CUS1, a suppressor of cold-sensitive U2 snRNA mutations, is a novel yeast splicing factor homologous to human SAP 145

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The function of U2 snRNA in splicing is mediated by the proteins of the U2 small nuclear ribonucleoprotein. To identify proteins that influence the function of U2 snRNA we carried out a screen for mutations in *Saccharomyces cerevisiae* that suppress the cold-sensitive growth defect of a mutation in U2 stem loop IIa, a structure important for the stable association of the U2 snRNP with pre-mRNA. The screen identified three dominant suppressor genes, one of which, *CUS1-54*, encodes an essential splicing protein required for U2 snRNP addition to the spliceosome. The suppressor protein rescues the spliceosome assembly defect of the mutant U2 in vitro, indicating that suppression is direct. Allele specificity tests show that the suppressor does not simply bypass the requirement for U2 stem loop IIa. Extra copies of wild-type *CUS1*, but not *CUS1-54*, suppress the temperature-sensitive *prp11* and *prp5* mutations, linking *CUS1* protein to a subset of other factors required at the same step of spliceosome assembly. *CUS1* is homologous to SAP 145, a component of the mammalian U2 snRNP that interacts with pre-mRNA. The yeast genome also encodes a homolog of human SAP 49, a protein that interacts strongly with both SAP 145 and pre-mRNA, underscoring the conservation of U2 snRNP proteins that function in spliceosome assembly.

[*Key Words*: U2 snRNA; *Saccharomyces cerevisiae*; spliceosome assembly; pre-mRNA]

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The spliceosome is the large ribonucleoprotein particle responsible for removal of introns from nuclear pre-mRNA transcripts. Spliceosome assembly and function are complex and dynamic, involving multiple transient protein–protein, protein–RNA and RNA–RNA interactions. The spliceosome is built on an intron-containing transcript by the sequential recognition of conserved sequence elements near the reactive sites. A transcript first becomes committed to the splicing pathway by stable association with the U1 small nuclear ribonucleoprotein (snRNP) to form the commitment complex, a step that does not require ATP. Stable binding of U2 snRNP to the commitment complex near the pre-mRNA branchpoint requires ATP and forms the prespliceosome. With the binding of U5/U4.U6 tri-snRNP the spliceosome is assembled but must be activated before the cleavage and ligation reactions begin (for review, see Moore et al. 1993; Newman 1994; Nilsen 1994; Ares and Weiser 1995).

The recognition of an intron and choice of splice sites are likely accomplished during the early steps of spliceosome assembly and may hold the key to the regulation of splicing. As the first ATP-requiring step, the stable addition of the U2 snRNP at the branchpoint is an attractive potential site of regulation. The RNA moiety of the U2 snRNP plays two roles in this step. A region called the branchpoint interaction sequence forms base pairs with the pre-mRNA at the site of lariat formation (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989). A structure downstream of the branchpoint interaction sequence called stem–loop IIa is an essential U2 structural element required for the stable binding of U2 snRNP to the assembling spliceosome in yeast (Ares and Igel 1990; Zavanelli and Ares 1991). Mutations that destabilize the stem are cold-sensitive for growth and splicing in vivo and in vitro (Ares and Igel 1990; Zavanelli and Ares 1991) and can be suppressed by second mutations in U2 that improve the stability of stem IIa or destabilize competing alternative structures (Zavanelli et al. 1994). A search for genetic interactions between U2 and known splicing proteins identified four proteins that function together with stem–loop IIa of U2: PRP5, PRP9, PRP11, and PRP21 (Ruby et al. 1993; Wells and Ares 1994). Each of these four proteins is independently required for prespliceosome assembly (Lin et al. 1987; Arenas and Abelson 1993; Ruby et al. 1993). Genetic interactions indicate that the four function closely with one another (Chapon and Legrain 1992; Ruby et al. 1993) and PRP21 interacts in the two-hybrid system with both PRP9 and PRP11 (Legrain and Chapon 1993; Legrain et al. 1993).

In mammals two multisubunit splicing factors designated SF3a and SF3b (Brosi et al. 1993b) are components of the U2 snRNP and are required for spliceosome as-

sembly. Similarities between the three yeast U2 snRNP-associated proteins PRP9, PRP11, and PRP21 and components of SF3a indicate that SF3a structure and function are conserved between yeast and mammals (Beggs 1993; Behrens et al. 1993b; Krämer 1993; Hodges and Beggs 1994; Newman 1994). SF3a^{66}/SAP 62 (spliceosome-associated protein 62) is related to PRP11 by sequence similarity and immunological cross-reactivity (Bennett and Reed 1993). SF3a^{60}/SAP 61 is related to PRP9 by sequence similarity, immunological cross-reactivity, and functional domain exchange (Behrens et al. 1993a; Bennett and Reed 1993; Brosi et al. 1993a; Chiara et al. 1994; Krämer et al. 1994). SF3a^{120}/SAP 114 is related to PRP21 by sequence similarity (Krämer et al. 1995). Protein–protein interactions detected among PRP9, PRP11, and PRP21 (Legrain and Chapon 1993; Legrain et al. 1993) are similar to those observed among the SF3a subunits (Bennett and Reed 1993; Chiara et al. 1994; Krämer et al. 1995). Despite the similarity in SF3a from yeast and mammals, there has been no evidence for a yeast homolog of SF3b.

To identify factors that influence the function of U2 RNA, we carried out a screen for mutations that suppress the cold-sensitive growth defect of the U2 G53A mutation in stem–loop IIa, a structure known to be essential for U2 function (Ares and Igel 1990). The screen yielded three different cold-sensitive U2 suppressor genes, *CUS1*, *CUS2*, and *CUS3*. One of these, *CUS1*, encodes an essential splicing protein that is required for U2 snRNP addition to the spliceosome. The *CUS1-54* suppressor rescues the cold-sensitive defect of U2 RNA at the step of stable U2 snRNP binding to pre-mRNA, indicating that rescue involves a direct rather than a bypass mechanism. The spectrum of U2 alleles suppressed by *CUS1-54* indicates that stem–loop IIa of U2 RNA is an important target of the suppressor activity. Wild-type *CUS1*, but not the *CUS1-54* mutant, mediates extra copy suppression of temperature-sensitive *Prp11* and *Prp5* mutations, linking the *CUS1* protein to a subset of other factors required at the same step of spliceosome assembly. As described here and in Gozani et al. (this issue), CUS1 shares a large region of homology with SAP 145, a protein component of the mammalian U2 snRNP (Staknis and Reed 1994). The yeast genome also encodes a homolog of SAP 49, a human protein that interacts with SAP 145 (Champion-Arnaud and Reed 1994; Gozani et al., this issue), demonstrating that key elements of SF3b are conserved in structure and function.

Results

Genetic analysis of the CUS *genes*

To identify mutations that increase the function of U2 snRNA, we carried out a genetic screen for suppressors of the cold-sensitive U2 mutation G53A (Ares and Igel 1990). Yeast cells carrying a U2 gene disruption in the chromosome and the cold-sensitive U2 snRNA gene on a *LYS2* plasmid were treated with ethyl methanesulfonate (EMS) and cold-resistant colonies were isolated. Of $\sim 1.4\times10^5$ viable colonies screened, 105 were cold resistant. Each colony was crossed to a wild-type strain carrying a U2 disruption and a new copy of the cold-sensitive U2 gene on a *LEU2* plasmid. The cold-sensitive growth phenotype of the resulting diploids was examined, and all were found to be cold resistant, indicating that they contain dominant or codominant suppressors. The diploids were then streaked on α-aminoadipate to select for the loss of the *LYS2* plasmid carrying the original U2 gene present during exposure of the cell to mutagen. Diploids that became cold sensitive upon loss of the *LYS2* plasmid were shown to contain revertants of the original cold-sensitive U2 mutation or second-site U2 mutations that suppress the RNA folding defect of the original mutation (Zavanelli et al. 1994). Diploids that remained cold resistant were judged to contain dominant extragenic suppressors. Of the 36 strains in this class, the 16 strains containing the strongest suppressors were analyzed in detail. All were backcrossed to wild type at least twice, and the suppressor in each case segregated as a single Mendelian gene. None of the suppressor mutations carried a severe growth defect in combination with wild-type U2. We called these the *CUS* genes.

The suppressors were sorted into gene groups by linkage analysis, rather than by complementation, because they are dominant and lack obvious recessive growth defects. Pairs of strains containing independently isolated suppressors were crossed to each other, the resulting diploids were dissected, and spores were tested for cold resistance. In the case where the two suppressor mutations are in the same gene (tightly linked), very few if any cold-sensitive spores will emerge from the cross. If the suppressor mutations are in different genes (likely to be unlinked), most tetrads (the abundant tetratype class) will contain one cold-sensitive spore (Sherman and Wakem 1991). We used these distinct patterns to determine that 12 isolates were in the same gene (*CUS1*), 3 isolates were unlinked to *CUS1* but tightly linked to each other at a second locus (*CUS2*), and 1 isolate was unlinked to either of the first two mutations (*CUS3*). The 12 *CUS1* isolates represent at least six independent events based on their appearance in different mutagenesis experiments. All three of the *CUS2* mutations were isolated independently. The growth phenotypes of *CUS*$^+$, *CUS1-54*, *CUS2-9*, and *CUS3-10* in combination with wild-type U2 or the cold-sensitive U2 G53A mutation are shown in Figure 1.

Isolation of CUS1 *and identification of the suppressor mutation*

To clone the *CUS1* gene, a genomic library was constructed using a *LEU2* centromere plasmid and DNA from a *CUS1-54* strain carrying a cold-sensitive U2 gene. The library was introduced into a *leu2*$^-$, *CUS*$^+$ yeast strain carrying a U2 disruption and the cold-sensitive U2 gene on a *LYS2* plasmid. Transformants that grew at 18°C on medium lacking leucine were isolated, and the *LEU2* library plasmid was recovered from each and re-

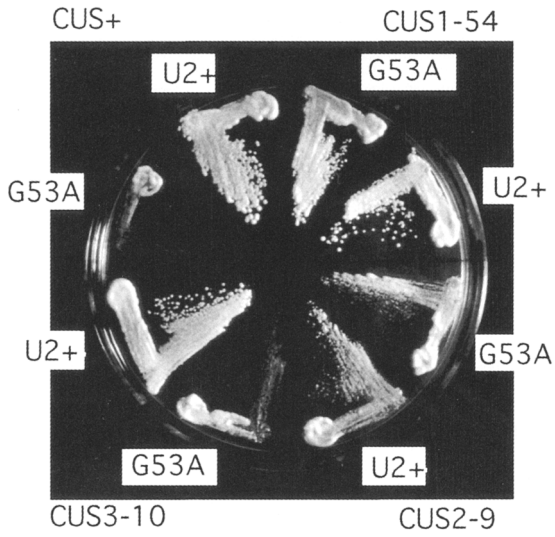

Figure 1. Suppression of cold-sensitive growth in mutagenized strains. Growth phenotypes of *CUS*$^+$, *CUS1-54*, *CUS2-9*, and *CUS3-10* in combination with wild-type U2 or the cold-sensitive U2 G53A mutation. Strains were streaked on YEPD and incubated for 4 days at 18°C.

transformed into the original recipient strain. Three plasmids that uniformly suppressed the cold-sensitive growth phenotype upon retransformation were analyzed by restriction endonuclease digestion and were found to contain overlapping regions of the yeast genome. A subcloned 2055 base pair *Xho*I–*Hin*dIII fragment suppressed cold sensitivity and was sequenced, revealing an open reading frame that predicts a basic protein of 436 amino acids (Fig. 2; GenBank accession no. U27016).

To confirm that the cloned locus represents the *CUS1-54* suppressor gene, we tested for genetic linkage between *CUS1-54* and the cloned sequences using strains carrying the cold-sensitive U2 mutation. A genomic fragment from downstream of the cloned gene was placed in an integrative vector carrying the *LEU2* marker and was used to target the insertion of the *LEU2* gene into the chromosome next to the endogenous wild-type copy of the cloned gene, without disrupting it, thus creating a Cus$^+$ (cold sensitive), Leu$^+$ strain with LEU2 tightly linked to *CUS1*$^+$. This strain was crossed to a *leu2*$^-$ *CUS1-54* (suppressed cold-resistant) strain. We dissected 15 tetrads, all of which were parental ditypes (two cold-sensitive, Leu$^+$ spores and two cold-resistant, Leu$^-$ spores), indicating that recombination between the *LEU2* marker integrated at the cloned locus and the *CUS1-54* mutation did not occur. We conclude that the cloned genomic fragment is identical to the *CUS1-54* suppressor.

Because we cloned and sequenced the mutant suppressor gene, we were unsure of the nature of the suppressor mutation. After we completed the suppressor sequence, a file containing the sequence of yeast chromosome XIII cosmid 9408 was deposited in the data base (GenBank accession no. Z48756). The two sequences matched at every position except one (1042 in Fig. 2), which predicted the replacement of the glutamic acid at position 181 with lysine within the *CUS1-54* coding region (Fig. 2). To prove that this difference represents the suppressor mutation, we analyzed cloned *CUS1* PCR products derived from the DNA of our wild-type strain and found G at position 1042. We created a clone of the wild-type allele *CUS1*$^+$ by replacing A1042 of the *CUS1-54* allele with G using site-directed mutagenesis. As demonstrated below, the wild-type gene carried on a centromere plasmid does not suppress cold sensitivity. We conclude that alteration of the wild-type *CUS1*$^+$ gene to the *CUS1-54* suppressor of U2 cold sensitivity occurs by replacement of glutamate 181 by lysine. Analysis of PCR

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CTCGAGATAT ATAAATGTTA CAGAAGGTAA GATCCATGCA AGAGGCTAAT GCGTTTTTCT TTTTTTTTTT TAGAGTGCGA TATCTTGTCA TAAGTTTACA  100
CAATGGGACA AAGGAAGCTA AAACATGAGT GCAAAAGAAG GGCCCATGTT TGTCGCCCTA TGCATTTAGC CAATCATCTT TTTTTTTTGC GTGATCTTTA  200
TCAATCGTGA TTCTCTGCCT TCTGAGTGAA AAAAGTCATG ATCTTCGCGT TATTGCAGCG GAATTTGCCG ACAGGACCTA GGTCTTGAGA AAGAAAAGTG  300
ATAGGGTAAG TAATGGAAGA AGAGGGCACA GGTCCAATCA CGCAGCTTCA TTACAGGGCT ATAATTGCAA GAATAATAAC TTTCAACCAT ATAGAAATCC  400
AATACAGTAT ATTGGTATTG TTTAGAAAGG TATTAAGAAG GCAGTAGCAT ACAGAGTATA TATCTATAGG CAGAGCATCA GATCATAAAG ATAGTGGTGA  500
A ATG GCT AGA ACC AAA AGT AGG AAG CGT TCC GGA AAT AAC CAA AAT AAG AAT GCG TCT GTG GTA AAT AAT AAG GCT GAG ATC  582
  M  A  R  T  K  S  R  K  R  S  G  N  N  Q  N  K  N  A  S  V  V  N  N  K  A  E  I   27
GCC GCC ATG ATT GAT GCA AGA AGG CTT GAG CAA AAG AAA AAG GGT GGG GTG ACT AAC AGC AAA GGA AAG ACG AAT AAG GTA  663
  A  A  M  I  D  A  R  R  L  E  Q  K  K  K  G  G  V  T  N  S  K  G  K  T  N  K  V   54
GTG GAT GCG AAG TTA GAA AAA GAG TTC AAA GAC GTT TTA CAA CGA TTT CAA GTG CAA GAG AAT GAT ACG CCG AAG GAA ATC  744
  V  D  A  K  L  E  K  E  F  K  D  V  L  Q  R  F  Q  V  Q  E  N  D  T  P  K  E  I   81
ACG AAA GAT GAA AAG AAC AAT CAT GTC GTT ATT GTT GAG AAA AAC CCA GTT ATG AAC AGA AAG CAT ACA GCA GAA GAT GAA  825
  T  K  D  E  K  N  N  H  V  V  I  V  E  K  N  P  V  M  N  R  K  H  T  A  E  D  E   108
TTG GAG GAT ACA CCC TCG GAT GGT ATT GAG GAG CAT CTA TCG GCA AGA AAA CGT AGG AAG ACT GAA AAA CCT TCA CTT TCG  906
  L  E  D  T  P  S  D  G  I  E  E  H  L  S  A  R  K  R  R  K  T  E  K  P  S  L  S   135
CAG TTA AAA AGC CAG GTA CCG TAT CCT CAA ATT ATA GAA TGG TAC GAT TGT GAC GCA AGA TAT CCA GGC TTA CTA GCG TCA  987
  Q  L  K  S  Q  V  P  Y  P  Q  I  I  E  W  Y  D  C  D  A  R  Y  P  G  L  L  A  S   162
                                                    (A1042)
ATT AAG TGC ACC AAA AAT GTC ATT CCT GTT CCA AGC CAC TGG CAG TCC AAG AAG GAA TAT CTA TCT GGC CGT TCT CTG TTA 1068
  I  K  C  T  K  N  V  I  P  V  P  S  H  W  Q  S  K  K  E  Y  L  S  G  R  S  L  L   189
                                                    (K181)
GGT AAA AGA CCT TTT GAA CTT CCT GAC ATT ATC AAG AAG ACA AAT ATA GAA CAA ATG AGA TCG ACG CTT CCG CAA AGC GGA 1149
  G  K  R  P  F  E  L  P  D  I  I  K  K  T  N  I  E  Q  M  R  S  T  L  P  Q  S  G   216
CTG GAT GGT CAA GAT GAA AAG TCA CTA AAG GAG GCC TCA AGA GCA AGA GTG CAG CCG AAA ATG GGC GCC TTG GAT TTG GAT 1230
  L  D  G  Q  D  E  K  S  L  K  E  A  S  R  A  R  V  Q  P  K  M  G  A  L  D  L  D   243
TAC AAG AAG TTA CAC GAT GTG TTT TTC AAG ATA GGA GCC AAC TGG AAA CCT GAC CAT TTG TTA TGC TTC GGT GAC GTC TAT 1311
  Y  K  K  L  H  D  V  F  F  K  I  G  A  N  W  K  P  D  H  L  L  C  F  G  D  V  Y   270
TAT GAA AAT AGG AAT CTT TTC GAG GAA ACG AAC TGG AAA AGA ATG GTT GAT CAT AAG AGA CCG GGG AGA ATT AGC CAA GAG 1392
  Y  E  N  R  N  L  F  E  E  T  N  W  K  R  M  V  D  H  K  R  P  G  R  I  S  Q  E   297
CTT CGT GCC ATA ATG AAT TTA CCC GAA GGT CAA CTA CCA CCA TGG TGT ATG AAG ATG AAA GAC ATT GGA TTA CCT ACG GGA 1473
  L  R  A  I  M  N  L  P  E  G  Q  L  P  P  W  C  M  K  M  K  D  I  G  L  P  T  G   324
TAT CCT GAT CTG AAA ATT GCC GGC TTG AAT TGG GAT ATA ACG AAT TTA AAA GGC GAC GTT TAC GGA AAA ATA ATC CCT AAT 1554
  Y  P  D  L  K  I  A  G  L  N  W  D  I  T  N  L  K  G  D  V  Y  G  K  I  I  P  N   351
CAC CAT TCA AGG TCC AAG AAA CAG GGT AGA AAC TAT TTT GGC GCA TTG ATC TCA TTT GAA ACC CCT GAA TTT GAA AAT TCA 1635
  H  H  S  R  S  K  K  Q  G  R  N  Y  F  G  A  L  I  S  F  E  T  P  E  F  E  N  S   378
AAA GAG GAT ACA CAG GCG AAT GCG GAA AAT GGG CGC CAA GAT GAC AAA ATC GAT GAT GAA GTA GAG CAT AAG TTA GAT CAC 1716
  K  E  D  T  Q  A  N  A  E  N  G  R  Q  D  D  K  I  D  D  E  V  E  H  K  L  D  H   405
TTC CAG GAA GAC ATA TCC GAA GTG ACA AGC GCA GAA GAA AAA CTT GAG AGA AAC GAA GAG GAA TCG GAG AAG CAA CTA TAT 1797
  F  Q  E  D  I  S  E  V  T  S  A  E  E  K  L  E  R  N  E  E  E  S  E  K  Q  L  Y   432
ACT GTA TTG AAA T AACTACTAAA TATCGCATAG GTACTTCAGT TTTACAAAAT TTTACATGTG GTACTATTTC TCACGTACGA GTTTTTTTCT  1890
  T  V  L  K
TTTTTAAATG CGGCCCTAAA CCATGCTGAA ATTTTTCATG AGCT
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Figure 2. The sequence of the 1934-bp *Xho*I–*Sac*I fragment containing the *CUS1* coding region. Predicted amino acid sequence of CUS1 is shown below the nucleotide sequence. Nucleotide and amino acid (in italics) positions are indicated at *right*. The positions of the mutation in the gene and the predicted amino acid substitution in the protein are underlined. In wild-type *CUS1*, position 1042 is a G and results in a glutamate at amino acid position 181; in the suppressor gene *CUS1-54* there is an A at 1042 resulting in a lysine.

products derived from each of the remaining 11 *CUS1* suppressor isolates shows that they all carry the same G1042 to A mutation.

CUS1 *is an essential splicing factor required for prespliceosome formation*

The genetic interaction between *CUS1-54* and mutant U2 snRNA suggested that the product of the wild-type *CUS1* gene might be a splicing factor. To determine whether *CUS1* is an essential gene and to begin studying the role of the *CUS1* gene product in splicing, we deleted a large segment of the *CUS1* coding region and replaced it with the selectable *HIS3* gene. This deletion construct was used to replace one of the *CUS1*$^+$ alleles in a diploid yeast strain by one-step gene disruption (Rothstein 1991). Southern analysis confirmed that correct replacement occurred, and dissection of tetrads obtained by sporulating the diploid produced 2:0 segregation of viable spores, none of which was His$^+$, indicating that disruption of *CUS1* is lethal. The *cus1::HIS3* null allele can be complemented by either the wild-type *CUS1*$^+$ gene or the *CUS1-54* suppressor gene.

To determine whether *CUS1* is required for splicing, we constructed a *GAL1:CUS1* fusion and integrated it at the chromosomal locus of *CUS1*, such that the endogenous gene was deleted and replaced with a *GAL*-regulated copy of *CUS1*. To examine the effect of *CUS1* protein depletion on splicing, we shifted strains carrying the *GAL*-regulated *CUS1* gene to media containing glucose, isolated RNA, and measured changes in splicing and snRNA levels. Upon repression of the integrated, *GAL*-regulated *CUS1* gene, growth is blocked and unspliced U3 RNA accumulates, indicating that loss of *CUS1* gene expression inhibits splicing (Fig. 3A). In addition, a slight, reproducible increase in the level of U2 snRNA relative to U1, U4, U5, and U6 snRNAs is also observed (data not shown). We conclude that the *CUS1* gene product is required for efficient splicing in vivo but is not required to maintain wild-type U2 RNA stability.

To determine the step in splicing at which the *CUS1* gene product is first required, we prepared splicing extracts depleted of CUS1 from cells carrying the *GAL1:CUS1* fusion shifted to glucose (Fig. 3B). When pre-mRNA is added to extracts depleted of CUS1 there is an accumulation of commitment complexes compared to a wild-type extract (cf. CC in lanes 1 and 2) but a dramatic decrease in the U2 snRNP containing prespliceosome and spliceosome complexes (cf. PS and SP in lanes 1 and 2). This indicates a requirement for CUS1 following commitment complex formation and prior to U2 snRNP addition. To provide evidence that the deficiency in the extract is attributable to loss of CUS1 we created a *GAL1:CUS1* gene with a double tag (Peterson et al. 1994) composed of the hemagglutinin (HA) epitope (Kolodziej and Young 1991) followed by six histidines (Hochuli 1990) at the carboxyl terminus of the *CUS1* protein (CUS1–tag). The tagged gene complements the *cus1::HIS3* disruption (on galactose), indicating that the tag does not block the function of the protein (data not

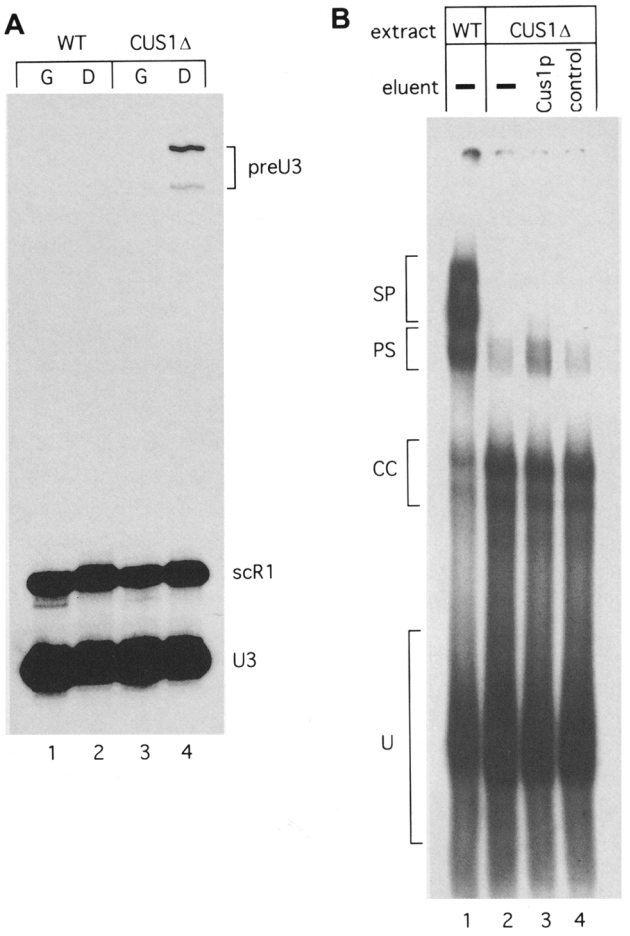

Figure 3. CUS1 is an essential splicing factor required for prespliceosome formation. (*A*) Depletion of CUS1 results in accumulation of unspliced RNA. Total RNA was isolated from a strain carrying a glucose-repressible chromosomal *CUS1* gene, SWY2, (lanes *3,4*) or from an isogenic control strain, HI227, (lanes *1,2*) following 7 hr of logarithmic growth at 30°C in media containing either glucose (D, lanes *2,4*) or galactose (G, lanes *1,3*). The levels of spliced and unspliced U3 were measured by primer extension using an oligonucleotide complementary to the second exon of the U3A and U3B genes. An oligonucleotide complementary to scR1, a cytoplasmic RNA not involved in splicing (Felici et al. 1989), was included in each reaction as an internal control for RNA amounts. Samples are HI227 in YPGal (lane *1*), HI227 in YEPD (lane *2*), SWY2 in YPGal (lane *3*), and SWY2 in YEPD (lane *4*). (*B*) Splicing complex formation in extracts depleted of CUS1. Splicing extract from cells genetically depleted of CUS1 (CUS1Δ) was incubated with labeled actin pre-mRNA and complexes were analyzed on nondenaturing polyacrylamide–agarose gels. Nickel column eluents from yeast strains expressing tagged CUS1 (lane *3*) or a control eluent (lane *4*) were added to depleted extracts to test their ability to reconstitute splicing complexes. Samples are as follows: Wild-type extract (lane *1*); CUS1-depleted extract (lane *2*); CUS1-depleted extract plus tagged CUS1 eluent (lane *3*), CUS1-depleted extract plus control eluent (lane *4*). The complexes are indicated by brackets as follows: Nonspecific complexes (U); commitment complexes (CC); prespliceosomes (PS); and splicesomes (SP).

shown). Using nickel chelate chromatography (Hochuli 1990), we isolated protein from cells expressing CUS1–tag and tested its ability to restore prespliceosome formation in the CUS1 depleted extract (Fig. 3B, lanes 3,4). Buffer alone (lane 2) or the eluent from the untagged extract (lane 4) did not stimulate prespliceosome formation, whereas the eluent derived from cells expressing CUS1–tag (lane 3) reproducibly stimulated the formation of prespliceosomes. The magnitude of the reconstitution is only about twofold, but stimulation of prespliceosome formation is never observed with fractions prepared from untagged extracts (cf. lanes 3 and 4). There could be several explanations for the inefficiency of reconstitution. First, although we can easily detect the tagged protein on blots after denaturation, both immunoprecipitation and nickel chelate affinity purification of the native protein are inefficient, suggesting that the epitope tag is not readily accessible in the native protein. Second, the effect of genetic depletion of CUS1 on the levels of other gene products is unknown. Proteins or RNAs that may require interaction with CUS1 for their stability or appearance in the extract may be reduced, and thus even purified CUS1 may be inadequate to reconstitute completely. The extracts are prepared under native conditions; thus it is possible that the reconstituting activity depends on the recovery of CUS1–tag as part of a complex with other proteins. Numerous proteins can be detected in these fractions by silver staining SDS gels (data not shown). Nonetheless, the accumulation of commitment complexes in CUS1-depleted extracts and the reconstitution of prespliceosomes by partially purified CUS1 demonstrates that CUS1 is required for U2 snRNP binding to pre-mRNA during spliceosome assembly.

CUS1-54 *suppresses U2 splicing defects in vivo and in vitro*

To investigate the mechanism by which the *CUS1-54* protein suppresses the cold-sensitive growth defect of the U2 G53A mutation, we compared splicing efficiency at 18°C in isogenic strains expressing either the wild-type or mutant U2 and carrying a plasmid with either the wild-type *CUS1*$^+$ gene or the *CUS1-54* gene, in addition to a chromosomal copy of *CUS1*$^+$. RNA was isolated from each of four strains and subjected to primer extension analysis using a U3 oligonucleotide to detect unspliced pre-U3A and pre-U3B (Fig. 4A). Mutation of U2 G53A causes accumulation of unspliced U3 after a shift to 18°C (Zavanelli and Ares 1991; Fig. 4A, cf. lanes 1 and 3). Presence of the *CUS1-54* plasmid almost completely suppresses the splicing defect caused by the U2 G53A mutation (cf. lane 4 with lanes 3 and 1) and causes no splicing defect in cells expressing wild-type U2 (lane 2). We conclude that suppression by *CUS1-54* of the growth defect caused by mutation of U2 G53A is mediated through a restoration of splicing efficiency in vivo.

A cold-sensitive defect in U2 function caused by the G53A mutation can be observed in splicing extracts (Zavanelli and Ares 1991). The defect results in slow or inefficient conversion of commitment complexes to splic-

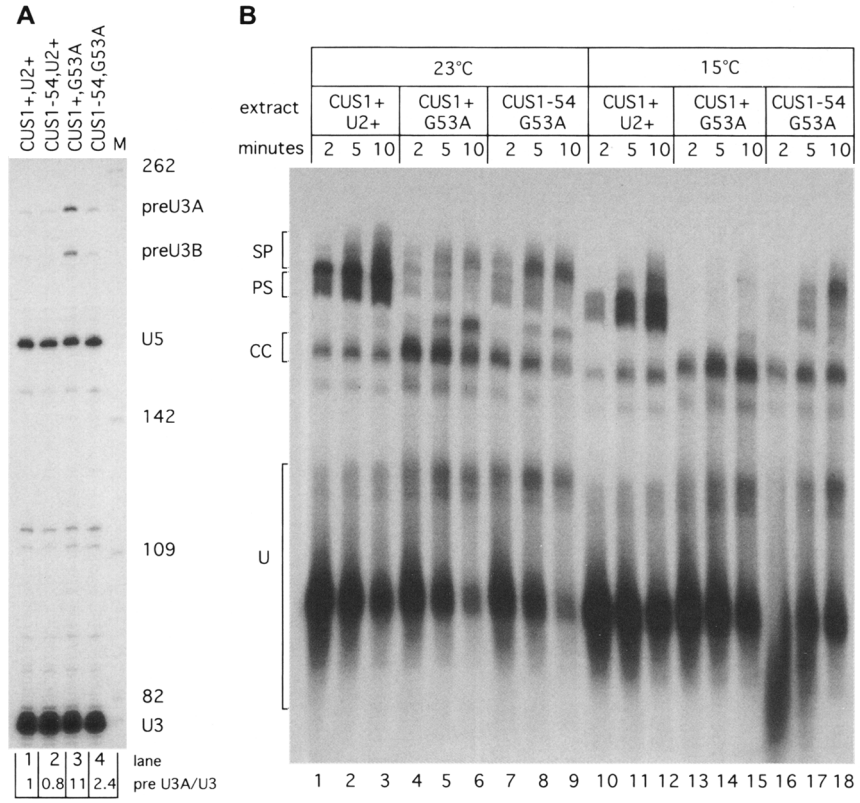

Figure 4. Suppression of cold-sensitive splicing and spliceosome assembly. (*A*) The in vivo splicing defect of G53A is suppressed by *CUS1-54*. RNA isolated from strains expressing only wild-type or G53A U2 at 18°C in combination with *CUS1* or *CUS1-54* was subjected to primer extension analysis using a primer that recognizes both spliced and unspliced forms of U3. A U5 primer was included as an internal control. Primer extension products were quantified using a Molecular Dynamics PhosphorImager, and the ratio of preU3A to U3 was calculated. The value of this ratio for wild type was set at 1.0, and the values of the remaining ratios were determined relative to the wild-type value. (*B*) Cold-sensitive spliceosome assembly is suppressed by *CUS1-54*. Splicing extracts carrying only wild-type or G53A U2 in combination with either CUS1 or CUS1-54 were incubated with radiolabeled actin pre-mRNA under splicing conditions at 15°C or 23°C. Aliquots of 5 μl were removed to quench at the times indicated and analyzed on a nondenaturing polyacrylamide-agarose gel. The complexes are indicated by brackets as follows: Nonspecific complexes (U); commitment complexes (CC); prespliceosomes (PS); and splicesomes (SP).

ing complexes containing the U2 snRNP (prespliceosomes and then subsequently spliceosomes), especially at low temperatures (Zavanelli and Ares 1991). To determine whether *CUS1-54*-mediated suppression occurs at the level of prespliceosome formation, we analyzed splicing extracts from different strains for the ability to form U2-containing splicing complexes at different temperatures in vitro, using native gel electrophoresis (Fig. 4B). Extracts from the wild-type strain form prespliceosomes efficiently after incubation at either 23°C or 15°C (lanes 1–3, 10–12). Extracts from strains carrying the U2–G53A mutation form prespliceosomes at a lower rate than wild type at 23°C, and very poorly at 15°C, with increased accumulation of commitment complexes (cf. lanes 4–6 with lanes 13–15). Extracts made from U2 G53A cells carrying the *CUS1-54* plasmid form prespliceosomes at 23°C and are more efficient in prespliceosome formation at 15°C, compared with extracts made from cells carrying the *CUS1*$^+$ plasmid (cf. lanes 4–6 with 13–15 and lanes 7–9 with 16–18). The defect in prespliceosome formation caused by mutation of U2 G53A is suppressed in extracts containing *CUS1-54* gene product, but not *CUS1*$^+$ gene product, indicating that the mechanism of *CUS1-54* suppression in vitro is through enhancement of a spliceosome assembly step that leads to association of the U2 snRNP with commitment complexes.

The secondary structure of U2 G53A RNA is perturbed in the bulk of the U2 snRNP population in vivo, such that stem–loop IIa is not formed, and loop IIa is paired to a conserved region of complementarity downstream (Zavanelli and Ares 1991; Zavanelli et al. 1994). The growth and in vitro splicing defects of G53A and other mutations that destabilize stem IIa, as well as many aspects of the RNA folding defect, can be suppressed by second mutations in U2 snRNA that disrupt the competing alternative structures that interfere with stem IIa formation (Zavanelli et al. 1994). We used in vivo RNA structure probing to assess the effect of *CUS1-54* on the folding of the bulk U2 snRNA and observed no effect of the suppressor on the folding pattern of the U2 G53A RNA (data not shown). We conclude that *CUS1-54* does not suppress U2 G53A cold-sensitive growth and prespliceosome formation defects by restoring the wild-type distribution of folded forms of U2 snRNA in the cell.

A discrete subset of U2 snRNA mutations is suppressed by CUS1-54

Both the *CUS1* protein and U2 stem–loop IIa are required for the stable association of U2 snRNP with pre-mRNA (Fig. 3B; Zavanelli and Ares 1991), suggesting that suppression occurs when they act together at the same step in prespliceosome formation. However, a formal interpretation of the efficient dominant suppression by *CUS1-54* is that the requirement for the stem–loop has been bypassed by the suppressor mutation. To address this possibility and to obtain clues to the mechanism by which *CUS1-54* suppresses U2 G53A, we tested *CUS1-54* for suppression of other U2 mutations (Fig. 5). In addition to G53A, *CUS1-54* suppresses the more severe phenotype of G53C. The pairing partner of G53 in the stem IIa is C62 (Ares and Igel 1990); the cold-sensitive phenotype of mutation C62U is also suppressed. Mutation of C62 to G is not suppressed by *CUS1-54* however, although this mutation can be suppressed by second U2 RNA mutations that change G53 to C, restoring the 53–62 base pair (Ares and Igel 1990), or G100 to A, destroying the conserved complementarity to loop IIa (Zavanelli et al. 1994). This result suggests that the mechanism of suppression by *CUS1-54* is less efficient

A

temperature	18°C		26°C		30°C	
U2	*CUS1*	*CUS1-54*	*CUS1*	*CUS1-54*	*CUS1*	*CUS1-54*
WT	++++	++++	++++	++++	++++	++++
G53A	–	+++	++	++++	++	++++
G53C	–	++	–	++	–	+
C62U	–	+++	+	++++	+++	++++
C62G	–	–	–	–	–	–
A61Δ	++	++	++	++	++	++
BTL	–	–	–	–	–	–
tmA	–	–	–	–	–	–
tmA'	–	–	–	–	–	–
tmC'	+++	+++	++++	++++	++++	++++
U44A	–	–	+	+	+	+
U44G	–	–	–	–	–	–
U33Δ	++++	++++	+++	+++	++	+++
C29Δ	+	+	–	–	–	–

B

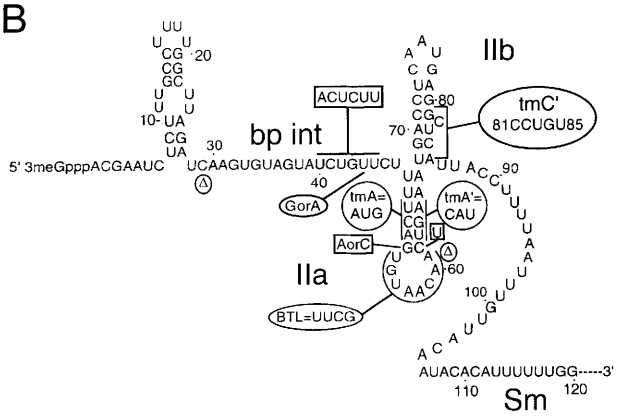

Figure 5. Allele-specific suppression of U2 mutants by *CUS1-54*. (*A*) *CUS1*$^+$ and *CUS1-54* were tested for suppression of a series of U2 mutations with growth phenotypes. Growth was assayed on YEPD after 3 days at 26°C and 30°C and 5 days at 18°C and is indicated as follows: Wild-type growth (++++); intermediate growth (+++) to (+); no growth (–). (*B*) Secondary structure model of the 5′ end of U2 snRNA (Ares and Igel 1990) including the sequence of triple mutations in stem IIa and IIb and of the tetraloop used to replace loop IIa in the mutant BTL. Mutations suppressed by *CUS1-54* are boxed; mutations that are not suppressed are circled. A collection of double and triple mutations in nucleotides 40–45 are also suppressed by *CUS1-54*. A40C43 is included as a representative of these mutations (Yan and Ares 1996).

than or qualitatively different from that of the intragenic suppressors.

CUS1-54 does not act to bypass the requirement for U2 snRNA or for stem–loop IIa in splicing, because it fails to suppress the lethal tetraloop replacement of loop IIa or the lethal triple mutations in stem IIa (Fig. 5; Ares and Igel 1990). This shows that suppression takes place by enhancing rather than replacing the function of this part of U2 snRNA. *CUS1-54* does not simply enhance splicing at lower temperatures, because it fails to suppress other mild (tmC′) or severe (U44A, U44G) cold-sensitive mutations in U2 snRNA. *CUS1-54* is particularly effective in the rescue of the stem IIa mutations but fails to significantly enhance the function of a single base deletion in loop IIa (A61Δ) or either of two mutations upstream of the branchpoint interaction region (U33Δ, C29Δ). We conclude that *CUS1-54* suppresses defects in U2 in large part through stem–loop IIa and does not simply bypass U2 function or generally enhance splicing at low temperature.

Extra copies of CUS1$^+$ *but not* CUS1-54 *suppress the temperature-sensitive growth defects of* prp5 *and* prp11

Synthetic lethal interactions have been observed between U2 snRNA and four proteins required for prespliceosome assembly (Ruby et al. 1993; Wells and Ares 1994). One of these is the DEAD-box protein PRP5 (Dalbadie-McFarland and Abelson 1990), and the other three (PRP9, PRP11, and PRP21) comprise the yeast homolog of SF3a (Beggs 1993; Krämer 1993; Hodges and Beggs 1994; Newman 1994). To determine which if any of these proteins might interact with *CUS1*, we introduced *CUS1*$^+$ or *CUS1-54* on centromere plasmids into strains carrying different temperature-sensitive *prp* mutations. We found that extra copies of *CUS1*$^+$ partially suppress the temperature-sensitive growth defect of *prp5-1* and *prp11-1*, but not *prp4-1*, *prp9-1*, or *prp21-1* (Table 1). This result suggests that CUS1 interacts with the putative RNA helicase PRP5 as well as the yeast homolog of SF3a and is consistent with the interpretation that CUS1 functions with U2 snRNA at the point of stable addition of U2 snRNP to the pre-mRNA. Surprisingly the *CUS1-54* allele, which so effectively suppresses U2 mutations, fails to rescue any of the *prp* mutations. The diametric effects of *CUS1-54* on suppression of U2 snRNA mutations and extra-copy rescue of *prp5* and *prp11* indicate that the increased efficiency of interaction between U2 snRNA and CUS1 comes at a cost to the interaction with PRP5 and yeast SF3a.

CUS1 *is a yeast homolog of the human U2 snRNP protein SAP 145*

The wild-type *CUS1* protein is estimated to be ~50 kD in molecular mass, basic at neutral pH (estimated pI=8.7), and rich in charged residues (6.9% Asp, 9.4% Glu, 11.7% Lys, and 5.7% Arg). With the exception of a potential coiled-coil domain at the carboxyl terminus

Table 1. *Suppression of* prp5 *and* prp11 *by extra copies of* CUS1$^+$

Strain	Plasmid	Temperature 26°C	30°C	34°C	36°C
Wild type	pRS316	+ + +	+ + +	+ + +	+ + +
	pRS316*CUS1-54*	+ + +	+ + +	+ + +	+ + +
	pRS316*CUS1*$^+$	+ + +	+ + +	+ + +	+ + +
prp4-1	pRS316	+ + +	−	−	−
	pRS316*CUS1-54*	+ + +	−	−	−
	pRS316*CUS1*$^+$	+ + +	−	−	−
prp5-1	pRS316	+ + +	+ +	+	−
	pRS316*CUS1-54*	+ + +	+ +	+	−
	pRS316*CUS1*$^+$	+ + +	+ +	+ +	+
prp9-1	pRS316	+ + +	−	−	−
	pRS316*CUS1-54*	+ + +	−	−	−
	pRS316*CUS1*$^+$	+ + +	−	−	−
prp11-1	pRS316	+ + +	+ +	−	−
	pRS316*CUS1-54*	+ + +	+ +	−	−
	pRS316*CUS1*$^+$	+ + +	+ + +	+	−
prp21-1	pRS316	+ + +	−	−	−
	pRS316*CUS1-54*	+ + +	−	−	−
	pRS316*CUS1*$^+$	+ + +	−	−	−

CUS1$^+$ and *CUS1-54* carried on centromere plasmids were tested for their ability to suppress the temperature-sensitive splicing mutants *prp4*, *prp5*, *prp9*, *prp11*; and *prp21*. Growth was assayed on YEPD after 3 days at 26°C, 30°C, 34°C or 36°C and is indicated as follows: (+ + +) Wild type growth; (+ + to +) intermediate growth; (−) no growth.

(Lupas et al. 1991), the protein lacks motifs that would link it convincingly to any of the known families of RNA-binding proteins. Furthermore, with the exception of the human, worm, and *Arabidopsis* sequences of unknown function discussed below, filtered BLAST searches fail to recognize homologous proteins of known function in the public data base. Recent efforts toward the cloning of human cDNAs encoding proteins associated with functional splicing complexes have led to the identification of a full-length cDNA encoding SAP 145, a U2 snRNP-associated protein (see Gozani et al., this issue). Alignment of the *CUS1* protein sequence with the sequence of SAP 145 (Fig. 6) shows that the two genes share a ~200-amino-acid region of significant similarity (43% identical, 65% similar), including the site of the suppressor substitution (Fig. 6). The region of significant similarity occupies the central parts of both proteins.

Comparison of the CUS1 and SAP 145 sequences shows that CUS1 residue glutamate-181, which is changed to lysine in the CUS1-54 suppressor protein, unambiguously aligns with lysine-487 in SAP 145. Thus, the human protein provides a phylogenetic example of the functionality of lysine at this position but offers no solid clues to the mechanism of suppression. Comparing the sequences of human U2 with those of the suppressible yeast U2 mutants does not suggest any RNA base whose recognition might be satisfied by the replacement of a potential hydrogen bond acceptor (E) with a donor (K). The suppressor substitution occurs in a residue sur-

Figure 6. Homology between CUS1 and SAP 145. (*A*) Amino acid sequence alignment of CUS1 and SAP 145. Sequences were aligned using results from the BLAST program (Altschul et al. 1990), as delivered (using the default settings) by the sequence search e-mail server at the National Library of Medicine. The ungapped segments of similarity were stitched together by visual inspection. Position of the suppressor substitution is indicated. (*B*) A segment of homology between CUS1 and SAP 145 is present in a *C. elegans* protein. BLAST identified a *C. elegans* protein with homology to CUS1 and SAP 145 in a region of SAP 145 required for a protein–protein interaction. The proteins were aligned using BLOCKMAKER (Henikoff et al. 1995) via the Baylor College of Medicine Search Launcher at http://dot-.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html. An expressed *Arabidopsis* homolog of CUS1/SAP 145 (GenBank H36362) used for the alignment is not shown. A consensus is listed below. Similar amino acids (I = L = V = M, D = E, K = R) are indicated by a colon. Hydrophobic (≠) and charged (+) residues are indicated.

rounded by conserved amino acids (H175, W176, K179, Y182, and L183). Structure predictions (Burkhard and Sander 1993) performed on each protein individually agree that the sequences surrounding residue 181 (or 487) are likely to be α-helical. If this is the case, the side chains of the conserved amino acids would be positioned on one face of the helix, with residue 181 (487) on the opposite face. Even in the absence of other evidence for a specific protein secondary structure, the substitution of glutamate with lysine could be expected to increase the general affinity of the protein for RNA.

Sequences from two other organisms have significant homology to CUS1. An expressed *Arabidopsis* sequence (GenBank accession no. H36362) is very likely a SAP 145 homolog. A protein from *Caenorhabditis elegans* (SWISSPROT accession no. P34656) has a significant match to CUS1 and SAP 145 only in the carboxy-terminal segment of the homology between CUS1 and SAP 145 (Fig. 6B). The worm protein has a structural arrangement distinct from CUS1 or SAP 145 and contains a retroviral type Cys-Cys-His-Cys zinc finger motif amino-terminal to its region of similarity with CUS1 (data not shown). This suggests that the homology between CUS1 and SAP 145 consists of at least two parts, an amino-terminal part containing the suppressor mutation and found so far only in CUS1 and SAP 145, and a carboxy-terminal part which may be a modular protein structural element found in otherwise unrelated proteins. A protein–protein interaction between SAP 49 and SAP 145 has been mapped by the Far Western technique (Champion-Arnaud and Reed 1994; Gozani et al., this issue). Sequences in SAP 145 required for this interaction lie between residues 556 and 694 (Gozani et al., this issue), a segment that contains this structure. A second possibility is that the carboxy-terminal glutamate-rich segment is involved in the interaction with SAP 49, as this element lies partly within the 556–694 region (Gozani et al., this issue). CUS1 also has a carboxy-terminal glutamate-rich region that scores very highly in a coiled-coil prediction program, designed to detect potential regions of protein–protein interaction (Lupas et al. 1991). The glutamate-rich region of SAP 145 does not score as highly in potential to form coiled coils. We did not attempt to align the glutamate-rich regions.

A yeast homolog of SAP 49

The similarity between CUS1 and SAP 145, and the strong protein–protein interaction between SAP 145 and SAP 49 (Champion-Arnaud and Reed 1994; see Gozani et al., this issue), predicted a yeast homolog of SAP 49. Recently, sequences from yeast (GenBank accession no. X90565) and *C. elegans* (SWISSPROT accession no. Q09442) have been released that match SAP 49 substantially more significantly than other RRM-containing proteins (Birney et al. 1993; Burd and Dreyfuss 1994) in BLAST searches. We aligned the sequences using CLUSTAL W (Fig. 7; Thompson et al. 1994). Over most of 160 of the first 200 amino acids, the yeast and human proteins are 40%–50% identical and 60%–70% similar. Patches of extreme similarity between the two proteins follow the RNP1 of the first RRM (62–71 yeast, 66–75 human), and RRM2 (residues 175–190 in yeast; 169–184 in human). A carboxy-terminal proline-rich domain shared by the human and *C. elegans* proteins is absent in yeast. The proline-rich region is dispensable for the protein–protein interaction between SAP 49 and SAP 145 (Champion-Arnaud and Reed 1994). Based on this observation, we conclude that yeast encodes a protein homologous to SAP 49. We call this yeast gene *HSH49*, for human SAP homolog 49. Experiments will be necessary

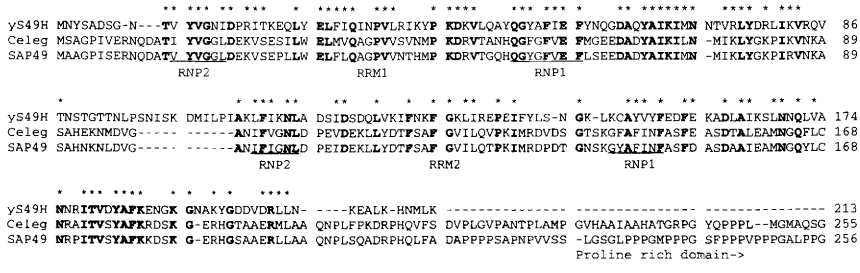

Figure 7. A yeast homolog of human SAP 49. A filtered BLAST search was performed (both seg and xnu filters were used) using the human SAP 49 sequence. The yeast and *C. elegans* SAP 49 homologs were aligned using CLUSTAL W (Thompson et al. 1994), through the Baylor College of Medicine Search Launcher. The alignment was not adjusted, except that the proline-rich carboxy-terminal regions present in the human (to residue 424) and worm (to residue 379) proteins, but absent in yeast, have been omitted. Identical residues are in boldface type; residues that are similar or identical between the human and yeast protein have asterisks. The RNP1 and RNP2 sequences of each of the two RRMs are underlined.

to determine whether and how this protein functions in collaboration with CUS1 during splicing.

Discussion

Using a genetic screen for suppressors of a mutation in U2 snRNA, we have identified a novel yeast gene *CUS1-54*, which overcomes defects in U2 snRNA. The suppressor was selected to rescue a mutation in stem–loop IIa of U2 snRNA, an essential structure required for the stable binding of the U2 snRNP to pre-mRNA during spliceosome assembly (Ares and Igel 1990; Zavanelli and Ares 1991). Rescue of U2 function by CUS1-54 occurs at the defective step (Fig. 4B) and does not bypass the requirement for U2 stem–loop IIa (Fig. 5). This suggests that the suppressor protein acts with stem–loop IIa to overcome the block to spliceosome assembly caused by mutation of the RNA. Consistent with this interpretation is evidence that the wild-type protein CUS1 is required for the addition of wild-type U2 snRNP to the assembling spliceosome (Fig. 3B). The wild-type *CUS1* gene is essential and its expression is required for splicing in vivo (Fig. 3A). Finally, CUS1 is homologous to SAP 145, a human U2-associated spliceosomal protein (Fig. 6; Gozani et al., this issue). The similarity between SAP 145 and CUS1 is strengthened by the identification of a yeast gene, *HSH49*, homologous to SAP 49 (Fig. 7), a human U2 protein that binds tightly to SAP 145 (Champion-Arnaud and Reed 1994).

Conserved protein structure in the spliceosome

Based on these similarities, we propose that yeast has an SF3b-like factor that includes CUS1 (and HSH49). The pattern of U2 mutations suppressed by *CUS1-54* (Fig. 5), the role of U2 stem–loop IIa in splicing (Zavanelli and Ares 1991), and the structural parallels between CUS1 (and HSH49) and SAP 145 (and SAP 49) suggest that stem–loop IIa is a critical part of the SF3b binding site. Given the presence of conserved RRMs in HSH49 and SAP 49 (Fig. 7), as well as the conservation of stem–loop IIa RNA sequence (Ares and Igel 1990), it seems possible that one of the RRMs may bind stem–loop IIa directly or in conjunction with CUS1. The allele-specific pattern of synthetic lethality observed between U2 mutations and temperature-sensitive alleles of the genes encoding yeast SF3a (*PRP9, PRP11, PRP21*) and *PRP5* supports this idea (Wells and Ares 1994) and suggests further that the binding detected between these proteins and U2 RNA (Abovich et al. 1990, 1994; Wells and Ares 1994) may be mediated through CUS1. Because mammalian SF3a depends on SF3b to bind U2 (Brosi et al. 1993a), the yeast SF3a homolog (composed of PRP9, PRP11, and PRP21) would be predicted to depend on the binding of CUS1 (and HSH49) for its association with yeast U2.

What is the mechanism of suppression?

How does mutation of CUS1 suppress U2 RNA defects? Second mutations elsewhere in U2 also suppress the defects of certain stem IIa mutants; these intragenic suppressors act to improve the stability of stem IIa relative to competing alternative U2 RNA structures (Zavanelli et al. 1994). Because of this parallel, and because the suppressor protein has little if any deleterious effect on growth or splicing, we propose that suppression occurs by increasing the efficiency with which the mutant U2 RNA is utilized in splicing. In this model the suppressor protein stimulates either the rate of refolding or recognition of U2 snRNA, relieving the block to splicing caused when the mutant U2 snRNP fails to be incorporated into spliceosomes rapidly enough to maintain cell growth (Zavanelli et al. 1994). Suppression by CUS1-54 could reflect direct binding to U2 snRNA, indirect binding to U2 through protein–protein interactions with U2 binding proteins, or stimulating the activity of the U2 snRNP by some other means. We favor the idea that CUS1 binds U2 snRNA, either directly or in conjunction with other proteins, and that the glutamate-to-lysine change caused by the *CUS1-54* mutation improves binding of the protein to U2 RNA.

Mutations that increase the affinity of protein for nucleic acids are not unprecedented. Such mutations have been isolated in several well-studied nucleic acid-binding proteins and many alter negatively charged amino acids, rendering them neutral or positively charged. In glutaminyl–tRNA synthetase, mutations of Asp-235 (to Asn or Gly) cause mischarging by increasing the affinity of the enzyme for noncognate tRNAs without loss of affinity for the cognate tRNA (Perona et al. 1989; Sher-

man et al. 1995), thus reducing selectivity. The same forces may be at play in λ repressor and catabolite activator protein, where several suppressors of operator mutations increase the binding of the respective protein to the target DNA at the expense of sequence discrimination (Nelson and Sauer 1985; Ebright et al. 1987; Benson et al. 1992). Similarly, the CUS1-54 suppressor protein may rescue U2 function by binding mutant U2 more tightly but may have lost some ability to discriminate between wild-type U2 and other RNAs. Several proteins have been shown to increase the rate of RNA structural interconversion in vitro (Coetzee et al. 1994; Herschlag et al. 1994); it is also possible the suppressor enhances the rate of interconversion between the two differently folded forms of U2 snRNA.

A clue that *CUS1-54* suppression of U2 defects bears as a cost a subtle loss of function can be found in the genetic interactions between *CUS1*, and *PRP5* and *PRP11*. Extra copies of *CUS1*$^+$ suppress the growth defects of *PRP5* and *PRP11* (Table 1), demonstrating functional interactions between yeast SF3a, PRP5, and CUS1, a putative SF3b component. Extra-copy rescue of the *prp* mutations is not supported by *CUS1-54*, implying that the glutamate-to-lysine substitution disrupts an interaction between CUS1 and SF3a and PRP5. A simple explanation for the diametric effects of the suppressor mutation is that there is a balance between the affinity of CUS1 for U2 snRNA on the one hand and the function of CUS1 with SF3a and PRP5 on the other. Although SF3a can bind U2 only in the presence of SF3b, and SF3b can bind U2 on its own (Brosi et al. 1993a), the two factors cochromatograph through several steps (Krämer and Utans 1991) and are probably associated in vivo. If SF3b alone binds U2 with higher affinity than SF3a+b, then the affinity of CUS1 for mutant U2 RNA could be increased simply by reducing the interaction between CUS1 and SF3a or PRP5. The suppressor protein would bind mutant U2 RNAs more tightly but would interact less avidly with SF3a and PRP5.

CUS1/SAP 145 may interact with both U2 RNA and the pre-mRNA

Our experiments in yeast are focused on the interaction between U2 RNA and CUS1; however, the human homolog of CUS1, SAP 145, is one of an intriguing set of proteins that can be photochemically cross-linked to the pre-mRNA branchpoint region within spliceosomes (see Gozani et al., this issue). Using labeled pre-mRNA and immunoprecipitation with the 12CA5 antibody, we tried but were unable to detect cross-linking of CUS1–tag to pre-mRNA in yeast splicing extracts. We can monitor the splicing of an intron with a UACUAAC to UACAAAC mutation by a U2 suppressor RNA with the compensatory G37U mutation (Parker et al. 1987); however, supplementation with *CUS1-54* did not improve the efficiency of splicing of the mutant intron, with or without the branchpoint suppressor U2. Our attempts to coprecipitate U2 RNA with antibodies against the HA determinant in CUS1–tag suggest that either CUS1 is less tightly associated with U2 than the Sm D protein controls (Roy et al. 1995), or that the HA tag on CUS1 is much less accessible. These observations do not exclude the possibility that CUS1 is positioned a fixed distance from the pre-mRNA branchpoint in the yeast spliceosome. Instead, the pattern of suppression of U2 mutants and (Fig. 5) in vitro rescue of mutant U2 (Fig. 3B) by *CUS1-54* argue for a role for CUS1/SAP 145 in mediating U2 RNA–protein interactions required to activate the U2 snRNP for spliceosome assembly. Taken together with the observations of Gozani et al. (this issue), a consistent hypothesis must include the establishment of early interactions between U2 RNA and CUS1/SAP 145 (Brosi et al. 1993a), and later interactions between pre-mRNA near the branchpoint and CUS1/SAP 145 as part of the U2 snRNP (Staknis and Reed 1994; Gozani et al., this issue). Future experiments will determine how the different protein components of the splicing apparatus position both the substrate and the small nuclear RNAs at their proper places in the spliceosome.

Materials and methods

Genetic screen and cloning

Yeast strains were constructed and analyzed genetically by standard procedures (Sherman et al. 1986; Ausubel et al. 1987; Guthrie and Fink 1991). Yeast cells carrying a chromosomal U2 disruption and the cold-sensitive U2–G53A mutation on a *LYS2* plasmid were treated with EMS to 90% lethality and plated on rich medium at 18°C as described previously (Zavanelli et al. 1994). Genomic DNA was partially digested with *Sau*3A, size selected on agarose gels and ligated with *Bam*HI digested pRS315 (Sikorski and Hieter 1989), then electroporated into *Escherichia coli* according to Rose and Broach (1991). Library DNA isolated from *E. coli* was used to transform a yeast strain carrying the U2 disruption and a cold-sensitive U2 plasmid. Leu$^+$, cold-resistant yeast colonies were picked, and DNA was isolated (Ausubel et al. 1987) and retransformed into *E. coli*. Individual library plasmids identified in this way were rescreened by retransformation into yeast; those that uniformly gave rise to cold-resistant transformants were analyzed by restriction mapping and subcloning. To sequence the *Xho*I fragment carrying *CUS1-54*, we cloned it in either orientation in pGEM7Zf+ (Promega, Madison, WI) and constructed and sequenced a deletion plasmid series for each with exonuclease III (Erase-a-base, Promega, Madison, WI).

To confirm that the cloned locus was the same as the *CUS1-54* gene we subcloned a *Sac*I–*Xho*I fragment from downstream of the putative suppressor open reading frame (so that it would not contain the suppressor mutation) into the integrative *LEU2* plasmid pRS305 (Sikorski and Hieter 1989). This plasmid was cut with *Bsm*I at a unique site within the insert and used to transform a cold-sensitive strain, selecting for *LEU2*. Southern blotting showed that the *LEU2* gene was integrated in the chromosome downstream of the putative suppressor locus. This strain was crossed to a *leu2*$^-$ suppressor strain and tetrads were dissected to determine whether recombination could occur between the *LEU2* marker and the suppressor mutation.

The *CUS1-54* gene was used to regenerate the wild-type *CUS1*$^+$ gene by oligonucleotide-directed mutagenesis (Kunkel 1985). The lethal deletion disruption allele of *CUS1* was constructed by replacing a *Bsm*I fragment spanning nucleotides 555–1659 and containing the suppressor mutation within the

CUS1 open reading frame, with a *Bam*HI fragment carrying the *HIS3* gene. *URA3*- or *TRP1*-marked centromere plasmids carrying the wild-type or suppressor genes were constructed by ligation of the *CUS1* or *CUS1-54* *Xho*I–*Sac*I fragment into pRS314 or pRS316 (Sikorski and Hieter 1989) cut with *Xho*I and *Sac*I. Galactose-regulated tagged versions of *CUS1* and *CUS1-54* were constructed by amplifying the coding regions by PCR using primers designed to introduce *Not*I restriction sites at either end of the PCR product (lightning, 5′-ATATATATGCGGCCGCTATAGGCAGAGCATCAGA-3′; thunder, 5′-ATTATAATGCGGCCGCTTTCAATACAGTATATAGTTG-3′). The products were ligated into the *Not*I site of pTAG, resulting in the in-frame addition of three alanine residues followed by the 9-amino-acid HA epitope (Kolodziej and Young 1991) and 6 histidines at the carboxyl terminus of CUS1 (pGCT) or CUS1-54 (pGEKT). The cloned PCR fragments were sequenced completely in each plasmid. Both fusion constructs complement the *CUS1* knockout, and the tagged *CUS1-54* construct suppresses the cold-sensitive U2 mutation. pTAG was constructed as a general utility vector for *GAL*-regulated expression of tagged proteins in yeast (G. Voeltz and M. Ares, unpubl.) by amplifying and cloning the fused HA–6HIS tag described by Peterson et al. (1994) into pYES1.2 (Invitrogen, San Diego, CA). The plasmids carrying the mutant alleles of U2 are as described (Ares and Igel 1990; Yan and Ares 1996).

Yeast strains

Strains carrying U2 disruption and U2 mutations on plasmids have been described (Ares and Igel 1990; Zavanelli et al. 1994). The temperature-sensitive *prp* strains SRY4-1b, SRY5-1b, SRY9-1c, SRY11-1c, and SRY21-1b have also been described (Ruby et al. 1993). Other strains are derived from IH1097 (*MAT**a**, leu2-3, 112, ura3-52, pep4-3, prb1, prc1*). BJ81 is a derivative of IH1097 that carries a glucose-repressible U2 gene in the chromosome (Miraglia et al. 1991). HI227 carries the same markers as IH1097 but is also deleted for the *HIS3* and *LYS2* genes. SWY1 was constructed from HI227 by first transforming with a functional copy of the *CUS1-54* gene on a *URA3* plasmid (pGEKT) then replacing the chromosomal copy of *CUS1* with the deletion–disruption allele described above. SWY2 carries a glucose-repressible *CUS1* gene in the chromosome and was constructed from HI227 by replacing the chromosomal copy of *CUS1* with an allele, *GAL1:CUS1*, in which the endogenous promoter has been replaced by the *GAL1* promoter from pYES1.2 (Invitrogen). To integrate the *GAL1:CUS1* construct, the yeast 2μ region and an internal fragment from *CUS1* were removed from pGCT. The plasmid was then digested with *Kpn*I, which cuts upstream of the *CUS1* deletion and used to transform HI227. Transformants were selected on media containing galactose and lacking uracil. Integration at the *CUS1* locus was confirmed by Southern blotting for both SWY1 and SWY2.

RNA isolation and analysis

To determine the efficiency of splicing in cells depleted of CUS1, strains SWY2 (carrying the glucose-repressible chromosomal *CUS1* gene) or an isogenic control (HI227) were grown to $OD_{600}=2$ at 30°C in YPGal and diluted into 50 ml of either YPGal or YEPD to a final $OD_{600}=0.05$. After 7 hr of growth, cells reached $OD_{600}=0.4$–0.8, and were harvested. To determine whether *CUS1-54* suppresses the cold-sensitive splicing defect of U2 G53A, derivatives of BJ81 were grown to $OD_{600}=0.5$ at 30°C and shifted to 18°C. Cells were harvested after 2 hr of growth at 18°C. RNA was isolated as described previously (Ares and Igel 1990) and resuspended in DEPC-treated water at a nucleic acid concentration of 1 mg/ml. Primer extensions were performed on 5 μg of total RNA using ^{32}P-end-labeled oligonucleotides as described previously (Ares and Igel 1990). Annealing was carried out at 50°C. The sequences of the oligonucleotides used for primer extension are as follows: U3A+B, 5′-CCAAGTTGGATTCAGTGGCTC-3′; scR1-108, 5′GGCGTGCAATCCGTGTCT3′; YU5, 5′AAGTTCCAAAAAATATGGCAAGC3′.

In vitro splicing

Whole-cell extracts were prepared as described by Lin et al. (1985) from derivatives of BJ81 grown in YEPD and were assayed as described by McPheeters et al. (1989). Actin pre-mRNA substrate was synthesized in vitro essentially as described by Milligan and Uhlenbeck (1989) and gel purified on a 6% denaturing acrylamide gel. The template for substrate synthesis is a wild-type actin intron and is described by Zavanelli and Ares (1991). CUS1-depleted extracts were made by growing a preculture of SWY2 to $OD_{600}=4.0$ in YPGal and diluting to $OD_{600}=0.03$ in 1 liter of YEPD; cells were harvested after 14 hr at 30°C.

To assay for cold-sensitive complex formation, 25μl splicing reactions were assembled on ice and incubated at the indicated temperatures. At each time point 5μl aliquots were removed and mixed with an equal volume of ice-cold R* buffer (50 mM HEPES at pH 7.6, 2 mM MgOAc) with 2 mg/ml of total yeast RNA, and incubated for 10 min on ice, then 2.5 μl of 5× loading dye (2.5× TBE, 50% glycerol, bromophenol blue) was added, and the reactions were loaded on a native gel as described (Séraphin and Rosbash 1989, 1991). Gels were run at 100 V for 20 hrs at 4°C.

CUS1–tag protein fractions used to reconstitute CUS1-depleted extracts were made as follows. Yeast strain 334 (Hovland et al. 1989) carrying either the *GAL1:CUS1*–tag construct (pGCT) or the vector alone (pTAG) were grown to $OD_{600}=4$ in 1 liter of YEPD to $OD_{600}=2$, and 50 ml of 40% galactose was added to induce the galactose-regulated promoter. After 3 hr, cells were pelleted, washed once with sorbitol buffer (1 M sorbitol, 10 mM $MgCl_2$, 50 mM Tris-HCl at pH=8.0), resuspended in buffer MD (10 mM HEPES at pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM PMSF, 0.5 μg/ml of leupeptin, 0.75 μg/ml of pepstatin), and broken by vortexing with glass beads (3×30 sec at 4°C), with 2 min between vortexing. The lysates were allowed to settle for 5 min on ice, and the supernatant was removed to an Oakridge tube and centrifuged at 37K for 1 hr. The clear supernatant was then incubated in batch with 750 μl of NiNTA–agarose (QIAGEN, Chatsworth, CA,) for 2–3 hr at 4°C with slow rotation, washed four times with buffer MD with 10 mM imidazole, (pH 7.9). Bound protein was eluted with 250 μl of buffer MD with 250 mM imidazole, (pH 7.9), on ice for 20 min with occasional mixing and concentrated fivefold with a Microcon 30 microconcentrator (Amicon, Beverly, MA). Western blot analysis using polyclonal antibody 12CA5 (BAbCO, Emeryville, CA) was used to assay for the presence of the tagged protein.

Reconstitution reactions were performed by mixing 1 μl of eluent (1 mg/ml) with 1 μl of 20 mM ATP, 2 μl of 5× splicing salts, 1 μl of 2 mM *E. coli* rRNA, and 4 μl of splicing extract, and incubating at 23°C for 5 min. Then 1 μl of actin pre-mRNA substrate (5–10 fmoles) was added, and the reactions were incubated for an additional 20 min. The reactions were quenched as described above by adding 10 μl of ice cold R*, incubating for 10 min. on ice, followed by the addition of 5 μl of 5× loading dye. Reactions were run on a native gel for 20 hrs as described above.

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