BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 92–99 (1996)
ARTICLE NO. 1135

Cloning of a Human Homolog of the *Drosophila Minibrain*/Rat Dyrk Gene from ''the Down Syndrome Critical Region'' of Chromosome 21[1]

Nobuaki Shindoh,* Jun Kudoh,* Hideto Maeda,* Akiko Yamaki,† Shinsei Minoshima,* Yoshiko Shimizu,† and Nobuyoshi Shimizu*,[2]

**Department of Molecular Biology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan; and †Department of Clinical Genetics, School of Health Sciences, Kyorin University, 476 Miyashitacho, Hachioji, Tokyo 192, Japan*

Received June 24, 1996

To isolate genes responsible for some features of Down syndrome, we performed exon trapping experiments using a series of cosmid clones derived from ''the Down syndrome critical region'' of chromosome 21 and isolated six exons which are highly homologous to the sequence of *Drosophila minibrain* (*mnb*) gene. The *Drosophila mnb* gene encodes a serine/threonine protein kinase that is required in distinct neuroblast proliferation centers during postembryonic neurogenesis. Using one of these six exons as a probe, we isolated cDNA clones for human homolog of *Drosophila mnb* gene (MNB) from a fetal brain cDNA library. Human MNB cDNA encodes a protein of 754 amino acids with a nuclear targeting sequence and a catalytic domain common to the serine/threonine-specific protein kinase. The human MNB protein strikingly resembles the recently discovered rat Dyrk protein kinase with a dual specificity. The MNB mRNA is expressed in various tissues including fetal and adult brains. The remarkable similarity of human MNB protein to *Drosophila mnb* and rat Dyrk proteins implies that human MNB protein may play a significant role in a signaling pathway regulating nuclear functions of neuronal cell proliferation, contributing to certain features of Down syndrome.

Down syndrome (Trisomy 21) is the most frequent birth defect and is a major cause of mental retardation and congenital heart disease (1). Besides a characteristic set of facial and physical features, Down syndrome is associated with defects of the immune and endocrine systems, an increased rate of leukemia and early onset Alzheimer disease (1). Although little is known about the mechanism by which trisomy 21 interferes with normal development, the increased dosage of the chromosomal elements clearly implicates altered levels of gene expression as a causative factor, but individual genes whose over-expression is particularly important have not been identified.

Studies of cases with partial trisomy of chromosome 21 have suggested that the 2-Mb region around locus D21S55 which is located between loci CBR and ERG is particularly important in the etiology of Down syndrome (2-4). Therefore, we have chosen to focus on this ''Down syndrome critical region'' and performed exon trapping experiments using a series of cosmid clones isolated from this chromosomal region (5, unpublished results). We isolated over 160 exons with unique sequences and the database search showed that six exons are highly homologous to *Drosophila minibrain* (*mnb*) gene.

Drosophila mnb gene encodes a serine/threonine protein kinase that is essential for the proliferation of distinct neuronal cell types during postembryonic neurogenesis (6). The mutations of *mnb* gene cause the size reduction of the adult optic lobes and central brain hemispheres

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D85759.

[2] To whom correspondence should be addressed. Fax: 81-3-3351-2370. E-mail: shimizu@dmb.med.keio.ac.jp.

0006-291X/96 $18.00

in comparison with wild-type flies (7). Since the *mnb* protein shares sequence similarities with protein kinases involved in the regulation of cell division, it has been suggested that the *mnb* kinase would be related to proper proliferation of neuronal progeny on optic lobes and central brain hemispheres during postembryonic neurogenesis (6).

We report here the isolation and characterization of the human homolog of *Drosophila mnb* gene which is designated MNB. Moreover, we show the expression pattern of MNB gene in various human tissues and discuss the possible involvement in the pathogenesis of Down syndrome.

MATERIALS AND METHODS

Exon trapping. A series of cosmid clones were isolated from chromosome 21-specific KU21D cosmid library using a set of ordered YAC clones (8) which covered ''the Down syndrome critical region'' (D21S17-D21S396-D21S55-ERG-ETS2) as hybridization probes (Kudoh *et al.,* in preparation). Among these cosmids, 884 clones were assigned to 19 subregions. Approximately 500 cosmids from subregions 7 to 18 (D21S396-D21S55-ERG) were used for exon trapping experiments essentially as described previously (9; Gibco/BRL manual 18449-017). The nucleotide sequencing of trapped putative exons was carried out by the dye terminator method using AmpliTaq DNA polymerase and analyzed on a DNA sequencer (Applied Biosystems, 373A). Nucleotide and predicted amino acid homologies were analyzed with FASTA, BLASTN and BLASTX searches against the non-redundant sequence database.

cDNA isolation and sequencing. One exon, 17B04, was used to screen a λgt10 cDNA library prepared from human fetal brain (Clontech). DNA sequencing was carried out by primer walking for the PCR products amplified from isolated single plaques by the dye terminator method and analyzed as described above.

Northern blot analysis of MNB. Human multiple tissue Northern blots (Clontech) containing 2μg poly(A)$^+$ mRNA from various adult and fetal tissues were hybridized with probes according to the recommended protocol. A 583-bp fragment (nt 410-992 in Fig. 3) was amplified by PCR using a pair of primers 17B04F (5′-GGGAGACGATTCTAG-TCATA-3′; nt 410-429) and 26C01R (5′-CCTCTGCCCCAACTGACAAG-3′; nt 992-973) from the cDNA clone MNB32. The amplified DNA was used as a probe after labeling with [α-^{32}P]dCTP by random priming (10). These Northern blots were reprobed with a control 2.0-kb human β-actin cDNA.

RESULTS

Isolation of exons and cDNA clones. We have isolated over 160 putative exons from ''the Down syndrome critical region'' of human chromosome 21 by exon trapping method. Homology search of these exons using public nucleotide sequence database and FASTA program revealed that four exons (17B04, m1618-42, 26C01 and m1618-05) have striking nucleotide sequence homology (65.5% to 75.1% identity) with the *Drosophila minibrain* (*mnb*) gene (6) (Fig. 1A). Furthermore, homology search using BLASTX program indicated two additional exons (17B07 and m1618-38) have significant amino acid sequence homology (77.4% and 62.5% identity) with the *Drosophila mnb* protein (Fig. 1B). Homologous region between six trapped exons of human MNB and *Drosophila mnb* protein correspond to amino acid residues from 18 to 354 in *Drosophila mnb* protein (Fig. 2). These results indicated that we found human homolog of *Drosophila mnb* gene.

Using one of these exons, 17B04, as a probe, we screened a λgt10 cDNA library prepared from human fetal brain and isolated one cDNA clone (designated MNB32) containing a 1.9-kb insert from 6×10^5 plaques. The sequence analysis of MNB32 cDNA clone revealed it contains all the six exons but lacks 3′-terminal portion of coding region (Fig. 2). Then, we used MNB32 cDNA clone as a probe for secondary screening and obtained additional twelve cDNA clones from 6×10^5 plaques. The sequence analysis revealed that the clone MNB38 contains a 1.3-kb insert extending 0.9 kb from the 3′-terminus of MNB32 (Fig. 2). These cDNA sequence information allowed us to identify an additional exon (m1618-07) which showed no homology with *Drosophila mnb* gene from the initial 160 putative exons (Fig. 2).

Analysis of the nucleotide and amino acid sequence. The nucleotide sequence and the deduced amino acid sequence of MNB are shown in Fig. 3. The open reading frame of MNB is 2262-bp long beginning at nucleotide 69 and ending at nucleotide 2330, and encodes a

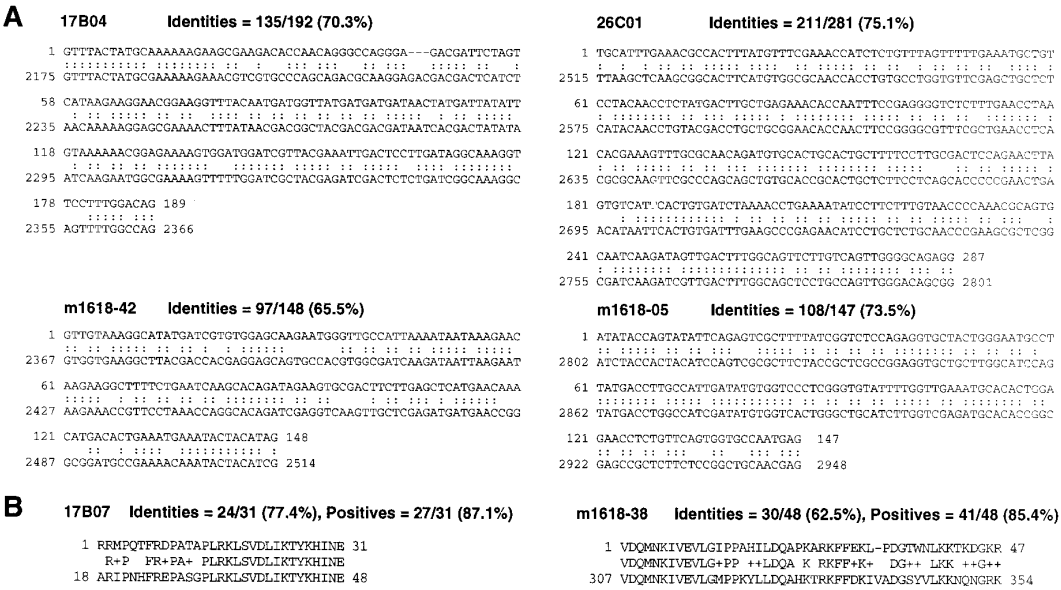

FIG. 1. Nucleotide and predicted amino acid sequence homology of the six trapped human exons to the *Drosophila mnb* gene. (**A**) Nucleotide sequences of the four human exons (17B04, m1618-42, 26C01 and m1618-05) and those of the *Drosophila mnb* gene (accession number X70794; 6) are shown in upper and lower lines, respectively. Predicted amino acid sequences of these four exons showed identities (78%–93%) with those of the *Drosophila mnb* protein kinase (Fig.5). (**B**) Predicted amino acid sequences of the two human exons (17B07 and m1618-38) and those of the *Drosophila mnb* protein are shown in upper and lower lines, respectively. Identical amino acids and conservative changes (+) are shown in middle line.

polypeptide of 754 amino acids with calculated molecular weight of 84,552. Fig. 4 illustrates a schematic comparison of the amino acid sequence of human MNB with *Drosophila mnb* protein which has been identified as three different forms due to alternative splicing (6). Human MNB protein has all conserved regions (subdomains I-XI) of the catalytic domain (amino

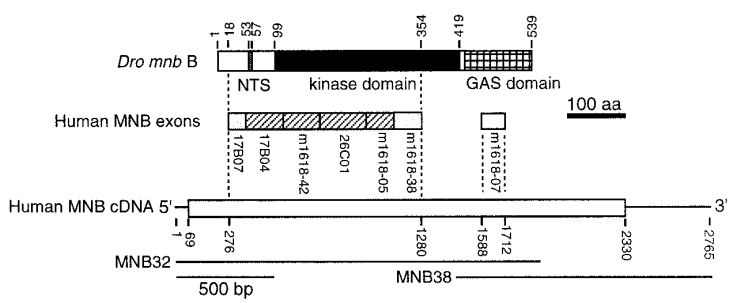

FIG. 2. Human MNB cDNA clones. The open bar represents the translated portion of the cDNA. Hatched boxes represent the four human MNB exons (17B04, m1618-42, 26C01 and m1618-05) which showed nucleotide sequence similarity to the *Drosophila mnb* gene (Fig. 1A). Two stippled exons (17B07 and m1618-38) showed predicted amino acid sequence similarity to the *Drosophila mnb* protein (Fig. 1B). Amino acid sequences encoded by six trapped exons of human MNB correspond to amino acid residues from 18 to 354 in *Drosophila mnb* protein in which distinct structural motifs are noted, including the potential nuclear targeting sequence (NTS), kinase catalytic domain, and a GAS domain which is rich in glycine, alanine and serine. Nucleotide sequences of six trapped exons were found in human MNB cDNA sequence at position 276 to 1280 (Fig. 3). Another exon m1618-07 was found to be unique to human MNB gene and has no similarity with *Drosophila mnb* gene.

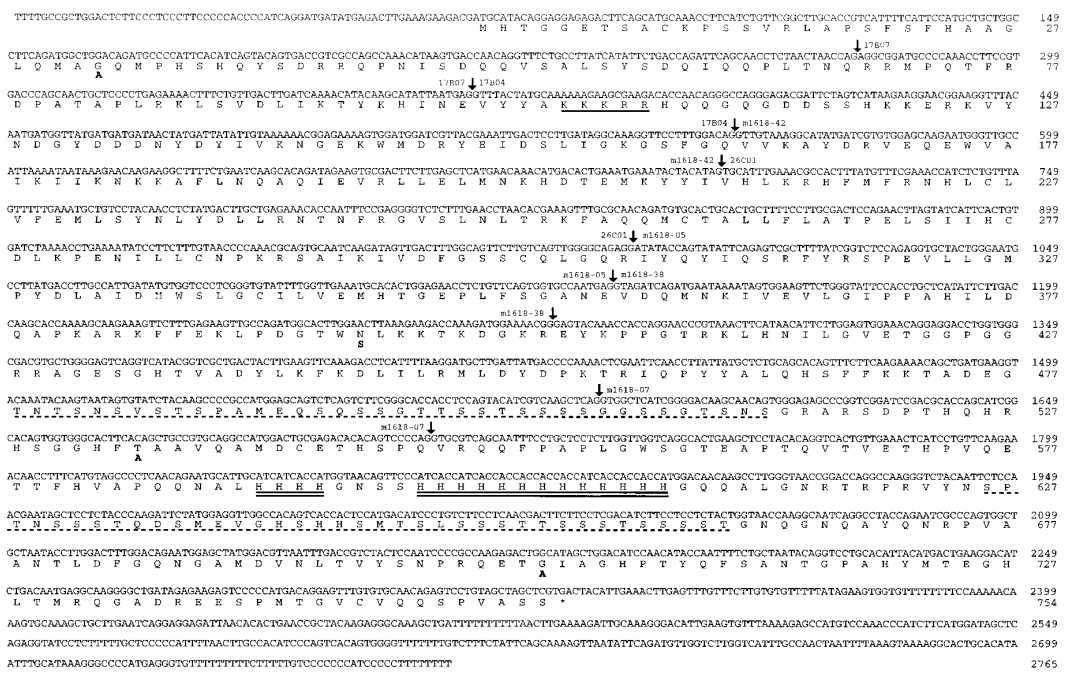

FIG. 3. Nucleotide sequence of the human MNB cDNA and deduced amino acid sequence. The nucleotide and deduced amino acid sequences of the human MNB cDNA coding strand are both numbered at the first residue. Borders of the seven trapped exons are indicated by arrows. The open reading frame consisting of 754 amino acid residues starts at nucleotide 69 and terminates at nucleotide 2330. Potential nuclear targeting sequence (*underline*), serine/threonine-rich regions (*dashed underline*) and histidine repeats (*double underline*) are indicated. Four amino acid residues replaced in rat Dyrk (accession number X79769; 18) are shown in boldface under human MNB amino acid sequence.

acids 150-470) of protein kinase (11) which shows 84% identity in 321 amino acids with *Drosophila mnb* protein (Figs. 4 and 5). The region (amino acids 69-149) of MNB protein flanking the N-terminus of its catalytic domain also shows high similarity (80% identical amino acids) with *Drosophila mnb* protein (Figs. 4 and 5). Within this conserved region, we found a nuclear targeting sequence (12), KKKRR (amino acids 105-109) (Figs. 3 and 5). Although amino acids 108-124 also match to a bipartite nuclear targeting sequence (13),

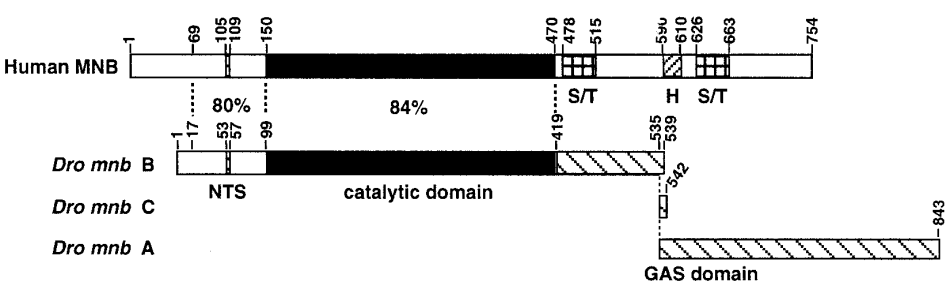

FIG. 4. Schematic comparison of human MNB and *Drosophila mnb* proteins. Distinct structural motifs are noted, including the potential nuclear targeting sequence (NTS), kinase catalytic domain, serine/threonine-rich region (S/T), histidine repeats (H). There are three different forms (A, B and C) of *Drosophila mnb* proteins which are generated by alternative splicing (6). In this case, a GAS domain which is rich in glycine, alanine and serine is noted.

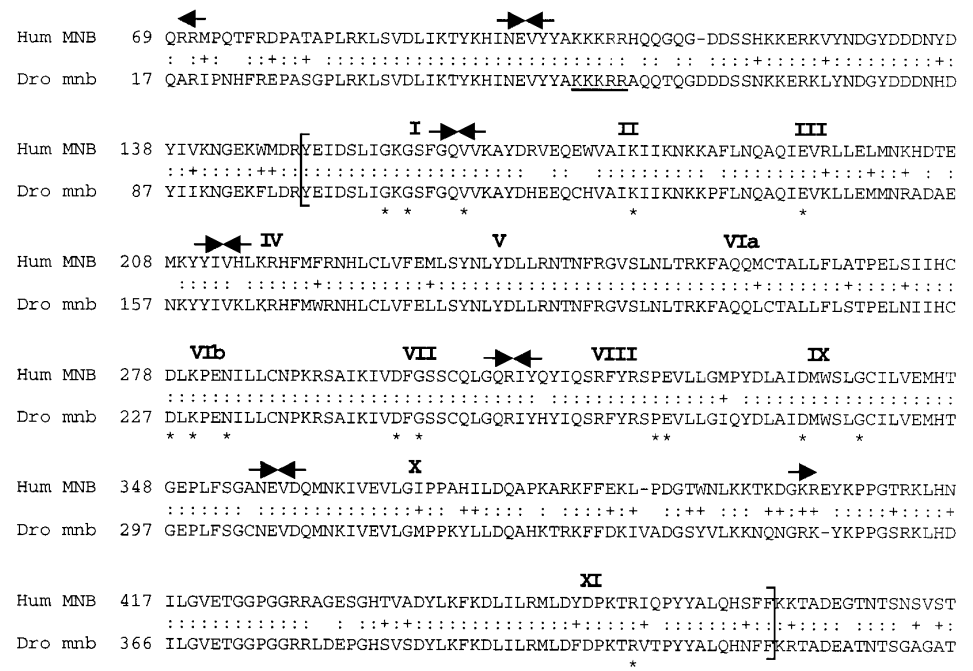

FIG. 5. Alignment of the amino acid sequence of the homologous regions of human MNB and *Drosophila mnb* proteins. The homology alignments shows the identical amino acids (:) and the conserved amino acids (+). Amino acid sequences encoded by six trapped exons of human MNB homolog correspond to amino acid boundaries of [18–48], [49–112], [113–162/], [/162–257], [258–306], and [307–354] of the *Drosophila mnb* protein, respectively. Positions of these human exon boundaries are indicated by arrows. The kinase subdomains (I–XI) according to the classification of Hanks (11) are shown on the top. Asteristics indicate highly conserved amino acids in the catalytic domain (indicated by brackets) of serine/threonine kinase (11). Potential nuclear targeting sequence is underlined.

$RR(X)_{10}HKKER$, *Drosophila mnb* protein (amino acids 56-73) has a longer spacer in corresponding sequence, $RR(X)_{11}NKKER$.

The C-terminus portion of human MNB protein has no homology with *Drosophila mnb* protein (Fig. 4). In *Drosophila mnb* protein, the C-terminus region is known as a GAS domain which is rich in glycine, alanine and serine (Fig. 4). Instead, the C-terminus region of human MNB protein is highly rich in serine and threonine (28% of 284 amino acid residues), and particularly there are two serine/threonine-rich domains (Thr-478 to Ser-515 and Ser-626 to Thr-663) containing 63% (24/38) and 66% (25/38) serine/threonine, respectively (Figs. 3 and 4). Moreover, there are two histidine repeats (His-590 to His-593 and His-598 to His-610) between two serine/threonine-rich domains (Figs. 3 and 4).

Expression of the MNB gene. An MNB cDNA fragment was used as a probe for Northern blot analysis of RNAs from various human tissues (Fig. 6). We detected two transcripts of approximately 6.0 kb and 7.5 kb in size. The expression of 6.0-kb transcript was detected in all tissues examined including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland and bone marrow. In the adult tissues, the highest level of expression was detected in skeletal muscle and testis, and the lower level of expression was found in lung, liver and kidney (Fig. 6A). In contrast, relatively higher level of expression was detected in fetal lung and fetal kidney (Fig. 6B). In the brain, 6.0-kb mRNA was expressed ubiquitously (Fig. 6C). A second

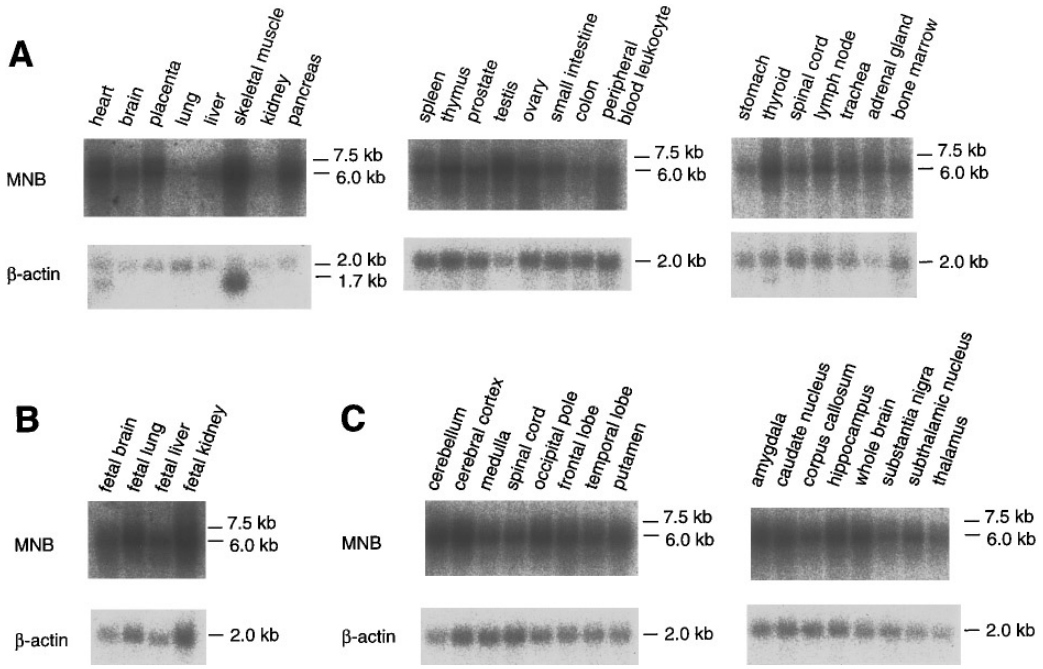

FIG. 6. Northern blot analysis of human MNB expression in various tissues. Upper panels show hybridizations with the 583-bp PCR fragment of the human MNB cDNA. Lower panels show control hybridizations with a 2.0-kb human β-actin cDNA probe. (**A**) Northern blot analysis of human adult mRNAs from a number of different tissues. (**B**) Northern blot analysis of human fetal mRNAs from 4 tissues. (**C**) Northern blot analysis of mRNAs from a number of different tissues of human brain.

transcript of about 7.5 kb was weakly expressed but obvious in the adult heart, placenta, spleen and testis (Fig. 6A).

DISCUSSION

We have isolated cDNAs for the human homolog of *Drosophila mnb* gene by screening a human fetal brain cDNA library with a putative exon which was isolated using exon trapping method. *Drosophila mnb* mutant was identified with a reduced brain volume and characterized by a reduction in the number of neuronal cells in the optic lobes and the central brain hemispheres (6,7). This specific loss of neurons might be expected to cause behavioral abnormality that exhibited absence of olfactory learning, aberrant visual fixation and decreased locomotor activity of freely walking (14,15). *Drosophila mnb* gene encodes a serine/threonine protein kinase which is expressed in the neuroblast proliferation centers during postembryonic neurogenesis (6). The catalytic domain of *mnb* protein kinase shares a sequence similarity to the kinases involved in the regulation of cell growth and cell cycle. Therefore, it is suggested that the *mnb* protein kinase may control the cell cycle events which are required for the regulation of the number of neuronal cells in optic lobes (6).

The catalytic domain of human MNB protein is highly conserved with *mnb* protein at amino acid level, while outside the catalytic domain shows no homology except for the region flanking the N-terminus of the catalytic domain which contains a potential nuclear targeting sequence (Figs. 4 and 5). The C-terminus portion of the MNB protein has a unique structure with two serine/threonine-rich domains and histidine repeats whose functions are as yet unknown. In addition, database search showed that several protein kinases share high sequence similarity

with a catalytic domain of MNB. These protein kinases include yeast Yak1 protein kinase (38% identity in 338 amino acids overlap), mammalian CLK protein kinase (32% identity in 339 amino acids overlap) that are involved in the regulation of the cell cycle (16,17). Recently, cDNA of Dyrk (Dual-specificity Yak1-related kinase) was cloned from a rat brain cDNA library (18). The amino acid sequence of Dyrk is almost the same as MNB, except that four amino acid residues Gly-32, Asn-395, Thr-534 and Gly-704 in MNB were replaced with Ala-32, Ser-404, Ala-543 and Ala-713 in Dyrk (accession number X79769), respectively (Fig. 3). Alignment of the nucleotide sequences of MNB and Dyrk revealed that Dyrk has an insertion of 27 bp encoding 9 amino acids VMPDIVMLQ which was reported as an alternatively spliced segment in Dyrk (18). Position of this insertion, between Gln-69 and Arg-70 of MNB, just fit to 5′-border of exon 17B07. The high similarity in the overall nucleotide sequence (93.7% identity in 2747 nt overlap) as well as in the coding region (99.5% identical amino acids and 94.1% identical nucleotides) between MNB and Dyrk suggests that Dyrk is a rat homolog of MNB. Dyrk was reported as a novel dual specificity protein kinase, phosphorylating both tyrosine and serine/threonine residues in its sequence (autophosphorylation) and in exogenous substrate (18). Its kinase activity depends on the presence of tyrosine residues (Tyr-310 and Tyr-312 in MNB) between subdomains VII and VIII (activating loop) and it was supposed that the activation of Dyrk depends on the phosphorylation of one or both of these tyrosines (18). They speculated that Dyrk is a component of a signaling pathway, possibly mediating the specific phosphorylation of transcription factors within the nucleus, because all other kinases that are regulated by tyrosine phosphorylation between subdomains VII and VIII, *e.g.* MAPK/ERK, JNK, and GSK3β, are components of signaling pathways that transduce extracellular signals to a nuclear phosphorylation of transcription factors (18). It is highly likely that MNB, human homolog of rat Dyrk, has the same characteristics as Dyrk.

Two MNB mRNAs with approximate size of 6.0 kb and 7.5 kb were detected on Northern blots. A 6.0-kb mRNA was expressed in all the tissues examined, while the expression of 7.5-kb mRNA was restricted in some tissues. In addition, we have performed Northern blot analysis for mouse embryos at age of 7, 11, 15 and 17 days, and detected two transcripts of 4.0 kb and 6.0 kb in all the developing embryos (data not shown). This observation indicates that MNB gene is expressed during the mammalian embryogenesis.

MNB cDNA was isolated using one of the exons trapped from ''the Down syndrome critical region'' located between two loci LA68 and ERG on chromosome 21. In order to determine the precise location of MNB gene within this region, we performed hybridization of the MNB cDNA clones to the *Eco*RI fragments of cosmid/PAC clones which were aligned in ''the Down syndrome critical region'' with a high-resolution physical map (19). The most 5′-portion of cDNA hybridized to a cosmid clone D73H11 and the most 3′-portion hybridized to a cosmid clone D104C9 (data not shown; refer to Fig. 5 of reference 19). This result together with published (20-22) and our unpublished results indicated that MNB gene locates to a region between maker D21S394 and D21S55 within the Down syndrome region in the order of (cen)-CBR-SIM2-HCS-TPRD-MNB-KCNJ6 (also known as KCNJ7 or GIRK2)-ERG-ETS2. The mouse homolog of *mnb* (*Mnb*) has recently been mapped between *Cbr* and *Pcp-4* within the syntenic region between mouse chromosome 16 and human chromosome 21 (23).

The remarkable similarity of human MNB protein to *Drosophila mnb* and rat Dyrk proteins implicates that human MNB protein may play a significant role in a signaling pathway regulating nuclear functions of cell proliferation. It is possible to suppose that increased amount of MNB protein caused by trisomy 21 may show pleiotropic effects in many tissues becauce MNB gene is ubiquitously expressed. It is also possible to suppose that apparent features may be seen in only restricted tissues, especially in neuronal cells by analogy with *Drosophila mnb* mutant.

It has been suggested that there are certain parallels between major phenotypes of mouse

trisomy 16 and those of human trisomy 21 (24,25). Therefore, the studies using knockout mouse and/or trisomy mouse of MNB gene will provide a clue to understand the involvement of MNB gene in the normal brain development and the pathogenesis of Down syndrome.

ACKNOWLEDGMENTS

We thank M. Asahina for technical assistance and H. Harigai for excellent assistance in manuscript preparation. We also thank Drs. K. Osoegawa and E. Soeda for their contribution to mapping of MNB gene. This work was supported in part by Grants-in-Aid for a Creative Basic Research (Human Genome Program) and Scientific Research from the Ministry of Education, Science and Culture of Japan, and by the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan.

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