Interaction of U2AF^{65} RS Region with Pre-mRNA of Branch Point and Promotion Base Pairing with U2 snRNA

Juan Valcárcel,* Rajesh K. Gaur, Ravinder Singh, Michael R. Green†

The mammalian splicing factor U2AF^{65} binds to the polypyrimidine tract adjacent to the 3′ splice site and promotes assembly of U2 small nuclear ribonucleoprotein on the upstream branch point, an interaction that involves base pairing with U2 small nuclear RNA (snRNA). U2AF^{65} contains an RNA binding domain, required for interaction with the polypyrimidine tract, and an arginine-serine–rich (RS) region, required for U2 snRNP recruitment and splicing. Here it is reported that binding of U2AF^{65} to the polypyrimidine tract directed the RS domain to contact the branch point and promoted U2 snRNA–branch point base pairing even in the absence of other splicing factors. Analysis of RS domain mutants indicated that the ability of U2AF^{65} to contact the branch point, to promote the U2 snRNA–branch point interaction, and to support splicing are related activities, requiring only a few basic amino acids. Thus, the U2AF^{65} RS domain plays a direct role in modulating spliceosomal RNA-RNA interactions.

Early during pre-mRNA splicing, U2 small nuclear ribonucleoprotein (snRNP) binds to the pre-mRNA branch point (*1*), an interaction that involves base pairing with a short sequence in U2 snRNA (*2*) and requires several auxiliary factors (*3*), including U2AF (*4*). The essential 65-kD subunit of U2AF (U2AF^{65}) has a bipartite structure comprising an RNA binding domain (RBD) and an RS region (*5*). The U2AF^{65} RBD is composed of three ribonucleoprotein consensus (RNP-CS) motifs (*6*) and specifically recognizes the polypyrimidine (Py) tract located between the branch point and the 3′ splice site (*5*, *7*, *8*). The RS domain is required for U2AF^{65} function (*5*), and when fused to a splicing repressor, Sex-lethal, converts it to a splicing activator (*7*).

As a first step toward understanding the function of the U2AF^{65} RS region (Fig. 1A), we prepared a series of RS derivatives and tested their activity in a U2AF-depleted nuclear extract (*7*, *9*). Substitution of the U2AF^{65} RS region by heterologous RS domains present in functionally unrelated splicing factors resulted in chimeric proteins with U2AF activity (Fig. 1B). Because these RS regions differ in size, distribution of RS dipeptides, and content of other amino acids, we conclude that the primary sequence of the U2AF^{65} RS region is not essential.

Progressive NH_2-terminal deletion of the U2AF^{65} RS region, which contains 10 RS dipeptides and stretches of basic amino acids (Fig. 1A), resulted in a concomitant decrease in activity (Fig. 1C). This result indicates that a small number of RS dipeptides or basic amino acids can provide RS function. Substitution of U2AF^{65} amino acids 1 through 94 by seven RS dipeptides supported splicing (Fig. 1D), confirming this observation.

We next analyzed a series of amino acid substitutions of the arginine or serine residues in the "synthetic" (RS)7 domain. Substitution of arginines by lysines did not compromise U2AF activity, whereas activity was abolished by alanine substitution (Fig. 1E). Surprisingly, there was no requirement for serine, which could be substituted by threonine, alanine, glycine, or methionine but not by negatively charged aspartic acid residues (Fig. 1, E and F). Taken together, these results suggest that the function provided by the RS domain depends solely on positive charge.

We have recently shown that when bound to the Py tract through the RNP-CS motifs, U2AF^{65} also contacts the branch point (*10*). Because the arginine-rich regions of several other proteins function by contacting RNA (*6*), we tested whether the RS region was the portion of U2AF^{65} that mediates branch point contact. A pre-mRNA specifically labeled at the branch point (*10*, *11*) became cross-linked to a ~65-kD protein in a nuclear extract but not in a U2AF-depleted nuclear extract (Fig. 2A). Recombinant glutathione-S-transferase (GST)–U2AF^{65} (93 kD), but not a derivative lacking the RS region (83 kD), was cross-linked when added to the depleted extract. Thus, the RS domain is required for the U2AF^{65}–branch point interaction. Similar results were obtained when the experiment was performed with purified fusion proteins (Fig. 2B), indicating that recognition of the branch point by the natural or synthetic RS domains is an intrinsic property of U2AF^{65} and does not

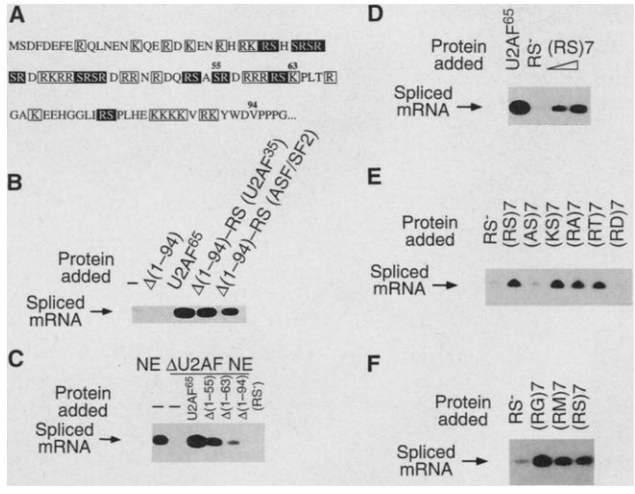

Fig. 1. Structure and function analysis of U2AF^{65} RS domain. (**A**) Primary sequence of 98 NH_2-terminal amino acids of U2AF^{65} (*5*, *32*). Black boxes, RS dipeptides; empty boxes, basic amino acids. (**B**) Heterologous RS domains can functionally substitute for U2AF^{65} RS region. U2AF^{65} amino acids 1 to 94 were substituted by the RS domains of either U2AF^{35} or ASF/SF2 (*33*). These chimeric proteins were expressed in and purified from *Escherichia coli* as GST fusions and their U2AF activity was tested in U2AF-depleted nuclear extracts (*7*) at an optimal concentration of 50 ng/μl. (**C**) Activity of U2AF^{65} NH_2-terminal deletion mutants. Recombinant U2AF^{65} and deletion mutants of the indicated residues (*34*) were tested as in (B). NE, nuclear extract; ΔU2AF NE, U2AF-depleted nuclear extract. (**D**) A synthetic RS domain can provide U2AF activity. A U2AF^{65} derivative was prepared in which the 94 NH_2-terminal residues were substituted by seven RS dipeptides [(RS)7] (*35*), and its activity was tested as in (B) at concentrations of 25 (left) and 50 ng/μl (right). RS$^-$ corresponds to U2AF^{65} Δ(1–94). (**E** and **F**) Mutagenesis of the synthetic RS domain. A series of U2AF^{65} derivatives in which either the arginine or the serine residues of the synthetic RS region are substituted by other amino acids were prepared and tested as in (D), at a concentration of 50 ng/μl.

Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical Center, 373 Plantation Street, Worcester, MA 01605, USA.

*Present address: Gene Expression Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

†To whom correspondence should be addressed.

require other spliceosomal components. Chemical cleavage of the cross-linked protein at Trp^{92} confirmed that the RS domain is the region of $U2AF^{65}$ that contacts the branch point (Fig. 2D). Neither $U2AF^{65}$ nor the (RS)7 mutant was cross-linked to a pre-mRNA that was labeled 20 nucleotides upstream of the branch point (*12*) (Fig. 2B) or to a substrate without a Py tract (*10*, *12*), indicating that the interaction is restricted to the branch point region and requires Py tract–bound $U2AF^{65}$. As expected (*5*, *8*), the RS domain did not affect binding of $U2AF^{65}$ to the Py tract, which occurs through the RNP-CS motifs (Fig. 2B).

Analysis of the RS region derivatives described in Fig. 1, E and F, revealed a strong correlation between splicing activity and branch point interaction (Fig. 2C). Collectively, these results indicate that binding of $U2AF^{65}$ to the Py tract positions the RS region to contact the branch point, and that this interaction is functionally related to splicing.

Because the $U2AF^{65}$ RS domain contacts the branch point, and the branch point forms base pairs with U2 snRNA, we investigated whether the $U2AF^{65}$ RS region is required for the U2 snRNA–branch point interaction. A psoralen cross-linking assay was used to detect U2 snRNA–pre-mRNA and U1 snRNA–pre-mRNA interactions in nuclear extract (*13*). The U2 snRNA–pre-mRNA interaction was not detected in a U2AF-depleted nuclear extract and was restored by addition of $U2AF^{65}$ but not by a $U2AF^{65}$ derivative lacking the RS domain (Fig. 3A). In contrast, base pairing between U1 snRNA and the 5′ splice site was unaffected by $U2AF^{65}$ depletion. We conclude that the RS domain of $U2AF^{65}$ is required to establish base pairing between U2 snRNA and the pre-mRNA. Unexpectedly, the U2-U6 snRNA interaction was also reduced upon U2AF depletion and partially restored upon addition of recombinant $U2AF^{65}$, suggesting that $U2AF^{65}$ may also modulate other spliceosomal RNA-RNA interactions (*14*).

To analyze whether promotion of U2 snRNA–branch point base pairing is a direct effect of the RS domain or requires other components (for example, a U2 snRNP polypeptide), we carried out psoralen cross-linking in a reaction mixture containing only pre-mRNA, U2 snRNA, and recombinant $U2AF^{65}$. A psoralen-dependent cross-link between U2 snRNA and the pre-mRNA was observed (Fig. 3B). Under identical conditions, a U1 snRNA–pre-mRNA cross-link was not detected. Formation of the U2 snRNA–pre-mRNA cross-link required (Fig. 3C) (i) an intact pre-mRNA branch point and Py tract, (ii) the U2 snRNA branch point recognition sequence (BPRS), and (iii) both the RS region and the RBD domains of $U2AF^{65}$. Primer extension analysis (Fig. 3D) mapped the cross-link to the pre-mRNA branch point and the U2 snRNA BPRS. Taken together, these data indicate that the interaction observed in this reconstituted system recapitulates that occurring during splicing. As shown in Fig. 3E, only those RS derivatives that supported splicing (Fig. 1, E and F) and contacted the branch point (Fig. 2C) facilitated U2 snRNA–branch point association.

A need to stabilize the mammalian U2 snRNA–pre-mRNA branch point interaction is anticipated because (i) the base-paired region is short, (ii) most mammalian branch points deviate from the consensus at several positions, and (iii) the branch point adenosine is probably bulged (*2*, *15*), further destabilizing the helix. On the basis of several independent lines of evidence, we propose that the $U2AF^{65}$ RS domain stabilizes base pairing immediately upstream of the $U2AF^{65}$ binding site, the Py tract (Fig. 4). First, a functional $U2AF^{65}$ RS region is determined solely by a limited number of basic residues, arguing against a highly specific interaction such as a protein-protein contact (*16*). Second, the target of the $U2AF^{65}$ RS domain is the pre-mRNA branch point. Third, the $U2AF^{65}$ RS domain promotes U2 snRNA–pre-mRNA association. And fourth, analysis of RS region mutants shows a close correlation between branch point contact, promotion of U2 snRNA–branch point base pairing, and splicing. Our model posits that $U2AF^{65}$ promotes the U2 snRNA–branch point interaction in an adenosine triphosphate (ATP)–independent manner. Indeed, Wassarman and Steitz (*13*) have shown in a nuclear extract that base pairing between U2 snRNA and the branch point occurs before ATP hydrolysis.

Whereas our mutational analysis and previous data (*5*, *17*, *18*) suggest that the interaction between the RS domain and pre-mRNA is not sequence-specific, our site-specific cross-linking experiments show that the interaction is restricted to fewer than 20 nucleotides from the Py tract. Thus, targeting of a nonspecific RNA-protein contact by specific recognition of the Py tract defines a pre-mRNA region for U2 snRNA binding. These considerations can explain why the primary constraint on mammalian branch point selection is distance from the Py tract (*19*).

We propose that the $U2AF^{65}$ RS region

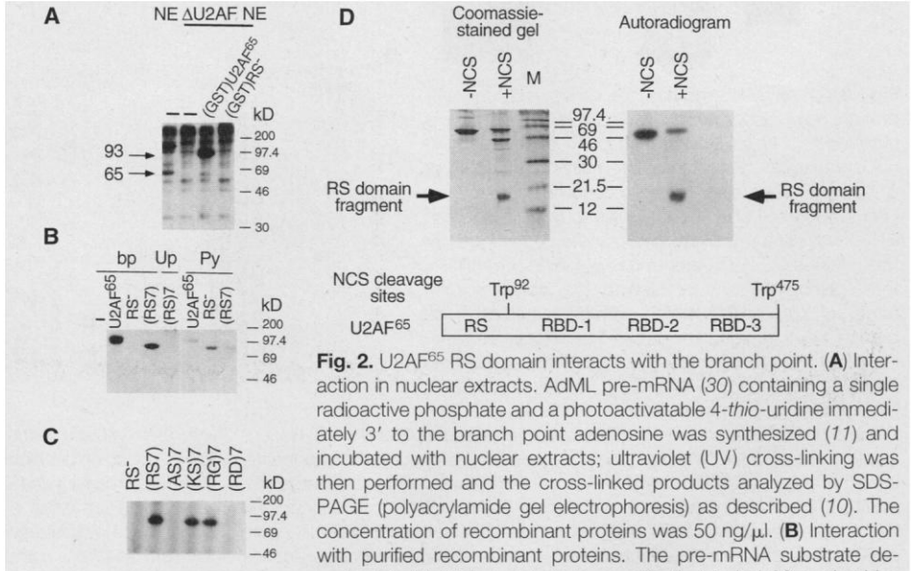

Fig. 2. $U2AF^{65}$ RS domain interacts with the branch point. (**A**) Interaction in nuclear extracts. AdML pre-mRNA (*30*) containing a single radioactive phosphate and a photoactivatable 4-*thio*-uridine immediately 3′ to the branch point adenosine was synthesized (*11*) and incubated with nuclear extracts; ultraviolet (UV) cross-linking was then performed and the cross-linked products analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) as described (*10*). The concentration of recombinant proteins was 50 ng/μl. (**B**) Interaction with purified recombinant proteins. The pre-mRNA substrate described in (A) (Bp), a substrate specifically labeled 20 nucleotides upstream of the branch point (Up), or a labeled RNA corresponding to the polypyrimidine tract (Py) was incubated with the indicated recombinant proteins (50 ng/μl) in buffer D plus 0.1 M KCl (*29*) and processed as described in (A). (**C**) Interaction of $U2AF^{65}$ RS domain mutants with the branch point. Cross-linking assays were performed as in (B) with recombinant $U2AF^{65}$ derivatives described in Fig. 1, E and F. (**D**) $U2AF^{65}$ RS domain directly contacts the branch point. Recombinant $U2AF^{65}$ (*36*) was UV cross-linked to the branch point as in (B), the cross-linked adduct was cleaved with the tryptophan-specific reagent *N*-chlorosuccinimide (NCS) as described (*37*), and the products fractionated on a 15%-polyacrylamide gel, which was stained with Coomassie blue (left) and autoradiographed (right). The positions of tryptophan residues in $U2AF^{65}$ are indicated (bottom). Cleavage with NCS generates two polypeptides with predicted molecular sizes of 10.3 kD (which corresponds to the RS region and has an anomalous mobility because of its high content of positively charged amino acids) and 43 kD (which corresponds to $U2AF^{65}$ RNP-CS domains). Lane M, molecular size markers; RBD, RNA binding domain.

promotes RNA-RNA association in the same way that basic peptides accelerate nucleic acid hybridization (*20*): the basic amino acid side chains interact with and neutralize the negatively charged phosphates, thereby facilitating approximation of the two strands. A U2AF^{65} arginine residue or residues could also stabilize the bulged adenosine; stabilization of an RNA bulge by arginine is an important feature of the human immunodeficiency virus Tat-TAR interaction (*21*).

Previous studies have shown that the RS domains present in splicing factors of the SR family (*22*) mediate protein-protein interactions (*23*). Our results suggest that the target of the U2AF^{65} RS region is the pre-mRNA branch point, and not a protein. Other differences between these two types of RS domains have also been reported. For example, whereas the activity of the U2AF^{65} RS domain depends solely on a few basic amino acids, the RS domain of ASF-SF2 is sensitive to small deletions (*24*) and even to conservative substitutions of arginines and serines (*25*). Second, properties associated with the RS domain of SR proteins, such as insolubility at high magne-

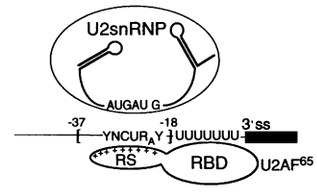

Fig. 4. A role for U2AF^{65} RS domain in U2 snRNP recruitment. When U2AF^{65} binds to the Py tract through its RBD, the region of the pre-mRNA immediately upstream from the Py tract is contacted by the positively charged arginine-serine–rich domain (RS). This interaction stabilizes base pairing between U2 snRNA and the loose mammalian branch point consensus, thus defining a region in the pre-mRNA for U2 snRNP recruitment. This model offers an explanation for the natural occurrence of branch points −18 to −37 nucleotides from the 3′ splice site (*19*). The Py tract is represented by a stretch of U's, the branch point by its consensus (Y, pyrimidine; N, any nucleotide; R, purine), and the BPRS in U2 sRNA by its sequence (GUAGUA). The adenosine forming the 2′-5′ branch is bulged out from the base-paired region.

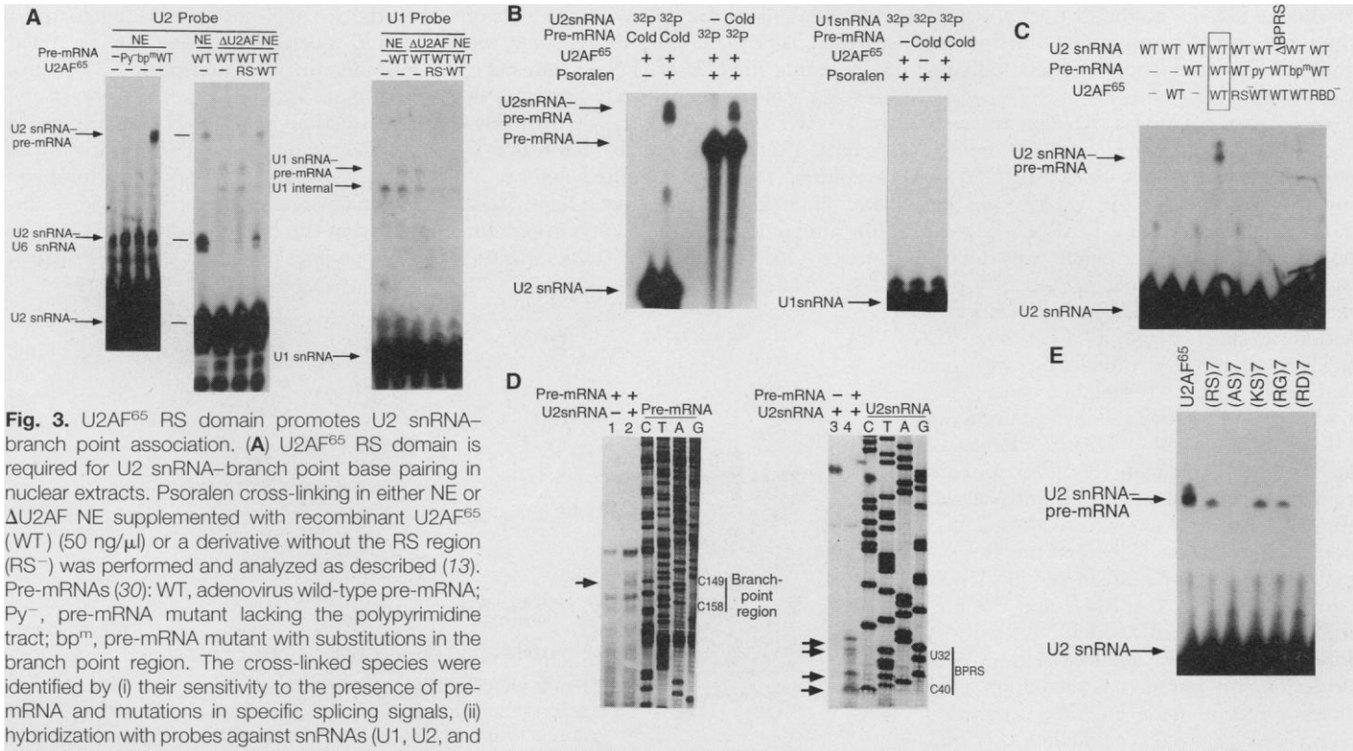

Fig. 3. U2AF^{65} RS domain promotes U2 snRNA–branch point association. (**A**) U2AF^{65} RS domain is required for U2 snRNA–branch point base pairing in nuclear extracts. Psoralen cross-linking in either NE or ΔU2AF NE supplemented with recombinant U2AF^{65} (WT) (50 ng/μl) or a derivative without the RS region (RS$^-$) was performed and analyzed as described (*13*). Pre-mRNAs (*30*): WT, adenovirus wild-type pre-mRNA; Py$^-$, pre-mRNA mutant lacking the polypyrimidine tract; bp^m, pre-mRNA mutant with substitutions in the branch point region. The cross-linked species were identified by (i) their sensitivity to the presence of pre-mRNA and mutations in specific splicing signals, (ii) hybridization with probes against snRNAs (U1, U2, and U6) and pre-mRNA, and (iii) comparison with previous reports (*13*). (**B**) Reconstitution of the U2 snRNA–branch point association with purified components. In vitro–transcribed [^{32}P-labeled (^{32}P) or nonradioactive (cold)] adenovirus pre-mRNA, U2 (or U1) snRNA, and recombinant purified U2AF^{65} were incubated (*38*) in the absence or presence of psoralen and UV-irradiated; the RNAs were purified and analyzed by electrophoresis on 5% denaturing polyacrylamide gels and autoradiography. The product of cross-linking between U2 snRNA and pre-mRNA is indicated. (**C**) The U2 snRNA–pre-mRNA interaction observed in the reconstituted system mimics the interaction observed in nuclear extracts and depends on the U2AF^{65} RS region. Assays were performed as in (B) with ^{32}P-labeled U2 snRNA or derivatives and nonradioactive pre-mRNAs. Pre-mRNAs (*30*) are as in (A). U2 snRNAs (*31*): WT, wild-type U2 snRNA; ΔBPRS, U2 snRNA derivative with a deletion in the BPRS. U2AF^{65} recombinant proteins: WT, wild-type GST-U2AF^{65}; RS$^-$, GST-U2AF^{65} Δ(1–94); RBD$^-$, GST fused to amino acids 1 to 63 of U2AF^{65}. (**D**) Cross-linking in the reconstituted system occurs between the pre-mRNA branch point region and the U2 snRNA BPRS. The nucleotides and area covering the branch point and BPRS in which psoralen cross-links have been identified between these two RNAs in nuclear extracts (*13*) are indicated. Primer extension assays with 5′-labeled oligodeoxyribonucleotides complementary to positions 202 to 220 of the pre-mRNA (left) or positions 54 to 71 of U2 snRNA (right) were performed on isolated, cross-linked U2 snRNA–pre-mRNA adducts obtained as in (C) (lanes 2 and 4) or on either pre-mRNA alone (lane 1) or U2 snRNA alone (lane 3), which were incubated with U2AF^{65} in the presence of psoralen, irradiated with UV light under the same conditions used to obtain the U2 snRNA–pre-mRNA cross-links, and recovered from the gel. As noted (*13*), psoralen modifies the pre-mRNA in the absence of nuclear extract components, generating mono-adducts that give rise to a background of primer extension stops in the absence of intermolecular base-pairing interactions. The arrow in the left panel indicates the primer extension stop in the pre-mRNA, which is induced by the presence of U2 snRNA. This position (C149) is adjacent to the nucleotide that studies in nuclear extracts (*13*) have predicted to form the most prominent psoralen cross-link with U2 snRNA. Several pre-mRNA–induced primer extension stops (*39*) can be identified in the BPRS of U2 snRNA. Note that the sequence ladder corresponds to a U2 mutant gene with an A-to-T transversion at position 39. (**E**) Activities of U2AF^{65} RS domain mutants in promoting U2 snRNA–branch point interactions. The activity associated with RS mutant derivatives described in Fig. 1, E and F, was analyzed as in (C).

sium concentrations (*22*) and reactivity to specific antibodies (*26*), are not shared by U2AF^{65}. Finally, studies reporting protein-protein interactions among SR proteins failed to detect interactions between the U2AF^{65} RS region and other splicing factors (*23*, *27*). Taken together, these observations suggest that there are two distinct classes of RS domains.

The orchestrated formation and disruption of short RNA-RNA helices is a common theme in pre-mRNA splicing (*28*) and probably in other processes that involve RNA. Here we have described a mechanism by which a spliceosomal RNA-RNA base-pairing interaction can be regulated by a protein.

REFERENCES AND NOTES

1. M. J. Moore, C. C. Query, P. A. Sharp, in *The RNA World*, R. F. Gesteland and J. F. Atkins, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), p. 303.
2. R. Parker, P. G. Siciliano, C. Guthrie, *Cell* **49**, 229 (1987); J. Y. Wu and J. L. Manley, *Genes Dev.* **3**, 1553 (1989); Y. Zhuang and A. M. Weiner, *ibid.*, p. 1545.
3. P. E. Hodges and J. D. Beggs, *Curr. Biol.* **4**, 264 (1994).
4. B. Ruskin, P. D. Zamore, M. R. Green, *Cell* **52**, 207 (1988); P. D. Zamore and M. R. Green, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9243 (1989).
5. P. D. Zamore, J. G. Patton, M. R. Green, *Nature* **355**, 609 (1992).
6. C. G. Burd and G. Dreyfuss, *Science* **265**, 615 (1994).
7. J. Valcárcel, R. Singh, P. D. Zamore, M. R. Green, *Nature* **362**, 171 (1993).
8. R. Singh, J. Valcárcel, M. R. Green, *Science* **268**, 1173 (1995).
9. Modifications in the RS region did not affect binding to the Py tract (*12*) (see also Fig. 2B), as expected from previous studies (*5*, *8*).
10. R. K. Gaur, J. Valcárcel, M. R. Green, *RNA* **1**, 407 (1995).
11. M. J. Moore and P. A. Sharp, *Science* **256**, 992 (1992).
12. J. Valcárcel and M. R. Green, unpublished observations.
13. D. A. Wassarman and J. A. Steitz, *Science* **257**, 1918 (1992).
14. Interestingly, the region in U6 that forms base pairs with U2 is immediately upstream of a pyrimidine stretch [T.-P. Hausner, L. M. Giglio, A. M. Weiner, *Genes Dev.* **4**, 2146 (1990)], suggesting that U2AF^{65} could bind to this sequence to modulate the U2-U6 interaction. Alternatively, this effect could be due to the general annealing activity associated with the U2AF^{65} RS region (*18*).
15. C. C. Query, M. J. Moore, P. A. Sharp, *Genes Dev.* **8**, 587 (1994).
16. Even protein-protein interaction motifs composed predominantly of a single amino acid, such as acidic transcription activation and proline-rich SH3 domains, contain additional types of residues also critical for activity [S. J. Triezenberg, *Curr. Opin. Genet. Dev.* **5**, 190 (1995); T. Pawson and J. Schlessinger, *Curr. Biol.* **3**, 434 (1993)].
17. P. Zuo and J. L. Manley, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3363 (1994).
18. C.-G. Lee, P. D. Zamore, M. R. Green, J. Hurtwitz, *J. Biol. Chem.* **268**, 13472 (1993).
19. M. R. Green, *Annu. Rev. Genet.* **20**, 671 (1986); C. W. J. Smith and B. Nadal-Ginard, *Cell* **56**, 749 (1989).
20. M. Feughelman *et al.*, *Nature* **175**, 834 (1955).
21. B. Calnan *et al.*, *Science* **252**, 1167 (1991).
22. A. M. Zahler, W. S. Lane, J. A. Stolk, M. B. Roth, *Genes Dev.* **6**, 837 (1992).
23. J. Wu and T. Maniatis, *Cell* **75**, 1061 (1993); J. Kohtz *et al.*, *Nature* **368**, 119 (1994); H. Amrein, M. L. Hedley, T. Maniatis, *Cell* **76**, 735 (1994).
24. P. Zuo and J. L. Manley, *EMBO J.* **12**, 4727 (1993).
25. J. F. Cáceres and A. R. Krainer, *ibid.*, p. 4715.
26. M. B. Roth, A. M. Zahler, J. A. Stolk, *J. Cell Biol.* **115**, 587 (1991).
27. Y. Ono, M. Ohno, Y. Shimura, *Mol. Cell. Biol.* **14**, 7611 (1994); J. Fleckner and M. R. Green, unpublished observations.
28. H. Madhani and C. Guthrie, *Annu. Rev. Genet.* **28**, 1 (1994).
29. Buffer D: 20 mM Hepes (pH 8.0), 0.2 mM EDTA, 20% glycerol, 0.05% NP-40, and 1 mM dithiothreitol.
30. Plasmid pMINX [M. Zillmann *et al.*, *Mol. Cell. Biol.* **8**, 814 (1988)] was used to generate pPy-MINX (pre-mRNA positions 165 to 177 substituted by AUCCAGGAAUUCUAAUACGACUCACUAU) and pbp^m-MINX (pre-mRNA positions 150 to 160 substituted by UAUGAUAGUGA). The corresponding pre-mRNAs were generated by in vitro transcription of Bam HI–digested templates.
31. Plasmids pT7U2 [A. M. Kleinschmidt *et al.*, *Nucleic Acids Res.* **17**, 4817 (1989)], pT7U2ΔBPRS (deletion of U2 snRNA positions 37 to 42), and pHU1A [J. R. Patton, R. J. Patterson, T. Pederson, *Mol. Cell. Biol.* **7**, 4030 (1987)] were used to transcribe U2 snRNA (positions 1 to 71), ΔBPRS, and U1 snRNA, respectively.
32. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
33. RS (U2AF^{35}): positions 561 to 723 of U2AF^{35} cDNA [M. Zhang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8769 (1992)]. RS (SF2): positions 887 to 1039 of ASF/SF2 cDNA [H. Ge *et al.*, *Cell* **66**, 373 (1991); A. R. Krainer *et al.*, *ibid.*, p. 383].
34. U2AF^{65} Δ(1–55) was prepared by deletion of a Bam HI–Xma I fragment from pGEX3X-U2AF^{65} (*5*) and religation after Klenow treatment. U2AF^{65} Δ(1–63) has been described (*5*). U2AF^{65} Δ(1–94) was prepared by inserting a U2AF^{65} cDNA fragment encoding amino acids 94 to 475, flanked by Bam HI and Eco RI, into the same sites in pGEX-2T [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. Digestion of this construct with Bam HI was used to obtain all the RS mutant derivatives.
35. Inserts were obtained by annealing complementary oligodeoxyribonucleotides corresponding to sequences encoding the RS repeats or mutant derivatives.
36. U2AF^{65} was cloned in plasmid pGEX-CS [T. D. Parks *et al.*, *Anal. Biochem.* **216**, 413 (1994)] to generate a GST-U2AF^{65} fusion with a TEV protease cleavage site between the two polypeptides. The fusion protein was expressed in bacteria, purified, and cleaved with TEV protease as described by Parks *et al.* (*ibid.*).
37. M. Mirfakhrai and A. M. Weiner, *Nucleic Acids Res.* **21**, 3591 (1993).
38. Reactions (20 μl) containing 75% buffer D (*29*), 75 mM KCl, 10 fmol of adenovirus-major-late (AdML) promotor derived pre-mRNAs (*30*), 50 fmol of U2 snRNA, ΔBPRS or U1 snRNA (*31*), recombinant U2AF^{65} or derivatives (50 ng/μl), psoralen (20 μg/ml), and pGEM3 plasmid (47 ng/μl) were irradiated at 25°C with 366-nm ultraviolet light (UVP Inc. lamp, at a distance of 3.5 cm from the samples) for 30 min. Under these conditions, U2AF^{65} promoted interactions between U2 snRNA and a variety of pre-mRNAs from *Drosophila* to human genes.
39. Primer extension mapping of psoralen cross-links has been shown to give rise to more than one primer extension stop from a single cross-link [G. Ericson and P. Wollenzien, *Anal. Biochem.* **174**, 215 (1988); J. Teare and P. Wollenzien, *Nucleic Acids Res.* **18**, 855 (1990)].

40. We thank M. Jacobson and T. Pederson for the U1 snRNA clone, L. Chiang and J. Kielty for technical support, T. O'Toole for secretarial assistance, and F. Gebauer, K. Neugebauer, and members of the Green lab for advice and comments on the manuscript. Supported by European Molecular Biology Organization and Spanish Ministerio de Educación y Ciencia fellowships to J.V., Leukemia Society of America postdoctoral and special fellowships to R.S., and a grant from the National Institutes of Health to M.R.G.

25 June 1996; accepted 30 July 1996

Small-Conductance, Calcium-Activated Potassium Channels from Mammalian Brain

M. Köhler, B. Hirschberg, C. T. Bond, J. M. Kinzie, N. V. Marrion, J. Maylie, J. P. Adelman*

Members of a previously unidentified family of potassium channel subunits were cloned from rat and human brain. The messenger RNAs encoding these subunits were widely expressed in brain with distinct yet overlapping patterns, as well as in several peripheral tissues. Expression of the messenger RNAs in *Xenopus* oocytes resulted in calcium-activated, voltage-independent potassium channels. The channels that formed from the various subunits displayed differential sensitivity to apamin and tubocurare. The distribution, function, and pharmacology of these channels are consistent with the SK class of small-conductance, calcium-activated potassium channels, which contribute to the afterhyperpolarization in central neurons and other cell types.

Action potentials in vertebrate neuronsare followed by an afterhyperpolarization (AHP) that may persist for several seconds and may have profound consequences for the firing pattern of the neuron. The AHP has several components. The fast component (fAHP) helps to repolarize the action potential and regulates spike interval, whereas subsequent slow components (sAHP) underlie spike-frequency adaptation (*1*–*5*).

Each component of the AHP is kinetically distinct and is mediated by different Ca^{2+}-activated K^+ channels. The large-conductance (100 to 200 pS), voltage- and Ca^{2+}-activated K^+ channels (BK channels) underlie the fAHP (*6*, *7*), which develops rapidly (1 to 2 ms) and decays within tens