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MOLECULAR CLONING OF HUMAN HIPPOCALCIN cDNA AND CHROMOSOMAL MAPPING OF ITS GENE*

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SUMMARY: We have isolated a cDNA clone encoding human hippocalcin from a human hippocampus cDNA library. This clone (hHLP1) consists of 840 nucleotides, including the entire open reading frame of 582 nucleotides, 10 nucleotides of the 5' leader and 248 nucleotides of the 3' noncoding regions. Comparison of the human hippocalcin sequence with the corresponding rat sequence revealed an amino acid identity of 100% and nucleotide identity of 92%. Northern blot analysis showed that a single transcript at a position corresponding to 2.0 kb was detected only in the brain. The human hippocalcin gene was mapped to chromosome 1 by amplification of a human hippocalcin-specific DNA fragment on DNA from human-rodent somatic cell hybrids by using the polymerase chain reaction.

Hippocalcin is a 23-kDa Ca^{2+}-binding protein with three EF-hand structures, recently identified in the rat hippocampus (1). The primary structure of rat hippocalcin is composed of 193 amino acid residues, and it has a striking sequence homology to those of chick visinin (2), bovine recoverin (3) and frog S-modulin (4), which are located in the photoreceptor cells and regulate photo-signal transduction systems via prolonging the light-activation of cyclic GMP hydrolysis in a Ca^{2+}-sensitive manner (5, 6, 7). Hippocalcin binds three moles of Ca^{2+} per mole of protein at submicromolar Ca^{2+} levels, and is associated with the plasma membrane in a Ca^{2+}-dependent manner (8). Hippocalcin is myristoylated at its NH_2-terminal glycine residue, a key event in terms of its membrane-association property (8). In the adult rat brain, hippocalcin is expressed abundantly in the pyramidal cells of the hippocampus and weakly in the Purkinje cells of the cerebellum and pyramidal cells of the cerebral cortex (9). In these

*Sequence data of human hippocalcin have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession No. D16593. Sequence data of rat hippocalcin (D12573) have been corrected and the correct data have been deposited under the same accession number.

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cells, hippocalcin is located in the cytoplasm and plasma membrane of the cell body and dendrites. In the developing rat brain, hippocalcin expression in the hippocampus increases with age, whereas its expression in the cerebral cortex and cerebellum is higher in the early postnatal ages than in older ages (10). These results suggest that the expression of hippocalcin is strictly controlled by both cell type and the developmental process, and that hippocalcin plays a role in neuronal differentiation in the early stages of development and may be related to other neuronal functions in the adult brain.

Isolation of the cDNA for human hippocalcin should be useful for investigating the function and expression of this protein in the human brain and the possible relationship of hippocalcin to human neurological disorders. In the present study, we screened a human hippocampus cDNA library using rat hippocalcin cDNA (1) as a probe, and isolated human hippocalcin cDNA. Tissue distribution of human hippocalcin mRNA in normal human tissue and the chromosomal location of the gene were subsequently determined.

MATERIALS AND METHODS

Materials: Restriction endonucleases, Taq DNA polymerase and other modifying enzymes were obtained from Takara Shuzo, a human hippocampus lambda-ZAP II cDNA library and an ExAssist/SOLR Excision System from Stratagene, [α-^{32}P]dATP (110 TBq/mmol) from Amersham Japan, Sequenase Ver. 2.0 kit from United States Biochem., the human multi-tissue Northern blot filter from Clontech, the genomic DNAs of 20 human-rodent somatic hybrid cell lines from Bios Lab. Oligonucleotides were synthesized on a model 392A DNA synthesizer (Applied Biosystems). All other chemicals were of the highest grade available.

Screening of cDNA Library: Approximately 1.6 x 10^6 plaques from a human hippocampus cDNA library were screened. The polymerase chain reaction (PCR) was performed between nucleotide residues 1-582 of rat hippocalcin cDNA (1) by using two synthetic primers to which the *Eco*RI site was added. The resulting PCR product was subcloned into the *Eco*RI site of the plasmid pBluescript II SK(-) to yield the plasmid pBHE1. The *Eco*RI fragment from pBHE1 was labeled by the multi-priming method (11) and used as a probe. Hybridization was carried out at 40°C for 20 h in a solution containing 5 x SSC, 5 x Denhardt's solution, 100 μg of denatured salmon sperm DNA per ml, 0.1% SDS, 50% formamide and the probe (2 x 10^6 cpm/ml). After hybridization, the filters were washed twice with 0.1 x SSC containing 0.1% SDS at 45°C for 30 min. The filters were then dried and autoradiographed at -80°C for 2 days.

DNA Sequencing: The plasmid pBluescript SK(-) carrying the cloned cDNA was obtained from *Escherichia coli* strain SOLR™ co-infected with the cloned phage and ExAssist helper phage according to the Stratagene protocol. The nucleotide sequences were determined by the dideoxy chain termination method (12) with [α-^{32}P]dATP and a Sequenase Ver. 2.0 Kit. The double-strand plasmid was first sequenced from both strands by using two vector primers which flanked the cDNA insert. The complete sequence was obtained by custom primer-directed DNA sequencing using specific primers complementary to the internal cDNA sequence.

Northern Blot Analysis: The human multi-tissue Northern blot filter was used. PCR was performed between nucleotide residues 410-554 of human hippocalcin cDNA (Fig. 1) by using synthetic oligonucleotide primers. The resulting PCR product was blunt-ended with T4 DNA polymerase and subcloned into the *Hinc* II site of the plasmid pBluescript II SK(-) to yield the plasmid pBBR11. The *Xho* I-*Hind* III fragment from pBBR11 was labeled by the multi-priming method (11) and used as a probe. Hybridization was carried out at 42°C for 20 h in a solution containing 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1% SDS, 250 μg of denatured salmon sperm DNA per ml and the probe (2 x 10^7 cpm/ml). After hybridization, the filter was washed twice with 0.2 x SSC containing 0.1% SDS at 50°C for 30 min. The filter was then dried and exposed to Kodak XAR film at -80°C for 4 days.

Chromosomal Mapping: Chromosomal mapping was performed by discordancy analysis using DNAs from 20 human-rodent somatic cell hybrids plus human, hamster and mouse control DNA. PCR was performed to amplify a human hippocalcin gene-specific fragment (nucleotide residues 18-136). The synthetic oligonucleotide primers (the sense primer CAAGCTGTGGTCCGAGATGT and the antisense primer CCACATAGAAGATTCCTGTG) were designed from human hippocalcin cDNA and introduced two nucleotide substitutions (underline) to amplify specifically the human gene in human-rodent somatic hybrids. The size of the amplification product was determined by using 5% polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

A human hippocampus cDNA library was screened with a rat hippocalcin cDNA probe (1) under low stringency hybridization conditions. Eight independent cDNA clones were obtained and classified into five species based on the restriction enzyme maps, and were expediently designated as hHLP1-5. As shown in Fig. 1, clone hHLP1 consisted of 840

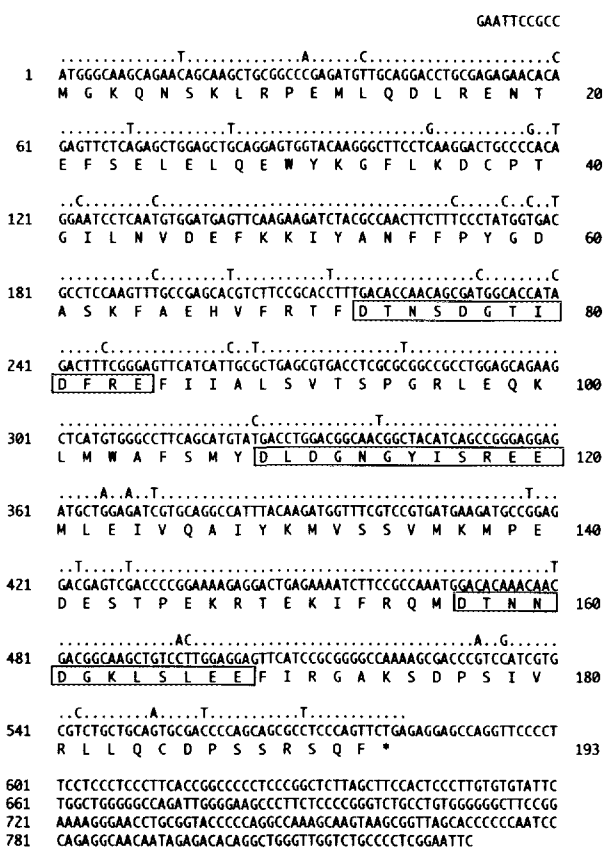

Fig. 1. Nucleotide sequence of human hippocalcin cDNA and its deduced amino acid sequence. The nucleotide sequence (second line) and the deduced amino acid sequence (third line) are numbered beginning with the first nucleotide of the translational initiation codon (left side) and the initiator methionine (right side), respectively. The nucleotide sequence is compared with the corresponding rat sequence (first line). Rat nucleotides are shown only when they are different from human nucleotides. Identical nucleotides in rat are shown with dots. Three putative calcium-binding domains of the EF-hand structure are boxed. * Indicates termination codon.

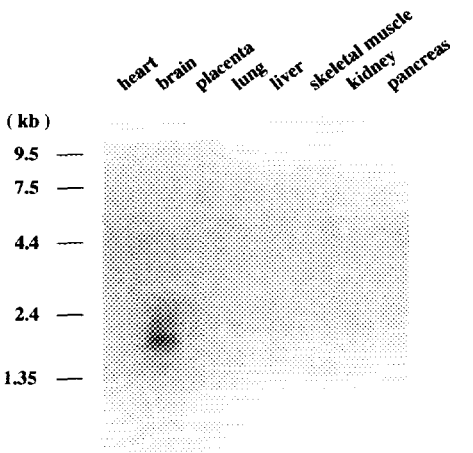

Fig. 2. Northern blot analysis of human hippocalcin mRNA in human tissues. A Northern blot filter containing 4 μg of poly (A)$^+$ RNA per lane from various human tissues was hybridized with the ^{32}P-labeled human hippocalcin-specific probe. Positions of RNA size markers are indicated on the left. A transcript of approximately 2.0 kb is seen only in the brain.

nucleotides including the entire open reading frame of 582 nucleotides, 10 nucleotides of the 5' leader and 248 nucleotides of the 3' noncoding regions. Clone hHLP1 encodes a protein of 193 amino acid residues, a calculated molecular mass of 22,426 daltons and an isoelectric point of 4.72. The predicted amino acid sequence was 100% homologous to that of rat hippocalcin (1). The nucleotide sequence of the open reading frame of clone hHLP1 was 92% homologous to that of rat hippocalcin (1). Therefore, we defined clone hHLP1 as a human hippocalcin cDNA clone.

Expression of the human hippocalcin gene was studied by using a human multi-tissue Northern blot filter and a human hippocalcin specific probe (nucleotide residues 442-662). As shown in Fig. 2, a single transcript at a position corresponding to 2.0 kb was detected in the brain. The size of the mRNA was similar to that of rat hippocalcin (1), but different from those of other homologous proteins, such as bovine neurocalcin (13), rat neural visinin-like proteins 1-3 (14), and chicken visinin-like protein (15). No significant signal was detected in the heart, placenta, lung, liver, skeletal muscle, kidney or pancreas. These results indicate that human hippocalcin is expressed only in the brain.

Chromosomal mapping was performed by discordancy analysis using DNAs from 20 human-rodent somatic cell hybrids plus human, hamster and mouse control DNA. PCR was performed to amplify a human hippocalcin gene-specific fragment (nucleotide residues 18-136). As shown in Table 1, among the hybrid cell lines examined, 100% concordance was seen for the cosegregation of the human hippocalcin band and the human chromosome 1. All other autosomes and sex chromosomes could be excluded by the presence of at least two discordant hybrids. These results indicate that the human hippocalcin gene maps to chromosome 1.

Table 1. Chromosomal mapping of the human hippocalcin gene

Cell Line	Hippocalcin	Chromosome Number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
010	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
016	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
212	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
423	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
683	+	*	-	-	-	D	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+	+	-	-
734	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
750	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-
756	-	-	-	-	-	D	+	+	-	-	-	-	*	+	+	-	-	-	-	+	+	+	-	-	+
803	-	-	-	-	*	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
811	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
852	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
867	+	+	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
909	-	-	-	-	-	D	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-
937	+	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	*	-	-	-	+	-	-	-
940	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
1006	-	-	-	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	-	+	-	+	-	-	*
1049	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
1079	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
1099	+	+	-	-	-	D	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-
Concordant +/+		4	0	0	0	4	0	0	0	0	0	0	1	2	3	0	0	1	1	3	0	3	2	0	0
-/-		16	15	14	14	6	14	14	13	15	15	15	15	13	13	14	14	15	14	13	14	14	15	14	13
Discordant +/-		0	4	4	4	0	4	4	4	4	4	4	3	2	1	4	4	3	3	1	4	1	2	4	4
-/+		0	1	2	2	10	2	2	3	1	1	1	1	3	3	2	2	1	2	3	2	2	1	2	3
Discordancy (%)		0	25	30	30	50	30	30	35	25	25	25	27	25	20	30	30	27	25	20	30	15	15	30	35

For each hybrid cell line, the presence (+) and absence (-) of each of the human chromosomes in the hybrid is indicated in the columns under Chromosome Number. "+" means >30% of the cells contain the given chromosome; "*" means 5-30% of the cells contain the given chromosome; "D" indicates multiple deletions in the given chromosome. In the column headed Hippocalcin, the presence (+) or absence (-) of the PCR product for the human hippocalcin gene in each cell line is indicated. Concordant hybrids have either retained (+/+) or lost (-/-) the human specific-hippocalcin band with a specific chromosome. Discordant hybrids have retained the human-specific hippocalcin band without a specific chromosome (+/-), or the reverse (-/+).

Six proteins homologous to hippocalcin have been found in the bovine brain (13, 16, 17), three in the rat brain (14) and one in the chicken tectum (15). We do not know the physiological function of these proteins; however, each of these proteins seems to be present in a different region of the brain and they are considered to share their roles with each other (14, 15, 18, 19). The present study demonstrates that at least five proteins are present in the human brain. To define the physiological nature of this brain-specific Ca^{2+}-binding protein family, it is necessary to identify the precise members of this family as well as to find their own functions. The human hippocalcin cDNA should be useful for studying the function and expression of this protein in the human brain. The mapping of the hippocalcin gene to chromosome 1 should also aid in considering it as a candidate gene for neurological disorders that map to the same chromosome. Characterization and chromosomal mapping of the human cDNA clones hHLP2-5 are now under investigation.

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