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Unusual Branch Point Selection in Processing of Human Growth Hormone Pre-mRNA

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Intron A of the human growth hormone gene does not contain an A residue within 56 nucleotides preceding the 3′ splice site. The analysis of the excised intron lariat revealed a C residue 28 nucleotides upstream from the 3′ splice site as the major branch acceptor nucleotide. Two additional minor branched nucleotides were identified as U residues at positions -22 and -36. An adenosine substitution at position -22 results in lariat formation solely to this nucleotide. Therefore, C and U residues can function efficiently as natural branch acceptors, but an A residue is preferred if available in the proper region. In addition, the data strongly reinforce the importance of the distance constraint for lariat formation. To explain selection of the branch acceptor nucleotide, potential base-pairing interactions of branch point sequences with the U2 RNA are discussed.

The excision of intervening sequences from nuclear pre-mRNAs has been shown to occur via RNA lariat intermediates (for a review, see reference 39). Primary structural requirements for the formation of lariats in nuclear pre-mRNAs involve sequences at the 5′ splice site and the polypyrimidine stretch preceding the 3′ splice site (1, 15, 46). In *Saccharomyces cerevisiae*, the invariant UACUAAC sequence 20 to 60 nucleotides upstream from the 3′ end of the intron was found to be necessary for lariat formation, whereby the third adenosine nucleotide functions as the branch acceptor (12, 29, 48). The search for a similar sequence in higher eucaryotic pre-mRNAs resulted initially in the proposal of a fairly degenerated consensus sequence (26). From further analyses of branch points of a number of naturally occurring genes and of genes with mutant introns, it became clear that no strict consensus sequence surrounding the branched nucleotide could be defined (40, 46, 50). However, the adenosine as branch acceptor nucleotide appeared to be conserved. Recently, an adenosine residue was also identified as the branch acceptor in group II introns (53, 57). In addition, there seemed to be a distance constraint for the branch acceptor, which for higher eucaryotic pre-mRNAs was always found within 18 to 40 nucleotides upstream from the 3′ splice site (40, 46, 50).

Selection of the branch point poses the problem of positioning the branch acceptor such that its 2′ OH group is in a sterically favorable position to attack the phosphodiester bond adjacent to the 5′ splice site. Recently, group II introns have been shown to be excised as lariats, and at least some of them are capable of self-splicing in vitro (44, 53, 57). This implies that the functions required for precise splicing are provided in *cis* and correlates well with a defined RNA secondary structure in group II introns (23, 33, 52). Moreover, similarities in the splicing mechanisms as well as in the intron boundaries suggest that nuclear and group II introns are evolutionarily related (7). However, the excision of nuclear introns is strongly dependent on *trans*-acting factors such as small nuclear ribonucleoproteins (snRNPs) and proteins, which might have replaced the requirement for intramolecular folding.

The question then arises whether the RNA or protein component of the snRNPs is involved in pre-mRNA recognition. There is considerable evidence that the 5′ end of U1 RNA is base pairing with the conserved sequence at the 5′ splice site, but, clearly, additional factors are necessary for precise cleavage at this site (2, 62; for a review, see reference 31). Several observations suggest that the complex recognizing the 3′ end of the intron includes U2, U4/U6, and U5 snRNPs (4–6, 8, 9). A protein which probably belongs to U5 snRNP was found to bind early in the reaction to the conserved polypyrimidine sequence near the 3′ splice site (17, 56). In contrast, U2 snRNP was found to bind quite late to sequences at the branch point region (5). Several models have been proposed for base pairing between U2 and pre-mRNA (26, 38), but the demonstration of the high degeneracy of the metazoan branch point sequences has not supported these models. In *S. cerevisiae*, the situation is much simpler owing to the invariant UACUAAC sequence. The identification of a U2 RNA equivalent in *S. cerevisiae* (3, 47) which has potential base pairing capabilities with the UAC UAAC sequence has prompted Parker et al. (41) to test this model by a direct genetic test. Base changes were introduced into U2-like RNA predicted to compensate for mutations in the UACUAAC sequence. In the two cases tested, improved splicing could be shown, indicating that the recognition of the UACUAAC box is indeed mediated partly by base pairing to the yeast U2 RNA analog.

During analysis of the in vitro splicing of a complete human growth hormone (hGH) pre-mRNA which contains four introns and an alternative splice site (20), it was noticed that intron A did not contain an adenosine residue within 56 nucleotides preceding the 3′ splice site. This indicated that either the distance constraint or the prediction of an adenosine as the natural acceptor nucleotide for branch formation could not be fulfilled in this case. Since the recognition of the 3′ region of the intron and the selection of the branch acceptor is an early and important step in splicing, the branched nucleotide in this intron was determined.

In this report we demonstrate that lariat formation in intron A of the hGH pre-mRNA occurs mainly at a C residue and to a lesser extent at two uridine residues. The results show that the distance of the branch acceptor from the 3′ splice site is a crucial parameter in lariat formation. Furthermore, the proposed interaction between branch point se-

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quences and a portion of the U2 RNA (41) is discussed. If base pairing to U2 RNA is indeed involved in the selection of branch points, the concept of overlapping recognition sequences on U2 RNA has to be introduced. In this model, shifting of the U2 branch point recognition sequence with respect to the branch acceptor is allowed. This modified model can account for the high variability of branch point sequences found in higher eucaryotes.

MATERIALS AND METHODS

Materials. Restriction enzymes, SP6 RNA polymerase, T4 DNA ligase, and *Hin*dIII linkers were from New England Biolabs, Schwalbach, Federal Republic of Germany. GpppG, nucleoside triphosphates (NTPs), deoxynucleoide triphosphates (dNTPs), polynucleotide kinase, and mung bean nuclease were from Pharmacia Molecular Biology Division, Uppsala, Sweden. Calf intestine alkaline phosphatase and snake venom phosphodiesterase were from Böhringer GmbH, Mannheim, Federal Republic of Germany. Snake venom phosphodiesterase was also purchased from Calbiochem-Behring, San Diego, Calif. DNA polymerase (Klenow fragment) was from P. H. Stehelin & Cie AG, Basel, Switzerland. Reverse transcriptase was provided by J. Beard, Life Sciences, St. Petersburg, Fla. [α-^{32}P]dNTPs (3,000 Ci/mmol), [α-^{32}P]UTP and [α-^{32}P]CTP (440 to 880 Ci/mmol), [α-^{32}P]ATP and [α-^{32}P]GTP (5,000 Ci/mmol), and human placental ribonuclease inhibitor were from Amersham Chemomedica, Vienna, Austria. Cellulose plates for thin-layer chromatography were from Schleicher & Schüll, Inc., Dassel, Federal Republic of Germany. Formamide was from Sigma Chemical Co., Munich, Federal Republic of Germany, and was recrystallized twice at 0°C. All other chemicals were of the highest purity available.

Construction of plasmids. All cloning steps were carried out essentially as described by Maniatis et al. (30). Plasmids pH10 and pH12 were constructed by inserting the *Hin*dIII fragment of pAAH5-hGH (20) into the vectors SP65 and SP64, respectively. Plasmid phCS3 (a gift of H. Hirt) is an SP64 derivative containing the *Bam*HI-*Eco*RI fragment of the hCS-3 gene (21).

Preparation of RNA substrates. For in vitro synthesis with SP6 RNA polymerase, DNA templates were linearized by excessive digestion with the appropriate restriction enzymes and transcribed by published procedures (32). For labeling RNA with [α-^{32}P]NTPs, the conditions described by Ruskin et al. (51) were used. Capping was accomplished by lowering the GTP concentration to 50 μM and adding 875 μM GpppG dinucleotide (10, 28). Radiolabeled RNA was stored at −20°C in 50% ethanol. For the primer extension analysis, nonradioactive RNA substrates were synthesized as described previously (19).

Preparation of extracts and splicing reactions. HeLa cell nuclear and cytoplasmic extracts were prepared from HeLa Ohio cells by the method of Dignam et al. (11) with the modifications described previously (20). Splicing incubation mixtures all contained 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–KOH (pH 7.9), 60 mM KCl, 0.12 mM dithiothreitol, 0.12 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 2.4 mM $MgCl_2$, 1.3 mM ATP, and 20 mM creatine phosphate; incubations took place at 30°C. With the SP6 RNAs, 50,000 to 500,000 cpm (Cerenkov) of radio-labeled RNA were incubated in 15 μl, and preparative reactions were performed in 200 μl. Incubations for primer extension analysis and recovery of RNA were carried out as described previously (19). Debranching reaction mixtures (49) contained 7.5 mM EDTA, 60% S100 extract, and 1,000 U of RNasin per ml, and the reactions took place in 20 μl for 2 to 3 h at 30°C.

Analysis of RNA. Preparation of the 3'-end-labeled 128-nucleotide (nt) *Msp*I-*Msp*I exon 2 primer and the 3'-end-labeled 83-nt *Taq*I-*Taq*I exon 5 primer and primer extension analysis were done as described previously (19). We eluted radiolabeled RNA from polyacrylamide gels by soaking the gel slice in 0.5 M ammonium acetate–0.1% sodium dodecyl sulfate–1 mM EDTA at 37°C overnight and recovering the RNA by ethanol precipitation.

Nuclease P1 digestions (27) took place in 10 μl of 60 mM ammonium acetate–0.1 mM zinc acetate (pH 5.3) for 1 to 2 h at 50°C with 1 μg of enzyme. Thin-layer chromatography on cellulose plates was performed as described previously (55) with solvent A (isobutyric acid, NH_4OH, H_2O [66:1:33, vol/vol/vol]) for the first dimension and solvent B (*t*-butanol, concentrated HCl, H_2O [70:15:15, vol/vol/vol]) for the second dimension. For the phosphatase-phosphodiesterase reaction sequence, radioactive trinucleotides were eluted from the plates with water, lyophilized, and incubated in 10 μl of 50 mM Tris–1 mM EDTA (pH 8.0) with 1 U of calf intestine alkaline phosphatase. The phosphatase was first inactivated by a 30-min incubation at 55°C and then extracted once with phenol-chloroform equilibrated with 50 mM Tris–1 mM EDTA (pH 7.5) and twice more with chloroform. The phosphodiesterase reaction was performed by adding 8 μl of water, 1 μl (12.5 μg) of yeast tRNA, and 1 μl (1 μg) of enzyme to the lyophilized material and incubating the mixture for 1.5 h at 37°C. Analytical plates were exposed to Kodak XAR films at −70°C with intensifying screens for 1 to 21 days, whereas preparative plates were exposed for 1 to 3 h at room temperature. Nonradioactive spots corresponding to the 5' monophosphates were detected under UV illumination.

RESULTS

It was previously shown that processing of hGH pre-mRNA requires modifications of the HeLa cell nuclear extract preparation procedure (20). After an unusually long lag phase of 1 h, splicing intermediates began to accumulate; analysis of the processing products showed that only the 3' splice sites of introns A and D were recognized efficiently. Neither of the two introns contains, within the proper distance from the 3' splice site, a sequence which resembles the proposed consensus sequence. The pre-mRNAs used to study branch point formation are shown in Fig. 1A, along with details of the lengths of exons and introns. The relevant sequences of the introns studied are depicted in Fig. 1B.

Identity of branched nucleotides in intron A. H12Hfl pre-mRNA was incubated for 2.5 h under conditions favorable for splicing the hGH pre-mRNA. Reverse transcription has been shown to stop at branched nucleotides (50). If the isolated RNA is treated with an extract containing a 2',5'-phosphodiesterase activity (49) prior to reverse transcription, the lariats are debranched. Primer extension analysis was performed by using a 3'-end-labeled 129-nucleotide *Msp*I-*Pvu*II primer homologous to exon 2 sequences. cDNA products isolated from reverse transcription on branched and debranched RNA were analyzed on a 6% polyacrylamide–8 M urea gel along with a dideoxy sequence generated on unprocessed pre-mRNA (Fig. 2). Two additional cDNA bands were generated during the splicing reaction; these bands disappeared again after debranching. Debranching of the spliced RNA also gave rise to a new cDNA product of

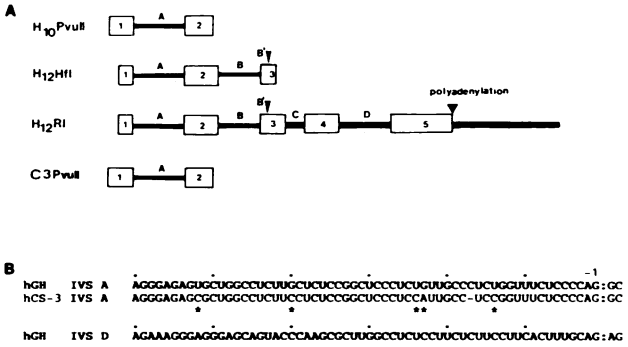


FIG. 1. Schematic diagram of SP6-generated pre-mRNAs of the hGH and hCS-3 genes used in this study. (A) Introns are denoted by capital letters; B′ is the intron released upon use of the alternative 3′ splice site of intron B. The lengths in nucleotides for the introns of the hGH are as follows: A, 259; B, 206; B′, 251; C, 93; D, 253 (54). Intron A of the hCS-3 gene is 254 nt long (21). Exons are denoted by Roman numerals. Exons 1 of H10PvuII, H12HfI, H12RI, and C3PvuII contain 125, 82, 82, and 104 nt, respectively; for H12RI, the lengths of the other exons in nucleotides are as follows: 2, 161; 3, 320; 4, 165; and 5, 303. The 3′ untranslated region is 540 nt. Exon 2 in H10PvuII and C3PvuII is 134 nt. (B) The sequence preceding the 3′ splice site of introns A and D of hGH and intron A of hCS-3 pre-mRNAs are shown. Differences in sequence between the hGH and hCS-3 pre-mRNAs are marked with asterisks.

393 nucleotides, resulting now from a stop at the 5′ splice site of the intron. A cDNA band of 216 nucleotides corresponding to the spliced product (E_1-E_2) was visible in both lanes. Comparison with the dideoxynucleotide sequence indicates that the two U residues at positions −22 and −36 upstream from the 3′ splice site are branch acceptors. The faint band visible at position −19 was not reproducibly seen in subsequent experiments (see Fig. 5A). Another strong cDNA band was visible at a C residue at position −28. Since this band was also weakly visible in the control lane and since incubation merely increased the intensity with no subsequent effect due to debranching, this nucleotide was initially not considered a branch acceptor. With a 3′- end-labeled, double-stranded, 41-base-pair *Msp*I-*Msp*I primer homologous to the last 34 nt of the intron and the first 7 nt of exon 2, no further branch points upstream from the last uridine identified above could be detected (our unpublished data).

The identification of two uridine residues as unusual branched nucleotides led us to investigate the branch sites of intron A more thoroughly. Since branched trinucleotides have been shown to be resistant to nuclease P1 digestion (59), a nuclease P1 analysis was performed on processed H12HfI pre-mRNA labeled with different [α-^{32}P]NTPs. If [α-^{32}P]CTP was used as the label, two nuclease-resistant products (termed X and Y) were visible after two-dimensional thin-layer-chromatography of the digestion products (Fig. 3A). If [α-^{32}P]UTP was used as the label, nuclease P1 digestion led to the production of product Y and an additional nuclease-resistant product, Z. No nuclease-resistant nucleotides were visible when the RNA was labeled with [α-^{32}P]ATP (Fig. 3A), even after prolonged exposures (data not shown). The fact that two branched nucleotides were labeled with [α-^{32}P]CTP was surprising, since only the uridine at position −36 was expected to be labeled in this case.

Further analysis of the nuclease P1-resistant trinucleotides was performed by first treating the isolated trinucleotides

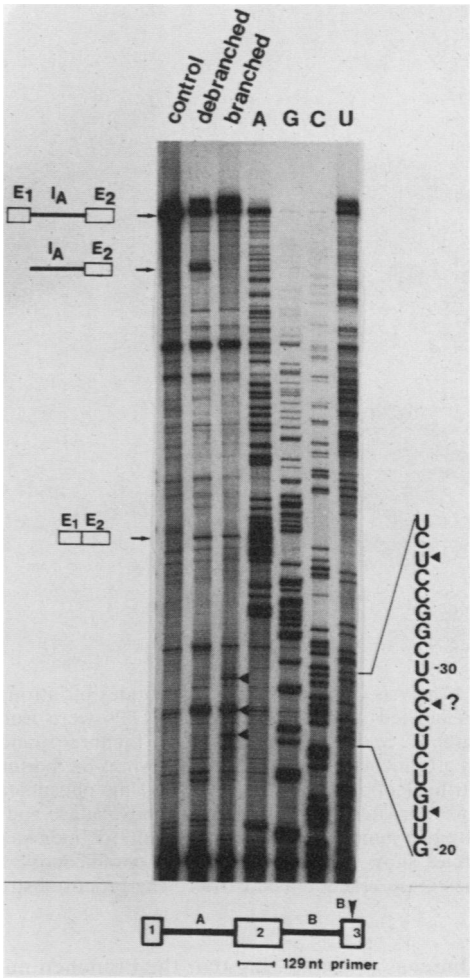


FIG. 2. Analysis of primer extension products synthesized on H12HfI RNA with a primer homologous to exon 2 sequences. The position of the 129-nucleotide, 3′-end-labeled *Msp*I-*Pvu*II primer is shown below the autoradiogram. Reverse transcription was performed with unprocessed (lane control) and processed (lane branched) RNA as template as described previously (19). Part of the processed RNA was treated with debranching extract prior to reverse transcription (lane debranched). The primer extension products were separated on a 6% denaturing polyacrylamide gel in parallel with cDNAs from dideoxy sequencing reactions performed on unprocessed H12HfI RNA (lanes A, G, C, and U; the notation corresponds to the sequence of the RNA). To the right, the RNA sequence covering nucleotides −20 to −38 upstream of the 3′ splice site of intron A is shown. Symbols: ◀, cDNA fragments generated during the splicing reaction; ?, this cDNA band is not sensitive to the debranching reaction. Schematic representations of unprocessed RNA, ligated E_1-E_2 product, and linear IVSA-E_2 (visible only after debranching) are shown to the left of the autoradiogram.

with alkaline phosphatase, inactivating the phosphatase, and subsequently digesting it with snake venom phosphodiesterase (Fig. 3B). The phosphatase should release the 5′ phosphate from the branch acceptor nucleotide, whereas the phosphodiesterase should release the 5′ nucleoside monophosphates from the 2′ and 3′ positions of the branch acceptor (59). The main branched trinucleotide X, which was not labeled with [α-^{32}P]UTP, was the most interesting one, since its appearance was not expected from the reverse transcriptase assay. After separation of the digestion products of X on thin-layer plates, two radioactive spots were

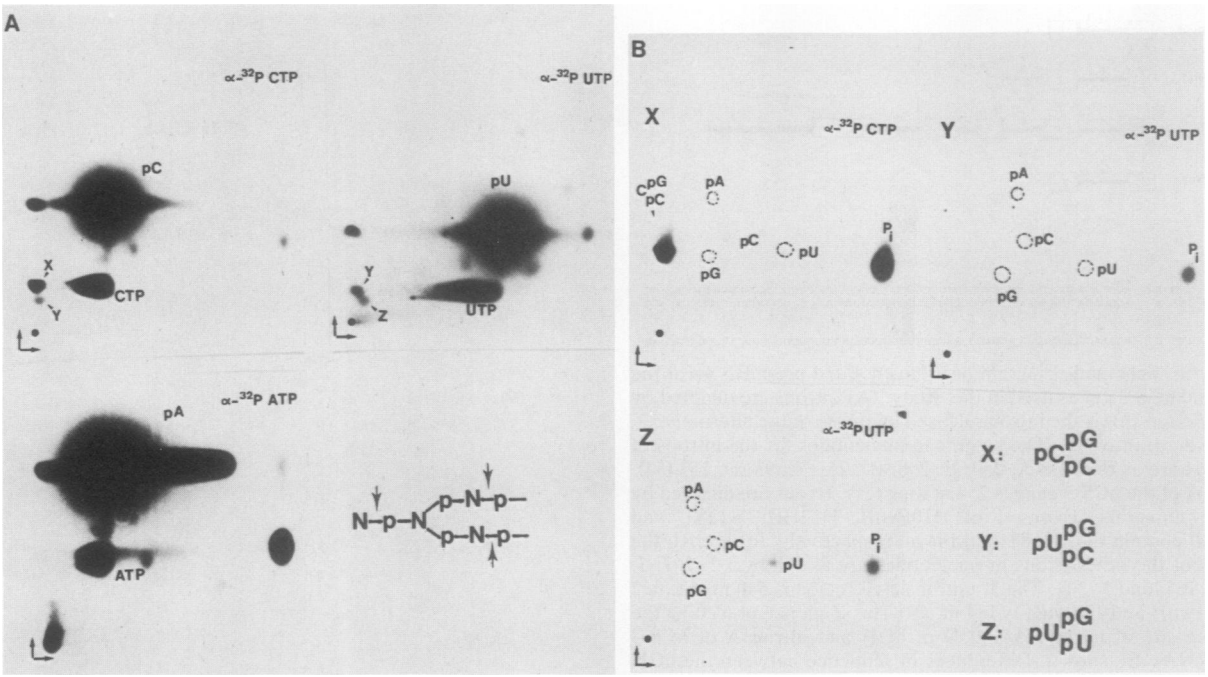


FIG. 3. Analysis of the branched nucleotides of intron A of hGH pre-mRNA. (A) Nuclease P1 analysis. RNAs from processed H12HfI pre-mRNA labeled with different [α-^{32}P]NTPs were isolated, digested with nuclease P1, and separated by two-dimensional thin-layer chromatography. Spots X, Y, and Z refer to the three branched trinucleotides visible in [α-^{32}P]CTP- and [α-^{32}P]UTP- labeled RNA. A general diagram of a branched trinucleotide is shown at the bottom right, with nuclease P1 cleavage sites indicated by arrows. (B) Analysis of the branched trinucleotides X, Y, and Z by alkaline phosphatase and snake venom phosphodiesterase digestion. The isolated trinucleotides X, Y, and Z were treated first with alkaline phosphatase and subsequently with snake venom phosphodiesterase. The reaction products were analyzed by two-dimensional chromatography. P_i indicates the radioactive phosphate released from the 5′ site of the branched nucleotide; broken circles show the positions of the nonradioactive 5′ monophosphates released from *S. cerevisiae* tRNA, which was added to the phosphodiesterase reaction as a carrier. The proposed structures for X, Y, and Z are shown at the bottom right.

visible. One was P_i, indicating that the branched nucleotide is a C residue. The other spot, close to the previous position of X, still contained a pC and must be X(-P_i), which is apparently resistant to phosphodiesterase. Since all three trinucleotides (X, Y, and Z) were radioactive when the RNA was labeled with [α-^{32}P]GTP (data not shown), the structure for X is pC^{pG}_{pC}. From both the sequence of the trinucleotide and the strong stop observed in the primer extension analysis at C residue −28, we conclude that this C residue is used as the major branch acceptor in intron A. At present, we do not know why this branch point can be neither debranched (Fig. 2 and 4) nor split with phosphodiesterase even when different batches of debranching extract or phosphodiesterase are used (our unpublished data).

A similar analysis was performed with products Y and Z. Y labeled with [α-^{32}P]UTP released only P_i and was also labeled with [α-^{32}P]CTP, suggesting the structure pU^{pG}_{pC} for Y. Z was labeled only with [α-^{32}P]UTP and should release P_i and pU in equimolar amounts upon digestion. The apparent surplus of P_i was due to a cross-contamination of spot Z with spot Y. The data suggest the structure pU^{pG}_{pU} for Z. From the combined information of the primer extension experiment and this analysis, we conclude that Y and Z result from branching at U residues at positions −22 and −36, respectively.

These results clearly demonstrate that cytidine and uridine residues can be used as efficient branch acceptors.

Activity in the second splicing step of lariats formed to cytidine and uridine residues. The use of different branch acceptors for lariat formation in intron A of the hGH pre-mRNA does not necessarily imply that all three possible intermediates are also active in the splicing step, namely, the ligation of the exons (22). To address this question, the intron and exon lariats from a splicing reaction of H10PvuII labeled with both [α-^{32}P]UTP and [α-^{32}P]CTP were isolated from denaturing polyacrylamide gels. In addition, the intron lariat of intron 1 of the human metallothionein (43) was isolated for comparison. In this 302-nucleotide intron, lariat formation occurs at an A residue at position −23 (K. Hartmuth, unpublished data). Parts of the various lariat RNAs were treated with debranching extract, and the products were analyzed on an 8% polyacrylamide–8 M urea gel (Fig. 4). All lariat RNAs consist of double bands resulting from exonucleolytic degradation during the splice incubation (50). The human metallothionein intron lariat was completely debranched, giving rise to the linearized intron of 302 nucleotides and additional shorter versions. In contrast, only part of the intron lariat and the exon lariat of hGH was debranched. In addition, a nuclease P1 analysis was performed with the intron lariat of hGH before and after the debranching reaction (Fig. 4). All three identified branched trinucleotides, X, Y, and Z, were present after two-dimensional separation of the digestion products on thin-layer plates. After debranching, most of Y and Z disappeared, whereas X was present with the same intensity as before. This is in accordance with the results from the primer extension analysis (Fig. 2), which showed that the C branch is resistant to debranching activity. These results prove that all three branch sites identified previously are used for

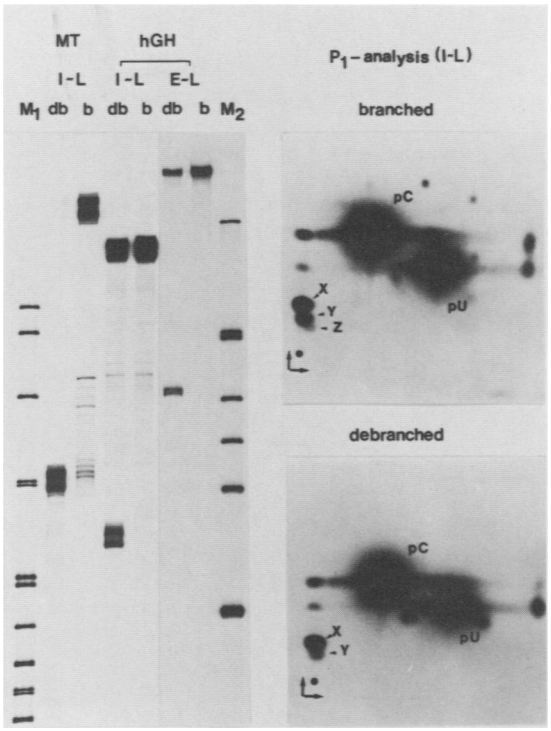


FIG. 4. Presence of both cytidine and uridine branches in the intron-lariat product and resistance of cytidine branch to debranching. Purified exon-lariat (E-L) and intron-lariat (I-L) RNAs resulting from processing of intron 1 of the hGH pre-mRNA (H10PvuII pre-mRNA double-labeled with [α-^{32}P]CTP and [α-^{32}P]UTP were incubated with debranching extract. For comparison, the intron resulting from correct processing of a human metallothionein pre-mRNA (MT) containing intron A was isolated and debranched. Branched (lanes b) and debranched (lanes db) RNAs were run in parallel on the 8% polyacrylamide–8 M urea gel shown on the left. Lane M1, ^{32}P-labeled *Msp*I-cleaved pBR322 DNA; lane M2, ^{32}P-labeled *Hin*fI-cleaved pBr322 DNA. On the right, the autoradiograms of the P1 analyses (see Materials and Methods) of the branched and debranched intron-lariats of intron 1 of the hGH pre-mRNA are shown, indicating that the branched nucleotides X, Y, and Z are present in the intron-lariat product. It further demonstrates that the C branch X is resistant to debranching activity.

productive lariat formation, resulting in the excision of the intron lariat and the ligation of the exons.

Branch acceptor nucleotide in intron A of the hCS-3. The results presented above imply that cytidine or uridine residues can be efficiently used as branch acceptors. To find whether they would also be used if an adenosine residue were present within 18 to 40 nucleotides upstream from the 3′ splice site, we analyzed lariat formation within intron 1 of the human chorionic somatomammotropin (hCS-3) pre-mRNA. The hGH and hCS-3 genes are about 93% homologous and are members of a multigene family (21). The first introns of the two genes exhibit only two differences in the nucleotide sequence in the region of probable branch formation: a G-to-A and a U-to-C change at positions −22 and −23, respectively (Fig. 1). The pre-mRNAs from C3PvuII and H12PvuII were used in parallel for a splice incubation under standard conditions for 2.5 h. Primer extension analysis on the spliced RNA products was performed with the ^{32}P-3′-end-labeled 129-nucleotide double-stranded *Msp*I-*Pvu*II primer with sequences homologous to exon 2. cDNA products were separated on a 6% polyacrylamide–8 M urea gel, and a dideoxynucleotide squence of the C3PvuII RNA was run in parallel (Fig. 5). Comparison of the resulting cDNA patterns of spliced and control RNA revealed the three branch acceptors of the hGH intron (cf. Fig. 5 and Fig. 2). Only one additional cDNA band is visible in hCS-3 RNA. The relevant sequence of the hCS-3 intron is shown on the right, and the branch acceptor can be identified as the adenosine residue at position −22. No increase in intensity can be observed in the region of the C-branch sequence, which is the main branch acceptor in the hGH intron. Since the sequences of the relevant regions (−18 to −40 nt preceding the 3′ splice site) of these two introns are otherwise homologous (Fig. 1), this result strongly suggests that an adenosine residue is the preferred acceptor if it is available in the proper region.

It has been noted previously that splicing of hGH pre-mRNA starts after an unusually long lag phase of 1 h, which is about twice the time needed by other pre-mRNAs in the HeLa cell nuclear extract (20, 25). To find whether the use of C and U residues rather than an A residue in lariat formation is in any way related to the long lag phase, ^{32}P-labeled C3PvuII and H12PvuII pre-mRNAs were incubated in parallel for various times under standard splicing conditions, and splicing products were separated on an 8% polyacrylamide–8 M urea gel (Fig. 5B). In both cases, splicing intermediates began to accumulate after 1 h of incubation. This indicates that features other than the nature of the branch acceptor nucleotide determines the duration of the lag phase.

Lariat formation in intron D of the hGH pre-mRNA. To determine the branched nucleotide in intron D, unlabeled full-length hGH pre-mRNA (H12RI) was incubated under standard splicing conditions and half of the reaction products were treated with debranching extract. Primer extension analysis was performed with an 83-nt ^{32}P-3′-end-labeled double-stranded *Taq*I-*Taq*I primer homologous to sequences within exon 5. The resulting cDNAs were run on an 8% polyacrylamide–8 M urea gel in parallel with a dideoxynucleotide sequence which was generated on unprocessed RNA. The strong band in Fig. 6 is due to an exon-skipping event, ligating exon 1 to exon 5 as shown previously (20). Three additional cDNAs, which were sensitive to the debranching reaction, were produced in the splicing reaction. This identifies the two A residues at positions −37 and −38 as the major branch points in this intron. In addition, a minor U branch point is present at position −24. Neither of the branch point sequences fits the proposed consensus sequence (see Discussion).

DISCUSSION

Cytidine and uridine are efficient branch acceptors. The analysis of the branch acceptor nucleotides of hGH pre-mRNA which has been spliced in vitro demonstrates that C and U can be efficiently used for lariat formation. Since it has been shown (61) that the branch acceptor nucleotide found in vivo is the same as that found in vitro for the large β-globin intron (40), it is generally assumed that in this respect in vitro splicing systems faithfully reflect the situation present in vivo.

Lariat formation is the first biochemical modification in splicing of pre-mRNA. In *S. cerevisiae*, the branch acceptor has always been found to be the third A in the conserved UACUAAC sequence (12, 29, 48). In contrast, the proposed consensus sequence in higher eucaroytes is highly degenerate, the only sequence constraint being an A as acceptor nucleotide (40, 46, 50). The use of an A as the branch

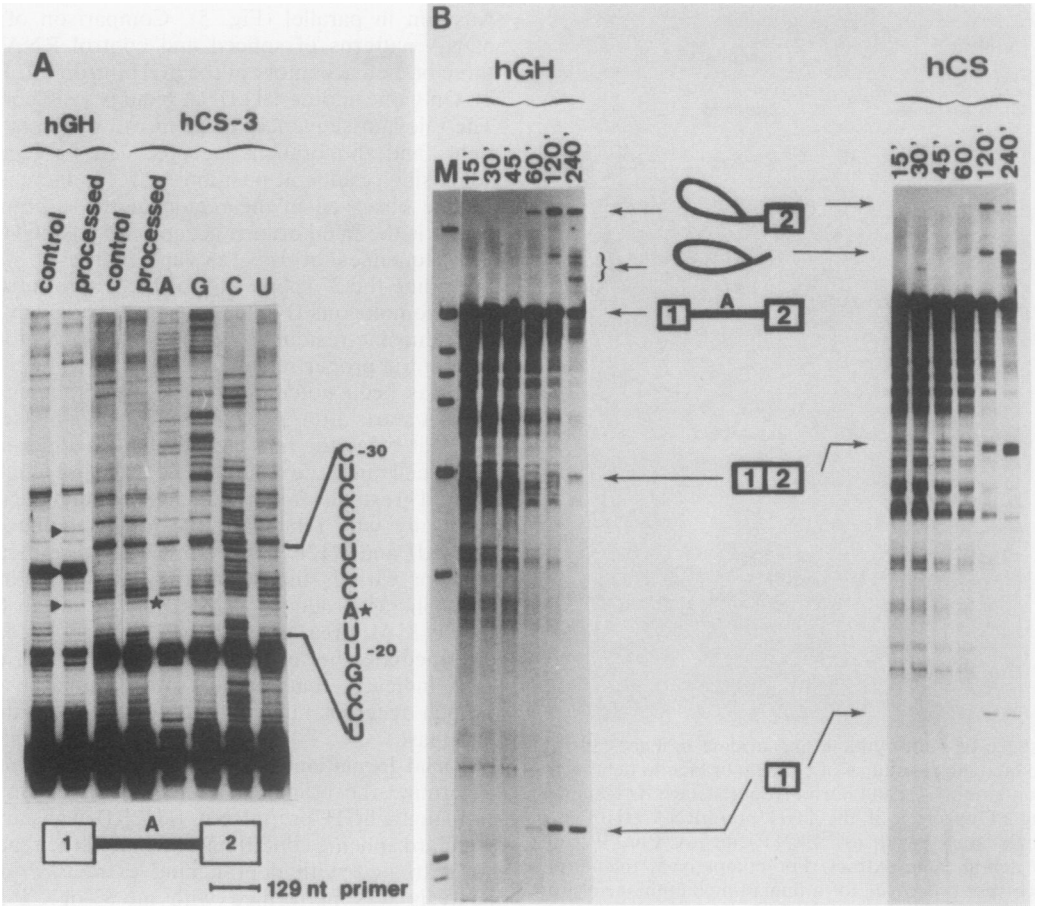


FIG. 5. (A) Identification of the branch acceptor nucleotide in intron 1 of the hCS-3 pre-mRNA. Primer extension analysis was performed on unprocessed (lanes control) and processed (lanes processed) H10PvuII and C3PvuII pre-mRNAs by using the 3′-end-labeled 129-nt *Msp*I-*Pvu*II primer homologous to sequences in exon 2 and analyzed as described in the legend to Fig. 2. Dideoxy sequencing reactions were performed on unprocessed hCS3 pre-mRNA (lanes A, G, C and U; the notation corresponds to the sequence of the RNA). The RNA sequence depicted on the right covers the region 16 to 30 nucleotides upstream of the 3′ splice site of intron A of the hCS3 pre-mRNA. Symbols; ▶, the three nucleotides used as branch acceptors in intron A of the hGH pre-mRNA (see also Fig. 2); *, the branch acceptor nucleotide in intron A of the hCS-3 pre-mRNA. (B) Processing of intron A of the hGH and hCS-3 pre-mRNAs exhibit an unusually long lag phase of 1 h. H12PvuII pre-mRNA of the hGH gene (left panel) and C3PvuII pre-mRNA of the hCS-3 gene (right panel) were incubated in parallel under splicing conditions (see Materials and Methods) for the times indicated. The RNA was recovered and analyzed on an 8% polyacrylamide–8 M urea gel. Lane M, ^{32}P-labeled *Hin*fI-cleaved pBR322. Between the panels, schematic diagrams of the precursors, intermediates, and reaction products are shown.

acceptor in protein-free lariat formation of group II introns has also been reported (53, 57). These observations are in accordance with the results of Wallace and Edmonds (59), who reported A as the predominant branched nucleotide in nuclear poly(A)$^+$ RNA. That the A residue is not a strict biochemical requirement for lariat formation was demonstrated by mutational analysis of the acceptor nucleotide in the conserved UACUAAC *S. cerevisiae* sequence (13, 24, 35, 58) and in two cases in higher eucaryotes (16, 22). However, all these mutations had pronounced effects on the rate of splicing, making it unlikely that such lariats would survive selection in vivo. Therefore, it was commonly accepted that lariat formation occurs to an A near the 3′ end of the intron. The demonstration of C and U as branch acceptors in intron 1 of the hGH pre-mRNA contradicts this assumption and suggests that C and U branches may be reasonably frequent in pre-mRNAs in vivo.

The observation that the C branch in the hGH pre-mRNA is resistant not only to debranching but also to digestion with snake venom phosphodiesterase is rather peculiar. Others have found that C branches are more resistant to debranchase (13, 22, 24), but we have consistently failed to detect any debranching even after prolonged incubation. Furthermore, testing several batches of snake venom phosphodiesterase did not yield cleavage of the phosphodiester bonds. Since snake venom phosphodiesterase is known to be inactive at modified nucleotides (55), we are currently investigating the possibility of a modification of this C branch in the HeLa cell extract.

The fact that the same amounts of C and U branches are found in the lariat intermediates and in the lariat products of intron 1 of the hGH pre-mRNA indicates that lariat formation to C and U residues is productive in the second step of splicing. This finding is in contrast to the effects of an A-to-U mutation of the branch acceptor in intron 2 of the rabbit β-globin, where the U branch was found to be highly inefficient in the second step of splicing (22). At present, these differences cannot be explained.

An adenosine residue is the preferred acceptor nucleotide if available within the proper region. There are two possible

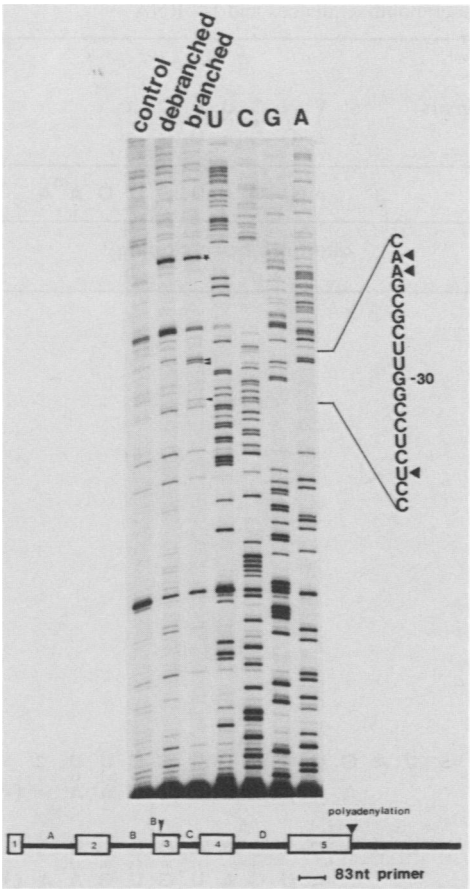


FIG. 6. Analysis of the branched nucleotides of intron D of the hGH pre-mRNA. Reverse transcription was performed on unprocessed (lane control) and processed (lane branched) H12RI RNA as template with a 3'-end-labeled 83-nucleotide *Taq*I-*Taq*I primer. Part of the processed RNA was treated with debranching extract (lane debranched) prior to reverse transcription. The cDNA products were separated on an 8% polyacrylamide 8 M urea gel in parallel with cDNAs from dideoxy sequencing reactions (lanes U, C, G, and A; the notation corresponds to the sequence of the RNA) performed on unprocessed H12RI pre-mRNA. The RNA sequence depicted on the right covers the region 22 to 39 nt upstream of the 3' splice site of intron D. Symbols, ◀, ◀, the three nucleotides used as branch acceptor in intron D of the hGH pre-mRNA; *, a cDNA product which has previously been identified as an exon skipping composed of exon 1 and exon 5 (20).

explanations for using C and U as acceptor nucleotides in intron A of the hGH pre-mRNA: (i) either the sequence around it represents an ideal structure for lariat formation or (ii) C and U are used only because no A is present in the region where the branch acceptor has to be selected. This question was resolved by investigating lariat formation in the hCS-3 pre-mRNA, which is closely related to the hGH pre-mRNA. Intron 1 of hCS-3 differs only in two nucleotides in the region of branch point formation (21). In addition to a U-to-C change at position −23, a G-to-A change occurs at position −22, exactly at the position of the weak U branch in the hGH gene (Fig. 1 and 2). Although the rest of the relevant sequence is unchanged (18 to 40 nt upstream from the 3' splice site), lariat formation takes place solely to the adenosine residue. Since the virtually unchanged C branch sequence at position −27 has proven to be a good branch acceptor sequence in the hGH intron (C at position −28), these data strongly suggest that an A is by far the preferred acceptor nucleotide. However, at present we cannot assess the influence on branch point selection of the nucleotide change at position −23 and the other scattered changes in the hCS-3 intron. The importance of the distance limit for branch selection is further corroborated, since the A at position −56 is not used in the hGH intron.

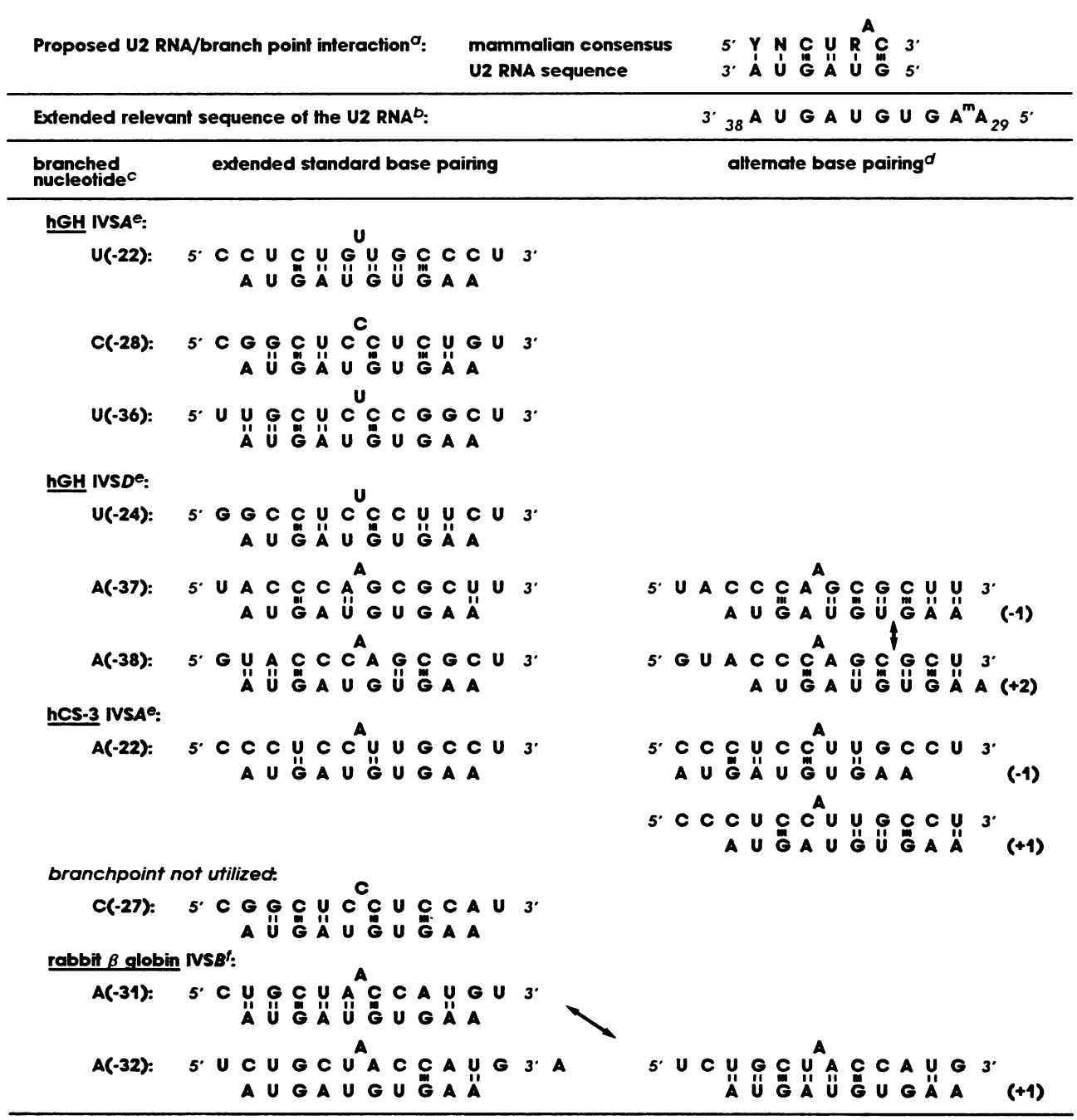
One explanation for the priority of A could be that splicing complexes assemble faster if an A is at the position of the branch acceptor. The observation that both pre-mRNAs exhibit the same unusually long lag phase (Fig. 5B) argues against this assumption.

Involvement of base pairing between U2 and branch point sequences in lariat formation. Self-splicing of group II introns has been shown to depend on a characteristic hybrid structure. In particular, the integrity of a helix structure near the 3' end of the intron containing the branch acceptor as a bulged adenosine is necessary for lariat formation (53, 57). It is thus conceivable that the architecture of the mammalian branch acceptor complex consisting of snRNPs and proteins substitutes for the hybrid structure seen in group II introns. Recently, genetic evidence was obtained that U2 RNA sequences in *S. cerevisiae* base pair to the highly conserved region surrounding the branch acceptor (41). Parker et al. (41) also proposed a similar interaction between a quite degenerate mammalian branch point consensus sequence (18) derived from 12 mapped branch points and the U2 sequence from A38 to G33 (Table 1) and suggest that the helix might be extended to G31 to U2 RNA. Since some of the branch point sequences identified by us did not fit into this scheme, we considered base pairing up to A29 so that the bulged nucleotide of the pre-mRNA is located in the middle of the helix. This region of the U2 is single stranded in both secondary structure models described (3, 5).

When estimating helix stabilities, a number of facts peculiar to RNA have to be taken into account. First, besides G · U pairs, there is evidence that A · G (36) and U · U (34a) might form base pairs. Second, terminal mismatches can contribute to helix stability depending on the type of stacking interaction (14). Finally, since the geometry of the bulged nucleotide is most probably an important feature of lariat formation, the intra- or extrahelical positioning of the bulged base has to be considered (34, 42). The conformation of bulged nucleotides in RNA is not yet known, but by measuring ethidium bromide intercalation near bulged A, C, and U in an RNA helix, it was shown that all three bulged nucleotides have the same pronounced effect on the conformation of the sugar-phosphate backbone (60). Thus, the nucleotide conformations either are identical or differ by only a small free energy. This correlates well with our result that C and U are also efficient branch acceptors. If the bulged nucleotide is stacked onto a helix, the A would provide more stacking energy than the C and U (14), and this difference could contribute to the preference for an A in lariat formation.

Table 1 shows the standard base-pairing interactions between nucleotides 29 and 38 of U2 RNA and the identified branch point regions of the hGH. The C and the two U branch point regions of intron 1 of hGH exhibit considerable base pairing. Inspection of all other C and U residues of the branch acceptor region of hGH intron A revealed less helix stability when they were base paired in the standard manner with the U2 sequence, except C(−19) (not shown), which exhibits more base pairs than the main branch acceptor at C(−28). This already suggest that base pairing to U2 cannot

TABLE 1. Compilation of base-pairing interactions for branch point sequences and U2 RNA



[a] Abbreviations: Y, pyrimidine; R, purine, N, any nucleotide (41).
[b] m refers to 2′-O-methylation (45).
[c] Numbers in parentheses refer to the distances of the acceptor nucleotide from the 3′ end of the intron.
[d] Numbers in parentheses refer to the shifts with respect to standard base pairing: shifts of the U2 RNA sequence toward the 5′ or 3′ end of the intron are termed − or +, respectively; double-headed arrows indicate equivalent configurations, differing only in the branch acceptor.
[e] This work.
[f] Reference 22.

be the sole factor which determines branch acceptor recognition.

hCS-3, although very similar to hGH and still containing the two main branch acceptor sequences, uses only the A at position −22 as branch acceptor. However, standard base pairing with the U2 RNA sequences yields only two U · G base pairs. To explain the exclusive use of A(−22) instead of C(−27) (Table 1) in terms of the theory discussed above, we have to propose shifting of the U2 sequence relative to the branch acceptor, revealing overlapping recognition sequences on U2 RNA with respect to the branch acceptor. Single-nucleotide shifts of the U2 sequence toward the 5′ or

the 3′ end of the intron are termed −1 or +1 recognition sequence, respectively (Table 1). For the hCS-3, both +1 and −1 shifts exhibit a much better fit than the standard. In any case, this must be energetically favored over the C(−27) branch, which is not used in hCS-3. Mutations of the C(−27) branch point sequence for a better fit to the recognition sequence should clarify whether better base pairing can activate C(−27).

Of the three branch acceptors in intron D of the hGH, only the minor U(−24) seems to conform to the proposed model, whereas complementarity of the standard U2 sequence to the two A branch point sequences at positions −37 and −38 are extremely poor. Again, the preference of the two A acceptors can be explained if shifting of the U2 recognition sequence is allowed.

Mutations of the branch acceptor itself generally resulted in the activation of cryptic A branch acceptors (40, 46, 50). The main exception was reported by Hornig et al. (22) for intron 2 of the rabbit β-globin. They showed that mutation of the branch acceptor to any of the other three nucleotides resulted in lariat formation to the mutated nucleotide, but not in an enhanced usage of the adjacent minor A branch. Inspection of the standard base pairing to U2 revealed that the major A(−31) indeed shows good base pairing, whereas A(−32) can base pair only twice. The 20% usage of A(−32) can be explained only if base pairing with the +1 recognition sequence is allowed. However, from this analysis, it is not clear why A(−32) is not exclusively used when A(−31) is mutated. This contrasts with our result from the hCS-3, in which a poor base pairing with a −1 recognition sequence of the A(−22) branch is preferred to a good standard base pairing at C(−27).

If this analysis is applied to other experimentally determined branch acceptors, the same general picture emerges (not shown). The concept of overlapping recognition sequences allows a reasonable base-pairing fit to U2 RNA for most branch point sequences. In this way, the high variability of branch point sequences observed in higher eucaryotes can be explained. This is in contrast to the situation found with U1 RNA, in which shifting of the recognition sequence of U1 RNA also results in a shift of the 5′ cleavage site of the intron (2). However, the analysis also shows that recognition and differential strength of branch points cannot be explained solely on the basis of the quality of base pairing to U2. This is not too surprising, since additional factors known to stabilize base pair interactions have not been taken into account. For example, protein-RNA interactions and coaxial helical stacking can stabilize even small RNA helices (37). Clearly, more information on the exact structure and composition of the branch point complex must be obtained before we can gain more insight into the mechanism of branch point selection.

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LITERATURE CITED

1. **Aebi, M., H. Hornig, R. A. Padgett, J. Reiser, and C. Weissmann.** 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell **47**:555–565.
2. **Aebi, M., H. Hornig, and C. Weissmann.** 1987. 5′ cleavage site in eukaryotic pre-mRNA splicing is determined by the overall 5′ splice region, not by the conserved 5′ GU. Cell **50**:237–246.
3. **Ares, M., Jr.** 1986. U2 RNA from yeast is unexpectedly large and contains homology to vertebrate U4, U5, and U6 small nuclear RNAs. Cell **47**:49–59.
4. **Berget, S. M., and B. L. Robberson.** 1986. U1, U2, and U4/U6 small nuclear ribonucleoproteins are required for *in vitro* splicing but not polyadenylation. Cell **46**:691–696.
5. **Black, D. L., B. Chabot, and J. A. Steitz.** 1985. U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell **42**:737–750.
6. **Black, D. L., and J. A. Steitz.** 1986. Pre-mRNA splicing *in vitro* requires intact U4/U6 small nuclear ribonucleoprotein. Cell **46**:697–704.
7. **Cech, T. R.** 1986. The generality of self-splicing RNA: relationship to nuclear mRNA splicing. Cell **44**:207–210.
8. **Chabot, B., D. L. Black, D. M. LeMaster, and J. A. Steitz.** 1985. The 3′ splice site of pre-mRNA is recognized by a small nuclear ribonucleoprotein. Science **230**:1344–1349.
9. **Chabot, B., and J. A. Steitz.** 1987. Multiple interactions between the splicing substrate and small nuclear ribonucleoproteins in spliceosomes. Mol. Cell. Biol. **7**:281–293.
10. **Contreras, R., H. Cheroutre, W. Degrave, and W. Fiers.** 1982. Simple, efficient *in vitro* synthesis of capped RNA useful for direct expression of cloned eukaryotic genes. Nucleic Acids Res. **10**:6353–6362.
11. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
12. **Domdey, H., B. Apostol, R. J. Lin, A. Newman, E. Brody, and J. Abelson.** 1984. Lariat structures are *in vivo* intermediates in yeast pre-mRNA splicing. Cell **39**:611–621.
13. **Fouser, L. A., and J. D. Friesen.** 1986. Mutations in a yeast intron demonstrate the importance of specific conserved nucleotides for the two stages of nuclear mRNA splicing. Cell **45**: 81–93.
14. **Freier, S. M., R. Kierzek, J. A. Jaeger, N. Sugimoto, M. H. Caruthers, T. Neilson, and D. H. Turner.** 1986. Improved free energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA **83**:9373–9377.
15. **Frendewey, D., and W. Keller.** 1985. Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences. Cell **42**:355–367.
16. **Freyer, G. A., J. Arenas, K. K. Perkins, H. M. Furneaux, L. Pick, B. Young, R. J. Roberts, and J. Hurwitz.** 1987. *In vitro* formation of a lariat structure containing a G2′-5′G linkage. J. Biol. Chem. **262**:4267–4273.
17. **Gerke, V., and J. A. Steitz.** 1986. A protein associated with small nuclear ribonucleoprotein particles recognizes the 3′ splice site of premessenger RNA. Cell **47**:973–984.
18. **Green, M. R.** 1986. Pre-mRNA splicing. Annu. Rev. Genet. **20**: 671–708.
19. **Hartmuth, K., and A. Barta.** 1986. *In vitro* processing of a plant pre-mRNA in a HeLa cell nuclear extract. Nucleic Acids Res. **14**:7513–7528.
20. **Hartmuth, K., and A. Barta.** 1987. *In vitro* processing of the human growth hormone primary transcript. Nucleic Acids Res. **15**:7005–7025.
21. **Hirt, H., J. Kimelman, M. Birnbaum, E. Chen, P. H. Seeburg, N. L. Eberhardt, and A. Barta.** 1987. The human growth hormone gene locus: structure, evolution and allelic variations. DNA **6**:59–70.
22. **Hornig, H., M. Aebi, and C. Weissmann.** 1986. Effect of mutations at the lariat branch acceptor site on alpha-globin pre-mRNA splicing *in vitro*. Nature (London) **324**:589–591.
23. **Jacquier, A., and F. Michel.** 1987. Multiple exon-binding sites in class II self-splicing introns. Cell **50**:17–29.
24. **Jacquier, A., and M. Rosbash.** 1986. RNA splicing and intron turnover are greatly diminished by a mutant yeast branch point. Proc. Natl. Acad. Sci. USA **83**:5835–5839.

25. **Kaltwasser, E., S. G. Spitzer, and C. J. Goldenberg.** 1986. Assembly on an *in vitro* splicing reaction of a mouse insulin messenger RNA precursor into a 60–40S ribonucleoprotein complex. Nucleic Acids Res. **14**:3687–3701.
26. **Keller, E. B., and W. A. Noon.** 1984. Intron splicing: a conserved internal signal in introns of animal pre-mRNAs. Proc. Natl. Acad. Sci. USA **81**:7417–7420.
27. **Konarska, M. M., P. J. Grabowski, R. A. Padgett, and P. A. Sharp.** 1985. Characterization of the branch site in lariat RNAs produced by splicing of mRNA precursors. Nature (London) **313**:552–557.
28. **Konarska, M. M., R. A. Padgett, and P. A. Sharp.** 1984. Recognition of cap structure in splicing *in vitro* of mRNA precursors. Cell **38**:731–736.
29. **Langford, C. J., F. J. C. Klinz, D. Donath, and D. Gallwitz.** 1984. Point mutations identify the conserved, intron-contained TACTAAC box as an essential splicing signal sequence in yeast. Cell **36**:645–653.
30. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Maniatis, T., and R. Reed.** 1987. The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature (London) **325**:673–678.
32. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
33. **Michel, F., and B. Dujon.** 1983. Conservation of RNA secondary structures in two intron families including mitochondrial-, chloroplast- and nuclear-encoded members. EMBO J. **2**:33–38.
34. **Morden, K. M., Y. G. Chu, F. H. Martin, and I. Tinoco, Jr.** 1983. Unpaired cytosine in the deoxyoligonucleotide duplex dCA3CA3G.dCT6G is outside of the helix. Biochemistry **22**: 5557–5563.
34a. **Mougel, M., F. Eyermann, E. Westhof, P. Romby, A. Expert-Bezançon, J.-P. Ebel, B. Ehresmann, and C. Ehresmann.** 1987. Binding of Escherichia coli ribosomal protein S8 to 16S rRNA. A model for the interaction and the tertiary structure of the RNA binding site. J. Mol. Biol. **198**:91–107.
35. **Newman, A. J., R. J. Lin, S. C. Cheng, and J. Abelson.** 1985. Molecular consequences of specific intron mutations on yeast mRNA splicing *in vivo* and *in vitro*. Cell **42**:335–344.
36. **Noller, H.** 1984. Structure of ribosomal RNA. Annu. Rev. Biochem. **53**:119–162.
37. **Noller, H. F., M. Asire, A. Barta, S. Douthwaite, T. Goldstein, R. R. Gutell, D. Moazed, J. Normanly, J. B. Prince, S. Stern, K. Triman, S. Turner, B. Van Stolk, V. Wheaton, B. Weiser, and C. R. Woese.** 1986. Studies on the structure and function of ribosomal RNA, p. 143–163. *In* B. Hardesty and G. Kramer (ed.), Structure, function, and genetics of ribosomes. Springer Verlag, New York.
38. **Ohshima, Y., M. Itoh, N. Okada, and T. Miyata.** 1981. Novel models for RNA splicing that involve a small nuclear RNA. Proc. Natl. Acad. Sci. USA **78**:4471–4474.
39. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. Annu. Rev. Biochem. **55**:119–150.
40. **Padgett, R. A., M. M. Konarska, M. Aebi, H. Horning, C. Weissmann, and P. A. Sharp.** 1985. Nonconsensus branch site sequences in the *in vitro* splicing of transcripts of mutant rabbit alpha-globin genes. Proc. Natl. Acad. Sci. USA **82**:8349–8353.
41. **Parker, R., P. G. Siliciano, and C. Guthrie.** 1987. Recognition of the TACTAAC box during mRNA splicing in yeast splicing involves base pairing to the U2-like snRNA. Cell **49**:229–239.
42. **Patel, D. J., S. A. Kozlowski, L. A. Marky, J. A. Rice, C. Broka, and K. Itakura.** 1982. Extra adenosine stacks into the self-complementary d(CGAGAATTCGCG) duplex in solution. Biochemistry **21**:445–451.
43. **Patzelt, E., E. Thalmann, K. Hartmuth, D. Blaas, and E. Küchler.** 1987. Assembly of pre-mRNA splicing complex is cap dependent. Nucleic Acids Res. **15**:1387–1399.
44. **Peebles, C. L., P. S. Perlman, K. L. Mecklenburg, M. L. Petrillo, J. H. Tabor, K. A. Jarell, and H. L. Cheng.** 1986. A self-splicing RNA excises and intron lariat. Cell **44**:213–223.
45. **Reddy, R.** 1986. Compilation of small RNA sequences. Nucleic Acids Res. **14**:61–72.
46. **Reed, R., and T. Maniatis.** 1985. Intron sequences involved in lariat formation during pre-mRNA splicing. Cell **41**:95–105.
47. **Riedel, N., J. A. Wise, H. Swerdlow, A. Mak, and C. Guthrie.** 1986. Small nuclear RNAs from *Saccharomyces cerevisiae*: unexpected diversity in abundance, size, and molecular complexity. Proc. Natl. Acad. Sci. USA **83**:8097–8101.
48. **Rodriguez, J. R., C. W. Pikielny, and M. Rosbash.** 1984. *In vivo* characterization of yeast mRNA processing intermediates. Cell **39**:603–610.
49. **Ruskin, B., and M. R. Green.** 1985. An RNA processing activity that debranches RNA lariats. Science **229**:135–140.
50. **Ruskin, B., J. M. Greene, and M. R. Green.** 1985. Cryptic branch point activation allows accurate *in vitro* splicing of human alpha-globin intron mutants. Cell **41**:833–844.
51. **Ruskin, B., A. R. Krainer, T. Maniatis, and M. R. Green.** 1984. Excision of an intact intron as a novel lariat structure during pre-mRNA splicing *in vitro*. Cell **38**:317–331.
52. **Schmelzer, C., C. Schmidt, K. May, and R. J. Schweyen.** 1983. Determination of functional domains in intron bI1 of yeast mitochondrial mutations and a nuclear suppressor. EMBO J. **2**:2047–2052.
53. **Schmelzer, C., and R. J. Schweyen.** 1986. Self-splicing of group II introns *in vitro*: mapping of the branch point and mutational inhibition of lariat formation. Cell **46**:557–565.
54. **Seeburg, P. H.** 1982. The human growth hormone gene family: nucleotide sequences show recent divergence and predict a new polypeptide hormone. DNA **1**:239–249.
55. **Silberklang, M., A. M. Gillum, and U. RajBhandary.** 1979. Use of *in vitro* ^{32}P labelling in the sequence analysis of nonradioactive tRNAs. Methods Enzymol. **59**:58–109.
56. **Tazi, J., C. Alibert, J. Temsamani, I. Reveillaud, G. Cathala, C. Brunel, and P. Jeanteur.** 1986. A protein that specifically recognizes the 3′ splice site of mammalian pre-mRNA introns is associated with a small nuclear ribonucleoprotein. Cell **47**:755–766.
57. **Van der Veen, R., A. C. Arnberg, G. Van der Horst, L. Bonen, H. F. Tabak, and L. A. Grivell.** 1986. Excised group II introns in yeast mitochondria are lariats and can be formed by self-splicing *in vitro*. Cell **44**:225–234.
58. **Vijayraghavan, U., R. Parker, J. Tamm, Y. Iimura, J. Rossi, J. Abelson, and C. Guthrie.** 1986. Mutations in conserved intron sequences affect multiple steps in the yeast splicing pathway, particularly assembly of the spliceosome. EMBO J. **5**:1688–1695.
59. **Wallace, J. C., and M. Edmonds.** 1983. Polyadenylated nuclear RNA contains branches. Proc. Natl. Acad. Sci. USA **80**:950–954.
60. **White, S. A., and D. E. Draper.** 1987. Single base bulges in small RNA hairpins enhance ethidium binding and promote an allosteric transition. Nucleic Acids Res. **15**:4049–4064.
61. **Zeitlin, S., and A. Efstratiadis.** 1984. *In vivo* splicing products of the rabbit β-globin pre-mRNA. Cell **39**:589–602.
62. **Zhuang, Y., and A. M. Weiner.** 1986. A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. Cell **46**:827–835.