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Regulation of Alternative Splicing in Vivo by Overexpression of Antagonistic Splicing Factors
Javier F. Cáceres, Stefan Stamm, David M. Helfman, Adrian R. Krainer*

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 may 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 individual SR proteins 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 cell types (12). 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 βthal 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 βthal 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
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 EIA gene. Transfection of the EIA
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).

Cotransfection of the SF2/ASF cDNA strongly activated the most proximal 5’ splice site, giving rise to the 13S mRNA (Figs. 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 SF2/ASF or 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 genespecific regulators, may in this manner control the expression of a wide variety of genes.

REFERENCES AND NOTES


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14. For transfection of HeLa cells, 5 μg of the 3′-thal plasmid plus 0.5 or 1 μg of pCG-SF2 (or pCG) was 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. Ausubel et al., eds., Wiley, New York, 1987, 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 (Epi- centre, 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 TCAAGGAAGAAGTATGCTGGTCTGA and CAGGAGTGACAGAGTCCCGGAC, as an exon 2 reverse primer. The oligos used for SF2/ASF detection were GACGCCATCCACGCTGTT, which is specific for the intron-containing region (UTR) derived from the pCG vector (24), and TGCTAGTCGCGCTTTGCG, which is specific for the 5′ UTR of the endogenous SF2/ASF gene, because these were used as forward primers, and GCTCTAGAAGAAGTCCGAC 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 detected in 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, but the proteins are indistinguishable.


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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 bio- logical activity. Determination of RNA se- condary 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 ribo- somal RNAs (rRNAs) (3), group I and group II self-splicing introns (4, 5), small nuclear RNAs (6), and the human immuno- deficiency virus (HIV) Rev response el- ement (7). In tRNAs, pairs of adjacent RNA helices share the same helix axis; this is the case for the aminoslyl 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 sys- tems for addressing questions of RNA struc- ture because their structural integrity is re-