Volume 17 Number 3 1989 Nucleic Acids Research

Role of the branch site/3'-splice site region in adenovirus-2 E1A pre-mRNA alternative splicing: evidence for 5'- and 3'-splice site co-operation

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Received November 23, 1988; Accepted January 3, 1989

ABSTRACT

The adenovirus E1A gene encodes five overlapping mRNAs which are processed by alternative RNA splicing from a common pre-mRNA. To characterize cis-acting sequence elements which are of importance for the alternative 5'-splice site selection deletion and substitution mutants within the intron that is common to all E1A mRNAs were constructed. Deletion of the wild-type E1A branch site/polypyrimidine tract resulted in activation of a functionally redundant sequence located within an A/T rich sequence just upstream of the normal E1A lariat branch site. Removal of both regulatory sequences abolished *in vivo* splicing completely and did not lead to activation of cryptic 3'-splice sites at other locations in the E1A pre-mRNA. Furthermore we show that the sequence around the E1A branch site/3'-splice site region may have a more direct effect on the efficiency by which the alternative E1A 5'-splice sites are selected. Replacing the E1A branch site/3'-splice site region with the corresponding sequence from the second intron of the rabbit ß-globin gene or the first intron of the major late transcription unit resulted in drastic changes in E1A 5'-splice site selection. For example, with the E1A/ß-globin hybrid gene the 9S mRNA became the most abundant E1A mRNA to accumulate. This contrasts with the wild-type E1A gene in which almost undetectable levels of 9S mRNA were produced in transient expression assays. Our results strongly suggest that a cooperative interaction between 5'- and 3'-splice sites on a pre-mRNA determines the outcome of alternative splicing.

INTRODUCTION

The development of soluble extracts that correctly excise introns from pre-mRNAs has led to a dramatic increase in our understanding of the mechanism and sequence specificity of RNA splicing. RNA splicing can be summarized as a two step reaction which takes place in a large nucleoprotein complex, the spliceosome. The first step consists of a cleavage at the 5'-splice site and a concomitant formation of a lariat by joining the 5'-end of the intron to the 2'-hydroxyl group of an A residue usually located between 20 and 40 nucleotides upstream of the 3'-splice site. The second step involves a cleavage at the 3'-splice site followed by exon ligation and release of the intron as a lariat (for a review see refs. 1 and 2)

Many viral and cellular pre-mRNAs contain multiple splice signals. This means that several mRNAs can be processed from a single transcription unit by alternative splicing. In many cases the selection of RNA splice sites have been shown to be controlled either in a temporal or tissue specific manner (reviewed in refs. 1 and 3). To characterize signals that are of importance for alternative RNA splicing we have undertaken a study of the splicing pathway of the early region 1A (E1A) mRNAs of adenovirus type 2 (Ad2). The E1A transcription unit encodes five over-

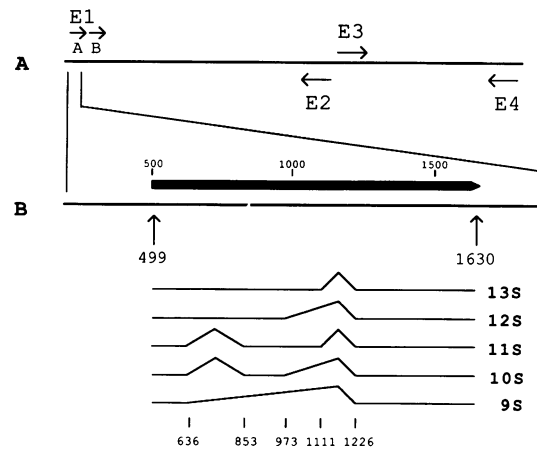


Figure 1.
Organization of early transcription units on the Ad2 genome.
A. A schematic drawing showing the location and direction of transcription of the regions expressed early after infection. Region E1 contain two promoters which subdivide it into E1A and E1B.
B. Enlargement of region E1A showing the structure of the five characterized E1A mRNAs. Numbering is in base-pair (bp) starting from the left end of the Ad2 genome (24).

lapping mRNAs, designated 9S, 10S, 11S, 12S, and 13S (Figure 1) (4, 5), which are processed by a complex alternative splicing pathway from a common pre-mRNA. The 9S, 12S and 13S mRNAs use the same 3´-splice site and vary from each other by using alternative 5´-splice sites (6, 7). The 10S and 11S mRNAs are expressed primarily late after infection and represent minor variants of the 12S and 13S mRNAs in which the 9S 5´-splice site has been connected to a minor 3´-splice site at position 853 (4, 5). During a lytic infection the E1A mRNAs accumulate with different kinetics. The 12S and 13S mRNAs are the most abundant species synthesized early after infection whereas the 9S mRNA becomes the predominant mRNA at late times (8). The accumulation of E1A mRNA is subjected to a post-transcriptional regulation, probably at the level of RNA splicing (9).

By sequence comparison, consensus sequences for the 5'-[AG/GT)(A/G)AGT] and the 3'-splice sites [$(Py)_{6-15}$NPyAG/G] have been determined (10, 11). Just upstream of the 3'-splice site polypyrimidine tract is the loosely conserved branchpoint sequence (PyNPyTPuAPy, 12, 13) which includes the A residue used for lariat formation. Deletion of the branch site fails to abolish splicing and usually leads to the use of cryptic branch sites (14, 15). On the other hand, shortening of the polypyrimidine tract results in a complete loss of splicing or selection of cryptic 3'-splice sites at other locations (14, 16, 17, 18). Kinetic experiments have suggested that one of the first steps in spliceosome assembly is the recognition of sequences upstream of the 3´-splice site by the U2 snRNP particle (19, 20); thus forming a stable U2 snRNP-pre-mRNA complex.

Recent studies have also indicated that the 5'-splice site enhances the efficiency of U2 snRNP-pre-mRNA complex formation (21, 22); suggesting an interaction between the 5'- and 3'-splice sites. In a previous report, we demonstrated that intron sequences in addition to the well conserved sequences at the 5'- and 3'-splice sites are important for efficient utilization of the E1A 13S mRNA 5'-splice site (23). Here we show that the nucleotide sequence at the E1A pre-mRNA branch site/3'-splice site region is important for the efficiency by which alternative E1A 5´-splice sites are selected. Our results suggest that a direct co-operative interaction between the 5'- and 3'-splice sites determines the outcome of alternative E1A pre-mRNA splicing.

MATERIAL AND METHODS

Plasmid construction

Deletion mutants were constructed as previously described (23). Plasmid pJU93L+ was constructed by ligating an octameric *Xho*I-linker to a blunt-ended *BstN*I-*Alu*I fragment which included the branch site/3´-splice site from the first intron of the Ad2 major late transcription unit (position 7051-7100, 24). This fragment was cloned into the *Xho*I cleavage site of plasmid pJU93. Plasmid pJU93ß+ was generated by the same strategy except that an *Alu*I-*Nci*I fragment including the branch site/3´-splice site from the large intron of the rabbit ß-globin gene (position 1021 - 1066 relative to the cap site; 25) was used. Plasmids pJU92S and pJU93S were constructed by inserting the synthetic double-stranded oligonucleotide

5´ TCGATGTCATACTTATCCTGC 3´
3´ ACAGTATGAATAGGACGAGCT 5´

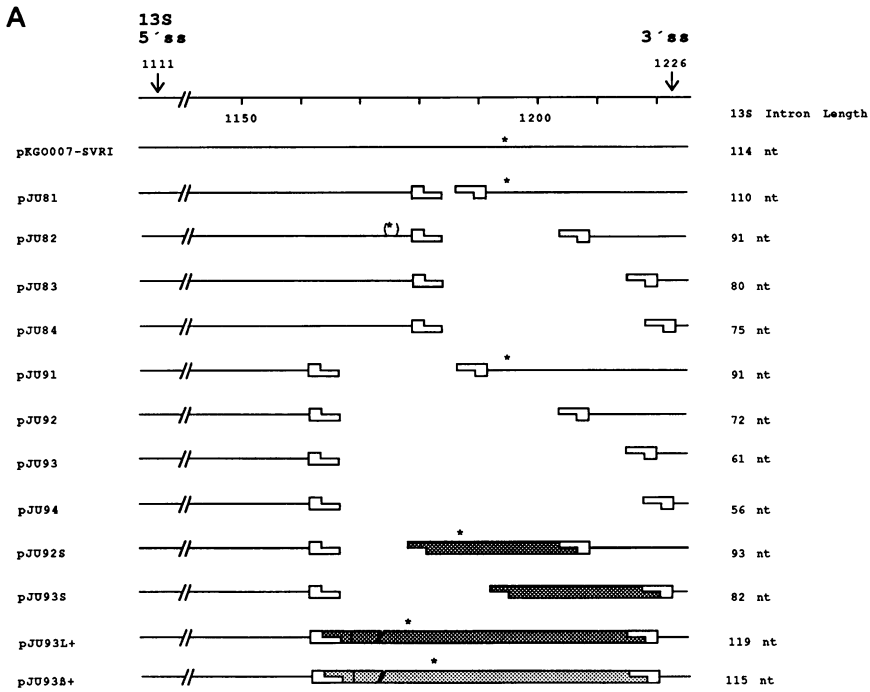
into the *Xho*I cleavage site of pJU92 and pJU93 (kindly provided by Kabigen AB, Stockholm). This oligonucleotide contains the branchpoint sequence from the first intron of the major late transcription unit (26).

DNA transfection and RNA preparation

Subconfluent monolayers of HeLa cells were transfected as previously described (23, 27), using the calcium phosphate co-precipitation technique (28, 29). A total of 13 µg plasmid DNA was added per 6 cm Petri dish. 3 µg of pSXß+ DNA, encoding the rabbit ß-globin gene (30), was always included as an internal control of the transfection efficiency. Approximately 50 hours post transfection cells were lysed by IsoB-Nonidet P-40 treatment and fractionated into cytoplasm and nuclei. Total cytoplasmic RNA was isolated by phenol extraction (31). 50 to 100 µg of RNA were obtained per 6 cm Petri dish.

S1 endonuclease analysis

S1-nuclease digestion and gel electrophoresis was performed as previously described (23, 32, 33). The relative amount of RNA was determined by densitometer scanning of autoradiograms using a Shimazu dual wavelength TLC scanner.


A
13S
5´ss
3´ss
1111
1226
1150
1200
13S Intron Length
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91 nt
pJU83
80 nt
pJU84
75 nt
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91 nt
pJU92
72 nt
pJU93
61 nt
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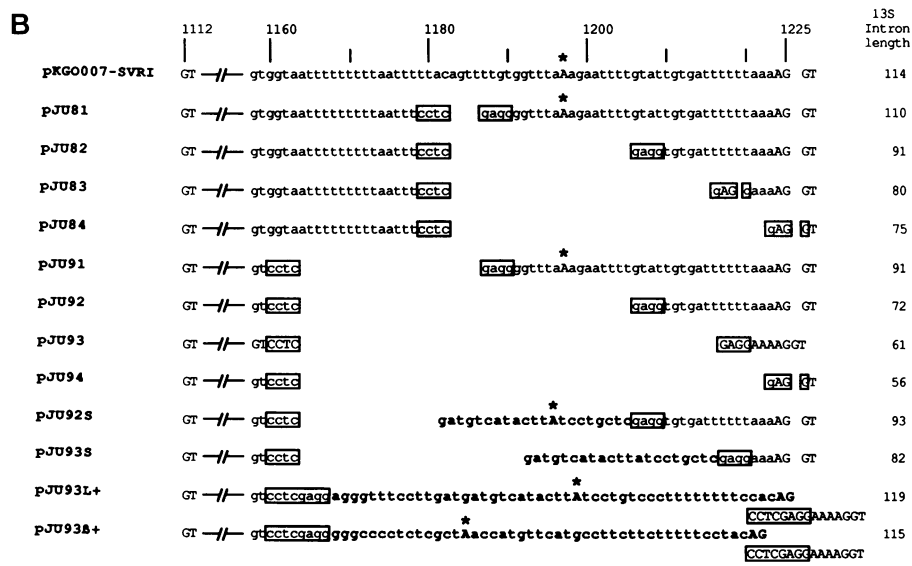
B
1112
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pJU93
pJU94
pJU92S
pJU93S
pJU93L+
pJU93S+

RESULTS

Deletion of the E1A branchpoint sequence and polypyrimidine tract does not inhibit E1A pre-mRNA alternative splicing.

To characterize cis-acting sequence elements important for alternative E1A pre-mRNA splicing we constructed a set of deletion/substitution mutants of plasmid pKGO007-SVRI (27). Four mutants extending from a midpoint of the 13S mRNA intron towards the common 3´-splice site were isolated (pJU81 to pJU84, Figure 2). Mutant pJU81, lacks 12 base pairs upstream of the E1A branchpoint sequence (9, 34); pJU82 lacks 31 base pairs which includes the normal E1A branchpoint sequence and part of the polypyrimidine tract; pJU83 lacks 42 base pairs which deletes both the branchpoint sequence and most of the polypyrimidine tract. The deletion in mutant pJU84 extends five nucleotides further than in pJU83 and also removes the conserved AG dinucleotide that defines the E1A 3´-splice site (Figure 2B). The end points of the deletions were fused such that they became separated by an octameric *Xho*I linker.

Mutant constructs were transfected into HeLa monolayer cells and the transiently expressed E1A mRNAs were characterized and quantitated by the S1-nuclease protection assay (32, 33). A small amount of plasmid pSXß+ (30) expressing the rabbit ß-globin mRNA under the control of the SV40 enhancer was included as a reference plasmid to measure the transfection efficiency (23, 35). The E1A proteins repress the activity of the SV40 enhancer (36, 37), however, under our experimental conditions this repression on pSXß+ expression is negligible (35).

Several reports have suggested that the polypyrimidine tract is important for the correct recognition of the 3´-splice site (18, 38). Surprisingly mutants pJU82 to pJU84 which lack various parts of the authentic 3'-splice site region produced wild-type levels of E1A mRNA (Figure 3A, Table 1). However, mutant transfected cells accumulated significantly reduced amounts of E1A 13S mRNA as compared to the wild-type E1A gene (pKGO007-SVRI) (Figure 3B). This reduction in 13S mRNA expression in pJU83 and pJU84 transfected cells is expected since these deletions shorten the 13S mRNA intron down to its minimal length (Figure 2A). We have previously shown that an intron of 78 nucleotides or less is very inefficiently spliced *in vivo* (23). The cytoplasmic steady-state level of E1A mRNA is constant in these experiments and therefore the

Figure 2.
Structure and sequence of plasmids used in this study.
A. Schematic diagram of plasmid clones constructed. The position of the E1A 13S 5´-splice site and the common 3´-splice site at position 1226 are indicated. Numbering is in bp from the left end of the genome. Hatched boxes denotes foreign sequences which have been inserted into some clones. The *Xho*I linker is indicated by ( ) and the position of the A residue used for lariat formation is indicated by an asterisk (*) when known.
B. Nucleotide sequence of deletion/insertion and substitution mutants in the E1A 13S mRNA intron. Exon sequences are written with capital letters and intron sequences are written in lower case letters except for the conserved GT-AG dinucleotides bordering the intron which also are written with capital letters. The *Xho*I linker is boxed in the figure and the A residues used for lariat formation are indicated by an asterisk (*).

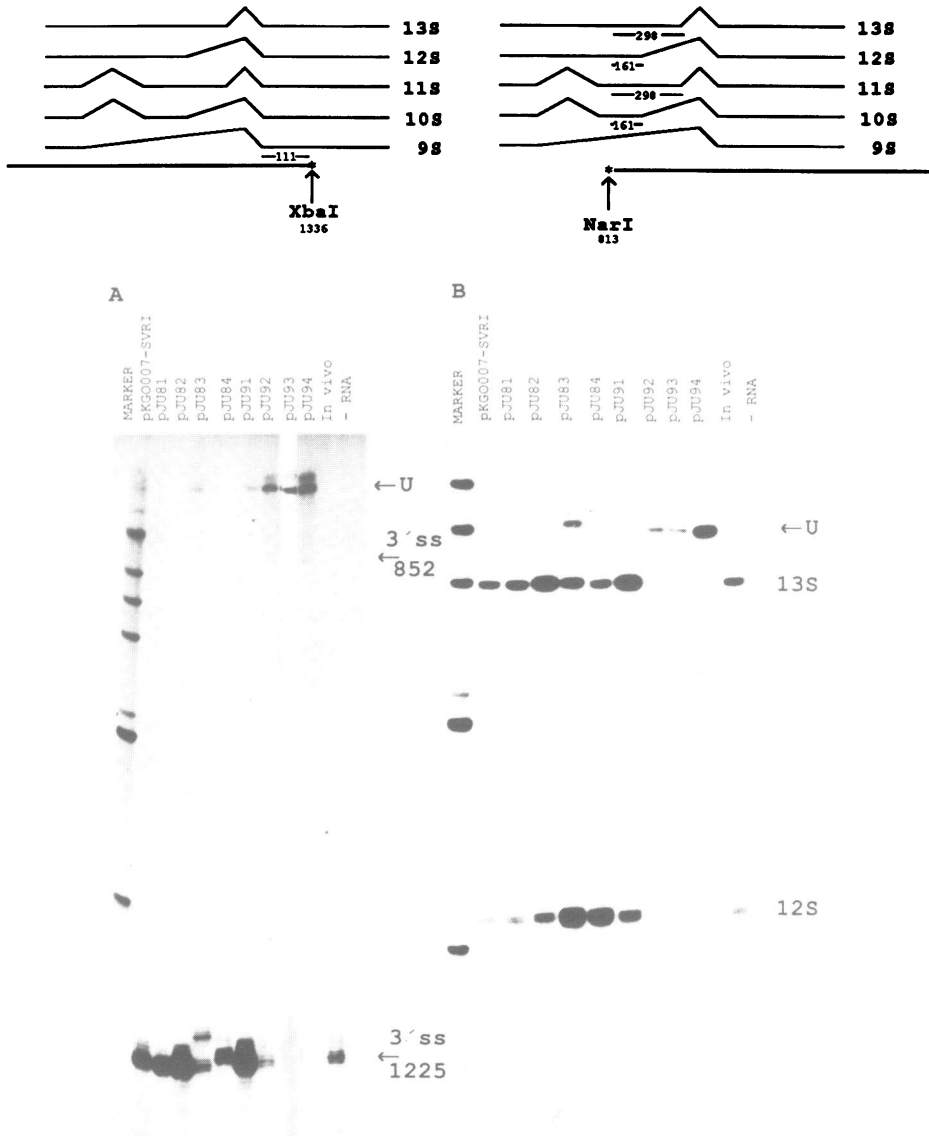

Figure 3.
Accumulation of E1A mRNA in cells transfected with mutant plasmids.
Total cytoplasmic RNA prepared from transfected HeLa cells was hybridized to the homologous DNA probe either 5´-end labeled at the *Xba*I cleavage site at position 1336 (panel A) or 3´-end labeled at the *Nar*I site at position 813 (panel B). Hybrids were digested with S1 endonuclease and the resistant fragments resolved on a 6% denaturing polyacrylamide gel. (U) denotes unspliced pre-mRNA and (ss) denotes splice site. Marker fragments were from pBR322. *In vivo*, total cytoplasmic RNA isolated early after an adenovirus infection.

decrease in 13S 5´-splice site utilization is compensated by an equivalent increase in E1A 12S 5´-splice site selection (Fig 3B and data not shown). From these results we conclude that the authentic E1A branchpoint sequence and polypyrimidine tract is dispensable for efficient E1A pre-mRNA splicing and that the intron must encode an alternative 3´-splice signal which is equally efficient as the wild-type sequence.

As shown in Figure 3A two 3´-splice sites, separated by six nucleotides, were utilized in mutant pJU83 transfected cells in about equal proportion. The downstream site corresponds to the normal E1A 3´-splice site at position 1226 and the upstream site corresponds to an AG dinucleotide contributed by the *Xho*I linker (Figure 2B). Both 3'-splice sites are used with approximately the same efficiency although the downstream AG dinucleotide is preceded by nine purines and therefore shows little homology to the 3'-splice site consensus (10, 11). The usage of two alternative 3´-splice sites in the same intron is rare in eukaryotic pre-mRNAs. Usually the AG dinucleotide located closest to the branchpoint sequence is used (reviewed in refs. 1 and 2). Mutant pJU84 which lacks the natural E1A AG dinucleotide efficiently utilizes the AG dinucleotide contributed by the *Xho*I linker as 3´-splice site (Figure 3A).

Requirement of a branchpoint sequence and a polypyrimidine tract for efficient E1A pre-mRNA splicing.

Clones pJU82 to pJU84 must possess an alternative 3'-splice signal since they efficiently accumulate cytoplasmic mRNA although they lack the characterized E1A branchpoint sequence and the authentic polypyrimidine tract. Examination of the DNA sequence preceding the 3´-splice site in these mutants revealed an A/T rich sequence which encodes two potential branch sites (TGGTAAT and TTTAAT) and a long polypyrimidine tract (Figure 2B). It is thus conceivable that this A/T rich sequence can functionally substitute for the natural E1A 3´-splice signal.

To test this hypothesis, we analyzed mRNA expression from deletion mutants, pJU91 to pJU94 which removed this A/T rich sequence (Figure 2B). As expected, plasmid pJU91, which encodes

Table 1. E1A mRNA accumulation in transfected HeLa-cells.

pKGO007-SVRI	1.0
pJU81	1.6
pJU82	1.0
pJU83	1.7
pJU84	1.1
pJU91	1.0
pJU92	0.04
pJU93	<0.04
pJU94	0.1
pJU92S	1.0
pJU93S	0.6
pJU93L+	0.7
pJU93ß+	1.1

Accumulation of E1A transcripts was quantitated by densitometer scanning of autoradiograms. The relative E1A mRNA level was calculated as the ratio between the signal of E1A to the that of rabit ß-globin control. The E1A mRNA expression in pKGO007-SVRI transfected cells was arbitrarily set at 1.0.

the natural E1A branch site and polypyrimidine tract produced wild-type levels of E1A mRNA (Figure 3B, Table 1). In contrast, E1A mRNA levels in cells transfected with plasmids pJU92 to pJU94 were drastically reduced when compared to the wild-type (Table 1; Figure 3A). Furthermore these mutants accumulated almost exclusively unspliced pre-mRNA (Figure 3A, 3B and data not shown). The loss of 13S mRNA splicing, can be attributed to a reduction of the 13S mRNA intron below its minimal length (23; Figure 2 A). However, the lack of 12S mRNA accumulation in pJU92 to pJU94 transfected cells can not be explained by creation of an unspliceable intron since the distance between the 12S 5′-splice site and the 3′-splice site is still more than 210 nucleotides in the mutant with the largest deletion (pJU84: Fig. 2A). We conclude that the A/T rich sequence between nucleotides 1162 and 1180 encodes an alternative strong 3′-splice signal. Removal of this sequence in plasmids pJU92 to pJU94 results in an unspliceable pre-mRNA which may have a low stability or alternatively is not transported to the cytoplasm.

Rescue of E1A pre-mRNA splicing by the insertion of a heterologous branch site.

To determine whether mutants pJU92 and pJU93 are defective in E1A pre-mRNA splicing because they lack regulatory signals required for 3′-splice site recognition we inserted a synthetic oligonucleotide corresponding to the branch site from the first intron of the major late transcription unit (26). By this strategy plasmids pJU92S and pJU93S were generated (Figure 2B). Care was taken to select an oligonucleotide that would encode as little as possible of the long polypyrimidine tract upstream of the second leader 3′-splice site (24, 26).

GATGTCATACTTATCCTGTCCCTTTTTTTTCCACAG-LEADER 2
Oligonucleotide

The results shown in Figure 4B, and quantitated in Table 1, demonstrated that the oligonucleotide was able to restore E1A mRNA accumulation to nearly wild-type levels in both pJU92S and pJU93S transfected cells. Surprisingly large quantities of 13S mRNA accumulated in pJU92S transfected cells. A quantitation of the mRNA expression demonstrated that the ratio of 13S to 12S mRNA increased from the wild-type value 5.5 to approximately 11 in pJU92S transfected cells (Fig. 5; Table 2). This was unexpected since the 13S mRNA intron in this plasmid is only 82 nucleotides long. We had expected it to be to short for efficient splicing (23) (also compare pJU83 and pJU92S in figures 3B and 4B two plasmids which have similar sized introns). Since the oligonucleotide was designed to encode a heterologous branch region these results suggest that sequences around the branch site may have an effect on the choice of alternative E1A 5′-splice sites. Interestingly the oligonucleotide restored RNA splicing only in pJU92S transfected cells. Plasmid pJU93S transfected cells accumulated elevated amounts of the unspliced pre-mRNA (Figure 4B and data not shown). We are currently analyzing this phenomenon in more detail.

Effect of heterologous 3′-splice signals on E1A pre-mRNA alternative splicing.

The alternative splicing of E1A mRNAs is regulated during the infectious cycle. Thus it was interesting to note that the spliced structure of E1A mRNA changed as the result of branch site

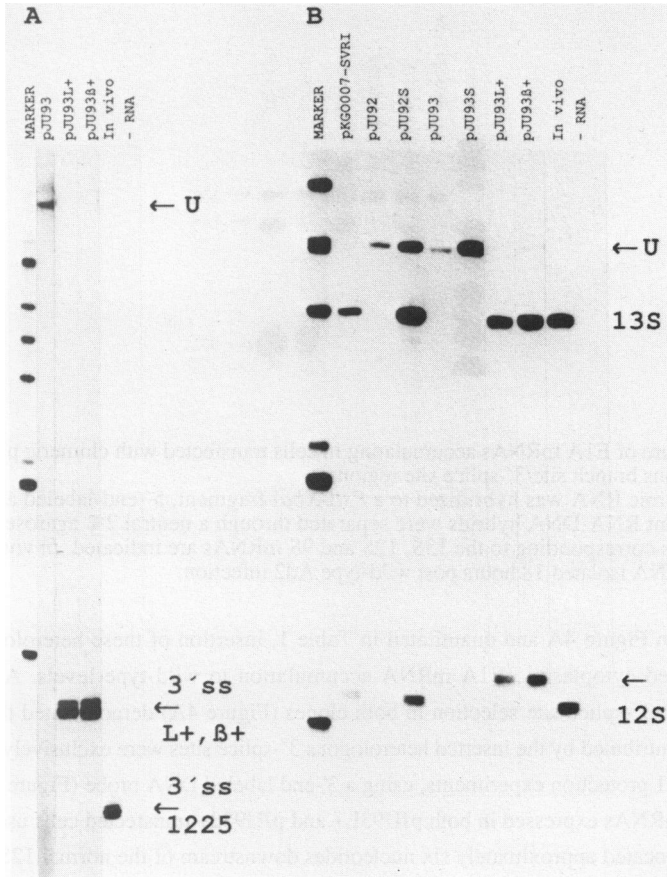

Figure 4.
Rescue of defective splicing mutants by insertion of heterologous branch sites/3´-splice site regions. Total cytoplasmic RNA prepared from transfected HeLa cells was hybridized to the homologous DNA probe either 5´-end labeled at the *Xba*I cleavage site at position 1336 (panel A) or 3´-end labeled at the *Nar*I site at position 813 (panel B). Hybrids were digested with S1 endonuclease and the resistant fragments resolved on a 6% denaturing polyacrylamide gel. (U) denotes unspliced pre-mRNA. In panel B, the arrow indicates an S1 artefact which is explained in the Results section. *In vivo*, total cytoplasmic RNA isolated early after an adenovirus infection

alteration in plasmid pJU92S (Fig. 5; Table 2).

To further define the function of the 3'-splice site in alternative splicing we constructed two additional clones, pJU93L+ and pJU93ß+. In these clones short DNA fragments (52 and 54 bp in length) encoding the branch site, polypyrimidine tract and AG dinucleotide from the first intron of the Ad2 major late transcription unit (pJU93L+; Figure 2) or the second intron of the rabbit ß-globin gene (pJU93ß+; Figure 2) were inserted into the *Xho*I site of plasmid pJU93.

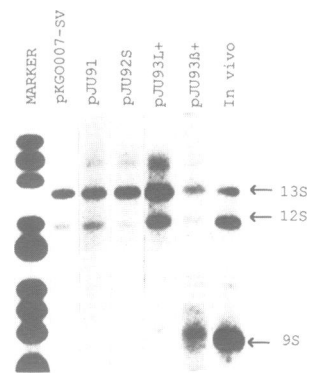

Figure 5.
Spliced structure of E1A mRNAs accumulating in cells transfected with chimeric plasmids encoding heterologous branch site/3´-splice site regions.
Total cytoplasmic RNA was hybridized to a *Pst*I-*Xba*I fragment, 5´-end-labeled at the *Xba*I site. The S1 resistant RNA-DNA hybrids were separated through a neutral 2% agarose gel. The position of hybrids corresponding to the 13S, 12S and 9S mRNAs are indicated. *In vivo* denotes total cytoplasmic RNA isolated 18 hours post wild-type Ad2 infection.

As shown in Figure 4A and quantitated in Table 1, insertion of these heterologous 3´-splice signals restored cytoplasmic E1A mRNA accumulation to wild-type levels. As expected an analysis of the 3´-splice site selection in both clones (Figure 4A) demonstrated that the AG dinucleotides contributed by the inserted heterologous 3´-splice sites were exclusively used.

The initial S1 protection experiments, using a 3'-end labeled DNA probe (Figure 4B), indicated that the 12S mRNAs expressed in both pJU93L+ and pJU93ß+ transfected cells used a novel 12S 5´-splice site located approximately six nucleotides downstream of the normal 12S 5'-splice site. However, an analysis of the precise structure of the 12S mRNAs expressed from these clones by restriction endonuclease cleavage of primer extension products (27) demonstrated that the novel nuclease protected bands were S1 artifacts (data not shown) caused by an extended complementarity between the DNA probe and the 12S mRNA.

The structure of the E1A mRNAs expressed in pJU93L+ and pJU93ß+ transfected cells were further analyzed by neutral agarose gel electrophoresis of S1 resistant fragments (27, 39). As

Table 2. Quantitation of E1A mRNA expression in cells transfected with E1A substitution mutants.

	13S	12S	9S
pKGO007-SVRI	83%	15%	~2%
pJU91	80%	18%	~2%
pJU92S	90%	8%	~2%
pJU93L+	55%	40%	5%
pJU93ß+	20%	10%	70%
Ad2 (18hpi)	13%	27%	60%

shown in Figure 5 and quantitated in Table 2 replacement of the complete E1A branch site/3'-splice signal with the corresponding sequence from the major late transcription unit (pJU93L+) resulted in an almost equal usage of the E1A 13S and 12S 5'-splice sites. Furthermore, insertion of the 3´-splice site from the rabbit ß-globin gene resulted in a dramatic switch to E1A 9S mRNA production. As a comparison the wild-type plasmid (pKGO007-SVRI) accumulated almost undetectable amounts of 9S mRNA under the same experimental conditions (Figure 5). Since the insertion of foreign sequences into pJU93 expanded the 13S mRNA intron from 61 to more than 115 nucleotides in length (Figure 2B) the observed changes in E1A mRNA accumulation should not be due to an unspliceable 13S mRNA intron. Taken together these results suggest that the sequences around the branch site/3'-splice site may directly determine the efficiency by which the alternative E1A 5'-splice sites are selected. Thus a co-operation between the 5'- and 3'-splice sites on the E1A pre-mRNA may take place during spliceosome formation.

DISCUSSION

A large body of evidence suggest that alternative RNA splicing is an important mechanism by which protein diversity is generated in eukaryotic cells (reviewed in ref. 3). Recently three spectacular examples have been presented where alternative splicing is used to turn on and off gene expression during Drosophila development (reviewed in ref. 40).

The adenovirus E1A gene represents one of the classic examples whereby differential pre-mRNA splicing is used to create multiple mRNAs which are translated into at least five proteins that have distinct biological activities (see refs, 4 and 5 for recent reviews). Experiments using protein and DNA synthesis inhibitors and viral mutants have implicated trans-acting factors which regulate E1A pre-mRNA splicing (for a review on E1A see ref. 41). Characterization of such factors and the cis-acting elements with which they interact is essential for our understanding of the mechanism of alternative RNA splicing.

We have previously presented evidence for a cis-acting signal located approximately 20 nucleotides downstream of the E1A 13S 5'-splice site which specifically promotes 13S mRNA accumulation (23). Here we characterize a second intronic signal which affects E1A pre-mRNA alternative splicing. We show that sequences around the E1A pre-mRNA branch site/3'-splice site strongly influence the efficiency by which the alternative E1A 5'-splice sites are selected. Deletion of the E1A branch site and polypyrimidine tract have little effect on E1A mRNA accumulation in transient expression experiments (pJU81 to pJU84; Table 1). However, expanding the deletion to include the A/T rich sequence located immediately upstream of the natural E1A branch site (positions 1162 to 1180; Figure 2B) resulted in an almost complete loss of cytoplasmic E1A mRNA (pJU92-94; 25-fold reduced compared to wild-type, Table 1) and did not result in the activation of cryptic 3´-splice signals on the E1A pre-mRNA. This A/T rich sequence contained 15 out of 19 pyrimidines and had furthermore two potential branch sites which closely matched the branch site consensus sequence (12, 13). Obviously it can functionally substitute for the E1A

branch site/polypyrimidine tract, during *in vivo* splicing.

Interestingly splicing of a hybrid E1A pre-mRNA consisting of the branch site from the first intron of the major late transcription unit and the E1A polypyrimidine tract-3´-splice site region (pJU92S; Fig. 3B) resulted in a more efficient utilization of the E1A 13S 5´-splice site compared to wild-type (Table 2). This was very unexpected since the 13S mRNA intron was only 82 nucleotides long in the particular pre-mRNA. We have previously shown that a 13S mRNA intron of 78 nucleotides or less is very inefficiently spliced (23). Deletions which reduce the 13S mRNA intron length down to or below its minimal length results in an increased 12S 5´-splice site utilization (see for example pJU83; Fig.3B and 23). Thus we would have expected pJU92S to accumulate predominantly 12S and not 13S mRNA. Taken together these results suggested that the sequences around the 3'-splice site influence the efficiency by which the alternative E1A 5'-splice site are selected.

To test this hypothesis further we constructed chimeric plasmids with heterologous branch site/3'-splice site regions inserted into the E1A gene. These plasmids were then assayed for changes in E1A 5'-splice site selection after transfection of HeLa-cells. Transient expression assays usually mimic the early infectious cycle (27) and accumulate almost undetectable levels of 9S mRNA (Figure 5). As shown in Figure 5 replacing the complete E1A branch site/3'-splice site region with the corresponding sequence from the major late transcription unit (pJU93L+; Fig. 2B) resulted in a significant shift in 5´-splice site selection. In this clone the E1A 13S and 12S 5'-splice sites were selected with an almost equal efficiency (Table 2). Even more remarkable was the event of replacing the normal E1A branch site/3'-splice site region with the corresponding region from the rabbit ß-globin gene (pJU93ß+; Fig. 2B) that resulted in an tremendous increase in 9S mRNA production (Table 2). A similar increase in 9S mRNA production can also be observed *in vivo* during a late adenovirus infection (Figure 5). Since the length of the 13S mRNA intron and the distance between the 13S 5´-splice site and the lariat branch nucleotide is similar in pJU93L+ pJU93ß+ and pKGO007-SVRI the relative decrease in 13S mRNA production can not be explained by creation of an unspliceable 13S mRNA intron. We interpret our results to indicate that sequences at the pre-mRNA branch site/3-splice site is critical for the efficiency by which alternative E1A 5'-splice sites are selected. In particular, the polypyrimidine tract/3´-splice site region appears to be important. In agreement with our results Fu et al.,(42) have recently shown that the polypyrimidine content of the SV40 early pre-mRNA 3´-splice site have an substantial effect on the efficiency by which the SV40 large-T and small-t 5´-splice sites are selected *in vivo*. At this stage we can of course not exclude the possibility that the observed shifts in E1A 5´-splice site selection is caused by an alteration of pre-mRNA secondary structure. Varying the salt concentration in HeLa cell nuclear extracts have been reported to partially reproduce the alternative E1A 5´-splice site selection (9). This suggests a role for pre-mRNA secondary structure (43) in E1A splicing.

Taken together our results indicate that the E1A 5'- and 3'-splice sites co-operate during spliceosome formation. The sequences at the 3´-splice site seems to determine which 5´-splice site

is used in the E1A pre-mRNA. Kinetic experiments have indicated that one of the first steps in spliceosome assembly is the binding of U2 snRNP to the pre-mRNA just upstream of the 3´-splice site (reviewed in refs. 1 and 2). This binding is facilitated by protein and/or RNP factors which interact with the 3'-splice site region (44, 45, 46). Thus it is possible that different splicing factors are required for U2 snRNP-pre-mRNA complex formation at different pre-mRNA 3´-splice sites. From this point of view, it is interesting to note that the snRNAs detectable on spliceosomes forming on the ß-globin and the adenovirus major late pre-mRNAs have been shown to vary in composition (49, 50). The 5'-splice site is believed to be recognized by a direct RNA-RNA complementarity between U1 snRNA and the pre-mRNA (19, 47). In accordance with these results many studies also show that U1 snRNA is a stable component of the active splicing complex (see ref. 48 and references therein). Our results suggest that the U2 snRNP-pre-mRNA complex formation involves, in addition to an interaction with the U1 snRNP particle, a direct recognition of the 5´-splice site sequence or the combined U1 snRNP/5´-splice site complex. In support of this hypothesis recent studies have shown that the presence of a 5´-splice site in the pre-mRNA promotes U2 snRNP complex formation at the 3´-splice site (21,22). Furthermore, in yeast, the pre-mRNA has been shown to be committed to the spliceosome pathway already before a stable U2 snRNA binding takes place (51). Formation of this committed complex requires both a 5'-splice site and the branch region again suggesting a long range interaction on the pre-mRNA during spliceosome formation. An intriguing possibility is of course that minor subspecies of U1 RNA (reviewed in ref. 52) recognize with a different affinity the alternative E1A 5´-splice sites; thus generating distinct U1 snRNP-pre-mRNA particles which can be discriminated by complex forming at the pre-mRNA 3´-splice site.

The results presented here imply that the shifts in E1A splice site selection taking place during the infectious cycle could in part be accomplished by virus induced modification of E1A 3´-splice site binding factors.

ACKNOWLEDGMENTS.

We thank Ulf Pettersson for continuous support, Kenn Zerivitz and Catharina Svensson for valuable comments on the manuscript. This work was supported by grants from the Swedish Cancer Society, the Swedish National Science Research Council, the Swedish Board for Technical Development and the Swedish Medical Research Council.

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