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Suppression of mammalian 5′ splice-site defects by U1 small nuclear RNAs from a distance

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ABSTRACT **One of the earliest events in the process of intron removal from mRNA precursors is the establishment of a base-pairing interaction between U1 small nuclear (sn) RNA and the 5′ splice site. Mutations at the 5′ splice site that prevent splicing can often be suppressed by coexpression of U1 snRNAs with compensatory changes, but in yeast, accurate splicing is not restored when the universally conserved first intron base is changed. In our mammalian system as well, such a mutation could not be suppressed, but the complementary U1 caused aberrant splicing 12 bases downstream. This result is reminiscent of observations in yeast that aberrant 5′ splice sites can be activated by U1 snRNA from a distance. Using a rapid, qualitative protein expression assay, we provide evidence that 5′ splice-site mutations can be suppressed in mammalian cells by U1 snRNAs with complementarity to a range of sequences upstream or downstream of the site. Our approach uncouples *in vivo* the commitment-activation step of mammalian splicing from the process of 5′ splice-site definition and as such will facilitate the genetic characterization of both.**

The accurate recognition of exon–intron boundaries in nuclear mRNA precursors (pre-mRNA) in complex organisms such as humans is a remarkable phenomenon in view of the generally limited conservation of the signals involved (for recent reviews, see refs. 1 and 2). In particular, the 5′ end of introns in most organisms is located within a 5′ splice-site consensus sequence [(C,A)AG/GU(A,G)AGU, where / indicates the cleavage site] that is highly conserved at just two positions (underlined); 5′ splice sites with no more than six matches to the consensus are not at all uncommon (3, 4). Selection of 3′ splice sites appears to be directed by a more complex signal, but the information content of the constituent elements, a poorly conserved branch-point sequence, a polypyrimidine tract of variable length, and an essential AG dinucleotide preceding the cleavage site, is limited.

Among the recent advances in our understanding of splice-site selection mechanisms was the proposal that exons, rather than introns, may represent the initial units of recognition for the splicing machinery (5). According to this so-called "exon definition" model, pairs of narrowly spaced (≤300 nt) upstream 3′ and downstream 5′ splice-site signals conspire to define the exons in a pre-mRNA; intron definition could be passive. Additional sequence elements may be involved to facilitate the necessary communication between splice sites across exons (6–8).

The selection of splice sites in the context of exons may be advantageous to higher eukaryotes, where multiple intervening sequences with enormous length heterogeneity may be encountered in individual pre-mRNAs. It is not immediately obvious, however, whether the same approach should benefit an organism such as the yeast *Saccharomyces cerevisiae*, which has been a valuable source of information on pre-mRNA splicing over the years (9–12). *S. cerevisiae* introns are comparatively small and have highly conserved 5′ splice-site and branch-point regions. Genes with introns are relatively rare in this yeast, multi-intron genes are sparse, and alternative splicing has not been observed (for reviews, see refs. 13 and 14). At least some of the presently identified mammalian splicing factors may function in exon definition and/or splice-site selection (6, 7, 15–20), but few yeast homologs have been detected to date (21, 22). These and other considerations argue that similarities between the two systems with respect to the mechanism of splice-site selection cannot be taken for granted.

The 5′ splice-site consensus sequence is complementary to U1 small nuclear (sn) RNA, and compensatory changes in U1 can suppress certain mutations that interfere with splicing (23–25). In yeast, however, splicing may occur outside the region of complementarity (24–27). At least in this organism, the cleavage site is therefore not defined relative to the base-paired region.

We have studied this issue in mammalian cells by transfection experiments. We initially found that, much like in yeast (24–27), a U1 snRNA with specificity for an upstream, mutationally silenced 5′ splice site activated a downstream aberrant site with minimal complementarity. To explore this observation, we determined whether splicing activity at a defective 5′ splice site could be restored by U1 snRNAs with complementarity to different sequences in the vicinity. Our analysis demonstrates that defective 5′ splice sites can regain activity when base pairing with U1 is established at any of a number of positions upstream or downstream of the site. This results in splicing of a skipped exon, which indicates that U1/pre-mRNA base-pairing functions *in vivo* at a stage analogous to the splicing-commitment step defined *in vitro* (for review, see ref. 2). The observed flexibility with respect to the position of U1 binding is consistent with an *in vivo* role of U1 in early events at the 3′ splice site across the exon (see refs. 15 and 28). At the same time, our data suggest that base pairing with U1 plays a minor role at best in the actual selection of mammalian 5′ splice sites.

MATERIALS AND METHODS

Plasmids. Mutant hGH.FSX (where hGH is human growth hormone) and U1 genes were generated by oligonucleotide-directed mutagenesis, as described (29) with minor modifications. Details will be provided on request. All mutant genes were sequenced through the PCR-derived region(s). pDNase is pDNaseQ106 in ref. 29. pRL.DNase was constructed from pDNase by replacing the upstream cytomegalovirus/intron region with the Rous sarcoma virus long terminal repeat from pRL.p21:GN (29). p22k was previously obtained by reverse

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Abbreviations: snRNA, small nuclear RNA; hGH, human growth hormone; RIP, radioimmunoprecipitation assay; RT-ds, reverse transcription/second-strand synthesis; IDX, intron D exon.

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transcription of cytoplasmic RNA from 293 cells transfected with phGH.FSX:G4A5 (29) and PCR with primers flanking the coding sequence. The amplified material was introduced into the polylinker region of vector pRK5 (30). Vector p22k+ was generated by insertion of a frame-shifted (FS) version of the alternative H-*ras* exon IDX (intron D exon) (31) into p22k using PCR-generated fragments. The upstream hGH/IDX junction was specified by a reverse PCR primer, and the downstream junction was obtained by reverse transcription-PCR with RNA from cells transfected with phGH.FSX:G4A5 in the presence of pU1-αG4A5 (29). The downstream IDX:cryp (cryp is an aberrant 5′ splice site 12 bases downstream of the authentic IDX site)/hGH junction was cloned after reverse transcription of RNA from C1/αC1-transfected cells with a 20-mer oligonucleotide complementary to hGH mRNA in the 3′ half of exon 4 (5′-CCTTTAGGAGGTCATAGACG-3′). Ten percent of the cDNA was amplified by PCR with a forward 23-mer of sequence identical to the 5′ end of IDX (5′-GGCAGCCGCTCTGGCTCTAGCTC-3′) and a reverse 23-mer complementary to a sequence at the center of exon 4 in hGH mRNA (5′-AGAGGCGCCGTACACCAGGCTGT-3′). The products were cloned into *Sma* I-digested plasmid pUC119 (32), and recombinants were characterized by restriction enzyme digestion and sequencing. Vector p22k.cryp was constructed by replacing the *Sma* I–*Bst*XI fragment that spans the IDX/hGH exon 4 junction in p22k+ by the corresponding fragment from one of the subclones containing the IDX:cryp/hGH junction.

Transfections and Protein Assay. Human 293 cells were transfected with equal amounts of phGH.FSX, pU1, and pDNase vector (5–10 μg) or 2–5 μg of hGH cDNA vector. The transfection procedure, [^{35}S]methionine labeling, immunoprecipitation, and subsequent manipulations were exactly as described (29).

Primer Extension, Reverse Transcription, Second-Strand Synthesis (RT-ds). Primer extension was done as described (29). For RT-ds, 30 μg of cytoplasmic RNA was reverse transcribed from an unlabeled 15-mer complementary to the 5′-terminal bases of the mRNA strand of hGH exon 4. The reaction was terminated by chloroform extraction, and the RNA was degraded with 0.2 M NaOH at 60°C for 15 min. After neutralization with HCl and ethanol precipitation, the products were resuspended in PCR buffer [20 mM Tris·HCl (pH 8.3)/50 mM KCl/2.5 mM $MgCl_2$/1 mM dithiothreitol/bovine serum albumin at 0.1 mg/ml], 30 pmol of a ^{32}P-labeled 24-mer corresponding in sequence to bases 18–41 of IDX was added, and the mixture was heated for 2 min at 95°C and incubated for 2 hr at 65°C under mineral oil. Second-strand synthesis was then done with *Taq* polymerase and the four deoxyribonucleotides at 0.5 mM each for 1 hr at 71°C.

RESULTS

To study the effect of U1 snRNA on 5′ splice-site activity *in vivo*, we use a chimeric, multi-intron mammalian reporter gene, generically termed hGH.FSX (Fig. 1), which is cotransfected with mutated U1 snRNA genes into cultured human 293 cells. The cells are labeled 2 days later with [^{35}S]methionine, and immunoprecipitated medium samples are analyzed by gel electrophoresis (29). The assay is used here to determine whether the alternative exon IDX^{FS} (31), derived from the human H-*ras* gene and present in hGH.FSX, is spliced. Accurate splicing results in the synthesis of a fusion protein, termed 22kD+ (22kD+IDX^{FS} in ref. 29). Mutations in the intron portion (positions 1–6) of the wild-type IDX splice donor (GCU/GUAAGU) generally interfere with IDX splicing (ref. 29; see also Fig. 4) and a smaller protein, termed 22kD, is formed. The assay provides an approximation of accurate IDX splicing activity, which is best reflected by the abundance of the 22kD+ protein in the

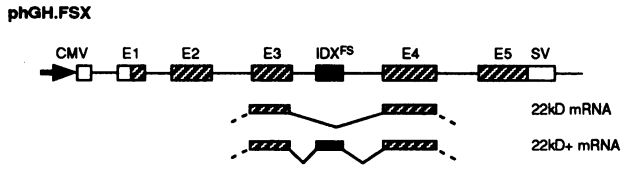

FIG. 1. Structure of the splicing reporter construct (29). phGH.FSX contains the hGH gene (hatched boxes and connecting lines) interrupted by the H-*ras*-derived, alternatively spliced exon IDX^{FS} (black box; ref. 31) and flanking DNA. The gene is preceded by an artificial intron and a transcription control region from cytomegalovirus (CMV; horizontal arrow). The two major alternative splicing pathways are depicted underneath. The 22kD+ protein is the major product of the vector with a wild-type IDX splice donor (see Fig. 4). E1–E5, hGH exons; SV, SV40 early poly(A) region; open boxes, untranslated sequences.

experimental sample relative to the total hGH protein level in the wild-type U1 control; the two hGH proteins are expressed at similar levels from cDNA vectors (unpublished work), and differential mRNA or protein stability are therefore not significant factors. On the other hand, decreases in the level of the 22kD protein, independent of any IDX splicing product, may be the better measure of overall commitment to IDX splicing, whether accurate or aberrant, productive or not. DNase protein is coexpressed as internal standard (29).

U1 Activates a Cryptic 5′ Splice Site from an Upstream Position. Vector phGH.FSX:C1 has the first-intron base (position 1) of the IDX splice donor changed from the universally conserved guanine to cytosine (29). Cotransfection of this reporter with the wild-type U1 gene (pU1-wt) resulted in the exclusive synthesis of 22kD hGH protein (Fig. 2, lane 2), but a larger protein appeared in the presence of pU1-αC1, which expresses a U1 snRNA with complete complementarity to the mutated IDX site (lane 3). The larger protein migrated slower than 22kD+ expressed from a cDNA vector (lane 4), suggesting it was not produced as a result of simple suppression of the splice-site mutation. The protein's identity was explored by reverse transcription of cytoplasmic RNA from C1/αC1-transfected cells and amplification of the resulting cDNA with IDX and hGH exon 4 primers. The amplified material was cloned, and each clone was found to contain the same insert. DNA sequencing localized a new 5′ splice site, termed cryp, 12 bases downstream of the authentic IDX site (Fig. 2 *Lower*). The IDX/hGH exon 4 junction in p22k+ was replaced by the new junction to generate cDNA expression vector p22k.cryp, which was found to express a protein that comigrates with the U1-αC1-induced protein, now termed 22kD-cryp (Fig. 2, lanes 5 and 6). These data indicated that U1-αC1 activated a silent, downstream splice site to which it had little complementarity. The observation was reminiscent of previous reports that aberrant cleavage in yeast at positions that lack complementarity to U1 was stimulated by U1 snRNAs with increased complementarity to a disabled site in the vicinity (24–27). The responsible mechanism has not been described in much detail, however.

A Splice-Donor Mutation Is Suppressed by an Intron-Specific U1 snRNA. A second effect of U1-αC1 can also be discerned in Fig. 2. Although the film was overexposed to bring out the 22kD-cryp band in lanes 3 and 5, a clear reduction in the total hGH protein signal in the presence of U1-αC1 is apparent. To avoid this complication, we used a reporter with a less severe mutation to determine whether U1 can activate defective splice sites from a distance. The $G^5 \rightarrow A$ mutation in the IDX splice donor in vector phGH.FSX:A5 (29) abolishes IDX splicing, but this defect is efficiently suppressed, unlike the C1 mutation, by coexpression of a U1 snRNA with complete complementarity to the site (U1-αA5; Fig. 3*A*, lanes 1, 2, and 4). Lane 3 of Fig. 3*A* shows that

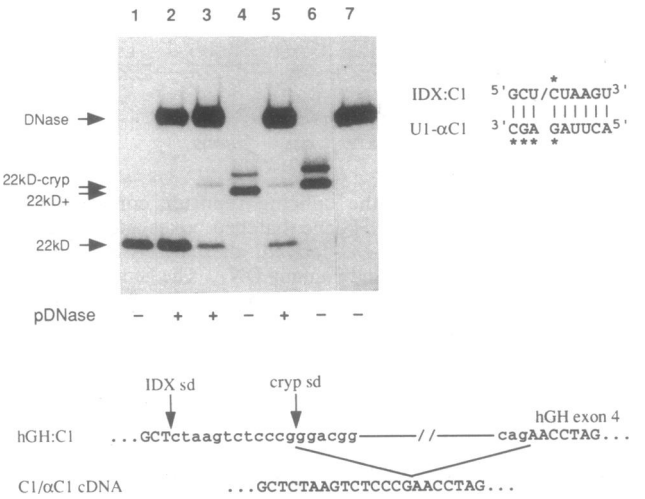

FIG. 2. Immunoprecipitation of labeled medium (radioimmunoprecipitation assay; RIP) from transiently transfected 293 cells. (*Upper*) phGH.FSX:C1 (29) was coexpressed with wild-type U1 (lane 2) or U1-αC1 (lanes 3 and 5). hGH cDNA expression vectors p22k (22kD expression; lane 1), p22k+ (22kD+ expression; lane 4), and p22k.cryp (22kD-cryp expression; lane 6) were transfected in parallel. Lane 7, pUC118. Vector pDNase (29) was included as shown underneath. Lanes 3 and 5 show the same sample, loaded twice to facilitate comparison with the cDNA products in lanes 4 and 6. hGH proteins often migrate as well separated doublets (e.g., lanes 4 and 6); the larger forms may represent uncleaved precursor leaked into the medium. The sequence of the IDX:C1 splice site (/ marks the normal IDX cleavage position) and positions 11–3 (left to right) of the complementary U1-αC1 are shown at right. ⋆, Changes relative to the wild-type IDX and U1 sequences; |, complementarity. (*Lower*) The sequence at the IDX/hGH exon 4 junction in cDNA from C1/αC1-transfected cells compared with the corresponding region in the gene (exons in capitals). The cryptic splice site is marked with an arrow (cryp sd).

Ul-α+4(A5), which is complementary to positions 2–10 of the downstream intron (Fig. 3*A*, *Right*; the intron region is referred to as the +4 target), induced the 22kD+ protein with significant efficiency as well. The, perhaps proportional, decrease in the level of the 22kD protein signifies a shift in splicing commitment from one pathway to another. Analysis of cytoplasmic RNA by reverse transcription and second-strand synthesis from a ^{32}P-labeled IDX primer (RT-ds, Fig. 3*B*) confirmed that accurate splicing occurred at the IDX splice donor in both cases but not in the U1-wt control (Fig. 3*C*). Fig. 3*D* validates these results by demonstrating that there was no significant variation in the total amount of reporter RNA expressed in the presence of the three U1s. Together, the data show that U1-α+4(A5) is an efficient suppressor of the G^5 → A mutation. Activating U1s with complementarity to sequences away from the eventual cleavage site are referred to here as shift-U1s.

The results in Fig. 4 (*Upper*) confirm that shift-U1-mediated 5′ splice-site activation depends on base-pair formation. Three different reporter genes were coexpressed with the same set of three shift-U1s (Fig. 4 *Lower*); wild-type U1 (lanes 3, 8, and 13) and a positive control (lanes 4, 9, and 14) were included in each case. U1-α+4(G4) had a complementary +4 target in G4 pre-mRNA and induced 22kD+ synthesis from the corresponding gene (lane 6). No 22kD+ protein was detected, however, when the same U1 was coexpressed with the A5 reporter, which mismatched U1-α+4(G4) at two internal positions (lane 11). Conversely, U1-α+4(A5) induced 22kD+ synthesis from the matched A5 but not from the mismatched G4 gene (lanes 12 and 7). The third gene contained a 4-nt insertion directly after the A5 splice site. The insertion disrupted the complementarity to

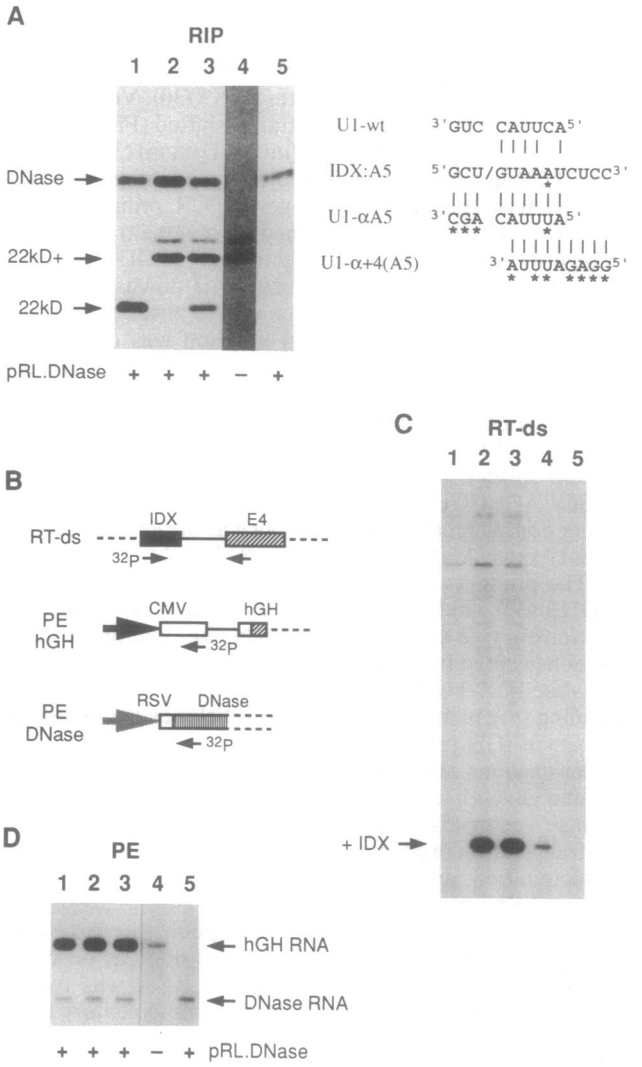

FIG. 3. RIP (*A*) and RNA analyses (*C* and *D*) of cells transfected in duplicate with phGH.FSX:A5 (29) and different U1 genes. pRL.-DNase, which expresses DNase cDNA from the Rous sarcoma virus (RSV) long terminal repeat, was cotransfected as shown. An alignment of positions 11–3 of the different U1s with the hGH.FSX:A5 pre-mRNA sequence is included in *A*. ⋆, Nucleotides that differ from the wild-type IDX or U1 sequence. Lane 4 was from a longer exposure than the other lanes. (*B*) RT-ds and primer extension (PE) strategies. Primers (^{32}P-labeled or unlabeled) are shown as small arrows. The PE primers are complementary to the first (untranslated) exon of phGH.FSX (see Fig. 1) or sequences just downstream of the DNase initiation codon in pRL.DNase. The RT-ds products marked in *C* (+IDX; 79 nt) result from accurate splicing at the IDX splice donor. In *D*, lanes 4 and 5 were from a longer exposure of the film than lanes 1–3. The product sizes are 126 (hGH mRNA) and 108 (DNase mRNA) nt. Lanes: 1–3, phGH.FSX:A5 + pU1-wt, pU1-αA5, and pU1-α+4(A5), respectively; 4, p22k+; 5, pUC118.

U1-α+4(A5) but created a 9-base complementarity to U1-α+4(G4) 4 bases further down. In this case, 22kD+ synthesis was induced by U1-α+4(G4) (lane 16) but not by U1-α+4(A5) (lane 17). Lanes 5 and 15 illustrate that a double mismatch at the two ends could be tolerated. As expected, however, the same U1 had no effect when it mismatched the reporter at two additional positions (lane 10). Together, the data strongly argued that significant base complementarity was required for the effect. The results with the third vector also indicated that the defective A5 site could be activated from a greater distance. It is not always clear here or below that the abundance of the 22kD+ protein is fully proportional to the decrease in 22kD signal (compare lanes 5 and 6). Accord-

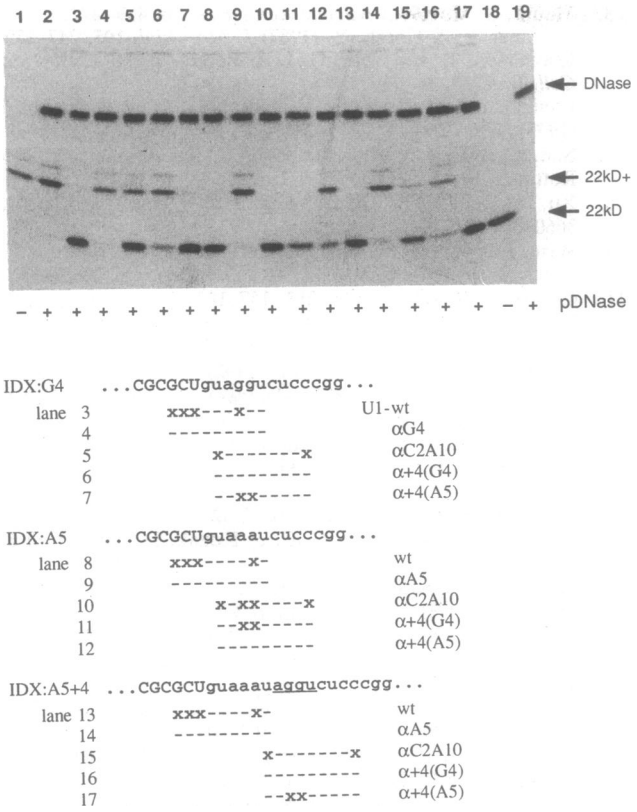

FIG. 4. (*Upper*) RIP analysis of medium from cells transiently transfected with the combinations of reporter and U1 genes illustrated below. Lanes: 1, p22k+; 2, phGH.FSX:wt + pU1-wt; 18, p22k; 19, pUC118. Lane 2 illustrates the activity of the wild-type IDX splice donor. (*Lower*) Sequences at the IDX (uppercase)/intron (lowercase) boundaries in three hGH.FSX reporter pre-mRNAs and complementarity (dashes) with positions 11–3 (left to right) of coexpressed U1s. Only the best alignments are shown. *x*, Mismatched positions. The IDX:G4 site differs from wild type by an $A^4 \rightarrow G$ change. The insertion in the A5+4 reporter is underlined.

ingly, accurate IDX splicing may be the common, but perhaps not always unique, consequence of shift-U1 coexpression with the reporters of Fig. 4.

Flexibility of Shift-U1 snRNAs. To begin to explore some of the possible constraints of the shift-U1 effect, we generated U1 genes complementary at bases 3–11 to a variety of sequences in the A5 reporter construct both upstream and downstream of the IDX splice site (Fig. 5 *Lower*). Remarkably, each U1 stimulated IDX splicing, although perhaps with variable efficiencies (Fig. 5 *Upper*). The specificity of the effect has been confirmed for a number of these U1s, and one example is included in Fig. 5 (lanes 15–17, compare with lane 2). As before, there appears to be a reduction in the combined signal of the two hGH bands in some of the lanes here (e.g., lane 14), suggesting that the corresponding U1s have additional effects.

DISCUSSION

We have used a simple protein assay to study splicing of a multi-intron mammalian pre-mRNA *in vivo*. These data demonstrate that U1 snRNA can activate defective mammalian 5′ splice sites through base pairing with sequences elsewhere. The same site was activated whether base pairing occurred nearby or further away, upstream or downstream of the site. This result suggests that activation and the definition of the 5′ cleavage site are separate events. The A5 and G4 substrates appear defective in the base-pairing-dependent recruitment of U1 to the IDX region but not in the interactions that define the 5′ splice site. In the C1/αC1 case, base pairing appears to establish a commitment to IDX splicing, as suggested by the substantial decrease in 22kD signal. *In vitro* studies have shown that U1 snRNA is a component of the earliest commitment complexes (33, 34), whereas the U5 and U6 small nuclear ribonucleoproteins (snRNPs), implicated in 5′ splice-site definition (e.g., refs. 35 and 36), are known to join the spliceosome at a later stage (2). Our work appears to uncouple the corresponding steps *in vivo*.

In yeast, base pairing of U1 snRNA with the 5′ splice site (or perhaps nearby sequences) is required for U2 snRNP

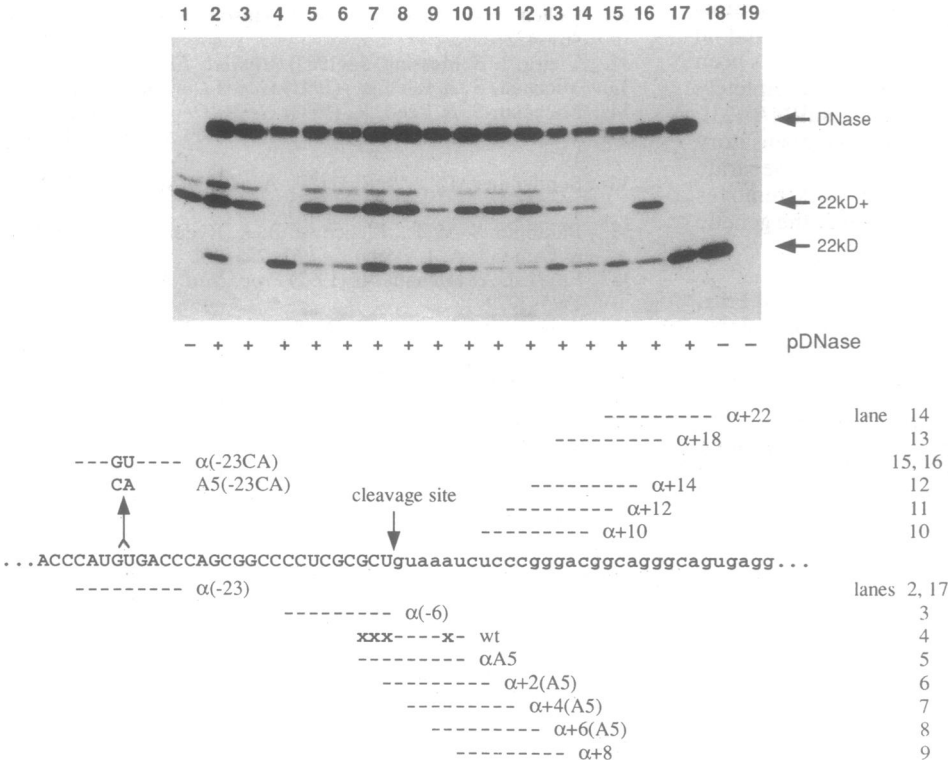

FIG. 5. (*Upper*) RIP analysis. phGH.FSX:A5 (lanes 2–15) or a derivative [phGH.FSX:A5(-23CA); lanes 16 and 17] were cotransfected with different U1 genes and protein profiles visualized as before. Lanes: 1, p22k+; 18, p22k; 19, pUC118. Other lanes are referenced underneath. (*Lower*) U1 genes with complementarity to different regions are represented above and below the sequence surrounding the IDX:A5 splice donor (exon in capitals). The corresponding lanes on the autoradiograph are indicated at right. The changes in phGH.FSX:A5(23CA) to test the specificity of U1-α(-23) are indicated at left. Also shown are the complementary changes in pU1-α(23CA).

binding to the branch region, an early step in spliceosome assembly (for review, see ref. 28). This requirement may not generally apply to mammalian systems, at least *in vitro* (28, 37, 38), but in one case, base pairing was shown to support a function of U1 snRNA that stimulates the association of splicing factor U2AF65 with the upstream 3′ splice-site region (15); U2AF is necessary for the recruitment of U2 snRNP (28, 39). We imagine that the A5 and G4 substrates have lost the ability to support such a function across the exon as a result of changes in the binding site for endogenous U1 and that shift-U1 activity reflects the rescue of this function. This proposal could explain the observed flexibility in the position of U1 RNA binding.

If, as suggested, 3′ splice sites are the principal targets of U1 snRNA, the position of U1 binding would not be expected to influence 5′ splice-site choice unless the activation and 5′ splice-site selection steps communicate. It is noteworthy in this regard that U1-α+12, which matches the cryp site, activates the upstream IDX:A5 site instead of the competing, proximal target. Thus, proximity to the U1 snRNA binding site fails to provide (absolute) priority during 5′ splice-site selection. This interpretation is consistent with the view, based largely on experiments with competing sites, that 5′ splice sites are selected principally on the basis of inherent strength (for review, see ref. 1). In yeast, base-pairing interactions with both U5 and U6 snRNAs are important for the selection of a 5′ cleavage site (27, 40–42), and 5′ splice-site strength may well depend, at least in part, on the quality of these interactions. This may also be true for mammalian 5′ splice sites, given the biochemical evidence that they, as well, interact with U5 and U6 snRNA (35, 36, 43). With respect to U1, our data and the analogous earlier work in yeast (24–27) argue that complementarity to U1 snRNA is not a decisive determinant of strength in either system. This conclusion is strongly supported by our recent observation that accurate splicing occurs in the presence of shift-U1s at 5′ splice sites that are complementary to endogenous U1 at just three positions (D.-Y. Hwang and J.B.C., unpublished results).

A genetic analysis of mammalian 5′ splice sites to explore the functional significance of the proposed base-pairing interactions with U1, U5, and U6 snRNAs is not straightforward because any single nucleotide may be involved in interactions with multiple snRNAs. For example, it has been shown that a change at position 3 is at best incompletely suppressed by a compensatory change in U1 snRNA (23). Instead, complete suppression may require compensatory changes in more than one snRNA, which introduces separate variables that are not easily controlled. Our work removes one of these variables and as such should simplify the genetic characterization of the remaining interactions.

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