SF2/ASF Autoregulation Involves Multiple Layers of Post-transcriptional and Translational Control

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Abstract

SF2/ASF is a prototypical SR protein, with important roles in splicing and other aspects of mRNA metabolism. \textit{SFRS1} (SF2/ASF) is a potent proto-oncogene with abnormal expression in many tumors. We found that SF2/ASF negatively autoregulates its expression to maintain homeostatic levels. We characterized six SF2/ASF alternatively spliced mRNA isoforms: the major isoform encodes full-length protein, whereas the others are either retained in the nucleus or degraded by NMD. Unproductive splicing accounts for only part of the autoregulation, which occurs primarily at the translational level. The effect is specific to SF2/ASF and requires RRM2. The ultraconserved 3'UTR is necessary and sufficient for downregulation. SF2/ASF overexpression shifts the distribution of target mRNA towards mono-ribosomes, and translational repression is partly independent of Dicer and a 5' cap. Thus, multiple post-transcriptional and translational mechanisms are involved in fine-tuning the expression of SF2/ASF.

Introduction

Alternative splicing is widespread: recent high-throughput RNA-sequencing analysis of tissue-specific splicing indicated that >90\% of human genes express multiple spliced isoforms\textsuperscript{1}. SF2/ASF is a prototypical SR protein that participates in both constitutive and alternative splicing\textsuperscript{2}. Additional functions of SF2/ASF extend to other aspects of mRNA metabolism, such as NMD (nonsense-mediated mRNA decay)\textsuperscript{3}, mRNA export\textsuperscript{4,5}, and translation\textsuperscript{6}.

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Although SF2/ASF levels vary widely among cell types, tight control of its expression is important for normal cell and organismal physiology. Knockdown of SF2/ASF results in genomic instability, cell-cycle arrest, and apoptosis. Knockout of SF2/ASF in cardiomyocytes results in defective postnatal heart remodeling in mice, due to incorrect CAMK2D splicing. Moderate (2-3 fold) overexpression of SF2/ASF is sufficient to transform immortal rodent fibroblasts, which then rapidly form sarcomas in nude mice. SF2/ASF also regulates alternative splicing of the MST1R (RON) proto-oncogene, inducing cell motility and invasion. SF2/ASF shows abnormal expression in many tumors, but little is known about how its expression is regulated, or why it is up-regulated in cancer, though gene amplification was found in some breast tumors.

Besides transcription, gene expression can be regulated at both post-transcriptional and translational levels. Alternative splicing can regulate gene expression by generating non-productive isoforms, such as mRNAs that are retained in the nucleus or are subject to NMD, or by encoding proteins with different functions. mRNA turnover and translation are also key control points for gene-expression regulation, frequently mediated by 3′UTR elements. For example, AU-rich elements (AREs) and associated proteins affect mRNA stability and translational efficiency. In addition, microRNAs (miRNAs) and small interfering RNAs (siRNAs) are important regulators of translation and mRNA decay.

Many splicing factors are regulated post-transcriptionally. In C. elegans, two SR proteins, SRp20 and SRp30b, have premature termination codon (PTC)-containing splicing isoforms, whose degradation depends on the smg genes. Likewise, the mammalian SR protein SC35, and the polypyrimidine-tract-binding protein (PTB) autoregulate by promoting the expression of unstable alternatively spliced mRNA isoforms that undergo NMD. SRp20, another SR protein, promotes expression from its own gene of a splicing isoform encoding a truncated protein, and SF2/ASF antagonizes this regulation. Recent reports described ultraconserved (UCR) elements in every member of the SR protein family, as well as in PTB. UCRs are present in regions that undergo alternatively splicing events that introduce PTCs, such that some of the resulting mRNAs are NMD targets. Thus, unproductive splicing can regulate SR protein expression.

Here we report that SF2/ASF negatively regulates its own expression, and we investigate the underlying mechanisms. We demonstrate that multiple layers of control, including alternative splicing and translational regulation, are involved in this homeostatic process.

**Results**

**SF2/ASF autoregulation by negative feedback**

We placed an SF2/ASF cDNA under the control of the TRE-CMV promoter and transduced HeLa cells stably expressing the tetracycline trans-activator protein tTA (tet-off). In medium without tetracycline, SF2/ASF expression was turned on (Fig. 1a). An N-terminal T7 tag allowed separation of ectopic and endogenous SF2/ASF by SDS-PAGE. Western blotting revealed that expression of endogenous SF2/ASF was reduced by ~70% in T7-SF2/ASF overexpressing cells, compared to uninduced cells, whereas a β-catenin loading
control was unaffected (Fig. 1b). These data confirm that SF2/ASF autoregulates its expression, as reported for stable retroviral transduction of human, mouse, and rat cells\(^\text{11}\).

**Alternative splicing contributes to autoregulation**

We first examined whether SF2/ASF autoregulation occurs via alternative splicing, as previously proposed\(^\text{20,21}\). To identify all the isoforms expressed in HeLa cells, we amplified them by RT-PCR from total RNA using primers positioned at the ends of the first and last exon of the canonical isoform\(^\text{25,26}\) (Fig. 2a). We detected six isoforms, with the canonical one being by far the most abundant. Other human cell lines, such as HEK293 and IMR90, showed similar patterns of SF2/ASF mRNA isoforms (not shown).

Cloning and sequencing revealed that isoforms III–VI undergo excision of one or two introns in their 3′UTR, resulting in PTCs that should trigger NMD\(^\text{27}\). However, when we inhibited NMD with cycloheximide, only isoforms V and VI increased substantially (Fig. 2b).

To determine the subcellular localization of the various mRNA isoforms, we performed cell fractionation, and we extracted RNAs from nuclear and cytoplasmic fractions for RT-PCR analysis (Fig. 2c). Surprisingly, isoforms II, III, and IV were all retained in the nucleus, which could explain why isoforms III and IV escape NMD, as this pathway requires a round of cytoplasmic translation\(^\text{27}\).

To determine how SF2/ASF overexpression affects each isoform, we performed RT-PCR of endogenous SF2/ASF mRNAs using the same samples as in Figure 1. The reverse primer corresponds to the end of the 3′UTR, which is absent in the ectopic SF2/ASF cDNA. After induction of T7-SF2/ASF, isoforms III and VI increased markedly (Fig. 2d). The protein-coding isoform I decreased by ~30% (Fig. 2e), considerably less than the ~70% reduction at the protein level (Fig. 1b).

These data show that SF2/ASF modulates alternative splicing of its own transcript, and downregulates itself in part by decreasing the production of the protein-coding isoform and increasing the isoforms that are retained in the nucleus or degraded by NMD. However, this switch in alternative splicing does not fully account for the downregulation at the protein level, suggesting that additional mechanisms are involved in SF2/ASF autoregulation.

**Autoregulation is specific to SF2/ASF and requires RRM2**

To better understand the mechanisms underlying SF2/ASF homeostasis, we amplified the genomic segment of the transcribed region of \(SFRS1\) from human DNA by PCR, and subcloned it into pcDNA3.1+. To detect the proteins expressed from the transfected genomic construct, we added a V5 tag before the start codon, and omitted the natural 5′UTR (Fig. 3a). Except where indicated, we used V5-SF2/ASF as a reporter and co-expression of T7-SF2/ASF cDNA (including the coding exons but not the UTRs) to study SF2/ASF autoregulation. By co-expressing V5-tagged genomic SF2/ASF and T7-tagged SF2/ASF cDNA, we sought to recapitulate the autoregulation. We transiently co-transfected HeLa cells with a constant amount of genomic V5-SF2/ASF plasmid and increasing amounts of T7-SF2/ASF cDNA plasmid (Fig. 3b). Western blotting using V5 and T7 antibodies showed
that overexpression of SF2/ASF cDNA strongly repressed the protein expressed from genomic SF2/ASF in a dose-dependent manner.

By co-transfecting HeLa cells with equal amounts of V5 genomic SF2/ASF plasmid and T7-tagged cDNAs of SF2/ASF mutants or other SR proteins, we confirmed that downregulation is not via promoter competition, and established that it is SF2/ASF-specific and requires RRM2 (Fig. 3c). Co-expression of SC35 or two other SR proteins, SRp55 and SRp75, did not affect the expression level of SF2/ASF from the genomic construct (Fig. 3c, lane 13, and data not shown). SRp30c—the closest paralog of SF2/ASF—had lower expression than most of the other proteins, even when we transfected three times more plasmid; even after normalizing to the expression level, its effect was slight (see histogram below the gel). Considering that SF2/ASF-ΔRS was also weakly expressed, yet it strongly decreased V5-SF2/ASF expression, the effect of SRp30c, if any, is much less pronounced than that of SF2/ASF. Most of the SF2/ASF mutants, including RS-domain deletion (ΔRS), RRM1 deletion (ΔRRM1), and nuclear-retained SF2/ASF with the NRS signal from SC35 (NRS-SC35), retained the downregulation activity of SF2/ASF. Only the RRM2-deletion mutant (ΔRRM2) was defective in downregulation.

Using a forward primer corresponding to the V5 tag and a reverse primer in SF2/ASF exon 3 for radioactive RT-PCR, we specifically amplified the total mRNA expressed from the transfected genomic construct. Interestingly, the change in mRNA level was not always consistent with the downregulation of SF2/ASF protein expression. Overexpression of T7-SF2/ASF led to accumulation of unspliced pre-mRNA and a decrease in mature mRNA—a decrease in splicing efficiency previously observed with other splicing reporters. As the T7-SF2/ASF protein increased, the V5-SF2/ASF protein level decreased steeply, whereas the spliced mRNA decreased much more gradually (Fig. 3b and 3d, lanes 1-6). ΔRS did not cause a decrease in mRNA level, but still caused strong downregulation of SF2/ASF protein expression, whereas ΔRRM2 resulted in decreased mRNA, but no change at the protein level (Fig. 3c and 3d, lanes 9 and 11). Furthermore, two other SR proteins, SC35 and SRp30c, also markedly inhibited splicing and decreased the mature mRNA level, but did not markedly repress protein expression (Fig. 3c and 3d, lanes 13 and 14). Therefore, the changes in steady-state mRNA levels do not consistently account for the observed decrease at the protein level.

The 3’UTR is necessary and sufficient for autoregulation

To identify regions important for SF2/ASF autoregulation, we constructed a genomic version of SF2/ASF with all three coding-region introns precisely deleted (Fig. 4a). We co-transfected HeLa cells with wild-type or Δintron123 V5-SF2/ASF and T7-SF2/ASF cDNA. Western blotting showed that SF2/ASF still downregulated protein expression from this intronless construct (Fig. 4b, lanes 1-2). Therefore, splicing out the first three introns is not required for autoregulation. To eliminate further splicing within the 3’UTR, without changing its length, we also inactivated the two pairs of alternative splice sites in this region by mutating G to C at the +1 position of the 5’ splice sites, and mutating G to T at the -1 position of the 3’ splice sites. SF2/ASF still showed autoregulation with this construct (Fig. 4b, lanes 3-4). However, when we replaced the 3’UTR with bacterial sequences, but kept the
length constant, SF2/ASF no longer downregulated the protein expression (Fig. 4b, lanes 5-6).

Another construct, ΔUTR, replaces the entire 1.9-Kb 3′UTR of SF2/ASF with ~100 bp of vector sequence (Fig. 4a). This construct also gave very different results than the wild-type construct. First, the basal level of protein greatly increased (Supplementary Fig. 1b, lane 7). To obtain comparable expression, we transfected cells with only 1/5 as much DNA for this construct, and loaded half as much protein for Western analysis (Fig. 4b, lanes 7-8). Second, the protein expressed from this construct was not downregulated by overexpression of T7-SF2/ASF cDNA (Fig. 4b, lanes 7-8; Supplementary Fig. 1b, lanes 7-9). When we deleted both the 3′UTR and the first three introns, we obtained similar results as with ΔUTR (Supplementary Fig. 1b, lanes 10-12). The lower expression level of SF2/ASF with its natural 3′UTR may reflect further inhibition by endogenous SF2/ASF, and may also be a non-specific effect of 3′UTR length, as a bacterial-sequence 3′UTR of the same length gave comparable basal-level expression as the natural 3′UTR (Fig. 4b, lanes 5-6). In general, very long 3′UTRs tend to repress translation.28 Finally, in all cases, despite very large differences at the protein level, there was relatively little variation at the mRNA level (Fig. 4b).

To examine whether the 3′UTR of SF2/ASF is sufficient to repress expression in response to SF2/ASF overexpression, we subcloned the 3′UTR after the coding sequence of a Renilla luciferase reporter. We co-transfected reporter constructs with or without the SF2/ASF 3′UTR with T7-SF2/ASF cDNA or control vector into HeLa cells. We measured luciferase activity and performed radioactive RT-PCR of luciferase mRNA as a normalization control (Fig. 4c). The basal expression of luciferase with SF2/ASF's 3′UTR was approximately 60% of the control. Overexpression of SF2/ASF downregulated the luciferase reporter with the SF2/ASF 3′UTR to ~20%, but had no repressive effect with the control gene. Therefore, the SF2/ASF 3′UTR is both necessary and sufficient to mediate downregulation of gene expression by SF2/ASF overexpression.

The 3′UTR of SF2/ASF does not inhibit mRNA export
To address whether SF2/ASF inhibits nuclear export of its own mRNA, we performed subcellular fractionation after co-transfecting HeLa cells with either wild-type or Δintron123 V5-SF2/ASF and T7-SF2/ASF cDNA or control vector. Radioactive RT-PCR of GAPDH pre-mRNA, which is retained in the nucleus, confirmed the clean separation of nucleus and cytoplasm (Supplementary Fig. 2). The proportion of V5-SF2/ASF mRNA present in the cytoplasm was very similar with and without T7-SF2/ASF co-expression. Thus, SF2/ASF mRNA export is not inhibited by SF2/ASF overexpression, and is not the mechanism of SF2/ASF autoregulation.

SF2/ASF downregulates itself at the level of translation
Translation is a highly regulated process, and initiation is usually the rate-limiting step.29 To determine whether SF2/ASF autoregulation involves decreased translational efficiency, we performed in vitro translation in HeLa cell extract. We in vitro transcribed luciferase-reporter mRNAs with or without the SF2/ASF 3′UTR, and in some cases added a poly(A) tail (Supplementary Fig. 3a). We incubated equal amounts of mRNAs in the extract, and
measured luciferase activity (Supplementary Fig. 3b). Translation of the 3′UTR-containing mRNA was less efficient, consistently with the above transfection result (Fig. 4c). However, there was little if any change when we added purified recombinant SF2/ASF—expressed in bacteria or in mammalian cells (Supplementary Fig. 3b). Therefore, we could not recapitulate the autoregulation of SF2/ASF in vitro. However, the same translation extract did respond to SF2/ASF addition when we assayed for ESE-dependent stimulation (not shown), as previously reported6, suggesting that different mechanisms underlie positive and negative control of translation by SF2/ASF.

This negative result in vitro does not rule out translation inhibition as the mechanism of SF2/ASF autoregulation. Therefore, we next used sucrose gradients to directly analyze the distribution of reporter mRNAs on polyribosomes in vivo. We co-transfected HeLa cells with V5-SF2/ASF Δintron123 and either T7-SF2/ASF cDNA or control vector. We also co-transfected as an internal control a Renilla-luciferase reporter with a bacterial-sequence 3′UTR of the same length. After 48 h, we fractionated cytoplasmic extracts on 10%-50% sucrose gradients, and detected V5-SF2/ASF mRNA in each fraction by radioactive RT-PCR (Fig. 5). In contrast to the control endogenous GAPDH mRNA, which peaked in the heavy polyribosome fractions, the main peak of V5-SF2/ASF mRNA or Rluc-pucUTR control-reporter mRNA was in the monoribosome fractions (Fig. 5a,b, left panels). This distribution is consistent with the repressive effect of the long 3′UTRs. An additional, broad peak of V5-SF2/ASF mRNA sedimented with polyribosomes. Co-expression of T7-SF2/ASF shifted this broad peak towards the monoribosome fractions, indicating that SF2/ASF reduced the translation efficiency of V5-SF2/ASF with the natural 3′UTR (Fig. 5a,b, right panels). The difference between the two distribution profiles is significant (p=0.028, Kolmogorov-Smirnov test). In contrast, the distribution of the Rluc-pucUTR control mRNA was not changed by SF2/ASF overexpression, consistent with our finding that SF2/ASF did not reduce the luciferase activity in the presence of the bacterial-sequence 3′UTR (see below, Fig. 6b). Treatment of cells with puromycin confirmed that sedimentation of the mRNAs in denser fractions indeed reflected polysome association (Fig. 5c).

Potential contribution of miRNAs to autoregulation

miRNAs regulate gene expression by controlling the translation or stability of target mRNAs. TargetScan predicts multiple putative miRNA targets in the 3′UTR of SF2/ASF (not shown). Dicer is an enzyme required for miRNA maturation31. To examine the role of miRNAs in SF2/ASF autoregulation, we used Dicer-disrupted or -knockout cell lines. We first used DicerEx5/Ex5 RKO cells, in which exon 5 of human Dicer is disrupted, interrupting the helicase domain32. We co-transfected V5-SF2/ASF with T7-SF2/ASF cDNA or control vector into wild-type or DicerEx5/Ex5 RKO cells. Western blotting showed that SF2/ASF downregulated itself in both wild-type and Dicer-disrupted cells (Supplementary Fig. 4a). However, because the biogenesis of some miRNAs is not disrupted in these cells32, the potential involvement of some miRNA(s) in SF2/ASF autoregulation could not be ruled out. We therefore used Dicer-null mouse ES cells, which have compromised proliferation but are viable33. The Dicer gene is completely knocked out in these cells, and the biogenesis of all miRNAs is thought to be fully disrupted. We performed
similar co-transfection experiments as above with Dicer−/− and control Dicer+/− ES cells, and observed downregulation in both cases, although there was less repression in Dicer-null cells, perhaps due to their reduced proliferation (Supplementary Fig. 4b). This experiment suggests that miRNA-mediated gene repression may contribute to SF2/ASF autoregulation, though not as the main mechanism.

**Effect of cap-dependent versus IRES-dependent translation**

We next examined whether 3′UTR-mediated translational repression of SF2/ASF can occur in the context of internal ribosome entry site (IRES)-dependent translation initiation. Translation driven by different viral IRES elements requires distinct subsets of the initiation factors necessary for cap-dependent translation.34 The encephalomyocarditis virus (EMCV) IRES requires most initiation factors, except for the cap-binding protein eIF4E. The hepatitis-C virus (HCV) IRES only requires eIF3 and eIF2. Finally, the cricket-paralysis virus (CrPV) IRES bypasses the requirement for all the initiation factors. We placed these IRES sequences 5′ of a Renilla luciferase reporter with or without the SF2/ASF 3′UTR (Fig. 6a). We inserted a hairpin structure upstream of each IRES to block ribosomes initiating at the 5′ cap and ensure IRES-dependent initiation.35 We co-transfected the various reporter constructs into HeLa cells together with control pCGT vector or T7-SF2/ASF cDNA. 40 h later, we measured luciferase activity and carried out radioactive RT-PCR of luciferase mRNA as a normalization control (Fig. 6b). As with cap-dependent translation, with the EMCV or the HCV IRES, SF2/ASF repressed translation in a manner that depended on the natural 3′UTR of SF2/ASF. In contrast, CrPV-IRES-dependent translation was not repressed by SF2/ASF overexpression (Fig. 6b). We conclude that SF2/ASF autoregulation takes place at the translation-initiation step, and that eIF3 and/or eIF2 may be involved in this effect.

**Discussion**

Negative autoregulation is an effective mechanism for homeostatic control of gene expression. SF2/ASF is an abundant and highly conserved RNA-binding protein with multiple functions and oncogenic potential, whose expression level needs to be precisely controlled for normal cell physiology. Post-transcriptional regulation of splicing factors can be complex, involving multiple layers of control. For example, PTB antagonizes the expression of its paralog, nPTB, by promoting an NMD-targeted alternative splicing isoform, and possibly also by inhibiting translation of correctly spliced mRNA through an unknown mechanism.36,37 During neuronal differentiation, PTB expression is repressed by the neuron-specific miR-124, resulting in increased nPTB protein.37 nPTB expression is also repressed during myoblast differentiation by the muscle-specific miR-133.38 Our study shows that multiple levels of post-transcriptional and translational control are likewise involved in fine-tuning SF2/ASF expression.

We identified and characterized six alternatively spliced mRNA isoforms of SF2/ASF in HeLa cells, of which isoforms IV and VI are not shown in the UCSC or ENSEMBL browsers. The major isoform, I, encodes full-length protein, and has a long 3′UTR.39 Isoform II, which retains the third intron, was previously reported.39 A third isoform was also described in these studies, involving an alternative 3′ splice site in the third intron.
used a specific primer to amplify that isoform, but did not detect it in the cell lines we tested. Isoforms II and III retain the third intron, which changes the reading frame and results in a stop codon shortly after exon 3; this would result in a truncated protein without the C-terminal RS domain. However, we found that these two isoforms are retained in the nucleus, and are therefore not translated. This explains why our SF2/ASF antibody, which recognizes an epitope near the N-terminus, fails to detect any smaller protein isoforms by Western blotting.

In general, intron-containing pre-mRNAs are retained in the nucleus, and only mature mRNAs are exported to the cytoplasm, preventing translation of incompletely processed messages. Interestingly, isoform IV retains one intron, compared to isoform V, and it remains nuclear; however, the major isoform I retains that plus one additional intron, but somehow is compatible with efficient nuclear export, which might involve potential RNA cis-acting elements that are recognized as export signals. Many retroviruses and some cellular mRNAs, such as Tap, employ this mechanism.

Isoforms III, IV, V, and VI are generated by splicing that removes one or two introns in the 3′UTR. Among these, isoforms V and VI are exported to the cytoplasm and accumulate after cycloheximide treatment, suggesting that they are NMD targets. Isoform V encodes the same full-length protein as isoform I, whereas isoform VI encodes a truncated protein lacking the RS domain. SF2/ASF overexpression upregulates the unproductive isoforms III and VI, and decreases the protein-encoding major isoform I, but only modestly. Quantitation of the changes at the mRNA and protein levels indicates that alternative splicing associated with NMD or nuclear retention only partly explains the autoregulation of SF2/ASF.

By co-transfecting a V5-tagged genomic SF2/ASF construct with a T7-tagged SF2/ASF cDNA, we recapitulated the autoregulation seen with endogenous SF2/ASF. Co-transfection experiments with different mutants showed that RRM2 is required, and the 3′UTR is the only critical cis-element for the regulation. The length of the 3′UTR affects basal expression, but is not responsible for autoregulation.

Post-transcriptional regulation is frequently mediated by RNA-protein interactions in the UTRs, and this is also where the two UCRs are located in SF2/ASF (Fig. 2a). We tried to map cis-element(s) required for downregulation, but were unable to narrow them down to well-defined sequences. First, when the 3′UTR was divided into four fragments, three still showed downregulation by SF2/ASF overexpression (Supplementary Fig. 5). Second, when each of the functional fragments was further subdivided, each subfragment gave much less or no repression (not shown). It appears that multiple elements in the 3′UTR are involved in SF2/ASF autoregulation, and the signals are dispersed and partially redundant. The roles of the two UCRs remain unclear, especially considering that the entire 3′UTR of SF2/ASF is ∼95% conserved between human and mouse.

A recent quantitative proteomics study showed that each miRNA has hundreds of target genes, but individual genes are only modestly repressed by a single miRNA. Therefore, several miRNAs might target multiple regions in this 3′UTR, with their combined action resulting in downregulation. However, the experiments with Dicer-disrupted or -knockout
cell lines suggest that miRNA-mediated repression is not the main mechanism of SF2/ASF autoregulation, although it may contribute to some extent. Indeed, miR7 was recently found to reduce SF2/ASF levels through a single binding site in the 3′UTR (Jun Zhu, personal communication).

Using a sucrose-gradient assay, we found that SF2/ASF overexpression reduces the translational efficiency of an SF2/ASF-3′UTR-containing mRNA reporter. However, we could not recapitulate the translation inhibition by adding purified SF2/ASF protein to a cell-free translation system. Possible reasons for this include: i) a component(s) required for translation inhibition might be lost during extract preparation; ii) SF2/ASF does not repress translation directly, but could instead affect alternative splicing of a translational regulator; iii) the substrate for translational regulation might be a 3′UTR in the form of mRNP generated by a defined pathway, involving transcription, processing, and export.

Translation is a cytoplasmic event, but surprisingly, a nuclear-retained version of SF2/ASF was still able to autoregulate (Fig. 3c). Perhaps nuclear SF2/ASF affects the mRNP composition of its own transcript, which in turn affects how efficiently it is translated in the cytoplasm. Nuclear events often determine the downstream cytoplasmic fate of mRNAs. It is also possible that SF2/ASF regulates translational control indirectly through its nuclear functions, such as splicing of a putative translational regulator's pre-mRNA. Finally, nuclear retention of the SF2/ASF-NRS variant might be slightly leaky. However, SF2/ASF can enhance translation of reporter mRNAs in a binding-site-dependent manner, which can be recapitulated in the cell-free system; this effect, which is reproducible in our hands (not shown), requires the shuttling activity of SF2/ASF, and the nuclear-retained mutant is no longer active.

Our experiments with viral IRES elements suggest that SF2/ASF translational autoregulation is cap-independent, and that eIF2 and/or eIF3 are important, although the exact mechanism remains unknown. On the other hand, SF2/ASF enhances cap-dependent translation by repressing the activity of 4E-BP, an inhibitor of eIF4E, and no enhancement was observed for IRES-dependent translation. Therefore, we believe that these two opposite effects of SF2/ASF in translation involve distinct mechanisms, and are not contradictory.

A recent study showed that SF2/ASF binds to its own transcript within the second UCR in the cytoplasm, and enhances polysome association. Although we observed neither translational repression nor activation by in vitro translation of a reporter with the SF2/ASF 3′UTR, it is possible that the long 3′UTR mediates complex positive as well as negative regulation, and that different mechanisms are dominant depending on the context.

SF2/ASF autoregulation is a complex process involving multiple mechanisms operating at different levels. We found that both alternative splicing and translation have contributing roles, and SF2/ASF translation itself may be negatively regulated at different steps by different factors. Multi-level regulation presumably serves to control SF2/ASF homeostasis more precisely. The relative contribution of each control mechanism might vary in different
tissues or physiological states. Conversely, particular control mechanisms may be disrupted in different tumors associated with SF2/ASF upregulation.\textsuperscript{11}

**Methods**

**Plasmids**

The T7-tagged SF2/ASF, SRp30c, and SC35 constructs are in the pCGT vector; SRp30c, SC35, SF2/ASF wild type, and the NRS variant have been described\textsuperscript{47, 48}. We used quick-change mutagenesis to construct the SF2/ASF ΔRRM1, ΔRRM2, and ΔRS mutants. We subcloned V5-tagged genomic SF2/ASF and V5-SF2/ASF ΔUTR into the EcoRI and XhoI sites of pcDNA3.1+ (Invitrogen). We used two-step cloning to construct V5-SF2/ASF Δintron123, pucUTR and UTR fragments A/B/C/D. First we cloned the V5-SF2/ASF cDNA coding region into pcDNA3.1+ via NheI and BamHI sites. Then we cloned the different 3′UTRs after the cDNA via BamHI/BglII and XhoI sites. We used a similar strategy to construct Rluc-SF2 UTR and Rluc-pucUTR. To mutate the splice sites in SF2/ASF 3′UTR we used site-directed mutagenesis. To construct the hp-IRES Renilla luciferase reporters, we used three-steps cloning. First, we inserted the hairpin sequence GCCUAGGCCGGAGCGCCCAGAUCUGGGCGCUCCGGCCUAGGC\textsuperscript{35} into pcDNA3.1+ via NheI and BamHI sites. We amplified the EMCV IRES by PCR from the pWZL vector (gift from Dr. Scott Lowe's lab). We amplified the HCV and CrPV IRES from pAR233 HCV la IRES and pAR237 CrPV IRES, respectively, which were generously provided by Dr. Vincent Racaniello (Columbia University). We cloned the IRES fragments after the hairpin using the BamHI and EcoRI sites. Finally, we inserted Renilla luciferase, with or without the SF2/ASF 3′UTR, after the IRES using the EcoRI and XhoI sites. We also subcloned T7-SF2/ASF cDNA into the XhoI and EcoRI sites of the inducible vector STP\textsuperscript{24}.

**Cell culture and transfection**

We cultured HeLa and RKO cells in DMEM supplemented with 10% (v/v) FBS, 100 U ml\textsuperscript{-1} penicillin and 100 μg ml\textsuperscript{-1} streptomycin. To transfect plasmids, we used Fugene 6 (Roche). We grew HeLa tet-off cells in the same medium, with 2 μg ml\textsuperscript{-1} tetracycline. To generate stable cell lines, we infected Hela tet-off cells with STP retroviral vectors with an SF2/ASF cDNA, and selected stable transductants with puromycin (2 μg ml\textsuperscript{-1}) for 72 h. To induce SF2/ASF, we placed the cells in medium lacking tetracycline. To grow ES cells, we used DMEM knockout medium containing 15% (v/v) FBS, 100 μM β-mercaptoethanol, 2 mM L-glutamine, 50 U ml\textsuperscript{-1} penicillin, 40 ug ml\textsuperscript{-1} streptomycin, and 1000 U ml\textsuperscript{-1} LIF (Chemicon). We seeded the ES cells on plates coated with gelatin (Chemicon), and transfected them with Lipofectamine 2000 (Invitrogen).

**Western blotting**

48 h after transfection, we harvested the cells and lysed them in Laemmli buffer. The primary antibodies included β-catenin (Sigma), SF2/ASF (mAb AK96), T7 tag (Novagen), and V5 tag (Invitrogen). The secondary antibodies were goat anti-mouse IgG (H+L) HRP-conjugated (Pierce), labeled with yellow-fluorescent Alexa Fluor 532 dye (Invitrogen), or with IRDye 800CW (LI-COR). For detection we used an ECL kit (Roche), an Image Reader.
RNA isolation and RT-PCR

To isolate total RNA, we used Trizol (Invitrogen) and treatment with RQ1 DNase I (Promega). For first-strand cDNA synthesis, we used random hexamers and Super Script II reverse transcriptase (Invitrogen). For regular PCR we used AmpliTaq (Roche); to amplify all the SF2/ASF isoforms we used rTth (Roche) and Vent (New England Biolabs) DNA polymerases. For radioactive PCR, we added γ-32P-dCTP and amplified for 24 cycles. We separated the PCR products on 6% non-denaturing polyacrylamide gels, and detected them with the Image Reader FLA-5100. Primers: GAPDH-F (5′-AAGGTTAGGTCGGAGTCAACGG-3′), GAPDH-R (5′-CCACTTGAATTTTGAGAGATCTC-3′), SF2-e1F (5′-ACATCGACCTCAAGAATCGCCGC-3′), SF2-e4R (5′-GGCGAGGAATCCACTCCTATG-3′), SF2-e3F (5′-GACTGAGTGGAGTTGTACCG-3′), SF2-e3R (5′-TCCACGACACCATTCATCTCG-3′), V5-F (5′-GGCAAGCCCATCCCTAACCC-3′), Rluc-F (5′-GACTTCGAAAGTTTATGATCC-3′), Rluc-R (5′-GCTCATAGCTATAATGAAATGCC-3′).

Cell fractionation

We lysed cells in gentle lysis buffer (10 mM HEPES pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) NP-40). We pelleted the nuclei at 2300 g for 5 min at 4 °C, and transferred the supernatant (cytoplasm) to another tube. We washed the nuclei once with the same buffer and repelleted them. To extract RNA, we added Trizol to the pellet and the first supernatant.

Luciferase reporter assay

We lysed HeLa cells using passive lysis buffer (Promega) and measured the levels of Renilla luciferase using Promega’s Dual Luciferase Assay Kit and a Monolight 2010 luminometer (Analytical Luminescence Laboratory). To extract the RNA, we added Trizol to the remaining lysates.

In vitro translation assay

We prepared translation-competent HeLa cell-free extracts as described30. We linearized the Renilla luciferase reporter construct with XhoI and used it as a template for in vitro transcription with T7 RNA polymerase using an mMessage mMMachine Kit (Ambion). We added a poly (A) tail using a Poly (A) Tailing Kit (Ambion). Translation reactions included 20 ng of reporter mRNA with or without 200 ng of recombinant SF2/ASF protein, purified from bacteria or 293E cells26,49, and were incubated at 37 °C for 30 min. We added 50 μl of passive lysis buffer (Promega) to stop the reactions. We measured luciferase activity with a Dual Luciferase Assay Kit (Promega).
Sucrose gradient assay

We used one 150-mm plate of cells for each assay. We prepared 10% (w/v) and 50% sucrose in 20 mM Tris-HCl pH 7.4, 100 mM KCl, 5 mM MgCl₂. We split transfected cells once, 12 h before harvesting. We treated HeLa cells with 50 μg ml⁻¹ cycloheximide at 37 °C for 20 min. We washed the cells with ice-cold PBS containing 50 μg ml⁻¹ cycloheximide and lysed in 500 μl of polisome-extraction buffer (20 mM Tris pH 7.5, 5 mM MgCl₂, 100 mM KCl, 0.5% (v/v) NP-40, 100 U of RNasin (Promega)). Where indicated, we added puromycin (100 μg ml⁻¹) 1 h before harvesting, and omitted cycloheximide. We spun the lysates at 13,000 g for 10 min, after a 10-min incubation on ice. Then, we layered 500 μl of each cytoplasmic lysate onto 10-50% sucrose gradients and centrifuged at 4 °C in a Sorvall SW41 rotor at 36,000 rpm for 2 h. We collected fractions from the top using a BioComp gradient master, while measuring the OD at 254 nm. We treated fractions with 1% (w/v) SDS and 150-200 μg ml⁻¹ Proteinase K (Roche). We extracted RNA with phenol/chloroform/isoamyl alcohol (25:24:1), treated it with RQ1 DNase I (Promega), and analyzed it by RT-PCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
HeLa tet-off cells with inducible SF2/ASF overexpression. (a) Western blot analysis of SF2/ASF before and after induction, using an antibody that recognizes both endogenous and epitope-tagged SF2/ASF. (b) Quantification of endogenous SF2/ASF protein before and after induction. Error bars show standard deviations (SD); n = 3.
Figure 2.
Alternative splicing of SF2/ASF. (a) Six alternative splicing isoforms were identified by RT-PCR with primers a and c, followed by cloning and sequencing. Their structures are shown in the diagrams, with the genomic scale shown at the top. The primers are indicated by arrows below the isoform diagrams. Grey represents protein-coding regions; black denotes the UTRs. The two bars below the genomic scale represent the positions of UCRs. The correspondence between exon sequences and the domain structure of the protein—including two RNA-recognition motifs (RRM) and an arginine-serine-rich (RS) domain—is shown at the bottom of the panel. (b) Cycloheximide treatment was used to
inhibit NMD. Radioactive RT-PCR with primers b and c was performed to detect the changes of all the alternative splicing isoforms. (c) Cell fractionation was performed to separate nucleus and cytoplasm. RNA from cells before fractionation and from nuclear and cytoplasmic fractions was extracted for radioactive RT-PCR with primers b and c. T: total; N: nucleus; C: cytoplasm. (d) RT-PCR of RNAs from the same cell samples as in Figure 1, before and after SF2/ASF induction. The bottom panel shows amplification of a region common to all the isoforms, using primers d and e. (e) Quantification of the SF2/ASF protein-coding mRNA, isoform I, before and after induction. Error bars show SD; n = 3. t-test, P < 0.04
Figure 3.
Expression of SF2/ASF from a genomic construct. (a) Diagrams of the V5-tagged SF2/ASF genomic construct and T7-tagged SF2/ASF cDNA construct. The V5 epitope tag is indicated by an open circle, and the T7 tag by an open hexagon. RT-PCR primers used in panel d are indicated by arrows. (b) V5-tagged genomic SF2/ASF was co-transfected with increasing amounts of T7-SF2/ASF cDNA into HeLa cells. After 48 h, protein and RNA were isolated, and Western blotting was performed with both V5 and T7 antibodies, with β-catenin as a normalization control. (c) Genomic V5-SF2/ASF was co-transfected with various T7-tagged SF2/ASF mutants and other SR protein cDNAs. Western blotting was performed with both V5 and T7 antibodies. The histogram shows the quantification of the relative V5-SF2/ASF expression level. The level of V5-tagged SF2/ASF was measured and normalized to that of each T7-tagged protein, with wild-type T7-SF2/ASF as the standard. The level of V5-SF2/ASF co-transfected with empty vector was set at 1. (d) RT-PCR of V5-SF2/ASF with
one primer in the V5 tag and the other in SF2/ASF exon 3. The band corresponding to spliced mRNA is indicated by an arrow. *, RNAs that retained one or more introns.
Figure 4.
The 3′UTR is necessary and sufficient for SF2/ASF autoregulation. (a) Diagrams of the genomic V5-SF2/ASF mutants. Grey represents the coding region; black represents the natural 3′UTR; white represents a heterologous sequence of the same length; and the thin light-grey bar represents the 3′UTR sequences from the vector; the V5 tag is denoted by an open circle; the grey vertical lines in the 3′UTR represent inactivating mutations of the alternative 5′ and 3′ splice sites. (b) HeLa cells were co-transfected with V5-SF2/ASF genomic mutants and T7-SF2/ASF cDNA. Western blotting was performed to detect SF2/ASF expressed from the genomic construct using V5 antibody, from the cDNA using
T7 antibody, and endogenous β-catenin was detected as a loading control. RT-PCR was carried out using the same primers as in Fig. 3d, with GAPDH as a reference. Deletion of the 3′UTR results in much more efficient translation, so in lanes 7 and 8 we transfected only 1/5 as much reporter plasmid, and loaded 1/2 as much total protein. (c) Luciferase reporter assay. The 3′UTR of SF2/ASF was fused to a Renilla luciferase reporter gene. The reporter was co-transfected into HeLa cells with control vector or SF2/ASF cDNA. Luciferase activity was measured and normalized to the luciferase mRNA level determined by radioactive RT-PCR. The relative luciferase activity of pcDNA-Rluc in the absence of SF2/ASF was set at 100%. The error bars show SD; n = 3. t-test, P<0.0001.
Figure 5. SF2/ASF reduces the polysome association of its own mRNA. (a) Sucrose-gradient fractionation of cytoplasmic extracts from HeLa cells expressing V5-SF2/ASF Δintron123, Rluc-pucUTR, with (right panel) or without (left panel) T7-SF2/ASF cDNA. Top, UV absorbance (254 nm) profile. Middle and bottom panels, RNA extracted from each fraction was analyzed by radioactive RT-PCR to amplify V5-SF2/ASF, Rluc-pucUTR, and endogenous GAPDH mRNAs. (b) Quantitation of V5-SF2/ASF mRNA distribution in polysome gradients. Relative mRNA levels in each fraction were calculated as percentage of...
the total levels from all the fractions. Error bars indicate SD; n = 3. (c) Transfected HeLa cells were treated with puromycin for 1 h prior to lysis and fractionation. Gradient fractionation and analysis were done as in a.
Figure 6.
IRES-dependent translation assay. (a) Diagrams of EMCV, HCV, and CrPV IRES Renilla-luciferase reporter constructs, with or without the SF2/ASF 3′UTR (black box) or a heterologous sequence of the same length (white box). A hairpin was placed upstream of each IRES to inhibit cap-dependent translation. (b) Luciferase assay. The various Renilla-luciferase reporter constructs were co-transfected into HeLa cells with empty vector or T7-SF2/ASF cDNA. Luciferase activity was normalized to the luciferase mRNA level, measured by radioactive RT-PCR, as in Fig. 4c, and the percent change in the presence of SF2/ASF, compared to the activity in the absence of SF2/ASF, was plotted. The error bars show SD; n = 3.