J. Biochem. 131, 861–868 (2002)

Role of PTB-Like Protein, a Neuronal RNA-Binding Protein, during the Differentiation of PC12 Cells[1]

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Received April 2, 2002; accepted April 11, 2002

PTB-like protein (PTBLP) is a new homologue of pyrimidine tract binding protein (PTB), and has been cloned as a possible autoantigen in cancer-associated retinopathy. PTBLP has two functional domains, the nuclear localization signal and the RNA recognition motifs (RRMs). Full-length PTBLP (PTBLP-L) has four RRMs, and its alternative splicing product (PTBLP-S) lacks the third and fourth RRMs. Although PTBLPs are expressed in neuronal tissues, the function of PTBLPs has not been determined. We have studed whether PTBLP plays a role in neuronal differentiation using PC12 cells. During the process of nerve growth factor-induced neuronal differentiation of PC12 cells, PTBLP-L was down-regulated whereas PTBLP-S was up-regulated. Transfection of PTBLP-L into PC12 cells led to the suppression of neuronal differentiation. In PTBLP-S transfected cells, however, this suppression was not evident. When both PTBLP-L and PTBLP-S were co-transfected, the suppressive effect of PTBLP-L decreased. In differentiated cells, PTBLP-S localized in the nucleus and PTBLP-L was found dispersed throughout the cytoplasm and neuronal growth cone. These findings suggest that PTBLP-L acts as a negative regulator of neuronal differentiation and PTBLP-S acts as a competitor of PTBLP-L.

Key words: nerve growth factor, neuronal differentiation, PC12, polypyrimidine tract binding protein, RNA binding protein.

Neuronal RNA-binding proteins play important roles in the post-transcriptional regulation of gene expression, *e.g.*, alternative splicing, RNA transport, and local translation (*1, 2*). Several families of neuronal RNA-binding proteins have been described. The mammalian ELAV-like neuronal RNA binding proteins, called Hu proteins, have been identified as autoantigens against human paraneoplastic encephaloneuropathies associated with small-cell lung carcinomas (*3*). HuA (HuR), HuB (Hel-N1), HuC (Ple-21), and HuD have been cloned and characterized (*3–6*). One of the major functions of the Hu proteins is to regulate mRNA metabolism by binding to RNA stability elements (*5, 7–11*).

Musashi is another example of a neuronal RNA-binding protein family, and members of this family can be classified on the basis of their homology to *Drosophila* proteins (*12–14*). Musashi is known to play a role in two successive asymmetric divisions of sensory organ precursor cells (*12*). Nova-1 is another neuron-specific RNA binding protein that regulates alternative splicing (*15–18*).

Polypyrimidine tract binding protein (PTB) is a well-known RNA-binding protein that is ubiquitously distributed in mammalian cells. PTB plays a role in the splicing of RNA and in the regulation of translation (*19–21*). We have recently cloned a homologue of the PTB from human and rat cDNA libraries, and have shown that it is a possible autoantigen of cancer-associated retinopathy (*22, 23*). This new protein was named PTB-like protein (PTBLP) (*22*). The amino acid sequence of PTBLP shows strong homology with PTB, and the functional domains of the PTB, *e.g.*, nuclear localization signal (NLS) and RNA recognition motifs (RRMs), are highly conserved in PTBLP. Unlike PTB, PTBLP is not a ubiquitous protein but is expressed mainly in neuronal cells (*22*). Recently, mouse PTBLP was reported to interact with Nova-1 (*24*).

In our previous studies, some variants of PTBLP were isolated during the amplification of the full-length rat PTBLP cDNA clone. The originally found PTBLP-L has four RRMs, and PTBLP-S, an alternative spliced variant, lacks the third and fourth RRMs of PTBLP-L. The RNA binding ability of PTBLP-S is much weaker than that of PTBLP-L (*23*). It is known that the PTB also has four RRMs and the third and the fourth RRMs are necessary for RNA binding and specificity (*25, 26*). PTB also has a sequence variation at nucleotide 921 generated by alternative splicing (*20*), but the function of this variant is not clear. Although it has been shown that mouse PTBLP interacts

[1] This work was partially supported by Grants-in-Aid 13671829 for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; NGF, nerve growth factor; PBS, phosphate-buffered saline; PTB, polypyrimidine-tract binding protein; RRM, RNA recognition motif; RT-PCR, reverse transcriptase–polymerase chain reaction.

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with and inhibits the ability of Nova to activate exon selection in neurons (*24*), the exact function of PTBLP has not yet been determined.

The purpose of this study was to study the possible role played by PTBLP in neuronal differentiation using phenochromocytoma cell line (PC12) cells (*27, 28*).

MATERIALS AND METHODS

PC12 Cells—PC12 cells were obtained from the Riken cell bank (Tsukuba, Japan), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 5% horse serum, and 100 units/ml penicillin G at 37°C under 5% CO_2. All culture media, sera, and antibiotics were obtained from Gibco-BRL (Grand Island, NY, USA).

Neuronal Differentiation of PC12 Cells—Two days after seeding at 2×10^4 cells/cm^2 in 6-well plates, PC12 cells were cultured in DMEM with 50 ng/ml of nerve growth factor (2.5S NGF, Promega, Madison, WI, USA) to induce differentiation. The morphological changes of the cells were observed with an IX70 epifluorescence microscope (Olympus, Tokyo). Microphotographs were taken at designated times by the IX70 and SenSys™ (Photometrics, München, Germany). The composite Hoffman and fluorescent images were prepared by the IPLab (Scanalytics, Faifax, VA, USA). The total number of cells and the number of differentiated cells were counted on the microphotographs. Neuronal differentiation was judged to have occurred by neurite extension (*29–32*), and cells bearing neurites longer than two cell diameters were counted as neurite-positive and differentiated (*33*). The results were expressed as means ± standard error of the means (SE).

Immunostaining—PC12 cells were seeded at 2×10^4 cells/cm^2 on a chamber slide (Iwaki, Tokyo). Twenty-four hours later, NGF was added to induced neuronal differentiation. On day 3 after the addition of NGF, the cells were fixed in 4% paraformaldehyde-PBS and incubated with rabbit anti-PTBLP antibody at a dilution of 1:50 with 5% goat serum-PBST containing 0.3% Triton X-100. After washing twice with PBST containing 0.3% Triton X-100, FITC-conjugated anti–rabbit IgG antibody (Dako, Kyoto) was applied as the second antibody. Analysis was performed with a confocal microscope (LSM410, Zeiss, Jena, Germany). The anti-PTBLP antibody was generated using a 19 amino acid peptide of the PTBLP N-terminal sequence (*22*).

RT-PCR—mRNA was prepared from PC12 cells using a Micro-FastTrack Kit™ (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The following primers were used.

Total PTBLP: 5′-GCTGTGACACCTCATCTTCGTAAC-3′ (sense). 5′-AGCATTAGGAATAGCCAAAGGACTG-3′(antisense). These were used to amplify a 600-bp fragment of rat PTBLP cDNA (amplification, 37 cycles).

PTBLP-L and PTBLP-S: 5′-GTCCTTTGGCTATTCCTAATGCTG-3′ (sense). 5′-TTCTGCCCATTAAGATGATTCATG-3′ (antisense). These were used to amplify 272-bp (PTBLP-L) and 238-bp (PTBLP-S) fragments of the rat PTBLP cDNA species (amplification, 40 cycles).

GAPDH: 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ (sense). 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (antisense). These were used to amplify a 983-bp rat GAPDH cDNA (35-1017 of GenBank: M17701, amplification, 37 cycles).

PCR products were electrophoresed in a 2% agarose gel. The nucleotide sequences of the PCR products were determined by direct sequencing using a Taq Dye Terminator Cycle Sequencing kit and an auto-sequencer ABI Prism™ 310 Genetic Analyzer (PE Biosystems, Foster City, CA, USA).

Western Blot Analysis—Proteins were extracted from PC12 cells, and the solutions were mixed with equal volumes of 2% SDS in sample buffer and electrophoresed in a 10% polyacrylamide gel. The proteins were transferred to nitrocellulose membranes, and the membranes were blocked in 10% blocking reagent (Roche Molecular Biochemicals, Mannheim, Germany), 0.05% sodium azide in 0.05% Tween 20, and Tris-buffered saline (TBST). They were then incubated with the anti-PTBLP antibody at a dilution of 1:200 with 1% BSA in TBS at 4°C for 12 h. After washing in TBST, the membranes were incubated with alkaline phosphatase-conjugated goat anti–rabbit IgG secondary antibody at a dilution of 1:5,000 for 1 h. The final reaction products were visualized with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Construction of Expression Vectors—The cDNA clones of full-length mouse PTBLP (PTBLP-L) and the alternatively spliced variant, PTBLP-S, were subcloned into the mammalian expression vector pEGFP. pEGFP was made by removing the internal ribosome entry site of pIRES-EGFP vector (Clontech Laboratories, Palo Alto, CA, USA). The pEGFP vector carries a CMV promoter. PTBLP-L and -S were expressed fused to the N-terminus of enhanced green fluorescent protein (EGFP, excitation maximum, 489 nm; emission maximum, 508 nm). For co-expression of PTBLP-L and -S, PTBLP-L was subcloned into the mammalian expression vector pDsRed1-N1. The pDsRed1-N1 vector, which carries a CMV promoter, was obtained from Clontech. In this case, PTBLP-L was expressed fused to the N-terminus of a red fluorescent protein (DsRed1, excitation maximum, 558 nm; emission maximum, 583 nm).

Transfection—Transfection of cDNA was performed by Lipofectamine2000™ (Gibco-BRL). Briefly, PC12 cells were cultured to subconfluency in 0.5 ml of normal medium without antibiotics on 24-well plates. Two micrograms of DNA was combined with 3 μl of Lipofectoamine 2000™ in 100 μl of Opti-MEM® medium (Gibco-BRL) without serum or antibiotics and incubated at room temperature for 20 min. This mixture was added directly to each well, and incubated for 2 days at 37°C under 5% CO_2.

Neuronal Differentiation of Transfected PC12 Cells—Transfected PC12 cells were cultured in DMEM, and reseeded after 2 days at 2×10^4 cells/cm^2 in 6-well plates. Twenty four hours later, NGF was added and the morphological changes in the transfected cells were monitored as described above. Each treatment group comprised 6 independent wells, and 6 fields were randomly chosen from each well for microphotographs. The total number of cells and the number of neuronal differentiated cells were counted in survey reporter protein (EGFP/DsRed1) expressed cells from the microphotographs. The ratio of neuronal differentiated cells was calculated. The results were expressed as mean ± SE. Scheffé's post hoc test was used to assess the differences after ANOVA. p values less than 0.05

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were considered to be statistically significant.

Expression of Neurofilaments in Transfected PC12 Cells—Transfected PC12 cells were cultured in DMEM, and reseeded after 2 days at 1×10^4 cells/cm^2 in 24-well plates. Twenty-four hours later, NGF was added and the cells were harvested 3 days after the addition of NGF. The cells were fixed and subsequently permeabilized with an Intrastain kit (Dako), which allows easier detection of intracellular antigens by flow cytometry. The cells were then incubated with mouse anti-neurofilament H antibody (Chemicon International Inc., Temecula, CA, USA) at a dilution of 1:200 with 1% horse serum-PBS. After the cells were washed in 1% horse serum-PBS for 5 min, they were incubated with secondary anti–mouse antibody–conjugated rhodamine (Dako) diluted 1:30 in 1% horse serum-PBS. After incubation for 15 minutes, they were washed once for 5 minutes in 1% horse serum-PBS and fixed again with 1% paraformaldehyde. EGFP and rhodamine-positive cells were detected using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and the ratio of both rhodamine and EGFP-positive cells to EGFP-positive cells were evaluated with CELLQuest™ (Becton Dickinson). Three or four independent cells were examined. Results are expressed as means ± SE. Student's *t*-test was used to assess the significance of the differences. *p* values less than 0.05 were considered to be statistically significant.

RESULTS

Localization of PTBLP in PC12 Cells—PTBLP immunoreactivity was detected in the nuclei of untreated PC12 cells (Fig. 1). Several hours after the addition of NGF, the cells became spindle-shaped and flattened, and formed short cytoplasmic extensions (spikes). After 1 day of NGF exposure, long and branching neurites were produced, and thus, the cells became increasingly more neuronal in appearance. By 2 days, about 70% to 80% of the cells had neurites (data not shown). In NGF-treated PC12 cells that underwent neuronal differentiation, immunostaining for PTBLP was also detected in the nucleus. In addition, diffuse immunostaining was observed in the cytoplasm and in the neuronal growth cone (Fig. 1B).

Expression of PTBLP in PC12 Cells during NGF-Induced Differentiation—The expression of PTBLP, PTBLP-L and -S was analyzed semi-quantitatively by RT-PCR analysis during the differentiation process of PC12 cells. The mRNA expression for total PTBLP was higher 2 days after the addition of NGF (Fig. 2A). In the first 2 days, PC12 cells underwent changes such as neurite extension, and up-regulation of total PTBLP. To confirm the dynamic changes in both PTBLP-L and -S during this period, RT-PCR was performed using discriminative primers. In PCR by these primers, three multiple bands were found. The upper band forms a hetero duplex with PTBLP-L and -S (*23*). Over 2 days, the expression of PTBLP-L decreased in a time-dependent fashion while the expression of PTBLP-S increased (Fig. 2B).

Western blot analysis indicated the apparent molecular mass of PTBLP-L to be 58 kDa and that of PTBLP-S to be

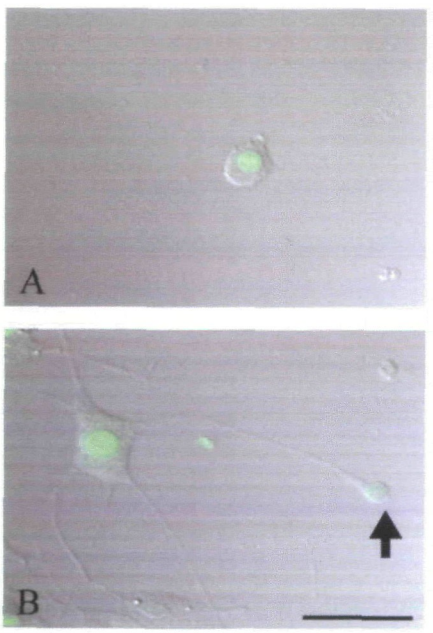

Fig. 1. **Confocal microscopic analysis of PC12 cells immunostained with anti-PTBLP antibody.** A: Non-treated PC12 cells. Immunostaining is observed in the nucleus. B: PC12 cells treated with NGF for 3 days. Weak cytoplasmic staining is observed in addition to the nuclear staining. The arrow indicates positive staining in the neuronal growth cone. Scale bar, 20 μm.

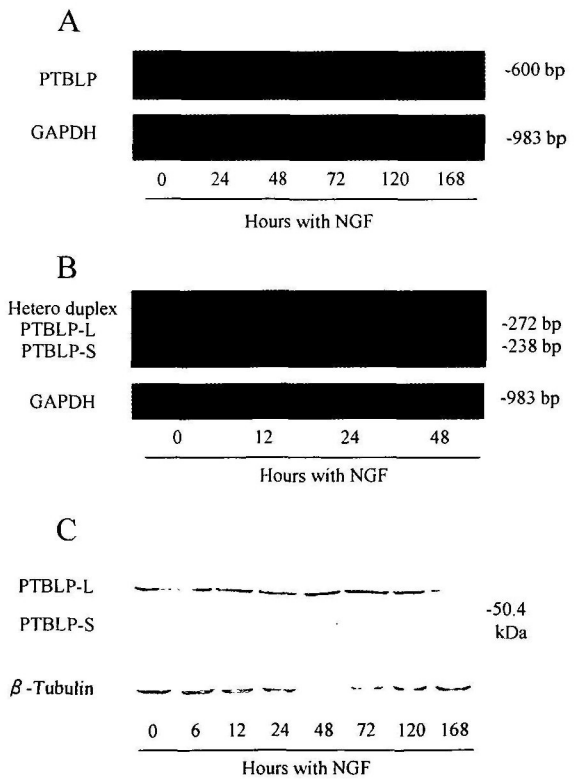

Fig. 2. **Expression of PTBLP in PC12 cells during neuronal differentiation induced by NGF.** A: RT-PCR of total PTBLP. B: RT-PCR of PTBLP-L and -S. RT-PCR was performed using specific primers for total PTBLP, PTBLP-L, -S and GAPDH genes. PCR products were electrophoresed in a 2% agarose gel. C: Western blot analysis of PTBLP-L and -S using anti-PTBLP antibodies. Samples were electrophoresed in a 10% polyacrylamide gel. Bands were visualized with BCIP/NBT.

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42 kDa (Fig. 2C). Without NGF-treatment, the expression of PTBLP-L at the protein level was much higher than that of PTBLP-S. The expression of PTBLP-S mRNA and protein increased for 2 to 3 days after the addition of NGF. PTBLP-L mRNA levels decreasd in a time-dependent manner whereas a small reduction in protein level was found at 168 h. The time difference in the expression levels of the mRNA and protein may be explained by the longer lifetime of the protein.

Effect of NGF on PTBLP-Transfected PC12 Cells—The efficiency of transfection of empty pEGFP vector was 31.3 ± 2.8%, that of pEGFP-PTBLP-L was 10.6 ± 1.3%, that of pEGFP-PTBLP-S was 9.4 ± 1.2%, that of empty pDsRed1 vector was 12.0 ± 1.0%, that of pDsRed1-PTBLP-L was 7.3 ± 0.7%, and that of pDsRed1-PTBLP-L and pEGFP-PTBLP-S were 6.1 ± 0.5%, (means ± SE, n = 6). EGFP was found diffusely in the cytoplasm of PC12 cells transfected with the empty pEGFP vector (Fig. 3A). The NGF-treated PC12 cells also showed diffuse cytoplasmic fluorescence (Fig. 3B). On the other hand, the EGFP of PC12 cells transfected with the EGFP-fused PTBLP-L or -S was localized in the nucleus (Fig. 3, C and E). These findings seem reasonable because both PTBLP-L and -S have nuclear localization signals (NLS). The transfection of PTBLP did not alter the shape of PC12 cells (Fig. 3, C and E). With NGF-treatment, EGFP-fused PTBLP-L and -S were still observed in the nucleus (Fig. 3, D and F). In PC12 cells transfected with empty pEGFP vector, approximately 70% of cells had neurites 3 days after the addition of NGF. This percentage is similar to that of cells without transfection. Similar results were found for PTBLP-S transfected cells, and the differences from cells transfected with empty pEGFP vector were not significant. However, in PTBLP-L transfected cells, the ratio of neurite-positive cells was only 20% 3 days after NGF treatment. PTBLP-L transfected cells showed a lower percentage of differentiation compared to cells transfected with the empty pEGFP vector and PTBLP-S

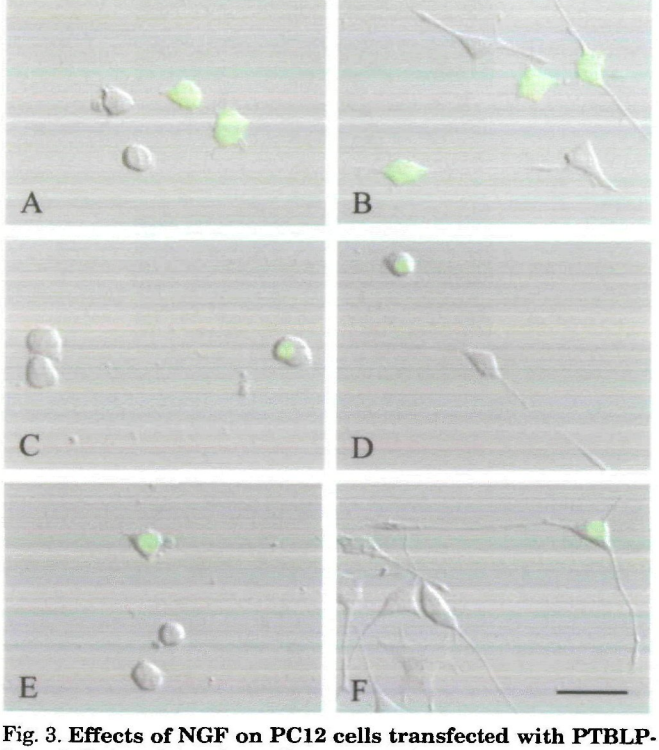

Fig. 3. **Effects of NGF on PC12 cells transfected with PTBLP-L and -S.** A and B: PC12 cells transfected with empty pEGFP vectors. C and D: PC12 cells transfected with PTBLP-L. E and F: PC12 cells transfected with PTBLP-S. A, C, and E: Control, no NGF. B, D, and F: Cells treated with NGF (50 ng/ml) for 3 days. Fluorescent signals were observed in the nucleus with or without NGF-treatment. Scale bar, 20 μm.

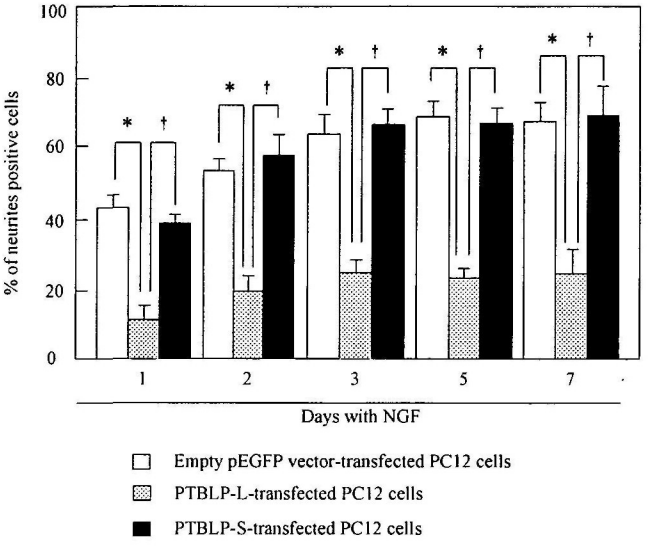

Fig. 4. **Time course of neurite-positive cells transfected with PTBLP-L and -S.** Values represent means ± SE. Data were obtained from 6 independent samples. Scheffé's host hoc test was used to assess the differences at each time after two-way ANOVA. There was no significant difference between PC12 cells transfected with PTBLP-S and empty vector. On the other hand, cells transfected with PTBLP-L showed a significantly lower percentage of neurite-positive cells compared to the empty-vector-transfected (*$p < 0.0001$) and PTBLP-S-transfected (†$p < 0.0001$) cells.

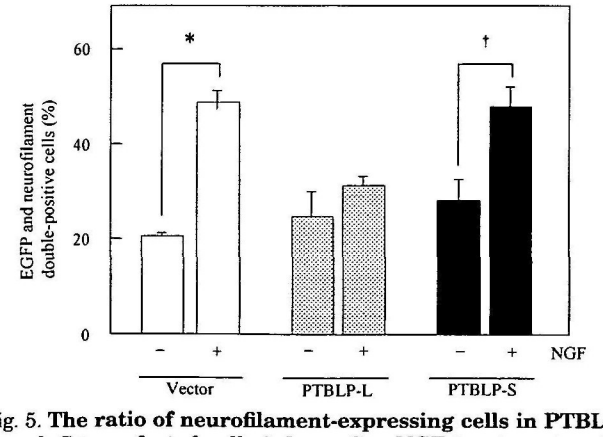

Fig. 5. **The ratio of neurofilament-expressing cells in PTBLP-L and -S transfected cells 3 days after NGF-treatment as determined by flow cytometry.** The values represent means ± SE. Data were obtained from 3 or 4 independent samples and compared by Student's t-test. In cells transfected with empty vector or PTBLP-S, the ratio of neurofilament-positive cells increased upon the addition of NGF (*$p < 0.05$; †$p < 0.05$). On the other hand, no up-regulation of neurofilaments by NGF addition was found in the PTBLP-L transfected cells.

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throughout the follow-up period of 7 days, and the differences were statistically significant (Fig. 4, $p < 0.0001$).

Expression of Neurofilaments on PTBLP-Transfected PC12 Cells—The evaluation of the expression of neurofilaments had two purposes: first, to confirm that the morphological changes in transfected PC12 cells caused by NGF were neuronal differentiation; and, second, to obtain data demonstrating that the NGF-induced neuronal differentiation is suppressed in PTBLP-L transfected PC12 cells (Fig. 4). Neurofilaments are known to be neuronal markers that are up-regulated during NGF-induced neuronal differentiation in PC12 cells (*34, 35*). In cells transfected with both empty pEGFP vector and PTBLP-S, the ratio of neurofilament-positive cells to EGFP-positive cells increased significantly upon the addition of NGF (Fig. 5). On the other hand, an increase in neurofilament-positive cells following NGF addition was not found for PTBLP-L transfected cells. These results agree with the results of the morphological study.

The question of whether PTBLP-L and -S induce cell death was also examined. Cells transfected with empty pEGFP vector, PTBLP-L or -S were stained with propidium iodide (PI, Wako Pure Chemicals, Osaka) 3 days after the addition of NGF, and the percentages of PI-positive cells to EGFP-positive cells were evaluated by flow cytometry. The percentage of PI-positive cells in the PTBLP-L transfected group and -S transfected group was approximately 10% of the EGFP-positive cells, and this was not significantly different from cells transfected with the empty pEGFP vector in spite of NGF-treatment (data not shown). These observations suggest that the transfection of PTBLP-L and -S does not induce apoptosis.

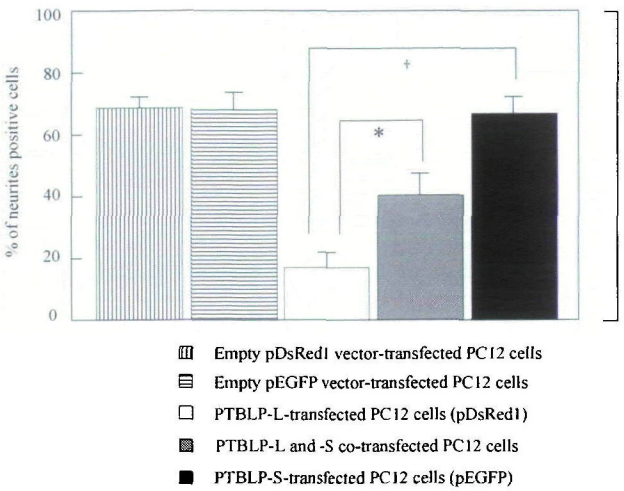

Fig. 7. **The ratio of neurite-positive cells 3 days after NGF-treatment.** The values represent means ± SE. Data were obtained from 6 independent samples. Scheffé's host hoc test was used to assess the differences after one-way ANOVA. Cells transfected with PTBLP-L showed a significantly lower percentage of neurite-positive cells compared to PTBLP-S-transfected cells (†$p < 0.0001$). The ratio of neurite-positive cells co-transfected with PTBLP-L and -S showed a significantly higher percentage of neurite-positive cells compared with PTBLP-L-transfected cells (*$p < 0.0001$) and a smaller percentage compared with PTBLP-S-transfected cells (no significant difference).

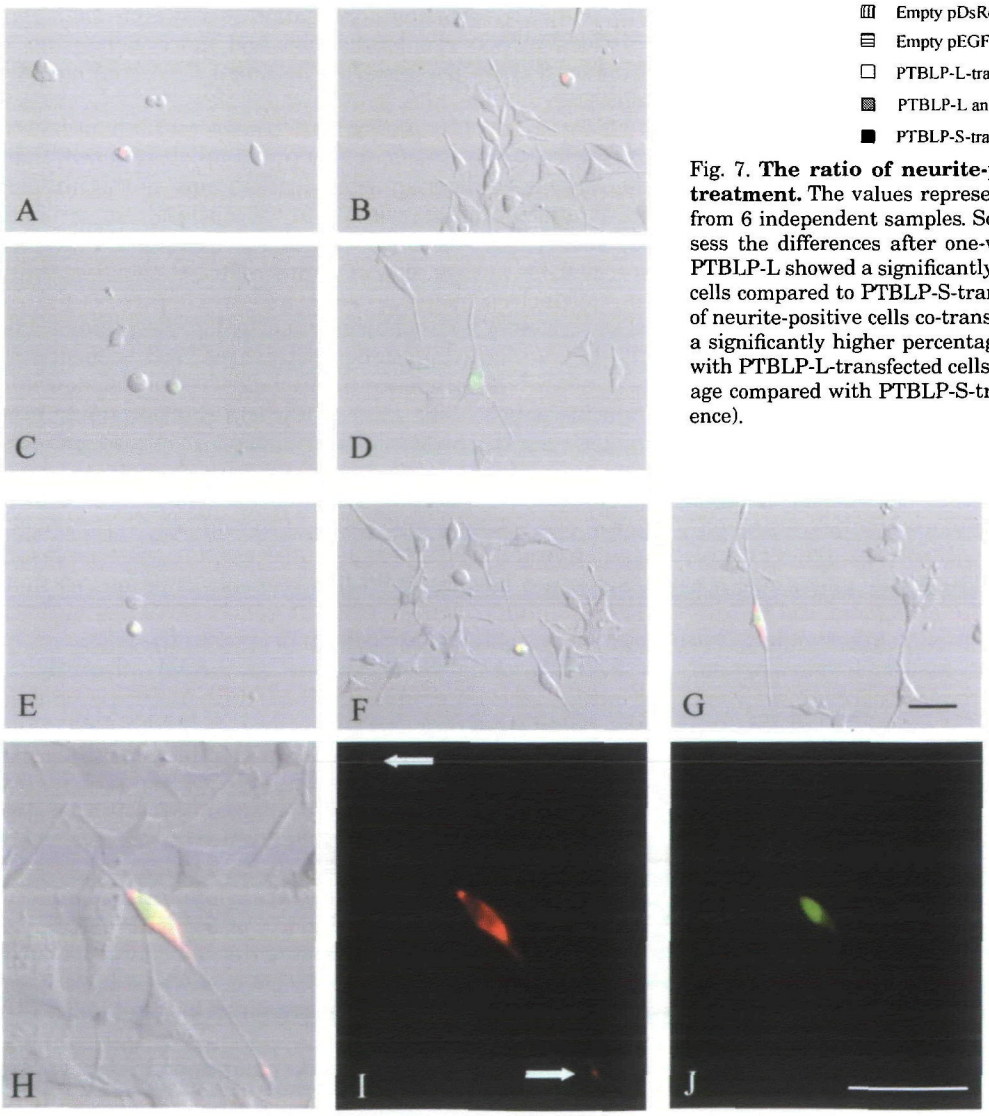

Fig. 6. **Effects of NGF on PC12 cells co-transfected with PTBLP-L and -S.** A and B: PC12 cells transfected with pDsRed1-PTBLP-L. C and D: PC12 cells transfected with pEGFP-PTBLP-S. E, F, and G: PC12 cells co-transfected with pDsRed1-PTBLP-L and pEGFP-PTBLP-S. A, C, and E: No treatment. B, D, and F: Cells were treated with NGF (50 ng/ml) for 3 days. H, I, and J: Higher magnification of cells co-transfected pDsRed1-PTBLP-L and pEGFP-PTBLP-S. I: Localization of DsRed1-fused PTBLP-L. Arrows indicate the distribution of PTBLP-L in the neuronal growth cone. J: Localization of EGFP-fused PTBLP-S. Scale bar, 20 μm.

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Co-Transfection of PTBLP-L and -S and Cellular Differentiation—In the co-transfection experiment, the red color of DsRed1 was used to identify PTBLP-L and the green color of EGFP was used to show PTBLP-S. Empty pDsRed1 vector did not alter the shape of PC12 cells (data not shown). PC12 cells transfected with DsRed1-fused PTBLP-L showed signal localization in the nucleus and the pattern did not change after NGF-treatment (Fig. 6, A and B). The percentage of neurite-positive cells was approximately 18% 3 days after NGF-treatment (Fig. 7). These findings were not different from those of EGFP-fused PTBLP-L (Fig. 3, C and D, and Fig. 4).

In the co-transfection experiments, PTBLP-L and -S were co-localized in the nucleus as shown by the orange color (Fig. 6E). Following NGF-treatment, co-transfected cells showed an intermediate response between PTBLP-L transfected cells and -S transfected cells. The percentage of neurite-positive cells 3 days after the addition of NGF was approximately 18% in PTBLP-L transfected cells, 43% in PTBLP-L and -S co-transfected cells, and 71% in PTBLP-S transfected cells (Fig. 7).

An interesting phenomenon was observed in this experiment. In pDsRed1-PTBLP-L and pEGFP-PTBLP-S transfected cells, fluorescent signals were detected in the nucleus with or without NGF-treatment (Fig. 6, A, B, C, and D). In PTBLP-L and -S co-transfected cells, however, the localization pattern of fluorescence was different between neurite-negative cells and neurite-positive cells. In the neurite-negative cells, both PTBLP-L and -S co-localized in the nucleus (Fig. 6F). On the other hand, the red fluorescent signals of PTBLP-L were located in the cytoplasm in neurite-positive, differentiated cells (Fig. 6, G and H) and in the neuronal growth cone (Fig. 6I, arrows). The green fluorescent signals of PTBLP-S remained in the nucleus (Fig. 6, H and J).

DISCUSSION

Localization and Dynamics of PTBLP—Two important findings were made in the experiments on the immunohistochemical localization of PTBLP. First, both PTBLP-L and -S were found to be nuclear proteins, which agrees with our previous immunohistochemical study on rat retina (*22*). Second, weak PTBLP-like immunoreactivities were observed in the cytoplasm and growth cone in differentiated PC12 cells. This suggests that PTBLP may play a role not only in the nucleus but also in the cytoplasm and growth cone during neuronal differentiation. Unfortunately, because PTBLP-L and -S show high molecular homology, no discriminative antibody could be found. Therefore, we were not able to differentiate the possible roles of PTBLP-L and -S from their subcellular localization. However, the expression of PTBLP-L was down-regulated and that of PTBLP-S was up-regulated during the neuronal differentiation process in PC12 cells. These results suggest that PTBLP-L and -S play reciprocal roles in the process.

Function of PTBLP-L and PTBLP-S—Transfection experiments were performed to study the roles played by the two proteins. We were not able to clone transfected cells, and comparative studies were done by counting the population of differentiated cells. Interestingly, we found that PTBLP-L transfected cells had a much lower percentage of neurite-positive cells, whereas, there was no difference in the percentage of neurite-positive cells between PTBLP-S transfected and empty vector-transfected PC12 cells following NGF-treatment. These results led us to conclude that PTBLP-L inhibits the NGF-induced neuronal differentiation of PC12 cells. The results of RT-PCR and western blot analysis support this conclusion. The results of the co-transfection experiments and the expression of neurofilaments were also supportive. PTBLP-S lacks the third and fourth RRM of PTBLP-L and the ability of PTBLP-S to bind mRNA is much weaker than that of PTBLP-L (*23*). Thus, the third and fourth RRM of PTBLP-L may play a role in neuronal differentiation.

Another important finding is that PTBLP-L was translocated from the nucleus into the cytoplasm and the growth cone in neurite-positive differentiated cells in the co-transfection experiments. The immunohistochemical data shown in Fig. 1 agree well with these observations. Because these phenomena were found only in neurite-positive cells, an association between cytoplasmic localization and neurite extension is suggested. It may well be that PTBLP-S is a competitor of PTBLP-L during the neuronal differentiation of PC12 cells, and the subcellular localization of the two proteins may determine cellular differentiation. The mammalian ELAV-like protein, HuD, has the ability to induce neurite outgrowth when over-expressed in PC12 cells. For this ability, cytoplasmic localization is required (*36*). The relationship between localization and function may be a common feature possessed by neuronal RNA-binding proteins.

Generally, nuclear proteins are transported into or out of the nucleus via a specific sequence (*37–39*). It is known that the nuclear localization signals (NLS) bind to the nuclear pore complex and translocate to the nucleoplasm through the Ran-GTPase cycle. In a similar way, nuclear export is specified by nuclear export signals (NESs) that reside in the ribonucleoprotein complexes and are characterized by a short stretch of hydrophobic, primarily leucine, amino acids (*39–41*). Nucleo-cytoplasmic shuttling of HuD is mediated by a novel NES sequence in the linker region for neurite-inducing activity (*36*). HuA (HuR) is a shuttle protein acting between the nucleus and cytoplasm. HuA may initially bind to AU-rich element–containing mRNAs in the nucleus, accompany them to the cytoplasm, and then return quickly to the nucleus after release from the mRNA (*7*). Although both HuA and PTBLP-L are translocated to the cytoplasm, they do not have an NES-like sequence. This means that there is an unknown shuttling system between the nucleus and the cytoplasm that does not depend on the NESs.

PTBLP-L May Bind to GAP-43 mRNA—Recently, a growth-associated protein (GAP-43) mRNA binding protein was identified in neonatal bovine brain cells (*42*). GAP-43 is a membrane-bound phosphoprotein found in neuronal cells and is characterized as an inducible marker protein for neural differentiation (*43*). In undifferentiated PC12 cells, GAP-43 mRNA degrades rapidly but becomes stable when cells are treated with NGF (*44–46*). The expression of GAP-43 is controlled partly by selective changes in the stability of its mRNA (*46, 47*). The stability of the GAP-43 mRNA has been shown to be controlled within its 3′-untranslated region (3′UTR) (*48–50*). The 3′UTR of mRNAs have been demonstrated to have biological functions, including the regulation of mRNA stability (*51–53*), mRNA translational efficiency (*54, 55*), and mRNA localization (*56–58*). The GAP-43 mRNA binding protein was identified as a trans-

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factor bound to an element of GAP-43 mRNA and purified by biochemical methods. It was shown that the protein binds to a 26-base pyrimidine-rich sequence in the 3′UTR of the GAP-43 mRNA. It is noteworthy that two partial amino acid sequences derived from the tryptic peptides of this protein completely match the amino acid sequence of PTBLP at amino acid positions 123–134 and 297–326. Even though the full-length amino acid sequence of the GAP-43 mRNA binding protein has not been reported, the protein seems to be identical to a bovine analogue of PTBLP. Additionally, because this protein was obtained by means of its mRNA binding ability, and its molecular size was 60 kDa (*42*), it is most likely that this protein is PTBLP-L. There are similar reports concerning Hu proteins. HuD protein binds to GAP-43 mRNA (*55*) and p21 mRNA (*59*) to stabilize these mRNA. There is a possibility that PTBLP-L binds to a 26-base pyrimidine-rich sequence in the 3′UTR of the GAP-43 mRNA and stabilizes the GAP-43 mRNA.

The present study reveals the roles of PTBLPs in the neuronal differentiation of PC12 cells. PTBLP-L acts as a negative regulator in neuronal differentiation and PTBLP-S acts as a competitor against PTBLP-L. The intracellular localizations of PTBLPs may be important in their function.

We wish to thank Dr. Tohru Kamata (Shinshu University, Matsumoto) for his helpful suggestions.

REFERENCES

1. Grabowski, P.J. (1998) Splicing regulation in neurons: tinkering with cell-specific control. *Cell* **92**, 709–712
2. Sakakibara, S. and Okano, H. (1997) Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. *J. Neurosci.* **17**, 8300–8312
3. 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
4. Sakai, K., Gofuku, M., Kitagawa, Y., Ogasawara, T., Hirose, G., Yamazaki, M., Koh, C.S., Yanagisawa, N., and Steinman, L. (1994) A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma. *Biochem. Biophys. Res. Commun.* **199**, 1200–1208
5. Levine, T.D., Gao, F., King, P.H., Andrews, L.G., and Keene, J.D. (1993) Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. *Mol. Cell Biol.* **13**, 3494–3504
6. Ma, W.J., Cheng, S., Campbell, C.A.W. and Furneaux, H. (1996) Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J. Biol. Chem.* **271**, 8144–8151
7. Fan, X.C. and Steitz, J.A. (1998) Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the *in vivo* stability of ARE-containing mRNAs. *EMBO J.* **17**, 3448–3460
8. Levy, N.S., Chung, S., Furneaux, H., and Levy, A.P. (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J. Biol. Chem.* **273**, 6417–6423
9. Myer, V.E., Fan, X.C., and Steitz, J.A. (1997) Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. *EMBO J.* **16**, 2130–2139
10. Ford, L.P., Watson, J., Keene, J.D., and Wilusz, J. (1999) ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. *Genes Dev.* **13**, 188–201
11. Peng, S.S., Chen, C.Y., Xu, N., and Shyu, A.B. (1998) RNA stabilization by the Aurich element binding protein, HuR, an ELAV protein. *EMBO J.* **17**, 3461–3470
12. 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
13. Richter, K., Good, P.J., and Dawid, I.B. (1990) A developmentally regulated, nervous system-specific gene in *Xenopus* encodes a putative RNA-binding protein. *New Biol.* **2**, 556–565
14. 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
15. Jensen, K.B., Dredge, B.K., Stefani, G., Zhong, R., Buckanovich, R.J., Okano, H.J., Yang, Y.Y., and Darnell, R.B. (2000) Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. *Neuron* **25**, 359–371
16. Grabowski, P.J. (2000) Genetic evidence for a Nova regulator of alternative splicing in the brain. *Neuron* **25**, 254–256
17. Buckanovich, R.J. and Darnell, R.B. (1997) The neuronal RNA binding protein Nova-1 recognizes specific RNA targets in vitro and in vivo. *Mol. Cell Biol.* **17**, 3194–3201
18. Yang, Y.Y., Yin, G.L., and Darnell, R.B. (1998) The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. *Proc. Natl. Acad. Sci. USA* **95**, 13254–13259
19. Brunel, F., Alzari, P.M., Ferrara, P., and Zakin, M.M. (1991) Cloning and sequencing of PYBP, a pyrimidine-rich specific single strand DNA-binding protein. *Nucleic Acids Res.* **19**, 5237–5245
20. Gil, A., Sharp, P.A., Jamison, S.F., and Garcia-Blanco, M.A. (1991) Characterization of cDNAs encoding the polypyrimidine tract-binding protein. *Genes Dev.* **5**, 1224–1236
21. Patton, J.G., Mayer, S.A., Tempst, P., and Nadal-Ginard, B. (1991) Characterization and molecular cloning of polypyrimidine tract-binding protein: a component of a complex necessary for pre-mRNA splicing. *Genes Dev.* **5**, 1237–1251
22. Kikuchi, T., Ichikawa, M., Arai, J., Tateiwa, H., Fu, L., Higuchi, K., and Yoshimura, N. (2000) Molecular cloning and characterization of a new neuron-specific homologue of rat polypyrimidine tract binding protein: PTB-like protein recognized by serum a patient with cancer-associated retinopathy. *J. Biochem.* **128**, 811–821
23. Tateiwa, H., Norimoto, G., Ichikawa, M., Kikuchi, T., and Yoshimura, N. (2001) Molecular cloning and characterization of human PTB-like protein: a possible retinal autoantigen of cancer-associated retinopathy. *J. Neuroimmunol.* **10**, 161–169
24. Polydorides, A.D., Okano, H.J., Yang, Y.Y., Stefani, G., and 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
25. Perez, I., McAfee, J.G., and Patton, J.G. (1997) Multiple RRMs contribute to RNA binding specificity and affinity for polypyrimidine tract binding protein. *Biochemistry* **36**, 11881–11890
26. Oh, Y.L., Hahm, B., Kim, Y.K., Lee, H.K., Lee, J.W., Song, O., Tsukiyama-Kohara, K., Kohara, M., Nomoto, A., and Jang, S.K. (1998) Determination of functional domains in polypyrimidine-tract-binding protein. *Biochem. J.* **331**, 169–175
27. Green, L.A. and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line od rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428
28. Green, L.A. and Tischler, A.S. (1982) PC12 cultures in neurobiological research. *Cell Neurobiol.* **3**, 373–414
29. Traina, G., Petrucci, C., Gargini, C., and Bagnoli, P. (1998) Somatostatin enhances neurite outgrowth in PC12 cells. *Dev. Brain Res.* **111**, 223–230
30. Gysbers, J.W. and Rathbone, M.P. (1996) GTP and guanosine synergistically enhance NGF-induced neurite outgrowth from

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PC12 cells. *Int. J. Dev. Neurosci.* **14**, 19–34
31. Rogers, M.V., Buensuceso, C., Montague, F., and Mahadevan, L. (1994) Vanadate stimulates differentiation and neurite outgrowth in rat pheochromocytoma PC12 cells and neurite extension in human neuroblastoma SH-SY5Y cells. *Neuroscience* **60**, 479–494
32. Ferriero, D.M., Sheldon, R.A., and Messing, R.O. (1994) Somatostatin enhances nerve growth factor-induced neurite outgrowth in PC12 cells. *Brain Res. Dev. Brain Res.* **80**, 13–18
33. Yanker, B.A., Benowitz, L.I., Villa-Komaroff, L., and Neve, R. (1990) Transfection of PC12 cells with the human GAP-43 gene: effects on neurite outgrowth and regeneration. *Mol. Brain Res.* **7**, 39–44
34. Lindenbaum, M.H., Carbonetto, S., Grosveld, F., Flavell, D., and Mushynski, W.E. (1988) Transcriptional and post-transcriptional effects of nerve growth factor on expression of the three neurofilament subunits in PC-12 cells. *J. Biol. Chem.* **263**, 5662–5667
35. Lee, V.M. and Page, C. (1984) The dynamics of nerve growth factor-induced neurofilament and vimentin filament expression and organization in PC12 cells. *J. Neurosci.* **4**, 1705–1714
36. Kawashima, K., Terashima, K., Yamamoto, K., Sakashita, E., and Sakamoto, H. (1999) Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. *Genes Cells* **4**, 667–683
37. Dingwall, C. and Laskey, R.A. (1991) Nuclear targeting sequences—a consensus? *Treands Biochem. Sci.* **16**, 478–481
38. Gorlich, D. and Mattaj, I.W. (1996) Nucleocytoplasmic transport. *Science* **271**, 1513–1518
39. Nakielny, S. and Dreyfuss, G. (1997) Nuclear export of proteins and RNAs. *Curr. Opin. Cell Biol.* **9**, 420–429
40. Elfgang, C., Rosorius, O., Hofer, L., Jaksche, H., Hauber, J., and Bevec, D. (1999) Evidence for specific nucleocytoplasmic transport pathways used by leucine-rich nuclear export signals. *Proc. Natl. Acad. Sci. USA* **96**, 6229–6234
41. Izaurralde, E. and Mattaj, I.W. (1995) RNA export. *Cell* **81**, 153–159
42. Irwin, N., Basekelandt, V., Goritchenko, L., and Benowitz, L.I. (1997) Identification of two proteins that bind to a pyrimidine-rich sequence in the 3′-untranslated region of GAP-43 mRNA. *Nucleic Acids Res.* **25**, 1281–1288
43. Skene, J.H. (1989) Axonal growth-associated proteins. *Annu. Rev. Neurosci.* **12**, 127–156
44. Nishizawa, K. and Okamoto, H. (1994) Mutation analysis of the role for the carboxyterminus encoding region in NGF-induced stabilization of GAP-43 mRNA. Biochem. *Biophys. Res. Commun.* **205**, 1380–1385
45. Nishizawa, K. (1994) NGF-induced stabilization of GAP-43 mRNA is mediated by both 3′ untranslated region and a segment encoding the carboxy-terminus peptide. *Biochem. Biophys. Res. Commun.* **200**, 789–796
46. Perrone-Bizzozero, N.I., Cansino, V.V., and Kohn, D.T. (1993) Posttranscriptional regulation of GAP-43 gene expression in PC12 cells through protein kinase Cdependent stabilization of the mRNA. *J. Cell Biol.* **120**, 1263–1270
47. Tasi, K.C., Cansino, V.V., Kohn, D.T., Neve, R.L., and Perrone-Bizzozero, N.I. (1997) Post-transcriptional regulation of the GAP-43 gene by specific sequences in the 3′ untranslated region of the mRNA. *J. Neurosci.* **17**, 1950–1958
48. Basi, G.S., Jacobson, R.D., Virag, I., Schilling, J., and Skene, J.P. (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. *Cell* **49**, 785–791
49. Kohn, D.T., Tsai, K.C., Cansino, V.V., Neve, R.L., and Perrone-Bizzzero, N.I. (1996) Role of highly conserved pyrimidine-rich sequences in the 3′ untranslated region of the GAP-43 mRNA in mRNA stability and RNA-protein interactions. *Mol. Brain Res.* **36**, 240–250
50. Chung, S., Eckrich, M., Perrone-Bizzozero, N., Kohn, D.T., and Furneaux, H. (1997) The Elav-like proteins bind to a conserved regulatory element in the 3′-untranslated region of GAP-43 mRNA. *J. Biol. Chem.* **272**, 6593–6598
51. Decker, C.J. and Parker, R. (1995) Diversity of cytoplasmic functions for the 3′ untranslated region of eukaryotic transcripts. *Curr. Opin. Cell Biol.* **7**, 386–392
52. Sachs, A.B. (1993) Messenger RNA degradation in eukaryotes. *Cell* **74**, 413–421
53. Shyu, A.B., Belasco, J.G., and Greenberg, M.E. (1991) Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. *Genes Dev.* **5**, 221–231
54. Kwon, Y.K. and Hecht, N.B. (1991) Cytoplasmic protein binding to highly conserved sequences in the 3′ untranslated region of mouse protamine 2 mRNA, a translationally regulated transcript of male germ cells. *Proc. Natl. Acad. Sci. USA* **88**, 3584–3588
55. Richter, J.D. (1991) Translational control during early development. *Bioessays* **13**, 179–183
56. Ding, D. and Lipshitz, H.D. (1993) Localized RNAs and their fuctions. *Bioessays* **15**, 651–658
57. Johnston, D.S. (1995) The intracellular localization of messenger RNAs. *Cell* **81**, 161–170
58. Wilhelm, J.E. and Vale, R.D. (1993) RNA on the move: the mRNA localization pathway. *J. Cell Biol.* **123**, 269–274
59. Joseph, B., Orlian, M., and Furneaux, H. (1998) p21(waf1) mRNA contains a conserved element in its 3′-untranslated region that is bound by the Elav-like mRNAstabilizing proteins. *J. Biol. Chem.* **273**, 205111–20516

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