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Characterization of the major altered leader sequence of late mRNA induced by SV40 deletion mutant d1-1811

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ABSTRACT

d1-1811 is a viable SV40 mutant with a 40 base pair deletion that includes the major wild-type capping site of late mRNA at map position 0.72. The late viral mRNAs induced by d1-1811 have now been further characterized by inversed S1-mapping analysis. The S1-resistant, ^{32}P-labeled RNA fragments derived from the leader region were examined by fingerprinting and by analysis of RNase T2-generated 5'-terminal cap structures. The results show that most if not all of the mutant leader fragments analyzed have their 5' terminus to the left of the d1-1811 deletion site, i.e., closer to the origin of DNA replication. The major altered leader fragment is a continuous transcript from the DNA in the region 0.716 to 0.761 map unit and its 5' terminus has been precisely mapped at nucleotide L290. The observation that the cap structure of the major altered leader is only a minor cap species in wild-type late RNA suggests regulation in the use of different capping sites in SV40.

INTRODUCTION

During the late stage of SV40 infection of monkey kidney cells, i.e., after the onset of viral DNA replication, two cytoplasmic late mRNA species are synthesized sedimenting at 16S and 19S (1, 2). Both species have been mapped on the genome (3, 4), and more recently it has been found that these viral mRNA molecules are composite structures comprising an untranslated "leader" segment and a coding sequence or "body". The sequence is not colinear with the DNA: each mRNA has been formed by at least one splicing event (5-8). The most abundant leader fragment is a sequence of 202(±1) nucleotides that is transcribed from the DNA region between 0.72 and 0.76 map unit and which is subsequently spliced 42(±1) nucleotides before the body of the VP1 gene (9-12; the uncertainty is because the exact position of splicing is ambiguous). Alternative leader fragments may also occur in

late viral RNA and these actually appear to constitute the most abundant leader segments in the 19S species (7, 13, 14).

Procedures have been described for creating viable SV40 deletion mutants in the late region of the genome (15-18). The deletions have been characterized by restriction mapping analysis; in some cases the exact size and location of the deletion has been precisely determined by nucleotide sequence analysis (18-20). It is remarkable that in some of these mutants the major late capping site, which maps at position 0.722 (21), is included in the deletion (18, 20). Although in some other systems cap formation seems to be directly linked with initiation of RNA transcription (22-26), deletion of the major capping site in SV40 does not result in loss of viability but at most only results in a decreased plaque size (18, 27).

Previously we have shown that deletion of the major capping site in the mutant dl-1811 results in the appearance of a variety of alternative capped 5' termini in the corresponding late mRNA (27). This suggests the presence of a number of alternative leader segments. Indeed, altered 16S and 19S mRNAs have been observed after infection with mutants that lack DNA segments of different size and at slightly different positions compared to the dl-1811 deletion (28). In this paper we report a further characterization of the dl-1811 late mRNA both by fingerprint analysis (9, 29) and by the S1 mapping procedure (30), followed again by fingerprinting and by cap analysis of the S1-resistant fragments. This enabled us to determine at the nucleotide level the major alternative leader segment present in the dl-1811 late mRNA and to identify the two related cap structures ^{7m}Gppp^{m}AmpG and ^{7m}Gppp^{m}AmpGmpA. These major terminal structures constitute about 40% of the total dl-1811 late mRNA cap content (27). Furthermore, the latter terminal trinucleotide sequence A-G-A permitted us to pinpoint unambiguously the 5' end of the alternative leader segment.

METHODS

Isolation and purification of ^{32}P-labeled cytoplasmic late viral mRNA from CV-1 monkey kidney cells infected by either wild-type (WT) SV40 or by dl-1811, fingerprint analysis of the puri-

fied RNA, and isolation and structure determination of the appropriate 5'-terminal capped structures have been described in detail previously (9, 27, 31). The Berk and Sharp technique (30) for mapping mRNA sequences on a genome, for pinpointing the 5' end of leader fragments and for positioning spliced-out regions has been applied in these studies in an inversed way, i.e., by using ^{32}P-labeled RNA and unlabeled DNA. The S1-resistant DNA·RNA hybrids were isolated by polyacrylamide gel electrophoresis and used for further characterization of the mRNA leader fragments by fingerprinting techniques.

Poly(A)-containing viral RNA was selected by a single hybridization step onto Sepharose-bound SV40 DNA, precipitated in the presence of carrier yeast ribosomal RNA, and dissolved in 80% deionized formamide, 0.4 M NaCl, 0.001 M EDTA and 0.04 M Pipes buffer, pH 6.4, along with the appropriate SV40 DNA fragment. The latter was obtained by digestion on a preparative scale of viral DNA isolated by Hirt extraction of infected cells, or was derived from plasmids consisting of segments of SV40 DNA integrated in pBR322 (details given below). In a typical experiment this solution (final vol. 60 µl) was heated for 15 min. at 85°C to completely denature the DNA and was then kept at 52°C for 3 h to allow formation of DNA·RNA hybrids. After this hybridization step the solution was diluted in 900 µl ice-cold S1 buffer containing 0.025 M NaAc, 0.25 M NaCl and 0.0045 M ZnAc, pH 4.4. 150 µl S1 nuclease (1 unit/µl) was added and the digestion was carried out for 30 min. at 37°C. The mixture was extracted with phenol, and the S1-resistant hybrids were precipitated, and fractionated on a polyacrylamide gel run in 0.09 M Tris-borate, 0.0025 M EDTA, pH 8.4. The gel bands containing the DNA·RNA hybrids were visualized by autoradiography and were eluted with 1 M NaCl. Each fragment was redissolved in 5 µl 0.02 M Tris-Cl, 0.002 M EDTA, pH 7.4, heated for 10 min. at 85°C and quickly chilled on ice to keep the hybrids denatured. 1 µl of T1 ribonuclease (10 units/µl) was added and the solution was incubated for 1 h at 37°C. The digest was subsequently analyzed by minifingerprinting (29). To isolate the 5'-terminal cap structures of S1-resistant RNA fragments, DNA·RNA hybrids were eluted and denatured as described above and digested with ribonuclease T2 and

bacterial alkaline phosphatase as reported previously (31).

RESULTS

Characterization of d1-1811 late mRNA by minifingerprinting

Twice-hybridized ^{32}P-labeled d1-1811 late mRNA was digested with T1 ribonuclease and a two-dimensional fingerprint was made. The d1-1811-specific T1 pattern is shown in Figure 1. The profile is homologous to the WT RNA fingerprint except for only one additional spot which occurs in about molar amount in the d1-1811

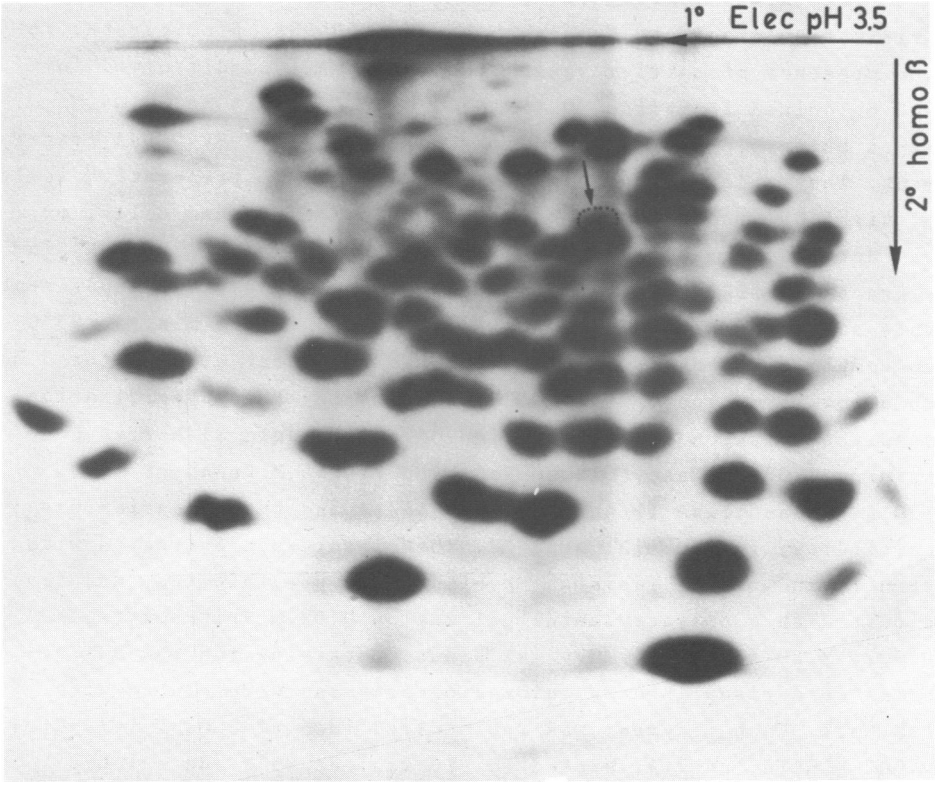


Fig. 1 : T1-ribonuclease fingerprint of d1-1811 late mRNA. Two-dimensional fractionation on PEI-cellulose thin-layer plates (20 cm x 20 cm) was carried out according to the procedure of Volckaert et al. (29) of a T1 hydrolysate of total cytoplasmic late mRNA of d1-1811. The arrow indicates the additional spot (T1 oligonucleotide 243) present in the d1-1811 pattern which represents the unique product with composition $(U_2,C_2,A\text{-}C,A\text{-}A\text{-}C)G$.

pattern. This additional product (T1 oligonucleotide 243; cf. legend to Figure 5) was analyzed with ribonuclease A (not shown) and its double-digestion products (U_x,C_y,A-C,A-A-C)G correspond to the sequence U-A-C-C-U-A-A-C-C-G, which is the predicted T1 oligonucleotide on the basis of the SV40 DNA nucleotide sequence (32) and the extent of the dl-1811 deletion (27). The first Up residue of this oligonucleotide (at L296) is part of the KpnI restriction site at map position 0.716 while the pG residue is derived from beyond the deletion (at L345) and corresponds to the position next to the HpaII site at 0.726 map unit. The presence of this oligonucleotide in about molar quantity (the resolution was not sufficient to allow rigorous quantitation) suggests that most if not all of the dl-1811 leader fragments extend into the 5' side of the deletion site and that the variety of cap structures associated with dl-1811 late mRNA (27) is predominantly derived from an area of DNA preceding the position of the major WT capping site (i.e., from a region closer to the origin of DNA replication around 0.67 map unit). The preceding characteristic T1 oligonucleotides, UUCUUUCCG (N° 530) and CCUCAG (N° 131) (cf. Figure 5), were not revealed as independent spots in the T1 fingerprint of total dl-1811 late mRNA, as they were only partially resolved from related sequence isomers. However, their presence, although in variable amounts, was revealed by pancreatic analysis of the appropriate mixed T1-oligonucleotide spots. Other unique T1 oligonucleotides located more upstream (i.e. towards map position 0.67) were not readily detectable in the fingerprint of total dl-1811 mRNA and were considered to be appreciably submolar compared to the relative abundance of the normal WT leader oligonucleotide sequences.

Isolation of the major WT leader segment by inversed S1 mapping

In order to specifically isolate the leader fragments of the viral RNA, an inversed S1-mapping procedure was used (see Methods section). Total poly(A)-containing RNA was first hybridized to immobilized SV40 DNA in order to decrease the background of the gel pattern; this step is almost essential when subsequent mapping of the oligonucleotides of the protected RNA fragment is intended. The SV40-specific RNA was then hybridized to the Hind III restric-

tion fragment C (0.646 to 0.860 map unit) and treated with S1 nuclease according to the Berk and Sharp (30) procedure. The gel pattern obtained with WT SV40 RNA is shown in Figure 2a. One major band (N° 5) is apparent indicating the presence of a major late leader fragment, whose size and T1-fingerprint is in complete agreement with previous results derived from oligonucleotide mapping (9, 10) and reverse transcription (11, 12) of late viral RNA. Slower migrating bands were also analyzed by T1 mapping and were shown to contain RNA sequences protected by the Hind II + III fragment D (i.e., part of the coding portion of the 19S mRNA) and, furthermore, RNA fragments including the major leader segment in addition to sequences corresponding to the Hind-D fragment. This means that besides the major WT SV40 leader sequence common to both 16S and 19S RNA but mainly derived from the former (9-13), some specific RNA fragments were picked up which were derived exclusively from the 19S mRNA. Minor leader fragments shorter than the major 202(±1) nucleotide long sequence and corresponding to those observed by reverse transcription (13, 14) may have been lost in the background smear of the gel.

Isolation and characterization of the major d1-1811 leader sequence by inversed S1 mapping

As the mutant d1-1811 late mRNA starts with a wide variety of 5'-terminal cap structures (27), a great diversity of leader fragments was expected. The major 5'-terminal leader fragments have now been further characterized by reversed S1-mapping. To circumvent the additional complexity of obtaining RNA segments protected entirely or partially by coding portions of the DNA during hybridization, the d1-1811 DNA segment comprising Hind II + III restriction fragments C and L (0.646 to 0.756 map unit) was inserted into plasmid pBR322 under CIII physical containment conditions. The resulting plasmid, pSCL12, was then used as a leader-specific probe in the S1-mapping experiments with d1-1811 mRNA (a detailed description of the construction of pSCL12 will be published elsewhere). Figure 2b shows the polyacrylamide gel of ^{32}P-labeled d1-1811 late mRNA after hybridization to pSCL12 DNA and subsequent S1 nuclease digestion. A number of successive bands of nearly the same size may be seen; however, one particular species, N° 3, seems to be more abundant compared to the ad-

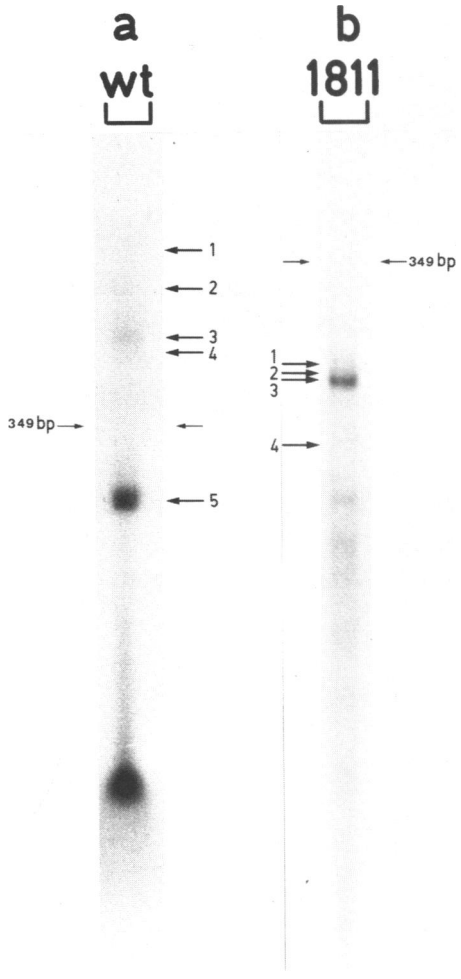


Fig. 2 : Fractionation by electrophoresis of ^{32}P-labeled S1-resistant RNA·DNA hybrids.
(a) Separation on a 4% polyacrylamide gel of WT SV40 late mRNA hybridized in 60 λ hybrid buffer (see Methods section) onto 3 µg of SV40 Hind-C DNA in the presence of 60 µg carrier plasmid DNA and 30 µg carrier yeast RNA. (b) Separation on a 12% polyacrylamide gel of dl-1811 late mRNA hybridized in 20 λ hybrid buffer onto 25 µg PstI-cleaved pSCL12 plasmid DNA (which contains the SV40 Hind II + III fragments C + L) in the presence of 12 µg carrier yeast RNA. Note that the S1 digestion conditions (see Methods section) have been adapted to the quantity of nucleic acid material present in the initial hybridization step. In this particular case, for example, 50 units of S1 nuclease were used in 300 µl S1 buffer. The size marker used is a S1-treated MS2 RNA·DNA hybrid of chain length 349 basepairs.

jacent compounds. All the bands were eluted and characterized by T1 fingerprinting. Several of them (N° 1, 2 and 3) were also analyzed with respect to cap content. Figure 3a and 3b shows the fingerprint and corresponding oligonucleotide identification, respectively, of the major band N° 3. Beginning at the 3' terminus of the leader fragment and going in the 5' direction (see Figure 5), all expected oligonucleotides, including the aforementioned T1 oligonucleotide 243 which spans the deletion site, are present on the fingerprint. However, oligonucleotide 131, which precedes oligonucleotide 243 by only 3 nucleotides, was definitely absent from band N° 3, as were all the remaining T1 products extending further in the 5' direction. These results allow the localization of the 5' end of the major mutant leader to a rather narrow area neighboring the KpnI restriction site at position 0.716. Total degradation of another aliquot of this leader segment RNA with T2 ribonuclease and bacterial alkaline phosphatase (Figure 4) revealed two prominent cap structures identified by position as ^{7m}Gppp^{m}AmpG and ^{7m}Gppp^{m}AmpGmpA. This

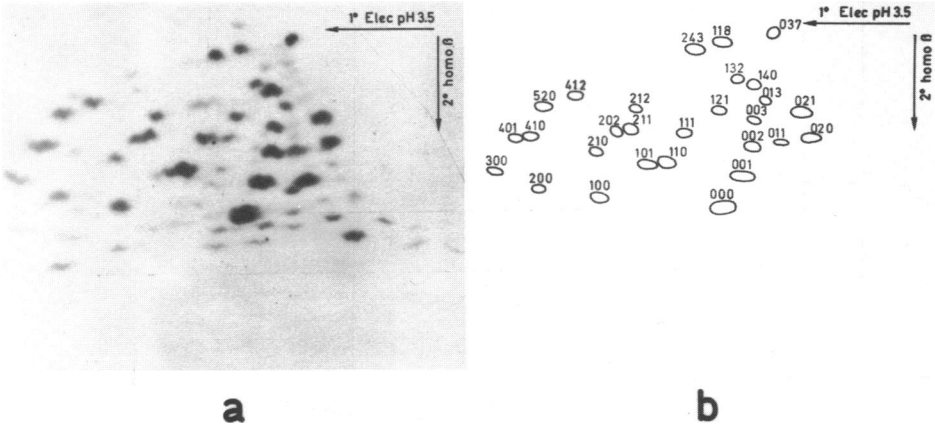


Fig. 3 : Characterization of the major leader fragment of d1-1811 late mRNA.
(a) A portion of the S1-resistant RNA·DNA hybrid band N° 3 in Figure 2b was digested with T1 ribonuclease (see Methods section) and fractionated in two dimensions on PEI-cellulose thin-layer plates (20 cm x 20 cm). (b) Schematic diagram identifying the oligonucleotide spots in (a). A standard nomenclature of three digits indicating the number of uridine, cytidine and adenosine residues, respectively, is used for identifying the T1-spots (38).

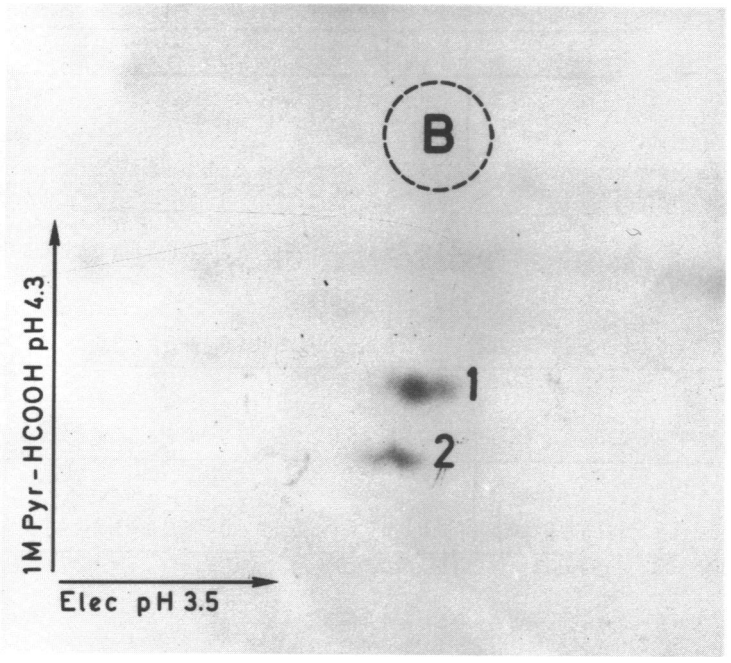


Fig. 4 : Isolation and identification of the cap structures from the major d1-1811 leader fragment.
Another aliquot of the S1-resistant band N° 3 (see Figure 2b) was treated with T2 ribonuclease and bacterial alkaline phosphatase and separated by electrophoresis at pH 3.4 in the first dimension and chromatography in 1 M pyridine-formic acid (pH 4.3) in the second dimension (31). B denotes the position of the blue dye marker xylene cyanol FF. The identity of the capped products ^{7m}Gppp^{m}AmpG (cap I) and ^{7m}Gppp^{m}AmpGmpA (cap II) was deduced from their specific position on the map relative to the blue dye marker; these spots correspond to spots 5 and 7 in Figure 3 of ref. 27, which describes the characterization of all the different d1-1811 capped termini.

couple of cap I and corresponding cap II structures is indeed identical to the major 5' cap terminus of total d1-1811 late mRNA as previously characterized (27).

Based on the trinucleotide sequence A-G-A, we concluded from these results that the capped nucleotide of the major d1-1811 leader fragment must correspond to position L290 of the late region of the genome (Figure 5; nucleotides are numbered as described in ref. 20). L290 lies upstream from the major WT capping

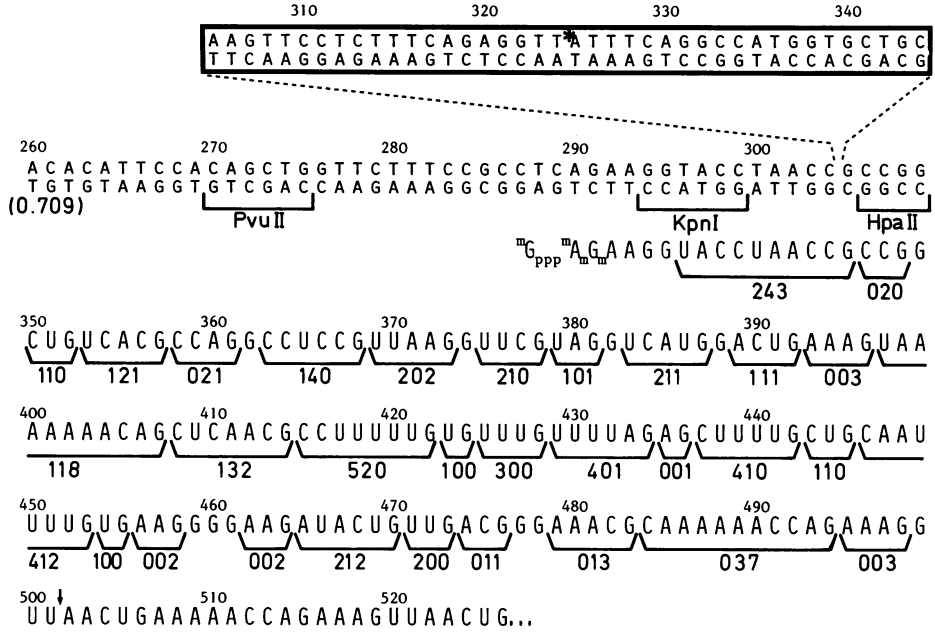


Fig. 5 : Nucleotide sequence of part of the Hind II + III restriction fragment C from the late region of dl-1811 DNA (27) and of the corresponding 5'-terminal sequence of the major altered leader fragment of the viral late mRNA. Nucleotide numbers are based on a total WT SV40 sequence of 5243 basepairs according to the system of Fiers et al. (32) subsequently corrected for the late region (20). The recognition sequences of relevant restriction endonucleases are enclosed in brackets. The 40 basepair dl-1811 deletion is shown in the box and the site of the deletion, between nucleotides L304 and L344, is indicated (20, 27). The position of the major WT capping site (21) is marked with an asterisk.

The major leader fragment of dl-1811 late mRNA starts at WT position L290 and is colinear with the DNA sequence up to nucleotide L526(±1)(map position 0.761), where a donor splicing signal is encountered. The T1 oligonucleotides are indicated below the RNA sequence and they are named in a code which gives the base composition of the respective products (see legend to Figure 3). These oligonucleotides may be seen on the T1 fingerprint in Figure 3. The arrow marks the end of the DNA fragment used for hybridization, i.e., the Hind II cutting site at position 0.756.

site, and the mutant leader sequence is colinear with the mutant DNA sequence (27) from L290 up to the position where splicing occurs (at 0.761 map unit, i.e., nucleotide L526 ± 1). This means that the length of the major mutant leader sequence (Figure 2b,

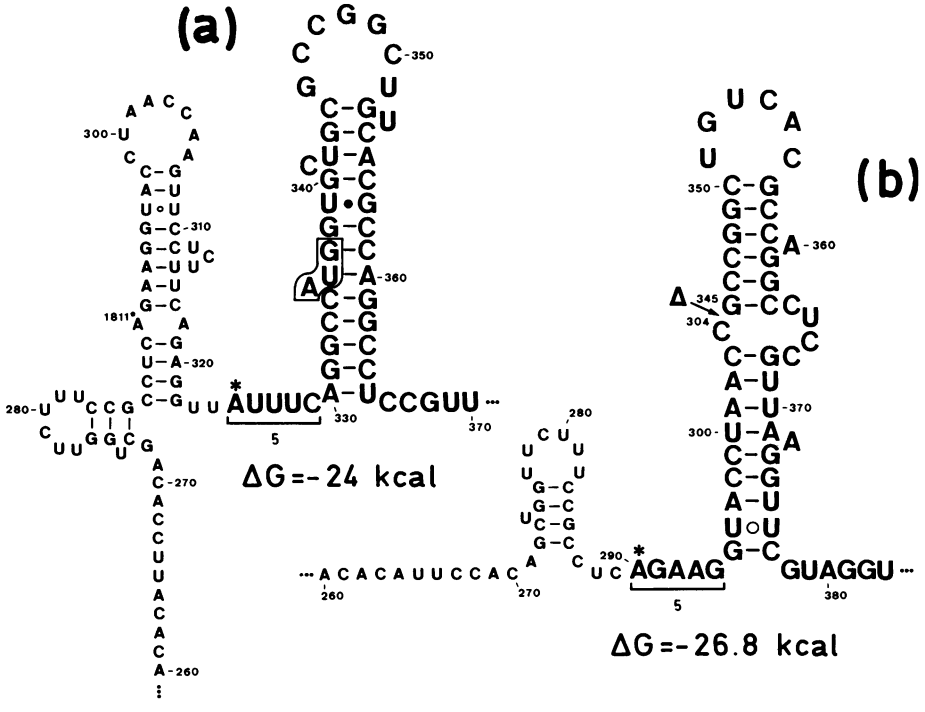
band 3) amounts to 197(±1) nucleotides.

Analysis of the other bands revealed the presence of additional A-cap structures characterized previously (cf. ref. 27) and the occurrence of specific T1 oligonucleotides derived from the DNA region preceding the aforementioned major dl-1811 capping site (evidence not shown). However, as there was too little radioactivity present in these minor S1-resistant bands, unambiguous characterization proved difficult and only visual inspection of the two-dimensional fractionation patterns was possible. From these data we nevertheless were able to conclude that all the alternative leader fragments analysed (5 bands) extend further in the 5' direction than the major mutant leader species (i.e., the 5' terminus was located closer to the origin of DNA replication). However, considering the variety of trinucleotides which could correspond to the 5'-cap sequences (27), we were not able to pinpoint the 5' terminus of these additional leader segments.

DISCUSSION

The most prominent leader sequence in dl-1811 late mRNA was characterized both by fingerprinting and by cap structure analysis of the material purified by the Berk and Sharp S1-procedure (30). Under the experimental conditions used, the RNA·DNA hybrids were found to be fairly stable and showed little if any breathing at the ends. Indeed, even after extensive digestion with S1 nuclease the 3'-terminal T1 products were present in molar amount on the fingerprint. The same holds for the 5'-terminal cap structures, which did not disturb hybridization and in fact allowed isolation of capped termini from S1-digested RNA·DNA hybrids. Indeed, we found that the major leader fragment in the mutant mRNA starts at nucleotide L290 (map position 0.716) and is colinear with the dl-1811 DNA sequence up to the major donor splicing site of WT RNA at nucleotide L526(±1) (map position 0.761). One consequence of the deletion in dl-1811 is that the coding potential of the open reading frame present in the WT leader fragment is lost: the presumptive initiation codon of this "agnogene" is comprised within the dl-1811 deletion (cf. Figure 5) and the reading frame is not restored in the mutant

leader segments by any preceding initiation codon. It follows that the potential product of the agnogene, if it is indeed produced by the WT virus, is not essential for viability under tissue-culture conditions. Indeed, the non-essential nature of this region of the genome is well known (15-18). The slower growth rate of d1-1811, as manifested by the smaller size of the plaques (27), may perhaps be due to a slightly decreased level of late mRNA synthesis and/or its different 5'-terminal structure. The donor splicing site in the leader sequence, viz the tetranucleotide A-G-G-U corresponding to nucleotides L293-296 (map position 0.716) (13, 14), is present in the major mutant mRNA, but apparently the intra-leader splicing event has not occurred, because the RNA started only a few bases earlier. Possibly adjacent capping and/or methylation distorts the splicing recognition signal and prevents the RNA from being spliced at this position, due, perhaps, to absence of a proper secondary structure (33). In contrast, mutant leader fragments which begin much more upstream do undergo splicing at this position, as has been tentatively de-



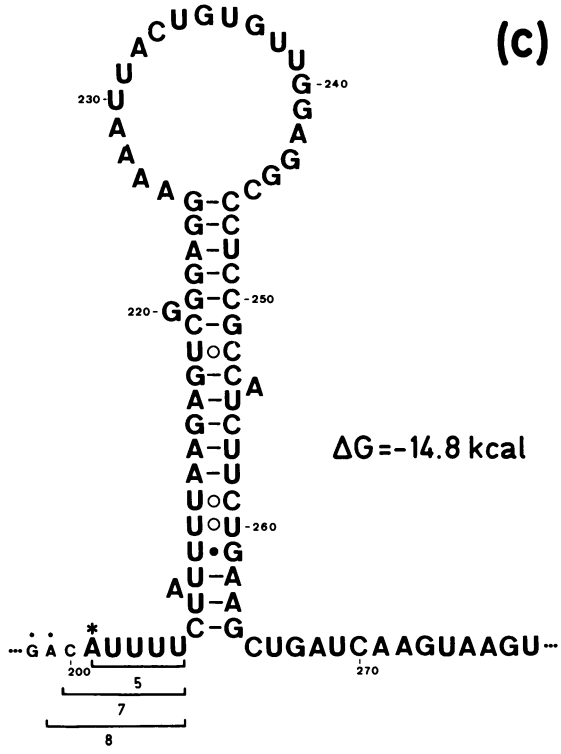


Fig. 6 : Proposed secondary structure models for the RNA sequence corresponding to the 5'-terminal major leader fragment(s) and the preceding, possibly transcribed, nucleotide sequence of (a) WT SV40 late mRNA, (b) dl-1811 late mRNA, and (c) polyoma virus late mRNA. The SV40 nucleotides are numbered as in Figure 5 and hence are identical for both WT and dl-1811 DNA (and corresponding RNA). The 40 nucleotide deletion is indicated by an arrow in (b). Polyoma nucleotide numbers were taken from Flavell et al. (35). Free energies of the hairpin structures were calculated according to the stability rules of Tinoco et al. (39). The small contribution made by a G·U basepair to the stability of a helix is indicated by a dot. The symbol ° between two bases indicates that they occur opposite each other in the helix but that the union does not add to the stability of the structure (40). The positions of the major capping sites are marked by an asterisk.
Note that in all the examples the major capping site precedes a stable (G·C)-rich hairpin structure by exactly 5 nucleotides. However, in the WT RNA sequence, the major dl-1811 capping site (marked by 1811*) is part of the stem of a hairpin loop. The boxed AUG sequence in (a) is the initiation codon of the conjectural agnogene.

duced from visual interpretation of T1 fingerprints of the alternative d1-1811 minor leader segments.

It is not unexpected that the most prominent cap I and II structure found on d1-1811 late mRNA corresponds to the 5' termini of the fragment found in the major band of S1-resistant leader RNA. It may be noted that this particular capped end is only a minor component in the diversity of WT SV40 cap structures (13, 34, and our unpublished data) and more particularly it seems that only one 19S leader species but no 16S species starts at this position (13). Favoring of the capping site at nucleotide L290 in d1-1811 as compared to the relative use of this position in WT SV40 suggests that the appearance of different caps is subject to regulation or depends on local configuration of the mRNA. Indeed, the relative abundance of different caps may be directly dependent on structural or functional requirements. A secondary structure model for part of the WT SV40 leader sequence (Figure 6) shows that the major WT capping site is located at the base of a stable hairpin, whereas nucleotide L290 is fully involved in base-pairing in the stem of the preceding loop. Also, some minor WT capping sites may be found in single-stranded regions preceding similar (G·C)-rich loop structures (our unpublished results). Remarkably the structure model for the d1-1811 RNA sequence shows that now the position of the major mutant capping site at nucleotide L290 lies free and at exactly the same distance (5 nucleotides) from an analogous (G·C)-rich hairpin. In the case of polyoma virus, which is distantly related to SV40, several cap structures in the late mRNA corresponding to consecutive purine positions in the DNA sequence have been identified (35). These may conceivably be derived by a stuttering effect of the polymerase II during initiation of transcription (35). The variety of capping sites in SV40 late RNA and especially their relative broad distribution over the DNA sequence suggests a more complicated mechanism for their formation than simple stuttering. Possibly, the RNA polymerase starts at many different positions in a defined region and only those RNA molecules with a hairpin near their 5' end become stabilized against rapid degradation and are conserved. Alternatively, initiation of RNA synthesis may occur more proximal to the origin of DNA synthesis and processing

enzymes may subsequently recognize the proposed hairpin loops (Figure 6), whereupon caps may be formed after further phosphorylation at the processed 5' ends, analogous to, e.g., the capping system of vaccinia virus (36, 37). It is of interest that also in polyoma virus mRNA a loop structure following (and specifying ?) the capping site can be drawn. However, this structure is less stable (and less G·C-rich) than the proposed SV40 hairpins and may perhaps be responsible for rather imprecise processing, thereby giving rise to multiple caps around the same position (35). More direct experimental approaches are needed to distinguish unambiguously between these two possibilities for generating the diversity of capped 5'-termini in the papova virus late mRNAs.

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