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DEVELOPMENTAL CHANGE IN SUBCELLULAR LOCATION OF BP-1 PROTEIN WITH AN ABILITY TO INTERACT WITH BOTH IDENTIFIER SEQUENCE AND ITS BRAIN- SPECIFIC TRANSCRIPT, BC-1 RNA

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Summary: Identifier sequences are transcribed to generate a brain-specific BC-1 RNA present as a ribonucleoprotein particle in the dendrites and somata of neurons. This ribonucleoprotein particle contains an identifier sequence-binding protein (Bp-1 protein). We report here the purification of BC-1 RNA and demonstrate that Bp-1 protein interacts directly with the RNA. We also demonstrate an accumulation of Bp-1 protein in the nucleus of brain cells from mouse fetus and newborns that precedes the postnatal increase in BC-1 RNA. Cytoplasmic Bp-1 protein present in a complex with BC-1 RNA increases postnatally with a concomitant decrease in nuclear Bp-1 protein. These observations suggest that Bp-1 protein may play a role(s) in the synthesis and nuclear export of BC-1 RNA.

BC-1 RNA is a brain-specific small RNA transcript of identifier (ID) sequences by RNA polymerase III (pol III)(1,2) and is one of the dendritic RNAs(3). We recently demonstrated that the RNA is complexed with proteins to form a 10S ribonucleoprotein particle (BC-1 RNP)(4). These proteins may govern possible neuronal cell-specific functions of BC-1 RNP such as transport of mRNAs to dendrites and may also be involved in their translation on site(3-6). In a previous report, we demonstrated that the RNP contains an ID sequence-binding protein (Bp-1 protein)(6). The binding region is located between split promoter sequences for pol III within the ID sequences and contains three-repetitive GCAAG/CTTGC pentamer motifs (see also Fig.1). The following

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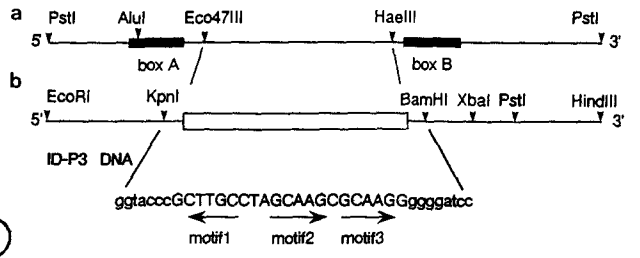

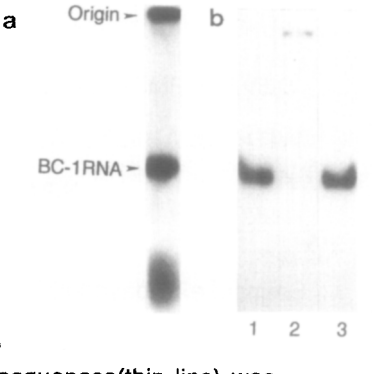

Fig.1 (a) An *Eco*47III/*Hae*III fragment (ID-P3 DNA) of ID sequences(thin line) was cloned into the *Sma*I site of pUC18 DNA. Boxes A and B indicate promoter sequences for pol III. (b) A *Kpn*I/*Bam*HI fragment of the construct labeled with γ-[^{32}P]ATP was used as a probe. Thin line : pUC 18 DNA. Uppercase letters : nucleotide sequences of the ID sequences. Arrows: GCAAG/CTTGC motifs.

Fig.2 (a) Poly(A)$^+$ RNA in the postribosomal fraction of brain homogenates was purified and labeled with [^{32}P]pCp. Radiolabeled RNA was passed over an oligo(dT) cellulose column and separated on a polyacrylamide gel containing urea. The position of BC-1 RNA is indicated. (b) BC-1 RNA purified by gel electrophoresis in (a) was heated in a solution containing 100 mM NaCl at 60°C in the presence (lanes 2 and 3) or absence (lane 1) of DNA, and subjected to electrophoresis on a 10% non-denaturing gel. DNA included : a coding strand of the ID sequences cloned in a single-stranded M13 DNA (lane 2) or single-stranded M13 vector DNA (lane 3).

evidence for certain promoter sequences and a transcription factor for pol III suggests that these pentamer motifs, along with Bp-1 protein, may play a role(s) in the brain-specific transcription of the ID sequences: 1. An array of the pentamer motifs has the structural feature of repeated guanine residues about 5-bp apart in the noncoding strand, which is characteristic of the promoter sequences of many other genes transcribed by pol III(7). 2. Of the pol III transcription factors, *Xenopus* oocyte- specific transcription factor (TFIIIA) for 5S ribosomal RNA (rRNA) genes associates with 5S RNA to form a 7S RNP(8). To play a role(s) in the synthesis of BC-1 RNA, the Bp-1 protein should be present also in the nucleus of brain cells, although it was originally found in the cytoplasmic BC-1 RNP(6). In this context, it is interesting to examine whether Bp-1 protein changes in amount during pre- and postnatal development, since BC-1 RNA greatly increases during the first month after birth(9,10).

We also suggested(6) that the Bp-1 protein has the ability to interact directly with BC-1 RNA, since brain RNA containing BC-1 RNA competed with a [^{32}P]ID sequence probe (ID-P3 DNA, Fig.1) for Bp-1 protein. However, since the competitor RNAs used included those other than BC-1 RNA, it remains to be confirmed whether BC-1 RNA itself was responsible for the competition observed, although this is likely. Hence, in this

report, we first purified BC-1 RNA and used it as a probe to unequivocally show the ability of Bp-1 protein to interact directly with BC-1 RNA.

MATERIALS AND METHODS

Materials: Inbred ddY strain mice were purchased from Sankyo Laboratory.
Preparation of [^{32}P]ID sequence probe: A [^{32}P]probe (ID-P3 DNA, Fig.1) was prepared as described previously(6).
Preparation of [^{32}P]BC-1 RNA: A postribosomal fraction of brain homogenates was prepared as described previously (4). Its poly(A)$^+$ RNA was purified by oligo(dT) cellulose column chromatography (11). RNA retained on the column was then labeled with 5'- [^{32}P] cytidine 3', 5'- bis (phosphate) ([^{32}P]pCp) by RNA ligase according to Bruce and Uhlenbeck(12). Radiolabeled BC-1 RNA was purified again by oligo(dT) cellulose column chromatography, followed by purification on a 10% polyacrylamide gel containing 4.5 M urea.
Preparation of extract of subcellular fractions of brain cells: Brain homogenates were successively spun at 1000xg and 15000xg as described(4) and the resulting postmitochondrial supernatant was referred to as the cytoplasmic extract. Nuclear extract was prepared essentially according to Gorski et al.(13). Purified nuclei were lysed and extracted with a solution containing 550 mM KCl. The lysate was centrifuged and the resulting supernatant was dialyzed and used as a nuclear extract.
Purification of Bp-1 protein: Bp-1 protein was successively purified from brain homogenates with a glycerol gradient, DE 52 column, hydroxylapatite column and heparin agarose column as described previously (6). At the last step of purification, Bp-1 protein was partially dissociated from BC-1 RNA and retained on the heparin column. This Bp-1 protein was used in a gel shift assay.
Gel shift assay: Gel shift assay was performed essentially according to Singh et al. (14) with some modifications (6).

RESULTS AND DISCUSSION

BC-1 RNA obtained from the postribosomal fraction of brain homogenates was labeled with [^{32}P]pCp and purified again on an oligo(dT) cellulose column. Retained RNA was then separated on a polyacrylamide gel. Fig.2-a shows that two discrete RNAs were resolved within the gel. The RNA with a higher molecular mass seems to be BC-1 RNA, since this was found to disappear when hybridized with a single-stranded DNA containing a coding strand of the ID sequences(r-4 DNA)(15) prior to gel electrophoresis (Fig.2-b, lane 2). Furthermore, it had a mobility identical to that of BC-1 RNA, which was analyzed on a Northern gel run in parallel (data not shown). The lower molecular mass RNA seen in Fig.2-a was also hybridizable with r-4 DNA, indicating that this RNA may be a degradation product of BC-1 RNA. We used a [^{32}P]BC-1 RNA thus obtained as a probe to examine whether Bp-1 protein binds to BC-1 RNA. The Bp-1 protein used was successively purified from brain homogenates with a glycerol gradient and three different columns. In agreement with previous studies (6), this

protein binds to [^{32}P]ID-P3 DNA (Fig. 3-a). Fig.3-b shows that the Bp-1 protein also binds to [^{32}P]BC-1 RNA (lane 1) and that an excess amount of ID-P3 DNA competed with [^{32}P]BC-1 RNA for Bp-1 protein more effectively than did nonspecific competitor DNA (lanes 2-7). Fig.3-a,b supports our previous findings(6) and demonstrates that Bp-1 protein associates directly with BC-1 RNA as well as with the ID sequences.

We next analyzed the expression of Bp-1 protein in the subcellular fractions of brain cells during pre- and postnatal development of mice. Cytoplasmic extract and nuclear extract were prepared and analyzed in a gel shift assay using a [^{32}P]ID-P3 DNA as a probe. Fig.4-a shows that Bp-1 protein level was high in the brain cell nucleus from mice of prenatal day 3 through postnatal day 4 , then decreased and remained constant thereafter. In contrast, its cytoplasmic counterpart increased with a developmental time-course similar to that of BC-1 RNA (compare Fig.4-b with c). Fig.3-b indicates that this cytoplasmic Bp-1 protein associates directly with BC-1 RNA in the RNP. In fact, no significant amount of Bp-1 protein was detected in the cytoplasmic extracts without RNase treatment (data not shown)(6). In contrast, Bp-1 protein in the nucleus does not seem to associate with RNA, since similar Bp-1 activity was detected in nuclear extracts with or without treatment with RNase A. Moreover, no BC-1 RNA was detected in the extracts used (data not shown). These observations in turn indicate that cytoplasmic contamination of the purified nuclei was negligible, if any at all. Fig.4- a,c demon-

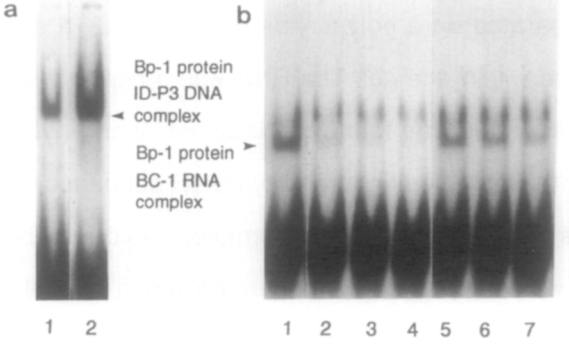

Fig.3 (a) Bp-1 protein retained on a heparin agarose column was analyzed in a gel shift assay using a [^{32}P]ID-P3 DNA as a probe (lane 1). The 10S BC-1 RNP digested with 100 μg/ml RNase A at 37°C for 10 min was used as a control (lane 2). All reactions contained 500 ng of poly(dI-dC)poly(dI-dC). (b) [^{32}P] BC-1 RNA was incubated with the Bp-1 protein under the same conditions as those of (a) except that 100 units of RNase inhibitor from human placenta was included. Competitor DNAs additionally included were ID-P3 DNA (lanes 2-4) and salmon sperm DNA (lanes 5-7). Amounts of additional DNAs included were 0 ng (lane 1), 2.5 ng (lanes 2 and 5), 5 ng (lanes 3 and 6) and 10 ng (lanes 4 and 7).

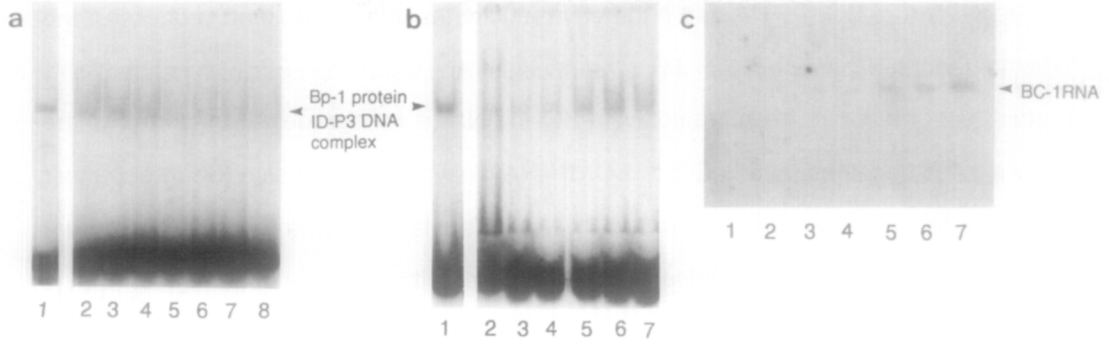

Fig.4 (a) Nuclear extracts were prepared from brains of different ages and analyzed in a gel shift assay using a [^{32}P] ID-P3 DNA (4000 cpm) as a probe. Reactions 2-8 contained 5 μg of nuclear extract and 4 μg of poly(dI-dC)poly(dI-dC). Lane 1 contained 5 μg of 10S BC-1 RNP digested with RNase A and 0.5 μg of poly(dI-dC)poly(dI-dC). Developmental ages were days -3, 0, 4, 10, 18, 30 and 100 (lanes 2-8, respectively). (b) Cytoplasmic extracts from brains corresponding to those in (a) were analyzed under the same conditions as those of (a) except that lanes 2-7 contained 10 μg of extracts treated with 100 μg/ml of RNase A at 37°C for 10 min and 1 μg of poly(dI-dC)poly(dI-dC). Developmental ages were days -3, 0, 4, 10, 18 and 30 (lanes 2-7, respectively). Lane 1 contained 5 μg of 10S BC-1 RNP as a control. (c) Northern blot analysis of total cytoplasmic RNA from brains of different ages using [^{32}P]pABr-4 DNA (14) as a probe. All reactions contained 10 μg of RNA. Developmental ages were days -3, 0, 4, 10, 18, 30 and 100 (lanes 1-7, respectively).

strates that the postnatal increase in cytoplasmic BC-1 RNA is preceded by a nuclear accumulation of Bp-1 protein. This accumulation in brains of mouse fetus and newborns seems to be relevant to the transcriptional competence of the ID sequences during postnatal development. It is important to determine whether Bp-1 protein associates only with the ID sequence(s) that is active in transcription. Furthermore, since the decrease in the level of Bp-1 protein in the nucleus is accompanied by an increase in the cytoplasm (Fig.4-a,b), Bp-1 protein may bind to a newly synthesized BC-1 RNA and migrate out of the nucleus with it. This Bp-1 protein seems to remain in a complex with BC-1 RNA in the somato-dendritic compartment after exported from the nucleus, since no Bp-1 protein present in the free state was found in the postribosomal fraction of brain homogenates (6). Taken together, this suggests that Bp-1 protein may play a dual role in transcription and nuclear export of RNA. Guddat et al. (16) reported that a transcription factor for 5S rRNA genes, TFIIIA binds to 5S rRNA and mediates its nuclear export.

Tiedge et al.(3) recently reported that BC-1 RNA is a member of the dendritic RNAs. The molecular basis for the selective import of BC-1 RNP into dendrites is unknown, however, as described above, Bp-1 protein appears to be involved in the regulation of

nuclear export of BC-1 RNA and it thereby participates in the transport process of the RNP to dendrites that originates from the nucleus. An interesting possibility is that Bp-1 protein (either alone or in a complex with BC-1 RNA) is retrogradely transported to the nucleus, and becomes recruited for BC-1 RNA biosynthesis and also for an enhancer activity of the ID sequences for pol II (17). More detailed analysis of the Bp-1 protein will provide information on the biological significance of its presence in dendrites.

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