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Polyadenylation Site-specific Differences in the Activity of the Neuronal βCstF-64 Protein in PC-12 Cells

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Abstract

Recent genome-wide analyses have implicated alternative polyadenylation — the process of regulated mRNA 3′ end formation — as a critical mechanism that influences multiple steps of mRNA metabolism in addition to increasing the protein-coding capacity of the genome. Although the functional consequences of alternative polyadenylation are well known, protein factors that regulate this process are poorly characterized. Previously, we described an evolutionarily conserved family of neuronal splice variants of the CstF-64 mRNA, βCstF-64, that we hypothesized to function in alternative polyadenylation in the nervous system. In the present study, we show that βCstF-64 mRNA and protein expression increase in response to nerve growth factor (NGF), concomitant with differentiation of adrenal PC-12 cells into a neuronal phenotype, suggesting a role for βCstF-64 in neuronal gene expression. Using PC-12 cells as model, we show that βCstF-64 is a bona fide polyadenylation protein, as evidenced by its association with the CstF complex, and by its ability to stimulate polyadenylation of luciferase reporter mRNA. Using luciferase assays, we show that βCstF-64 stimulates polyadenylation equivalently at the two weak poly(A) sites of the β-adducin mRNA. Notably, we demonstrate that the activity of βCstF-64 is less than CstF-64 on a strong polyadenylation signal, suggesting polyadenylation site-specific differences in the activity of the βCstF-64 protein. Our data address the polyadenylation functions of βCstF-64 for the first time, and provide initial insights into the mechanism of alternative poly(A) site selection in the nervous system.

Keywords

alternative polyadenylation; alternative splicing; neuronal gene expression; CstF-64; β-adducin

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Conflict of interest
The authors declare no conflicts of interest.

1. Introduction

Nuclear polyadenylation is the co-transcriptional process that adds a poly(A) tail to the 3′ ends of most mRNAs [1,2,3]. Alternative polyadenylation — polyadenylation at sites that change either the coding region or the 3′ untranslated region of mRNAs [4,5] — is important for diverse gene expression in most tissues [6], notably in brain and neural tissues [7,8,9,10,11]. Additionally, crosstalk between splicing and polyadenylation contributes to brain mRNA isoform diversity [12], suggesting interacting roles.

The CstF-64 polyadenylation protein (gene symbol *CSTF2*) is the 64,000 M_r RNA-binding component of the cleavage stimulation factor (CstF) that binds to the downstream sequence element (DSE) of the pre-mRNA prior to cleavage and polyadenylation [1,2,13]. Many studies have shown that CstF-64 plays a central role in mRNA 3′ end formation and hence gene expression [3,14]. As potential participants in control of alternative polyadenylation in brain, we discovered a family of splice variants of the CstF-64 polyadenylation mRNA between exons 8 and 9 (Figure 1A). Because they all arise in the same region of the gene, we called these variants collectively “βCstF-64” [15]. The βCstF-64 splice isoforms result from inclusion of one or both (usually both) tissue-specific exons in CstF-64 between exons 8 and 9. When both exons are included, a new 49 amino acid domain is included within the proline- and glycine-rich domain of CstF-64 that we call the “beta domain.” The beta domain is expressed exclusively in brain and peripheral neurons, and is conserved in all vertebrates. These features suggested that βCstF-64 has an ancient and important function in vertebrate neuronal function.

Another variant of CstF-64, τCstF-64 (gene symbol *CSTF2T*) is also expressed in mammalian brain as well as in testis [16,17,18]. Thus, at least three isoforms of CstF-64 are expressed in brain: two splice variants from *CSTF2* on the X chromosome (CstF-64 and βCstF-64), and τCstF-64 from a paralogous gene (*CSTF2T*) on an autosome [6,15]. This supports a strong role for various forms of CstF-64 in regulation of neuronal gene expression.

In characterizing βCstF-64, we found that it was expressed in neuronal-like cell lines including rat adrenal medulla pheochromocytoma PC-12 cells [15]. βCstF-64 mRNA expression increased in PC-12 cells after treatment with nerve growth factor (NGF) in reduced serum, attendant with differentiation of these cells into a neuronal phenotype [19]. Because βCstF-64 levels increase during PC-12 cell differentiation into the neuronal phenotype, we chose to examine its functions in undifferentiated and differentiated PC-12 cells. Here, we demonstrate that ectopically expressed βCstF-64 participates in CstF-mediated polyadenylation in PC-12 cells and show that the activity of βCstF-64 is distinct from CstF-64 in a polyadenylation-site specific manner. Using luciferase assays, we show that βCstF-64 enhances the activity of constructs containing polyadenylation signals from β-adducin (gene symbol *Add2*), a neuronally-expressed cytoskeletal gene that exhibits multiple sites of polyadenylation [20,21]. In these assays, βCstF-64 supported polyadenylation activity equivalently at the two polyadenylation sites of the β-adducin mRNA, as did CstF-64. Interestingly, the level at which βCstF-64 supported polyadenylation was less than CstF-64 on a strong polyadenylation signal, suggesting that the beta domain reduced polyadenylation efficiency and that the activity of βCstF-64 is different than CstF-64 on these sites. Taken together, data presented in this study indicate that βCstF-64 modulates mRNA polyadenylation in a site-specific manner, thus providing a mechanism of regulated alternative poly(A) site selection in the nervous system.

2. Materials and methods

2.1 Cell Culture and Transfection

PC-12 cells (ATCC, CRL-1721 from *Rattus norvegicus*) were grown at 37°C in 5% CO_2 in Dulbecco's Minimal Eagle Media (DMEM; CellGro, Manassas, VA) containing 10% equine serum (Hyclone, Logan, UT), 5% cosmic calf serum (Hyclone, Logan, UT), and 1% penicillin/streptomycin (Gibco, Carlsbad, CA). For NGF treatment, PC-12 cells (3×10^6 cells) were plated on 14 cm plastic dishes (Nunc, Rochester, NY) coated with 50 μg/mL poly-D-lysine (Sigma, St. Louis, MO) and cultured in 20 mL DMEM containing 2% serum for 3 hours. After 3 hours, nerve growth factor (NGF, Promega, Madison, WI) was added to a final concentration of 50 ng/mL. For culturing PC-12 cells in 2% serum-containing DMEM, cells (6×10^6) were seeded on 14 cm plastic dishes (Nunc) coated with 100 μg/mL poly-D-lysine (Sigma). As an alternative protocol for NGF treatment, PC-12 cells (3×10^6 cells) were plated on 14 cm plastic dishes (Nunc) coated with 50 μg/mL poly-D-lysine (Sigma) and cultured in 20 mL DMEM containing 15% serum. All transfections in PC-12 cells were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at 3 μL/μg of transfected DNA. Cells were harvested 48 hours post-transfection by rinsing with ice-cold PBS, and lysed in SDS loading buffer.

2.2 Antibodies

The following antibodies were used in this study: anti-α-tubulin and anti-FLAG antibodies (Sigma, St. Louis, MO); mouse monoclonal anti-CstF-64 (3A7, [17]); rabbit anti-βCstF-64 [15]; mouse monoclonal anti-CstF-77 (Abnova, Walnut, CA, catalog #H00001479-M01); and rabbit polyclonal anti-CstF-77 (Bethyl, Montgomery, TX, catalog #A301-096A).

2.3 Animal Studies

Animal experiments were performed at Texas Tech University Health Sciences Center in accordance with protocols that were approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee, and following NIH guidelines.

2.4 RNA Analysis and RT-PCR

Total RNA from mouse brain, rat brain, rat spleen, undifferentiated and NGF-differentiated PC-12 cells, at the indicated time points were extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA), and treated with TurboDNase (Ambion). Equal amounts of DNased RNA (4 μg) from each tissue sample were used to synthesize cDNA using SuperScriptII Reverse Transcriptase and oligo(dT) according to the manufacturer's protocol (Invitrogen). Polymerase chain reaction (PCR, 30 cycles) was conducted using the indicated primers (see Table 1) in an Air Thermocycler (Idaho Technologies, Salt Lake City, UT). Amplicons were resolved by electrophoresis on a 1% agarose gel. Ethidium bromide stained RT-PCR products were excised from the gel (Qiagen, Valencia, CA), cloned using Topo II system (Invitrogen), and identified by DNA sequence analysis.

2.5 3′ RACE analysis

For 3′ RACE, total RNAs from rat brain, rat spleen, undifferentiated and NGF-differentiated PC-12 cells were extracted and treated with DNase as described [15]. DNA-free RNA (1 μg) was used to synthesize cDNA with the SMART RACE cDNA amplification kit (BD Biosciences, Mountainview, CA). The cDNA was diluted with 100 μL Tricine-EDTA buffer and 2 μL of the diluted cDNA used for the first round of PCR. The first round of PCR (30 cycles) was conducted using Gene-Specific Primer (GSP; see Table 1) and Universal Primer Mix A in an Air Thermocycler (Idaho Technologies). The PCR reaction from the first round was diluted 1:10 in ddH_2O and 2 μL was used for the second PCR reaction. The second

round of PCR (30 cycles) was conducted using Nested Gene-Specific Primer (NGSP; Table 1) and Nested Universal Primer Mix in an Air Thermocycler (Idaho Technologies). The PCR products were resolved and sequenced.

2.6 Cloning and Plasmids

The plasmids 3×FLAG-MS2, 3×FLAG-MS2-CstF-64, and 3×FLAG-MS2-βCstF-64 were described in Shankarling, et al. [15]. The plasmids SV40-SL-Luc and SV40-SL-Luc (AGGAGA) were from Hockert, et al. [14,22]. The plasmid SL-Luc_{pA1} was created as follows: a 350 nt RACE product corresponding to the pA1 site of β-adducin mRNA was cloned into TOPOII vector (Invitrogen) to generate pA1-TopoII plasmid. A 150 nt region flanked by EcoRI and MfeI sites was amplified from the pA1-TopoII plasmid using primers pA1F and pA1R (see Table 1) and the PCR product digested with EcoRI and MfeI restriction enzymes. The digested PCR product was gel purified (Qiagen, Valencia, CA) and ligated to similarly digested SV40-SL-Luc plasmid to give SL-Luc_{pA1}. The plasmid SL-Luc_{pA4} was created as follows: A double stranded oligonucleotide (6 ng) containing 150 nucleotides corresponding to the β-adducin pA4 upstream polyadenylation region flanked by EcoRI and HpaI cohesive ends (IDT, Coralville, IA) was ligated to a similarly digested SV40-SL-Luc plasmid (20 ng) to give SL-Luc_{pA4}. The sequence of the two single stranded oligonucleotides, pA4HpaIUltraF and pA4HpaIUltraR, used to generate the double stranded oligonucleotide are shown in Table 1. The plasmids pA1FULL-Luc and pA4FULL-Luc were created as follows: the MS2 loops from the pA1-SL-Luc and pA4-SL-Luc plasmids were excised by digestion with MfeI and HindIII restriction enzymes and the digested plasmid was gel purified (Qiagen). A double stranded oligonucleotide containing 120 nucleotides corresponding to the downstream U/GU-rich sequences of pA1 or pA4 sites of β-adducin mRNA was synthesized (IDT). The sequence of the single stranded oligonucleotides (pA1FULLUltraF, pA1FULLUltraR, pA4FULLUltraF, and pA4FULLUltraR) used to generate the double stranded oligonucleotides is shown in Table 1. The double stranded oligonucleotides containing cohesive MfeI and HindIII sites were ligated into similarly digested pA1-SL-Luc or pA4-SL-Luc plasmids to give pA1FULL-Luc or pA4FULL-Luc plasmids.

2.7 Protein Analysis

For protein analysis, whole cell extract was prepared from undifferentiated, NGF-differentiated, and from transiently transfected PC-12 cells and HeLa cells by rinsing cells twice with ice-cold PBS, lysed and sonicated in SDS-PAGE loading buffer. Preparation of mouse nuclear extract has been described before [15]. Protein concentration was measured using the bicinchoninic acid (Pierce, Rockford, IL) assay. Equal amounts of protein (30 μg) were resolved by 10% SDS-PAGE, and transferred to nitrocellulose membranes for immunoblotting. For immunoblotting using anti-CstF-64 antibody, membranes were blocked with Tris-buffered saline containing 0.2% Tween-20 (TBST) with 2% nonfat dry milk (TBST) for 2 hours and treated with anti-CstF-64 antibody at a dilution of 1:50. The anti-α-tubulin, anti-FLAG, anti-CstF-77 (monoclonal), and anti-CstF-77 (polyclonal) antibodies were used at dilutions of 1:5000, 1:6000, 1:200, and 1:1000 respectively. Membranes were subsequently treated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Pierce, Rockford, IL) at a dilution of 1:25000 and immunoreactive bands were visualized by chemiluminescence using the Pierce SuperSignal kit (Rockford, IL). All antibody dilutions were conducted in 2% nonfat dry milk in TBST.

2.8 Stem Loop Luciferase Assay for Polyadenylation (SLAP)

PC-12 cells were plated on 24 well plates at 200×10^3 per well and after 24 hours were transfected with the following plasmids: 8 ng of pGL3-control plasmid (Firefly luciferase plasmid, Promega), 48 ng of Renilla luciferase plasmid (SL-Luc_{pA1}, SL-Luc_{pA4}, SV40-SL-

Luc, and SV40-SL-LucAGGAGA), and 500 ng of either 3×FLAG-MS2 (denoted "reporter alone"), 3×FLAG-MS2-CstF-64 or 3×FLAG-MS2-βCstF-64 plasmids. Cells were harvested 48 hours post-transfection. Cell lysis was conducted using 1× Passive Lysis Buffer (Promega) and lysates subjected to Dual Luciferase Assay (Promega) using a Turner 20 luminometer according to the manufacturer's instructions. Each experimental plasmid (reporter alone, 3×FLAG-MS2-CstF-64 or βCstF-64) was transfected in triplicates and each triplicate repeated five times (for a total of 15 replicates per experimental plasmid). The relative luciferase values (ratio of Renilla to Firefly values) were an average of 15 replicates. Lysates from all 15 replicates for each experimental plasmid were pooled and denatured in SDS loading buffer. Equal volume (20 μL) from samples were resolved by SDS-PAGE and subjected to immunoblot analysis using anti-FLAG antibody to determine protein expression. Statistical analysis including ANOVA was conducted using InStat program (Graphpad Software Inc., Sandiego, CA). Luciferase assays using the pA1FULL-Luc or pA4FULL-luc plasmids were also conducted as described above.

2.9 Immunoprecipitation

PC-12 cells (6×10^6) were plated on 10 cm dishes coated with 50 μg/mL poly-D-Lysine and transfected next day with 10 μg of either 3×FLAG-MS2, 3×FLAG-MS2-CstF-64 or 3×FLAG-MS2-βCstF-64 encoding plasmids using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours post-transfection. For co-immunoprecipitation analysis, cells were washed with ice-cold phosphate buffered saline (PBS), lysed with 1mL hypotonic gentle lysis buffer (10mM Tris-HCl, 10mM NaCl, 2mM EDTA, 0.5% Triton-X-100), incubated on ice for 10 minutes, followed by addition of NaCl to 150mM. Cell lysates were centrifuged at 16,000g for 15 minutes. An aliquot of the supernatant (5% of the total volume) was used as "Total extract" control while the remaining was added to anti-FLAG antibody coupled to agarose resin and incubated overnight at 4°C. For co-immunoprecipitation analysis using anti-CstF-77 antibody (Bethyl), anti-CstF-77 antibody (3 μg/mg cell lysate) or control rabbit IgG was added to the cell lysate followed by addition of 100 μL of 20% Protein A-Sepharose suspension and incubated overnight at 4°C. Following overnight incubation, an aliquot of the supernatant (5% of the total volume) was used to analyze "unbound" proteins. The beads were washed extensively with NET2 buffer (50mM Tris, 150mM NaCl, 0.05% Triton-X-100) and "bound" proteins eluted by boiling in SDS buffer. The eluted proteins (50% of eluted proteins) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analysis using anti-FLAG or anti-CstF-77 antibodies.

2.10 Densitometry analysis

Densitometry analysis of RT-PCR and immunoblot images was performed using Image J analysis software (NIH).

3. Results

3.1 βCstF-64 expression increases in NGF-treated PC-12 cells

Previous research showed that βCstF-64 was expressed in several neuronal cell lines, and that its expression was induced in PC-12 cells treated with NGF and reduced serum [15]. In order to confirm that increased βCstF-64 expression was due to NGF treatment and not due to the serum reduction, PC-12 cells were cultured in DMEM with either 15% or 2% fetal bovine serum, either in the presence or absence of NGF (Figure 1B). Using primers that recognize exons 7–11 of rat *Cstf2* (primer pair C), both CstF-64 and low levels of βCstF-64 mRNA were detected in undifferentiated PC-12 cells cultured in 15% serum (Figure 1B, lane 1). Low levels of the alternatively spliced α-CstF-64 isoform [15] were detected as well (arrowhead). There was no increase in βCstF-64 mRNA levels in PC-12 cells grown in 2%

serum-containing medium (lane 2). However, upon treatment with NGF for 96 hours, βCstF-64 mRNA expression increased in cells grown in 2% serum-containing medium (lane 4), and in NGF-differentiated PC-12 cells grown in 15% serum-containing medium (lane 3). Densitometry analysis using Image J software indicated that the percentage of the isoform containing the βCstF-64-specific exons increased from ~19% in undifferentiated cells to ~94% in NGF-differentiated cells.

Similarly, we examined βCstF-64 protein expression in uninduced and NGF-differentiated PC-12 cells using an anti-βCstF-64 antibody (Figure 1C). Consistent with the increase in βCstF-64 mRNA expression, βCstF-64 protein expression increased in NGF-differentiated PC-12 cells grown in 2% serum-containing medium (lane 4) and in NGF-differentiated PC-12 cells grown in 15% serum-containing medium (lane 3), but not in PC-12 cells grown in 15% serum-containing medium lacking NGF (lane 1) or in 2% serum-containing medium lacking NGF (lane 2). Densitometry indicated that βCstF-64 protein levels increased 2.5 fold in NGF-treated PC-12 cells as compared to undifferentiated cells (normalized to actin expression). These experiments demonstrate that induction of βCstF-64 expression in PC-12 cells was due to NGF-stimulation and not due to serum withdrawal.

3.2 βCstF-64 expression in PC-12 cells increases in NGF-treated cells for up to four days

To investigate the time course of βCstF-64 induction, PC-12 cells were treated with NGF and RNA and protein isolated at 1, 2, 3 and 4 days after treatment. RT-PCR using primer pair C showed that the βCstF-64-specific band increased in intensity relative to the CstF-64 band starting at day 2 through day 4 post NGF treatment (Figure 1D, lanes 3–5). βCstF-64 protein expression showed a similar pattern (Figure 1E, top panel). CstF-64 and tubulin protein levels remained relatively unchanged over the same course (Figure 1E, middle and bottom panels). Densitometry indicated that the percentage of the isoform containing the βCstF-64-specific exons increased from ~50% in undifferentiated cells to ~90% in NGF-differentiated cells, while βCstF-64 protein levels increased ~3 fold in in NGF-treated PC-12 cells as compared to undifferentiated cells (normalized to actin expression). Note that the anti-CstF-64 antibody does not distinguish CstF-64 from βCstF-64 under these conditions [15].

3.3 Both CstF-64 and βCstF-64 proteins interact with CstF-77 in PC-12 cells

Recent studies have brought into question whether CstF-64 is involved in other processes in addition to mRNA polyadenylation [23]. Therefore, to test whether βCstF-64 was involved in polyadenylation, we investigated whether it interacted with another member of the polyadenylation complex, CstF-77 [24]. Unfortunately, the anti-βCstF-64 antibody was not suitable for immunoprecipitation (not shown). Therefore, we transfected 3×FLAG, 3×FLAG-CstF-64 or 3×FLAG-βCstF-64 expression constructs into PC-12 cells and performed co-immunoprecipitation analysis using the anti-FLAG antibody (Figure 2). Immunoprecipitation from cells transfected with the 3×FLAG construct (Figure 2A, upper panel, lanes 1–3) did not result in detectable CstF-77 in the bound fraction (Figure 2A, lower panel, lane 2), but substantial CstF-77 was detected in the unbound fraction (lane 3), demonstrating that endogenous CstF-77 did not interact non-specifically with the 3×FLAG moiety or the anti-FLAG agarose beads. Similarly, immunoprecipitation from cells transfected with either 3×FLAG-CstF-64 or 3×FLAG-βCstF-64 expression constructs showed that endogenous CstF-77 was associated with both 3×FLAG-CstF-64 and 3×FLAG-βCstF-64 proteins (Figure 2A, lower panel, lanes 5 and 8). Note that the transfection efficiency in PC-12 cells is approximately 10–20% (data not shown) and hence only a fraction of the endogenous CstF-77 is associated with the transfected 3×FLAG-CstF-64 and 3×FLAG-βCstF-64 proteins (lower panel, lanes 6 and 9). Identical results were obtained using HeLa cells (data not shown). These experiments suggested that βCstF-64 interacts

with CstF-77 as part of the CstF complex, as does CstF-64, thus suggesting that βCstF-64 could function in polyadenylation.

As further evidence that βCstF-64 behaved as a component of the polyadenylation machinery, we examined its cellular localization (Figure 2 B, C). Earlier studies showed that CstF-64 localization to the nucleus required direct interaction with CstF-77 [14,25]. Similar localization of FLAG-tagged βCstF-64 implies a similar interaction, further implying that βCstF-64 is part of the CstF complex.

3.4 Exogenously expressed βCstF-64 is less active in the stem-loop luciferase assay for polyadenylation (SLAP) than CstF-64

We developed a transfection-based assay to examine CstF-64 function in polyadenylation that we called the stem-loop luciferase assay for polyadenylation (SLAP); this assay correlates mRNA 3′ end formation with relative luciferase activity [14,22]. To further test whether βCstF-64 could function in polyadenylation, we used SLAP to compare MS2-CstF-64 with MS2-βCstF-64 in support of polyadenylation of the luciferase reporter in PC-12 cells (Figure 3). The SLAP method uses a luciferase reporter gene containing the strong polyadenylation signal from the SV-40 late transcript in which the DSE has been replaced with two copies of the stem-loop element from the MS2 bacteriophage coat-protein mRNA (SL-Luc, Figure 3B). Similarly, we created a modified CstF-64 protein that has the MS2 RNA-binding domain cloned in-frame (MS2-CstF-64, and MS2-βCstF-64, Figure 3A). Because these modifications couple SL-Luc expression to the requirement for the MS2-CstF-64 protein, we can use SLAP to examine how changes to the MS2-CstF-64 component result in changes to luciferase expression [14].

Undifferentiated PC-12 cells were transfected with the SL-Luc reporter (Figure 3C, bar 1), or with SL-Luc co-transfected with MS2-CstF-64 (bar 2) or MS2-βCstF-64 (bar 3). To account for transfection differences, relative luciferase unit (RLU) values are further normalized to MS2-CstF-64 protein expression by immunoblotting using an antibody that recognizes the FLAG epitope in each construct [14]. As expected, SL-Luc alone resulted in low normalized relative luciferase activity (bar 1), but co-transfection with MS2-CstF-64 resulted in greater activity (bar 2). We determined that the measurable value for RLU from the SL-Luc vector alone (bar 1) was due to endogenous polyadenylation activity, because mutation of the polyadenylation signal from AAUAAA to AGGAGA decreased that background activity more than 17.8-fold (bar 4).

The normalized RLU values obtained from cells transfected with MS2-βCstF-64 (bar 3) were 33% less than those obtained by transfection with MS2-CstF-64 construct (bar 2). These data suggest that βCstF-64 is able to support polyadenylation as does CstF-64, probably via cooperative interactions with other polyadenylation proteins that bind to the polyadenylation region. It further suggests that the beta domain in βCstF-64 reduces CstF-64 activity in polyadenylation in undifferentiated PC-12 cells.

3.5 βCstF-64 supports polyadenylation of brain-specific sites to the same extent as CstF-64

The SLAP system is based on the SV40 late polyadenylation signal that is typically described as a “strong” site [26]. Because strong sites might mask subtle differences in efficiency, we asked whether differences between βCstF-64 and CstF-64 might be detected using weaker, brain-specific polyadenylation sites. For this assessment, we chose two polyadenylation sites from the β-adducin gene (rat gene symbol: *Add2*), a cytoskeletal protein that is expressed mainly in brain and hematopoietic tissues [27]. *Add2* uses three polyadenylation sites in rat tissues, pA1, pA2–3 (which is less well conserved between

rodents and humans), and pA4 [20,21]. We characterized the proximal (pA1) and distal (pA4) sites (Figure 4A). Using 3′ RACE, we determined that the pA1 site was expressed in PC-12 cells, as well as in brain and spleen (Figure 4B, top panel). The pA4 site, in contrast, was expressed only in PC-12 cells and brain, but not in spleen (Figure 4B, bottom panel). Both pA1 and pA4 supported expression of luciferase when cloned in place of the polyadenylation signal, with pA1 being one-eighth as efficient as pA4 (Figure 4C, D). This suggested that the *Add2* polyadenylation signals might respond differentially to βCstF-64 in a polyadenylation assay.

The pA1 and pA4 upstream and polyadenylation regions are different; for example, the polyadenylation signal upstream of pA1 is AUUAAA and upstream of pA4 is AGUAAA [20]. In order to eliminate differences in the downstream regions, we inserted MS2 stem-loops for use in SLAP, allowing us to investigate the role of the upstream polyadenylation regions of the pA1 and pA4 site in the polyadenylation function of βCstF-64. PC-12 cells were co-transfected with SLAP constructs containing 150 nt of the upstream polyadenylation regions of pA1 or pA4 fused to MS2 loops (Figure 5A) along with Firefly luciferase plasmid to control for transfection efficiency. Cells were also co-transfected with the MS2 vector, MS2-CstF-64, or MS2-βCstF-64 (Figure 5B). In these experiments, approximately equal amount of MS2-CstF-64 and MS2-βCstF-64 proteins were expressed as determined by using the anti-FLAG antibody (Figure 5C); SLAP values were normalized to these protein levels [14,22]. Co-transfection of MS2-CstF-64 with SL-Luc_{pA1} resulted in a 2.5 fold increase in normalized SLAP activity (Figure 5B, bars 1 and 2). Identical results were observed upon co-transfection of the MS2-βCstF-64 (bar 3). Similarly, co-transfection of MS2-CstF-64 with SL-Luc_{pA4} resulted in a two-fold increase in normalized SLAP activity (bars 4 and 5) as did co-transfection with MS2-βCstF-64 (bar 6). These results suggest that βCstF-64 can promote polyadenylation with the same efficiency as CstF-64 using the “weak” polyadenylation sites from *Add2*. This is in contrast to the decreased support of polyadenylation of βCstF-64 for the “strong” SV40 polyadenylation signal (Figure 4).

4. Discussion

The nervous system employs alternative pre-mRNA splicing to increase protein diversity from a limited set of genes to carry out its complex and specialized functions [28]. It also employs extensive alternative polyadenylation to regulate these new transcripts [29]. Consistent with these requirements, a set of alternatively-spliced isoforms of CstF-64, collectively named βCstF-64, is expressed in vertebrate neurons [15]. The data presented here address the polyadenylation functions of βCstF-64 for the first time. We hypothesized that βCstF-64 functions in polyadenylation of neuronal mRNAs, and possibly in regulating alternative poly(A) site selection in the nervous system. While we have not yet found support for the latter hypothesis, we present here abundant support for the former.

PC-12 cells are a rat adrenal pheochromocytoma line that differentiates into neuronal-like cells upon treatment with NGF and serum reduction [19]. Previously, we determined that PC-12 cells express the alternatively spliced βCstF-64 mRNA isoforms [15], suggesting they might be useful in further exploring βCstF-64 function. Here, we confirmed that treatment with NGF was sufficient to cause an increase in βCstF-64 mRNA and protein expression in PC-12 cells (Figure 1). NGF binds to the receptor tyrosine kinase (TrkA) and neurotrophin receptors, mediating a wide range of functions including cell survival, apoptosis, neuronal phenotype, synaptic plasticity, and neural repair [30]. There is precedent for regulation of alternative splicing by NGF signaling: in PC-12 cells, NGF has been shown to induce alternative splicing of mRNAs encoding TrkA [31], neurofibromatosis type I (NF1, [32]), agrin [33], and neural cell adhesion molecule (N-CAM, [34]). This suggests that signal

transduction pathways activated by NGF impact the activity of splicing regulators that activate alternative splicing of CstF-64 and other pre-mRNAs [35]. Undifferentiated PC-12 cells lack axons and dendrites but start extending them after two days of NGF treatment and reach their peak at day 4 [19]. Our data indicate that the increase in βCstF-64 expression occurs within 24–48 hours after NGF treatment (Figure 1D, E). The concomitant increase in the expression of βCstF-64 during the time when PC-12 cells assume a neuronal phenotype supports our hypothesis that βCstF-64 participates in neuronal gene expression.

The possibility existed that the neuron-specific domain in βCstF-64 interfered with interaction with CstF-77 [14]. To the contrary, we observed that ectopic βCstF-64 interacted with CstF-77 to the same degree as did CstF-64 (Figure 2). Thus, we infer that βCstF-64 is part of the CstF complex in neuronal cells of the nervous system and participates in polyadenylation. The neuronal-specific domain of βCstF-64 does affect polyadenylation in PC-12 cells, however. Using SLAP, we determined that βCstF-64 resulted in less efficient polyadenylation of the strong SV40 polyadenylation site (Figure 3C). Because both the MS2-CstF-64 and MS2-βCstF-64 have identical RNA-binding domains in these constructs, this suggests that the βCstF-64 domain decreases efficiency of polyadenylation of strong sites in a way that does not directly alter binding. However, when less efficient sites such as those in β-adducin (Figure 4) were used in the same assay, polyadenylation efficiency was indistinguishable between CstF-64 and βCstF-64 (Figure 5). This suggests that when other features of polyadenylation sites have less influence, the βCstF-64 domain can be effective in controlling polyadenylation [5].

If both CstF-64 and βCstF-64 function similarly, then how might βCstF-64 be distinct from that of CstF-64? We hypothesize that the insertion of a 49 amino acid segment in the proline/glycine-rich domain, βCstF-64 enables it to interact with neuron-specific polyadenylation proteins or other neuronal proteins. Apart from βCstF-64, neuron-specific polyadenylation factors have not yet been discovered. However, there is evidence that cleavage factor I_m plays a role in neural differentiation in PC-12 cells [36]. Furthermore, neuronal splicing factors such as Hu [37] and Nova [12] influence alternative polyadenylation in the nervous system. Since there is great degree of coupling between splicing and polyadenylation machineries [38,39], it will be interesting to investigate whether βCstF-64 interacts with any of these neuronal splicing proteins.

We note that the pA4 site of β-adducin functions more efficiently than the pA1 site in PC-12 cells (Figure 4), possibly because the downstream U/GU-rich signal in the pA4 site is better defined and conserved when compared to that in the pA1 site. This finding agrees with recent work by Costessi and coworkers [21]. Our data show that NGF treatment of PC-12 cells also resulted in differential poly(A) site use in the β-adducin mRNA, reducing use of the upstream pA1 site and increasing use of the downstream pA4 site (Figure 4B). Unfortunately, transfection of βCstF-64 into PC-12 cells failed to alter β-adducin polyadenylation site use differently than did CstF-64 (not shown, but see Figure 5B). It is possible that βCstF-64 is more active on the pA4 site in differentiated PC-12 cells. However, differentiated PC-12 cells function poorly in transient transfection experiments, which has precluded us from using these cells to test this hypothesis. Global studies of alternative polyadenylation in brain and other tissues have revealed that patterns of polyadenylation involve choice between proximal and distal sites [40,41]. While our SLAP method is useful in dissecting an individual site, it does not distinguish between proximal and distal sites, and therefore might not detect such differences in a complex alternative polyadenylation system.

Three forms of CstF-64 are expressed in brain, CstF-64, βCstF-64, and τCstF-64 [18,42]. Ablation of *Cstf2t*, the gene encoding τCstF-64 in mice did not result in overt changes in their behavior [16,43]. This suggests that τCstF-64 is not critical for brain function.

However, βCstF-64 is found in all vertebrates [15], while τCstF-64 is limited to eutherian mammals [44]. This suggests that the function of βCstF-64 might be more important in providing specific neuronal functions. For example, regulated use of alternative poly(A) sites present in 3′ UTR of many neuronal mRNAs could result in changes in mRNA localization [45], stability [46], or translation [47,48]. These processes could impact neuronal processes such activity dependent mRNA translation and synaptic plasticity. Future experiments will lead us to understand its role in regulating alternative poly(A) site selection in the nervous system.

Acknowledgments

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List of Abbreviations

Add2	mouse gene encoding β-adducin
CstF	cleavage stimulation factor
CstF-64	cleavage stimulation factor, 64,000 M_r subunit
βCstF-64	brain-specific splice variant of CstF-64
CstF-77	cleavage stimulation factor, 77,000 M_r subunit
Cstf2	mouse or rat gene encoding CstF-64
CSTF2	human gene encoding CstF-64
CSTF2T	human gene encoding τCstF-64
DSE	downstream sequence element
DMEM	Dulbecco's Minimal Eagle Media
N-CAM	neural cell adhesion molecule
NF1	neurofibromatosis type 1
NGF	nerve growth factor
NGSP	nested gene-specific primer
3′ RACE	3′ rapid amplification of cDNA ends
RLU	relative luciferase unit
RT-PCR	reverse transcriptase-mediated polymerase chain reaction
SLAP	stem-loop luciferase assay for polyadenylation

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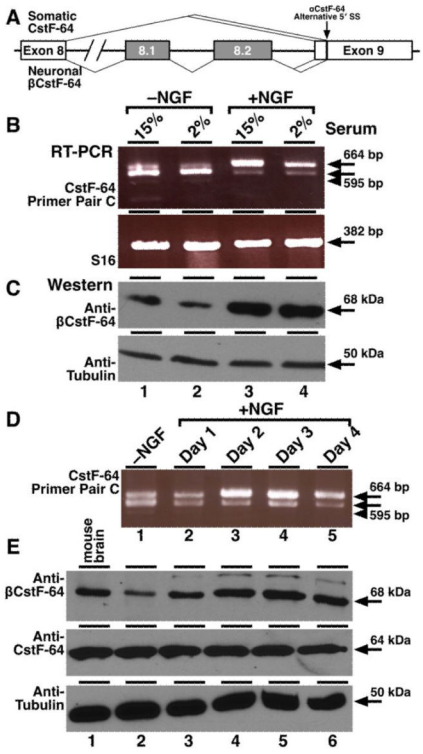

Figure 1.

The increase in βCstF-64 expression in differentiated PC-12 cells is due to NGF treatment and not serum reduction. **A.** Exon-intron diagram illustrating the somatic CstF-64 and neuronal βCstF-64 splicing patterns. Indicated are exons 8 and 9 (open boxes), the rodent-specific alternative 5′ splice site in exon 9, and the neuron-specific exons 8.1 and 8.2. **B.** βCstF-64 mRNA increases in NGF-differentiated PC-12 cells. (Upper panel) RT-PCR using CstF-64-specific primers was conducted using RNA isolated from undifferentiated PC-12 cells grown in either 15% (lane 1) or 2% (lane 2) serum-containing medium, or from NGF-treated PC-12 cells grown in either 15% (lane 3) or 2% (lane 4) serum-containing medium. RT-PCR of ribosomal S16 mRNA was performed to assess equal loading (lower panel). **C.** βCstF-64 protein expression increases in NGF-differentiated PC-12 cells. Total proteins were isolated from undifferentiated and differentiated PC-12 cells grown in DMEM medium containing the indicated amount of serum. Equal amount of protein (30μg) was loaded and resolved by SDS-PAGE and immunoblot analysis carried out using anti-βCstF-64 (upper panel) or anti-tubulin antibodies (lower panel). **D.** RT-PCR analysis using CstF-64-specific primers using RNA isolated from undifferentiated, and differentiated PC-12 cells at indicated time points. **E**. Immunoblot analysis of whole cell extracts from undifferentiated, and differentiated PC-12 cells using anti-βCstF-64 (upper panel), anti-CstF-64 (middle panel), and anti-tubulin (lower panel) antibodies. Lane 1 contains 30 μg of protein from mouse brain; lane 2, 30 μg of protein from undifferentiated PC-12 cells (as indicated in panel D); lanes 3–6 are 30 μg of protein from PC-12 cells after 1–4 days of treatment with NGF (as indicated in panel D). Note that the less intense band of slower migration seen in the anti-βCstF-64 panel of this gel was rarely seen in other experiments, and is likely an artifact.

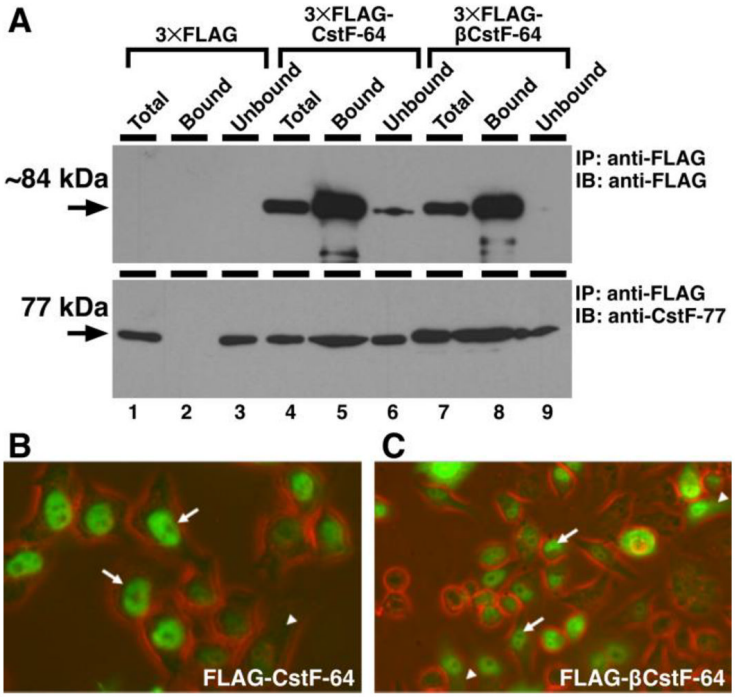

Figure 2.

βCstF-64 interacts with CstF-77 in PC-12 cells. **A)** PC-12 cells were seeded onto 10 cm dishes and transfected with 10 μg of 3×FLAG (lanes 1–3), 3×FLAG-CstF-64 (lanes 4–6) or 3×FLAG-βCstF-64 (lanes 7–9) using Lipofectamine 2000. Cells were harvested 48 hours post-transfection. An aliquot of cell lysate (5%) was used as "Total extract" control (upper and lower panels, lanes 1, 4, and 7) while the remaining was added to anti-FLAG antibody coupled to agarose resin and incubated overnight at 4°C. Beads were centrifuged and an aliquot of the supernatant (5%) was used to analyze "unbound" proteins (upper and lower panels, lanes 3, 6, and 9). After extensive washing with NET-2 buffer, "bound" proteins were eluted by boiling in SDS loading buffer. The eluted proteins (50%) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analysis (IB) using anti-FLAG (upper panel) or anti-CstF-77 antibodies (lower panel). The apparent molecular masses of the 3×FLAG-CstF-64 and βCstF-64 proteins and CstF-77 are indicated on left. **B, C**. Exogenously expressed CstF-64 and βCstF-64 proteins localize to the nucleus. PC-12 cells were grown on coverslips in 24-well plates and transfected with FLAG-MS2-CstF-64 (panel A) or FLAG-MS2-βCstF-64 using Lipofectamine 2000. After 48 hours, cells were fixed with paraformaldehyde, permeabilized with Triton-X-100 and incubated with anti-FLAG antibody for two hours. Secondary antibody conjugated to Alexa fluor 488 (green) was added and slides containing these coverslips were subsequently used for photomicrography. The images shown above are an overlay of the phase contrast (red) with the fluorescent signal emanating from the FLAG-CstF-64 and βCstF-64 proteins. Arrows indicate anti-FLAG staining of FLAG-CstF-64 (panel B) or FLAG-βCstF-64 (panel C), predominantly in nuclei; arrowheads denote less intense cytoplasmic staining, indicating a paucity of FLAG-CstF-64 (panel B) or FLAG-βCstF-64 (panel C) in cytoplasm.

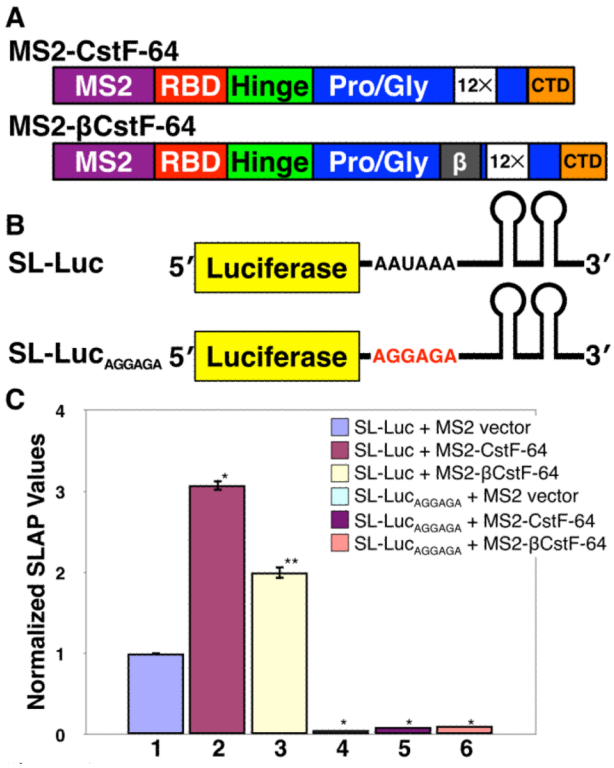

Figure 3.

Exogenously expressed βCstF-64 is less active than CstF-64 in SLAP. **A.** Illustration of the MS2-CstF-64, and MS2-βCstF-64 fusion proteins used in SLAP. **B**. Illustration of the reporter luciferase constructs used in SLAP, wherein the naturally occurring DSE is replaced by DNA encoding two copies of the stem-loop RNA element from the MS2 bacteriophage coat protein. The MS2 loops are fused to sequences upstream of the cleavage site containing AAUAAA (SL-Luc) or AGGAGA (SL-Luc$_{AGGAGA}$) and to Renilla luciferase coding region. **C**. SLAP in PC-12 cells using Renilla luciferase constructs containing either wild type SV40 late upstream polyadenylation region fused to MS2 loops (SL-Luc) or a mutated version (SL-Luc$_{AGGAGA}$). PC-12 cells were plated in 24 well plates and transfected with the indicated plasmids and with Firefly luciferase-encoding plasmid (as normalization control) using Lipofectamine 2000. Cells were transfected in triplicates and each triplicate was repeated five times. Cells were harvested 48 hours post transfection and assayed for luciferase activity. The Y-axes in the bar graphs display the fold change in Relative Luciferase Units (RLU; defined as the ratio of the Renilla, and Firefly luciferase activities) upon transfection with 3×FLAG-MS2-CstF-64 or 3×FLAG-MS2-βCstF-64-encoding plasmids. Error bars denote the 95% confidence interval of the results after ANOVA. Bars 1–3, and 3–6 refer to RLU values obtained using the SL-Luc, and SL-Luc$_{AGGAGA}$ reporters respectively. The co-transfected MS2-fusion proteins are indicated on right. The asterisks denote results that are statistically significant when compared to values obtained from MS2 vector alone (bar 1). The two asterisks denote that the difference between bars 2 and 3 is statistically significant.

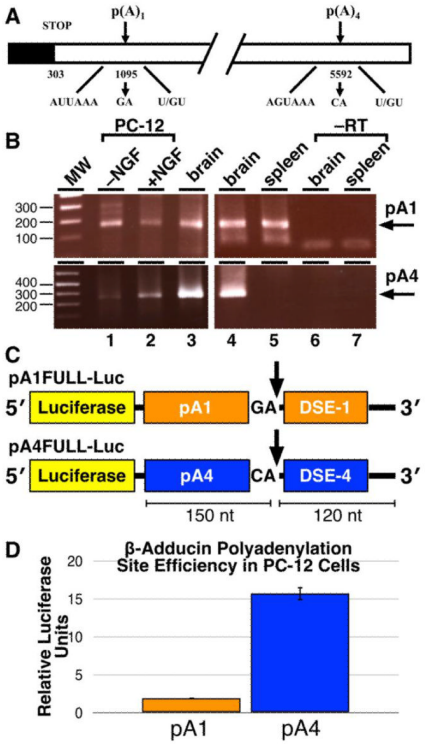

Figure 4.

The two poly(A) sites of the β-adducin mRNA are differentially used in PC-12 cells treated with NGF. **A.** Illustration of the 3′ UTR of β-adducin mRNA. The upstream and downstream poly(A) signals and the mRNA sequence at the cleavage sites (arrow) for pA1 and pA4 are indicated. **B.** 3′ RACE analysis of β-adducin mRNA using RNA from undifferentiated, and NGF-induced PC-12 cells, rat brain and spleen. RNA was isolated from indicated cells, cDNA synthesized by reverse transcription and subjected to two rounds of PCR using gene-specific and nested gene-specific primers for the pA1 and pA4 sites. "–RT" denotes PCR with no reverse transcriptase added during cDNA synthesis. **C.** Illustration of the Renilla Luciferase plasmids containing the polyadenylation signals of pA1 and pA4 site of β-adducin mRNA. A total of 270 nucleotides flanking either the pA1 or pA4 sites of β-adducin mRNA were cloned downstream of the Renilla Luciferase coding region (termed pA1FULL-Luc, pA4FULL-Luc). The upstream and downstream poly(A) signals and the mRNA sequence at the cleavage sites (arrow) for pA1 and pA4 are indicated. **D.** The pA4 polyadenylation region of the β-adducin mRNA supports more efficient polyadenylation than the pA1 polyadenylation region. Luciferase assay in PC-12 cells using pA1FULL-Luc or pA4FULL-Luc plasmids. PC-12 cells were plated in 24 well plates and transfected with the indicated plasmids and with firefly luciferase plasmid using Lipofectamine 2000. Cells were transfected in triplicates and each triplicate was repeated five times. Cells were harvested 48 hours post transfection and then cells extracts were assayed for luciferase activity. Bar graphs display the relative luciferase units obtained by dividing the Renilla luciferase units by firefly luciferase units. Error bars denote the 95% confidence interval of the results after ANOVA.

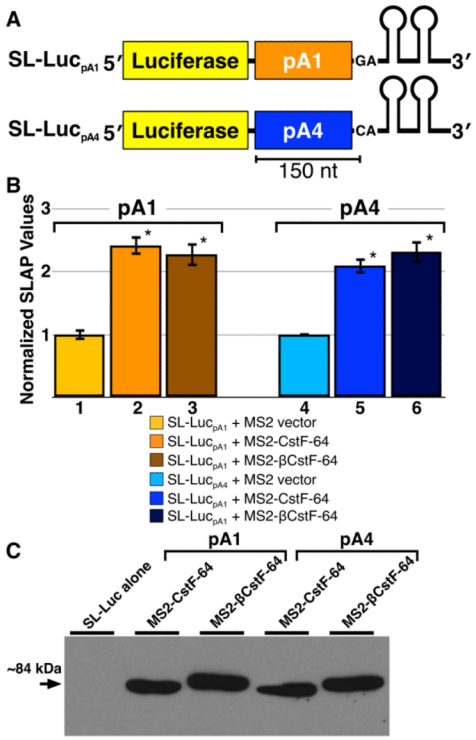

Figure 5.

βCstF-64 is as active as CstF-64 in SLAP using upstream polyadenylation regions of pA1 and pA4 of β-adducin mRNA. **A.** Illustration of Renilla luciferase constructs containing upstream polyadenylation regions of pA1 and pA4 sites of β-adducin mRNA fused to MS2 loops (SL-Luc$_{pA1}$, SL-Luc$_{pA4}$, respectively). **B**. PC-12 cells were plated in 24 well plates and transfected with the indicated plasmids and with Firefly luciferase plasmid using Lipofectamine 2000. SLAP was conducted as described in Figure 3. Bar graphs display the relative luciferase units obtained by dividing the Renilla luciferase units by firefly luciferase units. Error bars denote the 95% confidence interval of the results after ANOVA. The asterisks denote results that are statistically significant when compared to values obtained from MS2 vector alone (Bar 1). **C.** Immunoblot analysis using whole cell extracts prepared from cells transfected with constructs indicated in **B,** and probed with anti-FLAG antibody. The size of the exogenously expressed MS2-CstF-64 or MS2-βCstF-64 proteins is indicated on left.

Table 1

Primers used in RT-PCR and 3′ RACE analysis

Nucleotide sequences of the various primer pairs used in this study are indicated at right. The names of primer pairs along with their corresponding species are indicated at left in the table.

Species	Primer Name	Primer Sequence
Mouse, Rat	Pair C	5′ CAATGGCGCACCTCCTATGATG 3′ 5′ GGCACGGGCTTCCAGTCCT 3′
Rat	AddupAlGSP1	5′ CAAACACCAGGCACTGTCACGGAT 3′
Rat	AddupAlNGSP1	5′ GCTTCTCTCTTGGGTCCCTCCCC 3′
Rat	AddupAlNGSP2	5′ CTTGCACTCTCCCAAACACTCAACCTGAG 3′
Rat	AddupA4GSP1	5′ CCTTGTACATAGGCTGTCTGTGTT 3′
Rat	AddupA4NGSP1	5′ TATGCTAACACATCGAGCCGCTGC 3′
Rat	pA1F	5′ TGGATGGAAGAATTCTAAGAGCCAAC 3′
Rat	pA1R	5′ TTTTTTTTTTTTCAATTGCTACAGTTGGG 3′
Rat	pA4HpaIUltraF	5′ AATTCGATGAGAACTTTCCAAAGGCCTTTT GCTGCTTTGTGTCTGACTGTGTTTGGGGTG GACTCTGCTTGTGGGTTTCTCTGTGTGTGTGCC GTTATTTCACCTGCAGATCTTACCCTAGTA AACCAGATGTGCTCGCCGTT 3′
Rat	pA4HpaIUltraR	5′ AACGGCGAGCACATCTGGTTTACTAGGGTA AGATCTGCAGGTGAAATAACGGCACACACA CAGAGAAACCCACAAGCAGAGTCCACCCCA AACACAGTCAGACACAAAGCAGCAAAAGGC CTTTGGAAAGTTCTCATCG 3′
Rat	pA1FULLUltraF	5′ AAT TGG AAA AAG TCT TAT GCT CAG GGA AGA AGT CCT GGG ACA TTG AAA TCT GTT GCT GGC ATT GAG TTG TGC CAG GAG GTC AGA CAC CCC TTT GGC TTT GCA TGC TAT GCA GGG CTG TA 3′
Rat	pA1FULLUltraR	5′ AGCTTTACAGCCCTGCATAGCATGCAAAGC CAAAGGGGTGTCTGACCTCCTGGCACAACT CAATGCCAGCAACAGATTTCAATGTCCCAG GACTTCTTCCCTGAGCATAAGACTTTTTCC 3′
Rat	pA4FULLUltraF	5′ AATTGCTTCCCAGTGTGTGTGACCCATCTC TGTCGGCTCCTTGGGGGCTCCCGGGGAGGT GGAAGTTAAAAGGGGGCTCATGTATGCAGG TCACACTGGGAACTTAAACCCTTTTCA 3′
Rat	pA4FULLUltraR	5′ AGCTTTGAAAAGGGTTTAAGTTCCCAGTGT GACCTGCATACATGAGCCCCCTTTTAACTT CCACCTCCCCGGGAGCCCCCAAGGAGCCGA CAGAGATGGGTCACACACACTGGGAAGC 3′