In vitro splicing of mRNA precursors: 5′ cleavage site can be predicted from the interaction between the 5′ splice region and the 5′ terminus of U1 snRNA

Susanna Weber and Markus Aebi*

Institut für Molekularbiologie I, Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

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ABSTRACT

Combinations of different mutations within the 5′ splice region of the rabbit ß-globin large intron were analyzed for their effect on in vitro splicing. Based upon the complementarity of the 5′ splice region to the 5′ terminal region of the U1 snRNA, the exact location of the 5′ cleavage site of different mutants could be predicted and was experimentally confirmed. These findings add further strong support to the hypothesis (1) that the exact location of the 5′ cleavage site in pre-mRNA splicing of higher eukaryotes is determined by the overall 5′ splice region via the complementarity to the 5′ end of the U1 snRNA, and not by the strongly conserved GU dinucleotide.

INTRODUCTION

Intron sequences of higher eukaryotes are bounded by consensus sequences, (C/A)AG′GU(G/A)AUG at the 5′ and $(C/U)_{11}$NCAG′ at the 3′ splice site (2-4). Cleavage takes place immediately preceding the 5′ GU and following the 3′ AG, both of which are highly conserved. The splicing reaction proceeds in two stages. First, cleavage at the 5′ splice site occurs and the 5′ end of the intron is joined to the 2′ OH of an A residue of the branch region, 20 - 40 nucleotides upstream of the 3′ splice site. In the second step of the reaction, the 3′ OH of the upstream exon attacks the phosphodiester bond at the 3′ splice site, resulting in the joining of the exons and release of the lariat intron (for reviews, see 5,6).

It has been shown that several snRNP particles as well as non-snRNP factors are involved in the formation of the splicing complex, the spliceosome, (for reviews, see 7,8).

In particular, the complementarity of the 5′ end of the U1 snRNA to the 5′ splice region is essential for splice site recognition in vivo (9-11). We have recently proposed that this complementarity, and not the invariant GU dinucleotide determines the exact cleavage site within the 5′ splice region (1).

In this paper, we report on the analysis of different point mutations in the 5′ splice region of the large intron of the rabbit ß-globin gene. Our results confirm the finding that the complementarity of the 5′ end of the U1 snRNA to the 5′ splice region determines the exact cleavage site. However, we conclude that the selection of a 5′ splice region does not depend on this complementarity alone but is influenced by additional factors.

MATERIALS AND METHODS

All methods used are described (1,12).

RESULTS

We have described earlier (1) that the mutation 495 G-->T, which changes the invariant GT to TT (Figure 1) shifts the 5′ cleavage site upstream by one nucleotide (Figure 2a). Introduction of a second site mutation, namely 493 G-->A, reversed this shift. We postulated that the 5′ cleavage site is determined by the whole 5′ splice region via a base-pairing interaction with the 5′ end of the U1 snRNA (Figure 2a), perhaps as the phosphodiester bond opposite the C-C phosphodiester bond of the U1snRNA.

To explore this hypothesis we have constructed additional mutant 5′ splice regions and determined the resulting 5′ cleavage sites.

The Mutation 495 G-->C Leads to a Shift of the 5′ Cleavage Site Which Can Be Reversed by a Second Site Mutation

We have shown earlier that the mutation 495 G-->C leads to a shift of the 5′ cleavage site upstream by one nucleotide (1). We attributed this shift to a stronger base-pairing interaction between the 5′ splice region and the 5′ end of the U1 snRNA in a shifted frame (Figure 2a), in analogy to the situation with the mutation 495 G-->T (1). In order to test this hypothesis, we have introduced a second site mutation, namely 493 G-->A. This additional mutation increased the complementarity of the 5′ end of the U1 snRNA to the 5′ splice region in the original frame (-2.5 kcal) and reduced it in the shifted frame (+1.6 kcal) (Figure 2a).

In vitro splicing of the mutant 495 G-->C RNA yielded as major processed species the intermediates E2 and L_L-E3. In addition, formation of a small amount of the product E2E3 was observed (Figure 3a). Additional bands corresponding to cryptic splicing events were detected; a detailed analysis of the use of these cryptic splice sites is presented below. Introduction of the second site mutation 493 G-->A had two consequences: No fully spliced product E2E3 could be observed, instead, the two intermediates E2 and L_L-E3 accumulated (Figure 3a). The weak band corresponding to fully spliced product most probably resulted from cleavage at the cryptic 5′ splice site 498/499 (see below).

Primer extension analyses were performed in order to localize the 5′ cleavage site in the different mutated 5′ splice regions (Figure 4). Lariat product and lariat intermediate of wild-type yielded a 37 nucleotide cDNA, indicative of cleavage at the normal position, whereas a cDNA of 38 nucleotides showed that the 5′ cleavage site in the mutant 495 G-->C was shifted upstream by one nucleotide. This shift was reversed in the double mutant 493 G-->A/495 G-->C, as evidenced by the 37 nucleotide cDNA resulting from extension of the primer P10. Thus, this set of mutated 5′ splice regions confirmed our hypothesis that the cleavage site is determined by the complementarity of the whole 5′ splice region to the 5′ end of the U1 snRNA.

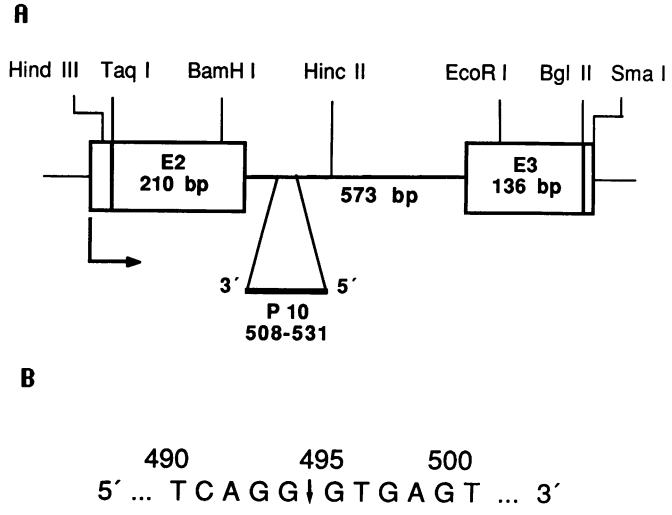


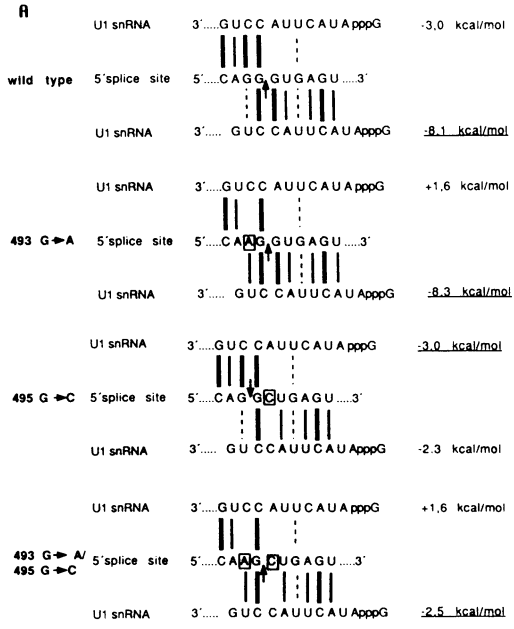
Figure 1 Partial Map of the Plasmid SP64 RchrßG (TaqI-BglII)
A) The TaqI-BglII fragment of the rabbit ß-globin gene (position 307 to 1196; 27) was cloned into the AccI - BamHI cleaved vector SP64 (22). Initiation of transcription by the SP6 polymerase is shown by an arrow. The location of the 24 nucleotide primer 10 is shown. Numbering is relative to the natural cap site of the rabbit ß-globin gene. The restriction sites relevant in this study are shown. B) The sequence of the 5' splice region of the large intron of the rabbit ß-globin gene. Numbering is relative to the natural cap site (27).

The 5′ Cleavage Site Can Be Predicted from the Complementarity of the 5′ Splice Region to the 5′ End of the U1 snRNA

As explained above, the mutation 495 G-->T led to an upstream shift of the 5′ cleavage site by one nucleotide. This shift could be reversed by the introduction of a second site mutation, namely 493 G-->A. We asked whether an appropriate third point mutation in the 5′ splice region would shift the 5′ cleavage site back to position -1. As shown in Figure 2b, the additional mutation 496 T-->A increased the complementarity of the 5′ end of the U1 snRNA to the 5′ splice region in a shifted frame (-1.3kcal) compared to the normal frame (-0.9kcal). We compared the splicing pattern of this triple mutant 493 G-->A/495 G-->T/496 T-->A to that of the relevant single or double mutants. As can be seen in Figure 3, wild-type and mutant 493 G-->A were spliced normally, however the single mutant 495 G-->T yielded spliced product E2E3, which resulted from the use of a cleavage site shifted upstream by one nucleotide (1 and below). In the case of the double mutant 493 G-->A/495 G-->T, accumulation of the intermediates E2 and L_L-E3 was detected. Weak bands corresponding to the spliced products E2E3 and L_L were observed, possibly as a result of cleavage at position -1 (see below). In the case of the triple mutant, overall splicing efficiency was reduced drastically, however, we observed bands corresponding to the

spliced products E2E3 and the lariat intron L_L , as well as the two intermediates E2 and L_L-E3. Additional bands with lower mobility than the normal lariat intermediate L_L-E_3 were attributed to lariat RNA originating from cleavage at cryptic splice sites, as in the case of the single mutant 496 G-->T. We did not try to assign each single band to a specific splicing event.

In order to determine the exact location of the 5´ cleavage site, primer extension was performed on the lariat product L_L and the lariat intermediate L_L-E3 (Figure 4). For wild-type and the two single mutants 493 G-->A and 496 T-->A, primer extension yielded a cDNA of 37 nucleotides, indicative of cleavage at the original site, whereas in the case of the mutant 495 G-->T, the major elongation product of 38 nucleotides confirmed the shifted cleavage site in this mutant. As expected, the double mutant 493 G-->A/495 G-->T was cleaved primarily at the original 5´ splice site (37 nucleotide extension product). A minor fraction of the RNA was cleaved at position -1, yielding a 38 nucleotide extension product. Cleavage at this position, in front of the GU dinucleotide would yield a lariat intermediate permissive for the second step of the splicing reaction. The fully spliced RNA detected for this mutant in Figure 3 probably results from cleavage at this position, since the major cleavage product would not allow exon joining, due to incorrect branch structure (1,13). The analysis of the triple mutant 493 G-->A/495 G-->T/496 T-->A yielded a 38 nucleotide cDNA. We concluded that the 5´ cleavage site in this mutant was at position -1 relative to the original cleavage site.



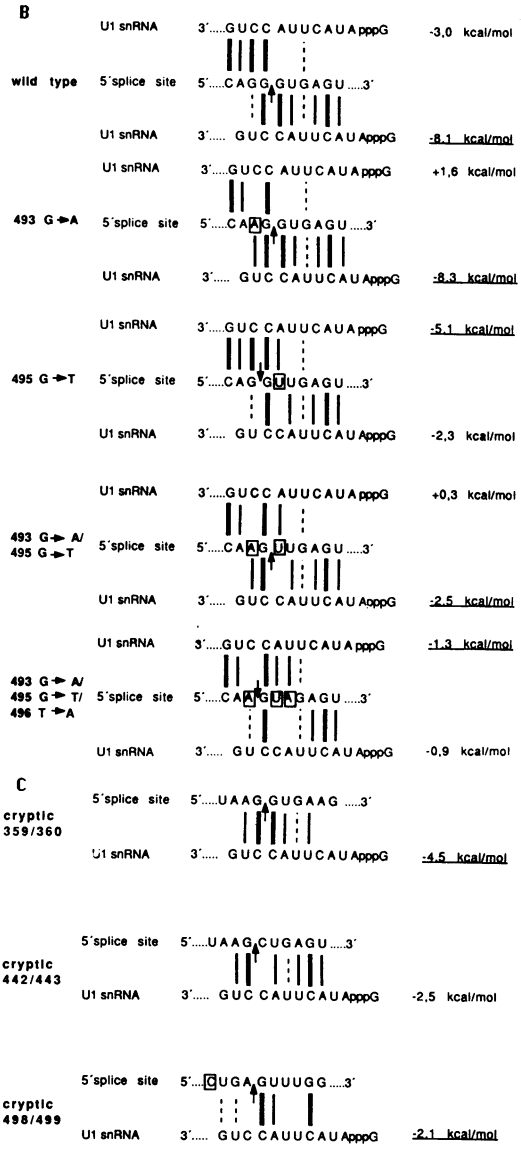


Figure 2 Scheme of U1 snRNA-5′ Splice Region Interaction
A and B) The base-pairing of the 5′ splice region of wild type and the different mutant RNAs is shown in the normal (lower) and the shifted (upper) frame. The mutated positions of the 5′ splice region are indicated. The experimentally determined 5′ cleavage site in each mutant is indicated by an arrow. G-C base pairing is indicated by thick bars, A-U base pairing by thin bars, and G-U base pairing by dashed bars. The free energy ΔG (calculated according to 26) for the U1 snRNA - 5′ splice site interactions are given. The ΔG values for the interaction proposed to be responsible for cleavage site determination are underlined. C) The base pairing of cryptic 5' splice regions and the 5' terminus of the U1 snRNA.

Activation of Cryptic Splice Sites is Dependent on the Sequence of the Original 5′ Splice Site

It is evident from the direct analysis of the splicing products of the different mutants that some mutations lead to the activation of cryptic 5′ splice sites (Figure 3). In order to localize these cryptic 5′ splice sites more precisely, S1 analysis was performed on the reaction products from the in vitro splicing reactions. A HindIII - EcoRI fragment, 3′ end-labeled at the HindIII site was used as a probe (Figure 5a). We prepared a specific probe for each mutant RNA, in order to avoid mismatches at the mutated positions. Figure 5a summarizes the expected products and the corresponding 5′ splice sites. The size differences of the protected fragments corresponding to cleavage at the original 5′ splice site between the wild-type and the different mutants were attributed to differences at the 3′ end of the RNA-DNA duplexes and altered S1 nuclease cleavage. We did not detect any cryptic 5′ splice sites other than those previously described (12,14). In this experiment we were not able to differentiate between cleavage at the normal 5′ cleavage site and cleavage at the -1 position (for the mutants 495 G-->T, 495 G-->C and 493 G-->A/495 G-->T/496 T-->A). Cleavage at the cryptic 5′ splice site 359/360 was expected to yield an S1 signal of 67 nucleotides, however, we observed a series of bands around this region, most probably due to nibbling by the S1 nuclease.

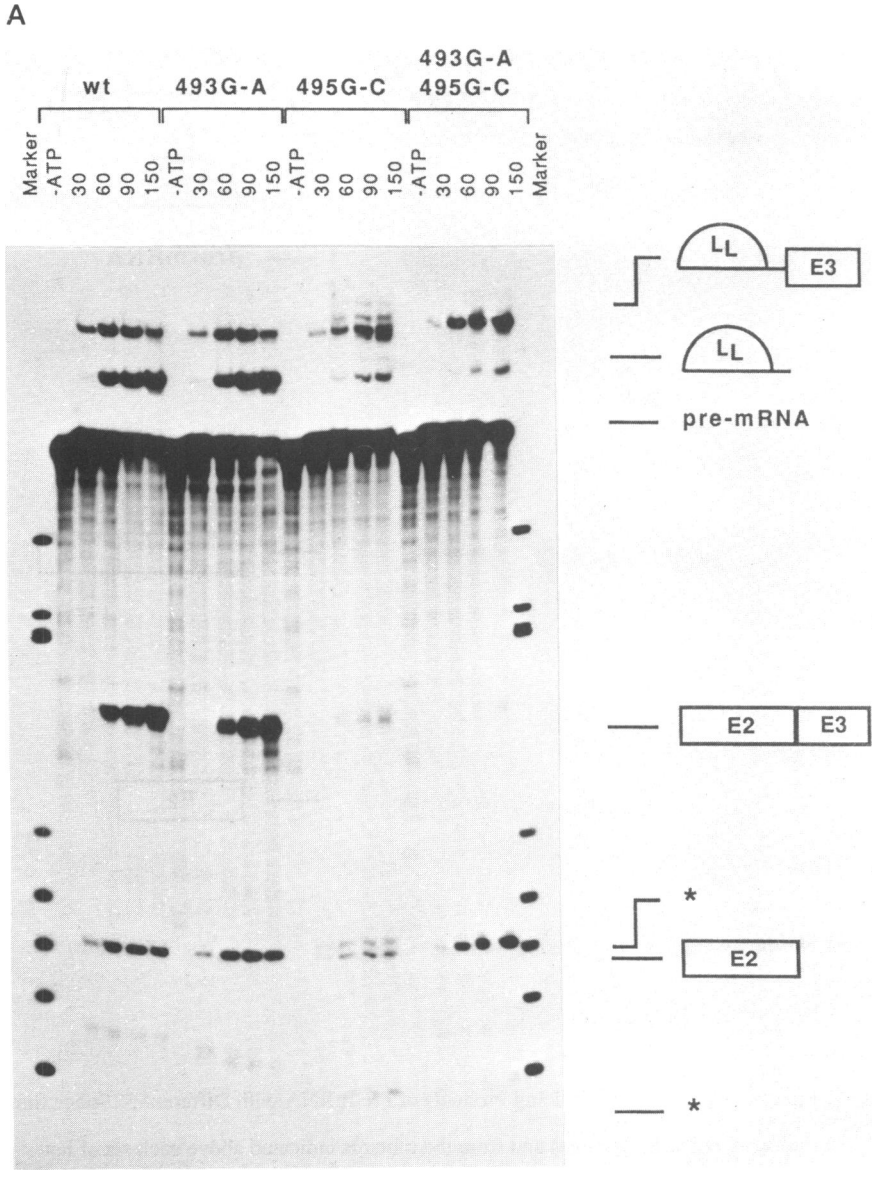
It is evident from Figure 5b that the activation of the different cryptic 5′ splice sites is dependent upon the sequence of the original 5′ splice site. The cryptic 5′ splice sites at positions 442/443 and 359/360 are activated the most in the case of the mutants 495 G-->C, 496 T-->A, 493 G-->A/495 G-->T/496 T-->A and 493 G-->A/495 G-->C: the less efficiently the original 5′ splice site is used, the more these cryptic sites are activated (Table 1). The cryptic splice site at position 498/499 is very prominent in the case of the mutant 495 G-->C, is weakly detected in the mutant 493 G-->A/495 G-->C but is absent in all other mutants where an activation of cryptic 5′ splice sites can be detected. We conclude that the activation of this cryptic splice site is associated with the mutation 495 G-->C.

DISCUSSION

The 5′ Cleavage Site Can be Predicted from the Complementarity of the 5′ Splice Region to the 5′ End of the U1 snRNA

The 5′ splice site consensus sequence (2-4) shows a striking complementarity to the 5′ terminal sequence of the U1 snRNA (15,16). There is strong evidence that this RNA (9,11,17,18) as well as the U1 snRNP (19-25) plays an essential role in the splicing process. We have further tested the hypothesis that the 5′ cleavage site is determined by the complementarity of the overall 5′ splice region to the 5′ end of the U1 snRNA (1). Based on the calculated values for the free energy ΔG of base-pairing (26) between the 5′ splice region (position 491 to 500; 27) and the 5 end of the U1 snRNA (position 3 to 11; 28), mutated 5′ splice regions were designed to lead either to a shift of the 5′ cleavage site or to reverse it (Table 1). The two new sets of mutated 5′ splice regions reported in this paper followed the predicted cleavage pattern, as did the previously

reported set of mutations, namely 495 G-->T and the double mutant 493 G-->A/495 G-->T (1). These results confirm our previous conclusion that the cleavage site is not critically determined by the GPy sequence, that precedes all natural 5´ cleavage sites (2-4), but by the whole 5´ splice region. In the case of the triple mutant 493 G-->A/495 G-->T/496 T-->A we have calculated a difference in the free energy ΔG of 0.4 kcal between the shifted (-1.3 kcal) and the normal (-0.9



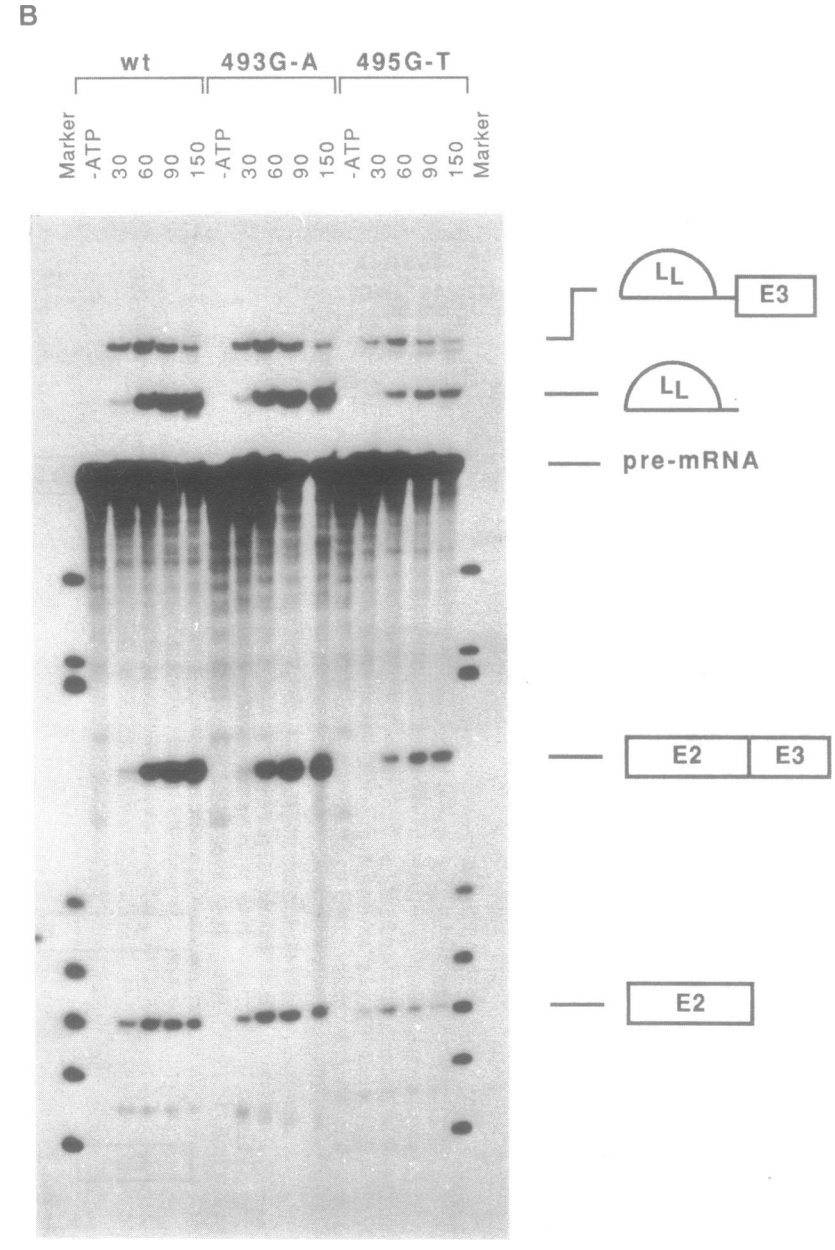
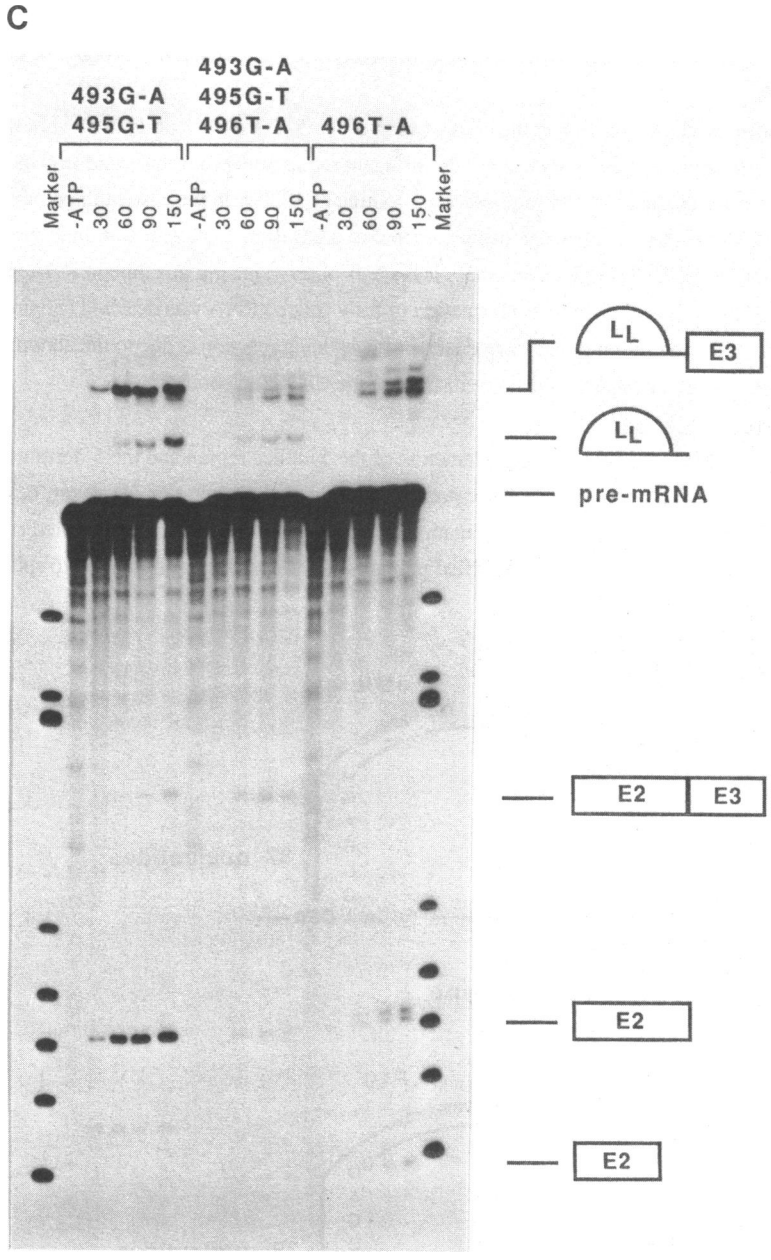


Figure 3 Analysis if the In Vitro Splicing Products of Pre-mRNA with Different 5´ Splice Site Mutations
A-C: Pre-mRNA from wild type (wt) and from the mutants indicated above each set of lanes was incubated in HeLa nuclear extract for the times indicated and analyzed on a 3.2% polyacrylamide



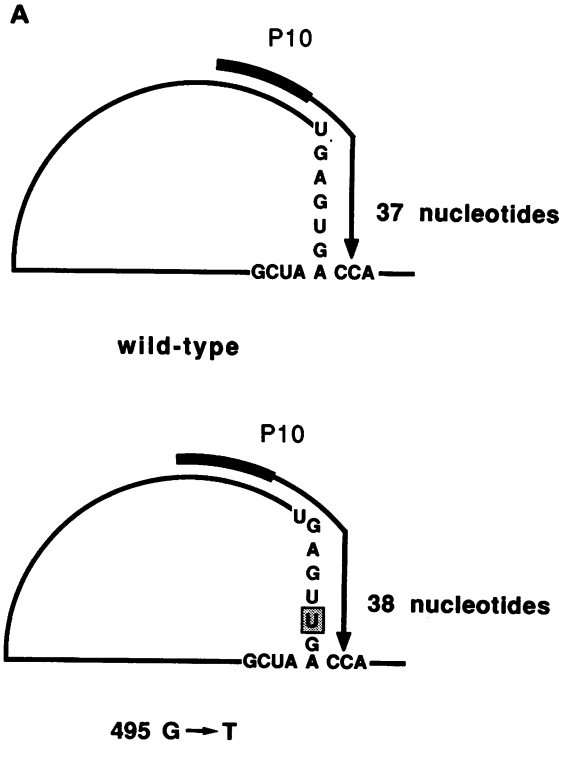
gel. -ATP indicates omission of ATP and creatine phosphate from the splicing reaction. * designates exon 2 arising from cleavage at cryptic 5' splice sites. Marker: 5'-^{32}P-labeled BspI-digested pBR327.

kcal) base-pairing interaction (Figure 2). Even though this seems to be a small difference in absolute terms, we observe cleavage almost exclusively at the energetically favoured -1 position (Figure 4b).

We have observed previously that the conservation of the 5′ GT dinucleotide in most of the naturally occurring 5′ splice sites reflects the stringent requirement for the second step of the splicing reaction, namely 3′ cleavage and exon joining (1,13,29). In the case of the double mutant 493 G-->A/495 G-->C, 5′ cleavage occurs in front of a CU dinucleotide, resulting in the branch structure A(2′-5′)CU. Though efficient 5′ cleavage is observed using this mutant RNA as substrate in the splicing reaction, no formation of fully spliced RNA was detected (Figure 3). We conclude that the inhibition of the second step of the splicing reaction is due to the abnormal branch structure resulting from cleavage in front of the CU dinucleotide.

Selection of the 5′ Splice Site

As discussed above, the base-pairing interaction of the 5′ splice region and the 5′ terminal sequences of the U1 snRNA allowed the prediction of the 5′ cleavage site. However, beside the shift in 5′ cleavage site, several of the mutations tested led to the activation of so called cryptic 5′ splice sites. These sites have been identified previously by the analysis of the in vivo splicing



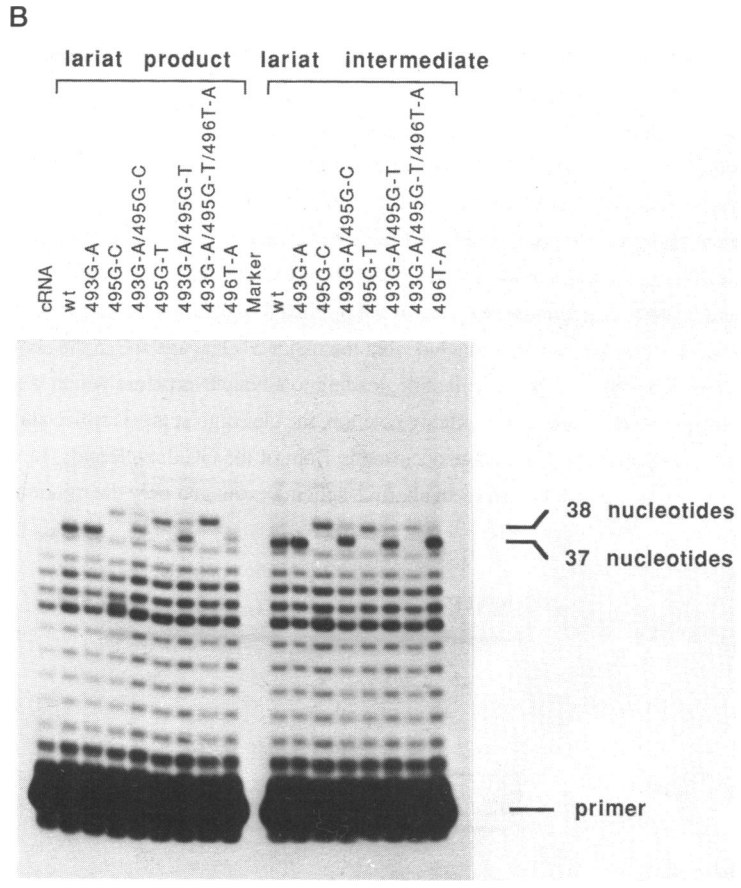
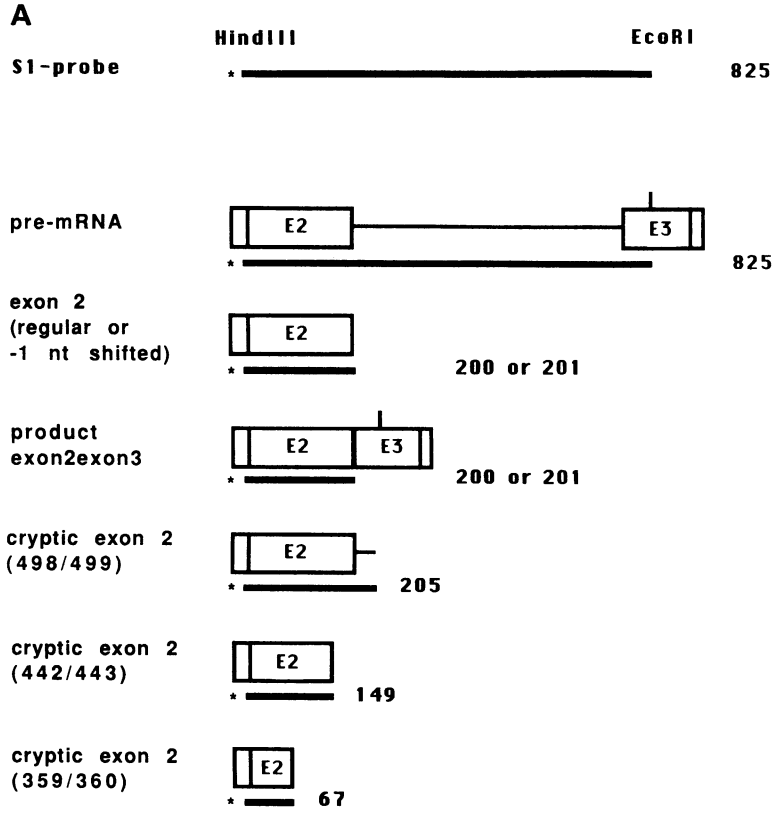


Figure 4 Primer Extension Analysis of Lariat Introns Resulting from In Vitro Splicing of Different 5´ Splice Site Mutant Pre-mRNAs
A) Lariat introns yield a 37 nucleotide extension product if 5´ cleavage ocured at the normal position, whereas 5´ cleavage at position -1 results in a 38 nucleotide extension product, as indicated in the case of the mutant 495 G-->T. B) ^{32}P-labeld pre-mRNA was spliced in vitro for 2 hr. Lariat intron ans lariat intermediate were isolated by PAGE and 2.5 fmol was used as template for reverse transcription, with ^{32}P 5´-labeled oligonucleotide primer 10 (see Figure 1). The yeast carrier RNA yielded a background of extension products in all lanes. Marker: ^{32}P 5´-endlabeled oligonucleotides, 34 and 45 nucleotides in length, visible after a longer exposure (not shown).

products of a 5´ splice site mutant (14). The cryptic 5´ splice site at position 498/499 (Figure 2) was detected only in conjunction with the G-->C transversion at position 495 (Figure 3 and Figure 5). The complementarity of this cryptic 5´ splice site to the 5´ end of the U1 snRNA reveals that the mutant C residue is located at position -4 relative to the cryptic splice site. This location allows base pairing interaction to the G at position 12 of the U1 snRNA. Although the

position -4 of the 5′ splice region is not conserved in the natural occuring 5′ splice sites, we propose that activation of this cryptic 5′ splice site is due to this increased complementarity and therefore restricted to this particular mutation.

The sequence 5′...A A G C U G A G U....3′ (position 441 to 448), previously recognized as a cryptic 5′ splice site (14), shows good complementarity to the 5′ end of the U1 snRNA (Figure 2c). However, the cleavage site would be predicted to be in front of the C nucleotide, in contrast to the cleavage found in vivo, in front of the first G nucleotide, which was deduced by sequencing cDNA of the appropriate mRNA. We did not localize the in vitro cleavage site at this splice site exactly, but the identical sequence at the original 5′ splice site led to a cleavage in front of the C nucleotide (see below). We conclude that the major 5′ cleavage site in the above cryptic sequence occurred in front of the C nucleotide, leading to a branch structure which is not permissive for the second step of the splicing reaction, the cleavage at the 3′ splice site and exon joining (1,13) and the minor 5′ cleavage occurred in front of the GC dinucleotide. In vivo, cleavage at the major site would lead to an abortive splicing event and only the minor cleavage in



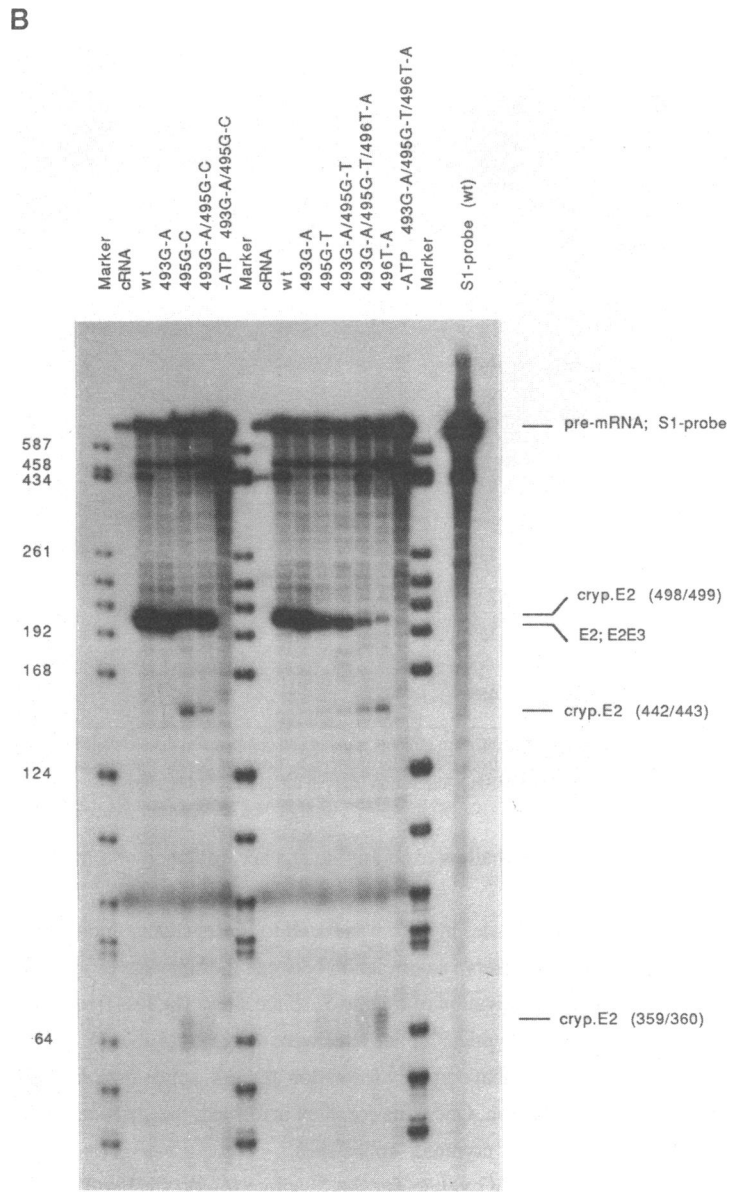


Figure 5 S1-Mapping of In Vitro Splicing Products.
A) Scheme of different splicing products and the corresponding signals after S1-mapping. The length of the signals is given in number of nucleotides.
B) ^{32}P-labeled pre-mRNA from wild type (wt) and the different mutants indicated above each lane was spliced in vitro for 2 hr and the splicing products were used for S1-mapping. For each mutant, the corresponding ^{32}P 3´-endlabeled HindIII*-EcoRI fragment was used. Marker: 5´-^{32}P-labeled, BspI digested pBR327.

Table 1: Summary of the Effects of Several Point Mutations in the 5´Splice Region

RNA	5´splice site	overall 5´cleavage efficiency relative to wild type	5´cleavage occurred at position: original 5´splice region normal	original 5´splice region shifted -1	cryptic 498/499 ΔG = -2.1 kcal	cryptic 442/443 ΔG = -2.5 kcal	cryptic 359/360 ΔG = -4.5 kcal
wild type	AGG·GUGAGU ΔG = -8.1 kcal	100%	++++	-	-	-	-
493G-A	AAG·GUGAGU ΔG = -8.3 kcal	118%	++++	-	-	-	-
495G-C	CAG·GCUGAG ΔG = -3.0 kcal	44%	-	++	++	++	++
493G-A/ 495G-C	AAG·CUGAGU ΔG = -2.5 kcal	61%	++++	±	±	+	+
495G-T	CAG·GUUGAGU ΔG = -5.1 kcal	64%	-	+++	-	±	±
493G-A/ 495G-T	AAG·UUGAGU ΔG = -2.5 kcal	58%	+++	±	-	±	±
493G-A/ 495G-T/ 496T-A	CAA·GUAGAGU ΔG = -1.3 kcal	19%	-	++	-	+	+
496T-A	AGG·GAGAGU ΔG = -4.5kcal	45%	++	±	-	++	++

The relative 5´cleavage efficiency of the different mutants is given and compared to the cleavage efficiency at cryptic 5´ splice sites. Arbitrary units calculated from the results obtained by direct or S1 analysis are used.The calculated ΔG-value for the 5´ splice region - U1 snRNA interaction is given for each 5´ splice site.

front of the GC dinucleotide would allow exon joining and accumulation of a mRNA-like product would therefore be detected.

The activation of cryptic 5´ splice sites in vitro was found to be dependent upon the sequence at the normal 5´ splice region. We observe a correlation between the cleavage efficiency in the normal 5´ splice region and the activation of cryptic 5´ splice sites: The less frequently normal cleavage occurred, the more the cryptic 5´ splice sites were activated (Table 1). This activation is best explained by the assumption that complex formation at the 3´ splice region (23,30) is the driving force in the splicing reaction. Once this complex is formed, the absence of the normal 5´ splice site leads to the activation of cryptic 5´ splice sites.

As outlined above, the calculated ΔG values for the 5´ splice region - U1 snRNA interaction allowed a prediction of the 5´ cleavage site in the normal 5´ splice region. However, these ΔG values do not explain the activation of cryptic 5´ splice sites: We observe a similar or even lower ΔG value for the cryptic 5´ splice regions compared to the original, albeit mutated site. Nevertheless, cleavage at the original 5´ splice site was favored over that at the cryptic 5´ splice sites (Table 1). This observation is of special importance in the case of the double mutant 493

G-->A/495 G-->C: These mutations converted the original splice region to 5'...A A G C U G A G U...3', a sequence which duplicates the one present in position 441 to 448, previously identified as a cryptic 5' splice site. However, 5' cleavage in this mutant RNA occurred preferentially in the original 5' splice region. The preferential use of the same sequence located at the normal 5' splice site rather than the cryptic site may be explained by the findings of Reed and Maniatis (31). These authors have shown that exon sequences may influence the selection of splice sites. In addition, they observed that in the case of duplicated 5' splice sites, the one closest to the 3' splice site was used preferentially. Irrespective of the explanation, we observe that the calculated ΔG values for the two cryptic 5' splice sites do not reflect the efficiency of cleavage these positions.

In the course of the spliceosome formation, U1 snRNP binding to the 5' splice region is one of the first steps observed (25). This binding of the U1 snRNP leads to a protection of the 5' splice region against RNAase T1 (24). The interaction of U1 snRNP with the U2 and/or U2, U4, U5, U6 snRNPs leading to the active, 60S spliceosome changes the protected region around the 5' splice site (24). This can be explained by an altered conformation of the U1 snRNP (25). Based on the results presented above, we postulate, that the base-pairing interaction of the 5' splice region and the 5' terminal sequences of the U1 snRNA is essential for determining the exact location of the 5' cleavage site and takes place in the active 60S spliceosome complex. However, for the initial binding of the U1 snRNP, this complementarity may be of minor importance and other factors, such as exon sequences or the tertiary structure of the pre-mRNA may influence the attachment of the U1 snRNP to a particular 5' splice region.

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*Present address: California Institute of Technology, Division of Biology 147-75, Pasadena, CA 91125, USA

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