J. Biochem. **133**, 615–623 (2003)
DOI: 10.1093/jb/mvg079

A Novel Rat Orthologue and Homologue for the *Drosophila crooked neck* Gene in Neural Stem Cells and Their Immediate Descendants

Naoki Amada[1,*], Tomoyuki Tezuka[1,*,†], Akila Mayeda[2], Kazuaki Araki[1], Nobuyuki Takei[1], Kazuo Todokoro[3] and Hiroyuki Nawa[1,‡]

[1]Molecular Neurobiology, Brain Research Institute, Niigata University, Asahimachi-dori 1-757, Niigata 951-8585; [2]Department of Biochemistry Molecular Biology, University of Miami School of Medicine, Miami, Florida 33136, USA; and [3]Laboratory of Molecular Cell Science, RIKEN institute, Ibaraki, 351-0198

Received February 4, 2003; accepted February 17, 2003

The crooked neck (*crn*) gene of *Drosophila melanogaster* encodes a scaffold protein carrying multiple tetratricopeptide repeat (TPR) motifs, and its mutation results in a reduction in the number of neuroblasts and lethality during larval stages. Here, we isolated two structurally related genes from a rat embryonic brain cDNA library. One gene is the rat orthologue of *crn*, which encodes 690 amino acids including 16 copies of TPR. The other gene, ATH55, encodes an 855 amino acid protein including 21 TPR motifs, which presumably represents a rat *crn* homologue and an orthologue of human XAB2. Both genes are highly expressed in embryonic brain but their expressions decrease during development. ATH55-like immunoreactivity is present in the ventricular zone and newly formed cortical plate, while CRN-like immunoreactivity is more abundant in a younger ventricular zone. In agreement, both proteins were found to be enriched in cultured neural stem cells and to decrease in response to cell differentiation signals. As indicated for the *yeast* CRN-like protein, ATH55 and CRN immunoreactivities were both recovered in the nuclear fraction and detected in the splicing complex carrying pre-mRNA. These findings suggest that both TPR-motif-containing proteins are involved in RNA processing of mammalian neural stem cells and their immediate descendants.

Key words: cortical layer, neural stem cell, splicing, tetratricopeptide repeat, XPA-binding protein 2.

Abbreviations: crn, crooked neck; EGF, epidermal growth factor; PCR, polymerase chain reaction; SSC, sodium chloride-sodium citrate buffer; TPR, tetratricopeptide repeats.

Much progress has been made toward understanding the molecular mechanisms underlying neural differentiation. To control individual neural cell fate, a large variety of transcriptional factors are sequentially expressed during neurogenesis as well as during subsequent neural differentiation into neuronal and glial linkages. In addition to such transcription factors that interact with DNA, various RNA binding proteins, such as Hu, musashi, PSF, and QKI, have also been identified in particular neural cell linkages and implicated in mammalian brain development (*1–4*). For example, PSF, named after the polypyrimidine-tract binding protein (PTB)-associating splicing factor, is highly enriched in embryonic neuroblasts and young neurons, where it regulates the second catalytic step of the splicing reaction (*5*, *6*). A gene, *qki,* that is responsible for the dysmyelination mutation called "quakingviable", is expressed in neural progenitor cells as well as in oligodendrocyte lineage cells, and regulates the subcellular localization of myelin basic protein mRNAs (*7*, *8*). In spite of their phenotypic association with neural differentiation or brain development, the biological functions of such RNA binding proteins in neural differentiation remain to be characterized. Recent investigations on *Drosophila* embryogenesis have provided clues to their functions(*9*, *10*). Various RNA binding proteins, such as Inscuteable and Staufen, play crucial roles in the asymmetric cell division of *Drosophila* neuroblasts: Staufen binds to and translocates *Prospero* mRNA in ganglion mother cells (*10*, *11*). Accordingly, RNA processing is now implicated in mammalian neural induction and/or differentiation.

Tetratricopeptide repeats (TPR) represent repetitive peptide motifs, each consisting of 34 amino acid residues that form double structures of alpha helix loops (*12*). These motifs are often found in the structures of cell cycle regulator genes in yeast, such as *cdc16, cdc23, cdc27*, which comprise the anaphase promoting complex (APC) (*13*). Individual motifs are suggested to interact with another TPR motif or with other proteins to make large protein complexes such as the APC. Recently, Nakatsu *et al*. identified another protein that carries multiple TPR motifs, XPA-binding protein 2 (XAB2) in HeLa cells (*14*). XAB2 interacts with various DNA repair-specific pro-

[*]Both authors contributed equally to this study.
[†]Present address: Mitsubishi Chemical Co, Yokohama Res. Ctr., 1000 Kamoshida-cho, Aoba-ku, Yakohama 227.
[‡]To whom correspondence should be addressed. E-mail: hnawa@bri.niigata-u.ac.jp

Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number NM139109 for rat ATH55 and NM053797 for rat CRN.

teins as well as with RNA polymerase II. The *Drosophila crooked neck* (*crn*) gene encodes a protein containing 16 copies of the TPR motif that is suggested to participate in pre-mRNA splicing (*15*, *16*). Human and yeast CRN-like proteins are also implicated in pre-mRNA splicing (*17*, *18*). The *crn* mutation in *Drosophila* causes abnormalities in the central and peripheral nervous systems. In addition, there are several proteins carrying TPR motifs that have been identified in the mammalian nervous system: TPR containing the Down syndrome gene product (TPR*D*), arylhydrocarbon interacting protein-like 1 (AIPL1), a novel candidate presenilin-1 interacting protein, and serine/threonine phosphatase PPT (*19–22*). All of these molecules contain only two or three copies of the TPR motif. The expression of TPRD and PPT is most enriched in the nervous system, especially in embryonic neuroblasts or stem cells, but for unknown reasons (*21*, *23*).

In the present study, we characterized the molecular structures and developmental regulation of two novel mammalian TPR-containing molecules, CRN and ATH55, in rat brain. ATH55 represents a rat orthologue of the human XAB2 (*14*). The potential involvement of ATH55 in a pre-mRNA splicing event was examined and is discussed with respect to neural differentiation.

MATERIALS AND METHODS

Differential Display Polymerase Chain Reaction—Rat neocortical cultures were prepared (*24*) and stimulated with 100 μM *N*-methy-D-aspartate (NMDA) in Mg-free Hanks buffer for 30 min, followed by incubation with complete medium for 7 h. A subsequent differential display polymerase chain reaction (PCR) was performed for the RNA extracted from the NMDA-stimulated and non-stimulated cultures according to the original procedure (*25*). In brief, total RNA was converted to cDNAs from a 10 μM anchor primer of oligo dT12N (N = dA, dC, or dG) with reverse transcriptase (Invitrogen Corp, Carlsbad, CA). Fragments of the resultant cDNAs were labeled and amplified in the presence of [^{32}P]dCTP by PCR. Both PCR products were subjected to polyacrylamide gel electrophoresis followed by autoradiography. PCR fragments that exhibited different strength of signals were subjected to DNA sequencing.

cDNA Library Screening—Two hundred thousand plaques of a lambda Zap library carrying embryonic rat brain cDNA (*26*) were screened with a labeled probe for the ATH55 PCR fragment or with a labeled probe for 50 mer synthetic oligonuleotides of the rat *crn* sequence. Hybridization was performed at 42°C in the presence of 50% formamide and a probe (2×10^6 cpm/ml), followed by stringent washing at 60°C with 0.3× sodium chloride-sodium citrate buffer (SSC). Fourteen clones were isolated for the ATH55 probe, and 21 clones for the *crn* probe. The largest cDNA in each group was subjected to automatic DNA sequencing. Both cDNAs were subcloned into an eukaryotic expression vector pCi (Promega Japan, Tokyo) to examine their expression.

RNA Analysis—Total RNA was extracted from adult male Sprague-Dawley rats (SLC, Shizuoka) (*27*). RNA samples were denatured in 50% formamide, 6% formaldehyde, 20 mM 3-(*N*-morpholino) propanesulfonic acid buffer (pH 7.0), and 1 mM EDTA, separated in a 1.5% formaldehyde-agarose gel, and transferred onto a nylon membrane. ^{32}P-labeled cDNA probes to ATH55 (the 984 nt *Bgl*I–*Bgl*I fragment) and to CRN mRNA (the 864-nt *Bam*H–*Bam*HI fragment) were generated using the Random primed DNA labeling kit (Roche Diagnostics, Tokyo). The probes (2×10^6 cpm/ml) were hybridized to filters for 20 h at 42°C in 50% formamide, 5x SSC, 5x Denhardt's solution, and 1% sodium dodecyl sulfate (SDS), washed with 0.1× SSC, 0.1% SDS at 60°C, and exposed to film.

Production of Antibodies—A cDNA fragment for the entire protein coding ATH55 (50–2617 nt) and a BamHI fragment (1241–2105 nt) for *CRN* were subcloned into a His-tagged expression vector, pET16b (Novagen, Madison, WI). The recombinant proteins were produced in *Escherichia coli*, BL21 strain, and purified on a nickel-conjugated affinity column (Invitrogen, Tokyo). The partially purified proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the protein bands for CRN and ATH55 were recovered. The recombinant proteins (1.0 mg) were emulsified with Freund's complete adjuvants and injected into rabbits every 2 weeks for 3 months (*28*). Serum batches with higher titers were subjected to antigen-affinity chromatography, which was performed using 1 mg of the recombinant protein and a 1-ml bed of Affi-Gel 10 (BioRad, Tokyo).

Cell Culture—Whole striatum of E16 rats was mechanically dissociated and plated onto poly-D-lysine and laminin-coated dishes at a density of $2–4 \times 10^3$ cells/ml. According to the method of Reynolds *et al.* (1992) (*29*), the dissociated cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing epidermal growth factor (EGF; Sigma Chemical Co., St. Louis, MO; 20 ng/ml). Cell spheres of growing progenitor cells were replated onto the coated dishes. More than 80% of the replated cells were immunoreactive to the antibody against nestin (data not shown). To induce neuronal or astroglial differentiation, EGF was replaced with brain-derived neurotrophic factor (BDNF; 50 ng/ml) or leukemia inhibitory factor (LIF; 10 ng/ml), respectively. Alternatively, the culture was stimulated with basic fibroblast growth factor (bFGF; 10 ng/ml). Human embryonic kidney cells (HEK293) were also cultured in DMEM containing 10% fetal bovine serum and transiently transfected with cDNA for ATH55 or CRN by the lipofection method.

Immunoblotting Analysis—Protein was denatured in sample buffer [20 mM Tris (pH 6.8), 2% SDS, and 0.7 M β-mercaptoethanol], separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Schleicher and Schull, Dassel, Germany). Immunoblots were treated with rabbit affinity purified anti-CRN antibody (1 μg/ml) or rabbit anti-ATH55 antiserum (1:1,000). Alternatively, anti-SF2/ASF monoclonal antibody (mAb96, gift from Dr. A.R. Krainer), anti-U1 70K protein monoclonal antibody (mAbH111, gift from Dr. R. Lührmann), rabbit anti-U5 snRNP 116 kDa protein antibody (gift from Dr. R. Lührmann), anti-neuron-specific enolase (NSE), anti-nestin antibody, or anti-GFAP antibody (all from Chemicon International, Temecula, CA) was used as a primary antibody. Immunoreactivity was visualized by chemiluminescence reaction (ECL kit; Amersham, Tokyo).

Immunohistochemistry—Whole brains of embryonic rats were fixed with 4% paraformaldehyde and 2% sucrose in

A

CRN

TPR-1	VAKVKNKAPAEVQIT--------AEQLLREAKERELELLPPP	(15-48)
(TPR-2)	PQQKITDE--EELND--------YKLRKRKTFEDNIRKNRTV	(49-80)
TPR-3	ISNWIKYAQWEESLK--------EIQRARSIYERALDVDYRN	(81-114)
TPR-4	ITLWLKYAEMEMKNR--------QVNHARNIWDRAITTLPRV	(115-148)
(TPR-5)	NQFWYKYTYMEEMLG--------NVAGARQVFERWMEWQPE-	(149-181)
(TPR-6)	EQAWHSYINFELRYK--------EVERARTIYERFVLVHPA-	(182-214)
TPR-7	VKNWIKYARFEEKHA--------YFAHARKVYERAVEFFGDE	(215-248)
TPR-8	EHLYVAFAKFEENQK--------EFERVRVIYKYALDRISKQ	(252-285)
TPR-9	QELFKNYTIFEKKFGDRRGIEDIIVDRRRRFQYEEVKANPHN	(288-329)
TPR-10	YDAWFDYLRLVESDA--------EADTVREVYERAIANVPPI	(330-363)
TPR-11	IYLWVNYALYEELEAK-------DPERTRQVYQASLELIPHK	(373-407)
TPR-12	AKMWLYYAQFEIRQK--------NLPFARRALGTSIGKCPKN	(412-445)
(TPR-13)	-KLFKGYIELELQLR--------EFDRCRKLYEKFLEFGPEN	(446-478)
TPR-14	CTSWIKFAELETILG--------DIERARAIYELAISQPRLD	(479-512)
(TPR-15)	EVLWKSYIDFEIEQE--------ETERTRNLYRQLLQRTQH-	(515-547)
TPR-16	VKVWISFAQFELSSGKEG-----SVAKCRQIYEEANKTMRNC	(548-584)

ATH55

(TPR-1)	VKCWLRYIEFKQGAPK----PRLNQLYERALKLLPCS	(35-67)
TPR-2	YKLWYRYLKARRAQVKH---RCVTDPAYEDVNNCHER	(68-101)
TPR-3	PRLWLDYCQFLMDQGRV---THTRRTFDRALRALPIT	(110-143)
TPR-4	SRIWPLYLRFLRSHPLP---ETAVRGYRRFLKLSPES	(146-179)
TPR-5	AEEYIEYLKSSDRLDEA---AQRLATVVNDERFVSKA	(180-213)
TPR-6	YQLWHELCDLISQNPDK---VQSLNVDAIIRGGLTRF	(218-251)
TPR-7	GKLWCSLADYYIRSGHF---EKARDVYEEAIRTVMTV	(256-289)
(TPR-8)	TQVFDSYAQFEESMIAA---KMETASELGREEEDD--	(293-324)
(TPR-9)	VDLELRLARFEQLISRR---PLLLNSVLLRQNPHH--	(325-356)
(TPR-10)	VHEWHKRVALHQ-GRPR---E-IINTYTEAVQTVDPF	(357-388)
(TPR-11)	HTLWVAFAKFYEDNGQL---DDARVILEKATKVN---	(395-425)
TPR-12	FKQVDDLASVWCQCGEL---ELRHENYDEALKLLRKA	(426-459)
TPR-13	LKVWSMLADLEESLGTF---QSTKAVYDRILDLRIAT	(484-517)
TPR-14	PQIVINYAMFLEEHKYF---EESFKAYERGISLFKWP	(518-551)
TPR-15	SDIWSTYLTKFISRYGGRKLERARDLFEQALDGCPPK	(554-590)
(TPR-16)	KTLYLLYAQLEEEWGLA---RHAMAVYDRATRA----	(593-622)
TPR-17	VEPAQQYDMFNIYIKRA---AEIYGVTHTRGIYQKAI	(623-656)
TPR-18	REMCLRFADMECKLGEI---DRARAIYSFCSQICDPR	(665-698)
TPR-19	GAFWQTWKDFEVRHGNE---DTIREMLRIRRSVQATY	(701-734)
TPR-20	SDLAPGQSGMDDMKLLE---QRAEQLAAEAERDQPPR	(757-790)
TPR-21	KIFFVRSDASREELAEL---AQQANPEEIQLGEDEDE	(794-827)

B

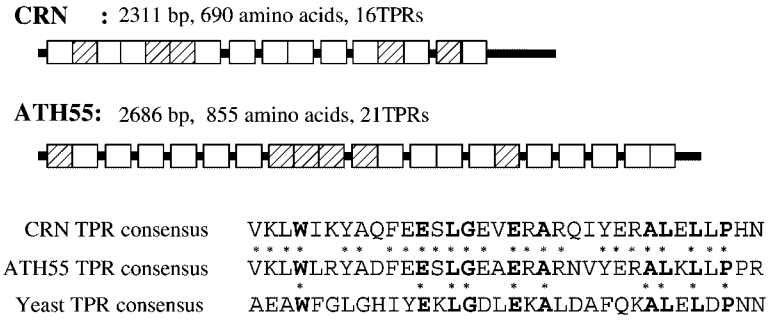

Fig. 1. **Rat CRN and ATH55 structures and their TPR motifs.** (A) Putative TPR motifs containing double α-helix-loops were aligned for in CRN and ATH55. Defective TPR motifs, whose lengths are shorter than 34 amino acid residues, are indicated in parentheses. (B) A typical TPR motif and degenerated TPR motif are shown schematically as open boxes and shaded boxes, respectively. The most frequently used amino acid residues in individual TPR motifs of CRN and ATH55 are shown as their TPR consensus sequence and compared to the consensus TPR sequence among yeast *cdc* proteins (*12*).

0.15 M phosphate buffer. Sagittal sections of the brain (10 μm thick) were made using a cryostat and dehydrated. Tissue sections were incubated with rabbit anti-CRN antibody (5 μg/ml) or rabbit anti-ATH55 antiserum (1:1,000) in the presence of 0.3% Triton X-100. After washing, sections were incubated with biotinylated goat anti-rabbit IgG diluted (1:200, Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized with the Avidin-Biotin system using 3,3′-diaminobenzidine as a substrate.

Nuclear Fractionation of HeLa Cells—HeLa cells were resuspended in 1 ml of 10 mM Tris buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM PMSF and homogenized by extrusion through a 23 gauge needle in the presence of 0.5% Triton X-100 (*30*). After centrifugation at 1,000 rpm for 10 min, precipitates were recovered as the nuclear fraction and the supernatant was recovered as the cytoplasmic fraction. Both fractions were used for immunoblotting as described above.

In Vitro Splicing and Immunoprecipitation—m7GpppG-capped ^{32}P-labeled β-globin pre-mRNA substrates were made by runoff transcription of linearized template DNA with SP6 RNA polymerase (*31*). A HeLa cell nuclear extract was prepared as described (*32*). *In vitro* splicing reactions (250 μl) containing 80 μl of S100 extract and 800 fmol of ^{32}P-labeled pre-mRNA were incubated at 30°C for 120 min (*31*). Immunoprecipitation (0.5 ml) was carried out with 25 μl of the splicing reaction mixture in

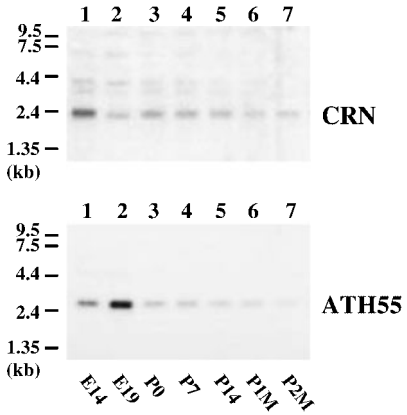

Fig. 2. **Developmental regulation of CRN and ATH55 expression.** Brain mRNA levels for CRN and ATH55 were examined during rat development by RNA blotting. Rat cerebral cortex was isolated from rats of various ages and total RNA was extracted. PolyA+RNA (1.8 μg) was separated in a 1.5% formamide agarose gel and blotted onto a nitrocellulose membrane. The RNA blot was probed with a *Bam*HI–*Bam*HI restriction fragment (864 bp) for CRN mRNA and a *Bgl*I–*Bgl*I (984bp) for ATH55 mRNA. Lanes 1–7 correspond to embryonic day E14 and E19, and postnatal day P0 and 7, 14, and postnatal month 1 and 2, respectively.

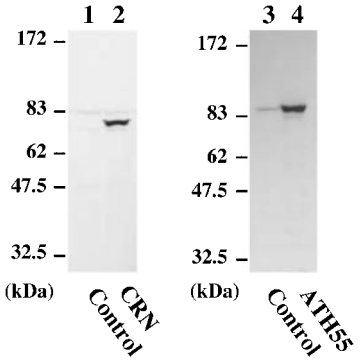

Fig. 3. **Detection of CRN and ATH55 proteins by immunoblotting.** A eukaryotic expression vector carrying rat CRN cDNA or ATH55 cDNA was transiently transfected into HEK293 cells. Cell lysates were prepared from the control HEK293 cells and the HEK293 cells transfected with either the CRN cDNA or ATH55 cDNA. Proteins were separated by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-CRN polyclonal antibody or anti-ATH55 polyclonal antiserum. Lanes 1 and 3, control HEK293 cells; lane 2, *crn*-transfected HEK293 cells; lane 4, ATH55-transfected HEK293 cells. Rat CRN cDNA encodes 690 amino acid residues with a calculated molecular weight of 83411 while ATH55 cDNA encodes 855 amino acids with a calculated molecular weight of 99992.

the presence of specific and control antibodies as described previously (*33*). The following antibodies were used: rabbit anti-ATH55 polyclonal antiserum (40 μl, 160 μl), rabbit anti-CRN polyclonal antiserum (40 μl, 160 μl), anti-SF2/ASF monoclonal antibody (mAb96; 8 μl ascites), anti-hnRNP A1 monoclonal antibody (9H10, 2 μl ascites), and anti–maltose binding protein (MalE) antiserum (2 μl) as a control. The RNA products of the immunoprecipitates were analyzed by electrophoresis in a 5.5% polyacrylamide/7 M urea gel and autoradiography.

RESULTS

We identified and cloned novel 2,686-bp and 2,311-bp cDNAs from an embryonic rat brain library, both of which encode multiple units of a 34-amino acid repeat similar to the tetratricopeptide repeat (TPR) (*12*). The 2,686-bp cDNA encodes a protein of 855 amino acid residues, designated ATH55, which shares 61% identity to *Drosophila melanogaster* CG6197 and 98% identity to the human XAB2 (*14*, *34*). The gene product of the 2,311-bp cDNA is a protein of 690 amino acid residues that shares 67% identity to the gene product of Drosophila *crn* and 28% identity to the ATH55 protein. The rat CRN protein contains 16 copies of the TPR motif, while the ATH55 protein carries at least 15 copies (Fig. 1A). If TPR with incomplete lengths are included, there are 21 copies of the TPR-like motif in the ATH55 protein. The core structure of the TPR motif of ATH55 is more homologous to the consensus sequence of the TPR of CRN than those of other TPR proteins in the *cdc*-family and the PPT family (Fig. 1B). RNA blotting analysis was performed on RNA prepared from developing rat neocortices. CRN mRNA levels were highest on embryonic day (E) 14 while ATH55 mRNA levels reached a peak on E19, and the levels of both decreased gradually with increasing age (Fig. 2).

To study their precise anatomic distributions, antibodies were raised in rabbits against recombinant ATH55 and CRN proteins. The anti-CRN antibody recognized a single ~80 kDa band protein and the anti-ATH55 antiserum marked an ~100 kDa protein in the cell lysates of HEK293 cells transfected with either CRN cDNA or ATH55 cDNA, respectively. The sizes are consistent with the molecular weights estimated from their nucleotide sequences (Fig. 3). There was a low level of endogenous ATH55 immunoreactivity in control HEK293 cells. To examine the anatomical distribution of the CRN and ATH55 proteins, we immunostained developing cerebrum of embryonic rats with the anti-CRN and anti-ATH55 antibodies. CRN-like immunoreactivity was detected in the ventricular zone (VZ) of the developing neocortex on E14 (Fig. 4). The immunoreactivity was localized in the VZ of the cortical plate on E16 and also in the newly formed cortical plate. When cortical migration ceased on E18, the immunoreactivity in the cortical plate began to decrease. The ATH55-like immunoreactivity exhibited a similar distribution and developmental regulation (Fig. 5). Its level was highest in the VZ of E14 embryos and also detectable in the outer cortical layer at E16–18. In comparison with the CRN-like immunoreactivity, however, the ATH55-like immunoreactivity was more intense and spread throughout the whole cortical layer at E16. The developmental changes in CRN or ATH55 immunoreactivity were similar to their patterns of mRNA expression.

To confirm the enrichment of CRN and ATH55 proteins in neuroblasts and/or neural stem cells, we prepared neural stem cell cultures from embryonic striatum according to Reynolds *et al* (*29*). Embryonic striatal cells were dissociated and grown under serum-free conditions in the presence of EGF. When the neural stem cell-enriched culture was stimulated with cell differentiation factors, the expression of CRN and ATH55 was signifi-

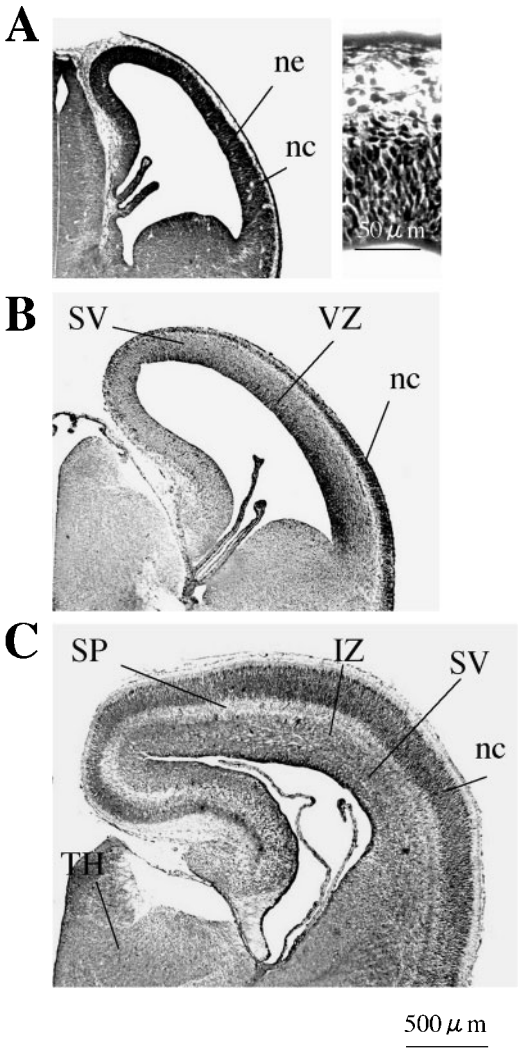

Fig. 4. **CRN-like immunoreactivity in developing rat cerebrum.** The distribution of CRN-like immunoreactivity was examined by immunohistochemical analysis. The whole cerebrum was isolated from embryonic rats (E14, E16, and 18 days) and fixed with paraformaldehyde in phosphate-buffer. Coronal sections (10 μm) were prepared and immunostained with the rabbit anti-CRN antibody. A magnified view of the cortical plate at E14 is shown on the right side. Abbreviations used; ne, neuroepithelium; SP, subplate; nc, neocortical plate; TH, thalamus; SV, subventricular zone; IZ, intermediate zone; VZ, ventricular zone.

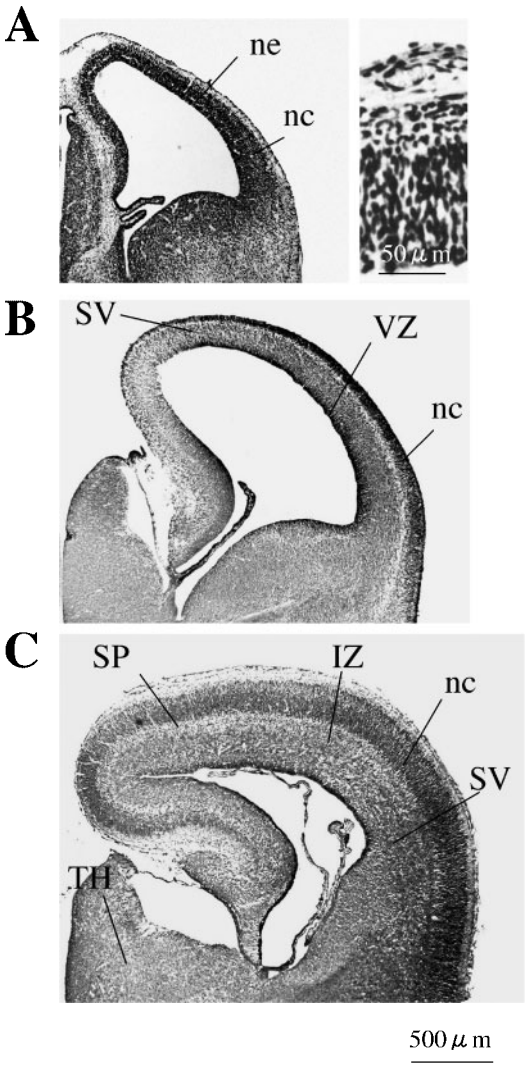

Fig. 5. **ATH55-like immunoreactivity in developing rat cerebrum.** The distribution of ATH55-like immunoreactivity was similarly examined using anti-ATH55 antiserum. See legend to Fig.4 for the abbreviations used.

cantly altered (Fig. 6). bFGF treatment, which is known to stimulate stem cell proliferation (*35*), maintained the basal level of nestin expression in the stem cell culture. bFGF treatment did not affect either the CRN or ATH 55 levels in comparison with their control levels. As reported by Yoshida *et al*. (*36*), LIF treatment markedly induced the expression of GFAP, a marker for astrocytes, and conversely down-regulated the expression of CRN and ATH55. In addition, a neuronal differentiation factor, BDNF (*37*), increased neuron-specific enolase (NSE) levels and inhibited the expression of both CRN and ATH55. These results suggest that both TPR-motif-containing proteins, CRN and ATH55, are most enriched in neural stem cells and might contribute to stem cell maintenance or differentiation.

Recently, Chung *et al*. (*17*) reported that the mammalian CRN is a component of the spliceosome and is involved in pre-mRNA splicing as proposed previously for a yeast *crn*-like gene product (*18*). We examined the possibility that ATH55 is also involved in splicing. First, we attempted to confirm the presence of the ATH55 and CRN proteins in HeLa cells and also examined the nuclear localization of CRN and ATH55 using other splicing factors as controls (Fig. 7A). In cultured HeLa cells, CRN and ATH55, as well as SF2/ASF, U1 and U5 snRNPs, were all enriched in the nuclear fraction rather than in the cytoplasmic fraction. U1 snRNP and U5 snRNP are essential splicing factors involved in the recognition of the 5′ splice site and flanking exon, respectively (*38*). SF2/ASF is an essential member of the SR protein family and is suggested to function in correct splice site selection by bridging between components bound to the 5′ and 3′ splice sites (*39*). To examine whether ATH55 and CRN are components of the spliceo-

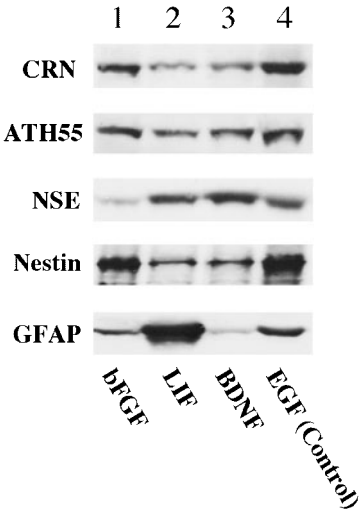

Fig. 6. **Regulation of CRN and ATH55 expression by growth factors in striatal neural stem cells.** Striatal neural stem cells were grown and concentrated from embryonic rat striatum using the neural sphere formation technique (*26*). Proliferating striatal neural stem cells were replated in poly-D-lysine coated dishes and grown in the presence of bFGF, LIF, BDNF, or EGF (control). The influnces of growth factors were examined by immunoblotting with the anti-CRN and anti-ATH55 antibodies as well as antibodies for a neuronal marker, NSE (47 kDa), a stem cell marker, nestin (198 kDa), and an astroglial marker, GFAP (50 kDa).

some, *in vitro* splicing of ^{32}P-labeled β-globin pre-mRNA was performed with Hela cell nuclear extract (*32*). RNA splicing products were immunoprecipitated with the anti-ATH55 and anti-CRN antisera together with control antibodies (Fig. 7B). The anti-SF2/ASF antibody mainly pulled down the fully spliced RNA products and the anti-hnRNP A1 antibody immunoprecipitated both the fully spliced RNA products and a lariat form of intron RNA (*40*). We found that the anti-ATH55 antiserum immunoprecipitated all the splicing products of β-globin pre-mRNA. The results suggest that ATH55 is a component of the active spliceosome. These splicing products were also precipitated with anti-CRN antiserum, although less efficiently, which may be due to the lower affinity of the antibody (data not shown). It might be noteworthy that the ATH55 immune complexes appeared to include a relatively higher amount of the first exon fragment in comparison with SF2/ASF immune complexes or hnRNP A1 immune complexes.

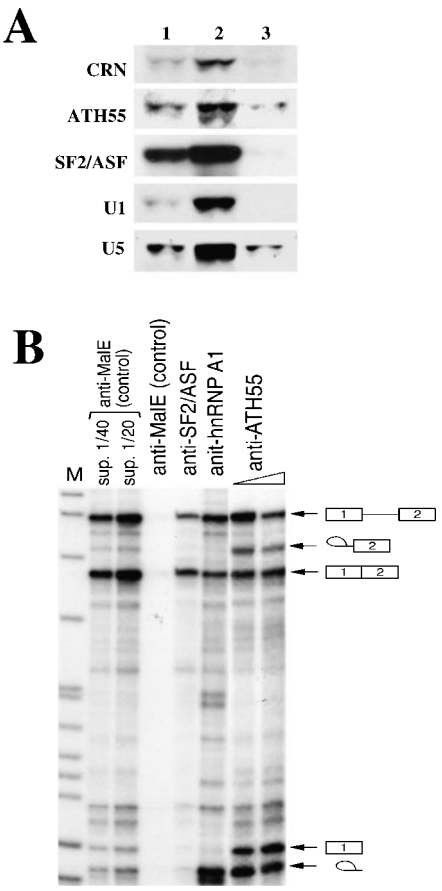

Fig. 7. **Nuclear localization of CRN and ATH55 in HeLa cells and their association with splicing complexes.** (A) Nuclear and cytoplasmic fractions of cultured HeLa cell lysates were prepared and subjected to immunoblotting with antibodies against CRN and ATH55, as well as the splicing factors; SF2/ASF (33 kDa), U1 (70 kDa), and U5 (116 kDa). Nuclei and cytoplasmic fractions of cultured Hela cells were prepared and subjected to immunoblotting with the anti-CRN antibody and antiserum for ATH55 as well as antibodies for splicing factors; SF2/ASF (33 kDa), U1 snRNP protein (70 kDa) and U5 snRNP protein (116 kDa). Lanes 1, 2 and 3 represent the total cell lysate, nuclear fraction and cytoplasmic fraction, respectively. (B) Immunoprecipitation of splicing reactions. *In vitro* splicing reactions with labeled β-globin pre-mRNA were incubated and immunoprecipitated with the indicated antibodies immobilized on protein G-Sepharose. RNA was extracted from the pellets and analyzed by denaturing PAGE and autoradiography. Portions (1/20 and 1/40) of the total RNA recovered from the supernatant (sup) of an immunoprecipitation with the anti-MalE antiserum (control) reflect the initial relative abundances of the pre-mRNA and splicing products (whose structures are shown schematically on the right). M: ^{32}P-labeled pBR322/HpaII DNA marker.

DISCUSSION

In the present investigation, we characterize the molecular features of two novel TPR-motif-containing proteins, CRN and ATH55, which are expressed in rat brain, predominantly in neural stem cells and/or differentiating neuroblasts. Almost the entire structures of CRN and ATH55 are composed of the repeating 34 amino acid motif (TPR) and they carry 16 repeats and 21 repeats, respectively. Recently, a human orthologue of the *Drosophila crooked neck* gene has been identified (*41*). Consistent with the rat *crn* gene, the human *crn* gene comprises at least 3903 bp with alternative splicing variations and encodes mainly a 687-amino acid protein with 16 copies of the TPR motif. The TPR motif is suggested to mediate a protein-protein interaction and is present in various types of molecules, *e.g.*, proteins involved in the cell cycle (*cdc*16, *cdc*23, *cdc*27) (*42*, *43*), synaptic vesicle protein (SGT) (*44*), postsynaptic scaffold protein (Rapsyn) (*45*), motor protein (kinesin) (*46*), and molecules involved in RNA splicing (SYF1 and SYF2) (*47*). Among these, CRN and ATH55 share 39% and 28% homology to SYF3 and SYF1, respectively. SYF1 and SYF3 are yeast proteins that interact with other splicing components (*47*). Their structural similarity to ATH55

also implies their potential involvement in pre-mRNA splicing processes. The human orthologue of ATH55, designated XAB2, is known to associate with RNA polymerase II and various transcription-coupled DNA repair proteins including XPA, CSA and CSB (*14*). In this context, our evidence that mammalian ATH55/XAB2 is another stable component of the active spliceosome is of considerable interest. It remains to be determined how or why the three molecular processes of transcription, DNA repair and pre-mRNA splicing are coupled by ATH55/XAB2.

During cerebral cortical development, both the mRNA and protein levels of CRN and ATH55 reach the maximal levels in the late embryonic stages when neural stem cells proliferate and migrate in the cortical plate. Their expression gradually decreases in the postnatal period. When we compared the expression of CRN and ATH55 mRNAs in various neural and non-neural tissues of adult rats, their mRNA levels in the mature brain remained as strong as in several peripheral tissues, including liver and kidney that presumably contain many proliferating cells (data not shown). In this context, the lower expression of both TPR molecules in the periphery might rule out their involvement in conventional cell cycle processes. Rather, the fact that the testis as well as neural stem cells specifically express extraordinarily high levels of both CRN and ATH55 mRNA suggest their contribution to stem cell maintenance or differentiation (data not shown). In developing rat neocortex, however, the expression patterns of these TPR proteins appear to differ: CRN mRNA levels peak at E14 while ATH55 mRNA reach a maximum level at E19. In agreement, CRN-like immunoreactivity is more limited to young neural stem cells in VZ while the higher immunoreactivity for ATH55 remains in the newly formed neocortical plate. These observations imply that the TPR proteins, CRN and ATH55, are involved in pre-mRNA splicing but in different neural cell lineages. Strong ATH55-like immunoreactivity as well as CRN-like immunoreactivity were observed in the VZ and/or newly formed cortical plate at rat embryonic stages E14–E16, but diminished gradually during later neocortical maturation. In agreement, the stimulation of EGF or bFGF, but not BDNF or LIF, is essential to maintain in the higher expression of CRN and ATH55 proteins in cultured neural stem cells. Together, these observations presumably suggest novel functions of TPR-motif-containing molecules CRN and ATH55 in neural stem cells.

A variety of RNA binding proteins exist in developing neuroblasts and neural stem cells (*48*). For example, proteins in the Elav (embryonic lethal abnormal vision) or Musashi families are expressed in mature neurons and in neural stem cells, and associate with mRNA to stabilize or polarize mRNA (*48–50*). Elav expression is higher in differentiated mature neurons whereas Musashi expression is more restricted to undifferentiated neural precursor cells (*48*). Musashi is suggested to bind to the mRNA for numb, a Notch signal blocker, and inhibit its translation to facilitate Notch signaling and resultant stem cell proliferation (*51*). How CRN and ATH55 are involved in the maintenance or proliferation of neural stem cells *via* splicing remains a question. Although the functional significance of alternative splicing is still largely unknown in neural development, alternative splicing alters the biological activity of cell growth factors to influence cell differentiation. The FGF-5 gene produces two gene products by alternative splicing, FGF-5 (264 aa) and FGF-5S (121 aa) (*52*). The short form of FGF-5 (FGF-5S) binds to FGF receptors but antagonizes FGF-5 signaling (*52*). There is a similar scenario for angiopoietin-1, a factor for capillary formation (*53*). Future studies should provide an answer to the question of which splicing factor(s) interacts with CRN or ATH55 in neural stem cells and how this contributes to neural differentiation and development through RNA splicing.

We thank Sumitomo Pharmaceuticals for supplying the human recombinant BDNF, Drs. A.R. Krainer for anti-SF2/ASF antibody (mAb96), G. Dreyfuss for anti-hnRNP A1 antibody (mAb9H10), H. Okano for anti-Musashi antibody, H. Brahms and R. Lührmann for anti-U5-116 and anti-U1-70K (mAbH111) antibodies. We are also grateful to Dr. F. Tsukahara for advice. This work was supported by the Japanese Society for the Promotion of Science (RFTF-96L00203) and a Grant-in-Aid for Creative Scientific Research (to H.N.); and by the Florida Biomedical Research Program Grant (BM031) from F.D.H. (to A.M.). A.M. is a research member of the Sylvester Comprehensive Cancer Center.

REFERENCES

1. Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Wong, E., Henson, J., Posner, J.B., and Furneaux, H.M. (1991) HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell* **67**, 325–333
2. Nakamura, M., Okano, H., Blendy, J.A., and Montell, C. (1994) Musashi, a neural RNA-binding protein required for Drosophila adult external sensory organ development. *Neuron* **13**, 67–81
3. Patton, J.G., Porro, E.B., Galceran, J., Tempst, P., and Nadal-Ginard, B. (1993) Cloning and characterization of PSF, a novel pre-mRNA splicing factor. *Genes Dev.* **7**, 393–406
4. Hardy, R.J., Loushin, C.L., Friedrich, V.L.Jr., Chen, Q., Ebersole, T.A., Lazzarini, R.A., and Artzt, K. (1996) Neural cell type-specific expression of QKI proteins is altered in quakingviable mutant mice. *J. Neurosci.* **16**, 7941–7949
5. Gozani, O., Patton, J.G., and Reed, R. (1994) A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. *EMBO J.* **13**, 3356–3367
6. Chanas-Sacre, G., Mazy-Servais, C., Wattiez, R., Pirard, S., Rogister, B., Patton, J.G., Belachew, S., Malgrange, B., Moonen, G., and Leprince, P. (1999) Identification of PSF, the polypyrimidine tract-binding protein-associated splicing factor, as a developmentally regulated neuronal protein. *J. Neurosci. Res.* **57**, 62–73
7. Hardy, R.J. (1998) Molecular defects in the dysmyelinating mutant quaking. *J. Neurosci. Res.* **51**, 417–422
8. Li, Z., Zhang, Y., Li, D., and Feng, Y. (2000) Destabilization and mislocalization of myelin basic protein mRNAs in quaking dysmyelination lacking the QKI RNA-binding proteins. *J. Neurosci.* **20**, 4944–4953
9. Hirata, J., Nakagoshi, H., Nabeshima, Y., and Matsuzaki, F. (1995) Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. *Nature* **377**, 627–630
10. Li, P., Yang, X., Wasser, M., Cai, Y., and Chia, W. (1997) Inscuteable and Staufen mediate asymmetric localization and segregation of prospero RNA during Drosophila neuroblast cell divisions. *Cell* **90**, 437–447

11. Broadus, J., Fuerstenberg, S., and Doe, C.Q. (1998) Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. *Nature* **391**, 792–795
12. Sikorski, R.S., Boguski, M.S., Goebl, M., and Hieter, P. (1990) A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. *Cell* **60**, 307–317
13. Zachariae, W., Shin, T.H., Galova, M., Obermaier, B., and Nasmyth, K. (1996) Identification of subunits of the anaphase-promoting complex of Saccharomyces cerevisiae. *Science* **274**, 1201–1204
14. Nakatsu, Y., Asahina, H., Citterio, E., Rademakers, S., Vermeulen, W., Kamiuchi, S., Yeo, J.P., Khaw, M.C., Saijo, M., Kodo, N., Matsuda, T., Hoeijmakers, J.H., and Tanaka K. (2000) XAB2, a novel tetratricopeptide repeat protein involved in transcription-coupled DNA repair and transcription. *J. Biol. Chem.* **275**, 34931–34937
15. Zhang, K., Smouse, D., and Perrimon, N. (1991) The crooked neck gene of Drosophila contains a motif found in a family of yeast cell cycle genes. *Genes Dev.* **5**, 1080–1091
16. Raisin-Tani, S. and Leopold, P. (2002) Drosophila crooked-neck protein co-fractionates in a multiprotein complex with splicing factors. *Biochem. Biophys. Res. Commun.* **296**, 288–292
17. Chung, S., Zhou, Z., Huddleston, K.A., Harrison, D.A., Reed, R., Coleman, T.A., and Rymond, B.C. (2002) Crooked neck is a component of the human spliceosome and implicated in the splicing process. *Biochim. Biophys Acta* **1576**, 287–297
18. Chung, S., McLean, M.R., and Rymond, B.C. (1999) Yeast ortholog of the Drosophila crooked neck protein promotes spliceosome assembly through stable U4/U6.U5 snRNP addition. *RNA* **5**, 1042–1054
19. Ohira, M., Ootsuyama, A., Suzuki, E., Ichikawa, H., Seki, N., Nagase, T., Nomura, N., and Ohki, M. (1996) Identification of a novel human gene containing the tetratricopeptide repeat domain from the Down syndrome region of chromosome 21. *DNA Res.* **3**, 9–16
20. Sohocki, M.M., Bowne, S.J., Sullivan, L.S., Blackshaw, S., Cepko, C.L., Payne, A.M., Bhattacharya, S.S., Khaliq, S., Qasim Mehdi, S., Birch, D.G., Harrison, W.R., Elder, F.F., Heckenlively, J.R., and Daiger, S.P. (2000) Mutations in a new photoreceptor-pineal gene on 17p cause Leber congenital amaurosis. *Nat. Genet.* **24**, 79–83
21. Prihar, G., Gonzalez de Chavez, F., Baker, M., Crook, R., McGowan, E., Grover, A., Hardy, J., and Hutton, M. (1999) A novel candidate presenilin-1 interacting protein containing tetratricopeptide repeats. *Neuroreport* **10**, 1409–1415
22. Becker, W., Kentrup, H., Klumpp, S., Schultz, J.E., and Joost, H.G. (1994) Molecular cloning of a protein serine/threonine phosphatase containing a putative regulatory tetratricopeptide repeat domain. *J. Biol. Chem.* **269**, 22586–22592
23. Rachidi, M., Lopes, C., Gassanova, S., Sinet, P.M., Vekemans, M., Attie, T., Delezoide, A.L., and Delabar, J.M. (2000) Regional and cellular specificity of the expression of TPRD, the tetratricopeptide Down syndrome gene, during human embryonic development. *Mech. Dev.* **93**, 189–193
24. Narisawa-Saito, M., Silva, A.J., Yamaguchi, T., Hayashi, T., Yamamoto, T., and Nawa, H. (1999) Growth factor-mediated Fyn signaling regulates alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression in rodent neocortical neurons. *Proc. Natl Acad. Sci. USA* **96**, 2461–2466
25. Liang, P. and Pardee, A.B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967–971
26. Nakajima, Y., Iwakabe, H., Akazawa, C., Nawa, H., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1993) Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. *J. Biol. Chem.* **268**, 11868–11873
27. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1991) *Molecular Cloning: A Laboratory Mannual,* . Cold Spring Harbor Press, Cold Spring Harbor, NY
28. Araki, K., Megro, H., Kushiya, E., Takayama, C., Inoue, Y., and Mishina, M. (1993) Selective expression of the glutamate receptor channel delta 2 subunit in cerebellar Purkinje cells. *Biochem. Biophys. Res. Commun.* **197**, 1267–1276
29. Reynolds, B.A., Tetzlaff, W., and Weiss, S. (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* **12**, 4565–4574
30. Belgrader, P., Siegel, A.J., and Berezney, R. (1991) A comprehensive study on the isolation and characterization of the HeLa S3 nuclear matrix. *J. Cell Sci.* **98**, 281–291
31. Mayeda, A. and Krainer, A.R. (1999) Mammalian in vitro splicing assays. *Methods Mol. Biol.* **118**, 315–321
32. Mayeda, A. and Krainer, A.R. (1999) Preparation of HeLa cell nuclear and cytosolic S100 extracts for in vitro splicing. *Methods Mol. Biol.* **118**, 309–314
33. Hanamura, A., Cáceres, J.F., Mayeda, A, Franza, B.R.Jr., and Krainer, A.R. (1998) Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA* **4**, 430–444
34. GenBank Accession NM 137047; *Drosophila melanogaster* CG6197-PA, mRNA (2002)
35. Vescovi, A.L., Reynolds, B.A., Fraser, D.D., and Weiss, S. (1993) bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11**, 951–966
36. Yoshida, T., Satoh, M., Nakagaito, Y., Kuno, H., and Takeuchi, M. (1993) Cytokines affecting survival and differentiation of an astrocyte progenitor cell line. *Brain Res. Dev. Brain Res.* **76**, 147–150
37. Ahmed, S., Reynolds, B.A., and Weiss, S. (1995) BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. *J. Neurosci.* **15**, 5765–5778
38. Will, C.L. and Lührmann, R. (1997) Protein functions in pre-mRNA splicing. *Curr. Opin. Cell Biol.* **9**, 320–328
39. Graveley, B.R. (2000) Sorting out the complexity of SR protein functions. *RNA* **6**, 1197–1211
40. Mayeda, A., Badolato, J., Kobayashi, R., Zhang, M.Q., Gardiner, E.M., and Krainer, A.R. (1999) Purification and characterization of human RNPS1: a general activator of pre-mRNA splicing. *EMBO J.* **18**, 4560–4570
41. Lai, C.H., Chiu, J.Y., and Lin, W. (2001) Identification of the human crooked neck gene by comparative gene identification. *Biochim. Biophys Acta.* **1517**, 449–454
42. Hirano, T., Kinoshita, N., Morikawa, K., and Yanagida, M. (1990) Snap helix with knob and hole: essential repeats in S. pombe nuclear protein nuc2+. *Cell* **60**, 319–328
43. Lamb, J.R, Michaud, W.A., Sikorski, R.S., and Hieter, P.A. (1994) Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. *EMBO J.* **13**, 4321–4328
44. Tobaben, S., Thakur, P., Fernandez-Chacon, R., Sudhof, T.C., Rettig, J., and Stahl, B. (2001) A trimeric protein complex functions as a synaptic chaperone machine. *Neuron* **31**, 987–999
45. Ramarao, M.K., Bianchetta, M.J., Lanken, J., and Cohen, J.B. (2001) Role of rapsyn tetratricopeptide repeat and coiled-coil domains in self-association and nicotinic acetylcholine receptor clustering. *J. Biol. Chem.* **276**, 7475–7483
46. Gindhart, J.G.Jr. and Goldstein, L.S. (1996) Tetratrico peptide repeats are present in the kinesin light chain. *Trends Biochem. Sci.* **21**, 52–53
47. Ben-Yehuda, S., Dix, I., Russell, C.S., McGarvey, M., Beggs, J.D., and Kupiec, M. (2000) Genetic and physical interactions between factors involved in both cell cycle progression and pre-mRNA splicing in Saccharomyces cerevisiae. *Genetics* **156**, 1503–1517
48. Sakakibara, S., Imai, T., Hamaguchi, K., Okabe, M., Aruga, J., Nakajima, K., Yasutomi, D., Nagata, T., Kurihara, Y., Uesugi, S., Miyata, T., Ogawa, M., Mikoshiba, K., and Okano, H. (1996) Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. *Dev. Biol.* **176**, 230–242
49. Mobarak, C.D., Anderson, K.D., Morin, M., Beckel-Mitchener, A., Rogers, S.L., Furneaux, H., King, P., and Perrone-Bizzozero, N.I. (2000) The RNA-binding protein HuD is required

for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. *Mol. Biol. Cell* **11**, 3191–3203
50. Aranda-Abreu, G.E., Behar, L., Chung, S., Furneaux, H., and Ginzburg, I. (1993) Embryonic lethal abnormal vision-like RNA-binding proteins regulate neurite outgrowth and tau expression in PC12 cells. *J. Neurosci.* **19**, 6907–6917
51. Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M., and Okano, H. (2001) The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol. Cell. Biol.* **21**, 3888–3900
52. Ozawa, K., Suzuki, S., Asada, M., Tomooka, Y., Li, A.J., Yoneda, A., Komi, A., and Imamura, T. (1998) An alternatively spliced fibroblast growth factor (FGF)-5 mRNA is abundant in brain and translates into a partial agonist/antagonist for FGF-5 neurotrophic activity. *J. Biol. Chem.* **273**, 29262–29271
53. Huang, Y.Q., Li, J.J., and Karpatkin, S. (2000) Identification of a family of alternatively spliced mRNA species of angiopoietin-1. *Blood* **95**, 1993–1999