NSSRs/TASRs/SRp38s function as splicing modulators via binding to pre-mRNAs

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Abstract

The genes for neural-salient serine/arginine-rich (NSSR) proteins 1 and 2 have been cloned from a neuronal differentiated embryocarcinoma cell line, P19. NSSRs contain an RNA recognition motif (RRM) at the N-terminal and several SR rich regions at the C-terminal resembling RS domains. We found that NSSRs associated with U1-70k, and determined the exon inclusion activity of NSSRs' C-terminals. First, the RRM was changed to the MS2 coat protein (MS2CP) and then, MS2 RNA stem-loops were inserted in the middle of the exon N of the the clathrin light chain B minigene as an artificial exonic splicing enhancer to be recognized by the MS2CP. The modified exon N of the pre-mRNA was included by the MS2CP switched NSSR 1, but it was excluded by the MS2CP switched NSSR 2. Deletion analysis of the MS2CP switched NSSR1 suggested that the middle SR rich region was responsible for the activity of the modified exon N inclusion. Furthermore, the RRM domain of NSSRs recognized mRNAs. NSSRs were expressed in the nervous system, especially in cerebellar and hippocampal primordia, neopallial cortex, ventricular zone, retina, and olfactory epithelium and bulb at E15.5. Taken together, our results showed that NSSRs modulate alternative splicing via binding to pre-mRNAs during neural differentiation.

Introduction

Alternative splicing is one way of generating multiple gene products from a single gene at the post-transcriptional level. A survey of alternative splicing with exon junction microarrays suggests at least 74% of human multi-exon genes are alternatively spliced (Johnson et al. 2003). In fact, many neurotransmitter receptors, such as dopamine D2 receptor (Khan et al. 1998), mGlu5 (Mion et al. 2001), NMDA R1 (Kuppenbender et al. 1999), GlyRα2 (Polydorides et al. 2000), GABA(A) receptor γ 2 (Jensen et al. 2000), serotonin receptor (Heidmann et al. 1998), and GluR1-4 (Lambolez et al. 1996) genes, have splicing products that are expressed by specific types of neurons. In addition, many analyses indicated that the alternative splicing products of a single gene have different functions (Lopez 1995; Eide et al. 1996; Valenzuela et al. 1993).

Serine/arginine-rich (SR) proteins have RNA binding domains (RRM domains) at the N-terminal and an arginine/serine-rich domain (RS domain) at the C-terminal. They regulate alternative splicing by interacting with U1-70k and U2AF35, which determine 5' and 3' splicing sites, respectively (Ge and Manley 1990; Cao and Garcia-Blanco 1998; Wu and Maniatis 1993; Graveley 2000). On the other hand, exonic cis-acting elements can be often found in pre-mRNAs. The consensus sequences of many SR proteins have been determined, and they act as exonic cis-acting elements, which are known as exonic splicing enhancers (ESEs). In addition, after SR proteins recognize ESEs, the RS domains contact with the pre-mRNA branchpoints (Shen et al. 2004).

Previously, we reported two SR protein-like gene products, neural-salient serine/arginine-rich proteins 1 and 2 (NSSR1 and 2), which are generated from a single

gene, and are thought to be splicing isoforms (Komatsu et al. 1999). NSSR1 and 2 mRNAs are present at higher levels in the brain and testis than in other tissues. NSSR1 is expressed in the neural stage during the neuroectoderm differentiation of embryocarcinoma cells. The expression of NSSR 2 prevents the inclusion of either the Flip or Flop exons in the splicing of the GluR-B gene, resulting in an increase in the abnormal exon-skipping product. However, transient transfection with NSSR 1 promotes the inclusion of the Flip exon in the splicing of the GluR-B gene (Komatsu et al. 1999). Although NSSRs affect alternative splicing, the consensus sequence of RRM in NSSRs has not been determined.

Previously, Yang et al. cloned the genes for the SR proteins, TASR-1 and 2, as Translocated in Liposarcoma (TLS) associated proteins using the yeast two-hybrid system, and showed that both gene products can process E1a pre-mRNA (Yang et al.1998; Yang et al. 2000). Their results indicate that NSSR1 and 2 are identical to TASR-2 and 1, respectively. TLS-ERG leukemia fusion protein interacts with both TSARs and inhibits the RNA splicing mediated by TASRs (Yang et al. 2000). Shin and Manley cloned SR proteins with unexpected properties called SRp38. The dephosphorylated SRp38 accumulates by heat shock and inhibits splicing at an early step (Shin et al. 2004). In addition, during M phase SRp38 is dephosphorylate as well (Shin and Manley 2002). SRp38 and SRp38-2 are also identical to NSSR1 and 2, respectively.

Phage MS2 is a bacterial RNA virus that infects E. coli. The MS2 coat protein (MS2CP) controls the sequence-specific RNA encapsidation and repression of the replicase translation by binding to the RNA stem-loop structure of the nineteen nucleotides in the viral genome (Peabody and Ely 1992). Recently, the interaction between MS2CP

and MS2 RNA has been applied to the analysis of RNA-protein interaction, splicing, etc. For example, spliceosomes are isolated using the interaction between MS2CP and MS2 RNA (Zhou et al. 2002). According to the analysis of fusion proteins consisting of MS2CP and SR dipeptide repeat, a hybrid of *Drosphila doublesex* pre-mRNA and the MS2 stem loop RNA, *dsx*(70)M1, undergoes splicing in the presence of the fusion protein consisting of the MS2CP and fourteen RS dipeptides in vitro. However, a fusion of seven RS dipeptides is unable to activate the splicing (Philipps et al. 2003).

Clathrin light chain B is one of components of clathrin coated vesicle. There are two alternative splicing isoforms, a ubiquitous and brain-specific form. The brain-specific form has fifty-four additional nucleotides as a result of the exon N inclusion. A minigene consists of exon IV, N and V of the clathrin light chain B gene has been constructed to study neuron-specific alternative splicing (Stamm et al. 1992).

In this paper, we demonstrated associations of NSSRs with U1-70k. The switching of the NSSRs' RRM to MS2CP suggested that NSSRs can modulate alternative splicing via binding to pre-mRNAs. Deletion analysis of the MS2CP switched NSSR1 indicated that the middle of the NSSR1 specific SR rich region regulates the inclusion of the modified exon N of the clathrin light chain B minigene. Furthermore, the expression of NSSRs in neuronal cells during development was confirmed by in situ hybridization.

Results

The association of NSSRs with U1-70k

One of the functions of SR proteins is to select 5' splicing sites by binding to U1-70k through their SR domains. The hybrid protein consisting of MS2CP and seven RS dipeptides is ineffective to stimulate splicing activity against the target in vitro (Philipps et al. 2003). However, RS like domain of the C-terminals of NSSRs consist of several three RS dipeptides (Fig 4A). Therefore, we examined the association between NSSRs and U1-70k by yeast two hybrid assays. In the presence of NSSR1 and 2 fused with the GAL4 DNA binding domain (BD), U1-70k fused with the GAL4 activation domain (AD) rescued the auxotrophy, as well as in the presence of ASF/SF2 fused with the GAL4 DNA BD on the highest stringent condition (-Ade and –His). All of growing the colonies were β−galactosidase positive (data not shown). In contrast, BD and AD themselves did not rescue the auxotrophy in the presence of U1-70k fused with AD and NSSR fused with BD, respectively (Fig 1B).

The results of the two hybrid assays were confirmed in COS7 cells. The expression vectors for myc-tagged U1-70k and FLAG-tagged NSSRs were introduced into COS7 cells, and immunoprecipitation with anti-myc tag agarose was carried out in the presence of RNase A. NSSR1 and 2 were detected in the immunocomplexes of U1-70k (Fig 1C).

Regulation of the clathrin light chain B minigene containing stem loops of MS2 RNA by MS2 coat protein fused NSSRs' C-terminals.

In order to determine how NSSRs contribute to alternative splicing, the clathrin light chain B minigene was applied as a model for neuronal specific alternative splicing, because the exon N of the clathrin light chain B gene is included during the neuronal differentiation of P19 cells (Stamm et al. 1992). NSSRs themselves do not affect the clathrin light chain B minigene (supplement data). In addition, the recognition sequence of NSSR's RRM has not been determined. Therefore, the RRM motif of NSSR1 and 2 was replaced with an RNA binding protein, MS2CP. In order to localize in nuclei, three nuclear localization signals were inserted between the MS2CP and NSSR C-terminals (Fig 2A). Furthermore, a myc-tag was added to the C-terminals. As a result of the introduction of these expression vectors into COS7 cells, bands at appropriate sizes were detected by western blot analysis of the lysates using myc antibody. These results suggested that these expression vectors work in mammalian cells (Fig 2C).

The stem loops of MS2 RNA, which are recognized by MS2CP, were inserted into the middle of the exon N of the clathrin light chain B minigene driven by an SV40 promoter (Fig 2B). These expression vectors were transiently transfected into COS7, NIH 3T3 and N2a cells. Then, total RNA was extracted from these cells followed by RT-PCR with minigene specific primers to determine the splicing forms of their minigene's mRNA. No signal was detected when total RNA extracted from untransfected cells (Fig 3, supplement data). In the case of the original minigene, the neural-specific form was not detected in the absence of the NSSRs' C-terminals. However, both the neural and

non-neural forms were detected, when the MS2 stem-loops were inserted into the exon N (Fig 2D). The expression of the NSSR1's C-terminal fused with MS2CP further activated the inclusion of the exon N containing MS2 RNA, but the original exon N was not included at all (Fig 2D). In contrast, the NSSR2's C-terminal fused with MS2CP repressed the inclusion of the exon N containing MS2 RNA (Fig 2D). In addition, the activities of NSSRs' C-terminals were in a dose-dependent manner (Fig 3) and observed in NIH3T3, COS7 and N2a cell lines (Fig 2D and 5).

Deletion analysis of NSSR1 C-terminal

Deletion mutations of the C-terminal of NSSR1 were fused to MS2CP, and then applied to the clathrin light chain B minigene analysis using N2a and 3T3 cells. The C-terminal RS region of NSSR1 (amino acids 222-262) had no effect on either the inclusion or exclusion of the minigene. Although the common sequence among NSSR1 and 2, the C-terminal RS region of NSSR1 or both were deleted, the inclusion activity of the modified exon N was retained in 3T3 and N2a (Fig 4, 5).

The RNA binding activity of the RRM domain in NSSRs

Since SR proteins recognize ESEs by their RRM, the binding activity of NSSRs to exons is one of our interests. In order to elucidate this issue, a bacterial recombinant NSSRs' RRM was constructed. The recombinant contained a cellulose binding domain (CBD)-tag at N-terminal. Therefore, the NSSRs' RRM was trapped on cellulose resin. Western blot analysis revealed that about 0.5 μg of the full length

recombinant protein was absorbed on the resin (Fig 6). As a result of incubation of the resin with 20 μg of mRNA from murine brains, 10 ng of mRNAs was obtained from the resin (Fig 6, Table 2). RNAs at 89 bases in length were detected in both tRNAs and the RNAs obtained from the resin (Fig 6). Because the resin was pre-coated with yeast tRNAs, non-specific interaction probably occurred between tRNAs and the cellulose resin. The electropherogram of the murine brain mRNAs showed obvious contamination of 18s ribosomal RNA, but the RNAs obtained from the resin contained considerably less 18s ribosomal RNA (Fig 6). The results suggest that the NSSRs' RRM recognizes mRNA in a sequence specific manner.

In situ hybrydization analysis of NSSR1 and 2 in embryos

Since NSSRs fused with MS2CP regulated neuronal-specific alternative splicing, it is possible that NSSRs are components of a spliceosome that plays a role of neuron-specific alternative splicing. In addition, the expression of NSSR1 is observed in P19 cells during neuronal differentiation, suggesting participation of NSSRs in neural differentiation (Komatsu et al. 1999). Therefore, we examined the expression of NSSRs in vivo by in situ hybridization. For in situ hybridization, three probes (NSSRC, NSSA1 and NSSA2) were used. NSSRC recognizes mRNAs of both NSSR 1 and 2, while NSSA1 and NSSA2 are specific for NSSR 1 and 2, respectively. NSSR 1 and 2 mRNAs were prominently distributed in cerebellar and hippocampal primordium, neopallial cortex, and ventricular zone, but the rest of the CNS showed only weak expression of NSSRs at E15.5 (Fig 7). In addition, tooth germs, retina, olfactory epithelium and bulb, intestine and

salivary gland showed detectable levels of the mRNA of NSSRs by NSSRC and NSSA1 (Fig 8). Overall, a similar distribution of hybridization signal was detected by NSSRC, NSSA1 or NSSA2. However, the signal for NSSA2 was very faint, totally (Fig. 8A, B). Weak expression of NSSRs was detected in muscle, but the expression of NSSR mRNAs was less than the detection limit in other tissues (data not shown). In the tooth germ, NSSR 1 and 2 mRNAs were detected in the dental bulbs, which are derived from dental mesenchyme (Fig 8D). In contrast to the expression in mesenchymal cells, olfactory epithelium expressed NSSR mRNAs (Fig 8C). The expression of NSSR mRNAs was observed not only in the neural retina, but also in the equatorial region of the lens, where the anterior capsular epithelial cells are incorporated into the lens (Fig 8A). The sense probe as a negative control demonstrated that NSSRC, NSSA1 and NSSA2 hybridized to NSSR mRNAs specifically (data not shown).

Discussion

U1, U2, U4, U5, and U6 snRNPs play a major role in pre-mRNA splicing. U1 and U2 snRNPs bind to a pre-mRNA at the 5' splicing site and branch point region, respectively, and form an early spliceosome. This suggests that U1 and U2 snRNP determine the splice site. U1-70k is a component of U1 snRNP and interacts with the SR protein, ASF/SF2. Therefore, the recognition of ESEs by SR proteins is important in exon inclusion and selection of 5' splicing sites (Graveley. 2000). In the presence of the GAL4 AD fused U1-70k, the GAL BD fused NSSR1 and 2 rescued the auxotrophy. Although the activity of NSSR1 was much weaker compared to NSSR2 and ASF/SF2, the results were able to be confirmed by the immunoprecipitation from overexpressing cell lysates. Furthermore, the interaction between U1-70k and NSSR1 is shown by the recombinant proteins (Shin et al. 2004). According to the interaction analysis by the recombinants, the dephosphorylation form of NSSR1 binds to U1-70k much stronger than the phosphorylated form does (Shin et al. 2004). Therefore, NSSR1 might be phosphorylated in the yeasts. One of the roles of dephosphorylated NSSR1 is repression of splicing (Shin et al. 2004). However, the NSSR1 C-terminal stimulated the inclusion of the modified exon. Since the dephosphorylated form cannot be detected in the normally growing cells (Shin & Manley 2002; Shin et al. 2004), the inclusion activity of NSSR1 C-terminal is likely to be caused by the phosphorylated form.

Since the consensus sequence of the RRM of NSSRs is unknown, it is difficult to show the exon inclusion activity by direct binding to the ESE on pre-mRNAs. Therefore, the RRM of NSSRs was switched to MS2CP and then, the stem loops of the

MS2 RNA were inserted into the middle of the exon N of the clathrin light chain B minigine. In the case of the original minigene, the neural-specific form was not detected in NIH3T3 cells. However, both the neural and non-neural forms of the mRNA were detected when MS2 stem-loops were inserted into the exon N. As a result, both inclusion and exclusion activities were detected (Fig 2D). The replacement of the RRM of NSSRs with MS2CP revealed the exon inclusion and exclusion activities of the NSSR1 and 2 C-terminals in all cell lines examined, respectively (Fig 2D and 5). Although NSSR1 has short SR dipeptide repeats, NSSR1 is thought to act as an SR protein. Correspondingly, a fusion protein consisting of MS2CP and the RS domain of U1-70k has the efficient splicing activity of *dsx*(70)M1 despite rather low content of SR (Philipps et al. 2003). Their sequences other than the SR dipeptides may be capable of functioning as a splicing activation domain.

Since switching RRMs to MS2CP to recognize the artificial ESE, the stem loop of MS2 RNA, was successful, we speculate that ESE-dependent splicing regulation can be detected by this system even though the consensus sequences of RNA binding domains are unknown. Recently, contribution of RNA binding proteins that do not contain any RS domains to alternative splicing have been reported. Therefore, this system can be applied to those proteins.

Deletion analysis revealed that the amino acids 143-234 of NSSR1 contribute to the exon inclusion. Although SR rich sequences are thought to participate in their protein-protein interaction among them, the third C-terminal SR rich region of NSSR1 (the amino acids 222-262) was not necessary for the exon inclusion. Since the amino acids

143-234 of NSSR1 consist of the SR rich region and additional sequence, this additional sequence seems to play an important role in the inclusion of exons. For example, because SR repeats are too simple to recognize their specific partners, the additional sequence may engage in the specific recognition of NSSR1 binding proteins which contain SR rich sequences. One of the targets for the amino acids 143-234 of NSSR1 may be U1-70k, but we do not have the evidence for direct binding between NSSR1 and U1-70k. Since the association between U1-70k and NSSR1 was observed in yeasts and COS cells, it is possible that NSSR1 brings U1-70k to exons recognized by NSSRs' RRM and activates the 5' splicing sites. On the other hand, the C-terminal sequence of NSSR2 contains SR rich sequence and NSSR2 associated with U1-70k. However, the C-terminal sequence of NSSR2 inhibited the inclusion of the modified exon. Therefore, NSS2 may block access of U1-70k to 5' splicing site of recognizing exons.

In this study, the bacterial recombinant NSSRs' RRM bound mRNAs specifically. We also showed the association of NSSR with U1-70k. In addition, NSSRs localize in nuclear speckles (Komatsu et al. 1999). Taken together, NSSRs potentially bind exons of pre-mRNAs and participate in the regulation of alternative splicing.

Finally, in situ hybridization indicated that NSSRs were expressed in the brain during development, which is consistent with the expression of NSSR1 during neuronal differentiating P19 cells (Komatsu et al. 1999). In the ventricular zone, there are cells that are differentiating into neurons and glial cells. The dental bulb of tooth germs and salivary gland also express NSSRs, and cells in these regions are in the middle of differention at E15.5. The results of northern blot analysis of human mature tissues

showed higher expression of NSSR1 and 2 in skeletal muscle than brain (Clinton et al. 2002). Since many types of cells are under differentiation in fetal bodies, NSSRs may contribute to differentiation of the cells rather than maintenance of these cells. In contrast, skeletal muscle seems to utilize NSSRs for its maintenance.

NSSR1 was cloned as an SR protein that was expressed in neural differentiated P19 cells (Komatsu et al. 1999). However, we found that many tissues express NSSR1 during their differentitaion. Thus, we speculate that NSSR1 regulates transcriptional regulators shared in NSSR1 expressing cells to control their differentiation.

In this paper, we demonstrated the association of NSSR with U1-70k. In addition, we showed roles of NSSRs in the regulation of neural alternative splicing by the direct binding to pre-mRNAs and the expression of NSSRs in nervous systems during differentiation. The C-terminal of NSSR2 and middle RS region of NSSR1 can participate in the exclusion and inclusion of exons, respectively. These results suggested that NSSRs are SR proteins despite their atypical RS domain and regulate alternative splicing during neuronal differentiation. Furthermore, we demonstrated that the domain swapping analysis would be useful to analyze exon inclusion and exclusion activity of splicing associated proteins. In fact, many RNA binding proteins other than SR proteins have been found as a splicing regulator.

Experimental procedures

Plasmid construction

High fidelity DNA polymerase (Pyrobest DNA polymerase, TAKARA) was used to construct plasmids. The primers used in this study are listed in Table 1. In order to construct pMS2CNS1, 2 and their deletion mutants, pCMV/myc/nuc and pHybLex/Zeo-MS2 were purchased from Invitrogen. DNA fragments of the NSSR C-terminals and MS2CP were obtained by PCR with primers shown in Table 1. The fragments for MS2CP were inserted between the Not I and Pst I sites of pCMV/myc/nuc followed by linearization by PCR with the primers, MycnucF and MycnucR. The linearized plasmid was digested with Not I and the C-terminal of NSSRs were inserted. The clathrin light chain B minigene expression vector, pJS74, which was a generous gift from Stamm (Stamm et al. 1992), was amplified with the primers, CLR and CLF, to linearize the plasmid. The MS2 stem loops, which were obtained by digesting pRH3' with EcoR I and Sma I, were ligated with the linearized pJS74.

For the yeast two hybrid assays, NSSRs and ASF in their full lengths were inserted into pAS2.1 in frame between the EcoRI and Sal I sites. U1-70k was inserted into pACT2 in frame at the Sma I site.

In order to produce the NSSRs' RMM recombinant, the DNA fragments that code the RRM were amplified by high fidelity PCR with the primers, RRMF and RRMR. The fragments were subcloned at the EcoRI site of pYesTrp3. The obtained plasmids were digested with Hind III and Xho I to insert into pET-34b(+) that carried a cellulose binding domain tag (CBD tag, Novagen).

RT-PCR

Total RNAs were isolated from cells using TRIzol reagent (Invitrogen). One microgram of total RNA was used for each reaction. Complementary DNAs were synthesized from oligo dT primer using ImProm-ll Reverse Transcription System (Promega). The mRNAs derived from the clathrin light chain B minigene were amplified by PCR with the specific primers, SV40A and SS031, as described by Stamm et al. (Stamm et al. 1992).

Yeast two hybrid assay

The GAL4 DNA binding domain and GAL4 activation domain fusions were constructed with pAS2.1 and pACT2 (Clontech), respectively. The plasmids derived from pAS2.1 and pACT2 were introduced into AH109 and Y189 with Yeast Transformation System (Clontech), respectively. The obtained clones were mated and grown on SD plates lacking tryptophan, leucine, adenine and histidine to test the interaction between NSSRs and U1-70k. In this two hybrid system each reporter gene in yeasts was driven by different promoter elements, UAS-TATA. Furthermore, the reporter gene for Ade- was tighter than that for His-. The Yeast Protocol Handbook (Clontech) was used as a guide to handle yeasts.

Mammalian cell culture

COS 7 and NIH3T3 cells were grown in DMEM supplemented with 10% heat inactivated FCS. Neuro2a cells were grown in DMEM supplemented with 10% heat

inactivated FCS and none-essential amino acids solution (Sigma). Transfection was performed with Lipofectamine and plus regent (Invitrogen), as described in the product manual.

Immunoprecipitation and immunoblot analysis

Cells were suspended in PBS containing 0.05% Triton X-100 RS, 10μg/ml RNase A and protein inhibitor cocktails for mammalian cells (Nacalai tesque), and then frozen once to lyse. Myc-tagged U1-70K was collected with anti-myc monoclonal antibody agarose affinity gel (clone 9E10, Santa Cruz). The agarose gel was washed four times by the lysis buffer.

SDS-PAGE were performed as described by Laemmli, followed by blotting onto PVDF membranes. Anti-FLAG M2 monoclonal antibody (Sigma), anti-myc monoclonal antibody (Cell Signaling Technology) and anti-CBD・Tag rabbit polyclonal antibody (Novagen) were used as primary antibodies. HRP linked anti-mouse IgG and anti- rabbit IgG (Chemicon) were used as a secondary antibody. FLAG, myc and CBD tagged proteins were detected by ECL (Amersham Biosciences).

Production and extraction of the NSSRs' RRM recombinant protein

The NSSRs' RRM recombinant was produced in 100ml culture of *E. coli*., Rosetta(DE3)pLysS, by induction with 1mM IPTG in LB medium for three hours at □□˚C. The bacterial pellet was frozen once and then lysed with 50mM Tris-HCl buffer (pH8.0) containing 500mM NaCl, 0.1% Triton X-100 and 100μM PMSF. The lysate were

dialysed against 50mM Tris-HCl buffer (pH8.0) containing 500mM NaCl and 100μM PMSF by Spectra/Por CE (MWCO 300,000, SPECTRUM). The external solution were concentrated by ultrafiltration using Amicon Ultra-4 (MWCO 10,000, MILLIPOR).

Messenger RNA binding assay

Total RNA was extracted from seven-day-old murine brains by TRIzol and then, the mRNA was purified with mTRAP total kit (ACTIVE MOTIF). The extracted NSSRs' RRM recombinant were absorbed on 30mg of cellulose resin, CBinD 100 (Novagen). After three washes with 10mM Tris-HCl (pH8.0) containing 500mM NaCl, 1mM $MgCl_2$ and 0.2u/μl SUPERase•In (Ambion), the resin was resuspeneded in 300μl of the same buffer. Twenty micrograms of yeast tRNA were added into the suspension followed by incubation for five minutes. Then, 20μ□ of the murine brain mRNA were added. After incubation for ten minutes, the resin was washed four times with the same buffer. Next, the resin was resuspended with 100μl of 0.5% SDS and then, 500□l of TRIzol was added into the resin to purify the mRNA on the resin. The obtained RNA was analyzed by BIOANALYZER 2100 with RNA 6000 pico reagent kit (Agilent Technologies).

in situ hybridizaion

The NSSR1 and 2-specific probes for in situ hybridization were constructed using the fragments comprised of the bp 831-2669 of NSSR1 cDNA and the bp 681-2227 of NSSR2 cDNA, respectively. The common probe recognizing NSSR1 and 2 was

produced from the bp 1-229 fragment of NSSR1 cDNA (Fig 4 A). ICR mice were purchased from Charles River, Japan. DIG-labeled cRNAs were synthesized by T7 RNA polymerase. In situ hybridization on frozen cryosections was performed using methods described previously (Takahashi and Osumi 2002).

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References

Cao, W. & Garcia-Blanco, M.A. (1998) A serine/arginine-rich domain in the human U1-70k protein is necessary and sufficient for ASF/SF2 binding. *J. Biol. Chem.* 273, 20629-20635.

Clinton, J.M., Chansky, H.A., Odell, D.D. Zielinska-Kwiatkowaska, A., Hickstein D.D. & Yang, L. (2002) Characterization and expression of the human gene encoding two translocation liposarcoma protein-associated serine-arginine (TASR) proteins. *Gene* 284, 141-147

Eide, F.F., Vining, E.R., Eide, B.L., Zang, K, Wang, X.Y. & Reichardt, L.F. (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. *J. Neurosci.* 16, 3123-3129.

Ge, H. & Manley, J.L. (1990) A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. *Cell* 62, 25-34.

Graveley, B.R. (2000) Sorting out the complexity of SR protein functions. *RNA* 6, 1197-1211.

Heidmann, D.E., Szot, P., Kohen, R. & Hamblin, M.W. (1998) Function and distribution of three rat 5-hydroxytryptamine 7 (5-HT7) receptor isoforms produced by alternative splicing. *Neuropharmacology* 37, 1621-1632.

Jensen, K.B., Dredge, B.K., Stefani, G., *et al*. (2000) Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. *Neuron* 25, 359-371.

Johnson, M.J., Castle, J., Garrett-Engele, P., et al. (2003) Genome-wide survey

of human alternative pre-mRNA splicing with exon junction microarrays. Science 302, 2141-2144.

Khan, Z.U., Mrzljak, L., Gutierrez, A, de la Calle, A. & Goldman-Rakic, P.S. (1998) Prominence of the dopamine D2 short isoform in dopaminergic pathways. *Proc. Natl. Acad. Sci. USA* 95, 7731-7736.

Komatsu, M, Kominami, E, Arahata, K & Tsukahara, T. (1999) Cloning and characterization of two neural-salient serine/arginine-rich (NSSR) proteins involved in the regulation of alternative splicing in neurones. *Genes Cells* 4, 593-606.

Kuppenbender, K.D., Albers, D.S., Iadarola, M.J., Landwehrmeyer, G.B. & Standaert, D.G. (1999) Localization of alternatively spliced NMDAR1 glutamate receptor isoforms in rat striatal neurons. *J. Comp. Neurol.* 415, 204-217.

Lambolez, B., Ropert, N., Perrais, D., Rossier, J. & Hestrin S. (1996) Correlation between kinetics and RNA splicing of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in neocortical neurons. *Proc. Natl. Acad. Sci. USA* 93, 1797-1802.

Lopez, A.J. (1995) Developmental role of transcription factor isoforms generated by alternative splicing. *Dev. Biol.* 172, 396-411.

Mion, S., Corti, C., Neki, A., *et al*. (2001) Bidirectional regulation of neurite elaboration by alternatively spliced metabotropic glutamate receptor 5 (mGluR5) isoforms. *Mol. Cell. Neurosci.* 17, 957-972.

Peabody, D.S & Ely, K. R. (1992) Control of translational repression by protein-protein interactions. *Nucleic Acids Res.* 20, 1649-1655.

Philipps, D., Celotto, A.M., Wang, Q., Tarng, R.S. & Graveley, B.R. (2003) Arginine/serine repeats are sufficient to constitute a splicing activation domain. *Nucleic Acids Res.* 31, 6502-6508.

Polydorides, A.D., Okano, H.J., Yang, Y.Y., Stefani, G. & Darnell, R.B. (2000) A brain-enriched polypyrimidine tract-binding protein antagonizes the ability of Nova to regulate neuron-specific alternative splicing. *Proc. Natl. Acad. Sci. USA* 97, 6350-6355.

Shen, H., Kan J.L.C. & Green, M.R. (2004) Arginine-Serine-Rich domains bound at splicing enhancers contact the branchpoint to promote prespliceosome assembly. *Mol. Cell* 13, 367-376.

Shin, C., Feng, Y. & Manley, J.L. (2004) Dephospholylated SRp38 acts as a splicing repressor in response to heat shock. *Nature* 427, 553-558

Shin, C. & Manley, J.L. (2002) The SR protein SRp38 represses splicing in M phase cells. *Cell* 111, 407-4417

Stamm, S., Casper, D., Dinsmore, J., Kaufmann, C.A., Brosius, J.& Helfman, D.M. (1992) The clathrin light chain B: gene structure and neuron-specific splicing. *Nucleic Acids Res.* 20, 5097-5103.

Takahashi,M. & Osumi, N. (2002) Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains.

Valenzuela, D.M., Maisonpierre, P.C., Glass, D.J., *et al*. (1993) Alternative forms of rat TrkC with different functional capabilities. *Neuron* 10, 963-974.

Wu, J.Y. & Maniatis, T. (1993) A striking organization of a large family of human neural cadherin-like cell adhesion genes. *Cell* 75, 1061-1070.

Yang, L., Embree L.J,., Tsai, S. & Hickstein D.D. (1998) Oncoprotein TLS interacts with serine-arginine proteins involved in RNA splicing. *J. Biol. Chem.* 273, 27761-27764.

Yang, L., Embree, L.J. & Hickstein, D.D. (2000) TLS-ERG leukemia fusion protein inhibits RNA splicing mediated by serine-arginine proteins. *Mol. Cell. Biol.* 20, 3345-3354.

Zhou, Z., Licklider, L.J., Gygi, S.P. & Reed, R. (2002) Comprehensive proteomic analysis of the human spliceosome. *Nature* 419, 182-185.

Figure Legends

Figure 1. Schematic structures of NSSR proteins and interaction of NSSRs with U1-70k.

A. NSSR1 and 2 have an RNA recognition motif (RRM) at the N-terminal and several serine and arginine rich regions that are located at the C-terminal. B. NSSRs were inserted into pAS2-1 to fuse with the GAL4 BD and introduced into AH109. Y187 expressing the U1-70k fused with the GAL4 AD was mated with the transformed AH109. The obtained yeasts were grown in the liquid media until cells were saturated. The media were diluted 10, 100, and 1,000 times, and 5μl of each medium was plated onto a SD agar plate lacking adenine, histidine, leucine, and tryptophan. The interaction of U1-70K with ASF/SF2 was detected as previously reported. The coexpression of NSSR1 and U1-70K resulted in the slow growth of the yeasts, but no growth in the negative controls was observed under the highest stringency (-His –Ade). NSSR2 also rescued the growth of the yeast in the presence of U1-70k. C. Myc tagged U1-70k and FLAG tagged NSSRs were overexpressed in COS 7 cells by transient transfection, and the cells were lysed to perform immunoprecipitation with anti-myc antibody. The immunocomplexes included tagged NSSRs in the presence of U1-70k (top panel). The lysates were analyzed by Western Blot (WB) analysis to confirm the expression of FLAG-tagged NSSRs (middle panel) and myc-tagged U1-70k (bottom panel).

Figure 2. Effects of MS2 coat fused NSSRs' C-terminals on neural specific exon.

A. The RRM region of NSSRs was switched to the RNA bacteriophage coat protein (MSCP) in order to detect effects on alternative splicing (MS2CNS1 and MS2CNS2). To assure the nuclear localization of the RRM switched NSSRs, three nuclear localization signals (NLS) were inserted between MSCP and the C-terminals. B. To study the effects of NSSRs on the neuron specific alternative splicing, the clathrin light chain B minigene (pJS74), which contains exon IV, neuron specific exon N and exon V, was modified as a model for neuronal alternative splicing. MS2 RNA stem loops were inserted into the middle of the exon N to be recognized by MS2CNS1 and 2. C. The protein expression by MS2CNS1 and MS2CNS2 expression vectors was confirmed by transfection into COS 7 cells. The cell lysates were loaded onto SDS-PAGE, and transferred onto a sheet of PVDF membrane to detect the expressed proteins by anti-myc antibody. Appropriate sizes of bands were observed from the lysates other than the negative control (pDSRED). D. The NSSR C-terminals fused with MSCP and the clathrin light chain B minigenes were transfected into NIH3T3 and COS7 cells. The processed pre-mRNAs were amplified by RT-PCR with the minigene specific primers. The exon N of pJS74 was excluded in NIH3T3 cells according to other non-neural cells as reported previously (pJS74 lane 1-3). Some of the modified exon N were included in the mRNA in the absence of MSCP fused with C-terminals (pJSMS2r, lane 3). The NSSR1 C-terminal activated the inclusion of the modified exon N further, but the NSSR2 C-terminal inhibited the inclusion in NIH3T3 and COS7 cells. The results were reproducible and the detected bands were confirmed by sequencing.

Figure 3. The dose-dependency of the activity of NSSRs' C-terminals

The modified minigene and various amounts of the expression vectors were co-transfected into NIH3T3 cells. The total amounts of DNA were adjusted with the empty vector. The mRNAs derived from the miningene were amplified by RT-PCR followed by 3% agarose gel electrophoresis. The products were stained with ethidum bromide (A). The proportion of the exon N included fragments in the detected bands by the electrophoresis was shown in B (MS2CNS1) and C (MS2CNS2). The experiments were triplicated and the standard deviations are shown by error bars.

Figure 4. Deletion constructs of NSSR1 C-terminal.

A. The primary structure of NSSR 1. The RRM is shown in gray and three SR rich regions are underlined. B. Five partial fragments of the NSSR1 C-terminal were fused with MSCP. The 79-183 fragment of NSSR1 is the common region among NSSR1 and 2 C-terminal.

Figure 5. The exon inclusion activity of the deletion mutants of NSSR1 C-terminal.

The each deletion mutants and modified clathrin light chain B minigenes were introduced into N2a (A) and NIH3T3 (B) cells to detect their splicing regulation as described in Figure 2. The numerals at the bottom of lanes indicate the proportion of the exon N included fragments in the detected bands. MS2CNS1 and MS2CNS2 possessed the inclusion and exclusion activity, respectively in N2a cells as well as CCS7 and NIH3T3 cells (A). The 143-234 region of NSSR1 was necessary for the exon N inclusion in both

of N2a and NIH3T3 cells. The half of the cells into which the plasmids were introduced was lysed with the SDS sample buffer containing 5M urea and then, the lysates were analyzed by western blotting. The deletion mutants were detected with ant-myc antibody. The experiments were performed twice, and similar results were obtained.

Figure 6. The RNA binding activity of the NSSRs' RRM.

The bacterial recombinant of NSSRs' RRM tagged by a cellulose binding domain (CBD) at the N-terminal was absorbed on 30 mg of cellulose resin. One twentieth of the resin was analyzed by Western Blotting using ant-CBD antibody. The intensity of the band for the full length CBD-tagged NSSRs' RRM was equivalent to 16 ng of the purified CBD protein (A). Twenty micrograms of mRNA from murine brains were added into the resin which was pre-coated with 20μg of yeast tRNAs. After incubation for ten minutes, the resin was washed four times. The binding RNAs were extracted and analyzed with Agilent 2100 bioanalyzer (B). The electropherograms of the yeast tRNAs and murine brain mRNA used are shown in C.

Figure 7. In situ hybridazation analysis of NSSRs in the central nervous system at E15.5.

Complementary RNAs were prepared as a probe for in situ hybridization from common (NSSRC), NSSR1 specific (NSSA1) and NSSR2 specific (NSSA2) regions. A. The sequence shared with NSSR 1and 2 is shown in black. The gray area indicates the specific regions. B. The sagital section at E15.5. The telencephalon was labeled by the

NSSA1 probe. The hybridization signal in the neopallial cortex (arrow) was weaker than that in the ventricular zone of the telencephalon (arrowhead). The asterisk indicates the lateral ventricle, and the striatum is enclosed by the line. C. The NSSRC probe hybridized to the primitive cerebellar (arrow). The asterisk indicates the fourth ventricle. D. The transverse section of the hippocampal primordium was hybridized by the NSSRC probe. The area indicated by the arrow would form the hippocampus. The asterisk indicates the lateral ventricle.

Figure 8. The distrubution of NSSRs other than the central nervous system at E15.5 embryo.

A. The NSSRC probe hybridized to the retina. In addition to the retina, the equatorial region of lens expressed NSSR mRNAs, as indicated by the arrows. B. The hybridization to the retina with the NSSA2 probe resulted in a similar staining pattern to NSSRC probe. C. The coronal section was hybridized by the NSSA1 probe. The wall of the olfactory bulbs (arrows) and ectoderm around nasal cavity (asterisks) were positive. D. NSSR mRNAs were detected in the dental mesenchyme of the tooth germs, which are enclosed by the lines. At E15.5, the tooth germs grow into the bell stage. The bottom of the tooth germ at bell stage consists of mesenchymal cells (arrows). The arrow heads indicate the epithelium area. The salivary gland (E) and intestine (F) were hybridized by the NSSRC probe.

Supplementary data

The original clathrin light chain B minigene and flag-tagged NSSRs expression vectors were transiently expressed in NIH3T3 cells. The expression of NSSRs was analyzed by western blot analysis with anti-flag antibody (the top panel). As an internal control, β-tublin was detected (the middle panel). The spliced mRNA of minigne transcripts were amplified by RT-PCR with the specific primers. The bands at 250 bps were not contained the exon N. Thus, the exon N was not included in any condition.

TABLE 1 Oligonucleotides used in this study

Name	sequence (5í-> 3í)
For lineatization of pJS74	
CLR	tggtagaacgcttttgtcagcgatc
CLF	gcagccagatgctgataccattgg
For lineatization of pCMV/myc/nuc	
MycnucF	cggcggccgcagaacaaaaactcatctcagaagaa
MycnucR	cgggttttgttctgcggccgcatctaccttttct
For fragments of full and partial NSSRsí C-terminals	
NSFMS2	ccgcggccgcacgtcagattgaaatccagttcg
NS1RMS2	ggggcggccgcgtggccactggacttgggactag
NS2RMS2	ggggcggccgcgatcttttcttgaagtgtagtaag
NSF429	ggcgcggccgctcctagaaacagtagaccgactg
NSF666	cgcgcggccgcatatgaaaaggaatcaaggaaaa
NSR549	cccgcggccgctggtcttccagtcggtctactgt
NSR703	cgcgcggccgctctaggtggttctttttttccttg
For MS2CP fragment	
MSPST	gaactgcagatccatatggcttctaacttt
M2BsmBI	ccgccgtctctggcccagtagatgccggagttggc
For RRM fragment	
RRMF	ctggaattcccatgtccgataccctgcgcc
RRMR	ccgaattctatatcggtcataatcgtcata

Table II The Absorbed RNA by the NSSRs' RRM on the resin

	mRNA (ng)	tRNA (ng)
pET34	1.9	21
pET34-RRM	10	36

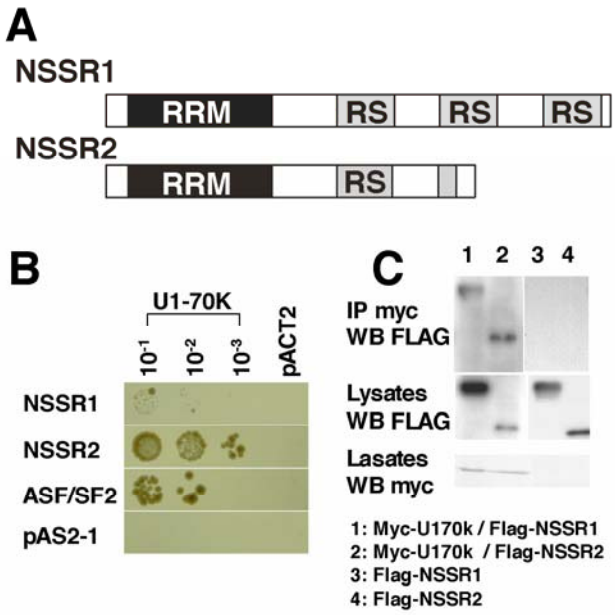

A
NSSR1
RRM
RS
RS
RS
NSSR2
RRM
RS
B
U1-70K
10-1
10-2
10-3
pACT2
NSSR1
NSSR2
ASF/SF2
pAS2-1
C
1 2 3 4
IP myc
WB FLAG
Lysates
WB FLAG
Lasates
WB myc
1: Myc-U170k / Flag-NSSR1
2: Myc-U170k / Flag-NSSR2
3: Flag-NSSR1
4: Flag-NSSR2

Figure1

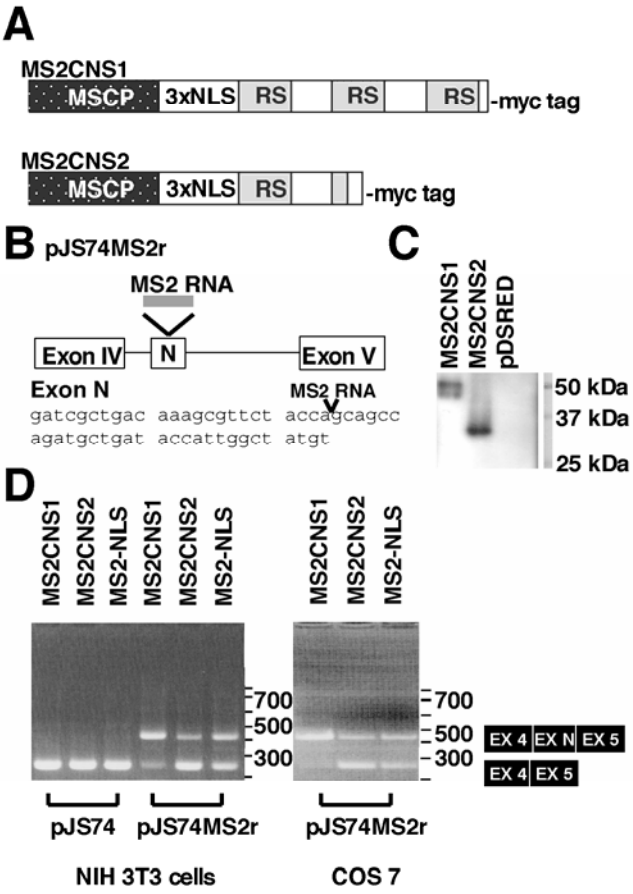

A
MS2CNS1
MSCP
3xNLS
RS
RS
RS
-myc tag
MS2CNS2
MSCP
3xNLS
RS
-myc tag
B
pJS74MS2r
MS2 RNA
Exon IV
N
Exon V
Exon N
MS2 RNA
gatcgctgac aaagcgttct accagcagcc
agatgctgat accattggct atgt
C
MS2CNS1
MS2CNS2
pDSRED
50 kDa
37 kDa
25 kDa
D
MS2CNS1
MS2CNS2
MS2-NLS
MS2CNS1
MS2CNS2
MS2-NLS
MS2CNS1
MS2CNS2
MS2-NLS
700
500
300
700
500
300
EX 4
EX N
EX 5
EX 4
EX 5
pJS74
pJS74MS2r
pJS74MS2r
NIH 3T3 cells
COS 7

Figure 2

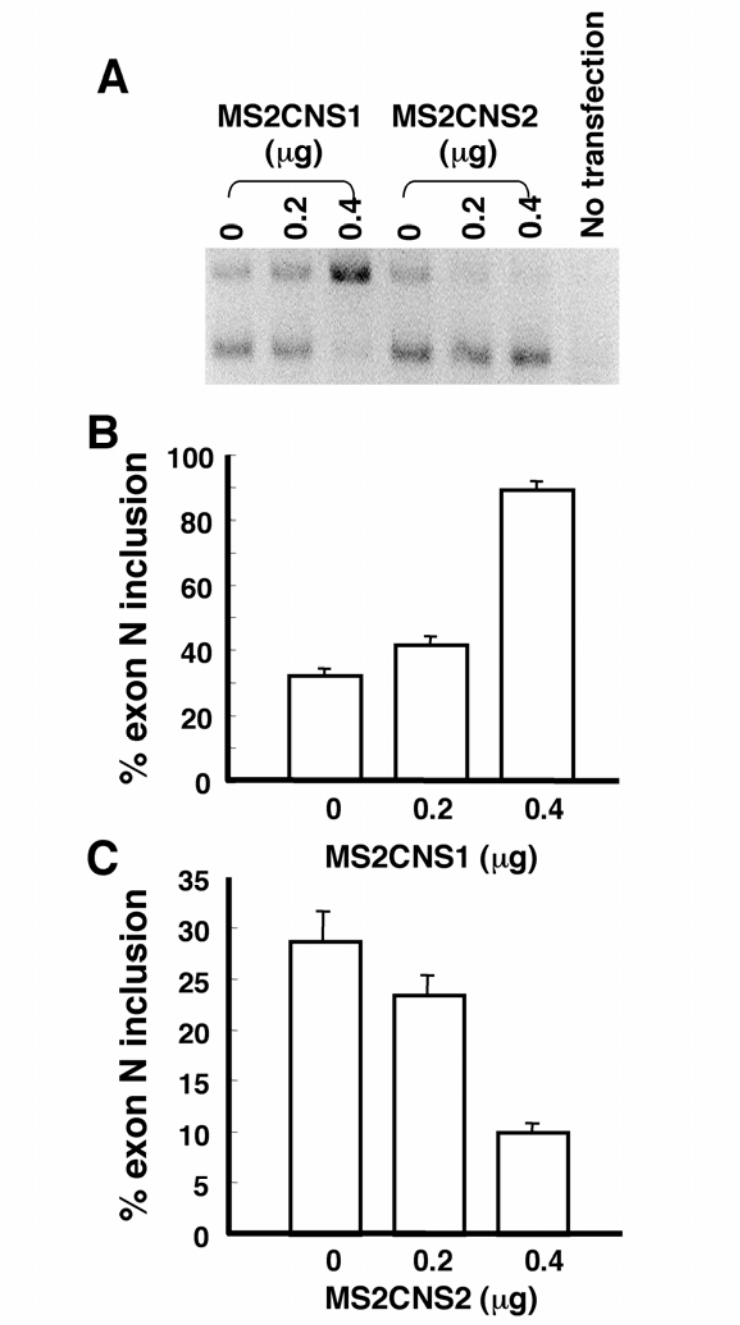

A
MS2CNS1 (μg)
MS2CNS2 (μg)
0
0.2
0.4
0
0.2
0.4
No transfection
B
% exon N inclusion
100
80
60
40
20
0
0
0.2
0.4
MS2CNS1 (μg)
C
% exon N inclusion
35
30
25
20
15
10
5
0
0
0.2
0.4
MS2CNS2 (μg)

Figure 3

A

```
        10          20          30          40          50
MSRYLRPPNT SLFVRNVADD TRSEDLRREF GRYGPIVDVY VPLDFYTRRP
        60          70          80          90         100
RGFAYVQFED VRDAEDALHN LDRKWICGRQ IEIQFAQGDR KTPNQMKAKE
       110         120         130         140         150
GRNVYSSSRY DDYDRYRRSR SRSYERRRSR SRSFDYNYRR SYSPRNSRPT
       160         170         180         190         200
GRPTRSRSHS DNDRFKHRNR SFSRSKSNSR SRSKSQPKKE MKAKSRSRSA
       210         220         230         240         250
SHTKTRGTSK TDSKTHYKSG SRYEKESRKK EPPRSKSQSR SQSRSRSKSR
       260
SRSWTSPKSS GH
```

B

Figure 4

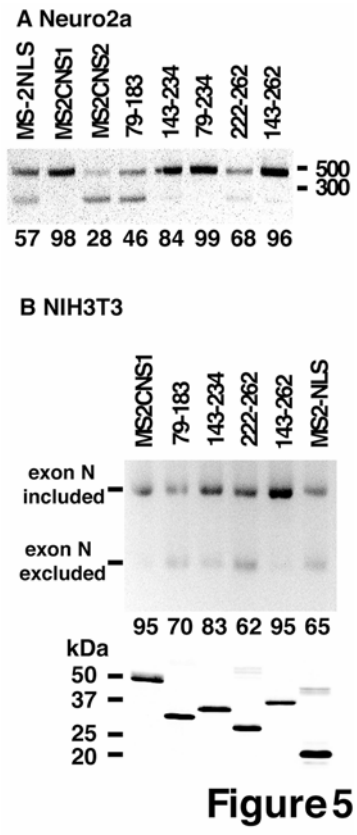
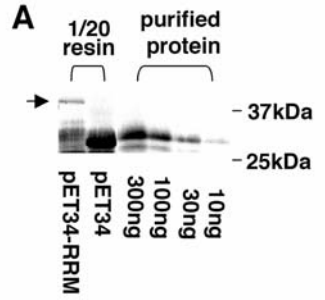
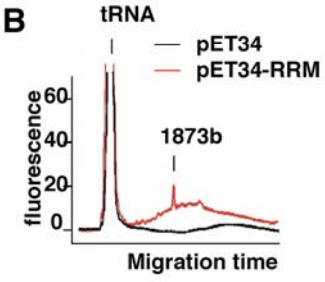

A Neuro2a
MS-2NLS
MS2CNS1
MS2CNS2
79-183
143-234
79-234
222-262
143-262
500
300
57 98 28 46 84 99 68 96
B NIH3T3
MS2CNS1
79-183
143-234
222-262
143-262
MS2-NLS
exon N included
exon N excluded
95 70 83 62 95 65
kDa
50
37
25
20

Figure 5


A
1/20 resin
purified protein
37kDa
25kDa
pET34-RRM
pET34
300ng
100ng
30ng
10ng


B
tRNA
pET34
pET34-RRM
1873b
fluorescence
60
40
20
0
Migration time

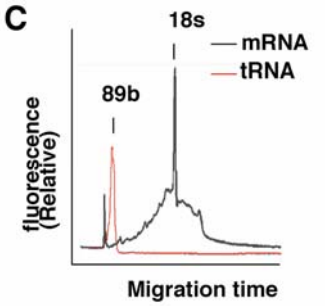

C
18s
mRNA
tRNA
89b
fluorescence (Relative)
Migration time

Figure 6