Spliing of the adenovirus-2 E1A 13S mRNA requires a minimal intron length and specific intron signals

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ABSTRACT
The adenovirus E1A region encodes three overlapping mRNAs, designated 9S, 12S and 13S. They differ from each other with regard to the length of the intron which is removed by RNA splicing. We have constructed E1A genes with deletions and insertions in the intervening sequence that is common to all three E1A mRNAs, in a search for signals which influence splicing of the 13S mRNA. Mutant plasmids were transfected into HeLa cells and the transiently expressed E1A mRNAs characterized by the SI protection assay. The results show that five upstream and 20 downstream nucleotides are sufficient to allow for a correct utilization of the 5'-splice junction for the E1A 13S mRNA. Moreover, we show that a minimal intron length of 78 nucleotides is required for efficient 13S mRNA splicing. The ability of mutants with large intron deletions to maturate a 13S mRNA could partially be restored by expanding the intron length with phage lambda sequences. However, in no case was the normal splicing efficiency obtained with these mutants. In contrast, one mutant in which sequences from the authentic 13S mRNA intron were used to expand the intron expressed almost normal levels of 13S mRNA, thus suggesting that signals which specifically promote 13S mRNA splicing exist.

INTRODUCTION
Studies of animal DNA viruses, like the human adenoviruses, have in a very important way contributed to our current understanding of the structure and expression of eucaryotic genes. For example, a phenomenon like RNA splicing was first discovered by studies of adenovirus mRNA (1,2) and later shown to be a characteristic shared by eucaryotes in general (3). The development of soluble extracts that correctly excise introns from mRNA precursors (pre-mRNAs) has led to a dramatic increase in our understanding of the mechanism of RNA splicing. Cofactors required for splicing have been characterized (4,5,6) and some remarkable intermediates which are formed during the splicing process have also been identified (7,8,9). However, the signals that direct the excision of intron sequences from pre-mRNAs are still poorly characterized. Consensus sequences have been derived for both 5'- and 3'-splice junctions (3,10) and small changes in and around the conserved GT
and AG dinucleotide pairs have in many cases been shown to cause aberrant or complete inhibition of splicing (11-15). The bulk of the intron sequences appears, furthermore, to be dispensable for a correct splicing provided a minimal intron length is retained (16).

To characterize, in more detail, the signals that direct the excision of intron sequences we have undertaken a study of the splicing pathway for the early region 1A (E1A) mRNAs of adenovirus type 2 (Ad2). The E1A transcription unit is of particular interest since it has been shown to encode functions which are required both for activation of early viral gene expression (17,18,19) and cell transformation (20,21,22).

The E1A region encodes three overlapping mRNAs, designated 9S, 12S, and 13S, which are generated by alternative splicing of a common nuclear precursor RNA (23,24,25) (Fig. 1). The expression of the different E1A mRNAs is subjected to a post transcriptional regulation during the infectious cycle. Thus, the 13S and 12S mRNAs are the most abundant species synthesized early after infection whereas the 9S mRNA predominates at late times (24,26,27). We have previously shown that splicing of the E1A mRNAs is non-sequential using the colinear transcript as the only precursor RNA (28). Here we extend these studies by examining the signals which are of importance for the generation of a correctly spliced 13S mRNA. We show that the intron must have a minimum
length to achieve efficient splicing and that the origin of the intron se-
quences, which separate the 5'- and 3'-splice junctions, is of importance for
the efficiency by which the 5'-splice site of the 13S mRNA is recognized by
the cellular processing machinery.

MATERIALS AND METHODS

Plasmid construction
All nucleotide numbers refer to positions in the sequence of the Ad2 genome
(29).

Deletion mutants pJU81 to pJU121. Plasmid pKG0-007 SV-RI DNA (28) was lin-
earized by cleavage with restriction endonucleases SmaI or XbaI and treated
with 0.5 units of exonuclease Bal-31 (New England Biolabs) per µg DNA in a
buffer containing 20 mM Tris-Hydrochloride (pH 8.0), 10 mM MgCl₂, 12 mM
CaCl₂, 0.6 M NaCl and 1 mM EDTA. At various intervals corresponding to
digestion of 20 base pairs (bp), samples of the reaction mixture were removed
and the digestion terminated by addition of EDTA to a final concentration of
20 mM. After repair of ragged ends using the Klenow fragment of DNA poly-
merase I an XhoI linker (dCCTCGAGG; New England Biolabs) was added by treat-
ment with T4 DNA ligase. Finally the molecules were recircularized and used
to transform the HB101 strain of E. coli to ampicillin resistance. One mutant
pJU9S (deletion extending from position 818 to 1190) was isolated from the
SmaI digestion products. Similarly a deletion mutant, pJUVIB (deletion ex-
tending from position 1209 to 1535), was isolated from the XbaI digestion
products. A third mutant, pJU44, having an XhoI site in the 13S mRNA Intron
was reconstructed by combining the 5'-half of region E1 (position 1 to 1208)
isolated from plasmid pJUVIB with the 3'-half (position 1191 to 2800) isolat-
ed from plasmid pJU9S. The remainder of the transformation mixture from the
XbaI digestion products was used to grow up a large scale plasmid prepara-
tion. This DNA was cleaved with restriction enzymes XmaI and XhoI and the
resulting fragment mixture separated through a 10% polyacrylamide gel. Frag-
ments corresponding to deletions of a desired length were eluted and cloned
into plasmid pJU44 similarly cleaved with XmaI and XhoI. Five clones, pJU81
to pJU121 (Fig. 2A), with deletions extending towards the 13S 5'-splice site
were characterized in detail by restriction endonuclease cleavage and DNA
sequence analysis.

Construction of insertion mutants pJU10Acc and pJU11Acc. Plasmid pKG0-007
SV-RI was cleaved with restriction endonuclease AccI (position 1106) and the
site converted to an XhoI site by linker addition as described above. The 231
bp XhoI-XbaI fragment (positions 1106-1337) was isolated by gel electrophoresis and inserted into clones pJU101 and pJU111 similarly cleaved with restriction endonucleases XhoI and XbaI.

Construction of insertion mutants pJU101λ and pJU111λ. Phage lambda DNA was digested with a mixture of restriction endonucleases AluI and HaeIII. XhoI linkers were added and the fragment mixture separated electrophoretically through a neutral 10% polyacrylamide gel. Fragments ranging in size between 40 and 60 bp were eluted (30) and cloned into the XhoI cleavage site of plasmids pJU101 and pJU111. Two clones were isolated at random and characterized in detail.

All cloning experiments connected with this study were carried out according to the guidelines of the Swedish Recombinant DNA Committee employing standard recombinant DNA technology (31).

DNA transfection and RNA preparation
Subconfluent monolayers of HeLa cells were transfected as previously described (28) using the calcium phosphate co-precipitation technique (32,33). A total of 13 μg plasmid DNA was added per 6 cm Petri dish. 3 μg of pSX8+ DNA, encoding rabbit β-globin (34), was always included as an internal control of the transfection efficiency. Approximately 50 hrs post transfection cells were lysed by IsoB-Nonidet P-40 extraction and fractionated into cytoplasm and nuclei. The total cytoplasmic RNA was isolated by phenol extraction (35). Usually 50 to 100 μg of RNA was obtained per 6 cm Petri dish.

SI endonuclease analysis.
Briefly, 10 μg of total cytoplasmic RNA isolated from transfected cells was hybridized over night at 55°C to the 5'- and 3'-end-labeled DNA fragments (36) indicated in the figure legends. SI nuclease cleavage and electrophoretic separation was through 6% polyacrylamide gels containing 8 M urea as previously described (28). In Fig. 5, where the spliced structure of the E1A transcripts were analyzed by neutral agarose gel electrophoresis (28,37), electrophoretic separation was through a 2% agarose gel. The electrophoresis buffer was in both cases 45 mM Tris-borate (pH 8.3), 0.5 mM EDTA. The relative amount of RNA was determined by densitometer scanning of autoradiograms using a Shimazu dual-wavelength TLC scanner model CS-930.

RESULTS
Analysis of 13S mRNA expression from plasmids with mutated E1A genes.
To establish the minimal intron sequences required for the formation of a correctly spliced E1A 13S mRNA, a series of deletion mutants were constructed

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by Bal-31 digestion of pKGO-007 SV-RI DNA (Fig. 2A). Five mutants with deletions of 12 to 79 bp extending from a midpoint of the 13S mRNA intron towards the 5'-splice junction of the 13S mRNA were isolated by this procedure (pJU81 to pJU121 Fig. 2A). Mutant pJU81, lacks 12 bp between positions 1179 and 1190; pJU91, 31 bp between 1160 and 1190; pJU101, 44 bp between 1147 and 1190; pJU111, 59 bp between 1132 an 1190; and pJU121, 79 bp from positions 1112 to 1190 (Fig. 3). The deletion in mutant pJU121 removes the conserved GT dinucleotide that is almost invariant at 5'-splice junctions (3,10). The end points of the deletions were fused by the addition of an octamer linker. As a result the actual intron length was in all mutants extended by eight nucleotides. This means that, for example, mutant pJU81, although

![Fig. 2. Structure and splice pattern of deletion and insertion mutants in the EIA 13S mRNA intron.](image)

A.) Schematic diagram of the plasmid clones used in this report. The positions of the 12S and 13S 5'-splice sites and the common 3'-splice site are indicated. Numbering is in bp from the left hand end of the genome. The number of bp deleted (△) as well as inserted (▽) in the different clones are shown. The deletion end-points have been joined by an XhoI linker (□). Open boxes denotes inserted phage lambda sequences. (×), indicates the position of the pm975 mutation (14).

B.) Quantitation of 12S and 13S mRNA expression in transfected cells. The values, which are expressed in percent, were determined by densitometric scanning of the autoradiogram shown in Fig. 5. (U), unspliced RNA. (a), RNAs which utilize an alternative 13S 5'-splice site (see Fig. 7).

C.) The ratio of 13S to 12S mRNA expression calculated from the values in panel B. Note that the pm975 derivatives does not express a 12S mRNA.
Fig. 3. Nucleotide sequence of deletion and phage lambda insertion mutants.

The illustrated sequence reads as the sense strand between positions 1100 and 1231 on the Ad2 genome. Exon sequences are shown in capital letters and the 13S mRNA intron in small letters. Deletion end-points were joined by an octameric XhoI linker which is shown boxed in the figure. The AccI cleavage site used for construction of mutants pJUI0Acc and pJU11Acc is underlined. The sequence of the phage lambda insert (41) in mutants pJUI01X (positions 36361-36404) and pJUI11X (positions 20146-20187) is indicated. The underlined sequence is contributed by the XhoI linker.

lacking 12 bp (Fig. 2A) has only suffered a net loss of four nucleotides length due to the linker insertion.

Mutant DNAs were transfected into HeLa cells and the transiently expressed E1A mRNAs were characterized and quantitated by S1 nuclease analysis (23), using 5'- and 3'-end-labeled DNA fragments as hybridization probes (36). A small amount of pSX8 DNA, expressing the rabbit B-globin mRNA under the transcriptional control of the SV40 enhancer (34), was always included as a reference plasmid to measure the transfection efficiency (38). Although the E1A proteins repress B-globin expression from SV40 enhancer bearing plasmids (38,39,40) the effect is negligible under our experimental conditions (38).

As shown in Fig. 4 and summarized in Table 1, introduction of deletions and insertions into the 13S mRNA intron induced only small changes in the cytoplasmic steady state level of E1A mRNAs. For unknown reasons mutant pJUI81 which lacks 12 intron nucleotides (Fig. 3) accumulated almost twice as much E1A transcripts as the wild type plasmid. However, an analysis of the spliced structure of the resulting E1A transcripts showed some drastic changes (Fig. 5). The shortest deletions (pJUI81, pJUI91) did not cause significant change of the ratio between spliced 13S and 12S mRNA, suggesting that the splicing efficiency was not greatly altered by insertion of the XhoI linker. Larger deletions, on the other hand, created more dramatic effects on the splicing pattern. Thus, mutant pJUI01, which lacked 44 nucleotides, showed a 30-fold reduction of 13S mRNA expression compared to pKGO-007 SV-RI (Fig. 2C), and the mutant pJU111, which lacked an additional 15 intron nucleotides,
Fig. 4. Effect of deletions and insertions on the accumulation of E1A transcripts.

Strategy for the S1 endonuclease analysis. For the detection of E1A transcripts (panel A) a 650 bp NarI-BstNI fragment (positions 810-1461), 5'-end-labeled at the BstNI cleavage site was used and for the detection of β-globin RNA (panel B) a clone 5'-end-labeled at the unique BamHI cleavage site located in the second exon (34) was used as a DNA probe.

C.) S1 endonuclease analysis of β-globin and E1A RNAs accumulated in HeLa cells transfected with 3μg of pSXB DNA and 10μg of the various E1A plasmids shown in Fig. 2A. The relative level of E1A transcripts (summarized in Table 1) was calculated as the ratio between the signal of the E1A RNAs and β-globin reference RNA.
Table 1. Expression of E1A mRNAs in transfected cells

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<th>Relative Transcript level</th>
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<tr>
<td>pKGO-007 SVRI</td>
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<td>pJU10 Acc</td>
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<td>pJU11 Acc</td>
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a) The accumulation of E1A transcripts was quantitated by densitometer scanning of the autoradiogram shown in Fig. 4. The relative transcript level was calculated as the ratio between the signal of E1A to that of rabbit β-globin. The expression in pKGO-007 SVRI transfected cells was arbitrary set at 1.

was completely defective in 13S mRNA expression (Fig. 2B). The results suggest therefore that more than 20 nucleotides downstream of the 5'-splice junction are required to allow the generation of a correctly spliced 13S mRNA.

Elimination of the 5'-splice site for the 12S mRNA.

Since the E1A nuclear precursor RNA generates three alternative mRNAs, by differential splicing, the loss of the 13S mRNA (pJU101 and pJU111, Fig. 5) may be caused by a preferential selection of the 5'-splice site of the 12S mRNA. In agreement with this hypothesis the loss of 13S mRNA expression leads to an increased level of 12S mRNA without a significant change in the amount of 9S mRNA (data not shown). In an attempt to force the splicing machinery to select the 5'-splice junction for the 13S mRNA more efficiently we introduced the Ad2 pm975 mutation, which efficiently prevents 12S mRNA splicing (13), into clones pKGO-007 SV-RI, pJU101 and pJU111.

To study the spliced structure of the E1A RNAs produced with our pm975 derivatives, the S1 nuclease resistant products were resolved by neutral agarose gel electrophoresis (28,37). Under appropriate conditions S1 endonuclease digestion will produce a nuclease resistant RNA-DNA hybrid which will vary in length depending on the size of the intron removed by RNA splicing. As shown in Fig. 6 elimination of the 12S mRNA splice junction from pKGO-007 SV-RI resulted in a more efficient use of the 5'-splice site of the 9S mRNA (pJU007pm975). However, elimination of the 5'-splice site of the 12S mRNA did not restore 13S mRNA synthesis in pJU101pm975 and pJU111pm975 transfected cells. This is particularly noteworthy in pJU101pm975 transfected...
Fig. 5. Changes in the expression of the E1A 13S and 12S mRNA in cells transfected with mutant plasmids.

A.) S1 nuclease mapping scheme. An approximately 2000 bp long NarI-HindIII fragment (positions 810-2800), 3'-end-labeled at the NarI cleavage site, was hybridized to 10μg of total cytoplasmic RNA isolated from transfected cells and treated with nuclease S1.

B.) Resistant material was separated by electrophoresis through a 6% denaturing polyacrylamide gel. The position of the S1 resistant fragment corresponding to the E1A 13S and 12S mRNAs are shown. Protected fragments corresponding to unspliced RNAs are shown within brackets (see also Fig. 7). In vivo RNA was isolated from Ad2 infected cells grown in the presence of AraC (25μg/ml) from 1 to 7 hpi. Marker fragments were from pBR322.
Fig. 6. Spliced structure of E1A RNAs accumulating in cells transfected with plasmids lacking a functional 12S 5'-splice site.

A.) Strategy for the S1 endonuclease analysis. An approximately 2000 bp long PstI-XbaI fragment (positions 3609 to 4363 in pBR322 connected to nucleotides 1 to 1340 in Ad2) was isolated from pKGO-007 DNA and 5'-end-labeled at the XbaI cleavage site. It was hybridized to 10µg of total cytoplasmic RNA isolated from transfected cells and treated with nuclease S1.

B.) The S1 resistant RNA-DNA hybrids were separated electrophoretically on a neutral 2% agarose gel (28,37). The position of hybrids corresponding to the 13S, 12S and 9S mRNAs are shown. The arrow indicates the protected fragment which results when the S1 nuclease cleaves the RNA strand opposite the nick in the DNA which corresponds to the splice junction in the mRNA (see also ref. 37).
cells as splicing of the 13S mRNA was only partially defective in the original mutant (pJU101, Fig. 5).

Restoration of splicing by insertion of phage lambda sequences into the 13S mRNA intron

The finding that deletion mutant pJU101 (Fig. 2A), leaving as much as 35 nucleotides downstream of the 5'-splice junction was only partially active, and mutant pJU111 leaving 20 nucleotides was completely defective in maturation of the 13S mRNA was at a first sight unexpected. For example, similar deletion studies have shown that six nucleotides at the 5'-end of the large intron of the rabbit β-globin pre-mRNA are sufficient to allow for a correct processing (16). However, the finding that an efficient splicing also requires a minimal intron length (16) prompted us to investigate a possible length dependence for the generation of the E1A 13S mRNA.

The intron was expanded by inserting random pieces of phage lambda DNA into mutants pJU101 and pJU111, thus generating mutants pJU101λ and pJU111λ (Fig. 2A). Mutant pJU101λ has an insert of 44 bp (position 36361-36404; 41) (Fig. 3) which, together with the bordering XhoI linker DNA, adds up to a total intron length of 130 nucleotides. Mutant pJU111λ has an insert of 42 bp (position 20146-20187; 41) (Fig. 3) resulting in a total intron length of 113 nucleotides. Mutant pJU101λ thus has a 13S mRNA intron which is 16 nucleotides longer than that of its wild type progenitor, whereas mutant pJU111λ has an intron which is one nucleotide shorter (Fig. 2A).

Figure 5 shows that mutant pJU111λ regained with a low efficiency the capacity to generate a correctly spliced 13S mRNA and that mutant pJU101λ increased the proportion 13S mRNA approximately 3-fold compared to the parental mutant pJU101 (Fig. 2C). The splicing defect could, therefore, be cured by expanding the intron length with a piece of foreign DNA. However, a quantitation of the amounts of 13S and 12S mRNA present in cells transfected with mutant DNA showed that the splicing defect was only partially relieved by the phage lambda sequences (Fig. 2B). In pKGO-007 SV-RI, pJU81 and pJU91 transfected cells the ratio between 13S and 12S mRNA varies between 2 and 3 (Fig. 2C). In pJU101λ and pJU111λ transfected cells the corresponding ratio was 0.6 and 0.3, respectively (Fig. 2C).

Interestingly a substantial fraction of the cytoplasmic RNA isolated from pJU111λ transfected cells (Fig. 5) accumulated as unspliced precursor RNA (see below).

Increased splicing efficiency by duplication of 13S mRNA intron sequences

Since expansion of the 13S mRNA intron with phage lambda sequences only lead
to a partial restoration of 13S mRNA splicing, we tested whether specific signals located within the intron were required for an optimal splicing efficiency. For these purposes the complete 13S mRNA intron was introduced into clones pJU101 and pJU111, thus generating mutants pJU10Acc and pJU11Acc (Fig. 2A). Since the AccI cleavage site used to construct these mutants is located within the part of the exon that is unique to the 13S mRNA (Figs 1, 2), both recombinants will have, in addition to the complete 13S mRNA intron, a duplication of the 13S 5'-splice junction and also five of the exon nucleotides. Thus pJU10Acc and pJU11Acc have four potentially active 5'-splice sites compared to three in the wild type parent (pKGO-007 SV-RI, Fig. 2A).

An analysis of the mRNAs produced in pJU10Acc and pJU11Acc transfected cells is shown in Fig. 5 and the quantitative data are summarized in Fig. 2B and 2C. The capacity to maturate a 13S mRNA was partially restored in both recombinants. Insertion of the full length intron into plasmid pJU111 allowed utilization of the 13S 5'-splice junction almost at wild type levels. Thus, the ratio between 13S and 12S mRNA increased from zero (pJU111) to 1.7 in pJU11Acc transfected cells. As shown above, insertion of a random lambda sequence resulted only in a partial restoration of the 13S mRNA splicing (pJU11x; 0.3). However, the inserted wild type intron in plasmid pJU10Acc led to only a slight increase in the utilization of the 5'-splice junction of the 13S mRNA (0.3), as compared to its parent pJU101 (0.2). In fact, introduction of a random phage lambda sequence restored 13S mRNA splicing more efficiently (pJU11x; 0.6).

Based on these results we conclude that a signal present in the 5'-half of the 13S mRNA intron under certain conditions facilitates 13S mRNA splicing.

Characterization of unspliced precursor RNAs
S1 nuclease analysis of RNA isolated from both pJU11x and pJU11Acc transfected cells revealed a protected fragment which had the length expected of a colinear precursor RNA (shown within brackets in Fig. 5). However, the fraction of unspliced versus spliced E1A RNA is much lower in pJU11Acc transfected cells (below 5%) as compared to pJU11 transfected cells (30%) (Fig. 2A, 5). These extended products may correspond to colinear pre-mRNAs which have been transported to the cytoplasm without splicing or alternatively RNAs which use cryptic 5'-splice sites in the inserted or duplicated DNA segments.

To discriminate between these two alternatives we repeated the S1 analysis using fragments isolated from the homologous plasmid as the hybridization
Fig. 7. Detailed analysis of the spliced and unspliced RNAs found in mutant pJU11Acc and pJU111λ transfected cells. 10μg of total cytoplasmic RNA was mixed and hybridized to the 3′-end-labeled Narl-HindIII DNA fragment described in Fig. 5, panel A. To discriminate between transcripts corresponding to colinear pre-mRNAs and RNAs which use cryptic 5′-splice sites the DNA probes were also prepared from the homologous plasmid DNA (pJU11Acc and pJU111λ). The S1 resistant material was separated electrophoretically through a 6% denaturing polyacrylamide gel. Arrow indicates the protected fragments of interest. Other conventions follow those in Fig. 4, 5 and 6.

probe. A 3′-end-labeled Narl-HindIII fragment (position 810-2800) was isolated both from pJU11Acc and pJU111λ DNA. As shown in Fig. 7, the extended transcripts present in cytoplasmic RNA isolated from pJU111λ transfected cells indeed corresponded to unspliced precursor RNAs, whereas the longer
RNAs found in pJU11Acq transfected cells represent a population of spliced transcripts which makes use of the 5'-splice site for the 13S mRNA which is duplicated in clone pJU11Acq. Thus, as little as five exon nucleotides are in this case sufficient to allow for a correct splicing at the 5'-splice junction of the 13S mRNA. However, this splice site, although active, is used at a very low frequency and is readily detectable only when the affinity for the upstream splice site has been impaired (compare with pJU10Acq; Fig. 2A, 5).

**DISCUSSION**

In the present communication we have investigated the requirement for specific intron sequences in the formation of a correctly spliced E1A 13S mRNA. Mutants with deletions and insertions in the part of the intron which is common to all three E1A mRNAs were created (Fig. 1) and the structure of the transiently expressed mRNAs characterized by the S1 protection assay.

Sequence studies of a large number of eucaryotic genes have demonstrated well conserved sequences located around both 5'- and 3'-splice junctions (3,10). These consensus sequences are very short and have in the case of 5'-splice sites been shown to correspond to the nonanucleotide (C/A)AG'T(A/G)AGT.

From our analysis of mutant pJU11Acq, which has a duplicated 5'-splice site for the 13S mRNA, it is obvious that no more than five nucleotides on the exon side of the splice junction are required in order to obtain a correct splicing. The truncated splice site is, however, used with a much lower efficiency than its upstream counterpart. Thus, it seems that exon sequences located further upstream, although not absolutely required for splicing, influence the splicing efficiency. The combined results from our analysis of deletion and insertion mutants (Fig. 2) shows that the signals which are absolutely required for the recognition of the E1A 13S 5'-splice junction are all located within a segment that does not extend more than five nucleotides upstream and 20 nucleotides downstream of the 5'-splice junction. Bearing in mind the fact that only the six 5'-proximal intron nucleotides are required for a normal splicing of the large intron in the rabbit G-globin pre-mRNA (16) and at the most 13 nucleotides in RNA polymerase II transcribed yeast introns (42) it seems very likely that our estimate for intron nucleotides will be narrowed down by future work.

The efficiency by which the 13S mRNA intron is excised is also influenced by the length and origin of the intron sequences. Thus a correct splicing requires a minimum intron length. Deletion mutants retaining 78 nucleotides
of the intron allowed for the formation of a functional 13S mRNA, although with a drastically reduced efficiency (Fig. 2C). Increasing the intron length to 91 nucleotides allowed for a normal splicing, whereas truncating it to 63 nucleotides completely abolished the utilization of the 13S 5'-splice junction. The ability to mature a normal 13S mRNA could be restored both by insertion of phage lambda DNA or specific intron sequences derived from region E1A. In summary these results show that an efficient splicing is dependent on a minimal intron length. A length which appears to exceed 78 nucleotides in the case of the E1A 13S mRNA.

Similar studies have shown that an intron length of more than 80 nucleotides is required in order to correctly splice the rabbit B-globin pre-mRNA (16). However, a general requirement for introns exceeding 78 nucleotides in length can not be argued from these results, since functional introns which are considerable shorter have been described in the literature (43,44,45). It seems, therefore, that for efficient splicing additional parameters such as flexibility and conformation of the pre-mRNA also are of importance. Alterations of both the intron length and sequence composition probably causes steric effects which reduce the splicing efficiency.

In contrast to the rabbit B-globin pre-mRNA (16) we find that the origin of the intron sequences play an important role for the efficiency by which a 5'-splice site is selected. Thus, insertion of phage lambda sequences into mutants with extreme intron deletions never restored the splicing efficiency to more than 10% of the normal level (Fig. 2C). Duplication of the authentic E1A 13S mRNA intron restored in one mutant (pJU11Acc) almost the wild type splicing efficiency, whereas insertion of the same DNA segment into mutant pJU101 led to a splicing efficiency which was not better than that obtained with a phage lambda sequence (compare pJU10Acc and pJU101; Fig. 2C).

It should be emphasized that in all insertion mutants examined the impairment on RNA splicing almost exclusively affects the maturation of the 13S mRNA. The overall E1A expression is only slightly altered in mutant transfected cells (Table 1) and therefore the decrease in 13S mRNA processing is almost completely compensated by an enhanced utilization of the 12S and 9S 5'-splice sites (Fig. 2 and data not shown). Hence, if we assume that the changes in plasmid structure do not lead to an enhanced transcription rate, or an increased stability of the E1A pre-mRNA, these results imply that a regulatory signal located in the 5'-half of the 13S mRNA intron specifically affects the efficiency by which the E1A 13S mRNA is synthesized. Obviously this signal may be of importance for the control of differential splicing of
the E1A mRNAs.

What is the nature of this signal? As argued above a likely explanation is that alterations of the intron sequences create an unfavorable secondary structure of the pre-mRNA which, in turn, may cause less flexibility of the intron. Alternatively, a specific regulatory sequence which selectively affects 13S mRNA splicing may be located in the 5'-half of the 13S mRNA intron. This sequence, which obviously is only partially defective in mutant pJU101, may cause a trapping of the processing enzymes at the inappropriate site and thereby preventing mutant pJU10Acc to restore the normal efficiency for 13S mRNA splicing. This phenomenon could, for example, occur if splicing involved a 5'– to 3'–directional scanning of the intron. A lateral diffusion would mean that the partially defective regulatory sequence in mutant pJU10Acc would be encountered before the wild type sequence in the duplicated E1A intron. Since RNA splicing results in the formation of lariat RNAs (7,8,9), the branch sequence which must exist may correspond to this regulatory signal. Theoretically the three alternative E1A mRNAs may use different branch sites during the splicing reaction. Deletion and insertion of foreign sequences into the 5'-half of the intron may therefore selectively cause a disadvantage for 13S mRNA splicing.

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