structure shows that residues 15 to 20 are part of α helix A and that residues 23 to 25 are involved in β sheet A. In T4 regA, changing Ala^{25} to asparagine results in a significant alteration of the binding affinity (*12*). The side chain of Ala^{25} is located in a hydrophobic environment generated by the side chains of β sheet A and helix D (Val^{32}, Ile^{104}, Leu^{114}, and Trp^{112}). Residue 25 is in the center of this β pleated sheet–forming region. It is likely that the integrity of this β sheet is maintained by the hydrophobic interactions involving the Ala^{25} side chain. Mutating Ala^{25} to valine appears to have no effect on the binding ability; this finding reinforces the interpretation that this hydrophobic binding domain is important for maintaining 3D structure.

Photochemical cross-linking to radioactive nucleotides, in combination with cyanogen bromide cleavage of the regA protein, led to the suggestion that two regions—residues 31 to 41 and 96 to 122—may be involved in RNA binding (*14*). Residues 31 to 41 are part of the β sheet system. In the COOH-terminal segment, residues 113 to 116 are involved in the same antiparallel β sheet system A that contains Ala^{25} as well as residues 31 to 35. Thus, several types of experiments have suggested that β sheet system A may be involved in RNA recognition. The mutational experiments mentioned above suggest that sequences in the NH_2-terminal region between Val^{15} and Ala^{25} are important for RNA binding (*13*). This region is found in α helix A, which serves to connect β sheet regions A and B.

Understanding the manner in which regA binds RNA in a sequence-specific manner requires the determination of the structure of the protein complexed to one of its RNA substrates. In view of the numerous and complex types of specific interactions exhibited by this protein, it is possible that the region involved in recognition is not simple and may span a large portion of the molecule. For example, the two β sheet regions A and B that are 25 Å apart could both be involved in RNA interactions, as could the α-helical segment connecting them. Although the experiments described above suggest that one or more of these regions of the regA molecule constitute a possible site of RNA recognition, the interactions found between the U1A protein and the U1 RNA (*9*) emphasize the importance of the RNP-1 and RNP-2 motifs. Hence, mutational experiments with regA should be conducted in which, for example, the sequences of the central β sheets are made identical to the RNP-1 and RNP-2 consensus sequences. Because regA binds to various mRNAs with different affinities (*3*), changes induced in binding affinities may yield insight into the regA recognition system.

REFERENCES AND NOTES

1. M. A. Rould *et al.*, *Nature* **352**, 213 (1991); J. Cavarelli *et al.*, *ibid.*, p. 181; V. Biou *et al.*, *Science* **263**, 1404 (1994).
2. C. G. Burd and G. Dreyfuss, *Science* **265**, 615 (1994).
3. E. S. Miller, J. D. Karam, E. Spicer, in *Molecular Biology of Bacteriophage T4*, J. D. Karam, Ed. (ASM Press, Washington, DC, 1994), pp. 193–205.
4. R. B. Winter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7822 (1987).
5. S. Unnithan *et al.*, *Nucleic Acids Res.* **18**, 7083 (1990); E. S. Miller, R. B. Winter, K. M. Campbell, S. D. Power, L. Gold, *J. Biol. Chem.* **260**, 13053 (1985).
6. Sedimentation equilibrium studies were carried out by Y. Kyogoku of Osaka University. In a dilute solution (0.03 mM) the molecular weight was close to the expected value of a dimer (personal communication).
7. K. Valegård *et al.*, *Nature* **345**, 36 (1990).
8. K. Nagai *et al.*, *ibid.* **348**, 515 (1990).
9. C. Oubridge, N. Ito, P. R. Evans, C.-H. Teo, K. Nagai, *ibid.* **372**, 432 (1994).
10. M. Trojanowska, E. S. Miller, J. Karam, G. Stormo, L. Gold, *Nucleic Acids Res.* **12**, 5979 (1984).
11. Single-letter abbreviations for the amino acid residues are as follows: E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; T, Thr; V, Val; Y, Tyr; and X, any amino acid.
12. K. R. Webster and E. K. Spicer, *J. Biol. Chem.* **265**, 19007 (1990).
13. C. E. Jozwik and E. S. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5053 (1992).
14. K. R. Webster *et al.*, *J. Biol. Chem.* **267**, 26097 (1992).
15. W. Steigmann, *PROTEIN: A Package of Crystallographic Programs for Analysis of Proteins* (Max Planck Institute for Biochemistry, Martinsried, Germany, 1982).
16. B. C. Wang, *Methods Enzymol.* **115**, 90 (1985).
17. T. A. Jones, in *Computational Crystallography*, D. Sayer, Ed. (Oxford Univ. Press, Oxford, 1982), pp. 303–317; J. W. Pflugrath, M. A. Saper, F. A. Quiocho, in *Methods and Applications in Crystallographic Computing*, S. Hall and E. Ashida, Eds. (Clarendon, Oxford, 1984), pp. 404–407.
18. A. T. Brünger, X-PLOR, Yale University, New Haven, CT.
19. P. J. Kraulis, *J. Appl. Crystallogr.* **24**, 946 (1991).

20. We thank A. M. deVos, N. Xuong, W. Royer, and R. Sweet for assistance in data collection and A. Herbert, K. Lowenhaupt, S. Woelfl, L. Su, J. Spitzner, and Y. Kyogoku for helpful discussions. Supported by grants from NIH, NSF, Human Frontier Science (HFS), and the U.S. Office of Naval Research (at MIT) and from HFS and NSF (at the University of Colorado).

4 November 1994; accepted 14 February 1995

Distinct Binding Specificities and Functions of Higher Eukaryotic Polypyrimidine Tract–Binding Proteins

Ravinder Singh, Juan Valcárcel, Michael R. Green

In higher eukaryotes, the polypyrimidine-tract (Py-tract) adjacent to the 3′ splice site is recognized by several proteins, including the essential splicing factor $U2AF^{65}$, the splicing regulator Sex-lethal (Sxl), and polypyrimidine tract–binding protein (PTB), whose function is unknown. Iterative in vitro genetic selection was used to show that these proteins have distinct sequence preferences. The uridine-rich degenerate sequences selected by $U2AF^{65}$ are similar to those present in the diverse array of natural metazoan Py-tracts. In contrast, the Sxl-consensus is a highly specific sequence, which can help explain the ability of Sxl to regulate splicing of *transformer* pre-mRNA and autoregulate splicing of its own pre-mRNA. The PTB-consensus is not a typical Py-tract; it can be found in certain alternatively spliced pre-mRNAs that undergo negative regulation. Here it is shown that PTB can regulate alternative splicing by selectively repressing 3′ splice sites that contain a PTB-binding site.

Several eukaryotic RNA-binding proteins preferentially interact with uridine-rich sequences and have thus been classified as Py-tract–binding proteins (*1*). Human $U2AF^{65}$ is an essential splicing factor that recognizes a wide variety of Py-tracts (*2*). *Drosophila* Sxl regulates 3′ splice-site switching of *transformer* (*tra*) pre-mRNA and exon skipping of its own pre-mRNA (*3*, *4*). The U-octamer (U_8C) sequence common to the non–sex-specific (NSS) Py-tract of *tra* and the male-specific Py-tract of *Sxl* pre-mRNA has been suggested to be the Sxl-binding site (*4*–*6*). PTB, also known as hnRNP I (*7*), was originally identified by its binding to the Py-tracts of adenoviral major late (Ad ML) and α-tropomyosin pre-mRNAs, and on this basis was proposed to be a splicing factor (*8*).

To gain insight into RNA recognition and function of these proteins, we performed iterative in vitro genetic selection (*9*). The sequences of 20 to 30 complementary DNA (cDNA) clones from each selected pool revealed that $U2AF^{65}$, Sxl, and PTB had distinct RNA sequence preferences (Fig. 1, A to C). The $U2AF^{65}$-selected sequences were enriched in uridines that were frequently interrupted by two to three cytidines [UUUUUU(U/C)-CC(C/U)UUUUUUCC]. The relative distribution of nucleotides in the $U2AF^{65}$-selected pool (11.5% A, 29.8% C, 7.3%

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

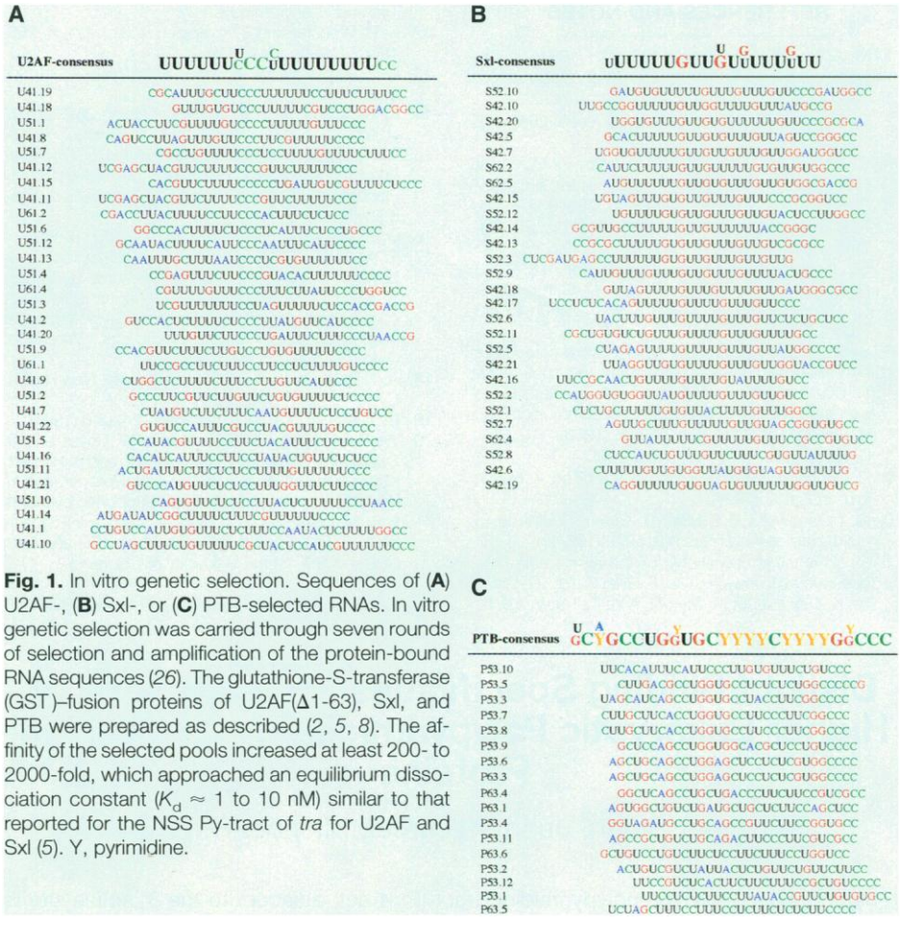

Fig. 1. In vitro genetic selection. Sequences of (**A**) U2AF-, (**B**) Sxl-, or (**C**) PTB-selected RNAs. In vitro genetic selection was carried through seven rounds of selection and amplification of the protein-bound RNA sequences (*26*). The glutathione-S-transferase (GST)–fusion proteins of U2AF(Δ1-63), Sxl, and PTB were prepared as described (*2*, *5*, *8*). The affinity of the selected pools increased at least 200- to 2000-fold, which approached an equilibrium dissociation constant ($K_d \approx 1$ to 10 nM) similar to that reported for the NSS Py-tract of *tra* for U2AF and Sxl (*5*). Y, pyrimidine.

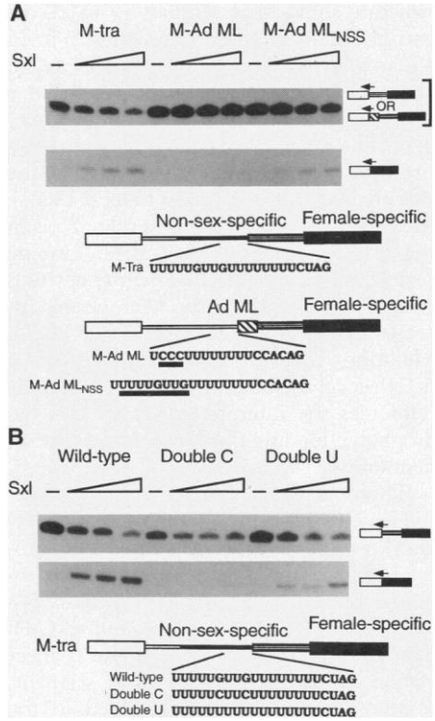

Fig. 2. Sequence requirements for Sxl responsiveness. (**A**) Sequences upstream of U-octamer are required for *tra* 3′ splice site switching by Sxl. Schematics of the chimeric pre-mRNAs, M-tra, M-Ad ML, and M-Ad ML$_{NSS}$ are shown (*27*). The NSS Py-tract/3′ splice site of *tra* was replaced by the Py-tract/3′ splice site of Ad ML gene in M-Ad ML (*12*). The CCC sequence of M-Ad ML was converted to UUUUGUUG sequence in M-Ad ML$_{NSS}$. The affinity of Sxl for the U_8C-containing Py-tract/3′ splice site of Ad ML pre-mRNA was approximately one-thirtieth that for the NSS Py-tract. (**B**) The two guanosines within the *tra* NSS Py-tract are important for Sxl regulation. Schematics of the wild-type, the double-C, and the double-U mutants of M-tra are shown. The concentrations of GST-Sxl were 3 μg/ml, 10 μg/ml, and 30 μg/ml. The pre-mRNAs were synthesized by SP6 RNA polymerase (*28*) and analyzed for 3′ splice site switching by Sxl in a HeLa nuclear extract (*29*). The spliced products were detected by primer extension with splice junction primers for M-tra as described (*5*). The splice junction primer for Ad ML 3′ splice site of M-Ad ML and M-Ad ML$_{NSS}$ pre-mRNAs was 5′-CCTCAACCGCGAGCG TTC-3′.

G, and 51% U) mirrored that of a collection of natural metazoan Py-tracts (9.8% A, 33.7% C, 9.8% G, and 46.7% U) (*10*). These findings are consistent with the role of U2AF^{65} as an essential splicing factor that must recognize the diverse array of natural Py-tracts (*2*, *11*).

The majority of Sxl-selected sequences comprised a stretch of 17 to 20 uridines interrupted by two to four guanosines (Fig. 1B). The Sxl-consensus, UUUUUGUU-(U/G)U(G/U)UUU(G/U)UU, is similar to the default, NSS Py-tract (UUUUUGUU-GUUUUUUUU) of *tra* pre-mRNA (*4*) and is more complex than a simple U-octamer, the proposed Sxl-binding site (*4*–*6*). We previously analyzed Sxl function in a 3′ splice site switch assay that used a pre-mRNA (M-tra) with two alternative 3′ splice sites: a proximal *tra* NSS 3′ splice site and a distal female-specific (FS) 3′ splice site (*5*). Under standard conditions, splicing was exclusively to the NSS 3′ splice site. Addition of Sxl decreased splicing to this site and concomitantly activated the alternative FS 3′ splice site (Fig. 2A). To verify the functional relevance of the more complex Sxl-consensus, we replaced the natural NSS Py-tract/3′ splice site of *tra* by the Ad ML Py-tract/3′ splice site (*12*), which contains a U-octamer. This M-Ad ML pre-mRNA was refractory to Sxl regulation (Fig. 2A), consistent with in vivo studies (*13*). Incorporation of the UUUUGUUG sequence upstream of the U-octamer rendered this pre-mRNA (M-Ad ML$_{NSS}$) Sxl-responsive.

Approximately 50% of the Sxl-selected clones contained a GUUG sequence (Fig. 1B) that is also present in the NSS Py-tract of *tra* (*4*). To analyze the role of the two guanosines, we converted them to cytidines or uridines. The double-cytidine mutation markedly impaired Sxl regulation, and the double-uridine mutation had a smaller, but reproducible effect (Fig. 2B). Together, these results indicate that the UUUUU(U/G)UU(U/G)UUUUUU-UU sequence, and not a U-octamer, confers Sxl responsiveness. This Sxl-consensus has been conserved among the NSS Py-tracts of five *Drosophila* species (*14*). The 19- to 23-nucleotide uridine-rich elements in the introns of *Sxl* pre-mRNA are involved in splicing autoregulation by Sxl (*15*). However, these sequences lack the GUUG element, which is present in *tra* NSS Py-tracts (*14*). Our in vitro selection (Fig. 1B) and mutational analyses (Fig. 2B) show that uridines can partially substitute for the two guanosines, which explains the binding of Sxl to these elements.

The majority of the PTB-selected clones contained the consensus sequence (U/G)-C(A/Y)GCCUG(Y/G)UGCYYYYCYY YYG(Y/G)CCC (Fig. 1C). As demonstrated by RNA-binding assay (Fig. 3A), U2AF^{65} and Sxl bound with high affinity to the Sxl-selected (Sxl-s) sequence (*16*), but failed to bind to the PTB-selected (PTB-s) sequence. Conversely, PTB bound efficiently to the PTB-selected sequence and the Ad ML Py-tract/3′ splice

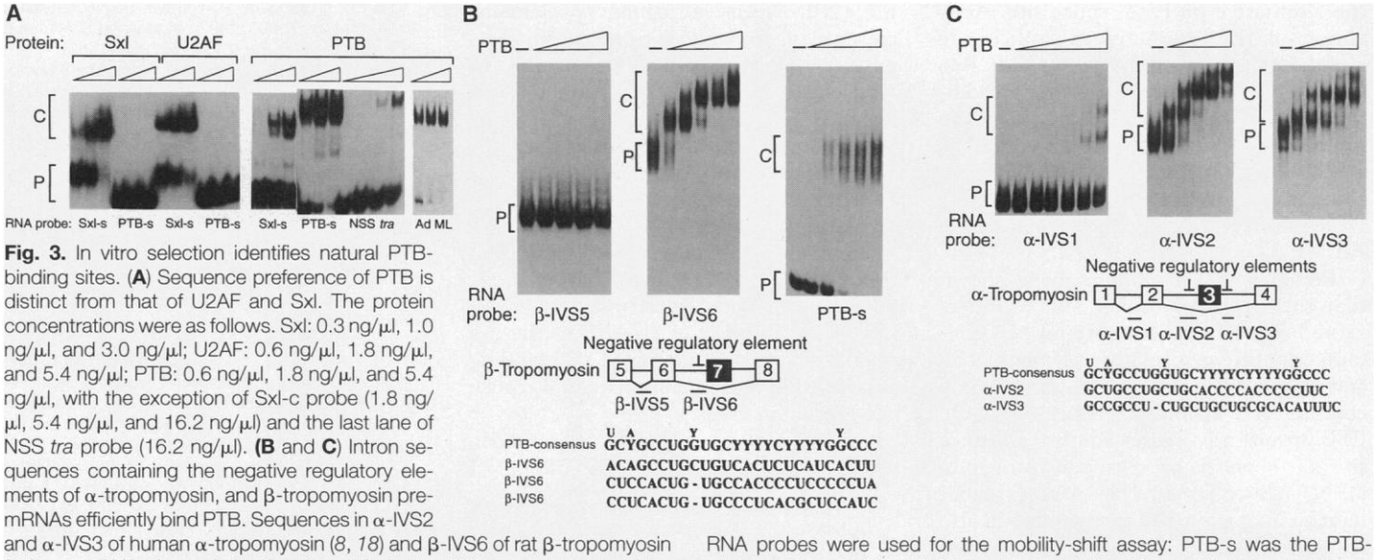

Fig. 3. In vitro selection identifies natural PTB-binding sites. (**A**) Sequence preference of PTB is distinct from that of U2AF and Sxl. The protein concentrations were as follows. Sxl: 0.3 ng/μl, 1.0 ng/μl, and 3.0 ng/μl; U2AF: 0.6 ng/μl, 1.8 ng/μl, and 5.4 ng/μl; PTB: 0.6 ng/μl, 1.8 ng/μl, and 5.4 ng/μl, with the exception of Sxl-c probe (1.8 ng/μl, 5.4 ng/μl, and 16.2 ng/μl) and the last lane of NSS *tra* probe (16.2 ng/μl). (**B** and **C**) Intron sequences containing the negative regulatory elements of α-tropomyosin, and β-tropomyosin pre-mRNAs efficiently bind PTB. Sequences in α-IVS2 and α-IVS3 of human α-tropomyosin (*8*, *18*) and β-IVS6 of rat β-tropomyosin (*18*) pre-mRNA showed significant match to the PTB-consensus and also corresponded to the previously identified negative regulatory elements in the α-tropomyosin (*17*) and β-tropomyosin (*18*). Additional PTB-consensus–related sequences are present in α-IVS2, α-IVS3, and β-IVS6 (*30*). C, RNA-protein complex; P, free probe. The PTB concentrations were 0.06 ng/μl, 0.18 ng/μl, 0.54 ng/μl, 1.6 ng/μl, and 4.8 ng/μl, with the exception of α-IVS1 (0.18 ng/μl, 0.54 ng/μl, 1.6 ng/μl, 4.8 ng/μl, and 14.2 ng/μl). The following RNA probes were used for the mobility-shift assay: PTB-s was the PTB-selected clone P53.3; α-IVS1 was the Xma I–Xho I fragment upstream of exon 2; α-IVS2 was the Asp 718–Pvu II fragment upstream of exon 3; α-IVS3 was the Pst I–Sty I fragment downstream of α-tropomyosin exon 3 (*25*); and β-IVS5 and β-IVS6 were nucleotides 1 to 258 and nucleotides 421 to 595, respectively, of β-tropomyosin (*18*). The RNA probes corresponding to Asp 718–Acc I fragment at the 5′ end of α-IVS2 and nucleotides 421 to 521 at the 5′ end of β-IVS6 also efficiently bound PTB.

site, with an affinity that was 15- to 30-fold higher than that for the Sxl-selected sequence and the *tra* NSS Py-tract/3′ splice site, which are both high-affinity U2AF^{65}- and Sxl-binding sites. These results confirm that PTB has a distinct RNA-binding specificity.

A sequence search revealed that intron sequences adjacent to exon 3 of α-tropomyosin and exon 7 of rat β-tropomyosin pre-mRNAs showed similarity to the PTB-consensus (Fig. 3, B and C). These pre-mRNAs undergo a similar type of alternative splicing (*17*, *18*). PTB bound the α-tropomyosin (α-IVS2) and β-tropomyosin (β-IVS6) pre-mRNA sequences with an affinity [K_d = 1 to 5 nM (where K_d is the dissociation constant)] similar to that for the PTB-selected sequence (Fig. 3, B and C). These introns may contain multiple PTB-binding sites, because additional RNA-protein complexes appeared as the PTB concentration was increased. The affinity of PTB was lower by a factor of at least 100 to 300 for the adjacent Py-tract/3′ splice site of exon 2 of α-tropomyosin (α-IVS1) and exon 6 of β-tropomyosin (β-IVS5) pre-mRNAs (Fig. 3, B and C).

To pursue this result, we used the 3′ splice site switch assay to ask how PTB affected pre-mRNA splicing in vitro. The M-tra and M-Ad ML pre-mRNAs contain two alternative 3′ splice sites: a proximal *tra* NSS or Ad ML 3′ splice site and a distal *tra* FS 3′ splice site. In a standard nuclear extract, only the proximal 3′ splice site of Ad ML pre-mRNA and the NSS 3′ splice site of *tra* pre-mRNA were used (Fig. 4A). Addition of PTB repressed the 3′ splice site of Ad ML pre-mRNA (M-Ad ML) in a concentration-dependent manner and concomitantly activated

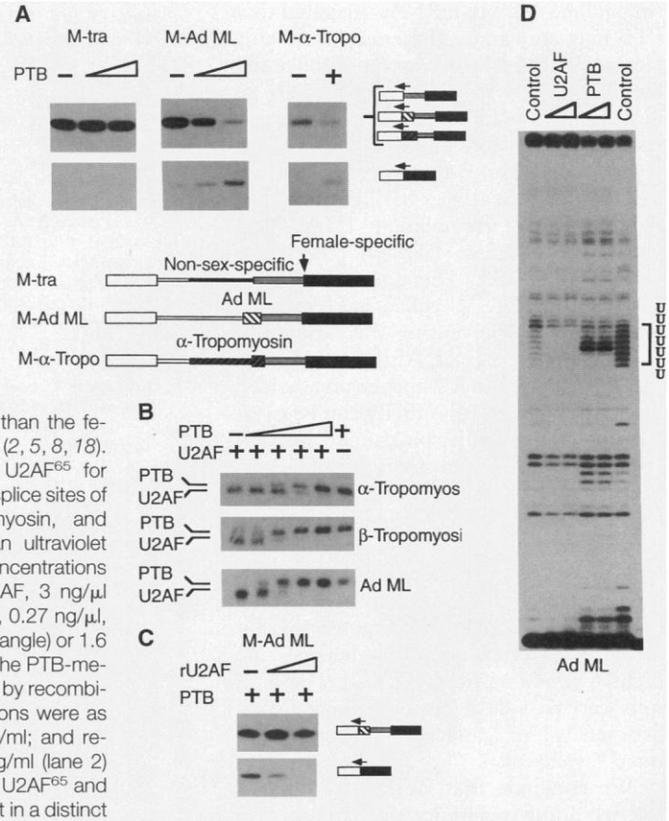

Fig. 4. PTB is a site-specific 3′ splice site repressor. (**A**) Site-specific 3′ splice site repression by PTB. The effect of PTB on the use of alternative 3′ splice sites was analyzed on three chimeric pre-mRNAs: M-tra, M-Ad ML, and M-α-Tropo (*31*). The concentrations of GST-PTB added to the splicing reactions were 22 μg/ml and 88 μg/ml. The proximal 3′ splice sites of *tra*, Ad ML, and α-tropomyosin exon 3 are stronger than the female-specific 3′ splice site (*2*, *5*, *8*, *18*). (**B**) PTB competes with U2AF^{65} for binding to the Py-tract/3′ splice sites of α-tropomyosin, β-tropomyosin, and Ad ML pre-mRNAs in an ultraviolet cross-linking assay (*5*). Concentrations were as follows: GST-U2AF, 3 ng/μl (+); GST-PTB, 0.09 ng/μl, 0.27 ng/μl, 0.81 ng/μl, or 2.4 ng/μl (triangle) or 1.6 ng/μl (+). (**C**) Reversal of the PTB-mediated 3′ splice site switch by recombinant U2AF^{65}. Concentrations were as follows: GST-PTB, 88 μg/ml; and recombinant U2AF^{65}, 20 μg/ml (lane 2) and 60 μg/ml (lane 3). (**D**) U2AF^{65} and PTB recognize the Py-tract in a distinct fashion. Transcripts containing the Ad ML Py-tract/3′ splice site (*12*) were incubated with U2AF^{65} or PTB, modified with CMCT, and the sites of modification detected by primer extension (*32*).

the alternative *tra* FS 3′ splice site. Activation of the alternative 3′ splice site eliminates the possibility that PTB is a nonspecific splicing inhibitor. As an additional control, PTB neither repressed the NSS 3′ splice site of *tra* (M-tra) nor activated the alternative FS 3′ splice site. Thus, in a crude HeLa cell nuclear extract, PTB selectively binds and represses the Ad ML 3′ splice site.

Previous in vivo studies have shown that the Py-tract/3′ splice site region of exon 3 of α-tropomyosin pre-mRNA contains a negative regulatory element (*17*), and we have shown that this same region contains a PTB-binding site (Fig. 3C). PTB specifically repressed the 3′ splice site of exon 3 of α-tropomyosin pre-mRNA (M-α-Tropo) (Fig. 4A). Efficient repression of exon 3 of α-tropomyosin also requires sequences in the downstream intron (*17*). These elements resembled the PTB-selected sequences and efficiently bound PTB (α-IVS3) (Fig. 3C). Taken together, these results strongly suggest that PTB is a negative regulator of α-tropomyosin pre-mRNA splicing.

The binding of PTB to the Py-tract/3′ splice sites of α-tropomyosin, β-tropomyosin, and Ad ML pre-mRNAs suggested that PTB may antagonize the essential splicing factor $U2AF^{65}$and thus repress 3′ splice site use. In support of this prediction, in an ultraviolet cross-linking assay PTB competed with $U2AF^{65}$ for binding to the Py-tracts of α-tropomyosin, β-tropomyosin, and Ad ML pre-mRNAs (Fig. 4B). Furthermore, the addition of recombinant $U2AF^{65}$ reversed the effect of PTB on pre-mRNA splicing (Fig. 4C). These results indicate that PTB mediates 3′ splice site repression most likely by preventing the binding of $U2AF^{65}$ to the Py-tract. Although PTB and $U2AF^{65}$ have distinct sequence preferences, their RNA-binding specificity can be overlapping—for example, on the Ad ML Py-tract. In a chemical footprinting assay, $U2AF^{65}$ and PTB gave rise to distinct patterns: whereas $U2AF^{65}$ protected each of the seven or eight consecutive uridines, PTB protected only the three uridines adjacent to the 3′ splice site (Fig. 4D). In fact, our in vitro selection data demonstrate that some of the PTB-selected sequences are highly pyrimidine-rich (Fig. 1C). These results can provide a basis for competition between $U2AF^{65}$ and PTB on certain Py-tract/3′ splice sites.

We conclude that PTB has a distinct RNA-binding specificity and can function as a sequence-specific 3′ splice site repressor. Another hnRNP protein, hnRNP A1, has also been shown to modulate splice site selection (*19*). Our results explain the previous inability to demonstrate a direct involvement of PTB in pre-mRNA splicing (*20*). Negative regulatory elements present in α- and β-tropomyosin pre-mRNAs, previously identified from in vivo experiments (*17*, *18*), bind PTB in vitro, strongly suggesting a role in splicing regulation. Our results do not exclude the possibility that other splicing factors may be required for complete regulation of tropomyosin pre-mRNA splicing (*21*).

In summary, although *Drosophila* Sxl and mammalian PTB recognize distinct sequences, they both appear to act by blocking the accessibility of $U2AF^{65}$ to the Py-tract. Thus, modulation of $U2AF^{65}$ binding appears to be a common mode of 3′ splice site regulation (*5*, *22*).

REFERENCES AND NOTES

1. M. R. Green, *Ann. Rev. Cell. Biol.* **7**, 559 (1991); M. J. Moore, C. C. Query, P. A. Sharp, in *RNA World*, R. F. Gestland and J. F. Atkins, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), pp. 1–30; G. Dreyfuss, M. J. Matunis, S. Piñol-Roma, C. G. Burd, *Ann. Rev. Biochem.* **62**, 289 (1993).
2. P. D. Zamore *et al.*, *Nature* **355**, 609 (1992).
3. B. S. Baker, *ibid.* **340**, 521 (1989); M. McKeown, *Ann. Rev. Cell Biol.* **8**, 133 (1992); L. R. Bell *et al.*, *Cell* **65**, 229 (1991); L. R. Bell *et al.*, *ibid.* **55**, 1037 (1988).
4. B. A. Sosnowski *et al.*, *Cell* **58**, 449 (1989).
5. J. Valcárcel *et al.*, *Nature* **362**, 171 (1993).
6. K. Inoue *et al.*, *ibid.* **344**, 461 (1990); M. E. Samuels *et al.*, *Mol. Cell. Biol.* **14**, 4975 (1994); J. Wang and L. R. Bell, *Genes Dev.* **8**, 2072 (1994). Sxl has also been shown to bind polyuridines containing fewer than eight uridine residues [E. Sakashita and H. Sakamoto, *Nucleic Acids Res.* **22**, 4082 (1994)].
7. A. Ghetti, S. Piñol-Roma, W. M. Michael, C. Morandi, G. Dreyfuss, *Nucleic Acids Res.* **20**, 3671 (1992).
8. M. A. García-Blanco, S. F. Jamison, P. A. Sharp, *Genes Dev.* **3**, 1874 (1989); A. Gil, P. A. Sharp, S. F. Jamison, M. A. García-Blanco, *ibid.* **5**, 1224 (1991); J. G. Patton, S. A. Mayer, P. Tempst, B. Nadal-Ginard, *ibid.*, p. 1237; J. Wang and T. Pederson, *Nucleic Acids Res.* **18**, 5995 (1990).
9. D. Irvine *et al.*, *J. Mol. Biol.* **222**, 739 (1991); J. W. Szostak, *Trends Biochem. Sci.* **17**, 89 (1992).
10. P. Senapathy, M. B. Shapiro, N. L. Harris, *Methods Enzymol.* **183**, 252 (1990). S. M. Mount *et al.*, *Nucleic Acids Res.* **20**, 4255 (1992).
11. R. Kanaar *et al.*, *Science* **262**, 569 (1993); J. Potashkin *et al.*, *ibid.*, p. 573.
12. M. L. Zapp and S. M. Berget, *Nucleic Acids Res.* **17**, 2655 (1989).
13. B. A. Sosnowski, D. D. Davis, R. T. Boggs, S. J. Madigan, M. McKeown, *Dev. Biol.* **161**, 302 (1994).
14. M. T. O'Neil and J. M. Belote, *Genetics* **131**, 113 (1992).
15. H. Sakamoto *et al.*, *Nucleic Acids Res.* **20**, 5533 (1992); J. I. Horabin and P. Schedl, *Mol. Cell. Biol.* **13**, 7734 (1993).
16. U2AF bound the U2AF- and the Sxl-selected sequences with similar affinity, whereas Sxl bound with approximately 10- to 20-fold higher affinity to the Sxl- than the U2AF-selected sequences.
17. C. Gooding, G. C. Roberts, G. Moreau, B. Nadal-Ginard, C. W. J. Smith, *EMBO J.* **13**, 3861 (1994).
18. B. Nadal-Ginard, C. W. J. Smith, J. G. Patton, R. G. Breitbart, *Adv. Enzyme Regul.* **31**, 261 (1991); W. Guo, G. J. Mulligan, S. Wormsley, D. M. Helfman, *Genes Dev.* **5**, 2096 (1991); G. J. Mulligan, W. Guo, S. Wormsley, D. M. Helfman, *J. Biol. Chem.* **267**, 25480 (1992); M. P. Mullen, C. W. J. Smith, J. G. Patton, B. Nadal-Ginard, *Genes Dev.* **5**, 642 (1991).
19. A. Mayeda and A. R. Krainer, *Cell* **68**, 365 (1992); J. F. Cáceres, S. Stamm, D. M. Helfman, A. R. Krainer, *Science* **265**, 1706 (1994).
20. S. F. Jamison, A. Crow, M. A. García-Blanco, *Mol. Cell. Biol.* **12**, 4279 (1992); J. G. Patton, E. B. Porro, J. Galceran, P. Tempst, B. Nadal-Ginard, *Genes Dev.* **7**, 393 (1993); M. Bennet, S. Michaud, J. Kingston, R. Reed, *ibid.* **6**, 1986 (1992).
21. T. Tsukahara, C. Casciato, D. M. Helfman, *Nucleic Acids Res.* **22**, 2318 (1994).
22. B. E. Hoffman and P. J. Grabowski, *Genes Dev.* **6**, 2554 (1992); M. Tian and T. Maniatis, *ibid.* **8**, 1703 (1994).
23. J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **15**, 8783 (1987).
24. R. K. Saiki, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, New York, 1990), pp. 13–20.
25. N. Ruiz-Opazo and B. Nadal-Ginard, *J. Biol. Chem.* **262**, 4755 (1987).
26. An oligonucleotide mixture [5′-GGTGATCAGATTCTGATCCA (N_{31}) TGAAGCTTGGATCCGTCGC-3′] containing a 31-nucleotide random sequence (31% G, 12% A, 32% T, 25% C by direct sequencing of 18 clones from pool 0) flanked by primer binding sites was chemically synthesized. Initially, T7 promoter was attached by means of the polymerase chain reaction (PCR) for five cycles (94°C, 1 min; 53°C, 1 min; and 72°C, 1 min) and one cycle (72°C, 10 min) with the use of 5′-GTAATACGACTCACTATAGGGTGATCAGATTCTGATCCA-3′ and 5′-GCGACGGATCCAAGCTTCA-3′ primers. Pool 0 RNA was obtained by in vitro transcription with T7 RNA polymerase (*23*) and incubated at 30°C for 30 min with U2AF, Sxl, or PTB in a 100-μl binding reaction [10 mM tris-HCl (pH 7. 5), 1 mM dithiothreitol (DTT), 50 mM KCl, RNasin (0.5 U μl^{-1}), bovine serum albumin (BSA, 0.09 $\mu g\ \mu l^{-1}$), 15 μg tRNA, and 30 μl recombinant protein (10 ng μl^{-1}) diluted in Buffer D]. The protein concentration was decreased by approximately one-third every two rounds. The protein-bound RNAs were recovered by a filter-binding assay in the initial three rounds and subsequently on a nondenaturing gel, reverse transcribed to cDNAs, and amplified (25 cycles) by PCR (*24*) through seven rounds. The Bcl I–Hind III cDNA fragments were cloned into pGEM3 vector (Bam HI–Hind III) (Promega, Madison, WI) and analyzed by dideoxy sequencing.
27. The M-Ad ML substrate was constructed by insertion of the Alu I fragment, which included the female-specific Py-tract/3′ splice site of *tra* (*5*), into the Hinc II site downstream of the 3′ splice site of Ad ML (*12*). With the use of oligonucleotide-directed mutagenesis, the CCC sequence of M-Ad ML was converted to UUUUGUUG sequence to obtain M-Ad ML_{NSS}, and the two guanosines within the NSS-Py-tract of M-tra were converted to cytosines (double C) or uridines (double U). The transcripts from Bam H1– or Xho I–linearized plasmids were analyzed for in vitro splicing (*5*).
28. D. A. Melton *et al.*, *Nucleic Acids Res.* **12**. 7035 (1984).
29. A. R. Krainer *et al.*, *Cell* **36**, 993 (1984).
30. PTB has also been shown to bind fibronectin pre-mRNA [P. Norton, *Nucleic Acids Res.* **22**, 3854 (1994)] and picornavirus RNA [C. W. T. Hellen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7642 (1993)].
31. M-α-Tropo substrate was constructed by replacement of the Bcl I–Nsi I fragment of M-tra (*5*) by the α-tropomyosin fragment (nt 1198 to 1479) (*25*), which contained the sequences flanking the 3′ splice site of exon 3. The sequence of the splice-junction primer for the 3′ splice site of exon 3 of M-α-Tropo was 5′-GCTCATCTTCCAGCGTTC-3′.
32. D. Moazed and H. F. Noller, *Cell* **47**, 985 (1986).

33. We thank J. G. Patton, J. Galcerán, and B. Nadal-Ginard for providing PTB and α-tropomyosin gene, D. Helfman for β-tropomyosin constructs, S. Gupta and R. Reddy for advice, members of M.R.G.'s laboratory for critically reading the manuscript, L. Chiang for technical assistance, and T. O'Toole for secretarial help. Supported by Leukemia Society of America Fellowship and Special Fellowship to R.S., European Molecular Biology Organization and Spanish Ministerio de Educación y Ciencia postdoctoral fellowships to J.V., and a grant from NIH to M.R.G.

17 January 1995; accepted 17 March 1995