Requirement of U12 snRNA for in Vivo Splicing of a Minor Class of Eukaryotic Nuclear Pre-mRNA Introns

Stephen L. Hall* and Richard A. Padgett†

A conserved sequence element in a minor class of eukaryotic pre–messenger RNA (pre-mRNA) introns was previously proposed to base pair with a complementary sequence in the U12 small nuclear RNA (snRNA) in a manner analogous to the pairing of U2 snRNA with the branch site sequence of the major class of introns. Here, mutations generated in this conserved sequence element block the splicing of a member of this minor intron class in vivo. This block was relieved by coexpression of a U12 snRNA containing compensatory mutations that restore the proposed base pairing interaction. These results show that this minor class of pre-mRNA introns is a distinct class existing alongside the major class of introns in animal genomes, and these results also establish an in vivo function for U12 snRNA.

A striking feature of eukaryotic nuclear pre-mRNA introns is that in most cases they clearly belong to a common conserved family in spite of the vast evolutionary distances covered by phylogeny and the sometimes extreme variation in the modes of splicing observed in various organisms. The most conserved feature of these introns is that they almost invariably begin with the dinucleotide GU and end with the dinucleotide AG. A similar conservation is seen with respect to the trans-acting snRNAs that play a major role in the splicing of these introns. In particular, homologs of the U2, U5, and U6 snRNAs occur throughout the eukaryotic family and show strong conservation of those regions that interact with the pre-mRNA splice sites and other snRNAs [for a review, see (*1*)].

Recently, however, a minor class has been recognized in which introns share a number of highly conserved features including the use of the terminal dinucleotides AU and AC. After comparing a number of examples of these introns and identifying the conserved sequences near the splice sites, we proposed that they constitute a distinct type of pre-mRNA intron that interacts with at least some distinct snRNAs in a spliceosomal structure (*2*). On the basis of complementarity with the conserved splice site elements, we proposed that the U11 and U12 snRNAs, which have no known functions, play a role in splicing these introns by forming Watson-Crick base pairs with sequences at the 5′ splice site and the presumptive branch site, respectively (Fig. 1). U11 and U12 snRNAs are low-abundance members of the Sm class of snRNAs and share features such as a hypermethylated 5′ cap structure, Sm protein binding sites, and various aspects of transcriptional regulation and processing with the more abundant snRNAs (*3*–*5*). They appear to be localized within the nucleus much like the U1 and U2 snRNAs are (*6*). Previous work has also shown that U11 and U12 are complexed with each other in vitro probably through protein interactions rather than through base pairing (*4*).

All of the above features suggested that U11 or U12 or both might play a role in splicing AU-AC introns, but there was no direct evidence for this model. Furthermore, although the conservation of the sequence elements at and near the splice sites of the known members of the AU-AC intron class suggested that they play an essential role in splicing, this too had not been tested experimentally.

A powerful method for establishing the existence of RNA-RNA interactions in vivo is the suppression of a loss-of-function mutation in one partner of a proposed base pairing interaction by introduction of compensatory mutations in the other proposed partner. Such experiments in the field of pre-mRNA splicing have established the in vivo requirement for U1 snRNA base pairing to the 5′ splice site (*7*, *8*) and the 3′ splice site (*9*), U2 snRNA base pairing to the branch site sequence (*10*), U6 snRNA base pairing to the 5′ splice site (*11*), U5 snRNA base pairing to the exon sequences immediately adjacent to the intron (*12*), and several base pairing interactions between U2 and U6 snRNAs (*13*).

The very high degree of conservation among the various members of the minor intron class suggested that the conserved sequence elements were likely to be very sensitive to mutation. To test this, we constructed an expression vector using sequences from the human *P120* gene in which the 99-nucleotide intron F is a member of the AU-AC class of introns (*14*). To ensure that all cis-acting elements that might be needed to splice this intron were present, we cloned a portion of the *P120* gene, which included parts of exons 5 and 8 and all of exons 6 and 7 and introns E, F, and G, into a mammalian cell expression vector downstream of a cytomegalovirus (CMV) promoter as shown in Fig. 2A. This four-exon expression construction was transfected into CHO cells, and the resulting RNA was assayed for splicing by reverse transcribing total cell RNA with a primer located in the pCB6 vector sequences downstream of the inserted *P120* gene fragment and amplifying the DNA with primers located in exons 6 and 7 flanking the minor class intron F. The wild-type *P120* minigene produces mainly spliced exons with little unspliced RNA detected (Fig. 3, lane 6). Other experiments (*15*) with more distal primers show that another major product is RNA in which exon 5 is spliced directly to exon 8, removing exons 6 and 7 and introns E, F, and G.

The branch site sequence of the AU-AC introns is much more highly conserved than is the branch site of GU-AG introns in animals. The conserved region also extends farther 5′ of the proposed branch point A residue in AU-AC introns than in GU-AG introns. There is, however, some resemblance of the TCCTTAAC sequence of the minor class introns to the preferred TAC-

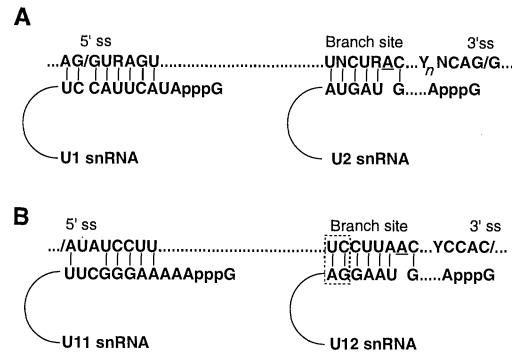

Fig. 1. Base pairing of snRNAs with major and minor intron splice sites. (**A**) The established base pairing interactions between U1 and U2 snRNAs and the 5′ splice site (ss) and the branch site, respectively, for the major class of introns (vertebrate consensus). The bulged branch site adenosine residue is underlined. N, nucleotide A, G, C, or T; R, a purine (A or G); Y, a pyrimidine (C or T). (**B**) The proposed base pairing interactions between U11 and U12 snRNAs with the analogous sequences of the AU-AC class of introns. The adenosine shown to be the branch site in vitro (*18*) is underlined. The base pairs in the U12 snRNA–branch site interaction that are mutated in this work are boxed.

Department of Molecular Biology, Cleveland Clinic Foundation, Cleveland, OH 44195, USA.

*Present address: University of Texas Southwestern Medical Center, Dallas, TX 75235, USA.
†To whom correspondence should be addressed.

TAAC sequence of major class introns. To provide evidence that the AU-AC introns were spliced by means of a non–U2 snRNA pathway, we mutated the two 5′ nucleotides of the TCCTTAAC sequence, which are poorly conserved in major class branch sites and would not be predicted to base pair to U2 snRNA in the minor class intron, to the sequence AGCTTAAC (mutant TC84/85AG, Fig. 2B). If U2 snRNA was interacting with this sequence by base pairing, these mutations would not be expected to affect splicing. However, no correctly spliced RNA was seen when this mutation was transfected into cells (Fig. 3, lane 8), suggesting that these nucleotides interact with a required splicing factor that is probably not U2 snRNA.

As noted above, we thought that U12 snRNA might base pair with this sequence on the basis of complementarity. In addition, it has recently been shown that the region of U12 snRNA proposed to base pair to the highly conserved branch site region of the AU-AC introns is equally highly conserved among the species examined (*5*). To test this proposed base pairing interaction, we made a U12 snRNA expression construct in which the U12 snRNA coding region replaces the U1 snRNA coding region of a functional U1 snRNA gene (*16*). Both wild-type U12 and a mutant U12 (GA24/25CT) that had a two–base pair mutation that restored base pairing to the TC84/85AG *P120* intron mutant were made and tested by cotransfection with the *P120* minigene into CHO cells. The cotransfection of the wild-type U12 snRNA gene had no effect on splicing of the wild-type *P120* intron nor did it rescue splicing of the TC84/85AG mutant (Fig. 3, lanes 10 and 12). Cotransfection of the U12 GA24/25CT mutant snRNA gene with the wild-type *P120* intron also had no effect on splicing (Fig. 3, lane 14). However, cotransfection of the U12 GA24/25CT mutant snRNA gene with the *P120* TC84/85AG mutant restored splicing of the *P120* AU-AC intron (Fig. 3, lane 16). Correct splicing of the product in lane 16 was confirmed by sequence analysis of the excised band. In other experiments (*15*) a different U12 mutant (GA24/25TC) having mutations at the same positions failed to rescue splicing of the *P120* TC84/85AG mutant, thus showing that the suppression is allele-specific and confirming the role of Watson-Crick base pairing in this interaction. To control for saturation of the polymerase chain reaction (PCR) assay, similar reactions were run for fewer cycles with results similar to those shown. The complete transfection and reverse transcriptase (RT)–PCR assay has been repeated several times with the same results. Reducing the ratio of *P120* to U12 construct in the transfections from 1:1 to 1:2 or 1:5 also gave similar results (*15*).

Our results show that the conserved branch site region of the AU-AC intron class is critical for proper splicing in vivo. This contrasts with GU-AG introns in animals where there is only modest conservation of this region and where many rather unlikely sequences appear to be able to serve as branch sites (*17*). The lack of any other conserved motif such as a polypyrimidine tract between the branch site sequence and the 3′ splice site suggests that this eight-nucleotide sequence is a major determinant of the 3′ splice site in AU-AC introns.

These results also establish the in vivo role of U12 snRNA in the splicing of this minor class of pre-mRNA introns. Recent in vitro results support this idea. Tarn and Steitz (*18*) have found that in vitro splicing of the *P120* intron F used here does not require U1, U2, or U6 snRNAs but does require U5 and U12 snRNAs. They have also shown that the predicted branch site A is the in vitro branch point for this intron. An overall similarity in the predicted secondary structure folds of U11 and U12 to U1 and U2 snRNAs, respectively, was noted previously (*3*). This similarity now extends, at least in the case of U12, to the level of function with U12 substituting for U2 in branch site binding mediated by base pairing. The base pairing can be drawn such that an adenosine can occupy a bulged position in the helix for both the major and minor intron classes, and in both cases this adenosine serves as the branch point (*18, 19*). The analogy of U12 with U2 snRNA can probably be extended to predict that U12 will interact with one or more additional snRNAs in the spliceosome by base pairing much as U2 snRNA interacts with U6 snRNA. Although some provocative potential interactions between U12 and U6 snRNAs can be modeled on paper [see (*5*) for examples], no evidence for U6 involvement in splicing the minor class of introns has emerged thus far. It is certainly possible that an as yet unidentified snRNA is the analog of U6 in the minor class spliceosome.

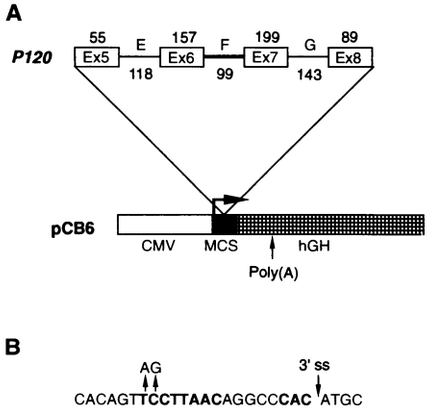

Fig. 2. Construction of the *P120* minigene. (**A**) The pCB6 vector contains the CMV immediate early promoter (CMV), a multiple cloning site region (MCS, shaded box), and the 3′ portion of the human growth hormone gene including the polyadenylation site and terminator sequences (hGH, hatched box). The human *P120* gene insert included exons 5 through 8 (open boxes) and the major class introns E and G and the AU-AC minor class intron F (thin and thick lines, respectively). The sizes of the exons and introns in base pairs are indicated above and below the elements, respectively. (**B**) The sequence of the *P120* intron F 3′ (ss) splice site region is shown with the conserved elements in bold. The position of the TC84/85AG double mutation is shown.

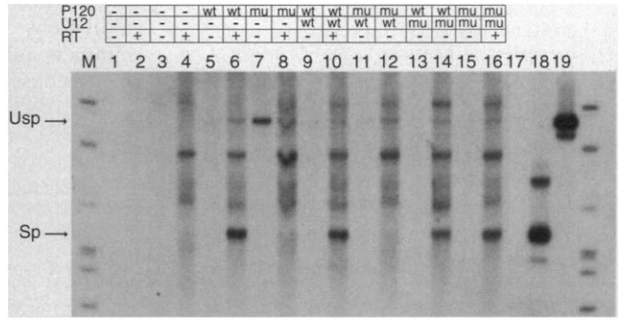

Fig. 3. In vivo splicing of transfected *P120* minigenes. CHO cells were transfected with the DNAs (*20*) as indicated. RNA was prepared and analyzed by reverse transcription followed by amplification of the exons flanking the AU-AC intron F (*21*). The products were separated by nondenaturing polyacrylamide gel electrophoresis. Odd numbered lanes marked RT minus were amplified without prior reverse transcription to control for contamination with DNA. CHO cells were transfected with the following DNAs: lanes 1 and 2, untransfected; lanes 3 and 4, pCB6 vector; lanes 5 and 6, wild-type (wt) *P120* minigene; lanes 7 and 8, *P120* mutant (mu) TC84/85AG minigene; lanes 9 and 10, wild-type *P120* plus wild-type U12 construct; lanes 11 and 12, *P120* mutant TC84/85AG plus wild-type U12 construct; lanes 13 and 14, wild-type *P120* plus U12 GA24/25CT mutant construct; lanes 15 and 16, *P120* mutant TC84/85AG plus U12 GA24/25CT mutant construct; lane 18, amplification products of *P120* complementary DNA; lane 19, amplification products of *P120* genomic clone DNA. The unspliced product DNA (Usp) is 201 base pairs long and the spliced product DNA (Sp) is 102 base pairs long. The band at the unspliced position in lane 7 is probably due to amplification of a small amount of plasmid or genomic DNA that escaped digestion with deoxyribonuclease I before reverse transcription.

REFERENCES AND NOTES

1. S. J. Baserga and J. A. Steitz, in *The RNA World*, R. F. Gesteland and J. F. Atkins, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), pp. 359–381.
2. S. L. Hall and R. A. Padgett, *J. Mol. Biol.* **239**, 357 (1994).
3. K. A. Montzka and J. A. Steitz, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8885 (1988).
4. K. Montzka Wassarman and J. A. Steitz, *Mol. Cell. Biol.* **12**, 1276 (1992).
5. W.-Y. Tarn, T. A. Yario, J. A. Steitz, *RNA* **1**, 644 (1995).
6. A. G. Matera and D. C. Ward, *J. Cell Biol.* **121**, 715 (1993).
7. Y. Zhuang and A. M. Weiner, *Cell* **46**, 827 (1986).
8. B. Séraphin, L. Kretzner, M. Rosbash, *EMBO J.* **7**, 2533 (1988); P. G. Siliciano and C. Guthrie, *Genes Dev.* **2**, 1258 (1988).
9. C. I. Reich, R. W. VanHoy, G. L. Porter, J. A. Wise, *Cell* **69**, 1159 (1992).
10. R. Parker, P. G. Siliciano, C. Guthrie, *ibid.* **49**, 229 (1987); Y. Zhuang and A. M. Weiner, *Genes Dev.* **3**, 1545 (1989); J. Wu and J. L. Manley, *ibid.*, p. 1553.
11. S. Kandels-Lewis and B. Séraphin, *Science* **262**, 2035 (1993); C. F. Lesser and C. Guthrie, *ibid.*, p. 1982.
12. A. J. Newman and C. Normán, *Cell* **68**, 743 (1992).
13. B. Datta and A. M. Weiner, *Nature* **352**, 821 (1991); J. Wu and J. L. Manley, *ibid.*, p. 818; H. D. Madhani and C. Guthrie, *Cell* **71**, 803 (1992); *Genes Dev.* **8**, 1071 (1994); J.-S. Sun and J. L. Manley, *ibid.* **9**, 843 (1995).
14. The human *P120* gene expression construct was prepared by PCR amplification from a genomic clone of the human *P120* gene (supplied by H. Busch) by using primers in exons 5 and 8 to generate a fragment containing nucleotides 6775 to 7632 (numbering from GenBank accession number M33132). This fragment contained exons 5 through 8 and introns E, F, and G, where intron F is the minor class intron. This fragment was cloned between the Kpn I and Hind III sites of the pCB6 mammalian expression vector [S. Andersson *et al.*, *J. Biol. Chem.* **264**, 8222 (1989)], which supplies a CMV promoter and termination and polyadenylation sites. Mutations were made either by site-directed mutagenesis of an M13 clone of this fragment followed by cloning in pCB6, or by PCR primer mutagenesis and subsequent cloning of the product in pCB6. All mutations were confirmed by sequence analysis.
15. S. L. Hall and R. A. Padgett, data not shown.
16. The human U12 snRNA expression construct was produced as described [U. Bond *et al.*, *Genes Dev.* **5**, 1709 (1991)]. Briefly, a PCR fragment of the human U12 snRNA gene (supplied by J. Steitz) (*5*) was cloned into the pUC13-U1 vector (*7*) such that the mature U12 snRNA sequences replaced the U1 snRNA sequences. U12 snRNA mutants were produced by substituting oligonucleotides containing the appropriate mutations for the PCR primer at the 5′ end of the U12 sequence and by cloning the fragments into the pUC13-U1 vector. Both the wild-type and mutant constructs have been sequenced through the 5′ and 3′ U1 snRNA gene sequences and the U12 snRNA sequences and are identical except for positions 24 and 25.
17. R. A. Padgett *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8349 (1985); B. Ruskin, J. M. Green, M. R. Green, *Cell* **41**, 833 (1985); K. K. Nelson and M. R. Green, *Genes Dev.* **3**, 1562 (1989).
18. W.-Y. Tarn and J. A. Steitz, *Cell,* in press.
19. R. A. Padgett, M. M. Konarska, P. J. Grabowski, S. F. Hardy, P. A. Sharp, *Science* **225**, 898 (1984); B. Ruskin, A. R. Krainer, T. Maniatis, M. R. Green, *Cell* **38**, 317 (1984); C. C. Query, M. J. Moore, P. A. Sharp, *Genes Dev.* **8**, 587 (1994).
20. The pCB6-based *P120* constructs and the pUC-U12 constructs were transfected into CHO cells with the hexadimethrine bromide (Polybrene)–mediated method as described [C. Brewer and M. G. Roth, *J. Cell. Biol.* **114**, 413 (1991)]. For these experiments, 20 μg of DNA (either 10 μg of each plasmid or 10 μg of *P120* construct with 10 μg of carrier DNA) was added to a 100-mm plate containing 1.5×10^6 to 2.0×10^6 CHO cells in 5 ml of growth medium containing hexadimethrine bromide (20 μg/ml). The cells were incubated with the DNA for 6 to 8 hours followed by a 5-min shock with 5 ml of complete medium containing 30% dimethyl sulfoxide. The plates were then washed, the medium replaced, and the plates incubated for 36 to 40 hours. RNA was then extracted from the cells with the Trizol reagent (Life Technologies) following the supplier's protocol.
21. Extracted RNA was treated with 2 units of deoxyribonuclease I (Life Technologies) for 45 min at 37°C to remove residual contamination with cellular DNA. The primer for reverse transcription (CCAAGGCCAGGAGAGGCACTCGGG) was complementary to the 3′ pCB6 vector–derived sequences that are unique to the transfected *P120* minigene. The primer (150 ng) was annealed to 2 μg of RNA in reverse transcriptase buffer (BRL) by heating to 70°C and slowly cooling to 45°C. Next, deoxynucleotide triphosphates (dNTPs) and 200 units of reverse transcriptase (Superscript II, BRL) were added (for a 20-μl reaction) and incubated for 60 min at 45°C. The samples were then treated with pancreatic ribonuclease A (10 μg) for 30 min at 37°C. The resulting DNA was amplified by PCR with primers in exons 6 (CTGCCCCCTGCTGGGGAGATG) and 7 (CCCAAAATCACGCAGAATTCC) with a hot start method in 25-μl reactions containing 3 μl of the reverse transcription reaction or a control reaction from which reverse transcriptase was omitted, 2.5 pmol of each primer, 0.2 mM each dNTP, 50 mM KCl, 10 mM tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, and 10 μCi of [α-^{32}P]deoxycytidine triphosphate (dCTP). PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C, 57°C, and 72°C for 1 min each.

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Requirement for cAMP-PKA Pathway Activation by M Phase–Promoting Factor in the Transition from Mitosis to Interphase

Domenico Grieco,* Antonio Porcellini, Enrico V. Avvedimento, Max E. Gottesman

Cell cycle progression in cycling *Xenopus* egg extracts is accompanied by fluctuations in the concentration of adenosine 3′,5′-monophosphate (cAMP) and in the activity of the cAMP-dependent protein kinase (PKA). The concentration of cAMP and the activity of PKA decrease at the onset of mitosis and increase at the transition between mitosis and interphase. Blocking the activation of PKA at metaphase prevented the transition into interphase; the activity of M phase–promoting factor (MPF; the cyclin B–p34^{cdc2} complex) remained high, and mitotic cyclins were not degraded. The arrest in mitosis was reversed by the reactivation of PKA. The inhibition of protein synthesis prevented the accumulation of cyclin and the oscillations of MPF, PKA, and cAMP. Addition of recombinant nondegradable cyclin B activated p34^{cdc2} and PKA and induced the degradation of full-length cyclin B. Addition of cyclin A activated p34^{cdc2} but not PKA, nor did it induce the degradation of full-length cyclin B. These findings suggest that cyclin degradation and exit from mitosis require MPF-dependent activation of the cAMP-PKA pathway.

A rapid succession of interphase and mitotic states characterizes the early embryonic divisions of many species. The onset of mitosis is induced by activation of MPF, a highly conserved complex consisting of a kinase, p34^{cdc2}, and an activating subunit (cyclin B1 or B2) (*1*). The transition into interphase is accompanied by the destruction of MPF by ubiquitin-mediated proteolysis of the cyclin component (*2*, *3*). Cyclin B synthesis plays a pivotal role in the cell cycle. It is the only protein whose synthesis is required to induce complete cycles of activation and inactivation of MPF in *Xenopus* (*4*). As cyclin accumulates and binds to p34^{cdc2}, a series of posttranslational modifications of the complex precede the activation of MPF. These reactions help to set the timing of the onset of mitosis (*5*). When p34^{cdc2} is fully activated by cyclin B, it induces cyclin degradation, whereas when it is activated by cyclin A, it does not (*6*, *7*). Activation of the cyclin degradation pathway is required to complete mitosis and to allow separation of sister chromatids at anaphase (*8*).

The biochemical oscillations of the *Xenopus* embryonic cell cycle can be reproduced in cytoplasmic extracts from fertilized eggs (*4*). Neither DNA synthesis nor mitotic spindle assembly is required for the cycles

D. Grieco, Dipartimento di Biologia e Patologia Cellulare e Molecolare "L. Califano" and Centro di Endocrinologia ed Oncologia Sperimentale del CNR, II Medical School, University of Naples, Via S. Pansini 5, Naples 80131, Italy, and Institute of Cancer Research, Columbia University, 701 West 168 Street, New York, NY 10032, USA.
A. Porcellini, Dipartimento di Biologia e Patologia Molecolare e Cellulare "L. Califano" and Centro di Endocrinologia ed Oncologia Sperimentale del CNR, II Medical School, University of Naples, Via S. Pansini 5, Naples 80131, Italy.
E. V. Avvedimento, Dipartimento di Medicina Sperimentale, Medical School, University of Reggio Calabria, Via T. Campanella 5, Catanzaro, Italy.
M. E. Gottesman, Institute of Cancer Research, Columbia University, 701 West 168 Street, New York, NY 10032, USA.

*To whom correspondence should be addressed.