U1 snRNA promotes the selection of nearby 5' splice sites by U6 snRNA in mammalian cells

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Communication between exon boundaries is a central feature of the exon definition model of pre-mRNA splice-site selection and an exon-bridging interaction involving U1 small nuclear RNA (snRNA) paired with the 5' splice site (5'ss) has been identified previously. It has become increasingly clear, however, that the 5'ss is not defined relative to the base-pairing interaction with U1, suggesting that a connection in the proposed line of communication between exon boundaries is missing. To explore this issue, we have first sought to characterize the role in mammalian 5'ss selection of a previously suggested base-pairing interaction with U6 snRNA. Using transfection experiments, we show that mutations at positions 5 and 6 of a 5'ss associated with an internal exon can be suppressed by compensatory changes in the first two positions of a conserved hexanucleotide of U6 RNA. The specificity of the effect was established by covariation experiments as well as by experiments with two splice sites arranged in tandem. Suppression of 5'ss mutations by U6 was more efficient when U1 could pair nearby than when pairing was restored further away and individual U1 RNAs stimulated U6-defined proximal sites more efficiently than distal sites. These results are interpreted to suggest that U1 acts to direct 5'ss choice by U6 to matching sequences nearby. Our work supports a central role for base-pairing with U6 snRNA in mammalian 5'ss selection and suggests how the interaction may be established properly despite the limited complementarity involved.

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Splicing of mRNA precursors (pre-mRNA) occurs by two cleavage-ligation reactions catalyzed by a complex structure, the spliceosome, which consists of five small nuclear RNAs (snRNAs) and an undetermined number of proteins [for reviews, see Green 1991; Moore et al. 1993]. The fidelity of splicing is determined fundamentally by three types of signals in pre-mRNA, but the information content of these signals, short and degenerate as they are in most organisms, appears insufficient to accomplish accurate splicing with acceptable efficiency. This is true in particular where numerous exons separated by often much larger introns must be spliced together in the correct order without losing the flexibility necessary for alternative splicing, an important source of protein diversity. Accordingly, the spliceosome is thought to serve not only as the catalyst of the splicing reaction steps, but also to translate the limited information presented by pre-mRNA into splicing specificity and orderliness.

The 5' splice-site (5'ss) consensus sequence is comple-

mentary to the 5' end of U1 snRNA and the two regions engage in base-pairing (for review, see Steitz et al. 1988) with the assistance of the general splicing factor SF2/ASF and perhaps others [Kohtz et al. 1994]. Association of U1 with pre-mRNA promotes binding of U2 small nuclear ribonucleoprotein (snRNP) to the branch region, but base-pairing with U1 is not the reference used to define the 5' cleavage site, at least in the yeast *Saccharomyces cerevisiae* (for review, see Rosbash and Séraphin 1991). Here, changes in U1 snRNA to compensate for mutations in the 5'ss can stimulate cleavage outside the paired region [Séraphin et al. 1988; Siliciano and Guthrie 1988; Séraphin and Rosbash 1990]. Recently, we described a similar observation for a mammalian gene where mutations in the 5' splice sites were suppressed in vivo by U1 snRNAs with complementarity to sequences away from the site [Cohen et al. 1994]. It has also been demonstrated recently that mammalian 5'ss can be selected in vitro in the absence of base-pairing with U1 [Crispino et al. 1994; Tarn and Steitz 1994], but a contribution to the fidelity of splicing was suggested [Tarn and Steitz 1994].

In yeast, base-pairing with both U5 and U6 snRNAs has been implicated in the definition of the 5' cleavage

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site (for review, see Madhani and Guthrie 1994). Genetic evidence has been presented that base-pairing between a conserved loop of U5 and exon nucleotides can play a vital role (Newman and Norman 1991, 1992). Similarly, cleavage specificity can be affected by base-pairing between a conserved ACAGAG hexanucleotide of U6 snRNA and intron nucleotides (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993). Because of the high degree of sequence conservation at the 5' ends of budding yeast introns (Woolford 1989; Rymond and Rosbash 1992), positions 2 and 4–6 of most 5' splice sites in this organism have the potential to pair with the first three bases and the fifth base of the hexanucleotide of U6. It has been demonstrated that mutations at position 5 of two different yeast introns can be suppressed partially by compensatory changes at the second position of the U6 hexanucleotide, and suppression was enhanced when complementarity was extended beyond the normal positions (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993). In addition, increased cleavage at an aberrant site when intron position 5 was changed could be rationalized in terms of a realignment with the conserved sequence of U6 (Lesser and Guthrie 1993).

Biochemical evidence supports the suggestion that the conserved loop of U5 interacts with exon nucleotides flanking the 5' cleavage site in mammalian cells as well (Wassarman and Steitz 1992; Wyatt et al. 1992; Sontheimer and Steitz 1993). As in yeast, this region is not well conserved among mammalian pre-mRNAs and the interaction is believed to involve unconventional base-pairs. Its significance is supported not only by analogy to yeast,

but also by experiments that followed the interaction through the splicing reaction and by transfection results showing that mutations in the loop of U5 can alter 5'ss choice (Cortes et al. 1993; Sontheimer and Steitz 1993). With respect to U6, biochemical data place the conserved hexanucleotide in close proximity to the 5' end of the intron at the time of the first chemical reaction (Wassarman and Steitz 1992; Sontheimer and Steitz 1993). Base-pairing involving positions 2, 5, and 6 of the intron and the first, second, and fifth position of the hexanucleotide was proposed on the basis of a nearby psoralen crosslink (Wassarman and Steitz 1992). The fifth base of the hexanucleotide was shown to interact with position 2 of the intron in lariat intermediate, but not pre-mRNA (Sontheimer and Steitz 1993); the two nucleotides can form a conventional A:U base-pair that may function in the second step of splicing without contributing to 5'ss recognition or cleavage. The genetic suppression experiments in yeast referred to above support the proposal that positions 5 and 6 pair with U6, and the first genetic evidence for pairing of position 5 with the hexanucleotide in a mammalian system was reported during the preparation of this paper (Sun and Manley 1995). Other than in yeast, position 4 of mammalian introns is not expected to pair with U6 because the consensus A (U in yeast) is not complementary to the third base of the U6 hexanucleotide (A in both cases).

We use transfection experiments to study selection of the alternative H-ras exon IDX placed in the context of a human growth hormone (hGH) gene (see Fig. 1). Previously, we reported that certain single-base changes in the 5'ss at the end of IDX (5'ss* in Fig. 1) that cause exon

Figure 1. Structure of the hGH.FSX splicing-reporter pre-mRNA and illustration of the major consequences of shift-U1 coexpression, as documented elsewhere (D.-Y. Hwang and J.B. Cohen, in prep.). Exons are shown as boxes (open boxes, untranslated regions; shaded boxes, hGH coding regions; solid box, IDX) and introns as connecting, horizontal lines; IDX is flanked by introns C1 and C2. U1-α+14 is a representative shift-U1 RNA. It is complementary at positions 3–11 to positions 12–20 of intron C2 (Cohen et al. 1994; overlined sequence at right), as symbolized by the dot (left). The main splicing products derived from wild-type (wt) pre-mRNA and three mutants with changes in the IDX-associated 5'ss (5'ss*) are shown schematically below the pre-mRNA; splicing is indicated by angular lines between exons. The sequences of the wild-type and mutated 5'ss in the different hGH.FSX expression vectors are shown at right; dashes indicate identity to the wild-type sequence and the 5'ss consensus sequence is included for comparison (M is C or A; R is A or G). The mutations abolish exon inclusion (cf. the two upper products at left). Coexpression of shift-U1s RNAs, such as U1-α+14, restores exon inclusion (third product; a small amount of this material is also formed when the CTC substrate is coexpressed with U1-α+14; see Fig. 2B) or causes exon inclusion with retention of the next intron ("IR" product). The last result suggested previously that shift-U1s stimulate the 3'ss across IDX at the expense of the 3'ss across intron C2. The specificity of U1-α+14 was demonstrated by the observation that mutation of positions 15–17 of intron C2 abolishes U1-α+14-promoted IDX(A5) inclusion, which is reversed by compensatory changes in U1-α+14 (D.-Y. Hwang and J.B. Cohen, in prep.).

skipping can be suppressed by coexpression of U1 snRNAs, termed shift-U1s, with complementarity to nearby sequences away from the site (e.g., U1- α + 14 in Fig. 1; see legend). We also referred to our observation that additional changes that decreased the complementarity with endogenous U1 to three of nine positions inhibited, but did not abolish accurate splicing (Cohen et al. 1994). Subsequent work has shown that shift-U1 RNAs, as well as U1 RNAs with restored complementarity to various mutated 5' splice sites, stimulated use of the 3' splice site across IDX at the expense of the 3' splice site across the flanking downstream intron (summarized in Fig. 1; D.-Y. Hwang and J.B. Cohen, in prep.).

The exon definition model proposes that exons, rather than introns, represent the initial units of recognition for the splicing machinery, and communication between exon boundaries is a central feature of the model (Roberson et al. 1990; Berget 1995). Previous studies focusing on the selection of an internal preprotachykinin exon have suggested that communication between exon boundaries is mediated by an exon-bridging interaction between U1 paired with the 5' splice site and the essential splicing factor U2AF65, which associates with the polypyrimidine tract preceding the opposite end of the exon (Hoffman and Grabowski 1992). Our own work as well has provided evidence for communication between U1 and the 3' splice site, but it also argued that the 5' splice site can be defined irrespective of the position of U1 binding (Cohen et al. 1994; D.-Y. Hwang and J.B. Cohen, in prep.). This suggested that the definition of exon boundaries with reference to one another must involve at least one other line of communication, either between U1 and the factors responsible for 5' splice site definition, or directly between these factors and the upstream 3' splice site. It is noteworthy in this context that communication between U1 and the preceding 3' splice site may play a role in the selection of 3' terminal exons as well. In the few cases examined in detail, U1 associates with sequences within the exon (Wassarman and Steitz 1993; Lutz and Alwine 1994), which indicates that here too, interaction of U1 with the 3' splice site cannot alone be sufficient for communication between exon boundaries.

To explore the unidentified link in the line of communication between exon boundaries suggested by the preceding considerations, we have first sought to obtain genetic evidence that U6 snRNA pairs with mammalian 5' splice sites and that this interaction contributes to 5' splice site selection in mammalian cells. The results demonstrate directly that base-pairing between positions 1 and 2 of the conserved hexanucleotide of U6 and positions 5 and 6 of a mammalian 5' splice site is necessary for efficient splicing; the data are also largely consistent with the suggestion that the 5' cleavage site is defined relative to this interaction. We exploit these observations to determine whether base-pairing with U1 influences 5' splice site selection by U6 and present evidence that the two factors communicate. On the basis of these results, we propose that U1 and U6 conspire to define mammalian 5' splice sites, with U1 serving to bring U6 into the vicinity of the site.

Results

Suppression of 5' splice site mutations by compensatory changes in U6

Previously, we observed that mutation of positions 4–6 of the 5' splice site of intron C2 in our hGH.FSX splicing-reporter gene to CTC or CAA (see Fig. 1) resulted in decreased use of the site, but did not eliminate shift-U1-dependent IDX splicing (D.-Y. Hwang and J.B. Cohen, in prep.; see also Fig. 2B, lane 3). Alignment of the wild-type 5' splice site sequence with the hexanucleotide of U6 in the currently favored register indicated a potential for base-pairing at positions 8 and 9 of the intron (Fig. 2A, top). To determine whether this could account for the residual use of the mutated sites, positions 8 and 9 in the CTC reporter gene were changed from TC to GA to generate mutant CTC · GA (Fig. 2A, middle). Protein analysis of transfected 293 and HeLa cells indicated that the efficiency of accurate splicing in the presence of U1- α + 14 was reduced further, whereas the use of an aberrant site downstream appeared largely unaffected (data not shown; see Fig. 3D for the CTC · GA pattern). This was consistent with base-pairing of positions 8 and/or 9 with U6 and with the suggestion that base-pairing with U6 is important for efficient splicing.

To pursue these suggestions, U6 snRNAs with increased complementarity to the CTC or CTC · GA sites (Fig. 2A) were coexpressed with the U1 and reporter genes together with a cDNA control vector expressing human DNase protein. The upper part of Figure 2B shows S1 nuclease-protection data corrected for differences in transfection efficiencies on the basis of the primer-extension results in the lower part of the figure. The data show that U6- α CTC significantly stimulated correct splicing at the end of IDX for both reporter genes and that U1- α + 14 was necessary to see this effect. Thus, in the presence of U1- α + 14, U6- α CTC increased the abundance of the small product marked at the side (Fig. 2B, cf. lanes 3 and 4; lanes 8 and 9), and this product comigrates with the sole product in the cDNA-control lane (p22k+, lane 5), indicating that it represents accurately spliced RNA (see also Fig. 3D). The larger product marked at the side represents unspliced RNA in the U1-wild-type lanes and the increased intensity of the signal at this position in the presence of U1- α + 14 (and U6-wt; lanes 3,8) is attributable to selective retention of intron C2, as documented elsewhere (D.-Y. Hwang and J.B. Cohen, in prep.; see Fig. 1). In the presence of U6- α CTC (lanes 4,9), this signal was reduced, suggesting that correct splicing as a result of U6- α CTC occurred at the expense of U1- α + 14-promoted retention of intron C2. The signal between the two major bands was also somewhat reduced and it is possible that accurate splicing occurred at the expense of aberrant 5' splice site selection as well, which would add to the abundance of the smallest product; at least some of the distinct bands between the two major products appear to result from the selection of aberrant 5' splice sites in intron C2 (see Fig. 3E). Together, these results were consistent with the suggestion that base-pairing with U6 stimulates accurate 5' splice site selection in mammalian cells in vivo.

Figure 2. Complementarity between U6 snRNA and the IDX-associated 5'ss region [5'ss*] and suppression of 5'ss mutations by compensatory base changes in U6. (A) (top) The conserved U6 hexanucleotide (numbered 1–6, corresponding to positions 41–46 relative to the 5' end of human U6 snRNA) is shown in alignment with the wild-type 5'ss* in the register suggested by mutation-suppression experiments in yeast (see introductory section). Complementarity is indicated by colons between the two lines, and two potential additional base pairs between the upstream region of U6 and the intron are also shown; complementary nucleotides in the intron are numbered, and the boundary between IDX and intron C2 is indicated by a slash (/). (Middle) Complementarity in the register at the top is reduced by the CTC mutation in the 5'ss and reduced further by additional changes at positions 8 and 9 (5'ss*: CTC · GA). Compensatory changes introduced into U6 are indicated above the U6-wt sequence; throughout, wild-type sequences are shown in bold and mutations in regular type. (Bottom) The structure of the two probes used in this work for S1 nuclease protection analyses is represented by horizontal lines (regions of complementarity to the reporter pre-mRNA) ending with downward tails (noncomplementary regions) below an illustration of a portion of the pre-mRNA; both probes were 3'-end labeled (circled x). (B) S1 nuclease protection analysis of cytoplasmic RNAs isolated 2

days after cotransfection of 293 cells with reporter hGH.FSX genes carrying the indicated changes in the 5'ss* and wild-type or mutant U1 and U6 genes. Dependent on the reporter, RNAs were analyzed with either a CTC or CTC · GA version of probe 1. One thirtieth of the CTC · GA probe used in the reactions is shown in the lane labeled probe. The amounts of reaction products loaded were adjusted according to the DNase signal in the primer extension at the bottom to correct for differences in transfection efficiencies. The regions of the reporter responsible for the main protection products are indicated to the left; the upper product is the result of protection of the complete reporter-specific portion of the probe; the lower product results from protection of the IDX portion only. p22k+ is a control vector expressing a cDNA corresponding to 22-kD + mRNA (Cohen et al. 1994). For the primer extension below the S1 image, a primer was used that recognizes sequences in the 5'-untranslated regions of hGH.FSX reporter mRNA as well as mRNA encoding DNase protein expressed from a cotransfected vector described elsewhere (pDNaseΔ10; D.-Y. Hwang and J.B. Cohen, in prep.). The last two lanes show RNAs from cells transfected separately with the DNase vector or one of the hGH.FSX vectors, respectively. (C) Comparison by S1 protection analysis, as in B, of RNAs from transfected 293 and HeLa cells. The CTC · GA version of probe 1 was used, and probe 2 was included in each reaction; 1/30 of the amount of each probe used in the reactions was loaded in the last lanes at the right. The U6-αCTC RNA from 293 cells was also reacted separately with each probe (probe 2, lane 8; probe 1, lane 9), and the protected probe 2 region is illustrated to the left (exon 5).

Figure 3. Specificity of compensatory changes in U6. (A) Alternative alignments of the CTC · GA site and U6-αCTC; mutations are in regular type. The upper alignment (1) is in the register shown in Fig. 2. The alignment in the register underneath (2) is particularly strong for the CTC site, which matches the altered hexanucleotide at all six positions (not shown). Nucleotide changes in U6-αCTC to test the alternative alignments are indicated by upward arrows; boldface type indicates reversion to wild type. (B) S1 nuclease protection analysis of RNAs from transfected 293 cells using probes 1 (CTC · GA version) and 2, as described in the legend to Fig. 2. Because of contamination, the original U1-α + 14/U6-αCTC transfection was lost and the RNA shown for this combination was from a separate transfection; the U1-α + 14/U6-αTCTC transfection was repeated in parallel, and the corresponding RNA is shown here for comparison next to RNA from the first U1-α + 14/U6-αTCTC transfection (lanes 10 and 11, respectively). (C) Positions of complementarity (alignment 1) in the hexanucleotide of normal and mutant U6 genes to the 5'ss*:CTC · GA sequence are indicated by dashes, and mismatched bases are shown. The arrows point to changes introduced into the 5'ss*:CTC · GA sequence to test the U6 mutants for allele specificity. (D) Analysis by immunoprecipitation of metabolically labeled, secreted proteins produced by the reporter genes and a cotransfected control vector, pDNase, 2 days after transfection of 293 cells. The procedure was as described previously (Cohen et al. 1993). To facilitate identification of the products, cDNA vectors expressing the 22-kD (p22k, lane 1) and 22-kD+ (lane 9) proteins were transfected separately. A third cDNA vector, pcryp + 12, was also included (lane 10); it represents an mRNA spliced at an aberrant 5'ss 12 bases into intron C2 (Cohen et al. 1994). (E) Suppression of the CTC · GA mutation by U6-αTC confirmed by S1 nuclease protection analysis using probe 1 (CTC · GA). RNAs were from transiently transfected 293 cells. pcryp2 + 12, pcryp2 + 22, and pcryp2 + 34 express cDNAs corresponding to CTC · GA mRNAs spliced at aberrant 5'ss 12, 22, and 34 bases, respectively, into intron C2. The IDX-exon 4 junctions in these cDNAs were isolated from pHGH.FSX:CTC · GA/pU1-α + 14-transfected cells (D.-Y. Hwang and J.B. Cohen, in prep.).

Figure 2C shows a comparison between 293 and HeLa cells. The comparison demonstrates that the preceding observations for U6 were not a peculiarity of 293 cells, which was a concern because these cells are known to express abnormally high levels of SF2/ASF (Ge and Manley 1990), a splicing factor implicated in (alternative) 5'ss selection (Horowitz and Krainer 1994; Kohtz et al. 1994; see also Crispino et al. 1994; Tarn and Steitz 1994). In this experiment, an hGH exon 5-specific probe was included in the S1 reactions to provide a reference signal (probe 2 of Fig. 2A). This was done on the basis of the primer-extension results at the bottom of Figure 2B, which showed that the amounts of reporter and control RNAs fluctuated in parallel in the presence of different U1 and U6 genes. It may be noted that in lanes 6 and 7 (293 cells), the largest product is more intense than in lanes 3 and 4 (HeLa cells). Results to be presented elsewhere suggest this is attributable to greater stimulation of the 3'ss of intron C1 by U1- α + 14 in 293 cells than in HeLa cells (D.-Y. Hwang and J.B. Cohen, in prep.). This could be a consequence of the different SF2/ASF levels in the two cell lines.

We also tested the effect of mutations introduced into U6 to restore complementarity with positions 8 and 9 of the CTC · GA substrate (see Fig. 2A). Surprisingly, these mutations reduced the stimulatory activity of U6- α CTC (Fig. 2B, lane 10). In isolation, they failed to promote correct splicing (Fig. 2B, lane 11). Preliminary results indicate that U6- α CTC · GA barely stimulates splicing at the CTC site, suggesting the mutant RNA may be inherently defective (data not shown). A recent report indicates that both of the U6 positions changed by the additional mutations participate in a newly discovered base-pairing interaction with U2 snRNA (Sun and Manley 1995), which could explain our results. However, we also considered the alternative possibility that U6- α CTC and the CTC · GA site align in a register different from the one shown in Figure 2A.

Specificity of base-pairing with U6

We did not identify an alternative base-pairing scheme that could easily explain the decreased activity of U6- α CTC · GA, but at the same time, alternative base-pairing schemes were not ruled out by the preceding experiments. For example, the hexanucleotide of U6- α CTC is complementary without interruption to positions 4–9 of the CTC site and when the sequences are aligned accordingly, complementarity between U6 and position 2 of the site is maintained (not shown). On the other hand, when the CTC · GA site is aligned in the same manner [U6- α CTC (2) in Fig. 3A], the uninterrupted complementarity is reduced to four positions and the difference with wild-type U6 is not dramatic; the difference between U6- α CTC and wild-type U6 is clearly greater in the original scheme [U6- α CTC (1) in Fig. 3A]. Therefore, to strengthen the suggestion that the original scheme is the correct one for the CTC · GA site and at the same time add to the evidence that base-pairing is in fact involved, positions 1, 2, and 4 of the altered hexanucleotide of

U6- α CTC were changed individually to distinguish the alternative alignments of Figure 3A. Positions 1 and 2 are paired with the CTC · GA site in the first scheme, but not in the second; conversely, position 4 is paired in the second scheme, but not in the first (Fig. 3A). The S1 protection results of Figure 3B demonstrate that activity was almost eliminated by a G $\rightarrow$ A change at position 1 of the hexanucleotide (U6- α CT; cf. lanes 7 and 8) or an A $\rightarrow$ U change at position 2 (U6- α CAC; lane 9), whereas substantial activity remained after a G $\rightarrow$ A change at position 4 (U6- α TCTC; lane 10). To verify that the lack of activity of the first two mutants reflected a loss of base-pairing potential, complementarity with each was restored by introducing compensatory changes into the reporter sequence (Fig. 3C). The protein results of Figure 3D confirm that U6- α CTC stimulates IDX inclusion with the CTC · GA substrate, as indicated by the production of the 22-kD + protein (cf. lanes 4 and 5; see also Fig. 1), whereas U6- α CT and U6- α CAC had only a small effect (lanes 6,7). When position 5 of the CTC · GA reporter was changed to A (CAC · GA), however, U6- α CAC stimulated (cf. lanes 13 and 14) and U6- α CTC had only a small effect (lane 15). Similarly, U6- α CT stimulated when the substrate had a C $\rightarrow$ U change at position 6 (CTT · GA; cf. lanes 18 and 19); G:U pairing at position 6 may explain the significant activity of U6- α CTC in this case (lane 20). These data support the conclusion that U6 pairs with the 5'ss according to alignment 1 by demonstrating the importance of complementarity between positions 5 and 6 of the 5'ss and U6 residues 2 and 1, respectively. At the same time, conventional base-pairing at position 4 appears to play little or no role, at least when positions 5 and 6 are paired. Thus, U6- α TC, which is complementary to positions 5 and 6 of the CTC · GA site, but mismatches the site at position 4 (A:C mismatch; Fig. 3C), appeared to induce IDX splicing as efficiently as U6- α CTC (Fig. 3D, cf. lanes 5 and 8). This is corroborated by the RNA analysis of Figure 3E (cf. lanes 4 and 7), which also indicates that the most prominent aberrant product seen here and in previous S1 experiments is due to U1- α + 14-promoted selection of a cryptic 5'ss 22 bases into the intron (cryp + 22; see legend). Note that the significance of the reduction in the intensities of the largest and cryp + 22 products in the U6- α TC lane is uncertain because of the absence of a standard for normalization. Together, the results are in complete agreement with the previous predictions that U6 interacts with positions 5 and 6, but not position 4, of the 5'ss in mammalian cells (Wassarman and Steitz 1992; Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993).

Contributions of base-pairing with U1 and U6 to 5'ss selection

To contrast the contributions to 5'ss activity of base-pairing with U1 and U6 RNAs and to explore the suggestion that base-pairing with U6 may contribute to 5'ss choice in mammalian cells, like it does in yeast (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993),

a plasmid was constructed with additional GT dinucleotides in intron C2 close to the CTC site (pGT+). The new sequence and its complementarity to different co-expressed U6 and U1 RNAs is illustrated in Figure 4A; the surroundings of the new GT dinucleotides lack significant complementarity to endogenous U1 or U6 RNAs. Figure 4B shows that irrespective of which U1 was expressed, U6- α CTC promoted accurate splicing at the end of IDX (bands labeled 5'ss*; lanes 2,5), whereas U6- α GT2 promoted the use of an aberrant 5'ss in the intron (GT2; lanes 3,6). The aberrant site has been mapped to the expected position in front of the second GT of the intron (GT2 in Fig. 4A; data not shown). Although it appears that U6- α GT2 also stimulated splicing at the 5'ss* and at another site between this and the GT2 site (lanes 3,6), the corresponding bands are artifacts of the S1 procedure, as indicated by a direct comparison with RNA from cells transfected with a GT2 cDNA expression vector (lanes 9,10). These results clearly demonstrate that base-pairing with U6, but not with U1, determines the 5' cleavage position.

Effects of U1 on 5'ss selection by U6

To explore whether U6 could connect the process of 5'ss selection with the exon-bridging line of communication involving U1, we determined whether U6-dependent 5'ss selection was sensitive to the position of base-pairing with U1. The results of Figure 5A show that the opposite effects of U6- α CTC on IDX splicing and intron retention (bands at the bottom and top) were more pronounced with U1- α CTC (cf. lanes 1 and 4) and U1- α GT2 (lanes 2,5) than with U1- α +14 (lanes 3,6), which binds farther away (see Fig. 5C). The difference was specific for 5'ss selection, as U1- α +14 stimulated the upstream 3'ss at least as efficiently as the other two U1 RNAs; this is indicated by the similar intensity of the largest band in lanes 1–3 (U6-wt lanes). To distinguish whether the difference was defined relative to U6, to some feature of the substrate other than the 5'ss, or whether it might reflect intrinsic differences between the U1 RNAs, the experiment of Figure 5B was performed. The results show that U1- α (-6), which is com-

Figure 4. 5'ss choice experiment. (A) Sequence at the 5' end of intron C2 in pGT+. Positions of complementarity to U1- α CTC and U6- α CTC are indicated by a horizontal line and a fork shape, respectively, above and below the sequence. A U1 gene with complete complementarity to intron positions 5–13 (U1- α GT2) and a corresponding U6 gene with mutations at the second and third positions of the hexanucleotide to match intron positions 11 and 12 (U6- α GT2) are also illustrated. The potential cleavage site in front of the second GT of the intron is termed GT2. (B) S1 nuclease protection analysis of RNAs from transiently transfected 293 cells using a GT+ version of probe 1 along with probe 2 (see Fig. 2A). The probe-2 protection product is indicated (exon 5) as well as the probe 1 products extending to the 5'ss* or the GT2 site. The two lanes to the right are from a separate experiment. pGT2 expresses a cDNA corresponding to mRNA spliced at the GT2 site instead of the 5'ss*.

Figure 5. Effects of the position of base-pairing with U1 on the efficiency of splicing at mutant U6-specified sites. (A,B,D) S1 protection analyses of RNAs from 293 cells transfected with pGT+ along with the indicated U1 and U6 vectors using probes 1 (GT+ version) and 2 (see Fig. 2A). In B, vector pDNaseE5, which contains a portion of hGH exon 5 in the 3'-untranslated region of the cDNA in the pDNase vector (details are provided in D.-Y. Hwang and J.B. Cohen, in prep.), was included in the pGT+ transfections. mRNA expressed from pDNaseE5 is responsible for the probe 2-protection product labeled DNase (control lanes not shown). The U1 RNAs expressed had 9-nucleotide complementarities to different sequences in the IDX/intron-C2 junction region of GT+ pre-mRNA, as illustrated in C.

plementary to the last nine positions of IDX (Fig. 5C), was more effective in combination with U6- α CTC, which activates the proximal 5'ss (5'ss*; lane 3), than with U6- α GT2, which activates the distal site (GT2; lane 5), whereas the reverse was observed for U1- $\alpha+14$ (lanes 4,6). This indicates cooperation between U1 and U6 and suggests, together with the results of Figure 5A, that the degree of cooperation is a function of the relative positions of the two snRNAs along the substrate. At the

same time, it appears that base-pairing of U1 with the splice site itself is not necessary for optimal cooperation, as U1- α CTC and U1- α GT2 were equally effective, irrespective of which site was selected by U6 (Figs. 4B and 5A).

It is noteworthy in Figure 5B that the overall abundance of IDX-containing RNA (upper and lower signals combined) was diminished when U1- $\alpha(-6)$ was coexpressed with U6- α GT2 (lane 5); a repeat experiment

showed the same effect. Although this could reflect some unexplained effect of U6- α GT2 on the activity of the upstream 3'ss, it is also possible that U1 can misguide U6. A new product is not seen, but it is conceivable that misguidance results in aberrant intermediates that are retained in the nucleus or in a lower processing rate of RNAs committed to the removal of intron C1. We have not yet tested these possibilities.

To strengthen the preceding conclusions, additional U1 RNAs were tested in combination with U6- α GT2. Figure 5D shows that U1- α +22, whose recognition sequence is removed farthest from the GT2 site (see Fig. 5C), was the least effective of the five U1s compared (lanes 5,10), which supports the suggestion that the distance between U1 and U6 is an important parameter. The difference between U1- α +14 on the one hand (Fig. 5D, lanes 4,9) and U1- α CTC and U1- α GT2 on the other (lanes 1 and 6 and lanes 2 and 7, respectively) is less pronounced here than in Figure 5A, which agrees with the results of Figure 5B. Perhaps most interestingly, U1- α GT3 was at least as effective as U1- α CTC or U1- α GT2 (lanes 3,8). This supports the suggestion that base-pairing of U1 with the 5'ss sequence in the usual register is not essential for optimal cooperation with U6. It should be noted that these different U1 RNAs are unable to promote IDX inclusion when their intended target sequences in the reporter pre-mRNA are missing or mu-

tated (D.-Y. Hwang and J.B. Cohen, in prep., and unpubl.).

To obtain further evidence that U1 promotes the selection of nearby recognition sites for U6, we determined whether the position of U1 binding could influence the choice between two competing U6- α CTC-dependent sites. The vector used in this experiment had the CTC·GA site, together with a portion of IDX, inserted between positions 18 and 19 of intron C2 in pGT+ (pGT+/CTC·GA; Fig. 6A); the competing sites were identical at the first eight intron positions. The results in Figure 6B show that the downstream site was used with substantial preference over the upstream site when the coexpressed U1 and U6 RNAs matched both sites (lane 2; cf. the two smallest products marked at the side). This was not unexpected as distal sites (relative to the exon, i.e., the larger product) are generally preferred in situations where functional 5' splice sites compete (for review, see Horowitz and Krainer 1994) and as SF2/ASF, which favors distal sites (Ge and Manley 1990; Krainer et al. 1990; but see also Wang and Manley 1995), is overexpressed in 293 cells (Ge and Manley 1990). The downstream site (larger product) was also favored in the presence of U1- α +14, which could pair nearby (lane 4), but the completely opposite situation was observed for U1- α GT2, whose complementary sequence was near the upstream site (Fig. 6B, lane 3). These results suggest that

Figure 6. The influence of nearby base-pairing with U1 on selection of competing 5' splice sites by U6. (A) Structure of the relevant portion of the competition-reporter plasmid pGT+/CTC·GA. Insertion of a fragment from the CTC·GA reporter into intron C2 of pGT+ (Insertion) resulted in a duplication of the last 32 nucleotides of IDX (truncated solid box), separated from IDX (solid box) by the GT+ intron sequence shown underneath. The insertion regenerated the normal CTC·GA version of intron C2 downstream and the sequence at the start of this intron is also shown. The complementary sequences for different U1 RNAs are underlined, and the positions of the recognition sites for U6- α CTC are indicated by the twin downward arrows above the schematic. (B) S1 nuclease protection results for RNAs from 293 cells transfected as indicated together with pDNase'E5, which was omitted in the p22k+ transfection. The same probe 2 was used as before, but probe 1 had the changes of pGT+/CTC·GA. The three larger products are labeled as in Fig. 5B; the products of probe 1 protection to the upstream or downstream site are indicated with corresponding illustrations.

the downstream site was selected by default and that base-pairing with U1 can drive the selection of an alternative site simply on the basis of proximity. Clearly, this agrees with the suggestion that U1 can direct U6 to the 5'ss. At the same time, possible association of U1 with both sites simultaneously had no obvious negative impact on selection of the default site, as the patterns for U1- α CTC and U1- α +14 were similar (Fig. 6B, lanes 2,4). Without altered U1 RNAs (Fig. 6B, lane 1), there was easily detectable activity at the downstream, but not the upstream site, supporting the suggestion that U6- α CTC selected the downstream site by default. The level of exon inclusion in the presence of just U6- α CTC appears higher here (Fig. 6B, lane 1) than before with other reporter constructs, but this has not been investigated further. As expected, a control experiment showed that neither competing site was selected when the different U1 genes were expressed without U6- α CTC (data not shown). Taken together, our results indicate U1 assists in the selection of 5' splice sites by U6.

Discussion

Previously, it was demonstrated that exon inclusion may be stimulated by an exon-spanning interaction involving U1 snRNA paired with sequences at the 3' boundary and U2AF65, which associates with a pyrimidine-rich sequence in the intron preceding the 5' boundary (Hoffman and Grabowski 1992). This interaction was proposed to permit the coordinate definition of exon boundaries, as demanded by the exon definition model, but it is unlikely that either factor represents an end-point in the line of communication between exon termini. For example, base-pairing with U1 is not sufficient to define the 5'ss.

Our previous work demonstrated that we could disrupt and reestablish communication between U1 and the upstream 3'ss by mutation of the 5'ss and introduction of U1 snRNAs with complementarity to sequences away from the 5'ss, respectively. To determine whether this connection feeds into the process of 5'ss definition, we have first sought to determine whether we could document 5'ss selection by U6 snRNA using a site (CTC · GA) that lacks significant complementarity to endogenous U1 or U6 RNAs. A shift-U1 RNA was used to establish communication with the upstream 3'ss, which had the advantage that interactions between the 5'ss and U6 RNA could be studied under conditions where base-pairing with U1 did not change. From primer-extension experiments with chain-terminating nucleotides, taking into account the relatively high (co-)transfection efficiency of 293 cells (Alwine 1985), we estimate that the introduced U1 and U6 snRNAs accumulate under our conditions to ~5% (U1) and 50% (U6) of the levels of the corresponding endogenous snRNAs in cells expressing the reporter (data not shown). Similar estimates have been reported by others (Zhuang and Weiner 1986; Datta and Weiner 1991). We also note that we have tested many shift-U1s for specificity and have never noticed any activity when the intended target site

was mutated or deleted (e.g., Cohen et al. 1994; see Fig. 1 legend).

Base-pairing with U6 stimulates 5'ss activity

Our results show that splicing at the CTC · GA site is stimulated dramatically by base-pairing with U6 and we have demonstrated by covariation experiments that this effect is specific. Furthermore, two different sites arranged in tandem were stimulated according to their pairing potential with U6, not U1 (Fig. 4B). The interaction involves the first and second nucleotides of the conserved hexanucleotide of U6, and perhaps the fifth as well, which is in agreement with predictions from previous genetic and biochemical studies in yeast and a mammalian system, respectively (Wassarman and Steitz 1992; Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993). We have no evidence at present that speaks to the importance of base-pairing between the fifth U6 base and position 2 of the 5'ss. A simple compensatory change in U6 did not suppress a T2 → A mutation in our reporter gene (not shown). There are conflicting reports on the consequences of this particular change in U6 (Datta and Weiner 1993; Wolff et al. 1994) and it is not clear whether our result implies additional roles for position 5 of the hexanucleotide, position 2 of the intron, or both.

In yeast, base-pairs outside the classical 5'ss signal can compensate for mismatches within (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993), but we could not demonstrate this in our situation. Disruption of two potential base-pairs outside the signal reduced splicing, but no evidence was obtained that the mutations could be suppressed by compensatory changes in U6. It remains to be determined whether this is attributable to disruption of the recently identified helix III between U6 and U2 snRNAs (Sun and Manley 1995) or whether positions 8 and 9 of our substrate support splicing by an unrelated mechanism.

The function of base-pairing with U6

Our results indicate that the identity of the first two positions of the conserved hexanucleotide of U6 is irrelevant to the stimulation of 5'ss activity as long as base-pairing is maintained. This suggests that this portion of the hexanucleotide serves largely, if not exclusively, to facilitate recognition of the 5'ss region by U6 and promote binding. Any additional role in subsequent stages of the splicing process appears unlikely if it is assumed that the spliceosome contains a single molecule of U6 RNA, which is not exchanged before the reaction is complete. The same is suggested by *in vitro* results showing that individual changes in the first two positions of the hexanucleotide of U6 do not prevent productive splicing (Wolff et al. 1994). The dedication of nucleotides in U6 to base-pairing with splice-site positions that are not universally conserved is reminiscent of the situations with both U1 and U2 snRNAs, and perhaps U5 as well (for reviews, see Green 1991; Moore et al. 1993; Madhani and

Guthrie 1994). It appears that individual mismatches with U1 can be accommodated as long as the balance remains significant, perhaps five positions of complementarity or greater. The interaction of U2 with the branchpoint region appears more forgiving, which may reflect the involvement of a number of proteins to recruit U2 and stabilize its interaction with pre-mRNA (for review, see Hodges and Beggs 1994). Where limited complementarity suffices, as appears to be the case for the interactions of both U2 and U6 with pre-mRNA, base-pairing may serve less as the basis of recognition globally and more to ensure the correct local fit and hold the two interacting RNAs together. It is possible that functions such as these are supported equally well by fortuitous complementarity elsewhere, which could be at the basis of the yeast results that mismatches between U6 and position 5 of the 5'ss can be suppressed by complementarity downstream (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993). Whereas proteins may coach U2 to the correct site, our work hints that U1 may assume this role with respect to U6 (see below).

Our results are consistent with the suggestion that U6 stimulates a unique site determined by base-pairing. It is less clear, however, whether U6 defines the 5' cleavage position. We observe a low level of splicing in the absence of foreign U6 RNAs at sites that lack complementarity to endogenous U6 (e.g., Fig. 4B, lane 4), suggesting other interactions contribute or may even be sufficient. For example, U5 snRNP and auxiliary factors, perhaps in addition to SR proteins, appear to be involved in 5'-cleavage site definition (Newman and Norman 1991, 1992; Cortes et al. 1993; Crispino et al. 1994; Kohtz et al. 1994; Tarn and Steitz 1994). It is possible that U1 also contributes at this stage, but the evidence is not strong. For example, the weak 5'ss* signal in the absence of altered U6 RNAs in Figures 4B and 5A,D is not specifically increased by the matching U1- α CTC as compared to other U1 RNAs. At best, there is a hint in Figure 4B (cf. lanes 1 and 4) that U1- α GT2 may enhance selection of the GT2 site. Thus, if U1 is involved at all, complementarity is not the key. In all, although the data may not show that base-pairing with U6 is critical for the definition of cleavage sites, it is clear that U6 determines whether or not a site will be used efficiently.

Cooperation between U1 and U6

Our comparison of U1 RNAs with complementarity to splice sites or neighboring sequences suggests that U1 acts in direct support of U6, not merely indirectly by stimulating the upstream 3'ss. Whereas different U1 RNAs appeared to interact with pre-mRNA with roughly comparable efficiencies, as suggested by the similar amounts of intron-retention product seen with each U1 alone, the efficacies of the two U6 mutants tested varied dependent on which U1 RNA was coexpressed (Fig. 5A,D). Importantly, we have also shown that the effects of two individual U1 RNAs varied dependent on which U6 mutant was coexpressed (Fig. 5B). In addition, we have shown that U1 can alter dramatically the selection

of competing recognition sites for U6 (Fig. 6). Together, these results strongly indicate that U1 facilitates the selection by U6 of sites for stimulation. Our data are generally consistent with a correlation between efficacy and the distance between U1 and U6, but within narrow limits, a surprising degree of flexibility without loss of activity was noted in the relative positions of the two snRNAs. On the basis of these results, we propose that U1 promotes association of U6 snRNP with pre-mRNA locally and that base-pairing both favors a particular U6/pre-mRNA alignment in this local environment and stabilizes the association once formed.

To the extent that we suggest that U1 permits specific interaction of splice sites with U6 and that this interaction determines the preferred cleavage position, our interpretation is in line with the evidence that base-pairing with U1 contributes to the fidelity of 5'ss choice (Tarn and Steitz 1994). However, our data argue that prereading of the actual site by U1 is not necessary and instead, they favor a more flexible, cooperative role for U1. The notion of cooperativity is in apparent conflict with the observation that U1 inhibits the assembly on a 5'ss consensus oligoribonucleotide of a multi-snRNP complex containing U6 (Konforti et al. 1993). However, our proposal is not conditional on continued pairing of U1 with the 5'ss at the time base-pairing with U6 is established. Instead, the U1/pre-mRNA helix probably unwinds as U6 moves in, a process referred to as a "handing-off" (Madhani and Guthrie 1994). Alternatively, U1 may vacate the 5'ss before U6 enters, but remain transiently associated with the area by protein factors such as SF2/ASF, which is capable of binding both the 5'ss region and U1 snRNP (Wu and Maniatis 1993; Kohtz et al. 1994; Zuo and Manley 1994); it is possible that the short oligoribonucleotide used by Konforti and co-workers (1993) would not support such a process. In fact, it is quite possible that the effect we ascribe to U1 is mediated through SF2/ASF or perhaps some other SR protein(s). Tarn and Steitz (1994) have suggested that SF2/ASF assists in recruiting U6 to the 5'ss, and our results could indicate that this as yet putative activity is sensitive to modulation by U1.

Exon definition and 5'ss identification

Previous work has indicated that splicing of IDX conforms to some of the hallmarks of the exon definition model. Thus, mutations in either splice site flanking the exon result in exon skipping and compensatory base changes in U1 to restore complementarity with the 5'ss region stimulate use of the upstream 3'ss (Cohen et al. 1989, 1994; D.-Y. Hwang and J.B. Cohen, in prep.). Although the mechanism is not fully understood, it seems clear that 3'ss selection is coupled to recognition of the branchpoint region by U2 snRNP (Smith et al. 1989; Moore et al. 1993) and communication between U1 and U2 across the exon, however indirect it may be (Hoffman and Grabowski 1992; Wu and Maniatis 1993), appears to be a given in a situation like ours (Berget 1995). U2 also interacts directly with U6 through base-pairing (Datta

and Weiner 1991; Wu and Manley 1991; Madhani and Guthrie 1992; Sun and Manley 1995), and in turn, U6 first interacts with the 5'ss and at a later stage with the branchpoint region (Wassarman and Steitz 1992; Sontheimer and Steitz 1993). The previous indications that U1 is not responsible for definition of the 5' cleavage site and that a 2-bp interaction with U6 provides the reference instead (for review, see Madhani and Guthrie 1994) raised the question how specificity is accomplished in 5'ss selection. In other words, how can U6 distinguish between correct and aberrant sites on the basis of minimal complementarity and how does it search for the correct site, perhaps while connected to the 3' region of the intron via U2? It further raised the question of how the position of the upstream 3'ss is acknowledged in the process of 5'ss selection, a prerequisite for exon definition (Robberson et al. 1990; Berget 1995), if not through U1. Our results suggest that U1 is in fact part of the answer. They are consistent with the interpretation that U1 communicates across the exon to promote definition of the upstream 3'ss and subsequently, or perhaps simultaneously, across the intron to coach U6 to the 5'ss. In this view, base-pairing with U1 determines in broad terms where splice junctions can be defined, whereas U6 picks the actual 5'ss from the menu of local possibilities. Generally, our results and those of others (Tarn and Steitz 1994) suggest active recruitment of U6 by factors also involved in communication across exons, like U1 and SR proteins, which may represent a previously missing, although perhaps suspected, link in the chain of events leading to precise exon selection.

Materials and methods

Plasmids

The hGH.FSX, U1, and DNase vectors and the procedures used to introduce mutations have been described (Cohen et al. 1993, 1994; D.-Y. Hwang and J.B. Cohen, in prep.). The same PCR-based procedure was used to generate pGT+, it has the 11 nucleotides between IDX and the first *SmaI* site of intron C2 (wild-type) replaced by the 18-nucleotide GT+ sequence shown in Figure 6A at the left and also in Figures 4A and 5C (intron positions 1–18). The essence of the strategy to generate competing splice sites in pGT+/CTC·GA is illustrated in Figure 6A; details are available on request.

Most of the hGH.FSX-related cDNA plasmids have also been described (Cohen et al. 1994; D.-Y. Hwang and J.B. Cohen, in prep.); pcryp+12 is p22k.cryp in Cohen et al. (1994). pcryp2+34 was constructed as described elsewhere for pcryp2+12 and pcryp2+22 (D.-Y. Hwang and J.B. Cohen, in prep.); the cDNA in this plasmid contains the first 34 nucleotides of intron C2 (CTC·GA) between IDX and exon 4. pGT2 was constructed in the same manner using RNA from 293 cells cotransfected with pGT+, pU1- α GT2, and pU6- α GT2.

A human U6 gene was cloned from genomic 293-cell DNA by PCR using forward primer U6-5'A (5'-GAAGAGGGCCTATT-CCCCATG-3') and reverse primer U6-3' (5'-TGCACCGGCG-TATAACGTGG-3'). The 442-bp amplified fragment containing ~250 and ~80 bp of 5'- and 3'-flanking sequences, respectively, was inserted into the *SmaI* site of pUC119 (Vieira and Messing 1987) and the sequence of the insert was determined. The sequence of our "wild-type" clone (phU6-wt) differs from

the sequence published by Kunkel and Pederson (1988) at positions -102 (T deletion), -11 (G $\rightarrow$ A substitution), and -1 (C $\rightarrow$ T); these may be PCR-related mutations. phU6-wt was used as template for site-directed mutagenesis of the U6-coding sequence by the same PCR-based approach referred to above; details will be provided on request. All new PCR-derived regions in our constructs were entirely sequenced.

Other procedures

All other procedures used here have been described and specific references can be found in D.-Y. Hwang and J.B. Cohen (in prep.). The 3' end-labeled IDX-specific S1 probes used here were the same as described elsewhere (D.-Y. Hwang and J.B. Cohen, in prep.), except for changes in the 5'ss region to match mutations or other alterations in the transfected reporter plasmids.

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