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PSF contacts exon 7 of SMN2 pre-mRNA to promote exon 7 inclusion

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disease and a leading cause of infant mortality. Deletions or mutations in SMN1, a gene that encodes SMN protein, which regulates assembly/disassembly of U snRNP and are suggested to direct axonal transport of β -actin mRNA in neurons, are responsible for most cases of SMA disease. However, human contain a second SMN gene called SMN2. Unlike SMN1, the majority of SMN2 mRNA doesn't include exon 7. Here we show that increased expression of PSF significantly promotes inclusion of exon 7 in the SMN2 and SMN1 mRNA, whereas reduced expression of PSF promotes exon 7 skipping in various cell lines and in fibroblast cells from SMA patients. In addition, we present evidence showing that PSF interacts with the GAAGGA enhancer on exon 7. We also demonstrate that a mutation in this enhancer abolishes the effects of PSF on the exon 7 splicing. Furthermore we show that the RNA target sequences of PSF and tra2 β on exon 7 are partially overlapped. These results lead us to conclude that PSF interacts with an enhancer on exon 7 to promote exon 7 splicing of SMN2 pre-mRNA.

Keywords

RNA splicing; Spinal Muscular Atrophy; PSF; exon 7 splicing; enhancer

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1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disease that affects more than 1:11,000 newborns. Type 1 SMA patients without respiratory support usually die before two years old. As a result, SMA is the leading cause of infant mortality [1, 2]. The motor neurons in the anterior horn of spinal cord are severely destructed in the type 1 SMA patients [3]. The genetic cause of SMA is the deletion or a mutation in the SMN1 gene [4]. There are two SMN genes in humans. On one hand, SMN1 is defective in SMA patients. SMN1 encodes a full-length functional SMN protein that regulates the assembly or disassembly of U snRNP complex and directs the β -actin mRNA transport in the neuronal cells [5-12]. On the other hand, the SMN2 gene remains intact in patients. But the SMN2 gene contains a few nucleotide differences from the SMN1 gene [13-15]. Consequently, SMN2 pre-mRNA is spliced mainly to an mRNA variant that lacks the canonical exon 7 and encodes a SMN Δ 7 protein, which does not oligomerize well and thus gets rapidly degraded so there is very little SMN Δ 7 protein [16]. SMA patients express predominantly the SMN Δ 7 protein with only a small amount of the functional full length SMN [17, 18].

Exon 7 splicing of SMN2 pre-mRNA can be regulated by multiple positive and negative factors. It has been shown that hnRNP A1 inhibits exon 7 inclusion of SMN2 pre-mRNA by contacting a C-T mutation of the SMN2 pre-mRNA on exon 7 [19, 20] whereas SRSF1 promotes the exon 7 inclusion through interacting with the C-T mutation [21]. Besides hnRNP A1 and SRSF1, tra2 β , SRSF9, hnRNP Q and Sam68 also promote exon 7 inclusion [22-25]. Moreover, the RNA sequences on exon 7, intron 6 and intron 7 are also identified as enhancers or inhibitors for the exon 7 splicing of the SMN2 pre-mRNA [26-28]. Inhibitor-targeting ASO (Anti-sense oligonucleotide) are efficient for modification of SMN2 splicing in SMA mouse model [29]. However, detailed mechanisms of exon 7 splicing in SMN2 pre-mRNA still remain elusive.

PSF (PTB-associated splicing factor) was initially identified as a protein that forms a complex with PTB (The Polypyrimidine Tract Binding Protein) [30, 31]. Since the PTB-PSF complex is required early in spliceosome formation and is essential in catalytic step II [30, 32, 33], PSF has been suggested to be important in regulation of pre-mRNA splicing. For example, PSF is known to regulate alternative splicing of the tau and CD45 pre-mRNA [34, 35]. PSF contacts purine rich sequence in the 3' site of U5 snRNA [36] and is able to mediate transcriptional activator-dependent stimulation of pre-mRNA processing. In addition to its role in pre-mRNA splicing, PSF also plays important roles in transcription and translation [37-40] as well as in tumor formation and cancer cell proliferation [41-43].

In this study, we identified PSF as a new regulator of SMN2 pre-mRNA splicing. We show that PSF promotes the inclusion of exon 7 in the SMN2 mRNA by interacting with the enhancer sequence (GAAGGA) on the exon 7. Furthermore, we demonstrate that the target sequences of PSF and tra2 β are partially overlapped. Our study provides additional clues on how SMN2 pre-mRNA splicing is regulated.

2. Materials and methods

2.1. Plasmid construction

All primers used for plasmid construction are listed in Table 1. Wild type SMN1 and SMN2 minigenes were constructed using PCR-amplification with human genomic DNA as a template. SMN1-S and SMN-2 mini-genes were generated by the overlapping PCR extension with the SMN1-GFP and SMN2-GFP plasmids as templates and SMNE6(B).F and SMNI6(D).R as primers in the first-step PCR reactions. In the second PCR reaction, the SMNI6(D).F and SMNE8(X).R primers were used with the human genomic DNA (Promega) as a template. The overlapped third PCR products were cloned into pcDNA3.1(+) plasmid using enzymes BamHI (TAKARA) and XhoI (TAKARA). To generate SMN2-E7-1, SMN2-E7-2, SMN2-E7-3 and SMN2-E7-UCC mutant mini-genes, we performed the site-directed mutagenesis using following primers; common primers (forward: SMNE6(B).F, Reverse: SMNE8(X).R), specific primers for SMN2-E7-1 (forward: E7-1.F, reverse: E7-1.R), SMN2-E7-2 (forward: E7-2.F, reverse: E7-2.R), SMN2-E7-3 (forward: E7-2.F, reverse: E7-2.R) and SMN2-E7-UCC (forward: E7-UCC.F, reverse: E7-UCC.R). All minigenes were validated by sequencing analysis (Cosmo Genetech).

2.2. Cell culture

C33A, 293A and neuroblastoma SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS). Human SMA type I fibroblast GM03813 (Coriell Repositories) cells were maintained in DMEM supplemented with 10% of non-inactivated FBS. All cells are maintained at 37°C under a humidified 5% CO₂ condition.

2.3. RT-PCR

RT-PCR analysis for exon 7 splicing of SMN2 and SMN1 pre-mRNA was performed as previously described [44]. Total RNA was extracted from mammalian cells by RiboEx reagent (Geneall) and ethanol precipitation. Reverse transcription was performed in a total volume of 20 µl, containing 1 µg RNA, 0.5 µg oligo-dT, dNTP mix (0.5 mM each dNTP), 6mM MgCl₂, 4 µl of 5X ImProm-II TM reaction buffer and 1 µl of ImProm-II TM reverse transcriptase (Promega). To detect the spliced forms from SMN1-L and SMN2-L minigenes, pCI.F and GFP-220AS.R Primers were used for PCR. The PCR primers for SMN2-E7-1, SMN2-E7-2, SMN2-E7-3 and SMN2-E7-UUA mutant minigenes are SMN.F and pcDNA.R. RT-PCR amplification of PSF and the control GAPDH was conducted with PSF.F, PSF.R, GAPDH.F and GAPDH.R as primers. PCR products were analyzed on 2% agarose gels with ethidium bromide solution (0.5 µg/ml). RT-PCR analysis of endogenous SMN1 and SMN2 exon 7 splicing were performed with primers located in exon 5 and exon 8. PCR products were digested with DdeI (NEB) and loaded onto 5% native polyacrylamide gels for detection.

2.4. Plasmid transfection

Transient transfection of cells was performed with polyethyleneimine (PEI) reagent. Cells were plated 24 h prior to transfection so that the density of cells reached ~50% on the day of transfection. A mixture that contains 0.5 µg SMN1 or SMN2 mini-gene plasmid and 0.5 µg PSF plasmid in the medium with PEI was applied to cells. 4 h later, media was replaced with fresh medium. Total RNA was extracted after 48 h transfection.

2.5. shRNA treatment

To produce shRNA lentivirus for the PSF gene, 0.5 µg of lentiviral pLKO.1 plasmids (openbiosystem) were co-transfected with 0.3 µg of PSPAX2 and PMD2G helper plasmids into 293T cells using PEI reagent. After incubation of cells for 12 h, growth media was changed to a fresh media. Another 24 h later, the supernatant containing the lentiviral particle were collected. To knockdown PSF expression, cells were incubated with media containing the lentivirus for 24 h and then in fresh media for another 48 h.

2.6. RNA pulldown assay

RNA pulldown assay was performed as previously described [45]. Streptavidin agarose beads (Upstate) that were washed three times with NETN buffer [100 mM NaCl, 200 mM Tris-Cl (pH8.0), 1 mM EDTA, 0.5% NP-40) supplemented with 200 mM PMSF and 0.5 mM DTT] were incubated with 5'-biotinylated RNA (Bioneer) for 60min at 4°C under rotation. The beads were then washed with NETN buffer to remove the unbound RNAs. Streptavidin beads with biotin labeled RNAs were incubated with HeLa nuclear extract by rotating at 4°C for 4 h. After washing the beads for seven times with NETN buffer, we added SDS-PAGE loading buffer to elute the RNA bound proteins. Samples were analyzed by western blotting using an anti-PSF and anti-myc antibody.

3. Results

3.1. Increased expression of PSF promotes exon 7 inclusion of the SMN2 mRNA

Because exon 7 of SMN2 pre-mRNA includes GAAGGA sequence, which resembles the PSF binding sites, we raised a possibility that PSF regulates SMN2 splicing. To determine whether PSF affects exon 7 splicing of SMN2 pre-mRNA, we first expressed PSF protein in 293A cells that were co-transfected with SMN2 minigenes. The first minigene we tested was the previously described SMN2-L [44], which contained exons 6-8, had a deletion of a large part of exon 8 and encompassed a GFP coding region as a reporter for the exon 7 inclusion (figure 1A) [44]. After 48 h transfection, we extracted RNA and then performed RT-PCR analysis for the exon 7 splicing of the RNA transcript from this minigene. The primers used were matched to the pCI-neo vector and the GFP coding region as shown by arrows in figure 1A. As expected, the SMN2-L minigene produced a predominant variant lacking the exon 7 (~98%) (figure 1A), which is consistent with our previous result and confirms that the minigene resembles nicely the endogenous SMN2 gene in SMA patients [44]. Figure 1B shows that increased expression of PSF significantly (~34%) promotes exon 7 inclusion into the SMN2 mRNA in the SMN2-L transfected 293A cells, compared with the control plasmid counterpart (figure 1B, lanes 1-3). To further explore whether the effect of PSF is

specific to 293A cells, we expressed the PSF plasmid in C33A and SH-SY5Y cells as, like the 293 cells, these two cell lines also express exclusively the exon 7 skipping form with a hardly-detectable level of the exon 7 containing variant. The results in figure 1B show that after expression of PSF, the exon 7 containing form is increased to ~7% and ~9% of the total mRNA levels in the C33A and SH-SY5Y cells respectively, demonstrating a universal effect of PSF on the splicing of the SMN2-L transcript. We next studied the effects of PSF on exon 7 splicing of the transcript from the SMN2-S minigene that contained a shorter intron 6 but the full length exon 8 (figure 1A). Figure 1C shows that PSF strongly promotes exon 7 inclusion into the SMN2 mRNA in 293A, C33A and SH-SY5Y cells (~62%, ~66% and ~60% independently). Collectively, these results lead us to a conclusion that PSF promotes exon 7 inclusion into the SMN2 mRNA, and that, although the effect are quantitatively different in two minigenes, the PSF effect is independent of the middle part of intron 6 and the downstream part of exon 8.

3.2. Increased expression of PSF promotes exon 7 inclusion of SMN1 pre-mRNA

Wondering whether the effects of PSF are related to the C to T base pair difference between the SMN2 gene and the SMN1 gene, we examined the effects of PSF on two SMN1 minigenes, SMM1-L and SMN1-S, which were constructed similarly to the SMN2-L and SMN2-S minigenes (figure 1A). SMN1-L minigene produced predominantly the exon 7 containing form in 293A, C33A and SH-SY5Y cells (figure 2A), thus recapitulating the exon 7 splicing of the endogenous SMN1 pre-mRNA. The results in figure 2A show that PSF significantly reduced the exon 7 skipped form, making the wild type form increased by ~22%, ~49% and ~16% of the total RNA level in 293A, C33A and SH-SY5Y cells. SMN1-S produces solely the exon 7 included form, making the SMN 7 form hardly detectable. Not surprisingly, figure 2B shows that the expression of PSF produces the exon 7 containing isoform exclusively. By combining the results of figures 1 and 2 together, we conclude that the effect of PSF on exon 7 splicing is not due to the point mutations in exon 7 of the SMN2 pre-mRNA.

3.3. Reduced expression of PSF promotes exon 7 skipping of SMN2 and SMN1 pre-mRNA

To further determine the effects of PSF on SMN splicing, we reduced the PSF expression by infection of the cells with viruses containing PSF shRNA. Because SMN2 exon 8 encompasses a DdeI restriction enzyme cleavage site but SMN1 lacks it (Figure 3A), RT-PCR products of the SMN1 and SMN2 mRNAs can be distinguished by their different sizes after DdeI cleavage. Our results revealed that the SMN1 mRNA is predominantly the exon 7 containing wild type form whereas the SMN2 mRNA is predominantly the exon 7 skipped form in the neuroblastoma SH-SY5Y cells (Figure 3B). Decreased expression of PSF by infection with shRNA-containing viruses increased the SMN1 exon 7 skipping form to ~54% of the total SMN1 RNA level from ~32% in neuroblastoma SH-SY5Y cells, (lanes 3, in Figure 3B). After the viral infection, the exon 7 included form is also significantly decreased in endogenous SMN2 pre-mRNA. form was also increased by ~8% of the total SMN2 mRNA level. These results confirm from the opposite angle that PSF promotes exon 7 inclusion in both SMN2 and SMN1 mRNAs.

3.4. PSF promotes exon 7 inclusion in SMA patient cells

To determine whether PSF also promote the exon 7 inclusion in the fibroblast cells from SMA patients, we ectopically expressed PSF in combination with the SMN2-L, SMN2-S, SMN1-L or SMN1-S minigene. Figure 4A showed that PSF significantly promoted the exon 7 containing form to ~39% and ~62% of the total SMN2 RNA level in the patient cells co-expressing the SMN2-L and SMN2-S minigenes, independently, which are consistent with the results in figure 1. As in non-patient cells, the co-expressed SMN1-L and SMN1-S minigenes produced solely the exon 7 containing form in the SMA patient cells (figure 4A, lanes 7 and 10), making the increase in the exon 7 containing form undetectable. After PSF expression, SMN2-L and SMN2-S minigenes produced solely the exon 7 containing form (figure 4A, lanes 3 and 6). Consistent with the results with minigenes, PSF overexpression increased 14% of the exon 7 inclusion form of the total endogenous SMN2 mRNA level (Figure 4B). We conclude that PSF promotes exon 7 inclusion in the SMN2 mRNA in SMA patient cells.

3.5. Deletion of an enhancer on exon 7 abolishes the effects of PSF

PSF was previously shown to promote or inhibit splicing by directly contacting RNA sequences [34, 36, 46]. For instance, the UGGAGAGGAAC sequence was shown to be contacted by PSF to promote splicing [36]. Sequence analysis indicated that a middle region of exon 7 contains a similar PSF contacting sequence (GAAGGA), which was previously shown to be an enhancer that can be targeted by tra2 β . To determine whether PSF also targets GAAGGA sequence on exon 7 to promote exon 7 splicing, we deleted GAAGGA from the exon 7 (referred as 7-3), with two other deletion mutants as controls, in which the upstream AAAAUC (7-1) or AAAAA (7-2) sequence were deleted (figure 5A). Figure 5B shows that 7-3 mutant produces exon 7 skipped form exclusively, whereas the increase of mRNA with exon 7 inclusion in the presence of PSF was barely detectable (lane 12). In contrast, splicing of exon 7 in the mutant 7-1 was still highly regulated by PSF (lane 6). It should be pointed out that the control plasmid did not promote exon 7 splicing either from 7-3 or from 7-1 (lanes 5). Interestingly, we observed that the mutant 7-2 minigene produced exon 7 retaining form exclusively (lane 7) regardless if PSF was expressed (lane 9). Collectively, these results strongly suggest that the GAAGGA sequence is the potential target of PSF in the exon 7 splicing of SMN2 pre-mRNA.

3.6. PSF contacts GAAGGA sequence on exon 7 to promote exon 7 inclusion

To further validate the target sequence of PSF on exon 7, we mutated the GAAGGA sequence into GAAUUA (referred to as UUA). As shown in figure 6A, the tra2 β protein still promoted exon 7 inclusion in mutant UUA to a significant level (lane 4), consistent with a previous report that GAA of the GAAGGA sequence was sufficient for the tra2 β protein to regulate splicing of SMN exon 7 [47]. In contrast, the exon 7 skipped form was exclusively expressed in the UUA mutant even when PSF (lane 3) is expressed. Thus the effect of PSF to promote exon 7 inclusion is abolished in the UUA mutant.

To determine whether PSF directly contacts the GAAGGA sequence in the exon 7 to promote its inclusion, we chemically synthesized 5' biotinylated AAGAAGGAAG and AAGAAUUAAAG RNA sequences. The wild type (GAAGGA) and the UUA mutant

(GAAUUA) sequences of the potential PSF binding sites were flanked with 2nt at both ends. We incubated biotin-labeled RNAs with HeLa nuclear extract. RNA-protein complexes were subsequently pulled-down with streptavidin beads. Presence of PSF in the complexes was examined by immunoblotting analysis with an anti-PSF antibody. The results in figure 6B show that PSF directly contacts the wild type RNA (lane 3), but not the UUA mutant RNA. Therefore, we conclude that PSF promotes exon 7 inclusion into the SMN2 mRNA through contacting the GAAGGA sequence in the exon 7.

4. Discussion

Spinal muscular atrophy is a devastating disease that kills thousands of children each year. But there is no effective treatment. There are two SMN genes in humans. SMN1 gene is deleted or mutated in SMA patients. SMN2 which has only a few nucleotide differences from SMN1 is still present in SMA patients. However, exon 7 is mostly spliced out from SMN2 mRNA due to one nucleotide difference on exon 7 between SMN1 and SMN2, producing the SMN⁷ protein. Consequently, presence of SMN2 allows the organism to survive and the low levels of SMN result in SMA. Extensive studies have been conducted in order to stimulate inclusion of exon 7 into SMN2 mRNA so that more full length functional SMN protein can be produced for the treatment of SMA disease. Great strides have been made during the last 10 years and several potential drugs are currently considered for or already under clinical trials. However, in order to better design drugs for SMA treatment by targeting exon 7 splicing, it is important to completely understand the mechanisms of exon 7 splicing.

In this study, we have investigated the basis by which SMN2 pre-mRNA splicing is regulated. We have shown that the increased expression of PSF promotes exon 7 inclusion in both SMN2 and SMN1 mRNA. Conversely, the reduced expression of PSF facilitates exon 7 skipping in SMN2 and SMN1 mRNA. The effects of PSF on the exon 7 splicing of SMN2 and SMN1 pre-mRNA are consistent in the fibroblast cells from SMA patients as well as other cells. We also show that PSF targets the purine rich sequence on exon 7. Indeed, abrogating the PSF binding by mutating the purine rich sequence on exon 7 inactivated the effects of PSF on exon 7 splicing in SMN2 pre-mRNA. Furthermore we demonstrate that the RNA target sequence of PSF and tra2 β are partially overlapped. The results that exon 7 splicing of both SMN2 and SMN1 is promoted by PSF indicate that the effects of PSF are not related to the nucleotide sequence differences between SMN1 and SMN2.

PSF has been reported to promote splicing and early spliceosome assembly in the in vitro analysis using immunodepletion and adding-back experiments [30, 32]. The basis by which PSF regulate splicing has remained elusive, and it is possible that various mechanisms are involved. In some cases PSF may recognize RNA secondary structure. For instance, PSF contacts the stem-loop structure at the 5' splice site downstream of tau exon 10, regulating tau splicing. In other cases, PSF is recruited to the exon splicing silencer complex to inhibit exon inclusion, indicating that PSF forms a large RNA-protein complex. PSF can also regulate alternative splicing through direct phosphorylation by GSK3 [34, 35, 46]. Whereas various RNA structures were recognized by PSF for its activity, we have shown in this

report that PSF contacts purine rich sequence on exon 7 of SMN genes to promote exon 7 inclusion of SMN2 pre-mRNA. The results are consistent with the previous reports that PSF contacts purine rich sequence on U5 snRNA [36].

It was previously shown that the enhancer on exon 7 was recognized by tra2 β protein to promote exon 7 inclusion of SMN2 pre-mRNA. We demonstrated in this study that this enhancer is also targeted by PSF. While our results demonstrate that PSF and tra2 β protein target partially overlapped RNA sequences on the enhancer, how one enhancer is recognized by multiple proteins remains to be elucidated. One possibility is that the enhancer complex that includes PSF and tra2 β is formed on the enhancer of exon 7 to promote spliceosome assembly. It is also possible that PSF and tra2 β contact the enhancer independently to facilitate exon 7 splicing. The dynamics of these two proteins to contact short RNA sequences with the similar effects needs to be determined in the future.

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Highlights

- PSF promotes exon 7 inclusion of SMN2 and SMN1 pre-mRNA.
- PSF contacts exon 7 to regulate SMN2 splicing.
- PSF and tra2 β contact overlapped sequence on exon 7 to promotes exon 7 inclusion.

Figure 1.

Increased PSF expression promotes exon 7 inclusion of SMN2 pre-mRNA. (A) The outlines of SMN2-L and SMN2-S minigenes are shown. Exons are shown as boxes and introns as lines. The primers used in the RT-PCR analysis are shown with arrows. Vector sequences are shown with dot lines. (B) RT-PCR analysis of exon 7 splicing of SMN2-L minigene was conducted in untreated 293A, C33A and SH-SY5Y cells or cells treated with pcDNA 3.1(+) plasmid or PSF expression plasmid. GAPDH was used as a loading control. Quantitation results are shown at the lower panel. Immunoblotting analysis with anti-Myc antibody was shown to demonstrate the ectopic expression of PSF, with an anti- α -tubulin antibody as controls.

(C) RT-PCR analysis of exon 7 splicing of SMN2-S minigene was conducted in untreated 293A, C33A and SH-SY5Y cells or cells treated with pcDNA3.1(+) plasmid or PSF expression plasmid. Quantitation of RT-PCR results is shown.

Figure 2.

Increased PSF expression promotes exon7 inclusion of SMN1 pre-mRNA. (A) RT-PCR analysis of exon 7 splicing of SMN1-L minigene was carried out in untreated 293A, C33A and SH-SY5Y cells or cells treated with pcDNA3.1(+) plasmid or PSF expression plasmid. GAPDH was used as a loading control. Quantitation results are shown at the lower panel. (B) RT-PCR analysis of exon 7 splicing of SMN1-S minigene was carried out in untreated 293A, C33A and SH-SY5Y cells or cells treated with pcDNA3.1(+) plasmid or PSF expression plasmid. Quantitation of RT-PCR results is shown.

Figure 3.

Reduced PSF expression promotes exon 7 skipping of SMN2 and SMN1 pre-mRNA. (A) Schematic outline of the strategy to separate the RT-PCR products of SMN1 and SMN2 pre-mRNA. The RT-PCR product of SMN2 pre-mRNA but not SMN1 pre-mRNA can be digested by DdeI at exon 8, producing shorter DNA fragments on agarose gels. The primers for PCR are shown as arrows. (B) RT-PCR analysis of Exon 7 inclusion and skipping of endogenous SMN1 and SMN2 pre-mRNAs was carried out with RNAs from untreated SH-SY5Y cells or cells treated with non-silencing shRNA virus or PSF shRNA virus. Reduced expression of PSF is demonstrated by RT-PCR and immunoblotting analysis. GAPDH and α -tubulin are the loading controls. Quantitation results are shown at the lower panel.

Figure 4.

PSF promotes exon 7 inclusion in SMA patient cells. (A) RT-PCR analysis of exon 7 splicing of minigenes was conducted in SMA patient cells that were transfected with SMN2-L, SMN2-S, SMN1-L and SMN1-S minigenes along with pcDNA3.1(+) plasmid, PSF plasmid or no control plasmid (-). GAPDH was used as the loading control. Quantitation results are shown at the lower panel. (B) RT-PCR analysis of exon 7 splicing from endogenous SMN2 pre-mRNA was conducted in SMA patient cells that are PSF overexpressed. Ectopic expression of PSF was indicated with anti-myc antibody, with α -tubulin as the loading control. Quantitation of exon 7 splicing in endogenous SMN2 pre-mRNA is shown in the lower panel.

Figure 5. Deletion of an enhancer on exon 7 abolishes the effects of PSF. (A) The RNA sequences of wild type exon 7; deletion mutant 7-1, 7-2 and 7-3 are shown. Deletions are indicated by dashes (-). (B) RT-PCR analysis of exon 7 splicing was conducted in cells that express wild type exon 7, mutant 7-1, 7-2 and 7-3 SMN2-S minigenes with co-transfection of either PSF, pcDNA3.1(+) plasmid. Quantitation results are shown at the lower panel.

Figure 6. PSF contacts GAAGGA sequence on exon 7 to promote exon 7 inclusion. (A) The RNA sequences of wild type and UUA mutant exon 7 are shown. The mutated sequences are underlined. RT-PCR analysis of exon 7 splicing was conducted in cells that express the UUA mutant SMN2-S minigene with PSF, Tra2β or pcDNA3.1(+) vector plasmid. Quantitation results are shown at the lower panel. (B) Interaction between PSF and exon 7 RNA sequences. Shown in the top panel are the sequences of biotin labeled wild type and UUA mutant RNAs. Biotin-labeled RNAs were incubated with HeLa nuclear extract. RNA-protein complexes were pulled down with streptavidin bead. PSF was examined by immunoblotting with anti-PSF antibody.

Table 1

List of primers.

Name	Sequences
SMNE6(B).F	5'-TACTCGGATCCATAATTCCCCACCACCTCC-3'
SMNI6(D).R	5'-GACCTCTAATCCCAGCTACGACAGGCGTGGTGGCAG-3'
SMNI6(D).F	5'-CTGCCACCACGCCTGTCGTAGCTGGGATTAGAGGTC-3'
SMNE8(X).R	5'-CTAACCTCGAGAACAGTACAATGAACAGCCATG-3'
E7-1.F	5'-ACAGGGTTTTAGACAAAAAGAAGGAAGG-3'
E7-1.R	5'-CCTTCCTTCTTTTGTCTAAAACCTGT-3'
E7-2.F	5'-TTTAGACAAAATCGAAGGAAGGTGCTC-3'
E7-2.R	5'-GAGCACCTTCCTTCGATTTTGTCTAAAA-3'
E7-3.F	5'-GACAAAATCAAAAAAGGTGCTCACATTC-3'
E7-3.R	5'-GAATGTGAGCACCTTTTTTGATTTTGTC-3'
E7-UCC.F	5'-GACAAAATCAAAAAAGAATCCAGGTGCTCACATTC-3'
E7-UCC.R	5'-GAATGTGAGCACCTGGATTCTTTTGATTTTGTC-3
Ex5.F	5'-CTATCATGCTGGCTGCCT-3'
Ex8.R	5'-CTACAACACCCTTCTCACAG-3'
pcI.R	5'-GCTAACGCAGTCAGTGCTTC-3'
SMNE6.F	5'-CTGAAGCACTGCACGCCGTAC-3'
pcDNA.R	5'-CTAGAAGGCACAGTCGAGGCT-3'
PSF.F	5'-AAGGCAAAGGATTCGGATT-3'
PSF.R	5'-TGGCTAAAGGCTTCTTCCAA-3'
GAPDH.F	5'-ACCACAGTCCATGCCATCA-3'
GAPDH.R	5'-TCCACCACCCTGTTGCTGTA-3'