Splicing 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 S1 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 autenthic 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
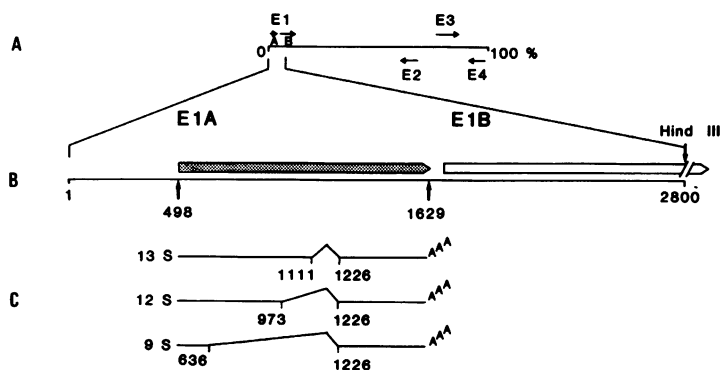
Fig. 1. Organization of the Ad2 genome.
 A.) A schematic drawing showing the locations and direction of transcrip-
tion of the four regions expressed early after infection. Region E1 contains
two promoters which subdivide it into region E1A and E1B.
 B.) Enlargement of a region located between co-ordinates 0 and 7.9 in the
Ad2 genome encoding region E1A and the 5'-portion of region E1B.
 C.) Structure of the mRNAs encoded by E1A as determined by DNA sequence
analysis of cDNA clones (46,47).

and AG dinucleotide pairs have in many cases been shown to cause abberant or

complete inhibition of splicing (11-15). The bulk of the intron sequences

appears, furthermore, to be dispensible 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 precur-

sor 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 pKGO-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 $MgCl_2$, 12 mM
$CaCl_2$, 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'-plice site
were characterized in detail by restriction endonuclease cleavage and DNA
sequence analysis.

Construction of insertion mutants pJU10Acc and pJU11Acc. Plasmid pKGO-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 electroph-
oresis 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 plas-
mids pJU101 and pJU111. Two clones were isolated at random and characterized
in detail.

   All cloning experiments connected with this study were carried out accor-
ding 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 de-
scribed (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 pSXβ+
DNA, encoding rabbit β-globin (34), was always included as an internal con-
trol of the transfection efficiency. Approximately 50 hrs post transfection
cells were lysed by IsoB-Nonidet P-40 extraction and fractionated into cyto-
plasm 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.

S1 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. S1 nuclease cleavage and electroph-
oretic 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 rela-
tive 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 miminal intron sequences required for the formation of a
correctly spliced E1A 13S mRNA, a series of deletion mutants were constructed

by Bal-31 digestion of pKGO-007 SV-RI DNA (Fig. 2A). Five mutants with delet-
ions 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 octameric XhoI
linker. As a result the actual intron length was in all mutants extended by
eight nucleotides. This means that, for example, mutant pJU81, although

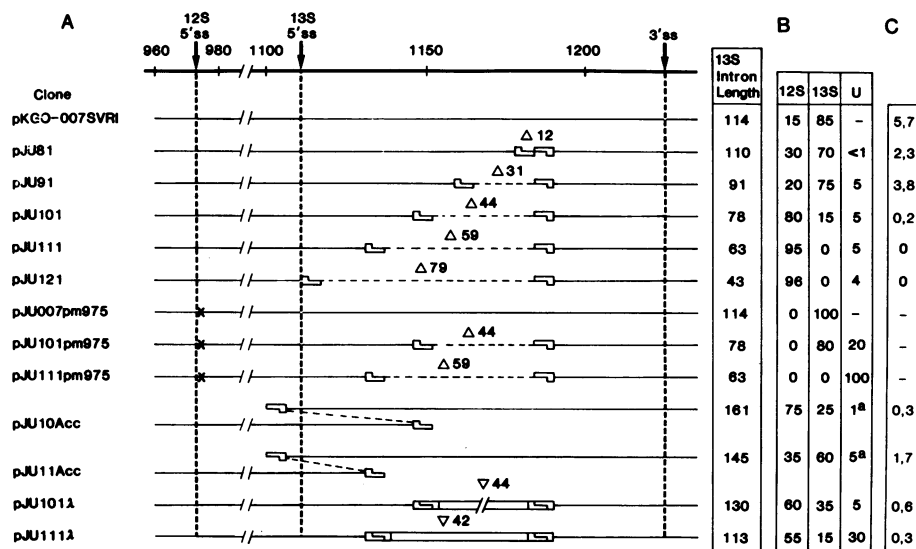| Clone	13S Intron Length	12S	13S	U	C
pKGO-007SVRI	114	15	85	–	5,7
pJU81	110	30	70	<1	2,3
pJU91	91	20	75	5	3,8
pJU101	78	80	15	5	0,2
pJU111	63	95	0	5	0
pJU121	43	96	0	4	0
pJU007pm975	114	0	100	–	–
pJU101pm975	78	0	80	20	–
pJU111pm975	63	0	0	100	–
pJU10Acc	161	75	25	1a	0,3
pJU11Acc	145	35	60	5a	1,7
pJU101λ	130	60	35	5	0,6
pJU111λ	113	55	15	30	0,3

Fig. 2. Structure and splice pattern of deletion and insertion mutants in the
E1A 13S mRNA intron.
   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 ($\triangle$) as well as inserted ($\bigtriangledown$) in the different clones
are shown. The deletion end-points have been joined by an XhoI linker ($\square$).
Open boxes denotes inserted phage lambda sequences. ($\times$), 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.
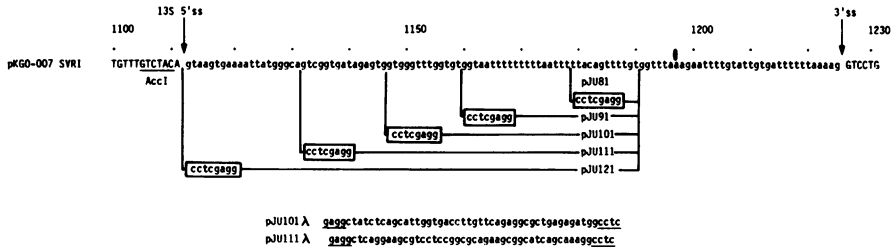
Fig. 3. Nucleotide sequence of deletion and phage lambda insertion mutants.
A.) The illustrated sequence reads as the sense strand between positions
1100 and 1231 on the Ad2 genome. Exon sequences are shown in capital letters
and the 13S mRNA intron in small letters. Deletion end-points were joined by
an octameric XhoI linker which is shown boxed in the figure. The AccI cleav-
age site used for construction of mutants pJU10Acc and pJU11Acc is under-
lined. The sequence of the phage lambda insert (41) in mutants pJU101 λ (pos-
itions 36361-36404) and pJU111λ (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 pSXβ⁺ DNA, expressing the rabbit β-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 β-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 pJU81
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 (pJU81, pJU91) 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 pJU101, 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,
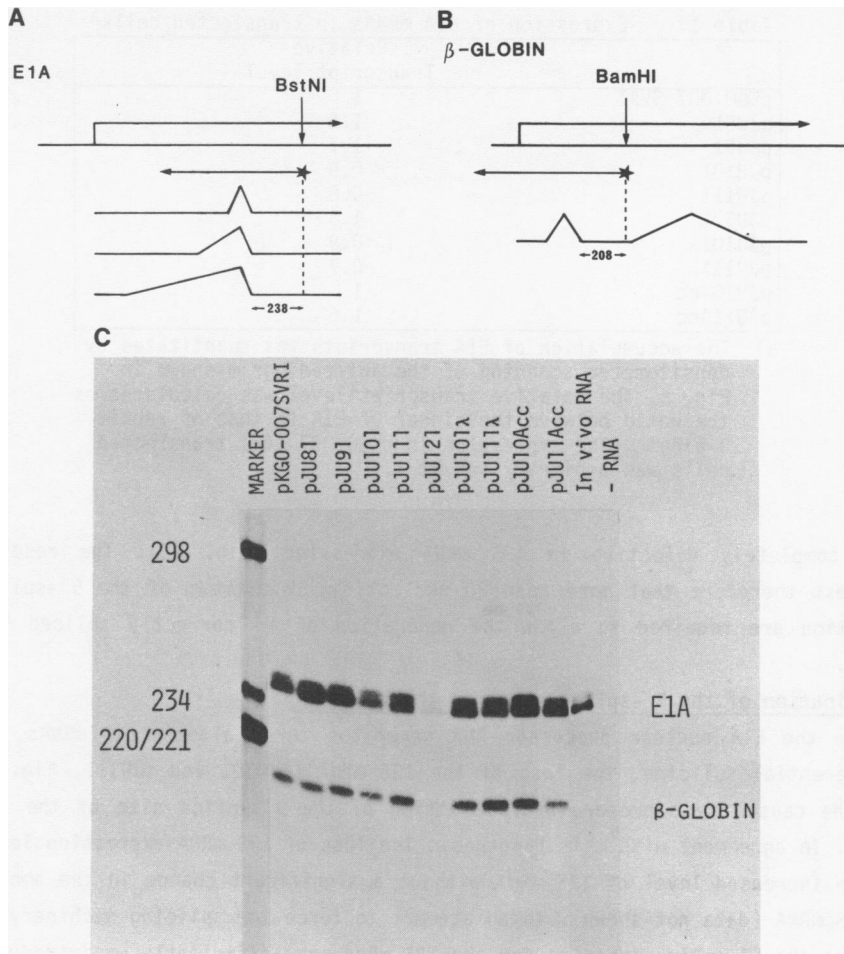
Fig. 4. Effect of deletions and insertions on the accumulation of E1A transcripts.
Strategy for the S1 endonuclease analysis. For the detection of E1A transcripts (panel A) a 650 bp NarI-BstNI fragment (positions 810-1461), 5'-end-labeled at the BstNI cleavage site was used and for the detection of ß-globin 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 ß-globin and E1A RNAs accumulated in HeLa cells transfected with 3µg of pSXß⁺ 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 ß-globin reference RNA.

Table 1.   Expression of E1A mRNAs in transfected cells[a]

	Relative Transcript level
pKGO-007 SVRI	1
pJU81	1.5
pJU91	1.1
pJU101	0.9
pJU111	0.8
pJU121	1.3
pJU101λ	0.9
pJU111λ	0.7
pJU10Acc	1
pJU11Acc	1.6

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-077 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 resistent products were resolved by neutral
agarose gel electrophoresis (28,37). Under appropriate conditions S1 endo-
nuclease digestion will produce a nuclease resistent 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 trans-
fected cells. This is particularly noteworthy in pJU101pm975 transfected
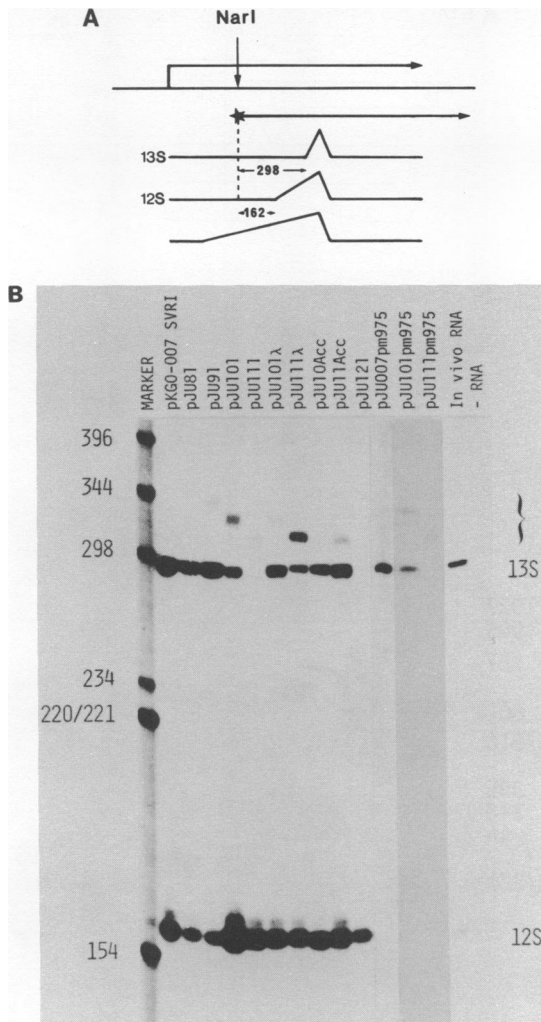
Fig. 5. Changes in the expression of the E1A 13S and 12S mRNA in cells trans-
fected with mutant plasmids.
   A.) S1 nuclease mapping scheme. An approximatly 2000 bp long NarI-HindIII
fragment (positions 810-2800), 3'-end-labeled at the NarI cleavage site, was
hybridized to 10µg of total cytoplasmic RNA isolated from transfected cells
and treated with nuclease S1.
   B.) Resistent material was separated by electrophoresis through a 6%
denaturing polyacrylamide gel. The position of the S1 resistent 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.
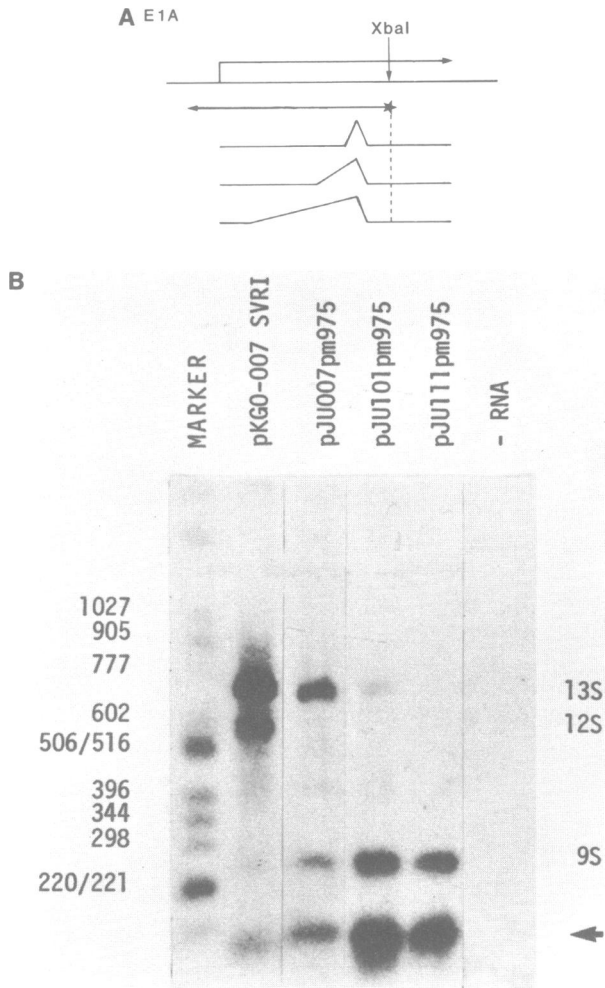
Fig. 6. Spliced structure of E1A RNAs accumulating in cells transfected with plasmids lacking a functional 12S 5'-splice site.

A.) Strategy for the S1 endonuclease analysis. An approximatly 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 cyto-plasmic RNA isolated from transfected cells and treated with nuclease S1.

B.) The S1 resistent 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 orig-
inal 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 matu-
ration 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 re-
quires 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 approximatly 3-fold compared to the par-
ental mutant pJU101 (Fig. 2C). The splicing defect could, therefore, be cured
by expanding the intron length with a piece of foreign DNA. However, a quan-
titation 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 trans-
fected 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 (pJU111λ; 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 (pJU101λ; 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 pJU111λ 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 pJU111 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
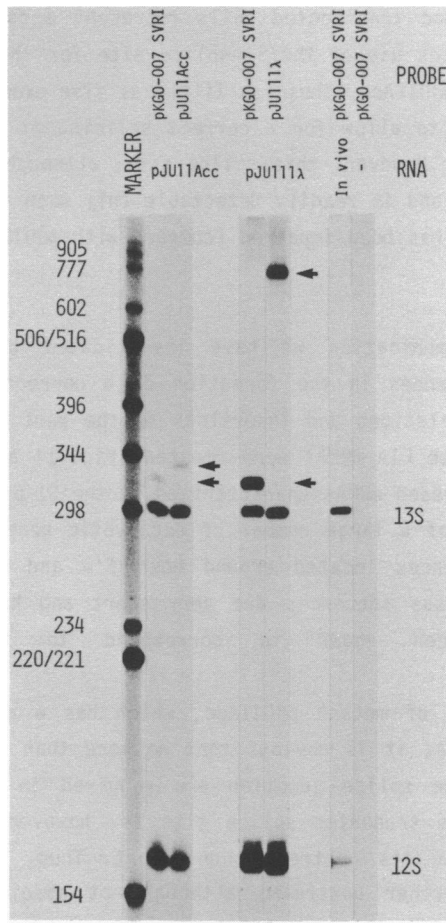
Fig. 7. Detailed analysis of the spliced and unspliced RNAs found in mutant pJU11Acc and pJU111λ transfected cells. 10µg of total cytoplasmic RNA was mixed and hybridized to the 3'-end-labeled NarI-HindIII DNA fragment de-scribed in Fig. 5, panel A. To discriminate between transcripts corresponding to 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 resistent 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 NarI-HindIII fragment (position 810-2800) was iso-lated 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 pJU11Acc 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 pJU11Acc. 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 pJU10Acc; 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'GT(A/G)AGT.

From our analysis of mutant pJU11Acc, 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 β-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 maturate 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 ß-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 ß-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 pJU101λ; 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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