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Genetics

UACUAAC is the preferred branch site for mammalian mRNA splicing

(alternative splicing/U2 small nuclear RNA)

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ABSTRACT **The conserved branch-site sequence UACUAAC is known to form base pairs with the complementary sequence GUAGUA in U2 small nuclear RNA (snRNA) during mRNA splicing in the yeast *Saccharomyces cerevisiae*. Although the GUAGUA element is conserved in mammalian U2 snRNA, mammalian branch sites conform only weakly to a YURAC consensus and can even be deleted without obvious effects on the efficiency of splicing *in vivo*. To understand why the GUAGUA element of U2 is conserved in evolution but the branch site is not, we have devised two different competitive assays for branch-site selection using the first intron of the human β-globin gene. We find that a sequence resembling UACUAAC is the most efficient branch site for mammalian mRNA splicing both *in vivo* and *in vitro*. Our results suggest that in mammals (*i*) U2 snRNA can form base pairs with the branch site and (*ii*) the interaction between U2 and the branch site can be augmented or replaced by an interaction between the spliceosome and some other element of the intron or exons, perhaps the conserved polypyrimidine tract located immediately upstream from the 3′ splice site.**

Although the general features of nuclear mRNA splicing have been conserved through evolution, there are many subtle differences between mRNA splicing in the lower eukaryotic yeast *Saccharomyces cerevisiae* and in mammals. One of the most puzzling differences is that the branch-site sequence UACUAA*C is invariant in *S. cerevisiae* but exhibits only a weak consensus YURA*C in mammals (*, "branchpoint" residue; Y, pyrimidine; R, purine). In fact, deletion or mutation of the normal mammalian branchpoint does not abolish splicing but rather leads to the use of cryptic branchpoints having little, if any, match to the consensus (1, 2). Equally surprising is the observation that the mammalian splicing apparatus will resort to using a cytidine or uridine residue as the branchpoint if the region immediately upstream from the 3′ splice site is devoid of adenosine residues (3).

The UACUAAC element in yeast has been shown by elegant genetic experiments to form base pairs with the sequence element GUAGUA in yeast U2 small nuclear RNA (snRNA) (4). This base-pairing interaction is thought to bulge the branchpoint adenosine in the mRNA precursor, thereby enabling the 2′-hydroxyl group of the adenosine to attack the 5′ splice junction in the initial step of splicing. A bulged branchpoint adenosine is also found in group II autocatalytic introns (5), which may have given rise to the modern mRNA splicing apparatus (6, 7). Because the first 100 nucleotides of yeast and mammalian U2 snRNA are highly conserved (8–10), including the GUAGUA sequence that recognizes the branch-site UACUAAC sequence in yeast, an apparent paradox arises. Why is the GUAGUA element in U2 snRNA conserved from yeast to mammals, while the branch site in the mRNA precursor is not? To resolve this paradox and to examine the sequence specificity of mammalian branch-site selection in detail, we have devised two different competition assays for branch-site selection. Both assays are based on the human β-globin first intron; one assay involves competition between identical duplicated 3′ splice sites, and the other involves competition between duplicated branch sites within a single intron. We find that the mammalian splicing apparatus prefers the branch site with the better match to UACUAAC both *in vivo* and *in vitro*, and, in particular, UACUAAC is a more efficient branch site than the natural human β-globin branch-site sequence CACUGAC.

MATERIALS AND METHODS

The parental construct for all recombinant constructions was the *Hin*dIII–*Bam*HI fragment of human β-globin excised from pSP64-Δ6 (11). For convenience, the cytidine residue at position 210 of the human β-globin gene was deleted to create a *Bgl* II site. The *Hin*dIII–*Bam*HI fragment containing the new *Bgl* II site was cloned into the M13 vector mp9 for site-directed mutagenesis (12) to generate branch-site mutants (Table 1). To generate duplication constructs with tandemly repeated 3′ splice sites for expression assays *in vivo*, a globin *Hin*dIII–*Bam*HI fragment was cloned between the *Hin*dIII and *Bcl* I sites of a simian virus 40 vector (13), and the *Hin*dIII–*Bgl* II fragment of this clone was then replaced with the globin *Hin*dIII–*Bam*HI fragment (Fig. 1*A*). Conditions for transfection, preparation of cellular RNA, and the S1 nuclease assay were as described (14). For *in vitro*-splicing assays, globin-duplication constructs were built between the *Hin*dIII and *Bam*HI sites of pSP64 using the same cloning strategy employed above to construct duplications between the *Hin*dIII and *Bgl* II sites of the simian virus 40 vector. Transcription of these templates with SP6 RNA polymerase and conditions for *in vitro* splicing have been described (13). "Debranching" reactions were as described (15).

RESULTS

To examine the sequence specificity of mammalian branch-site selection, we made a series of point mutations in the natural branch site of intron 1 of the human β-globin gene and tested the effect of these mutations on splicing both *in vivo* and *in vitro*. This intron was chosen because the natural CACUGAC branch site of the human β-globin intron 1 not only matches the mammalian consensus YURAC exactly but also resembles the yeast UACUAAC sequence (Fig. 1*B*); thus, there was reason to suspect that splicing of this intron might be unusually dependent on the quality of the branch site.

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Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle.

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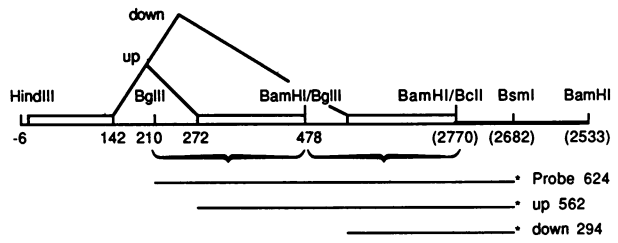
Table 1. Mutations in the normal and cryptic branch sites of the first intron of the human β-globin gene

Branch site	Sequence
1 (normal human)	CACUGAC
0	UACUGAC
2	UAGUGAC
4	UACGGAC
5	UUCUGAC
10 (yeast-like)	UACUAAC
11	UAUUAAC
12	UAGUAAC
13	UACGAAC
14	UACAAAC
15	UACCAAC
w (cryptic)	UGCCUAU
a	UGCUGAC
c	UGCGGAC
d	UGGGGAC
Mammalian consensus	YURAC

All mutants were generated by oligonucleotide-directed mutagenesis as previously described. Positions that match the yeast UACUAAC sequence in each branch-site sequence are in boldface. In the mammalian consensus Y = pyrimidine and R = purine. The normal and cryptic branch sites are indicated in Fig. 1.

Competitive Assay for Branch-Site Selection Using Alternative 3′ Splice Sites. For convenience, we first introduced a *Bgl* II site into intron 1 by deleting a single cytidine residue 24 nucleotides upstream from the natural branch site (position 210; Fig. 1*B*). We then generated duplication constructs with alternative 3′ splice sites by duplicating a 268-base-pair (bp) *Bgl* II–*Bam*HI fragment spanning the natural branch site of intron 1 and most of exon 2 (Fig. 1*A*). Our hope was that small changes in the relative efficiency of either 3′ splice site would cause much larger changes in the observed pattern of alternative 3′ splicing. A similar competition assay using alternative 5′ splice sites had previously enabled us to test for suppressor U1 snRNAs (16), and others have used the same strategy to examine the role of exon sequences in splicing *in vitro* (17).

A



B

agaaactgggcatgtggagacagagaagactcttgggtttctgatag
210

gcactgactctctctgcctattggtctattttcccacccttagGCTG
272

FIG. 1. (*A*, *Upper*) Duplication constructs are based on the human β-globin gene and contain a tandem duplication of the 3′ splice region of the first intron from position 210 to position 478. Splicing of the single 5′ splice site to either of the two alternative 3′ splice sites is indicated (up or down). All β-globin nucleotides are numbered relative to the transcriptional initiation site at position 1; simian virus 40 sequences are numbered in parentheses according to the standard map. Open boxes represent exon sequences, light lines represent β-globin sequences, heavy lines represent simian virus 40 vector sequences, and brackets represent the duplicated region. Note that neither of the hybrid restriction sites (*Bam*HI/*Bgl* II or *Bam*HI/*Bcl* I) represents a 5′ splice site. (*A*, *Lower*) Lengths of the S1 nuclease probe and protected fragments expected for splicing to the upstream or downstream 3′ splice site. (*B*) Sequence of the region surrounding the 3′ splice site of intron 1 of the human β-globin gene. Both the normal and cryptic branch sites are underlined. ★, Normal branchpoint; (★), cryptic branchpoint. Exon sequences are capitalized. To facilitate construction of the test genes described, the cytidine residue at position 210 was deleted to create a *Bgl* II site.

Competitive Assay *in Vivo*. For maximal expression *in vivo*, duplication constructs containing various combinations of normal and mutant 3′ splice regions (Table 1) were cloned into a simian virus 40 expression vector between the simian virus 40 early promoter and polyadenylylation signals. The constructs were introduced into HeLa cells by transfection, and relative use of the alternative 3′ splice sites was determined 48 hr later by S1 nuclease assay on total cytoplasmic RNA (Fig. 2). Using UACUAAC as the reference branch site at the downstream position, we varied the upstream branch site (Fig. 2*A*). All changes that reduced the match of the upstream branch site to UACUAAC, including the natural branch site (lane 1,10), also decreased use of the upstream 3′ splice site relative to the control (lane 10,10) with UACUAAC at both the upstream and downstream branch sites. In addition, the behavior of mutant branch sites supported the

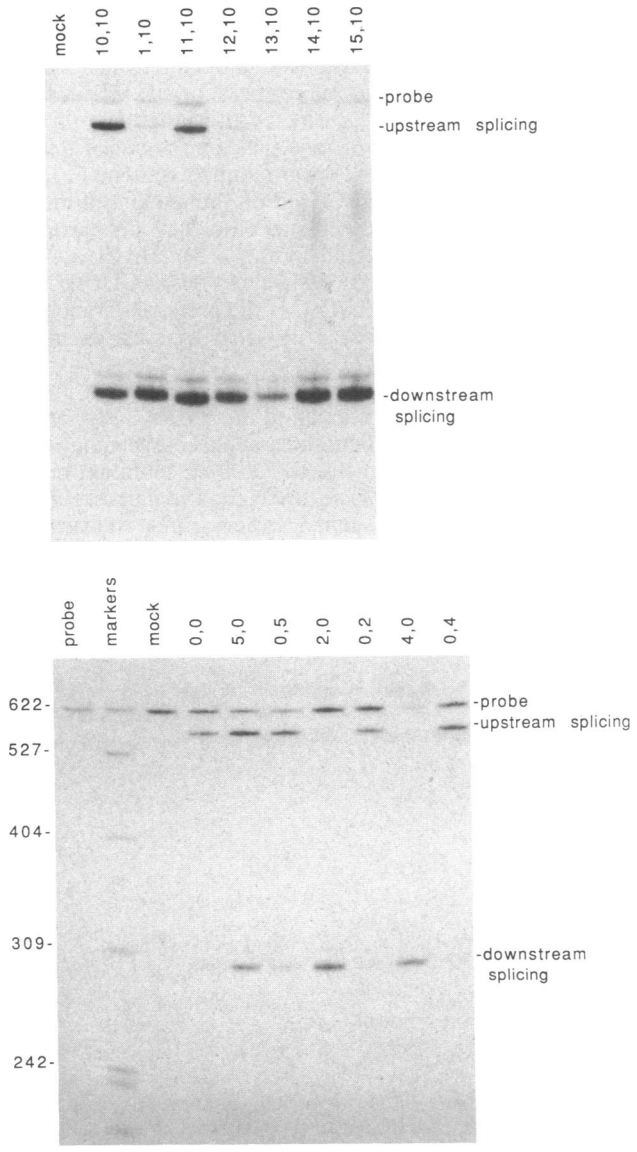


FIG. 2. Splicing of duplication constructs *in vivo* as determined by S1 nuclease assay. Upstream and downstream branch sites are indicated above each lane; for example, 1,10 indicates branch site 1 upstream and branch site 10 downstream (Table 1). Markers, *Hpa* II digest of pBR322; mock, transfection without DNA.

proposed mammalian branch site consensus YURAC (18, 19); a cytidine to uridine transition (lane 11,10) has less severe effects than a cytidine to guanosine transversion (lane 12,10) at the first position of the consensus, whereas any change from the conserved uridine at the second position (lanes 13,10; 14,10; and 15,10) affected splicing dramatically. To control for position effects, we exchanged the position of several mutants between the upstream and downstream 3′ splice sites (Fig. 2*B*). Two mutants behaved identically at either site (compare lane 2,0 with lane 0,2 and lane 4,0 with lane 0,4), but the effect of a third mutant was reduced at the upstream site (compare lane 5,0 with lane 0,5).

We do not understand why some pairs of mutants exhibit a position effect and others do not. Equally puzzling is the observation that the upstream branch site is preferred when UACUGAC is present at both sites (Fig. 2*B*, lane 0,0) but not when UACUAAC is present at both sites (Fig. 2*A*, lane 10, 10), although these two branch-site mutants function with nearly equal efficiency in the noncompetitive assay *in vitro* described below (Fig. 3).

Noncompetitive Assay *in Vitro*. To understand the basis for branch-site selection in the competitive assay *in vivo*, we initially examined the kinetics of *in vitro* splicing using a noncompetitive assay. Human β-globin constructs containing only a single 3′ splice site were cloned into the plasmid vector pSP64, and capped, uniformly labeled, splicing substrates were synthesized *in vitro* by transcription with bacteriophage SP6 RNA polymerase. The time course of splicing in a HeLa nuclear extract (20) was monitored after 0.5, 1, and 2 hr of incubation (Fig. 3). Based on the amount of mature product after 0.5 hr, branch-site efficiency decreases by about a factor of 10 overall in the order UACUAAC > UACUGAC ≅ CACUGAC (wild type) ≅ UAGUGAC >> UACGGAC. Thus, UACUAAC is the preferred branch site in the noncompetitive assay *in vitro* as well as in the competitive assay *in vivo*.

Consistent with the observation that mammalian introns frequently lack a credible match to the YURAC consensus, none of the branch-site mutations abolished splicing; however, the severe effect of the UACGGAC mutation on the rate of *in vitro* splicing was unexpected. The mutation at the fourth nucleotide of this sequence represents a transversion relative to both UACUAAC and the mammalian consensus YURAC. The unusual intron lariat seen for this mutant (Figs. 3 and 5) suggests that the same cryptic branch site is used as when the natural branch site is completely deleted (1).

Competitive Assay *in Vitro*. We asked whether the natural branch site of the β-globin first intron (branch site 1, CACUGAC) could compete *in vitro* with the most severe mutant (branch site 4, UACGGAC). Duplication constructs (Fig. 1*A*) corresponding to three different combinations of the natural and mutant branch sites were cloned into pSP64. Capped, uniformly labeled, transcripts generated by run-off transcription with SP6 RNA polymerase were then incubated in a HeLa nuclear extract. We observed little, if any, competition *in vitro* (Fig. 4). Instead, the upstream 3′ splice site was always used preferentially, regardless of whether the upstream branch site was efficient or defective in the noncompetitive *in vitro* assay (Fig. 3). This result implies that recognition of the branch site is not the primary determinant of 3′ splice-site use *in vitro*. Moreover, use of the natural branch site at the downstream position was almost unaffected by mutations in the upstream branch site, suggesting that the duplicated 3′ splice sites functioned independently *in vitro*. Differences between competition *in vivo* and *in vitro* could also be due to the structure of the substrate; the downstream 3′ splice site is followed by only 206 nucleotides of exon 2 in the *in vitro* transcripts but by 391 nucleotides in the *in vivo* transcripts ending at the simian virus 40 early polyadenylylation site (Fig. 1*A*, nucleotide 2586).

An Alternative Assay for Branch-Site Selection *in Vitro*. Deletion of the normal branch point of human β-globin intron 1 (Fig. 1*B*, asterisk) is known to activate a cryptic downstream branch site (Fig. 1*B*, asterisk in parentheses) (1). This suggested to us that two potential branch sites within a single intron might compete with each other. We therefore made a series of mutations in the normal and cryptic branch sites of human β-globin intron 1 (Fig. 1*B*, underlined nucleotides) and assayed splicing *in vitro*. We were able to determine which of the two competing branch sites was used because the mobility of an intron lariat is strongly dependent on the actual

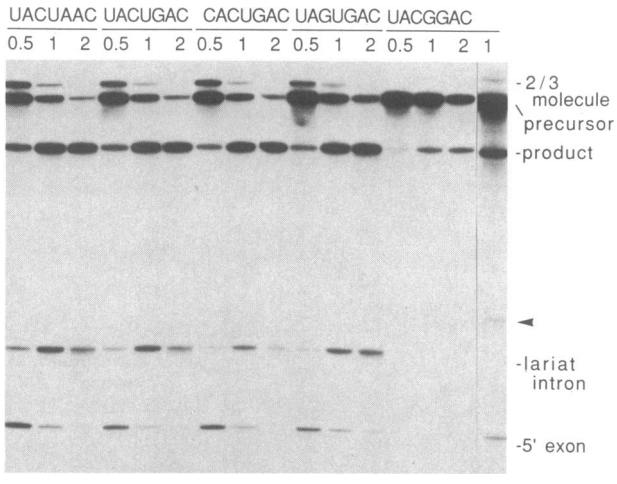


FIG. 3. Time course of *in vitro* splicing of human β-globin intron 1 containing mutations at the normal branch site. The arrow indicates where the intron lariat presumably formed at the cryptic branch site (1) is visible on longer exposure of the autoradiogram (far right lane); this lariat yields a linear intron of the expected length after debranching (data not shown). Each set of three lanes corresponds to 0.5-, 1-, and 2-hr splicing reactions. Products were resolved by electrophoresis through an 8% denaturing polyacrylamide gel.

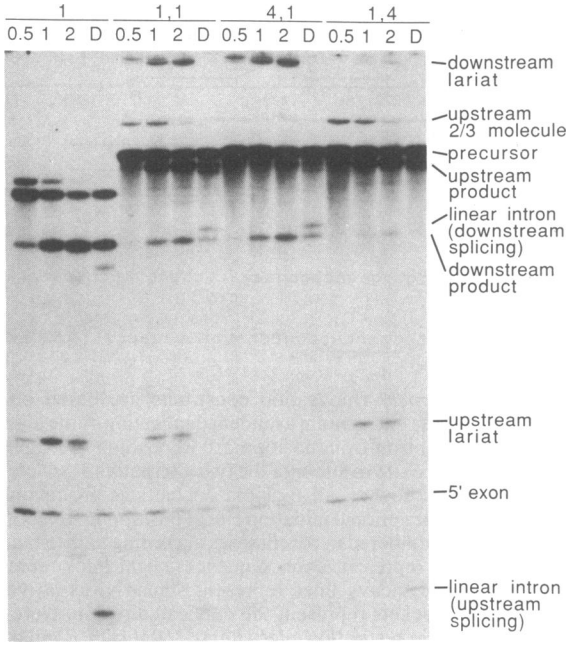


FIG. 4. Time course of *in vitro* splicing of duplication constructs. Upstream and downstream branch sites in the duplication constructs are designated as for Fig. 2. The splicing products derived from the parental unduplicated β-globin construct are shown as size markers (far left set of four lanes labeled 1). Products were resolved by electrophoresis through an 8% denaturing polyacrylamide gel. D, debranched products after a 1-hr splicing reaction.

branch site (Fig. 5). We found that the proximal branch site is preferentially used when both branch sites match the mammalian consensus (Fig. 5, lanes 0,a) or when the match of the upstream site to the consensus is decreased (Fig. 5, lanes 14,a and 15,a). The distal branch site is used, however, when the match of the proximal site to the consensus is decreased (Fig. 5, lanes 0,c and 0,d). This result demonstrates that match to the mammalian consensus YURAC can be a major determinant of splice-site selection when the competing branch sites are both an acceptable distance (3) from the functional 3′ splice site.

DISCUSSION

We have shown using two different competitive assays that the sequence UACUAAC is the preferred branch site for mammalian mRNA splicing both *in vitro* and *in vivo*. In particular, UACUAAC is a better branch site than the normal human β-globin branch site CACUGAC, even though both branch sites match the mammalian consensus YURAC.

In the yeast *S. cerevisiae*, the invariant UACUAAC branch site is known to form base pairs with the complementary sequence GUAGUA in U2 snRNA during mRNA splicing (4). Although the 5′ 100 nucleotides of U2 snRNA (including the GUAGUA sequence) are highly conserved from yeast to mammals (8–10), mammalian branch sites exhibit only a very weak consensus YURAC (3, 18, 19) and can even be deleted with apparent impunity (1, 2, 21). The obvious similarity between the mammalian branch-site consensus YURAC and the invariant yeast sequence UACUAAC suggested that mammalian U2 also might form base pairs with the branch site at some step in splicing; however, the branch-site deletion data implies that such base pairing is often weak or perhaps even optional. Consistent with this point of view, weak base pairing between the branch site and an extended region of U2 snRNA surrounding the GUAGUA element has been proposed to explain the use of nonconsensus branch sites in mammals (3).

Our demonstration that UACUAAC is the preferred mammalian branch site argues strongly that in mammals, as in yeast, U2 snRNA can recognize the branch site but that the criteria for recognition are relaxed. We realize, however, that two very different assays have been used to demonstrate an early step in yeast spliceosome assembly that precedes U2 small nuclear ribonucleoprotein particle (snRNP) binding but is sensitive to mutations in the UACUAAC element as well as the 5′ splice site (22, 23). These results, together with experiments demonstrating that U2 forms base pairs with UACUAAC in yeast (4), imply that UACUAAC is recognized at least twice in yeast mRNA splicing, once before and once during U2 binding. In light of the remarkable conservation of the mechanism of mRNA splicing from yeast to mammals (24), we cannot exclude the possibility that UACUAAC is the preferred mammalian branch site because it interacts with some other factor than U2; however, we consider this unlikely because the branch-site sequence is only weakly conserved (and sometimes dispensable) in mammals.

Why, then, is the GUAGUA sequence of U2 conserved from yeast to mammals when the branch-site sequence is not? One possibility is that GUAGUA is conserved because it plays more than one role in splicing. For example, GUAGUA might form base pairs with more than one sequence in the spliceosome during the splicing reaction. Such base pairing would be consistent with evidence that mRNA splicing may involve significant conformational changes in the spliceosome, as exemplified by dissociation (or destabilization) of the base-paired region between U4 and U6 snRNA after lariat formation (24–26). Alternatively, GUAGUA might be conserved because it is part of the catalytic site of the spliceosome. Similarities between the splicing of nuclear mRNA precursors and autocatalytic group II introns have previously been construed as evidence that mRNA splicing could, in part, be catalyzed by RNA (5–7).

Why do some mammalian introns match the consensus branch-site sequence better than others? We suggest that the interaction between U2 and the branch site can be augmented or replaced by an interaction between the spliceosome and the conserved polypyrimidine tract located between the branch site and the 3′ splice site (27) or some other element of the intron or exon (17, 28). In fact, a purified 70-kDa protein (29, 30), a factor known as U2AF (31), and the hnRNP (heterogeneous nuclear ribonucleoprotein particle) proteins A1, C, and D (32) are all able to recognize the polypyrimidine tract and the AG dinucleotide (but not the branch site) before U2 binding. Strong interactions between such factors and the region surrounding the 3′ splice site might compensate for weak base pairing between U2 and the branch site. In this way, relaxation of branch-site constraints in metazoans might have given rise to a more discriminating U2 snRNP attuned

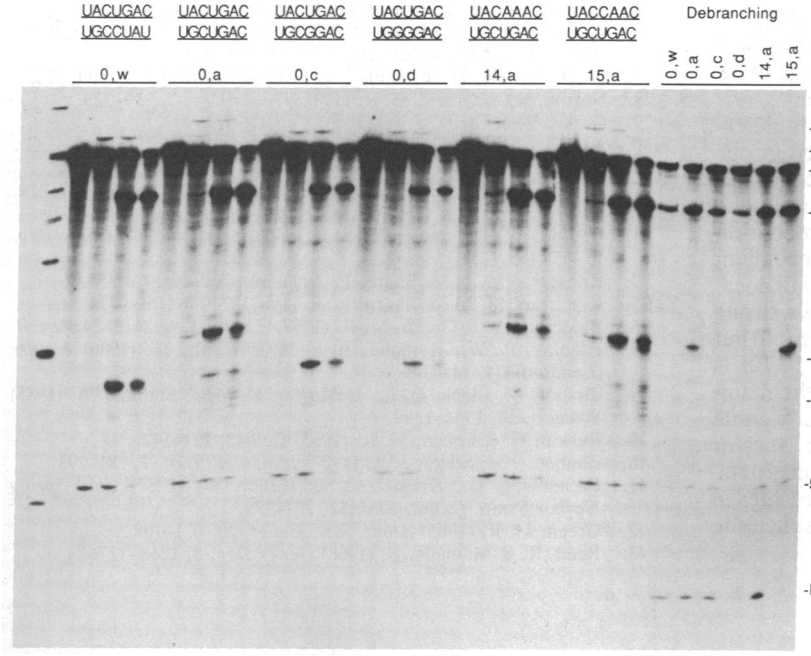


FIG. 5. Time course of *in vitro* splicing of a human β-globin intron 1 containing mutations at both the normal (distal) and cryptic (proximal) branch sites (see Fig. 1*B*). Each set of four lanes represents a 0-, 20-, 45-, and 90-min splicing reaction. The two competing branch sites are indicated on the top of each lane; for example, 0,a means distal branch site 0 and proximal branch site a (Table 1). Debranching reactions were performed after a 45-min splicing reaction to verify that the normal 5′ and 3′ splice sites were used in all cases (right lanes). We have consistently observed inefficient debranching at the proximal branch site except for construct 14,a. *Hin*fI digest of pBR322 (extreme left).

to fine distinctions between 3′ splice sites; thus relaxation would help to ensure accurate splicing of complex transcripts containing alternative exons or multiple introns of variable size. A discriminating U2 snRNP would be unnecessary in budding yeast, where introns are usually short and multiple introns are rare; here, selection for efficient splicing would lead to an invariant branch site. We note, however, that a mutation at the *RNA16* locus can relieve the growth defect caused by mutation from UACUAAC to UACUACC (33). Thus, even in yeast, U2 snRNA cannot be the only component involved in branch-site recognition.

We do not understand how the extent of base pairing between U2 and the branch site can influence the choice of alternative 3′ splice sites in the duplication constructs (Fig. 1*A*) or the ability of two branch sites *within* a single intron to compete with each other (Fig. 5). This depends on whether recognition of the branch site by U2 occurs early or late in the splicing reaction. If recognition occurs early, it could facilitate preferential assembly of the spliceosome on the 3′ splice site adjacent to the better branch site. If recognition occurs late, spliceosome assembly might begin on *both* 3′ splice sites, but the site adjacent to the better branch site would interact with the 5′ splice site preferentially; the partially assembled spliceosome on the unused 3′ splice site would then disassemble. Yet another possibility is that U2 might recognize the branch site both early and late. In fact, different branch sites could be used for initial assembly of the spliceosome and for the actual transesterification resulting in lariat formation (Y.Z. and A.M.W., unpublished work).

In both budding yeast and mammals, the U2 snRNP binds to the 3′ splice site very early in spliceosome assembly (25, 34–37), and U2 binding ultimately protects the branch site from ribonuclease digestion (38–40). However, neither deletion of the branch site (31) nor site-directed cleavage of the GUAGUA element in U2 (41) prevents binding of the U2 snRNP to the mRNA precursor *in vitro*, and this suggests that initial protection of the branch site by U2 may reflect sequence-independent wrapping of the mRNA precursor rather than actual base pairing between U2 and the branch site. Sequence-independent wrapping could also explain why the major constraint on branch-site selection in mammals is distance from the 3′ splice site (for review, see ref. 42). Remarkably, this distance constraint is so strong that a cytidine or even a uridine residue will serve as the branch-point if the region is devoid of adenosine residues (3). Thus spliceosome formation could be facilitated or stabilized, either early or late, by the ability of U2 snRNA to form base pairs with a good branch site within the narrow range of 18 and 40 nucleotides upstream from the 3′ splice site.

Although the quality of the branch site can clearly determine which of two alternative 3′ splice sites is used in our assays, we are unable to explain why position effects sometimes dominate the effect of branch-site mutations. In the competitive assay *in vivo* (Fig. 2), for example, competition between some mutants (2 and 0, 4 and 0) does not depend on position, whereas competition between others (5 and 0) does. The observation that mutant 0 (UACUGAC) exhibits a strong position effect in competition with itself (Fig. 2*B*, lane 0,0) but mutant 10 (UACUAAC) does not (Fig. 2*A*, lane 10,10) is especially puzzling, because both mutants match the mammalian consensus YURAC and are spliced with nearly equal efficiency in the noncompetitive assay *in vitro* (Fig. 3). Nor can we explain why, using essentially identical constructs, we observe competition between alternative 3′ splice sites *in vivo* (Fig. 2) but preferential use of the upstream 3′ splice site *in vitro* (Fig. 4).

Having developed two different assays for mammalian branch-site selection, one using duplicated 3′ splice sites (Figs. 2 and 4) and the other using duplicated branch sites within a single intron (Fig. 5), we are now in a position to ask whether branch-site mutations can be suppressed by a compensatory base change in U2 snRNA as has previously proved possible in yeast (4). The development of a suppressor U2 snRNA, together with the suppressor U1 snRNA already described (16), could open the way for a genetic analysis of the mechanism of mRNA splicing in mammals.

Note. Similar conclusions have been drawn by R. Reed and T. Maniatis (43) in a publication that appeared after this manuscript was completed.

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