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Cloning and Expression of a Rat Brain Basic Helix–Loop–Helix Factor[1]

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We cloned two rat cDNAs of brain basic helix–loop–helix factor 1 (BHF1). These have an identical coding region, contain 357 amino acids and exhibit 94.6% identity to MATH-2/NEX1 in the basic helix–loop–helix region. BHF1mRNAs are dominantly expressed in the brain, particularly in the cerebellum, in the adult bovine, rat and mouse. Two shorter BHF1mRNAs (1.6 kb and 1.8 kb) were also detected in the mouse embryo, and these decreased in the developmental process. These results suggest that BHF1 may play important roles in cerebellum-specific functions and development of neurons.

Basic helix–loop–helix (bHLH) proteins work as transcriptional factors to regulate myogenesis and neurogenesis. In muscle development, a family of myogenic regulatory factors with the bHLH domain, MyoD1, Myogenin, Myf-5, and MRF4 is involved both in myogenic determination and in myogenic differentiation. Each of these myogenic bHLH proteins is able to convert cells derived from all three embryonic layers to the myogenic lineage, and some members directly activate the transcription of muscle-specific genes (1,2). In neurogenesis, bHLH factors play an essential role in the early stages of neural development in *Drosophila.* The bHLH factors are encoded by the proneural genes, *achaete-scute* complex and *daughterless.* (3,4). In mammals, some homologues of the bHLH factors of *Drosophila* were isolated and were shown to play important roles in neuronal development (2,5). Mash1, a homologue of Drosophila proneural *achaete-scute* complex, was shown to be essential for the early development of olfactory and autonomic neurons. However, the remaining central nervous system was intact in the Mash1 knockout mouse and other unknown bHLH factors are believed to play essential roles in neural development and neural functions (6).

We report herein a rat brain basic helix–loop–helix factor 1 (BHF-1). This gene is dominantly expressed in the brain, particularly cerebellum in different species, from early development to the adult stage.

MATERIALS AND METHODS

A bovine genomic library was screened under the low stringency condition (the final washing condition: at 60°C, 30 mM NaCl, 3 mM Na_3citrate) by hybridization *in situ* with the 1,214-bp BamHI-EcoRI cDNA fragment of the bovine myosin I heavy chain (MIHC) as described previously (7). The 207 bp DdeI-XbaI cDNA fragment covering the ATP binding site of MIHC (7) was used with further hybridization analysis.

A rat cerebellum cDNA library (8) was screened by hybridization *in situ* with the 3.8 kb Hind III fragment of the bovine λ71 clone. The identified clones were subcloned in the pBluescript plasmid and sequenced by the dideoxy chain termination method using Sequenase 2.0 (U.S. Biochemical Corp.) with a [^{35}S] and [^{33}P] dCTP.

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GeneBank nucleotide sequence database with the following accession numbers: D82074 (p16) and D82075 (p93).

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Abbreviations: bHLH, basic helix–loop–helix; BHF1, brain basic helix–loop–helix factor 1; MIHC, myosin I heavy chain.

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Total RNA was extracted from cell lines and tissues of bovine, rat, and mouse by the method (9). Poly(A)$^+$RNA was purified with oligo-dT column and oligo-dT Latex. RNA blotting analysis was performed according to the procedure described (9).

RESULTS AND DISCUSSION

We screened a bovine genomic library by hybridization to isolate gene families of MIHC. In this process, we isolated one clone λ71. RNA blotting analysis with a 3.9 kbp Hind III fragment of the λ71 clone revealed the 2.4 kb and 3.9 kb specific bands and abundant expression in the cerebellum, and no signal of total RNA in other brain regions or the peripheral tissues. There was only a slight signal of ploy(A)$^+$RNA in the amygdala (Figure 1). This characteristic expression of the gene led to further analysis, although λ71 showed a weak hybridization signal, indicating a low possibility of belonging to MIHC gene families.

We screened 10^5 plaques of a rat cerebellum cDNA library with a 3.9 kbp Hind III fragment of the λ71 clone. Two types of cDNA clones, p16 and p93, were isolated and analyzed (Figure 2). The p16 clone had a long open reading frame, with the first ATG considered to be the translation start site. This open reading frame consists of 357 amino acids and its calculated molecular size was 40.0 kilodaltons. This was assigned as BHF1 (Brain basic helix–loop–helix factor 1), because of its basic helix–loop–helix (bHLH) region as shown later. BHF1 exhibited 94.6% identity to MATH-2/NEX-1, a mammalian Atonal related factor, in the basic helix–loop–helix region (10,11). BHF1 showed similarities to other neuronal bHLH factors, MATH-1 (60.7%), Atonal (53.6%), and MASH-1 (48.2%) in the bHLH region (Figure 3) (5,12,13). BHF1 showed poly-glutamic acid and aspartic acid (in the region between amino acid residues 58 and 78) with a leucine interruption at 66. This acidic region may be involved in transcriptional activation. BHF1 also showed a basic region (basic region 1) between the acidic region and the basic region of bHLH. The basic region 1 may interact with the acidic region of BHF1 and be related with the folding of the BHF1 protein. Half of the carboxyl-terminal of BHF1 is rich in proline (24.7% between amino acid residues 192 and 276). These proline residues may be involved in protein-protein interaction (14). The p93 clone has a long 5′-untranslated region. This untranslated region starts at nucleotide position 17 of the p16 clone and a long non-coding region (1484bp) was inserted between nucleotides 86 and 87 of the p16 clone. The long open reading frame and 3′-untranslated region of p93 were identical to those of p16, except for the polyadenylation site. The 3′-untranslated region of p93 and p16 had four RNA destabilizational signals (ATTTA), and this may suggest BHF1 is regulated by a rapid process. According to these structural analyses, the p93 clone seems to be a read-through product,

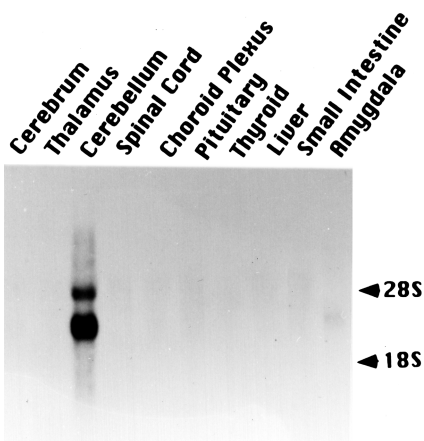

FIG. 1. RNA blotting analysis of bovine tissues. Each lane contains 10μg total RNA from bovine tissues, except for amygdala, which contains 2μg poly(A)$^+$RNA.

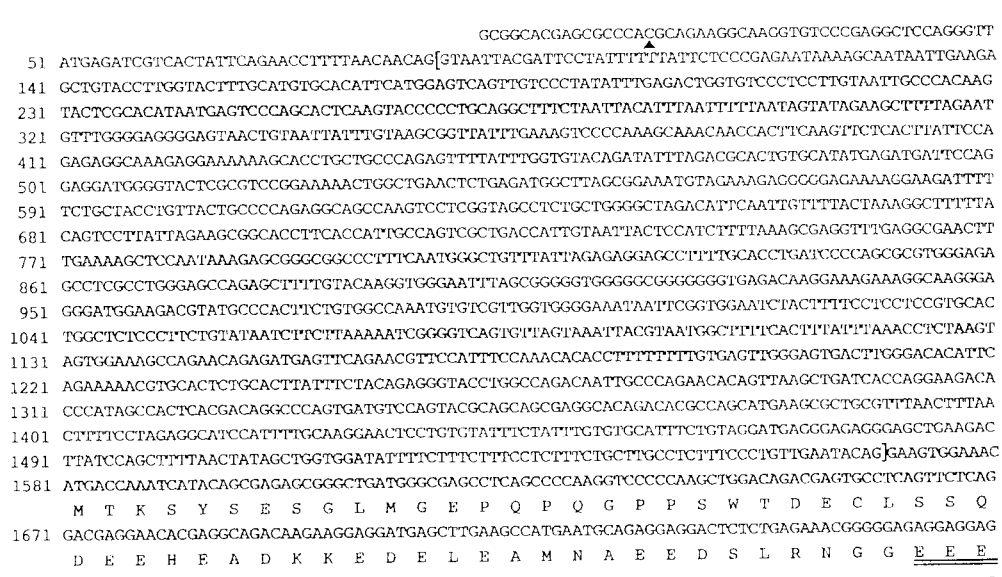

FIG. 2. Nucleotide and predicted amino acid sequences of rat BHF1 cDNAs. Nucleotide residues are numbered at the left column and the amino acid residues are numbered at the right column. The rat BHF1 p16 clone contains the sequence from nucleotide residue number 1 to 3435, except for the sequence from 87 to 1569, indicated by [ ]. The rat BHF1 p93 clone contains a sequence from nucleotide residue number 17 indicated by a *black triangle* to nucleotide residue number 3311 indicated by an *open triangle.* The amino acid numbers start at the putative initiation methionine assigned to the first ATG codon of p16 and of a long open reading frame of p93. Polyglutamic acid and aspartic acid is *underlined.* The helix–loop–helix region is *double underlined.* mRNA destabilization signals in the 3′ untranslated region are indicated by *upper dots.*

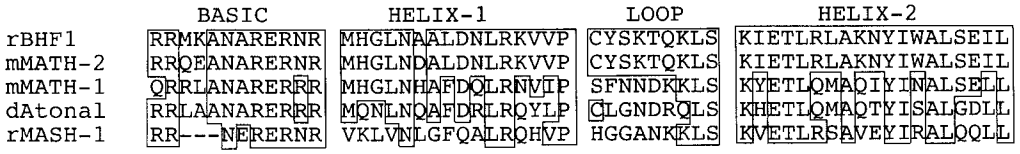

FIG. 3. Comparisons of basic helix–loop–helix regions of BHF1 and other bHLH factors. The positions of the basic region, the putative amphipathic helix 1 and 2, and the loop are shown *above.* The conserved residues between BHF1 and other bHLH factors are *boxed.*

comparing the p16 clone and the mouse genome (Maruyama H, et al in preparation). RNA blotting analysis revealed a large amount of 2.2 kb bands and less of 3.8 kb bands in the rat cerebellum, but no signal in the rat liver or cerebrum (Figure 4). The p16 and p93 clones were thought to correspond to these two bands, respectively.

More detailed analysis of tissue specific and developmental expression were performed in mice. In adult mice, the RNA blotting analysis revealed the presence of a major BHF1 transcript of 2.2 kb and a minor transcript of 3.6 kb in only the brain. The bands were detectable strongly in the cerebellum. In other brain regions, long exposure showed a faint band in the olfactory bulb. They were not detectable in other peripheral tissues examined (Figure 5a). In developmental analysis, the shorter bands were already present at day 8 of the mouse embryo. These bands were broad but the main bands showed 1.6 kb and 1.8 kb. Two longer bands, 2.2 kb and 3.6 kb, also existed but were less in amount than the two shorter bands. In progress of development, the two shorter BHF1mRNA gradually disappeared and the 2.2 kb BHF1mRNA increased. In the adult brain, the 2.2 kb BHF1mRNA was dominantly expressed and the two shorter BHF1mRNAs showed little signal (Figure 5b). Furthermore, BHF1mRNAs were observed in a similar pattern to the early developmental stage, in RNAs from mouse embryonal carcinoma cell lines, P19 and F9, and from the mouse neuroblastoma cell line, neuro2a (Figure 5b).

We cloned the BHF1 into the expression vector pcDNA3. When we transfected the BHF1 expression vector with or without E47, we could detect no activation of transcription of the promoter with seven repeats of E-box element (CAGGTG) (12) or NEX1 promoter (10) in either P19 or PC12 cells. In addition, the BHF1 expression vector was introduced into the P19 cell and selected with G418. In our preliminary results, the colonies which appeared showed no morpho-

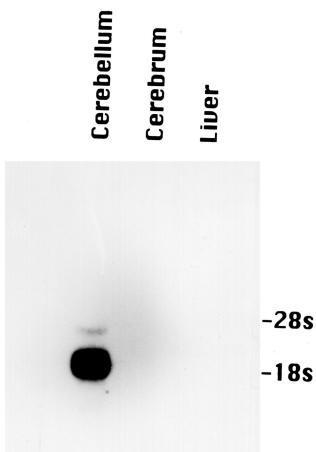

FIG. 4. RNA blotting analysis of rat tissues. 10μg total RNAs from the adult rat cerebellum, cerebrum, and liver were analyzed.

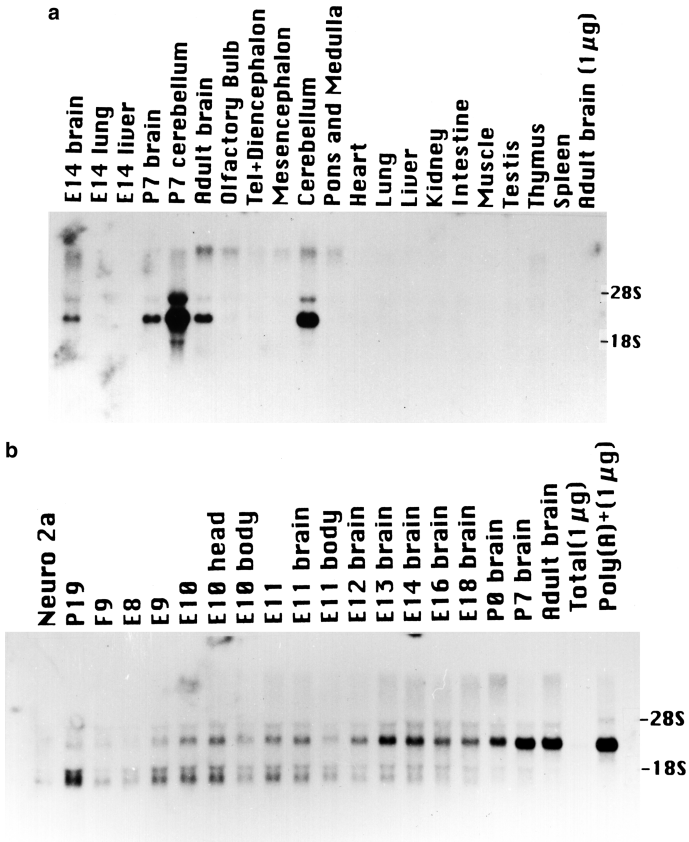

FIG. 5. RNA blotting analysis of BHF1 mRNA. (a) 10μg of total RNAs from various in embryo (E14), postneonatal (P7), and adult mice, and μg of total RNA from the brain in the adult mice were analyzed. (b) 10μg of total RNAs from various cell lines (Neuro2a, P19, and F9), whole mouse embryos (E8, E9, E10, E11), the head (E10), the bodies (E10 and E11), the brains of embryo (E11, 12, 13, 14, 16, 18), neonate (PO), 7-day-old (P7), adult mice, and 1μg of total and poly(A)$^+$RNA from the brains of adult mice were analyzed.

rogical change with or without bovine fetal serum in the medium, compared with untransfected P19 cells.

We cloned a rat basic helix–loop–helix factor 1 (BHF1). This clone is dominantly expressed in the cerebellum in the adult and the embryo. These RNA blotting analyses suggested that BHF1 plays important roles in cerebellum-specific functions and the development of neurons. Particularly, in the development process, the shorter RNAs were expressed in the early developmental stage, then gradually changed to the middle sized RNA, and in the adult the shorter RNAs had disappeared. These changes of BHF1 mRNAs suggest that the function of BHF1 in the embryo may be different from those in the adult. Further studies of the structural and functional analysis will lead to a better understanding of BHF1.

In additional note, while we were conducting functional analysis, reports on the hamster BETA 2 (15) and mouse NeuroD (16) were published. Rat BHF1 corresponds to a homologue of these.

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