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Genetic Test for Involvement of Intervening Sequences in Transport of Nuclear RNA

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The construction of a recombinant virus in the late region of simian virus 40 is presented. The small intervening sequence of late 19S RNA (0.760 to 0.765 map unit) was cloned and inserted into the *Eco*RI site (1.0 map unit) in the late region of simian virus 40. This is a mutant virus that now has two intervening sequences, one at the normal position (0.760 map unit) and another out of the context of its flanking sequence and now at 1.0 map unit. The recombinant appears poisonous, as repeated attempts to plaque it as a virus with a standard helper virus were unsuccessful. The transcription of this recombinant was, therefore, studied after direct DNA transfection onto CV-1 cells. Nuclease S1 analysis of mutant RNA indicates that the major nuclear transcript was a spliced but nuclear 16S RNA species. Normally, 16S RNA is not found in the nucleus. This result was shown to be an artifact of the DNA transfection protocol. When the glycerol shock was done after infection with virus, a similar alteration in the makeup of nuclear RNA was seen. A transient stock of this double-intron mutant was finally obtained, using a nonrevertable helper virus. The transcriptional analysis of this mutant showed that unspliced 19S RNA was not transported and remained within the nucleus, whereas spliced 19S and 16S RNAs were transported. We conclude that the retention of nuclear transcripts within the nucleus is not simply due to the presence of intronic sequences, as spliced 19S and 16S RNAs which contain the second intron were efficiently transported.

The process by which the nuclear transcripts of eucaryotic cells are transported into the cytoplasm remains poorly understood. It has been proposed that a differential transport mechanism may account for the control of a number of differentially regulated genes (9, 19, 22, 24, 32). Although other mechanisms are clearly involved (i.e., promoter activation), the existence of differential RNA transport has been documented.

The detailed characterization of the structure of nuclear versus cytoplasmic transcripts from a number of cloned cellular and viral genes has been determined (7, 11, 13, 17, 20, 23, 29, 30, 33). Inspections of these results suggest little that clearly distinguishes a nuclear from a cytoplasmic transcript. The locations of 5′ ends, 3′ ends, polyadenylation, and cap sites are frequently the same in the nuclear and cytoplasmic molecules. Some differences, however, are frequently, though not uniformly, observed. The most common difference is that of the spliced status of the nuclear versus cytoplasmic RNA. For the great majority of transcripts, nuclear RNAs are both unspliced and spliced, whereas cytoplasmic RNAs are almost completely spliced. The sea urchin histones are apparently one exception to this observation (18).

The transcriptional phenotype of a number of naturally occurring and constructed deletion mutants in both cellular and viral genes has been studied. Many of these mutants have been observed to affect both the splicing and the transport of nuclear RNA. The most detailed analysis of this nature to date has been performed with the DNA viruses, simian virus 40 (SV40), and adenovirus (5, 6, 8, 10, 11, 15, 16, 21, 36; R. T. White, Ph.D. thesis, Stanford University, Stanford, Calif., 1980). The initial results of these studies, however, offered no clear picture of what relationship existed between splicing and transport. The first deletion studied was in the late region of SV40 (15, 16). This mutant had deleted the intervening sequence for the late 16S RNA and was not able to transport nuclear RNA. This observation led to the proposal that RNA splicing and transport were coupled events. More recent reports dispute this interpretation as the transport of unspliced although mutant late SV40 and adenovirus RNA has been observed, indicating splicing and transport need not be directly coupled (6, 10, 36; White, Ph.D. thesis). Nevertheless, it remains that the common difference between most nuclear and cytoplasmic RNAs is the spliced status of the RNA.

This led us to propose a retention hypothesis for the nuclear localization of unspliced RNA (4, 35). It was proposed that nuclear RNAs are actively retained within the nucleus and that the removal of a retention signal, by splicing or deletion, allows transport to occur. The likely SV40 candidate for the retention signal would then be the small intervening sequence of the late 19S RNA (0.760 to 0.765 map unit).

To test this hypothesis, an insertion mutant in the late region of SV40 was constructed. A second intervening sequence was inserted into the body of the late transcript (at 1.0 map unit). This second intron has lost most of its normal flanking sequences, and it was expected that this intron could not be excised by splicing. If the 19S intron is recognized by cellular components as a primary sequence and is involved in retention, it is then predicted that such a mutant should produce a spliced but nuclear 19S and 16S RNA. An analysis of the transcripts made by this insertion mutant is presented, and the results demonstrate that the efficient transport of RNA which contains intronic sequences can occur.

MATERIALS AND METHODS

Cell culture and viruses. The history and protocols for virus growth in the CV-1 monkey kidney cell line have been described by Mertz and Berg (27). CV-1 cells were grown in the presence of 10% calf serum. The SVS strain of SV40 was used as a parent for mutant construction. The helper virus for virus growth at 40°C was *ts*A58 (31). The early deletion mutant, which was also used as a helper virus for growing mutants at 37°C, was p536. This deletion has lost sequences between 0.169 and 0.536 map unit and was kindly provided by L. Sompayrac.

Growth of plasmid DNA. The HB101 strain of *Escherichia coli* was used for growing all plasmid DNAs. Recombinant DNAs were constructed in either pBR322 or pBR325 as indicated in the text and grown as described previously (4).

Enzymes and label. The T4 DNA ligase, polynucleotide kinase, and restriction enzymes *Eco*RI, *Hin*cII, *Hin*dIII, *Hin*fI, and *Bam*HI were purchased from Bethesda Research Laboratories. Nuclease S1 and DNA pol I were from Boehringer Mannheim Corp. Exonuclease VII was purified from *E. coli* as described by Goff and Berg (12). [γ-^{32}P]ATP was purchased from ICN Pharmaceuticals, Inc. *Eco*RI linkers were from Collaborative Research, Inc.

DNA transfections. DNA transfections were performed as described by Graham and Van Der Eb (14) and as modified by Parker and Stark (28). Viral DNA (10 μg/plate of 2×10^7 cells) was diluted into 1 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (8 g of NaCl, 0.36 g of KCl, 0.16 g of $Na_2HPO_4 \cdot 7H_2O$, 1 g of glucose, 5 g of HEPES [pH 7.05] per liter of solution) and adjusted to 125 mM $CaCl_2$ from a 2 M stock solution. The solution was incubated for 20 min at room temperature. The fine precipitate which formed was overlaid onto CV-1 cells without rinsing the monolayer and incubated for 30 min at room temperature. The cells were then overlaid with Dulbecco medium and incubated for 4 h at 37°C. The medium was then removed, and the cells were shocked by overlaying 2 ml of HEPES-buffered saline with 15% (wt/vol) glycerol for 4 min. The glycerol solution was then removed, and the cell monolayer was rinsed twice, then overlaid with Dulbecco medium with 10% calf serum.

Cell fractionation and extraction of RNA. The fractionation of cells into cytoplasmic and nuclear fractions and the extraction of RNA have been described previously (34).

Preparation of DNA hybridization probes. Recombinant plasmid DNA was digested with *Bam*HI, and the overhanging 5' termini were then end labeled with [γ-^{32}P]ATP by the exchange reaction (2). The labeled DNA was then purified by electrophoresis on a 1.0% agarose gel, and stained bands were cut out and electroeluted. The specific activity of the DNA was about 2×10^6 cpm/μg.

Analysis of RNA. Virus-specific RNA was analyzed by the nuclease S1 method of Berk and Sharp (1), modified as described previously (35). Nuclease-resistant DNA was electrophoresed on 1.5% alkaline agarose gels (26). Dried gels were exposed to Kodak AR5 film at −70°C for the times indicated in the text in the presence of a Dupont Lightning-Plus intensifying screen.

RESULTS

Double-intron mutant construction. A schematic diagram of mutant inB11 construction is shown in Fig. 1. The small 19S intervening sequence (nucleotide no. 526 to 558, corrected numbers of Buchman et al. [3]) was isolated by first digesting SV40 DNA with *Hin*cII. The B fragment of this digest (no. 519 to 2,057) was purified by gel electrophoresis and then digested with *Sau*96I (no. 557). The 38-nucleotide fragment (no. 519 to 557) produced was repaired with DNA pol I and gel purified, and *Eco*RI linkers were attached with DNA ligase. After digestion with *Eco*RI, the 46-nucleotide product of the above reactions was ligated via the cohesive *Eco*RI termini into an *Eco*RI-linearized plasmid DNA which contained SV40 cloned into the *Bam*HI site of pBR322. This DNA was used to transform *E. coli* cells to ampicillin resistance. Colonies were picked and grown, and DNA was extracted. DNAs were then screened for the insertion of a 46-nucleotide fragment into the *Eco*RI site of SV40 DNA by the electrophoresis of a *Hin*cII plus *Hin*dIII digest. A candidate colony (inB11) was chosen for sequencing by the method of Maxam and Gilbert (25). The sequence was determined from the *Hin*fI site (no. 1,739) at 0.992 map unit. The determined sequence is shown in Fig. 2. It was determined that the second intron was inserted into the *Eco*RI site with the same polarity as the original small 19S intron. Furthermore, due to the DNA pol I repair, six nucleotides upstream from the 5' end of the leader-intron junction are also includ-

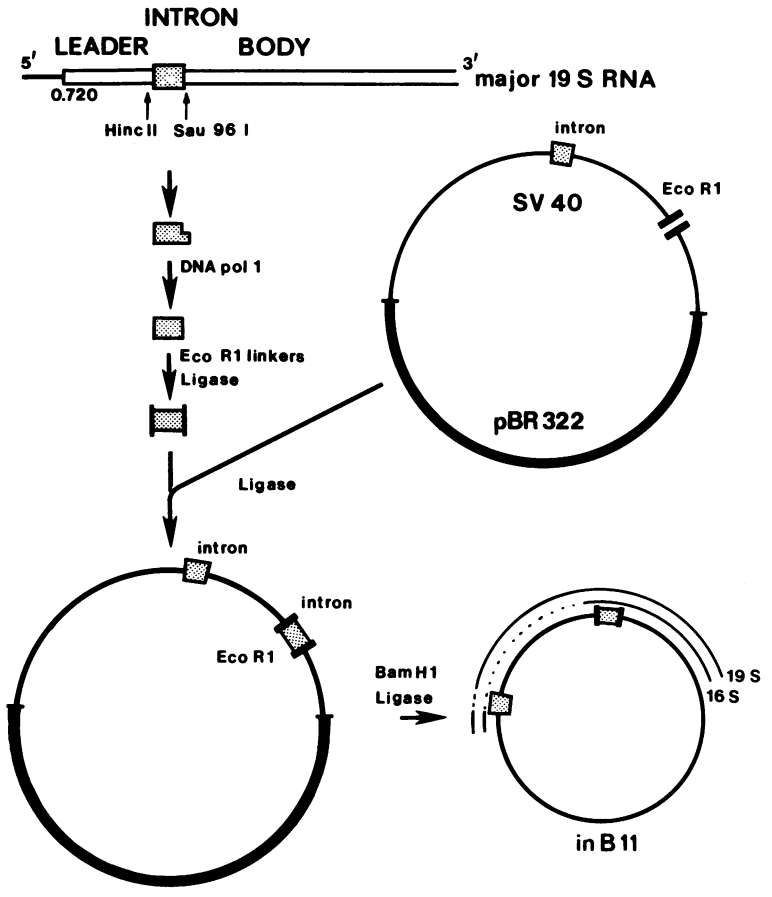

FIG. 1. Schematic summary of mutant inB11 construction. Shaded area indicates the small 19S intron (nucleotide no. 526 to 558). The mutant is an insertional mutant with a second intervening sequence at the *Eco*RI site.

ed. Only one nucleotide downstream from the 3' end of the intron-leader junction was included in the recombinant.

Inability of SV inB11 to complement and plaque with *ts*A58 helper virus. Five successive attempts were made to plaque SV inB11 as a virus with *ts*A58 as a helper virus at 40°C. DNA from the SV inB11/pBR322 plasmid was digested with *Bam*HI. The SV40 band was purified by gel electrophoresis. This DNA was then circularized with T4 DNA ligase. inB11 DNA (5 μg) was mixed with 0.5 μg of *ts*A58 DNA and transfected onto CV-1 cells. The cells were incubated at 40°C until a cytopathic effect was apparent (14 days). Virus was harvested and plaqued with *ts*A58 helper virus at 40°C. Plaques were picked and used to infect monolayers of CV-1 cells. After 14 days of incubation at 40°C, virus was harvested, and viral DNA was prepared. Viral DNA was then digested with *Hinc*II and *Hin*dIII and analyzed by polyacrylamide gel electrophoresis. On three attempts, a mixed virus stock was not obtained (data not shown). The virus that grew was determined to be a revertant of the helper virus, *ts*A58. On two other attempts, however, a mixture of a deletion mutant and helper virus was obtained. The restriction pattern of these DNAs is shown in Fig. 3. In

FIG. 2. Inserted sequence of inB11. Plasmid DNA was end labeled at the *Hin*fI site at nucleotide no. 1,739, and the sequence was determined as described by Maxam and Gilbert (25). The inserted sequence includes the small intron (nucleotide no. 526 to 558), as well as the six nucleotides upstream of the leader-intron junction and one nucleotide downstream of the intron-body junction.

both instances, it is seen that the double-intron-specific f band of the parental DNA is not present. Instead, two new bands are observed which migrate below the wild-type f band (349 nucleotides). Thus, the deletion mutants which grew with helper virus have each sustained a similar but distinct deletion which encompasses and includes the second intervening sequence. These results suggest that the presence of the second intron is not a stable or allowed insert and that its presence is selected against during growth. We have previously inserted other sequences into the *Eco*RI site of SV40. One such insert contains a 38-nucleotide lactose operator sequence from *E. coli*. This insert grew as a virus on our first and all subsequent attempts (L.P.V., unpublished data). Thus, the apparent inability of inB11 to grow as a virus with a *ts*A58 helper is due to the nature of the specific sequence inserted at the *Eco*RI site.

Transcription of inB11 after DNA transfection. Due to the inability of inB11 to grow as a virus plaque, it was necessary to examine inB11 transcription directly after DNA transfection onto CV-1 cells. DNA from the pinB11/pBR322 plasmid was digested with *Bam*HI. The viral DNA was gel purified and recircularized with DNA ligase. This DNA was transfected onto CV-1 cells as described above. After incubation (48 h, 37°C), cytoplasmic and nuclear fractions were prepared, and RNA was extracted. Virus-specific RNA was examined by the nuclease S1 method. As a control experiment, wild-type SV40 DNA cloned into the *Bam*HI site of pBR322 was also transfected onto CV-1 cells after the same protocol used for inB11, and transcripts were analyzed by the nuclease S1 procedure. The results of this experiment are shown in Fig. 4. The transcriptional pattern obtained from wild-type DNA transfection was not as expected (Fig. 4A). Because the end-labeled probe was at the position of the *Bam*HI cleavage (no. 2,533), spliced 19S and 16S RNA were expected to protect DNA of 1,975 and 1,070 nucleotides in

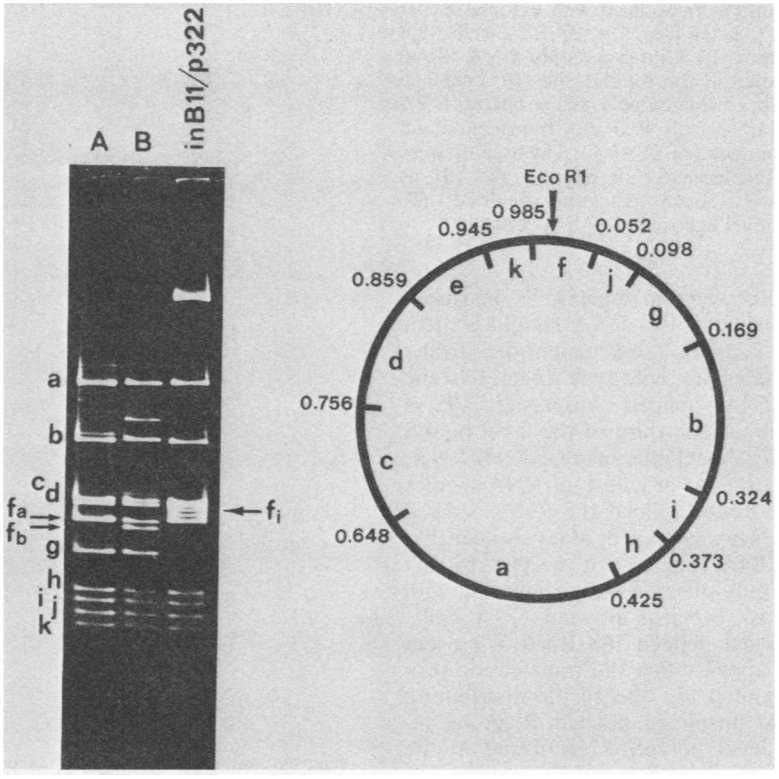

FIG. 3. Restriction analysis of plaque-purified mutant DNA. The parental plasmid mutant DNA, pinB11, was digested with *Hin*dIII and *Hin*cII. Shown is an ethidium bromide-stained polyacrylamide gradient gel (3 to 8%). The f band containing the inserted intron sequence is indicated by arrow f_i. Track B is the same digest of viral DNA that grew a mutant after cotransfection with *ts*A58 DNA. Arrow f_b indicates a mutant-specific f band that had deleted about 70 nucleotides. Track A is the pattern of another mutant that grew after cotransfection with *ts*A58. Arrow f_a indicates the mutant-specific f band which had deleted about 50 nucleotides.

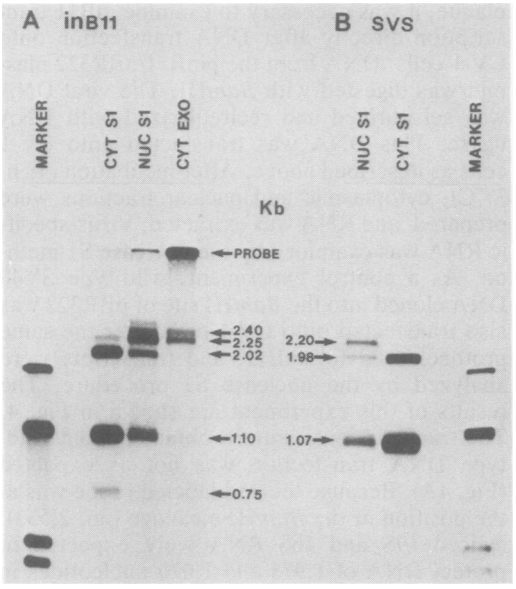

FIG. 4. Transcriptional analysis after DNA transfection. (A) inB11 DNA. inB11 DNA, end labeled at the *Bam*HI site, was hybridized with cytoplasmic or nuclear RNA extracted from 1×10^7 CV-1 cells 48 h after transfection. (B) Wild-type SV40 DNA. SV40 DNA, end labeled at the *Bam*HI site (10^5 cpm/0.05 μg), was hybridized with cytoplasmic or nuclear RNA extracted from CV-1 cells 48 h after transfection and analyzed by the nuclease S1 assay. The hybrids were digested with nuclease S1 or exonuclease VII as indicated. Shown is an autoradiogram of a dried 1.5% alkaline agarose gel exposed for 4 h at −70°C.

length, respectively, from nuclease S1 digestion. The major unspliced 19S RNA should protect DNA of from 2,208 to 2,396 nucleotides. In the cytoplasm, the major wild-type species is the spliced 16S RNA. Spliced cytoplasmic 19S is observed at about one third of the level of 16S RNA with little detectable unspliced 19S RNA. In the nucleus, the major wild-type RNA species is also seen to be the spliced 16S RNA. Spliced nuclear 19S RNA was present at levels near that of unspliced RNA. This is not the pattern that we have generally observed after infection with wild-type virus. In virus-infected CV-1 cells, very little nuclear spliced 16S RNA is present (23). Thus, it appears that the transfection procedure has significantly altered the distribution of spliced and unspliced nuclear RNA by increasing the level of 16S RNA found in the nucleus.

The transcripts seen after transfection with inB11 DNA are shown in Fig. 4A. If spliced 19S and 16S RNAs are synthesized without excising the second intron at 1.0 map unit, it was expected that bands of 2,023 and 1,118 nucleotides in length would be observed. The excision of the second intron at 1.0 map unit would produce a band 750 nucleotides long (*Eco*RI; 1,782 to *Bam*HI; 2,533). In the cytoplasm, spliced 16S and 19S RNAs that retained the second intron at 1.0 map unit were present at high levels. Also detected were trace amounts of RNA species which had lost the second intron at 1.0 map unit (a band at 750 nucleotides). In the nucleus, both the spliced 16S and 19S RNAs were present as were the unspliced 19S RNA (2,250 nucleotides). Even though spliced 19S and 16S RNAs were present in the nucleus at unusually high levels, unspliced 19S RNA remained strictly nuclear and was not observed in the cytoplasm.

It appeared likely that much of the altered pattern of nuclear transcripts (i.e., elevated levels of 16S RNA) could be attributed to the transfection procedure. To test this, CV-1 cells were infected with SV40 virus, then subjected the $CaPO_4$ and glycerol treatment as performed for DNA transfections. Forty-eight hours later, RNA was extracted and analyzed. It was observed (Fig. 5) that 16S RNA was now present at elevated levels in the nucleus.

After this confirmation that the transfection

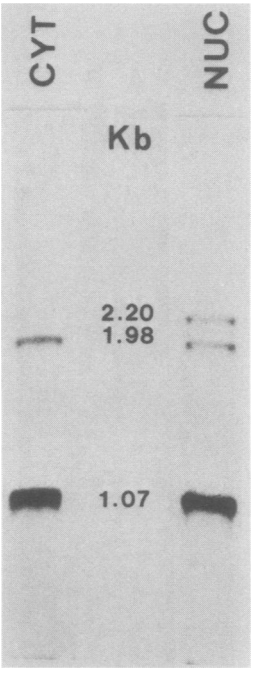

FIG. 5. Nuclease S1 analysis of RNA from virus-infected $CaPO_4$-shocked cells. Cytoplasmic and nuclear RNAs were extracted from 4×10^7 cells 48 h after infection with SV40 and analyzed as described in the legend to Fig. 4. Cells were subjected to the $CaPo_4$-glycerol shock at 2 h postinfection. The autoradiogram of cytoplasmic RNA was exposed for 2 h, and that of nuclear RNA was exposed for 5 h.

procedure perturbs the pattern of nuclear transcripts, we renewed our efforts to obtain a stock of inB11 virus so that we might study mutant transcription after virus infection. We have in the past successfully grown late mutants of SV40, using a large deletion of the early region as a helper virus (L.P.V., unpublished data). This deletion (p536) has lost 1,900 nucleotides from the early region, rendering it a nonrevertable helper virus. Furthermore, due to its small genome, this helper is inefficiently packaged into virions and allows the preferential propagation of full-sized late mutant genomes over those of the helper virus. Generally, this helper yields a ratio of about 5 to 10 parts mutant to 1 part helper DNA. Using this helper, we were able to obtain a transient unplaqued stock of inB11 virus. CV-1 cells (2×10^7) were transfected with 10 μg of purified recircularized inB11 DNA, along with 1 μg of p536 helper DNA. After 7 days of incubation at 37°C, virus and viral DNA were prepared from the monolayers. Restriction enzyme analysis of viral DNA indicated that the ratio of inB11 to helper p536 was about 1:5 (data not shown). It appears that the inB11 genome is not propagated efficiently as the helper DNA outgrew the mutant. This stock was used to infect cells at a high multiplicity for a transcriptional analysis. The nuclease S1 patterns obtained from this infection are shown in Fig. 6. Because the probe was inB11 DNA labeled at the *Bam*HI site, transcripts from the helper virus are seen as a single band at 750 nucleotides. Clearly, the great majority of RNA transcripts were from the helper virus (>95%). On longer exposures of the autoradiograms, however, the mutant inB11 transcripts became visible. In the cytoplasm, two species of 1,100 and 2,020 nucleotides long were present. These correspond to spliced 16S and 19S RNAs, which retain the second intron at map 1.0 map unit. It is of interest that the ratio of 16S to 19S RNA seen here differed substantially from the pattern seen after DNA transfection. In virus infection, 19S RNA was the most abundant species, whereas in DNA transfection, 16S RNA was most abundant. In the nucleus of virus-infected cells, the majority of transcripts were again from the helper virus (750 nucleotides). On a long exposure of the autoradiogram, the mutant transcripts corresponding to both spliced and unspliced 19S RNAs were seen (2,020 and 2,250 nucleotides). Clearly, the majority of unspliced mutant 19S RNAs are mainly nuclear.

DISCUSSION

The major question addressed by this study is the following. How does an additional intervening sequence, introduced out of the context of its normal flanking sequences, affect the transport phenotype of nuclear RNA? It was expected that the loss of most of the flanking sequences would prevent splicing out of the second intron, yielding a transcript that was spliced at the first intron but containing sequences (the second intron) which are normally spliced out. Such a transcript would therefore be subjected to the splicing machinery, yet retain intronic sequences directly testing the necessity of splicing per se versus the necessity of removing a specific portion of the transcript (the second intron) to transport the molecule into the cytoplasm.

The construction of this double-intron mutant was accomplished by standard recombinant DNA techniques and ultimately confirmed by

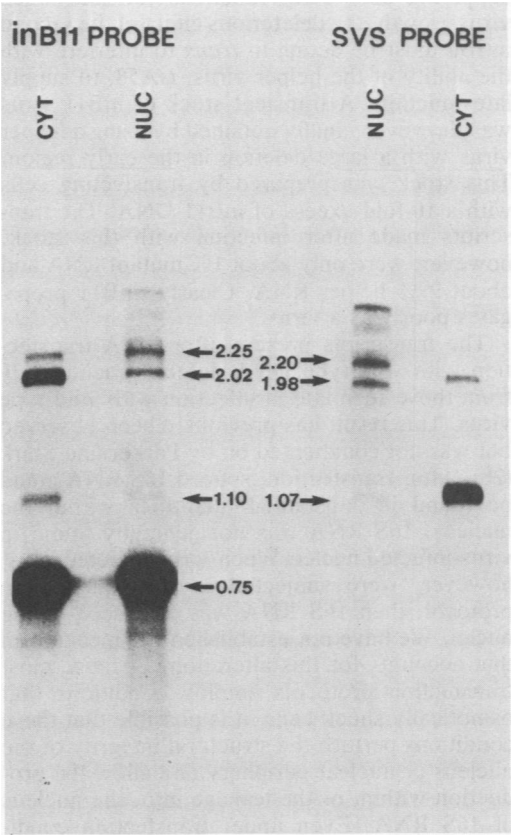

FIG. 6. Nuclease S1 analysis of transcripts synthesized after infection with a transient stock of inB11 virus. A stock of inB11 virus with p536 helper virus was prepared as described in the text and used to infect 2×10^7 CV-1 cells. At 48 h after infection, RNA was extracted and analyzed by the S1 nuclease assay. The nuclease S1 pattern, using the inB11 probe end labeled at the *Bam*HI site and the SV40 DNA probe end labeled at the *Bam*HI site, is shown. The autoradiograms were exposed for 24 and 2 h for the inB11 and SV40 probes, respectively.

DNA sequencing. This double-intron mutant is referred to as inB11.

The inability to grow inB11 as a virus plaque was initially puzzling. On only one previous occasion has it been observed that a late-region mutant grew poorly as a virus (5), but did grow. This behavior appears to be specific to the sequence of the insert, as the insertion of an *E. coli* lactose operator into the *Eco*RI site did not present any difficulties for virus growth. On the two occasions when a mutant virus did plaque along with the helper, the mutants had deleted the area spanning the inserted second intron. This implies the existence of a strong selective pressure against the presence of the second intervening sequence during growth in CV-1 cells. Because the inB11 mutant is presumably supplying only early functions for the mixed virus growth, the deleterious effect of the second intron must be acting in *trans* to interfere with the ability of the helper virus, *ts*A58, to supply late function. A transient stock of inB11 virus was, however, finally obtained by using a helper virus with a large deletion in the early region. This stock was prepared by transfecting cells with a 10-fold excess of inB11 DNA. The transcripts made after infection with this stock, however, were only about 1% mutant RNA and about 99% helper RNA. Clearly, inB11 propagates poorly as a virus.

The transcripts present after DNA transfection with wild-type DNA differed qualitatively from those seen late in infection with wild-type virus. This result has previously been observed but was not commented on by Parker and Stark (28). After transfection, spliced 16S RNA could be found in substantial quantities within the nucleus. 16S RNA was not generally found in virus-infected nuclei. When virus-infected cells, however, were subjected to the transfection protocol, then 16S RNA was also seen in the nuclei. We have not established the mechanism that accounts for this alteration. Because most transfection protocols employ conditions that osmotically shock cells, it is possible that these conditions perturb the structural integrity of the nucleus or nuclear periphery and allow the production within, or the leakage into, the nucleus of 16S RNA. Even under transfection conditions, however, unspliced 19S RNAs remained predominantly within the nucleus. This argues that the retention system is still operational.

The transcripts present after transfection with inB11 DNA showed several changes when compared with the wild type. The 16S- and 19S-like RNA made by the mutant had both been correctly spliced at the normal body positions (0.939 and 0.765 map units, respectively). The second intron at 1.0 map unit had, for the most part, not been excised from these transcripts. Although we observed a low level of RNA which did splice out the second intron, it appears that the six nucleotides upstream of the intron that were included in the inserted sequence were insufficient information to allow efficient splicing to occur. Thus, it is clear that the presence of intronic sequences did not interfere with the ability to transport these RNAs. It is also clear that at least one RNA species (unspliced 19S RNA) was not transported, indicating that the differential transport of mutant transcripts is conserved. Certain conclusions can be drawn from this result. The retention of nuclear RNA probably does not operate by the simple recognition of a primary sequence, as the only sequence difference between transported and untransported 19S RNA was the presence or absence of the 19S intron. Previous results on the behavior of mutants that affect transport are relevant to this study (5). Explanations accounting for the differential transport of RNA that employ kinetic phenomena appear to be inconsistent with the transport phenotype of some deletion mutants (i.e., mutants which don't transport). By exclusion, therefore, it appears that an active process is responsible for the nuclear localization of unspliced RNA. As this study has shown, this process does not operate by simple primary sequence recognition. It does remain as a possibility, however, that an intervening sequence cannot be efficiently recognized by the retention system at its new location due to an altered secondary structure of RNA.

It is curious that inB11 appears to code for the production of a half-spliced 16S RNA. This molecule has a 5′ end at what is normally the intron-body junction (the body acceptor site at 0.939 map unit). It is also possible that this apparently half-spliced molecule may have spliced out the second intron at 1.0 map unit. Our results do not allow us to distinguish between these possibilities.

The observation that the transcription pattern after virus infection differs quantitatively from that seen after viral DNA transfection should raise a note of caution in interpreting the transcriptional results obtained after DNA transfections.

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