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Segments of simian virus 40 DNA spanning most of the leader sequence of the major late viral messenger RNA are dispensable

(viable deletion mutants/nucleotide sequence of DNA/16S mRNA leader/splicing and capping/primate DNA ligase)

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ABSTRACT **A highly specific procedure for the isolation of deletion mutants is described. The size and location of the deletions can be predetermined. By this method a series of deletion mutants mapping within and near the untranslated 5′ leader sequence of the late 16S mRNA of simian virus 40 have been isolated. The boundaries of the deletions have been accurately determined by DNA sequence analysis. The deletions range from 20 to 223 nucleotides. All these deletion mutants are viable and grow without helper virus. The largest of these deletions removes the entire leader sequence except for six nucleotides at the 3′ end that are probably involved in covalent linkage with the 5′ end of the body of the mRNA located 937 nucleotides away on the genome. Three of the deletion mutants remove the 5′ end of the leader that normally bears the cap structure of the mRNA. A large segment immediately preceding the leader sequence is also removed in one of these mutants, ruling out the generation of the 5′ end of the mRNA via initiation of transcription at this point. The circularization of linear infecting DNA producing the DNA of the deletion mutants proceeds mainly by way of blunt end ligation *in vivo*.**

Late in productive infection by simian virus 40 (SV40), two species of viral RNAs accumulate in the cytoplasm of infected cells. Of these, 16S mRNA, the major species, codes for the major viral capsid protein, VP1; 19S mRNA, the minor species, is believed to code for the minor capsid proteins, VP2 and VP3. Comparison of the nucleotide sequence of late 16S mRNA with that of the complementary DNA strand by molecular hybridization (1, 2), electron microscopy (3), and nucleotide sequence analysis (4, 5) has shown that the RNA consists of two noncontiguous segments that are "spliced" or covalently linked. One of these is the 5′-end leader sequence transcribed from the portion of the genome located roughly between 0.72 and 0.76 map units; the other is the body of the mRNA derived from 0.935–0.175 map units of the genome.

The complete nucleotide sequence of SV40 DNA has been determined (6, 7). The 5′-end sequence of the 16S mRNA has been determined by analysis of reverse transcripts of the RNA made with suitable DNA primers (8). Comparison of the DNA and RNA sequences reveals that the leader sequence of 16S mRNA is 203 nucleotides long and that the 3′ end of the leader is spliced with the 5′ end of the body of the mRNA located 937 nucleotides away on the genome (8). SV40 late 16S mRNA is "capped" at its 5′ end (9). The cap structure is associated with the 5′ end of the leader sequence of the mRNA (1, 2). The AUG codon for initiation of synthesis of VP1 is located within the body of the mRNA at a distance 41 nucleotides downstream from the splice point (8, 10). The ribosome binding site for VP1 initiation is believed to lie immediately before this AUG codon (10). Thus, none of the codons for the synthesis of VP1 is contributed by the 5′ leader sequence; all of the VP1 codons are present within the body of the 16S mRNA.

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This report describes the construction and sequence analysis of viable deletion mutants of SV40 spanning the leader sequence of the late 16S mRNA.

MATERIALS AND METHODS

Materials. Wild-type SV40 strain 776 was the parent virus used for making the mutants reported in this study. Viruses were grown in BSC-1 or CV-1P cells. The following enzymes were purchased from commercial sources: restriction endonucleases (New England Biolabs or Bethesda Research Laboratories, Rockville, MD); *Escherichia coli* DNA polymerase I (Boehringer Mannheim); [γ-^{32}P]ATP, [α-^{32}P]dCTP, and [α-^{32}P]dTTP (ICN).

Preparation of Mutants. Form I SV40 DNA was cut by two different restriction endonucleases that cleave the DNA once each, producing fragments with "sticky" or single-stranded cohesive termini. The enzymes were chosen such that the cleavage sites lay within or very near the region of the DNA in which deletion mutants were desired. The cleavage resulted in the production of a larger "core" fragment and a smaller fragment. The fragments were separated by 1.4% agarose gel electrophoresis, visualized under ultraviolet light after being stained with ethidium bromide, and extracted by a gel extraction procedure published earlier (11). In a few instances (e.g., digestion with the enzymes *Kpn* I and *Hpa* II, which cut SV40 DNA at 0.717 and 0.727 map units, respectively), the larger DNA fragment could be used as such as the infecting DNA for isolation of deletion mutants.

In most instances the smaller product of the double digestion might contain additional sequences that we may not want to remove. In such cases the smaller fragment was cut by another restriction endonuclease, producing two or more fragments; one or both of the end pieces resulting from this secondary digestion, which may correspond to sequences that need to be kept in the DNA, were then ligated *in vitro* (with phage T4 DNA ligase) with the larger core fragment (produced earlier) by using their complementary cohesive termini. The map position of the fragments left out in this ligation would roughly correspond to the site of deletion in the mutant DNA. This procedure is illustrated by an example in Fig. 1.

The 16S mRNA leader region of SV40 DNA has a number of restriction sites (Fig. 2) that I have used to advantage in making a series of linear DNA molecules lacking sequences in this region by the procedure described above. The constituent fragments used in constructing these DNA molecules are listed in Table 1.

Cloning and Isolation of Mutants. BSC-1 or CV-1P cells were infected with linear DNA molecules constructed as described above, either alone or with DNA from tsA58, an early region mutant of SV40, in case a helper is needed. Only those

Abbreviation: SV40, simian virus 40.

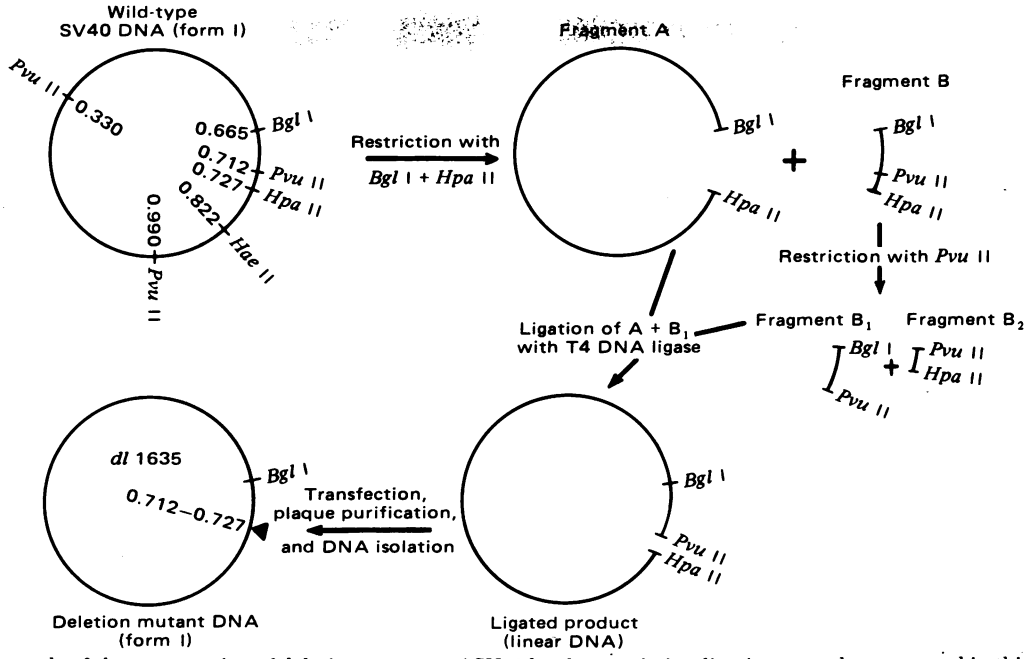

FIG. 1. An example of the construction of deletion mutants of SV40 by the restriction-ligation procedure reported in this study. All steps except the last one are *in vitro* reactions. The solid triangle denotes the location of the deletion. Form I DNA is circular DNA.

DNA molecules that become circular *in vivo* would be infectious. The infected cells were overlaid with agar medium (12), and virus plaques appearing 11–15 days after infection were picked, purified by replaquing, and grown in BSC-1 cells to prepare form I viral DNA by the Hirt procedure (13).

Deletion Mapping of Mutants. Form I viral DNA isolated from each mutant was incubated with *E. coli* DNA polymerase I in the presence of α-^{32}P-labeled deoxyribonucleoside triphosphates to synthesize full-length radioactive DNA *in vitro* by the "nick-translation" procedure of Maniatis *et al.* (14). The radioactive DNAs were digested with suitable restriction endonucleases and were compared with digests of wild-type DNA by electrophoresis on polyacrylamide gels. Fragments spanning the deletion might be missing or, if not, would be smaller than the corresponding wild-type DNA fragment and hence would move faster in the gels (15). (Occasionally the deletion might result in the production of larger fusion fragments.) The deletions were thus localized within certain restriction fragments of the DNA.

The boundaries of the deletions were then accurately determined by nucleotide sequence analysis. A convenient DNA fragment spanning the deletion was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and its sequence was determined through the deleted region by the procedure of Maxam and Gilbert (16).

RESULTS

Phenotype of Deletion Mutants. Because the leader sequence of the 16S mRNA contains its cap structure [cap structures are believed to be essential for mRNA translation (17)] and because it is a sequence that the cell bothers to covalently link with the coding sequences of the mRNA transcribed from a noncontiguous region of the genome, mutants having deletions in this region may be expected to be defective. However, they were all viable. They formed plaques and grew in the absence of helper virus. Growth characteristics of the mutants are listed in Table 2. The deletion mutants formed small or tiny plaques compared to the parent wild-type strain 776 which usually forms small plaques. Mutant plaques took 11–14 days after infection to appear compared to 10–12 days for the wild-type plaques. The mutants produced titers ranging from 1×10^7 to 5×10^8 plaque-forming units/ml of cell-free viral lysate compared to wild-type titers of up to 2×10^9 plaque-forming units/ml. All these variants indicate that although the deletion mutants are viable, they grow more slowly than wild-type SV40. In this respect they are comparable to viable deletion mutants located elsewhere in the genome of SV40 (18).

Restriction Mapping. *Hin*d(II + III) fragments spanning the 16S mRNA leader region were C (0.650–0.752 map units), L (0.752–0.757), M (0.757–0.761), and D (0.761–0.862) (refs.

Table 1. Substrates and products of *in vitro* ligations performed to construct deletion mutants

Map coordinates of DNA fragments ligated*			No. of ligations and termini linked by ligation	Map coordinates of product of ligation*	Mutant produced
Fragment A	Fragment B	Fragment C			
0.727–0.665(93.8%)	0.665–0.712(4.7%)	—	One; *Bgl* I ↔ *Bgl* I†	0.727–0.712(98.5%)	*dl* 1635
0.727–0.717(99.0%)‡	—	—	—	—	*dl* 1659
0.822–0.665(84.3%)	0.665–0.757(9.2%)	0.761–0.822(6.1%)	Two; *Bgl* I ↔ *Bgl* I† and *Hae* II ↔ *Hae* II§	0.761–0.757(99.6%)	*dl* 1613
0.822–0.727(90.5%)	0.757–0.822(6.5%)	—	One; *Hae* II ↔ *Hae* II§	0.757–0.727(97.0%)	*dl* 1626
0.822–0.717(89.5%)	0.761–0.822(6.1%)	—	One; *Hae* II ↔ *Hae* II§	0.761–0.717(95.6%)	*dl* 1661

* Numbers within parentheses indicate sizes of DNA molecules measured as percent of full-length SV40 DNA.
† These ligations restore the *Bgl* I cleavage site at map position 0.665.
‡ This fragment was not subjected to ligation and was used as such for infection.
§ These ligations restore the *Hae* II cleavage site at map position 0.822.

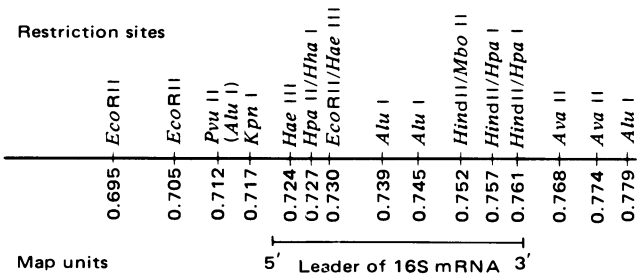

FIG. 2. Restriction sites spanning the late 16S mRNA leader sequence region of SV40 DNA. Map units refer to distances from the single *Eco*RI cleavage site in the DNA.

19 and 20). Comparison of the *Hin*d(II + III) digestion patterns (data not shown) of the wild-type and mutant DNAs indicated that *dl* 1659, *dl* 1635, and *dl* 1626 all have shorter C fragments, the amount of shortening being in the same order; in addition, *dl* 1626 lacks the small fragments L and M. The C, D, L, and M fragments of *dl* 1661 are all missing and the mutant produces a larger "fusion" fragment resulting from deletion of intervening *Hin*d(II + III) sites. *dl* 1613 lacks only the small fragment M. *Hin*d(II + III)-M is the same as *Hpa* I-D because the *Hpa* I cleavage sequence is a subset of the four possible *Hin*dII sequences (21). Because *dl* 1613 lacks only M and has the flanking *Hin*d(II + III) fragments intact, the only way the deletion could have been produced is by circularization via blunt end ligation of the two flush *Hpa* I termini of the infecting linear DNA.

Fragments F, G, and O produced by *Hae* III restriction endonuclease span the 16S mRNA leader (11, 22). Comparison of the *Hae* III digestion patterns of wild-type and mutant DNAs (Fig. 3) shows the following: *dl* 1659 and *dl* 1635 have a shorter G fragment whereas *dl* 1613 and *dl* 1626 have a shorter F fragment; *dl* 1661 lacks both F and G and, instead, has a larger fusion fragment. In addition, *dl* 1659, *dl* 1635, *dl* 1626, and *dl* 1661 lack the small O fragment (not shown).

Determination of Boundaries of Deletion Mutants by DNA Sequence Analysis. Appropriate DNA fragments spanning the deletions were labeled at their 5′ ends and recut with suitable restriction enzymes to separate the two labeled 5′ ends of each fragment. *Hae* III-G′ from *dl* 1659 and *dl* 1635 were recut with *Eco*RII; *Hae* III-F′ from *dl* 1626, which comigrated with *Hae* III-J (Fig. 3), was recut with *Alu* I and *Ava* II in order to separate their subfragments; *Eco*RII-D′ of *dl* 1661 was recut with either *Hae* II or *Ava* II. (*Ava* II + *Hpa* II)-F′ of *dl* 1613 was recut with *Alu* I. The nucleotide sequence of the appropriate subfragment spanning each deletion, now labeled in only one of its 5′ ends, was determined by the procedure of Maxam and Gilbert (16). Two representative gels

Table 2. Phenotype of the viable deletion mutants

Virus	Plaque morphology: Appearance, days after infection	Plaque morphology: Mean diameter,† mm	Growth properties*: Time for 90% CPE, days	Growth properties*: Yield of virus, PFU/ml lysate
WT 776‡	10–12	3.0	7	2.0×10^9
dl 1613	11–14	2.0	10	1.5×10^8
dl 1659	11–13	2.1	10	2.5×10^8
dl 1635	11–13	2.5	9	5.2×10^8
dl 1626	11–14	1.8	10	8.0×10^7
dl 1661	11–14	1.5	12	3.0×10^7

* Infections were at a multiplicity of 0.1 plaque-forming unit (PFU) per cell. CPE, cytopathic effect.
† Determined 13 days after infection.
‡ Wild-type strain 776.

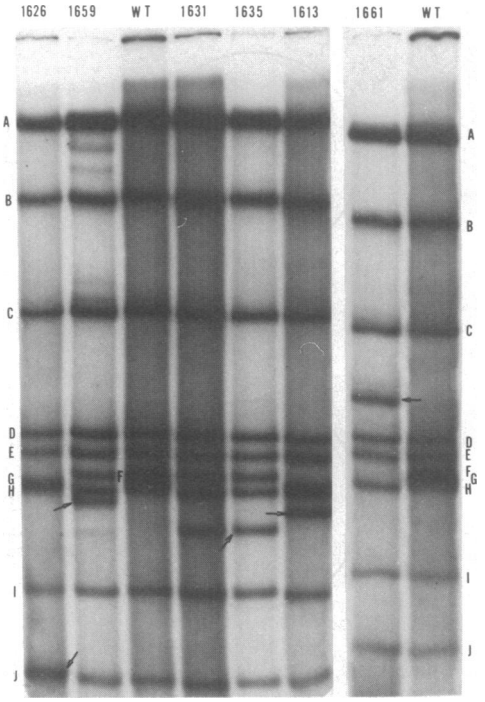

FIG. 3. Autoradiograph of *Hae* III digests of mutant and wild-type (WT) DNAs electrophoresed on 4% (5% for *dl* 1661) acrylamide gel. Fragments spanning the respective deletions are denoted by arrows. The shorter F′ fragment of *dl* 1626 comigrates with J; F and G are both absent in *dl* 1661 and a larger fusion fragment shown by arrow appears. *dl* 1635 and *dl* 1631 are identical.

corresponding to the sequences of *dl* 1626 and *dl* 1661 are shown in Figs. 4 and 5, respectively. The boundaries of all the deletion mutants reported in this study are shown in Fig. 6.

dl 1661 contains the largest deletion, amounting to 4.5% of the genome of SV40. It lacks a total of 223 nucleotides, corresponding to all but the last 6 nucleotides at the 3′-end splice point of the 16S mRNA leader plus a stretch of 26 nucleotides immediately preceding the 5′ end of the leader. *dl* 1626 lacks a total of 153 nucleotides (amounting to 3% of SV40 DNA) located internally within the leader. *dl* 1635 and *dl* 1659 lack the 5′ end of the leader and sequences on either side of it totalling 74 and 48 nucleotides, respectively. The deletion in *dl* 1635 reaches out the farthest to the left of the leader, removing a segment of 52 nucleotides immediately preceding its 5′ end. *dl* 1659 DNA has become circular in such a way that the *Hpa* II and *Kpn* I sites (that were at the two ends of the infecting linear DNA) are fully regenerated. [This has been confirmed by cleavage of the *dl* 1659 DNA with *Hpa* II and *Kpn* I (data not shown).] *dl* 1613 contains the smallest deletion–namely, 20 nucleotides near the 3′ end of the leader.

DISCUSSION

There are a few known methods for making deletion mutants that have been used to yield valuable data on the functioning of the genome of SV40. Carbon *et al.* (23) and Cole *et al.* (24) cut SV40 DNA with a restriction endonuclease that makes a single cleavage in the DNA, such as *Hpa* II, *Eco*RI, *Bam*HI, or *Hae* II, and subjected the linear DNA product to limited digestion with a 5′-exonuclease. Infection of CV-1P cells with these DNA preparations resulted in the production of mutants containing short deletions at the restriction site. Shenk *et al.* (18) have isolated viable deletion mutants of SV40 mapping at 0.54–0.59, 0.68–0.74, and 0.17–0.18 map unit regions of the genome by infection of CV-1P cells with linear DNA molecules

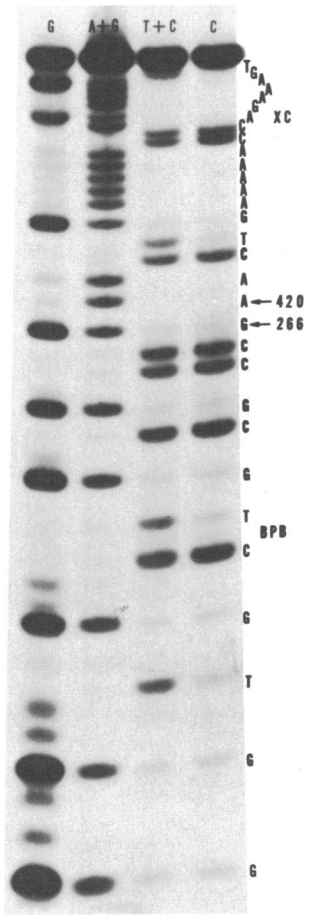

FIG. 4. Autoradiograph of a sequencing gel (20% acrylamide/7 M urea) of *Hae* III-F′ fragment of *dl* 1626. The labeled 5′ end corresponds to residue 251 (Fig. 6 and ref. 6). BPB and XC denote positions of the bromophenol blue and xylene cyanol marker dyes, respectively. The sequence is read from bottom to top and corresponds to the late strand of the fragment. Nucleotides 226 and 420 (indicated by arrows) become covalently linked in *dl* 1626 because of the deletion of 153 nucleotides between them.

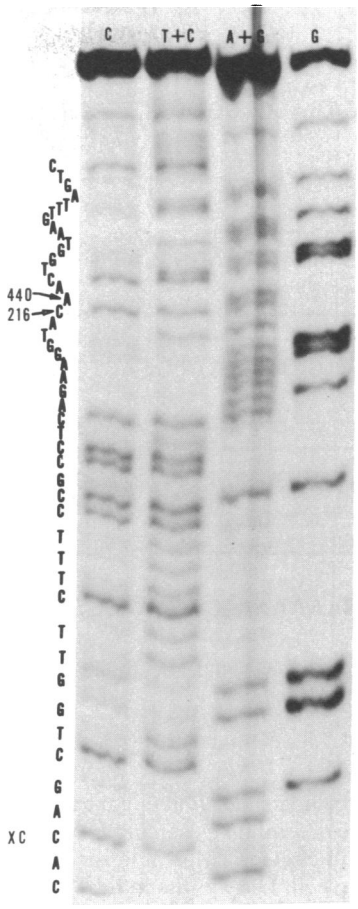

FIG. 5. Autoradiograph of a sequencing gel (12% acrylamide/7 M urea) of *Eco*RII-D′ fragment of *dl* 1661. The labeled 5′ end corresponds to residue 150 (ref. 6). The bromophenol blue marker dye was run out of the gel to resolve sequences somewhat away from the labeled 5′ end; XC denotes the position of the xylene cyanol marker dye. The sequence corresponds to the late strand of the fragment. Nucleotides 216 and 440 (indicated by arrows) become covalently linked in *dl* 1661 because of the deletion of 223 nucleotides between them.

obtained by random double-stranded cleavage with deoxyribonuclease I. Mertz and Berg (25) have isolated naturally occurring mutants at or near the single *Hpa* II site in the DNA by a selection procedure involving resistance to cleavage by *Hpa* II. Lai and Nathans (26) have infected BSC-1 cells with partial digestion products of SV40 DNA produced by restriction enzyme *Hin*dIII in the presence of suitable helper viruses and have isolated nonviable deletion mutants.

The procedure reported in this study has increased specificity and versatility in the sense that the location as well as the size of the deletion can be predetermined *in vitro*. The method is also universally applicable to any DNA molecule provided suitable restriction sites are present. DNA products are subjected to agarose gel electrophoresis before and after ligation, which makes sure that the background due to wild-type plaques is less than 10% of the total number. The boundaries of the deletions have been determined by DNA sequence analysis, and the deletions turn out just as they were intended to be.

I have made use of this procedure to make deletion mutants within the leader sequence of the 16S mRNA, a sequence whose function has not been investigated so far. The mutants are all viable. The largest of these deletions removes the entire leader but for six nucleotides at its 3′ end (Fig. 6). Assuming that the late 16S mRNA in this mutant is covalently linked, it would mean that only the sequence A-A-C-T-G-G, occurring at the 5′ end of the leader, is required for linkage with the body of the mRNA. The sequence A-A-C-T-G forms the 3′ end of the second copy of a 20-nucleotide-long tandem repeat occurring at the 3′ end of the leader (27, 6). My results show that the tandem repeat (but for the sequence A-A-C-T-G) is dispensable, ruling our any vital function for the repeat.

Three of the deletion mutants (namely, *dl* 1659, *dl* 1635, and *dl* 1661) remove, among other sequences, the 5′ end of the leader (and hence the 5′ end of the 16S mRNA itself), including the residue bearing the cap structure (Fig. 6). The 5′ cap structure is believed to be essential for mRNA translation (17). We presume that the 16S mRNAs of these mutants would still have a cap structure, albeit at a new location because the original location is deleted. It would be interesting to look at the nucleotide sequence at this (presumed) new location to see if it bears any resemblance to the original location; this would tell us if the cellular capping enzyme has any sequence specificity.

The transcription of the early or late nuclear SV40 RNAs are believed to be initiated at or near the origin of DNA replication mapping at 0.665 map units (6, 27, 28). The location of the 5′ end of the late 16S mRNA at 0.722 map units might be a result of RNA processing or a separate initiation of transcription at this location. I find that *dl* 1635 lacks, in addition to the 5′ terminus of the mRNA, a stretch of 52 nucleotides preceding it, thereby ruling out the idea of transcription initiation at this point and lending credence to the RNA processing hypothesis.

The late 19S mRNA is believed to be a heterogeneous population of several mRNAs which share the same body but have different leaders (6). A few of these are believed to have the same leader as the late 16S mRNA. The mutations described in this study might also affect the leaders of these 19S species.

In the present study, linear DNA molecules with known termini were used as the infecting DNAs that become circular *in vivo* to form the DNAs of the deletion mutants. The exact

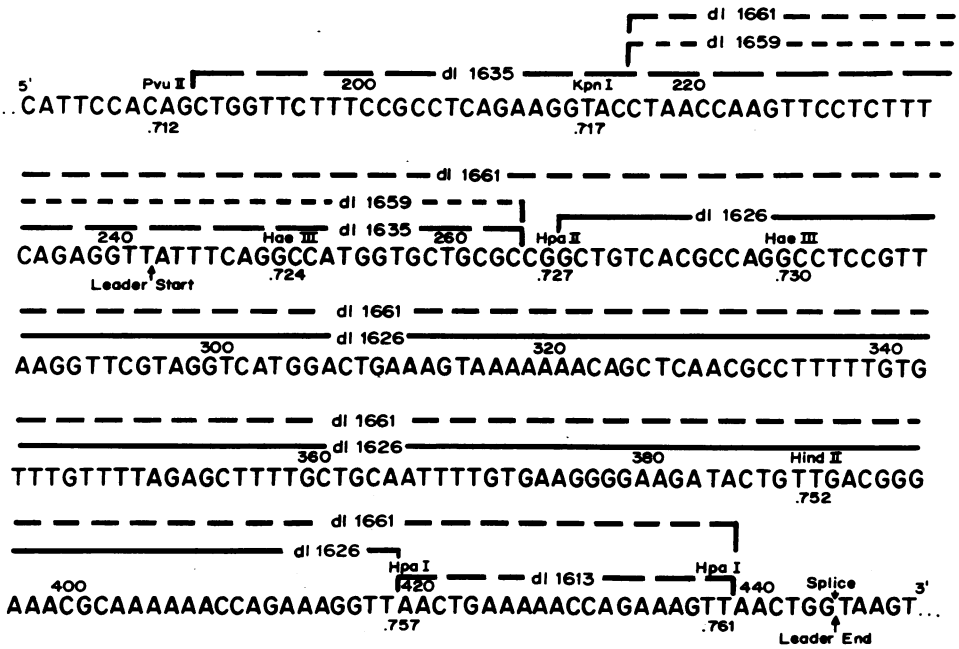

FIG. 6. Sequence of the late strand of SV40 DNA around the late 16S mRNA leader (6). The numbering system of Reddy *et al.* (6) is used. DNA segments deleted in the mutants reported in this study are denoted by distinctive lines drawn above the sequence. Some of the restriction sites falling within this sequence and their map positions are indicated above and below the sequence, respectively, to help with orientation of the sequence with the physical maps of SV40 DNA.

nucleotide sequences of the circularized deletion mutant DNAs at the sites of deletion have been determined, enabling us to take a close look at the mechanism of circularization of linear DNA molecules within a eukaryotic cell. Our results indicate that circularization can occur by way of blunt end ligation. Most restriction endonucleases cut DNA in such a way that short single-stranded termini are formed. Examination of the fused sequences in the mutants (Fig. 6) shows that 5′ protruding single-stranded termini are repaired *in vivo* by DNA synthesis to produce fully base-paired ends that are then circularized via ligation by a cellular DNA ligase. If one of the termini of the linear SV40 DNA happens to have a 3′ protruding single-stranded segment, such as the one produced by *Kpn* I (29), examination of the fused sequences (Fig. 6) indicates that circularization would proceed by end-to-end ligation of the strand containing the 3′ protruding end (a reaction that could be called semi-blunt end ligation) followed by gap filling and ligation of the opposite strand. Information on the ligation specificities of mammalian DNA ligases is lacking at present. My results show the presence of cellular DNA ligases capable of effecting the novel ligation reactions described above.

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