Prediction of tertiary structure of NSSRs’ RNA recognition motif and the RNA binding activity

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Abstract

RNAs possess potentials to become excellent bio-material because of their biochemical and biological activities. For instance, most RNA splicings are catalyzed by machinery including their own RNAs or other RNAs. The eukaryote machineries for splicing of pre-mRNA, which are called spliceosomes, are flexible and accurate for separating substrates. Although RNAs themselves catalyze the splicing, spliceosomes are supported by many proteins. Furthermore, a great accuracy is required for the alternative splicing because there are choices available, which must be regulated in tissue-specific and developmental manners. Neural-salient serine/arginine-rich (NSSR) proteins 1 and 2 are candidates for supporting the accuracy of the splicing. The features of their amino acid sequences suggest that NSSRs are SR proteins, which bind to pre-mRNA and determine the splicing site. Since SR proteins have a RNA recognition motif or motives (RRM or RRMs), which binds to RNA, we predicted the secondary and tertiary structures of NSSRs’ RRM by comparing them to RRMs of other proteins. The predicted structure suggested that the RNA binding activity of NSSRs’ RRM is similar to the poly A binding protein (PABP). Moreover, to detect the targets for NSSR, mRNAs were obtained by screening them from murine brains with bacterial recombinant NSSRs’ RRM and microarray experiments were conducted using these mRNAs. The results suggested that NSSRs bind specifically to particular pre-mRNAs and regulate the alternative splicing of the binding pre-mRNA.

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1. Introduction

Some RNAs have catalytic and biological activities; therefore, it is valuable to study them and to explore their applications for various fields, including material developments. Among these RNAs, self splicing is one of their catalytic activities. For example, many mRNAs in eukaryotic cells are produced when introns are spliced out from the pre-mRNAs, which are immediate copies of genomic DNA by transcription. This splicing is not an autocatalytic reaction, but catalytically active RNA components splice the pre-mRNA [1]. In this mechanism, many proteins cooperate to activate the catalytic activity. Among these proteins, SR proteins are included. The characteristics of the amino acid sequences of SR proteins are the presence of the RNA recognition motif/ motives (RRM/RRMs) at the N-terminal and the serin–arginine dipeptide sequence at the C-terminal. They recognize exons and bring the splicing machinery to the correct position to perform splicing at exact sites [2]. In addition, more than one mRNAs are produced from a single gene, due to different choices of exons on the gene in metazoan, which is known as alternative splicing. This alternative splicing is regulated in a tissue and developmental specific manner in living organisms. SR proteins also contribute to regulation of alternative splicing.

We previously reported that two genes for SR protein like polypeptides, neural salient serine/arginine-rich 1 and 2 (NSSRs 1 and 2), were cloned from a subtraction library of neuroectodermal differentiated P19 cells by degenerative PCR [3]. To characterize these genes, the conserved domain search was performed, which suggested the presence of an RRM in the N-terminal region of the products. Furthermore, their C-terminals consist of SR repetitive sequences.
However, there exists a difference between them; that is,
NSSR2 is shorter than NSSR1 due to alternative splicing. In
the expression levels, mRNAs for NSSR 1 and 2 are present
at higher levels in the brain and testis than in other tissues.
Unlike NSSR2, which is expressed before the differentiation
of the cells, NSSR1 is expressed in the neural stage during
the neuroectodermal differentiation of embryonal carci-
nomata cells, P19, suggesting that it is engaged in the produc-
tion of neuronal splicing products. In addition, the
homology search for NSSRs revealed that there exist high
similarities with many RNA binding proteins, including SR
proteins. Currently, it is known that the overexpression of
NSSRs regulate alternative splicing of GluR-B gene [3];
however, it is not yet clear how the RRM of NSSRs
contributes to the regulation of its splicing activities.

In recent years, the amount of protein structures analyzed
by NMR and X-ray crystallography has increased tremen-
dously. Among them, RRM is one of the well characterized
polypeptide structures, which consists of four β-sheets
strands and α-helix in between the strands (β/α/β/β/α/β)
[4–10]. A typical RRM is 90–100 amino acid long with two
conserved sequence motifs, RNP-1 [(K/R)-G-(F/Y)-(G/A)-
F-V-x-(F/Y)] and RNP-2 [(L/I)-(F/Y)-(V/L)-(G/K)-(G/N)-
(L/M)] [6]. Aromatic amino acids on RNP-1 and 2 interact
with bases of their target RNAs by stacking on the surface
of their RRM's. Their RNA binding activity is supported
primarily on their β sheet surface.

Microarrays are a very useful tool to analyze relative
amounts of mRNA in a high-throughput manner. They can
be applied for analyses for mRNA expression to understand
biological phenomena, diseases or diagnoses. Their basic
procedural scheme is that nucleic acids are labeled by
fluorescent dyes, such as Cy3 and Cy5, and the labeled
nucleic acids are hybridized with spotted probes on the
microarray slide to give fluorescent intensities, which
represent the amount of nucleic acids contained in the cells.

In this paper, we predicted the structure of the RRM in
NSSRs and showed its possibility of binding to an RNA.
Furthermore, using combination of recombinant technology
and microarray analysis, we successfully screened murine
brain mRNAs to find NSSRs’ targets.

2. Experimental procedure

2.1. Structure analyses of NSSRs’ RRM

The comparison of amino acid sequences was performed
using conserved domain database search at NCBI (http://
www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) [11]. For
the prediction of the secondary structure of NSSRs’ RRM,
PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/psiform.html)
was utilized [12]. The tertiary structure of NSSRs’ RRM
was computed through SWISS MODEL (http://swissmodel.
expasy.org/SWISS-MODEL.html) [13]. The structures
were visualized with Swiss PDB Viewer (http://kr.expasy.
org/)

2.2. Production and extraction of the NSSRs’ RRM recombinant protein

To produce an NSSRs’ RRM recombinant protein, the
dNA fragments that code the RRM were amplified by high
fidelity PCR (pyrobest, TAKARA) using primers, RRMR
5'-ctggaaatctccatggccagtcttgagcc-3' and RRMR 5'-
cgaaatctcatctgtatcatatcgc-3'. These fragments were
subcloned into the EcoRI site of pYesTrp3. The obtained
plasmids were digested with HindIII and Xhol to insert into
pET-34b (+), which carries a cellulose binding domain tag
(CBD tag, Novagen).

The NSSRs’ recombinant protein was produced in the
100 ml culture of E. coli, Rosetta(DE3)pLysS, by
induction with 1 mM IPTG in LB medium for three hours at
37 °C. The bacterial pellet was frozen once, resuspended
with PBS containing the protease inhibitor cocktail
(NACALAI TESQUE), and then lysed by sonication though
the treatment on ice (VIBRA CELL, SONIC and
MATERIAL). The lysate was concentrated by ultrafiltration
using Amicon Ultra-4 (MWCO 10,000, MILLIPOR), and
then applied onto cellulose resin to detect the RNA binding
activity of NSSR’s RRM-CBD fusion protein.

2.3. Messenger RNA binding assay

Total RNA was extracted from the bulk of murine brains
obtained from 5-day-old to adult mice using TRIzol
(Invitrogen) followed by purification using mTRAP total
kit (ACTIVE MOTIF). The purified recombinant NSSRs’
RRM-CBD fusion protein was mixed with mRNA fraction,
then absorbed on 5 mg of cellulose resin (CBinD 100,
Novagen), and then applied onto cellulose resin to detect the RNA binding
activity of NSSR’s RRM-CBD fusion protein.

2.4. SDS-PAGE and immunoblot analysis

SDS-PAGE was performed as described by Laemmli
[14]. The proteins fractionated by SDS-PAGE were stained
with CBB (Rapid Stain CBB kit, NACALAI TESQUE) or
transferred onto PVDF membranes. Anti-CBD-Tag rabbit
polyclonal antibody (Novagen) was used as a primary
antibody (×5000 dilution), and alkaline phosphatase linked
anti-rabbit IgG (Chemicon) was used as secondary antibody
(×10,000 dilution). The CBD-tagged proteins were detected
using BICP-NBT solution kit (NACALAI TESQUE).
2.5. Microarray analysis

The procedure for the microarray experiment was followed as described in the products manual. Briefly, the purified RNA was reverse transcribed using the T7 tagged oligo-dT primer and then, labeled RNA with Cy3 or Cy5 (Low RNA Input Fluorescent Linear Amplification Kit, Agilent). These labeled cRNAs were hybridized onto Mouse Oligo Microarray Kit (Agilent). Then, the hybridized microarray was scanned using CRBIO IIe (Hitachi Software Engineering). The scanned image was analyzed using ScanAlyze version 2.50 (http://rana.lbl.gov/EisenSoftware.htm).

3. Results and discussion

3.1. Secondary structure analyses

The secondary structure of NSSR 1 was predicted using PSIPRED. As a result, $\alpha$-helix structures were suggested in positions from 23 to 32 and from 61 to 71, while residues next to both helices at the C- and N-terminals were likely to form $\beta$-strand structures (Fig. 1). Because an RRM forms a $\beta$/$\alpha$/$\beta$/$\alpha$/$\beta$ structure from its N-terminal and is about 100 amino acids long, the prediction by PSIPRED suggested that NSSRs have a similar structure to other RRMs (Fig. 2). The sequence features of NSSRs were further explored by comparison with other proteins. The following proteins were selected for comparison: solution structures of U2AF65 (accession; 1U2FA), hnRNP D0 RBD 1 (accession; 1HD0) and 2 (accession; 1IQT), and mushashi1 RBD 1 (accession; 2MSS), all of which were analyzed by NMR [7–10]. Their structures include four-stranded antiparallel $\beta$-sheets backed by two $\alpha$-helices, which are characteristics of RRMs. The comparison among the NSSRs’ RRM and

Fig. 1. Predicted secondary structure of NSSR. The predicted secondary structure of NSSR 1 was produced from its full primary sequence by the PSIPRED program using its default settings. E and yellow arrows indicate $\beta$-strands, and $\alpha$-helices are shown by H and green columns. C indicates possibility of random coils. From S11 to Q84, $\beta$/$\alpha$/$\beta$/$\alpha$/$\beta$ alignment was observed for NSSR1 as well as typical RRMs. In the C-terminal of NSSRs’ RRM, two $\beta$-strands were predicted ($\beta$4N and $\beta$4C) (For interpretation of the reference to color in this legend, the reader is referred to the web version of this article.).

Fig. 2. Comparison of primary structure of NSSRs’ RRM with other RRMs characterized by NMR. The RRMs, whose structures were determined by NMR, were aligned with NSSRs’ RRM. Gray shaded regions and underlined regions form $\alpha$-helices and $\beta$-strands, respectively. Yellow shaded parts indicate amino acids whose exposed solvent surrounds the RRMs’ $\beta$-sheets. RNP-1 and 2 motifs are conserved on third and first $\beta$-strands, respectively, and aromatic amino acids indicated by asterisks are exposed to the outside of the proteins (For interpretation of the reference to color in this legend, the reader is referred to the web version of this article.).
above mentioned proteins revealed that the amino acid sequences S11, F13 and R15 of the first β-strand from N-terminal (β1), V37, D38, Y40 and P42 of the second β-strand from N-terminal (β2), and F53, Y55 and Q57 of the third β-strand from N-terminal (β3) in the NSSRs’ RRM are likely to be exposed to the solvent that surrounds them. β1 and 3 were similar to the corresponding β-sheets of the NSSRs’ RRM predicted by PSIPRED, which are reflecting RNP-1 and 2 motifs in β3 and 1 of RRMs, respectively. The third amino acids on β1 and β3 of RRMs are usually aromatic amino acids (F or Y) that are on the surface of the proteins and interact with bases of the target nucleic acids by stacking. The conservation of aromatic amino acids at the 13 and 55th positions from the N-terminal of the NSSRs’ RRM (F13 and Y55) suggested that these amino acids are also located on the surface of the protein and stack with the bases of the target nucleic acids. However, β2 and β4 (the fourth β-strand from N-terminal) were not well-conserved among the compared RRMs, especially, the β4 of hnRNP D0 RRM2 has an unusual structure [8]. Although only one β-strand follows after β2 in a typical RRM, there are two possible regions to form β-strands in hnRNP D0 RRM2; in which, the N-terminal region (YHNV) forms an additional antiparallel strand (β4) with β4 consisting of the C-terminal region (SKCEIKVA). According to the prediction by PSIPRED, a highly possible region to form β-strands next to C-terminal of α2 is divided into two by a random coil as well as hnRNP D0 RRM2 (Fig. 1). Therefore, the NSSRs’ RRM may fold its structure in a similar manner as hnRNP D0 RRM2 consisting of five stands, β1–4 and 4-does.

3.2. Analysis of the tertiary structure of the NSSRs’ RRM

The tertiary structure of the NSSRs’ RRM was built using SWISS-MODEL. The structure of the domain 1 in human poly A binding protein chain A (accession: 1CVJ_A) was used as a template [5]. As a result, the identity of the amino acid sequences between NSSRs’ RRM and 1CVJ_A was 34.6% (Fig. 3), and the total final energy for NSSRs’ RRM was ~5046.952 KJ/mol. From these results, NSSRs’ RRM may have a similar structure to a typical RRM (Fig. 4A). In Fig. 4C, F13, F53 and Y55 in NSSRs’ RRM, which correspond to the solvent exposed aromatic amino acids, were found to be exposed to the solvent. The tertiary structure of NSSRs’ RRM was constructed through SWISS-MODEL. The cartoons of the structures for RSSRs’ RRM and PABP domain 1 are shown in (A) and (B), respectively. The aromatic amino acids conserved in RNP-1 (F53 and Y55) and RNP-2 (F13) of NSSRs were exposed to the solvent surrounding them, (C), as well as those of PABP domain 1, (D).
acids in the RNP-1 and 2 of PABP (Y14, Y54 and Y56 in Fig. 4D), were faced on the outside of the \( \beta \)-sheet. The secondary structure suggest that the RRM of NSSRs has the additional \( \beta \)-strand at the C-terminal as well as the hnRNP D0 domain 1 structure. The upstream short strand of the \( \beta_4 \) region (KWI) built a \( \beta \)-sheet with the next \( \beta \)-strand (RQIEIQFA) which also built the \( \beta \)-sheet structure with \( \beta_1 \). This structure was also observed in PABP domain 1. The tertiary structure analysis suggests that the NSSRs’ RRM polypeptide is fold into a typical RRM structure in coincidence with the secondary structure prediction other than the additional \( \beta \)-strand next to \( \beta_4 \) (Fig. 4C).

The electrostatic potentials of NSSRs’ RRM was calculated and shown in Fig. 5. The highly negative region was found around the second \( \alpha \)-helix of NSSR’s RRM (Fig. 5A and B), while PABP domain 1 did not have such a large negative area around its second helix region (Fig. 5C). Furthermore, the highly negative region around the second \( \alpha \)-helix was absent in other RRMs (Figs. 2 and 3). Most RRMs recognize RNA on the surface of their \( \beta \)-sheets. Therefore, this unique feature of NSSRs’ RRM suggested that there exists a specific interaction with certain positively charged proteins or repulsion of nucleic acids interacting with their \( \beta \)-sheets. Since the surface of the \( \beta \)-sheet in NSSRs’ RRM was neutral as well as that of PABP.
domain 1, the β-sheet in NSSRs’ RRM may interact with bases of RNA by aliphatic and van der Waals interaction, base stacking, and hydrogen bond.

Next, the spatial positions of the amino acids which interact with RNA in PABP domains 1 and 2 were compared with those of equivalent NSSRs’ amino acids. In the case of PABP domains 1 and 2, phosphates of RNA interact with K104, R89, Y14, Y54, and Y140 (Fig. 3). Among the equivalent positions of NSSRs’ RRM, only R15, which corresponds to K104 in the domain 2 of PABP, potentially interacted with phosphate of RNAs. Therefore, the manner in which the residue supports the phosphate of an RNA backbone seemed to be different between NSSR and PABP.

In respect of amino acids recognizing adenines, counterparts of NSSRs to the amino acids supporting Ade2 and Ade6 in PABP showed considerable similarity suggesting NSSRs hold adenines at those positions (Fig. 6 A, B, G, H, Table 1). Totally, the amino acids, which stack with adenines in PABP, were well conserved, but most of the counterparts for the amino acids specified adenines in NSSRs may not recognize adenines (Fig. 6, Table 1). The equivalent amino acids of NSSRs to amino acids interacting with Ade5 were unclear, because the amino acids straddle both of RRM domains 1 and 2 in PABP.

### Table 1
Comparison of amino acids responsible to recognize adenines in PABP with NSSR

| Position | PABP Interaction | Specifying NSSR |
|----------|------------------|-----------------|
| Ade2     | W1s              | R15             |
|          | N105             | N16             |
| Y140     | O6–N6, adenine   | N6              |
| Ade3     | OH–O1p           | F53             |
|          | F102             | F13             |
| R179     | N15              | R90             |
| K104     | N6–N7, Purine    | S11             |
| Ade4     | N100             | D37             |
|          | Y142             | F85             |
| Ade6     | O–N6              | F85             |
| W86      | N–N1s            | Q87             |
| Q88      | F14              | F13             |
| Ade7     | L52               | R51             |
| M46      | O151–N6, Adenine | F45             |
| D45      | Y56               | D44             |
| Ade8     | S12               | S11             |
| S127     | Y55               | S59             |
|          | N6–N7, Purine    | S127            |
|          | O–N6c Adenine    | O–N6c Adenine   |

Candidates to interact with bases were predicted by the tertiary structures. The amino acids, which stack with adenines in PABP, were well conserved in NSSRs. The amino acids shown in bold potentially support adenines in a similar manner to PABP.

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**3.3. RNA Binding activity of NSSRs’ RRM**

The structural observation prompted us to explore the RNA binding activity of NSSR and the targets for NSSR. Generally, SR proteins recognize exons of pre-mRNA to include the selected exons into the spliced mRNA. By using this mechanism, it is possible to screen for the target of the specific splicing. Therefore, we tried to identify target RNAs of NSSRs’ RRM. The DNA fragment coding RRM of NSSR was fused with CBD to produce the recombinant protein. The NSSR’s RRM-CBD fusion protein was obtained from *E. coli* lysate, and absorbed on 5 mg of cellulose resin. We confirmed that 2.6 μg of the purified fusion protein were absorbed on the resin. As shown in Fig. 7A, a band at about 46 kDa was detected as a major protein on the resin. In addition, it was reactive to the anti-CBD antibody (Fig. 7B). These results suggested that the CBD fused NSSRs’ RRM was successfully produced and trapped on the cellulose resin.

The mRNAs from murine brains were added onto the immobilized NSSRs’ RRM-CBD fusion protein/cellulose column and washed four times with the binding buffer to eliminate unbound mRNAs. Next, the trapped mRNAs were extracted by denaturation. The recovered mRNAs were amplified and labeled with Cy5. The untreated murine brain mRNAs were labeled with Cy3 as a reference. Both labeled cRNAs were hybridized onto the mouse oligo microarray, and scanned with CRBIO IIe. The scanned image was analyzed by ScanAlyze.
Fig. 8 shows the distribution of the expression ratio for the microarray data. Channel 1 was the Cy5 fluorescence intensities for the mRNA from NSSRs’ RRM trapped on the resin, and Channel 2 was the Cy3 fluorescence intensities for the reference murine brain mRNA. A single peak was observed, but the distribution was skewed towards the higher ratio than towards the lower, which might suggest that some mRNAs were concentrated for NSSRs’ RRM trapped on the resin. The most concentrated RNA was the clone IMAGE: 3584181 (13-fold expression level increase compared to the reference), and 63 other RNAs were concentrated more than 4.9 times (Table 2). These RNAs coded many types of proteins, including structure proteins (e.g. neurofilament-L), kinases (e.g. Mapk12), secretory proteins (e.g. Chromogranin B) and RNA-binding proteins (e.g. mHuC-L). Many hypothetical genes and pseudogenes were also found among them. Chromogranin B was listed many times therefore the microarray analysis was likely to be reliable. The Database searches for these genes through Ensembl Genome Browser (http://www.ensembl.org/) revealed that 27.5% of the identified genes had alternative splicing isoforms. Although NSSRs were expected as an alternative splicing regulator, there were many genes, which were not suggested as splicing isoforms in the list. Therefore, the splicing isoforms of these genes may be unidentified so far. Another possibility is that NSSRs participate in not only alternative splicing but also constitutive splicing in the brain.

Table 2

| Accession no. | Description                                                                 | CH1   | CH2   | Ratio  | Products |
|---------------|------------------------------------------------------------------------------|-------|-------|--------|----------|
| BC018347      | Similar to translation initiation factor IF2, clone IMAGE: 3584181            | 2018  | 26180 | 13.0   | 1        |
| BC040015      | clone MGC: 7593 IMAGE: 3493893                                               | 2715  | 24,295| 8.9    | 1        |
| BC023025      | clone IMAGE:5362343                                                          | 1868  | 16,519| 8.8    | –        |
| AJ278123      | mRNA for putative synaptotagmin                                                 | 5852  | 49,917| 8.5    | 2        |
| AK046243      | Hypothetical LIM domain, Villain headpiece domain containing protein            | 5157  | 43,490| 8.4    | –        |
| M20480        | Brain neurofilament-L                                                         | 5602  | 46,434| 8.3    | 1        |
| NM_029842     | Similar to THYRO1000124 PROTEIN                                               | 4340  | 35,554| 8.2    | 1        |
| AK009959      | Ankyrin-like repeat protein                                                   | 4811  | 39,116| 8.1    | 1        |
| NM_010123     | Eukaryotic translation initiation factor 3 (Eif3)                              | 1531  | 11,798| 7.7    | 1        |
| NM_008527     | Killer cell lectin-like receptor subfamily B member 1C (Klr1b1c)              | 3792  | 28,994| 7.6    | 3        |
| AK005564      | Similar to 60S RIBOSOMAL PROTEIN L17 (L23)                                    | 6515  | 46,949| 7.2    | 1        |
| BC04722       | clone IMAGE:3582796                                                          | 2268  | 15,787| 7.0    | –        |
| AK046455      | Weakly similar to RIBOSOMAL PROTEIN L18A HOMOLOGUE                            | 2155  | 14,759| 6.8    | –        |
| BC047049      | Similar to UPF3 regulator of nonsense transcripts homolog B                   | 1608  | 10,922| 6.8    | 2        |
| NM_010880     | Nucleolin (Ncl), mRNA                                                         | 1796  | 12,188| 6.8    | 1        |
| BC018011      | clone MGC:19,122 IMAGE: 4210,911                                             | 2074  | 14,073| 6.8    | –        |
| NM_007694     | Chromogranin B (Chgb)                                                        | 7926  | 53,452| 6.7    | 1        |
| NM_027349     | SIMILAR TO S164 PROTEIN homolog                                               | 1768  | 11,752| 6.6    | 3        |
| NM_007694     | Chromogranin B (Chgb)                                                        | 8047  | 52,766| 6.6    | 1        |
| NM_028071     | Coacactosin-like protein (Clp-pending)                                        | 7832  | 51,071| 6.5    | 1        |
| NM_013871     | Mitogen-activated protein kinase 12 (Mapk 12)                                 | 3225  | 20,954| 6.5    | 1        |
| NM_023153     | RIKEN cDNA 0610040D20 gene (0610040D20Rik                                   | 6668  | 43,229| 6.5    | 1        |
| NM_007694     | Chromogranin B (Chgb)                                                        | 8401  | 54,294| 6.5    | 1        |
| NM_007786     | Casein kappa (Csak)                                                           | 2327  | 14,940| 6.4    | 1        |
| NM_007684     | Centrin 3 (Cetn 3)                                                           | 2487  | 15,797| 6.4    | 1        |
| NG_001389     | Hmgb1-rs13 pseudogene on chromosome X                                         | 1641  | 10,273| 6.3    | –        |

(continued on next page)
4. Conclusions

In this paper, computational methods were applied to predict the secondary and tertiary structures of NSSRs’ RRM from its amino acid sequence. Through the homology searches, it was suggested that the RNA binding activity of NSSRs’ RRM is similar to that of a typical RRM, PABP. Furthermore, the NSSR’s targets were screen from the murine brain by a combination of recombinant technology and microarray analysis. These results suggested that NSSRs contribute to choices of exons for the alternative splicing by directly binding to its target pre-mRNAs.

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