucleotide derived from SV40 RNA could only be resolved satisfactorily by the system illustrated in Fig. 2. Ring opening of the 7-methyl G and 2'-O methylation of the second residue can explain only part of the diversity observed; alternate spatial configuration in the polyphosphate-containing compounds (19) may also be involved.

Fig. 3 shows the pancreatic ribonuclease analysis of one of

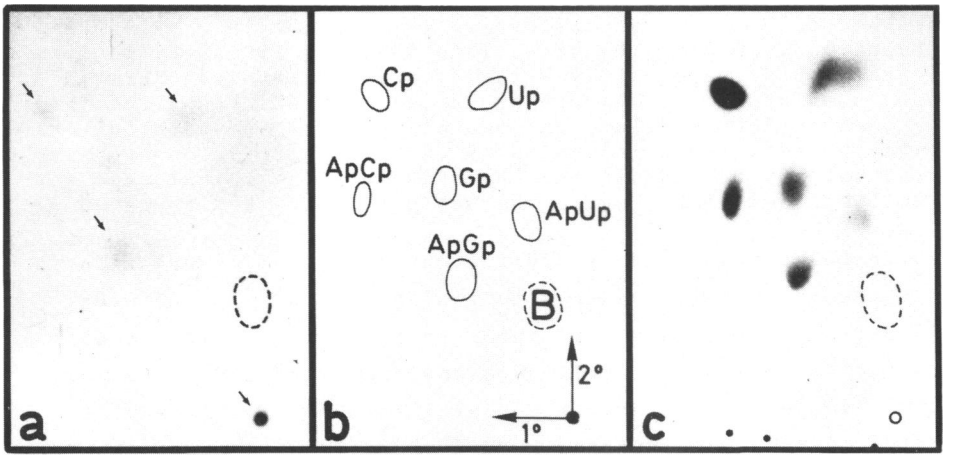

Figure 3 : Two-dimensional separation of a pancreatic ribonuclease-A digest of T_1 oligonucleotides on PEI-cellulose mini-plates (6 x 10 cm) (14). (a) One of the new products present in the fingerprint after specific enrichment (Fig. 2, panel b; all spots indicated by an arrow gave the same double-digestion pattern). The arrows indicate the four products obtained: Cp, Up, ApGp and material remaining at the origin (cf. Fig. 4). (b) Diagram to identify the double-digestion products. B denotes the position of the blue dye and O marks the origin. (c) Ribonuclease-A analysis of the oligonucleotide with composition (Up,2Cp,Ap)Gp (n° 121) which was recovered from a normal T_1 fingerprint of SV40 late mRNA.

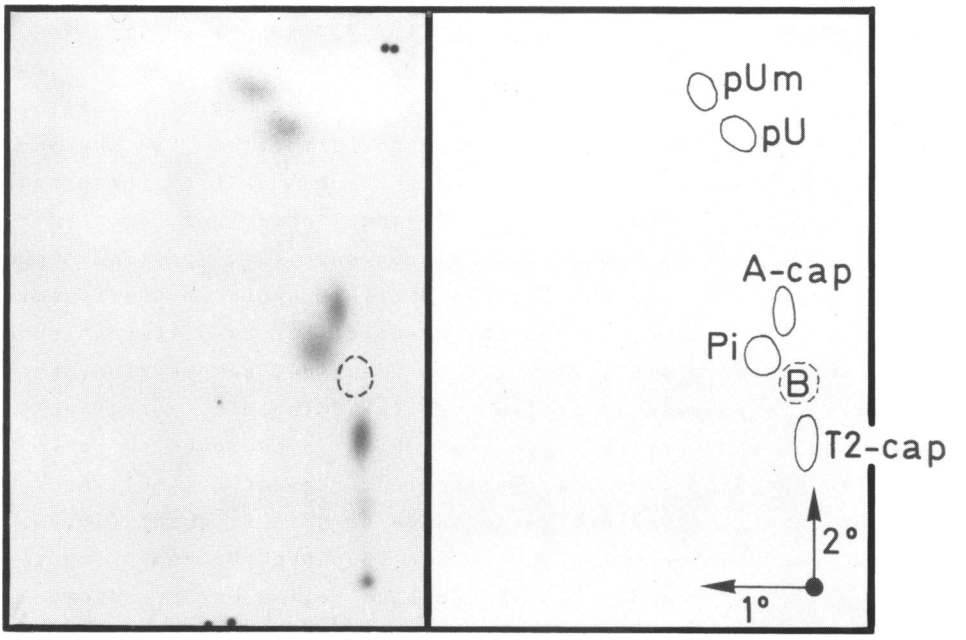

Figure 4 : Nuclease P_1 analysis of the material recovered from the origin of figure 3a. The material was eluted, digested with nuclease P_1 and the digest was fractionated two-dimensionally on PEI-cellulose miniplates (6 x 10 cm) (2). The diagram shows the position and identity of the different spots, as documented in a previous paper (2). A-cap refers to the P_1-generated structure $^{7m}Gppp^{6m}Am$; T_2-cap marks the spot which probably originates from incomplete digestion and may contain undegraded cap I ($^{7m}Gppp^{6m}AmpU$) and/or partially degraded cap II structure ($^{7m}Gppp^{6m}AmpUm$). B indicates the position of the blue dye marker and O corresponds to the origin.

the main new spots which appeared specifically on the map of the sorbitol peak. No significant differences could be seen between the miniplates of the several new spots (as described above). The enzymic hydrolysis products were Cp, Up and ApGp in addition to ribonuclease-A-resistant material which remained at the origin because of its high charge. The degradation products were identified by their position, and their identity was confirmed by alkaline hydrolysis (data not shown). The undegraded material at the origin was eluted and further analyzed by digestion with P_1 nuclease which released the following products: pU and to a lesser extent 2'-O-methyl-uridine-5'-phosphate (pUm); inorganic phosphate (Pi), derived from the 3'-terminal nucleotide; and

other undegraded products which in the system typically moved as cap structures (Fig. 4). These analyses point to an oligonucleotide which contains a mixed population of cap structures I and II, identified previously (2), and furthermore the double-digestion products Cp, Up and ApGp. We conclude from these analyses that the cap-containing T_1 oligonucleotide has the structure ^{7m}G(5')ppp(5')^{m}AmpUpUp(Up,Cp)ApGp and ^{7m}G(5')ppp(5')^{m}AmpUmp Up(Up,Cp)ApGp. The reason for the multiple spots in the fingerprints (Fig. 2) is not known for certain, but as different types of further analyses all indicate an identical composition, the diversity is presumably artifactual (cf. preceding paragraph).

The 5' start of the late SV40 mRNAs is believed to be located in the Hind II + III restriction fragment C (4-6). An inspection of the nucleotide sequence of this fragment (10,20, 21) reveals that the oligonucleotide UpUpApUpUpUpCpApGp (corresponding to position 306-314 of the late region but presented here as an RNA sequence; ref. 10) is the only T_1 product which is ApGp terminal and which carries the specfic sequence ApUpUp (derived from cap II; ref. 2) followed by pyrimidine nucleotides (Fig. 5). This T_1 oligonucleotide had previously been shown not to be present as such in the leader sequence (8). Its structure (containing ApUpUp) as well as its position in the total sequence relative to the known part of the leader, had already previously led to the suggestion that it might correspond to the 5' start of the SV40 late mRNAs (8). Indeed, we reported that the unique and discriminative oligonucleotide CpCpUpCpCpGp (code n° 140) was definitely present in the leader sequence. Therefore, the 5' end must extend further counterclockwise from nucleotide 343 (Fig. 5). The preceding oligonucleotides were presumably also present, but they were not considered to be discriminative, e.g., the T_1 products CpCpGp (n° 020) and CpCpApGp (n° 021), although unique in the molecule, are relatively short and may possibly be overestimated by slight contamination of the viral late mRNA. The T_1 product CpGp (n° 010) occurs twice: once in the leader sequence and once more in the Hind II + III fragment G of the coding region (10,22). The oligonucleotide with composition (Up,2Cp,Ap)Gp (n° 121) occurs three times in the 16S mRNA, but each time it is present as a different sequence isomer. Fortunately, these can easily be distinguished by analysis with

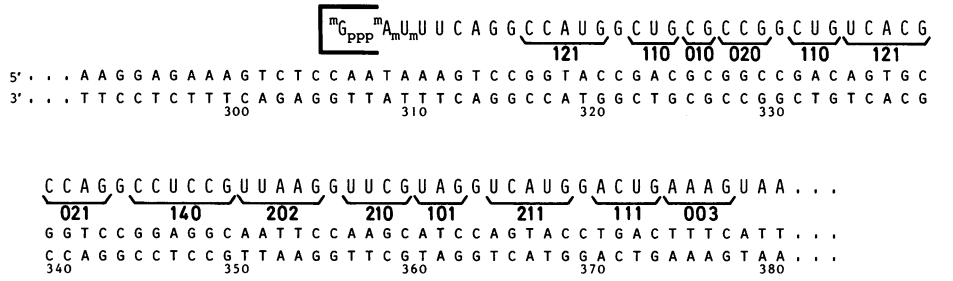

Figure 5 : Nucleotide sequence of part of the Hind II + III fragment C from the late region of SV40 DNA (10,20) and the corresponding leader sequence of the viral late RNAs determined by fingerprint analysis (8). The nucleotide numbers (below the sequence) refer to the position of the fragment in the total sequence of the SV40 DNA late region (10). The mRNA leader fragment starts at position 308. The oligoribonucleotide numbers (below the RNA sequence) are a code referring to the base composition of the corresponding T_1 products (33). All T_1 oligonucleotides following the cap-containing T_1 oligonucleotide have been identified in the fingerprint of the SV40 late mRNA.

ribonuclease A and/or U_2. Digestion with ribonuclease A (Fig. 3c) clearly shows the products ApCp and ApUp (both of which are specific for the isomers present in the leader sequence) in equimolar amount with the product ApGp derived from the isomer UpCpCpAp Gp (n° 121) present in the Hind II + III fragment K (10,23). Further confirmation of the structures is given by U_2 analysis, which releases CpCpAp, UpCpAp, and UpCpCpAp, respectively, from the 5' ends of the three sequence isomers in addition to their respective 3'-terminal products UpGp, CpGp and Gp. From these data it is evident that both sequence isomers CpCpApUpGp and UpCpApCpGp are present in the leader fragment, and hence that the latter extends continuously from the region previously characterized (nucleotide 501 to 344) to nucleotide 316 where it links to the capped oligonucleotide ^{7m}G(5')ppp(5')^{m}AmpU(m) pUpUpCpApGp. These results, obtained by detailed oligonucleotide analysis, proved that the sequence of the leader of the viral late RNAs in this region corresponds exactly to the DNA sequence of the genome.

DISCUSSION

The major 5'-terminal, cap-containing T_1 oligonucleotide of SV40 late mRNA has been isolated by chromatography on DBAE-cellulose followed by fingerprinting, and it was identified by

subsequent analysis with a number of nucleases. The rather unique structure of this oligonucleotide enabled us to localize unambiguously the 5' end of the late mRNAs on the DNA sequence at nucleotide L 308.

No capped oligonucleotide had so far been found upon analysis of all the main T_1 products present in the fingerprint of 16S mRNA, although the various T_1 oligonucleotides derived from the leader fragment were all present in molar amounts (8). From the present study, however, we learned that the cap-containing oligonucleotide appears on such fingerprints in a series of related spots, a diversity which presumably originates from artifacts (e.g., abnormal chromatographic mobility due to the polyphosphate segment). Each of these individual spots must have been considerably submolar compared to the regular products. The radioactivity counts were too low to accurately determine whether the sum of the 5 to 6 cap-containing oligonucleotides represents one molar equivalent. We had previously reported that the caps of the viral late RNAs are present in 0.5 molar ratio only (2). Moreover, after we realized that the presumably unique, cap-containing T_1 oligonucleotide was dispersed over at least 5 to 6 spots, the site of these spots in a normal T_1 fingerprint of 16S mRNA was rigorously reinvestigated, and evidence was obtained that, indeed, the capped oligonucleotide could be recovered from this particular area of the plate (data not shown).

The presence of the degradation products pU and pUm in ribonuclease-A-resistant material released from the capped oligonucleotide shows that a mixed population of cap structures I and II occurs in this oligonucleotide, thus demonstrating that both caps are derived from the same 5' end (i.e., the same sequence on the RNA gives rise to cap I and cap II and the latter is only a more methylated form of the former). Furthermore, these results also show that the multiple cap-spots present in the T_1 fingerprint are in the first instance not due to a different degree of methylation. Because the isolation of capped 5' termini from total SV40 late mRNA, which includes 19S, 16S and possibly even an 18S species, has revealed only one major capped oligonucleotide, it follows that the different late RNA species possess the same 5' terminus. It has recently been established that SV40 16S and 19S share a common 5'-terminal

leader sequence (5,6), and, therefore, our findings of a single prominent 5'-terminal cap for the common leader fragment is not unexpected.

Having established that the cap T_1-oligonucleotide has the structure ^{7m}G(5')ppp(5')^{m}AmpU(m)pUp(Up,Cp)ApGp, it follows from the known SV40 DNA sequence that this oligonucleotide is derived from nucleotide L 308 and the following nucleotides in clockwise direction (Fig. 5). Our previous studies had indicated that the leader sequence is initiated before nucleotide L 344 and extends continuously up to at least nucleotide 501, around which position the splicing event occurs (8). In view of these results, we have carefully analyzed again the T_1 fingerprint of the SV40 late mRNA, and we did find all the expected products in the region immediately following the capped T_1 oligonucleotide (Fig. 5). Therefore we can conclude that unlike the adenovirus-2 late mRNAs, which undergo multiple splicing events in the leader sequence (24,25), the leader of the SV40 mRNA is at least 194 nucleotides long and the segment between the cap and the start of the single splice corresponds to a continuous sequence of the DNA genome.

The only enzymic mechanisms of cap-formation which have so far been identified in eukaryotic cells involve modification of 5' triphosphate or diphosphate termini (26). There is no unambiguous evidence that the latter can be generated also from processed 5' ends. Therefore, the possibility should be seriously considered that caps correspond really to initiation points of transcription (26,27). If so, we have identified the major start of SV40 late RNA transcription.

In the case of prokaryotes, the initiation of transcription takes place at a distance of 6 to 7 nucleotides from a segment of 7 nucleotides which are rather well conserved in all promotors. On this basis, it could be proposed that the region on the SV40 genome before nucleotide L 308 corresponds to an eukaryotic promoter. This region would then be recognized by RNA polymerase II, which is responsible for all SV40-directed transcription. However, there are several reasons to believe that the situation may be more complex. First, it is well known that the start of late transcription depends on the initiation of DNA replication, and therefore the late transcriptional initiation complex may

be a peculiar structure (although continuous DNA replication is not required for maintenance of late transcription; refs. 28-30). Furthermore, many viable deletion mutants have been isolated which have the mutation around the Hpa II site or in the region 0.68 to 0.74 (31,32). Some of these mutants must have lost information from the leader sequence and some may even have lost the putative transcriptional initiation point identified in the present communication. If so, then the most likely explanation for the viability of these deletion mutants is that late transcription can initiate at alternative positions. It should be noted, in fact, that the SV40 late ribonuclease T_2 caps, ^{7m}G(5')ppp(5')^{m}AmpU and ^{7m}G(5')ppp(5')^{m}AmpUmpU, represented about 70 % or more of the entire T_2-resistant material isolated, but the origin of the remaining fraction is still unknown. In order to obtain information on these aspects, we are now characterizing in detail some of the aforementioned deletion mutants, both at the level of the DNA sequence and at the level of the induced late mRNA.

ACKNOWLEDGMENTS

We are indebted to Dr. A. Van de Voorde for discussions, and to José Van der Heyden for excellent technical help. G.H. thanks the *Nationaal Fonds voor Wetenschappelijk Onderzoek* of Belgium for a fellowship. This research was supported by grants from the *Kankerfonds* of the *Algemene Spaar- en Lijfrentekas* and from the *Geconcerteerde Akties* of the Belgian Ministry of Science.

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