Regulation of Alternative Splicing in Vivo by Overexpression of Antagonistic Splicing Factors

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(Pharmacia) column. Protein flow-through (0.5 g) was mixed with a DNA-affinity resin prepared by coupling synthetic, biotinylated DNA corresponding to the IL-4 response element of the gene encoding FcγR1 (5′-GTATTTCCCAGAAAAGGAAC-3′) to streptavidin agarose (Sigma). After binding (2 hours at 4°C), the affinity matrix was placed on a disposable column and washed sequentially with 10 ml of buffer C, 4 ml of buffer C supplemented with a mutated variant of the IL-4 response element (5′-GTATCACCCAGTCAAGGAAC-3′) at 0.2 mg/ml, and 10 ml of buffer C. Protein (40 μg) was eluted by exposure to 5 ml of buffer C supplemented with 0.35 M NaCl, dialyzed against buffer C, and placed on a 0.5-ml Q-Sepharose (Pharmacia) column. The column was washed with 5 ml of buffer C and protein (10 μg) was eluted with 1 ml of buffer C supplemented with 0.35 M NaCl.

9. Purified IL-4 Stat (Fig. 1) was subjected to SDS-gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore) [P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)]. The membrane was stained with Coomassie blue R-250 in 40% methanol and 0.1% acidic acid for 30 s and then destained for 5 min with 10% acetic acid in 50% methanol. The 100-kD IL-4 Stat protein was excised from the membrane and treated with 1 μl of methanol. Membrane-bound protein was alkylated with isopropylacetamide [H. C. Krutzsch and J. K. Inman, *Anal. Biochem.* **209**, 109 (1993); S. Wong, C. Grimley, A. Padua, J. H. Bourell, W. J. Henzel, *Techniques in Protein Chemistry IV* (Academic Press, New York, 1993), p. 371] and then digested in 50 μl of 0.1 M ammonium bicarbonate, 10% acetonitrile with 0.2 μg of lysine-C (Wako) at 37°C for 17 hours. The solution was then concentrated to 20 μl and directly injected onto a capillary high-performance liquid chromatogram. Peptides were separated on a C18 capillary column (0.32 by 100 mm) (LC Packing) developed with 0.1% aqueous trifluoroacetic acid as buffer A and acetonitrile containing 0.07% trifluoroacetic acid as buffer B [W. J. Henzel, J. H. Bourell, J. T. Stults, *Anal. Biochem.* **187**, 228 (1990)]. Isolated peptides were sequenced on a 470A Applied Biosystems sequencer. Sequence interpretation was performed on a DEC 5900 computer [W. J. Henzel, H. Rodriguez, C. Watanabe, *J. Chromatogr.* **404**, 41 (1987)].
10. Degenerate oligonucleotides corresponding to two sequenced peptides of IL-4 Stat were used for PCR amplification of cDNA prepared from Thp-1 cell mRNA. One primer 5′-AARATGTGYGARACNYTNAA-3′ (R was equimolar mix of G and A; Y was an equimolar mix of T and C; N was an equimolar mix of G, A, T, and C) corresponded to the peptide sequence NH_2-KMCETLN-COOH. The other primer 5′-CATYTGYTCNGGYTTRTARTG-3′ corresponded to the peptide sequence NH_2-HYKPEQM-COOH. Conditions for PCR amplification were 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min repeated for 35 cycles. The PCR reaction buffer consisted of 10 mM tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 10 pM of each primer and 20 ng of cDNA. The amplified product, 0.5 kb in length, was cloned and sequenced by the chain termination method.
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13. RNA blot hybridization with a uniformly labeled DNA probe prepared from IL-4 Stat cDNA and Multiple Tissue Northern Blot membranes (Clonetech) were used. Probe labeling, hybridization, and membrane washing were performed as described [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, (1989)].
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Regulation of Alternative Splicing in Vivo by Overexpression of Antagonistic Splicing Factors

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The opposing effects of SF2/ASF and heterogeneous nuclear ribonucleoprotein (hnRNP) A1 influence alternative splicing in vitro. SF2/ASF or hnRNP A1 complementary DNAs were transiently overexpressed in HeLa cells, and the effect on alternative splicing of several cotransfected reporter genes was measured. Increased expression of SF2/ASF activated proximal 5′ splice sites, promoted inclusion of a neuron-specific exon, and prevented abnormal exon skipping. Increased expression of hnRNP A1 activated distal 5′ splice sites. Therefore, variations in the intracellular levels of antagonistic splicing factors influence different modes of alternative splicing in vivo and may be a natural mechanism for tissue-specific or developmental regulation of gene expression.

Alternative splicing is a major mechanism for controlling the expression of cellular and viral genes. SF2/ASF and other members of the SR protein family have an activity required for general splicing in vitro and also regulate alternative splicing by promoting the use of proximal 5′ splice sites (*1*–*4*). This latter activity is counteracted in vitro by hnRNP A1, which promotes the use of distal 5′ splice sites (*5*, *6*). Thus, the antagonizing activities of SR proteins and hnRNP A1 are key determinants of alternative 5′ splice site selection in vitro. In addition, a small increase in the concentration of SF2/ASF prevents the inappropriate exon-skipping observed when certain precursor messenger RNAs (pre-mRNAs) are spliced in vitro (*7*). This property may reflect a mechanism by which SR proteins ensure the fidelity of splicing. Although any individual SR protein can complement an inactive splicing extract lacking all the SR proteins, differences have been detected in their ability to regulate alternative splicing of different pre-mRNAs in vitro (*8*, *9*). Therefore, the relative abundance of each SR protein and the molar ratio of each SR protein to hnRNP A1, or to other antagonists, may determine the patterns of alternative splicing of many genes expressed in a particular cell type. Tissue-specific variations in the total and relative amounts of SR proteins or their mRNAs have been described (*8*, *10*, *11*), and in addition the molar ratio of SF2/ASF to hnRNP A1 varies over a wide range in different rat tissues (*11*).

Whether changes in the relative amounts of SF2/ASF and hnRNP A1 can affect gene expression in vivo was not known. To address this question, we transiently overexpressed SF2/ASF or hnRNP A1 complementary DNAs (cDNAs) in HeLa cells and analyzed the splicing patterns of cotransfected reporter genes. We first analyzed a thalassemic allele of the human β-globin gene, whose splicing is responsive to changes in the concentration of SF2/ASF in vitro (*1*). This β^{thal} allele contains a G to A transition at position 1 of intron 1, which results in abnormally spliced mRNA both in vitro and in vivo (*12*, *13*). This mutation causes the activation of three cryptic 5′ splice sites that are completely silent in the wild-type allele (Fig. 1A).

When the β^{thal} gene was transiently transfected into HeLa cells (*14*), all three cryptic sites were used in roughly equal proportions (Fig. 1A). Upon cotransfection of a human SF2/ASF cDNA, a substantial change in the relative use of each cryptic

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site was observed, with the most proximal 5′ splice site (cryptic site 3) selected almost exclusively (Fig. 1A). This effect on 5′ splice site selection was the result of increased SF2/ASF expression, because cotransfection of the parent plasmid vector without the SF2/ASF coding sequence had no effect (Fig. 1A). SF2/ASF mRNA expressed from the cloned cDNA accumulated in large amounts in the transfected HeLa cell population (Fig. 1B) (*15*). Although the relative use of the three cryptic sites differs in vivo (*13*) (Fig. 1A) and in vitro (*1*, *12*), the increase in SF2/ASF causes a shift toward the most proximal 5′ splice site in both cases (*1*) (Fig. 1A).

Next, we tested the effect of SF2/ASF overexpression on the alternative splicing of rat clathrin light chain B. This gene has six exons, one of which, termed EN, is included only in neurons (*16*). Transfection of a clathrin minigene into HeLa cells generates predominantly the EN-skipped form (*16*). Upon overexpression of cotransfected SF2/ASF (Fig. 2B), the ratio of EN exon inclusion to exon skipping increased greatly (Fig. 2, C and D). Hence, in vivo overexpression of SF2/ASF can promote inclusion of a neuron-specific exon in a fibroblast.

The difference in exon EN inclusion between HeLa cells transfected with or without SF2/ASF is quantitatively smaller than that observed between neurons and HeLa cells transfected with only the clathrin minigene (*16*), and therefore the skipped form is still detectable (Fig. 2D). The partial extent of the switch suggests that exon EN inclusion is regulated in the brain by specific factors other than, or in addition to, SF2/ASF—for example, a different SR protein. However, a complete switch in splice site selection may not be achievable in a cotransfection experiment because the observed effect is dependent on the timing of expression of the reporter gene and of SF2/ASF, as well as on the stabilities of reporter mRNA isoforms that are expressed before substantial amounts of SF2/ASF protein accumulate in the nucleus.

Alternative splicing of rat β-tropomyosin pre-mRNA is regulated in a tissue-specific manner, in part by use of exon 6 in smooth muscle and nonmuscle cells and by use of exon 7 in skeletal muscle (*17*, *18*). Splicing of a β-tropomyosin minigene transcript in HeLa cell extracts or in transfected cells generates, in addition to the nonmuscle 5, 6, 8, 9 mRNA, an inappropriately spliced 5, 8, 9 mRNA as a consequence of abnormal skipping of exons 6 and 7 (*18*, *19*) (Fig. 3A). We tested whether increasing the amount of SF2/ASF in vivo can correct this abnormal splicing pattern.

Cotransfection of the SF2/ASF cDNA and the β-tropomyosin minigene resulted in a reduction in the amount of the exon-skipped 5, 8, 9 mRNA relative to the exon inclusion form (Fig. 3A) (*20*). Further analysis indicated that SF2/ASF overexpression stimulated inclusion of the fibroblast-specific exon 6 (Fig. 3B), whereas exon 7 inclusion was not detected (*21*). These results agree with in vitro experiments that showed that high concentrations of SF2/ASF can prevent inappropriate exon skipping (*7*) and further implicate SF2/ASF in the mechanism responsible for splicing fidelity in vivo. The fact that SF2/ASF did not activate the more proximal exon 7 is consistent with the notion that use of this exon may require different, or additional, muscle-specific regulators, such as another SR protein or an unrelated factor.

To address the role of hnRNP A1 in the regulation of alternative splicing in vivo, we transiently overexpressed the cDNA in HeLa cells and analyzed the pattern of alternative splicing of a cotransfected adenovirus *E1A* gene. Transfection of the *E1A*

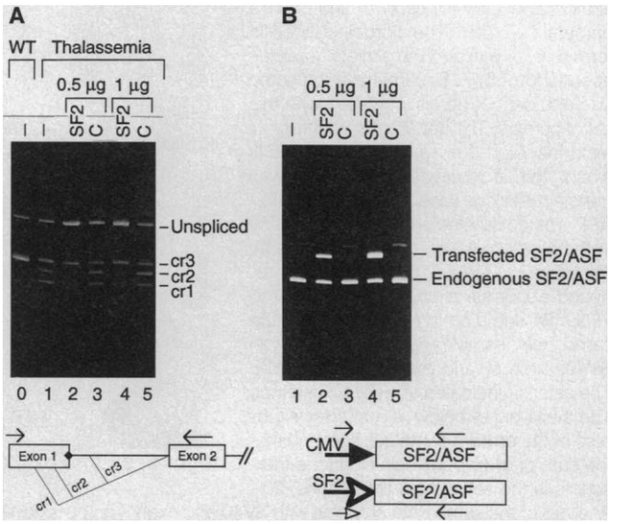

Fig. 1. Effect of SF2/ASF transient overexpression on β-thalassemia pre-mRNA splicing. (**A**) Top: Patterns of splicing of a human β^{thal} gene upon cotransfection of pCG-SF2 (lanes 2 and 4) or pCG (lanes 3 and 5). Lane 0 shows transfection of a β-globin wild-type (WT) allele (pUCβ128SV) (*25*). Bottom: Structure of a β^{thal} allele (*13*) of human β-globin pre-mRNA, showing three cryptic 5′ splice sites (cr1, cr2, and cr3) that are activated as a result of the mutation at the 5′ splice site and primers used for RT-PCR analysis. (**B**) Top: Detection of SF2/ASF expression in the same RNA samples as in (A). Bottom: Diagram of the promoters that drive expression of endogenous and transfected SF2/ASF and primers used for RT-PCR analysis. CMV, cytomegalovirus.

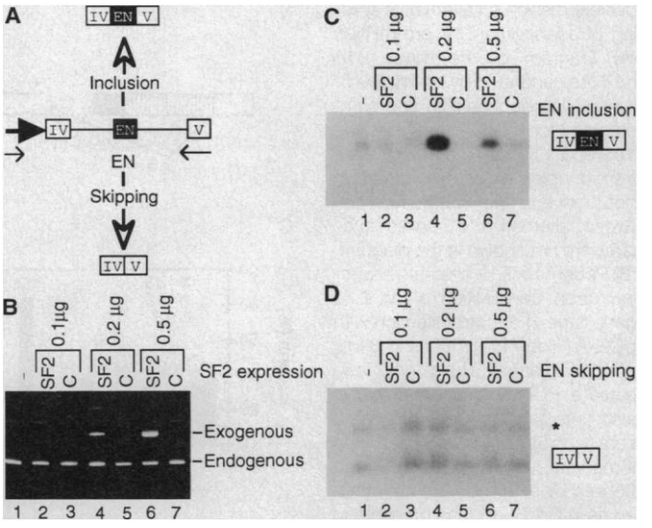

Fig. 2. Effect of SF2/ASF transient overexpression on alternative splicing of rat clathrin light chain B (LCB). (**A**) Diagram of the LCB minigene, which includes exon EN in neurons but excludes it in all other cell types, and primers used for amplification by RT-PCR. (**B**) Detection of SF2/ASF expression in transfected cells (see Fig. 1B diagram). (**C**) Detection of exon EN inclusion; lanes are as in (B). (**D**) Detection of exon EN skipping; lanes are as in (B). The upper band (indicated by an asterisk) represents a previously described heterodimer artifact (*16*). The LCB minigene contains the complete introns D (1.8 kb) and E (3.1 kb) of the LCB gene, as well as 108 and 45 nucleotides of exons IV and V, respectively, and the complete 54-nucleotide EN exon (*16*). The minigene was cloned into pΩ3 (*26*), in which transcription is driven by an SV40 promoter. HeLa cells were transfected with 4 μg of the LCB minigene and the indicated amounts of either pCG-SF2 or pCG. RNA samples were analyzed by RT-PCR with exon V reverse and SV40 forward primers. The RT-PCR products were detected by Southern (DNA) blotting with the [γ-^{32}P]adenosine triphosphate end-labeled oligonucleotide CTTCCGATGCCACATAGCCAATGG, which is EN-specific, as a probe for exon inclusion. The same blot was stripped and reprobed with an oligonucleotide complementary to the flanking exons IV and V, under hybridization conditions that detect only EN skipping (*16*).

gene alone, or cotransfection with the control plasmid, generated multiple mRNA isoforms that represent alternative 5′ splice site utilization (Fig. 4, B and C). Cotransfection of the hnRNP A1 cDNA resulted in a shift toward use of the most distal 5′ splice site (Fig. 4, B and C) to generate the 9S mRNA, which is characteristic of the late phase of adenovirus infection (*22*). This result is consistent with in vitro experiments showing that addition of hnRNP A1 causes activation of the E1A 9S 5′ splice site (*5*).

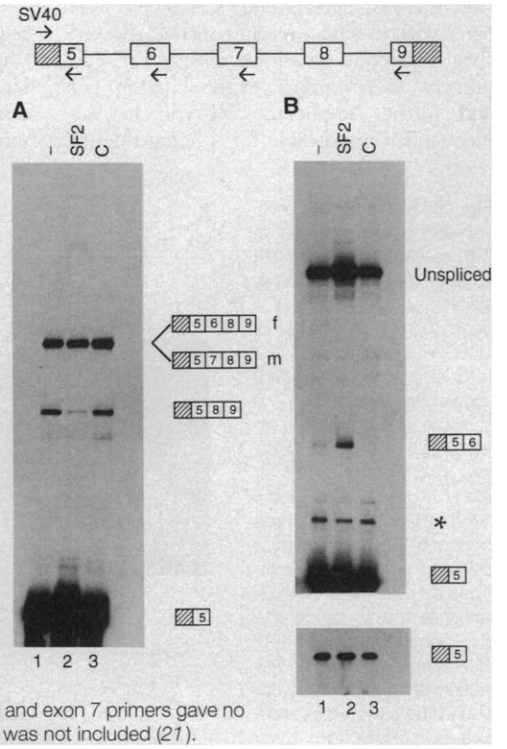

Fig. 3. Effect of SF2/ASF transient overexpression on rat β-tropomyosin alternative splicing. The top diagram shows the structure of the tropomyosin minigene and primers used for detection by RT-PCR. (**A**) Patterns of β-tropomyosin alternative splicing upon cotransfection of pCG-SF2 (lane 2) or pCG (lane 3). RNA samples were analyzed with SV40, exon 5, and exon 9 primers (*19*, *20*). The fibroblast-specific form 5, 6, 8, 9 (f) and the skeletal muscle–specific form 5, 7, 8, 9 (m) have the same size and cannot be distinguished with this set of primers. The apparent percentage of exon inclusion (f or m mRNAs) was 65% when the tropomyosin minigene was transfected alone (lane 1), 90% when SF2/ASF was cotransfected (lane 2), and 70% in the control pCG cotransfection (lane 3). Therefore, SF2/ASF overexpression reduced abnormal exon skipping by 20 to 25%. (**B**) Same as in (A), except that the same RNA samples were analyzed with SV40, exon 5, and exon 6 primers (*27*). The asterisk indicates an RT-PCR artifact. The small panel below is a shorter exposure of the bottom of the gel, showing that the sum of all tropomyosin minigene transcripts is the same in all three lanes (*20*). Analysis of the same RNA samples with SV40 and exon 7 primers gave no amplification products, indicating that exon 7 was not included (*21*).

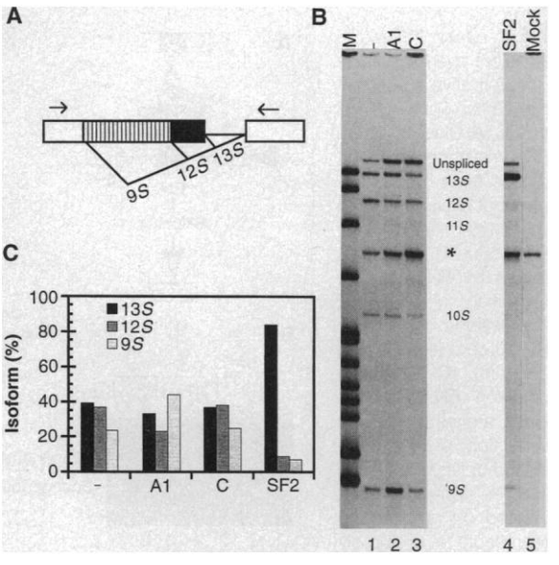

Fig. 4. Effect of hnRNP A1 transient overexpression on alternative splicing of adenovirus E1A pre-mRNA. (**A**) Diagram of the major E1A mRNAs generated by alternative 5′ splice site selection (*28*) and primers used for RT-PCR analysis. The 10*S* and 11*S* mRNAs, which arise from double splicing events that do not involve simple competition between alternative 5′ splice sites (*29*), are not shown in the diagram. (**B**) Patterns of E1A alternative splicing upon transfection of an *E1A* gene (lane 1) or cotransfection with pCG-A1 (lane 2), pCG (lane 3), or pCG-SF2 (lane 4). The asterisk indicates a PCR artifact band that is also detected in mock-transfected cells (lane 5). Lane M, pBR322/Hpa II markers. (**C**) The 13*S*, 12*S*, and 9*S* mRNA isoforms were quantitated as in Fig. 3, and the percentage of each isoform is shown. Transfections were carried out as described in Fig. 1, with 6 μg of the E1A expression plasmid pMTE1A (*30*). The hnRNP A1 expression plasmid was constructed by the amplification of a cDNA fragment from the plasmid pET9d-hnRNP A1 (*5*) with the forward primer GTCTCTAGATCAGAGTCTCCTAAAGA and a COOH-terminal hnRNP A1 reverse primer (*5*). The PCR product was digested with Xba I and Bam HI and subcloned into the corresponding sites of pCG (*24*). The resulting plasmid pCG-A1 contains the full-length natural hnRNP A1 coding sequence, except that the first three codons, Met-Ser-Lys, are replaced by Met-Ala-Ser-Arg. E1A mRNA detection was carried out with the exon 1 forward primer GTTTTCTCCTCCGAGCCGCTCCGA and the 5′ end-labeled exon 2 reverse primer CTCAGGCTCAGGTTCAGACACAGG.

Cotransfection of the SF2/ASF cDNA strongly activated the most proximal 5′ splice site, giving rise to the 13*S* mRNA (Fig. 4, B and C). Expression of transfected SF2/ASF or hnRNP A1 was verified by reverse transcription–polymerase chain reaction (RT-PCR) analysis of the corresponding samples (*21*). Expression of transfected hnRNP A1 was low, which may account for the relatively small extent of proximal to distal 5′ splice site switching (Fig. 4C). The hnRNP A1 protein is very abundant in HeLa cells, and its expression may be tightly regulated (*23*).

In summary, we show that overexpression of hnRNP A1 activated distal 5′ splice sites, whereas overexpression of SF2/ASF activated proximal 5′ splice sites, prevented abnormal exon skipping, and promoted exon inclusion. These effects are exactly as predicted from previous in vitro experiments with SF2/ASF and hnRNP A1. Our transfection experiments demonstrate that transient changes in the cellular ratio of SF2/ASF to hnRNP A1 can influence several different modes of alternative splicing in vivo. Recent experiments showed that the molar ratio of SF2/ASF to hnRNP A1 naturally varies over a very wide range in different rat tissues (*11*). Taken together, these results strongly suggest that in vivo regulation of the expression of many genes by alternative splicing may be accomplished at least in part by tissue-specific, developmentally regulated, physiological state–dependent, or virus-induced variations in the relative levels of one or more SR proteins and the antagonizing factor hnRNP A1. Changes in the nuclear concentration of a limited number of general splicing factors, rather than expression of numerous gene-specific regulators, may in this manner control the expression of a wide variety of genes.

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14. For transfection of HeLa cells, 5 μg of the β^{thal} plasmid and 0.5 or 1 μg of pCG-SF2 (or pCG) were added to a 60-mm dish of 60 to 75% confluent HeLa cells in the presence of 20 μg of lipofectin (Gibco-BRL). Cells were harvested 48 hours after transfection and lysed with NP-40, and total RNA was purified as described [M. Z. Gilman, *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Wiley, New York, 1988, vol. 1, pp. 4.1.2–4.1.3)]. Total RNA (200 ng) was analyzed by RT-PCR with thermostable DNA polymerase from *Thermus thermophilus* (Epicentre, Madison, WI). Aliquots of each RNA sample were analyzed with the appropriate set of β-globin or SF2/ASF primers. The oligonucleotides used for β-globin detection were TCAAACAGACACCATGGTGCACCTGACT, as an exon 1 forward primer, and CAGGAGTGGACAGATCCC, as an exon 2 reverse primer. The oligos used for SF2/ASF detection were GACGCCATCCACGCTGTT, which is specific for the 5′ untranslated region (UTR) derived from the pCG vector (*24*), and TCGAGTCCGCGCTTTTCG, which is specific for the 5′ UTR of the endogenous SF2/ASF gene; both of these were used as forward primers, and GCTTCGAGGAAACTCCAC was used as a reverse primer. All three SF2/ASF primers were present in the same reaction. In these and the following RT-PCR reactions, the different RNA species were amplified simultaneously with at least one common primer. In addition, the number of cycles was minimized to maintain linearity, and the relative abundance of the SF2/ASF mRNAs was verified by primer extension (*21*).
15. HeLa cells were separately transfected with a plasmid expressing the *Escherichia coli lacZ* gene [R. R. Spaete and E. S. Mocarski, *J. Virol.* **56**, 135 (1985)], followed by staining with X-gal to measure the transfection efficiency. Approximately 5% of the cells expressed the transfected DNA (*21*). Because similar amounts of SF2/ASF mRNA transcribed from endogenous and transfected genes were measured in the total cell population (Fig. 1B), we conclude that the overall expression of SF2/ASF mRNA in the transfected cells increased approximately 20-fold, on average. The relative efficiencies with which the endogenous and exogenous SF2/ASF mRNAs are translated are not known, because the proteins are indistinguishable.
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20. We transfected 5 μg of the tropomyosin minigene plasmid pSV40-p2 (*18*) into HeLa cells with or without SF2/ASF or control plasmids, as described (*14*). Aliquots of each RNA sample were analyzed by RT-PCR with a 5′ end-labeled forward primer homologous to the SV40 5′ UTR and a reverse primer complementary to either exon 9 (Fig. 3A) or to exon 6 (Fig. 3B), as described (*19*). A reverse primer complementary to exon 5 was also present in the RT-PCR reactions; the resulting amplification product serves to normalize with respect to all tropomyosin minigene transcripts (*19*). The RT-PCR products were quantitated on a Fujix BAS2000 PhosphorImager.
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25. The β-globin expression vector (pUCβ128SV) consists of a Hind III–Pst I fragment of wild-type human β-globin from πSVHPβΔ128 [R. Treisman, M. R. Green, T. Maniatis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7428 (1983)] subcloned into the corresponding sites of pUC19 and an SV40 Pvu II–Nco I enhancer fragment from the same plasmid subcloned into the Sma I–Hinc II sites. The β-thalassemia expression plasmid was constructed by the replacement of an Nco I–Bam HI β-globin fragment of pUCβΔ128SV with the corresponding fragment of the β^{thal} allele from SP64-HβΔ6IVS1-1A (*12*). The SF2/ASF expression plasmid pCG-SF2 was constructed by subcloning an Nde I (blunted)–Bam HI fragment of pET9c-SF2 (*3*) into pCG (*24*) cleaved with Xba I and Bam HI. The Xba I end was first treated with T4 DNA polymerase in the presence of deoxyadenosine triphosphate, followed by digestion with mung bean nuclease. The resulting plasmid contains the full-length natural SF2/ASF coding sequence.
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27. Detection of the unspliced pre-mRNA with the SV40 and exon 6 primers (Fig. 3B) but not with the SV40 and exon 9 primers (Fig. 3A) is probably due to inefficient amplification of the much longer product with the latter set of primers. Likewise, increased 5, 6 splicing upon SF2/ASF overexpression is more evident with the exon 6 primer (Fig. 3B), probably because RNA molecules that still retain one or more of the remaining introns amplify inefficiently with the exon 9 primer (Fig. 3A). We note that the amounts of unspliced pre-mRNA seldom remain unchanged when the selection of alternative splice sites is modulated by SF2/ASF or hnRNP A1, and this is also the case in vitro (*1*, *5*). This is probably because the overall splicing efficiency differs according to which splice sites have been selected.
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Coaxially Stacked RNA Helices in the Catalytic Center of the *Tetrahymena* Ribozyme

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Coaxial stacking of helical elements is a determinant of three-dimensional structure in RNA. In the catalytic center of the *Tetrahymena* group I intron, helices P4 and P6 are part of a tertiary structural domain that folds independently of the remainder of the intron. When P4 and P6 were fused with a phosphodiester linkage, the resulting RNA retained the detailed tertiary interactions characteristic of the native P4-P6 domain and even required lower magnesium ion concentrations for folding. These results indicate that P4 and P6 are coaxial in the P4-P6 domain and, therefore, in the native ribozyme. Helix fusion could provide a general method for identifying pairs of coaxially stacked helices in biological RNA molecules.

Many RNA molecules require specific three-dimensional structures for their biological activity. Determination of RNA secondary structure (base-paired helices, bulges, and hairpin loops) is now relatively straightforward (*1*). Bridging the gap from a secondary structure to a three-dimensional structure, on the other hand, remains problematic. The only biologically active RNAs whose structures have been resolved at the atomic level by x-ray crystallography are transfer RNAs (tRNAs) (*2*).

RNA secondary structures often have three or more helical elements diverging from a central wheel, as in the tRNA cloverleaf. Other examples are found in ribosomal RNAs (rRNAs) (*3*), group I and group II self-splicing introns (*4*, *5*), small nuclear RNAs (*6*), and the human immunodeficiency virus (HIV) Rev response element (*7*). In tRNA, pairs of adjacent RNA helices share the same helix axis; this is the case for the aminoacyl acceptor and T stems and also for the anticodon and D stems. Such arrangements are expected to be favorable because of the energetic contribution of base stacking. If coaxially stacked helices could be identified in an unknown RNA structure, then the overall architecture of the molecule would begin to become apparent, and a constraint for modeling would be provided.

Catalytic RNAs (ribozymes) provide systems for addressing questions of RNA structure because their structural integrity is re-

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