© 2000 Oxford University Press Human Molecular Genetics, 2000, Vol. 9, No. 9 1443–1452

Barhl1, a gene belonging to a new subfamily of mammalian homeobox genes, is expressed in migrating neurons of the CNS

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Received 7 February 2000; Revised and Accepted 27 March 2000

The *BarH1* and *BarH2* (*Bar*) *Drosophila* genes are homeobox-containing genes, which are required for the fate determination of external sensory organs in the fly. By means of a bioinformatic approach, we have identified in mouse and human two homeobox genes highly related to the *Bar Drosophila* genes, *Barhl1* and *Barhl2*. While *Barhl1* represents a novel gene, *Barhl2* turned out to correspond to the *mBH1* cDNA recently described in rat. We isolated and sequenced the full-length mouse *Barhl1* and mapped both the human *BARHL1* and *BARHL2* genes to chromosomes 9q34 and 1p22, respectively. Detailed analysis of the murine *Barhl1* expression pattern by *in situ* hybridization revealed that this transcript is exclusively expressed in restricted domains of the developing CNS, which suggests that this gene, similar to its *Drosophila* counterparts *BarH1* and *BarH2*, may play a crucial role in cell fate determination of neural structures. In particular, *Barhl1* showed specific domains of expression in the diencephalon and in the rhombencephalon where it was found to be expressed in migrating cells giving rise to the cerebellar external granular layer and to specific populations of dorsal sensory interneurons of the spinal cord. Thus, *Barhl1* function may be required for the generation of these specific subtypes of neuronal progenitors. Furthermore, the mapping assignment and the expression pattern make *BARHL1* an attractive positional candidate gene for a form of Joubert syndrome, a rare developmental anomaly of the cerebellum in humans.

INTRODUCTION

The development of the mammalian neural system is a very complex process that requires an ordered series of steps beginning with an initial growth phase of dividing precursor cells and the subsequent process of differentiation of post-mitotic cells. The molecular mechanisms controlling these events still remain largely elusive and involve many genes, most of which have not yet been identified and characterized. One way to gain insight into the genetic program of neural development in mammals is represented by the study of model organisms. In fact, many of the pathways involved in neurogenesis have been found to share similarities during evolution and numerous genes have been found to be conserved and to play similar roles even in distantly related species. In particular, the fruitfly *Drosophila melanogaster* is one of the most valuable model organisms to study development (1). Many genes playing a role in neurogenesis in the fly have been characterized at both the genetic and molecular levels (2,3). This has led to the understanding of some of the basic pathways underlying neural development in *Drosophila*, particularly of the sensory organs. The proneural genes such as the four members of the *Achaete–scute* complex, *daughterless* and *atonal*, act as positive regulators for sensory organ formation, as opposed to other genes like *hairy*, *Enhancer of split* and *extramacrochetae*, which act as negative regulators (for review see refs 4,5). A number of upstream regulators of proneural genes have also been identified and several of them are transcription factors which are required for the initial activation of proneural genes in sensory organs (6–9). Among these are the *BarH1* and *BarH2* genes. They are required for the fate determination of external sensory organs and for normal eye development in the fly (10–12). Recently, they have also been found to play a crucial role in regional pre-patterning of specific structures of the *Drosophila* nervous system, such as the notum. The *BarH1* and *BarH2* genes exert this function through an upstream regulation of the proneural *achaete–scute* genes (13).

We recently started a project aimed at the identification and characterization of novel human cDNAs similar to *Drosophila* mutant genes. By screening the Expressed Sequence Tag database (dbEST) using the BLAST software, we identified >200 such human cDNAs and we termed them *Drosophila*-related expressed sequences (DRES) (14,15). One of the DRES genes

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identified in the course of our dbEST screening, DRES115, was found to bear significant homology to the homeodomain of the *Drosophila* genes *BarH1* and *BarH2*. Here, we report a detailed characterization of the *Dres115* (*Barhl1*) gene including cDNA identification, sequence and mapping assignment in both human and mouse. In addition, we have isolated and mapped the human homolog of *mBH1* (*Barhl2*), a gene highly related to *Barhl1* which has been described previously in rat (16). A comprehensive study of *Barhl1* expression by RNA *in situ* hybridization revealed that this gene is expressed in discrete domains of the CNS, particularly in migrating progenitor cells, giving rise to the cerebellar external granular layer and to specific populations of dorsal sensory interneurons of the spinal cord.

RESULTS

cDNA identification and sequence analysis

By analyzing the DRES Search Engine (17), a collection of TBLASTN searches obtained using all *Drosophila* protein sequences as queries, we identified a mouse EST (GenBank accession no. W36375), significantly similar to the homeodomain of the *Drosophila* proteins BARH1 and BARH2 (GenBank accession nos B39369 and S27806, respectively). The EST W36375 was derived from the IMAGE mouse embryo cDNA clone 335997 and we named this clone DRES115. Due to its sequence similarity, the *DRES115* cDNA was renamed *BarH* (*Drosophila*)*-like 1* (*Barhl1*). Using the entire cDNA insert as a probe, we screened two different mouse cDNA libraries: an embryonic day 11.5 (E11.5) embryo and an embryonic carcinoma cDNA library. These screenings led to the identification of five cDNA clones, among which clone ME9 was found to span the entire *Barhl1* transcript (1593 bp). Sequence analysis of *Barhl1* revealed a single open reading frame of 984 bp (GenBank accession no. AJ237590). The putative initiation codon was identified at position 18 within a nucleotide sequence meeting Kozak's criteria (18). The open reading frame ends with a TGA stop codon at nucleotide 999. The *Barhl1* gene is therefore predicted to encode a protein product of 327 amino acids.

To identify the human *BARHL1* gene, we screened a human lambda genomic library and identified one positive clone which, by sequence analysis, was found to contain the last two exons of the human gene. By joining together these two exons, we obtained a partial *BARHL1* coding sequence of 518 bp containing the homeobox and the stop codon (GenBank accession no. AJ237816). The degree of sequence identity between the partial human *BARHL1* coding sequence and the corresponding murine sequence was 94% at the nucleotide level and 99% at the amino acid level (with only one amino acid change in the two proteins; Fig. 1A).

Sequence comparison analysis revealed that the *Barhl1* gene is a new member of the *bar* subgroup of homeobox genes, characterized by the presence of a tyrosine residue at position 49 (within the third helix) of the homeodomain rather than the more common phenylalanine. This class includes a limited number of proteins, namely the *Drosophila* BARH1 and BARH2, the *Caenorhabditis elegans* CEH-30, the hydra CNOX3, the mammalian BARX1 and BARX2, and the rat mBH1 proteins (Fig. 1B). The BARHL1 predicted protein shows the highest level of sequence similarity to the rat mBH1 protein and, as expected, to the *Drosophila* proteins BARH1 and BARH2. In particular, BARHL1 was found to be closely related to the mBH1 protein, recently described by Saito *et al.* (16), as the two proteins show 71% similarity and 67% identity. The stretch of highest sequence conservation between the two proteins was found in a region of 107 amino acids encompassing the homeodomain: only seven amino acid changes were detected in this portion. The degree of similarity is much lower in the N- and the C-termini (Fig. 1A); in the N-terminus, high similarity between the two proteins is found in two regions that have been designated by Saito *et al.* (16) as 'FIL' domains (FIL1 and FIL2 domains; Fig. 1A) because their consensus sequence is composed of phenylalanine, isoleucine and leucine. The degree of sequence similarity between the murine BARHL1 and the rat mBH1 in the N- and C-termini (very low compared with the almost complete identity found in the human and murine *Barhl1* genes in the C-terminal region of the proteins), as well as their different patterns of expression (see below), indicated that they are not orthologous genes but rather paralogous genes belonging to a new subclass of mammalian homeobox genes. We confirmed this hypothesis by PCR analysis on human and murine genomic DNA (Materials and Methods), by isolating a fragment of 208 bp corresponding to the human and murine homologs of the rat *mBH1* gene, hereafter termed *Barhl2* (HGNC-approved gene symbol). The human *BARHL2* fragment (GenBank accession no. AJ251753) shared 92 and 74% nucleotide identity with the rat *Barhl2* and the human *BARHL1* cDNA sequences, respectively.

The similarity of BARHL1 to the *Drosophila* BARH1 and BARH2, on the other hand, is mostly restricted to a region of 80 amino acids (Fig. 1A) including the homeodomain, and 20 amino acids immediately downstream (81% identity with BARH1 and 85% identity with BARH2). Also, the nematode protein CEH-30 shows a striking homology to BARHL1 in the same portion (70% identity). The degree of similarity between the murine BARHL1 and the murine BARX1 and BARX2 proteins is less significant (63 and 65% identity, respectively) and is restricted to the homeodomain. This indicates that the BARX proteins, previously reported to be very closely related to the *Bar Drosophila* genes, belong to a different subfamily of homeodomain-containing proteins which is also suggested by the different expression pattern (19,20).

Genomic mapping of *Barhl1* and *Barhl2*

To establish the mapping assignment of the human *BARHL1* gene, we performed a PCR analysis on the GeneBridge4 radiation hybrid panel (21) using a sequence tagged site generated in the genomic region downstream of the third coding exon of this gene. As a result, *BARHL1* was assigned to chromosome 9q34, between markers *WI-9685* and *WI-6494*. Interestingly, Saar *et al.* (22) recently reported on the genetic mapping of a locus for Joubert syndrome (a rare developmental disorder characterized by aplasia/hypoplasia of the cerebellar vermis) to 9q34, between the markers *D9S114* and *D9S1826*. The latter region overlaps with the *BARHL1* radiation hybrid (RH) mapping interval suggesting that *BARHL1* is a positional candidate gene for this form of Joubert syndrome.

We also decided to establish the localization of *Barhl1* in the mouse genome. By haplotype and linkage analysis of the BSS

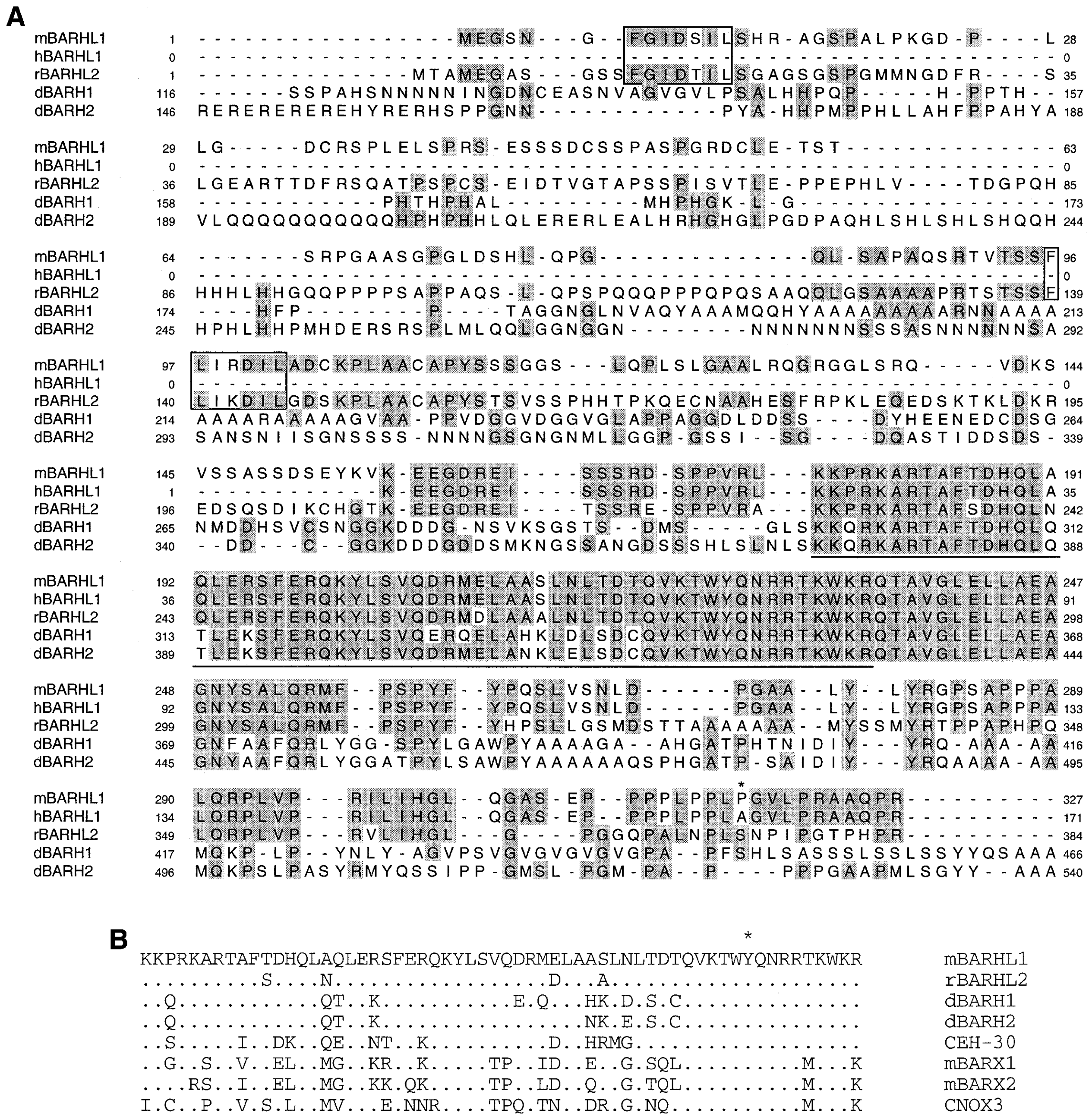

Figure 1. Sequence analysis of the *Barhl1* cDNA. (**A**) Sequence alignment of the murine BARHL1 predicted protein (mBARHL1) with the human partial BARHL1 protein (hBARHL1), the rat BARHL2 (rBARHL2) and the *Drosophila* BARH1 and BARH2 proteins (dBARH1 and dBARH2, respectively). Amino acids that are identical in two or more proteins are shaded. The homeodomain is underlined and the two 'FIL' domains are boxed. The asterisk indicates the only amino acid difference between the murine and the partial human BARHL1 proteins. (**B**) Amino acid sequence alignment of the BARHL1 murine homeodomain and other related homeodomains, namely the rat BARHL2, the *Drosophila* BARH1 and BARH2, the *C.elegans* CEH-30, the murine BARX1 and BARX2 (mBARX1 and mBARX2, respectively), and the hydra CNOX3. Residues identical to the BARHL1 protein are indicated by dots. All these homeodomains are characterized by the presence of a tyrosine residue at position 49 (see asterisk) instead of the more common phenylalanine.

backcross DNA panel generated and maintained at The Jackson Laboratory (Bar Harbor, ME), we mapped the *Barhl1* mouse gene to murine chromosome 2 (Fig. 2). Mapping of *Barhl1* was made possible by the existence of an *Eco*RI polymorphism between the parental DNAs (C57BL/6JEi and SPRET/Ei). Such polymorphism allowed us to distinguish BS heterozygotes from SS homozygotes in the N2 progeny of the cross. *Barhl1* maps between *D2Ertd217e* (q = 1.09; LOD = 25.3) distally and *D2Ucl22* (q = 9.78; LOD = 14.9) proximally and cosegregates with *Notch1*. This region of murine chromosome 2 has been found to be syntenic to human chromosome 9q34 because the human homolog of *Notch1* has been mapped to 9q34.3.

We also decided to establish the mapping assignment of *BARHL2*, the human homolog of the rat *mBH1* gene. For this purpose, we designed oligonucleotide primers on the *BARHL2* fragment and performed a PCR analysis on the GeneBridge4

Figure 2. Mapping of *Barhl1* in the mouse genome. (**A**) Haplotype and linkage analysis of *Barhl1* and flanking loci on mouse chromosome 2 through the analysis of the BSS backcross (Jackson Laboratory). The distinction between BS heterozygotes and SS homozygotes in the N2 progeny of the cross is made possible by the analysis of an *Eco*RI polymorphism (Materials and Methods). Empty squares indicate the *Mus spretus* allele; solid squares indicate the C57BL/6J allele; shaded squares signify that the genotype was not determined. Numbers to the right, between rows, indicate recombination fractions ± SE, and LOD scores. Columns show different haplotypes observed on chromosome 2. Numbers below columns define the number of individuals sharing each haplotype. (**B**) Position of *Barhl1* on chromosome 2 with respect to nearby markers independently mapped by others on the BSS backcross. Numbers on the left represent approximate genetic distances (cM) from the most centromeric chromosome 2 marker in this cross. The chromosomal location of a syntenic human gene (*NOTCH1*) and the two markers defining the *BARHL1* RH mapping interval (see text) is also indicated. The relative order of the human loci was determined by analysis of the Genetic Location Database (LDB; http://cedar.genetics.soton.ac.uk/public_html/ldb.html).

RH panel. This analysis revealed that the *BARHL2* gene maps to chromosome 1p22, between the markers *AFMA205XD5* and *WI-7719*.

Expression of the murine *Barhl1* gene during embryonic development

To determine the temporal and spatial expression of *Barhl1* during mouse development, we used *in situ* hybridization to histological sections and whole-mount preparations at several developmental stages. *Barhl1* expression was highly dynamic over the course of development but exclusively restricted to the CNS. The earliest embryonic day examined was E7.5 and the first expression was detected at 9.5 days' gestation in the caudal part of the diencephalic anlage. At later stages other domains of expression appeared in the mesencephalon, rhombencephalon and spinal cord. To interpret *Barhl1* expression, we used the terminology of the prosomeric model which suggests that the forebrain (prosencephalon) can be divided into two preneuromeric domains: the diencephalon and the secondary prosencephalon. These two structures can be regionally subdivided into three diencephalic (p1–p3) and three secondary prosencephalic (p4–p6) molecular compartments called prosomeres (23,24). In its alar components, the diencephalon consists of the pretectum (p1), dorsal thalamus and epithalamus (p2), and ventral thalamus (p3) (Fig. 3O). The secondary prosencephalon includes the telencephalic and optic vesicles, the preoptic area and the hypothalamus (Fig. 3O).

In the forebrain, *Barhl1* is expressed exclusively in a restricted domain of the caudal diencephalon at early neural tube stage (E9.5), and more specifically in the ventral area of p1 and p2 (Fig. 3A). By E10.5, *Barhl1* expression is detected along the neural tube in two separate rostral and caudal domains (Figs 3B, and 4A, D and G). The rostral diencephalic domain extends: (i) anteriorly, into the basal plate of prosomeres 3 and 4 (p3, p4), anlage of the mammillary area; (ii) dorsally, along the p2–p3 boundary corresponding to the zona limitans (ZL), which will separate the dorsal from the ventral thalamus; and (iii) posteriorly to the pretectum (p1) (Fig. 3B, E, F, G and M). The mammillary area corresponds to the only region where the mouse *Barhl1* gene is expressed in the ventral midline (p4 in Fig. 3B and E). In the pretectum, two distinct domains of expression can be recognized, one in the basal plate and the other in the most alar and caudal region (Fig. 3B and M). This latter expression, corresponding to the commissural area, will extend basally by E11.5 (Fig. 3C, K, N and O). At E10.5, the caudal domain extends from the midbrain/isthmic boundary, through the rhombencephalon (cerebellar plates and rhombic lips) to the entire extent of the spinal cord. In these structures, this gene is expressed as a longitudinal stripe along the most dorsal area of the alar plate, but not in the roof plate (Figs 3B and H, and 4A, D and G).

By E11.5 the expression pattern of *Barhl1* changes dynamically: a positive band appears in the mesencephalic basal plate (Fig. 3C, L, N and O), and in the spinal cord the expression expands laterally, following the ventral migrating cells from the dorsal alar plate to the boundary between the basal and alar plate (Fig. 4E and H). At E12.5, the expression shows another significant change of pattern: in the diencephalon, expression becomes mainly restricted to the mammillary region (basal p4) (Fig. 3D, I and J), whereas mesencephalic expression is now localized in the rostral alar plate in the presumptive area of the superior colliculus (Figs 3D and 4C). In the caudal rhombencephalon, a large lateral band of expression is observed and probably corresponds to the cells migrating ventrally, which will form the pontine and inferior olivary nuclear complex (25,26) (Figs 3D and 4C). At this stage, most of the expression in the spinal cord is confined to the mantle layer in a lateral band between the basal and alar plate (Figs 3D, and 4F and I) and this pattern will not change during later stages of development (data not shown).

The sites of expression at late embryonic stages (E16.5), besides the spinal cord, are the mantle layer of the inferior

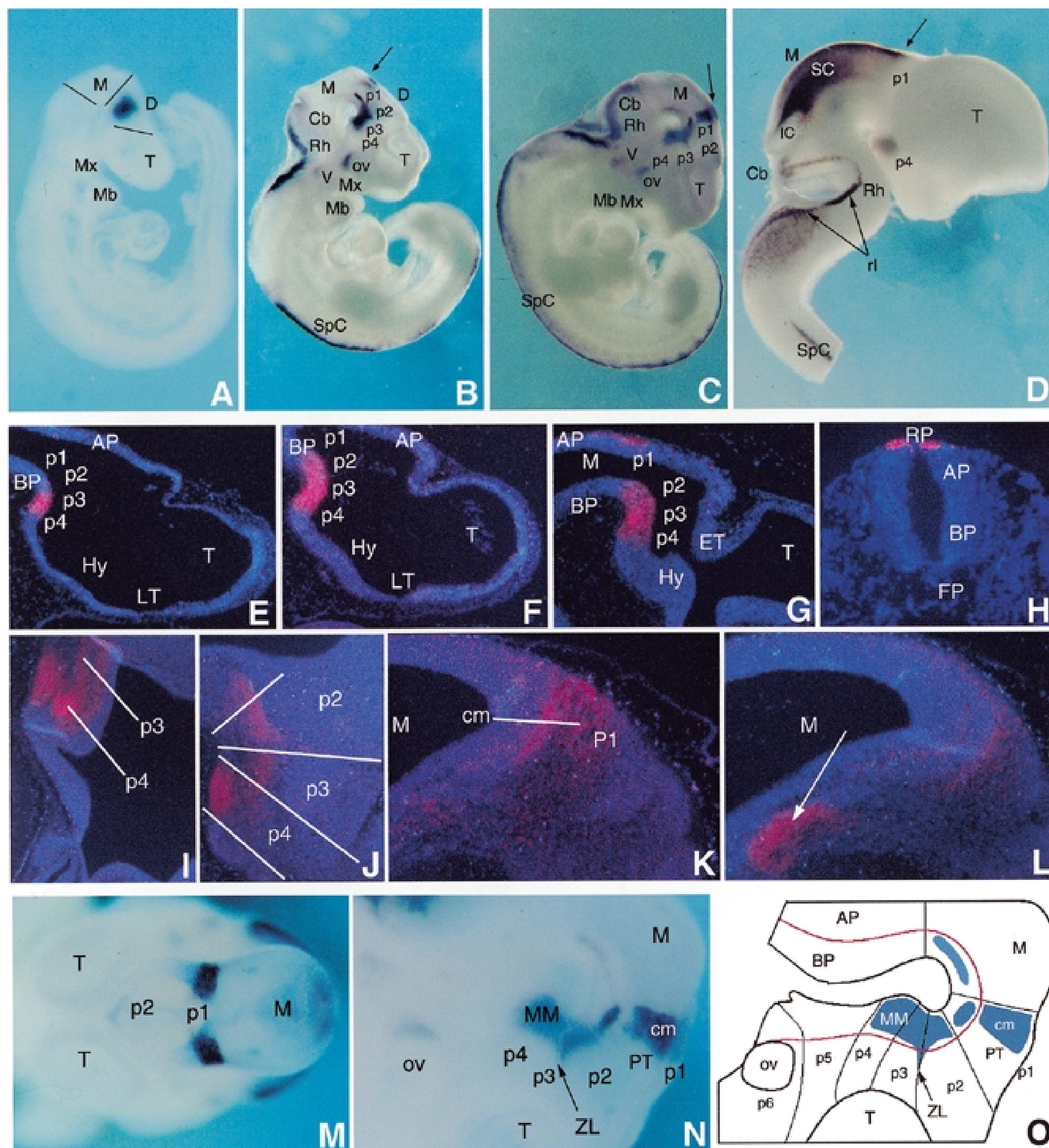

Figure 3. Expression of the murine *Barhl1* gene during embryonic development. The *Barhl1* transcript was detected by *in situ* hybridization in whole-mount preparations (**A–D** and **M**) and in tissue sections (**E–L**): *Barhl1* expression is visualized at E9.5 (A), E10.5 (B, E, F, G, H and M), E11.5 (C, K, L and N) and E12.5 (D, I and J). At E9.5 *Barhl1* expression is detected in the caudal part of the diencephalic anlage corresponding to the ventral area of prosomeres 1 and 2 (p1, p2) (A). At E10.5 *Barhl1* is expressed in two separate domains (B). The rostral diencephalic domain extends from the basal plate of prosomeres 3 and 4 (p3, p4) anteriorly, anlage of the mammillary area, to the pretectum (p1) (B, E, F and G) posteriorly. The mammillary area corresponds to the only region where the mouse *Barhl1* gene is expressed in the ventral midline [p4 in (B) and (E)]. The basal expression in P2 and P3 extends dorsally into the ZL between the anlage of the ventral and dorsal thalamus [best seen at E11.5 in (N) and (O)]. In the pretectum it is expressed in two distinct bands: one in the basal plate and the other in the most alar and caudal region (B and M); the arrows in (B) and (C) show the boundary between mesencephalon and diencephalon. In the caudal domain, extending from the midbrain/isthmic boundary to the entire extent of the spinal cord, *Barhl1* is expressed as a longitudinal stripe along the most dorsal area of the alar plate, but not in the roof plate (B and H). At E11.5 in the commissural area of the pretectum *Barhl1* expression extends basally and a positive band appears in the mesencephalic basal plate (C, K, L, N and O) corresponding to the ventral periaqueductal grey [see arrow in (L)]. The signal immediately posterior to the optic vesicle, detected at E10.5 (B) and E11.5 (C and N) seems to correspond to the primordium of the extra-ocular muscles. At E12.5 the rostral diencephalic expression becomes restricted to the mammillary region (basal p4) and prerubral tegmentum, encompassing retromammillar (basal p3), posterior tubercle (basal p2) and subpretectal (basal p1) tegmental zones (D, I and J). In the mesencephalon *Barhl1* expression is localized in the rostral alar plate in the presumptive area of the superior colliculus (D). At this stage in the caudal rhombencephalon a large stream of positive cells probably migrating ventrally is evident [see arrow in (D)]. While in the spinal cord expression is definitively confined to the mantle layer of a lateral band between the basal and alar plates. AP, alar plate; BP, basal plate; Cb, cerebellar plate; cm, posterior commissure; D, diencephalon; ET, eminentia thalami; FP, floor plate; Hy, hypothalamus; IC, inferior colliculus; LT, lamina terminalis; M, mesencephalon; MM, mammillary region; Mb, mandibulary component of the first branchial arch; Mx, maxillary component of the first branchial arch; ov, optic vesicle; PT, pretectum; Rh, rhombencephalon; rl, rhombic lips; RP, roof plate; SC, superior colliculus; SpC, spinal cord; T, telencephalon; V, fifth cranial nerve ganglia; ZL, zona limitans.

colliculus anlage, with a clear caudo–rostral gradient, the pontine nucleus and the cerebellar external granular layer (Fig. 5A and B). Another site of expression, only evident at this stage of development, is the sensory epithelium of the inner ear, where *Barhl1* expression is possibly restricted to the sensory hair cells (Fig. 5C).

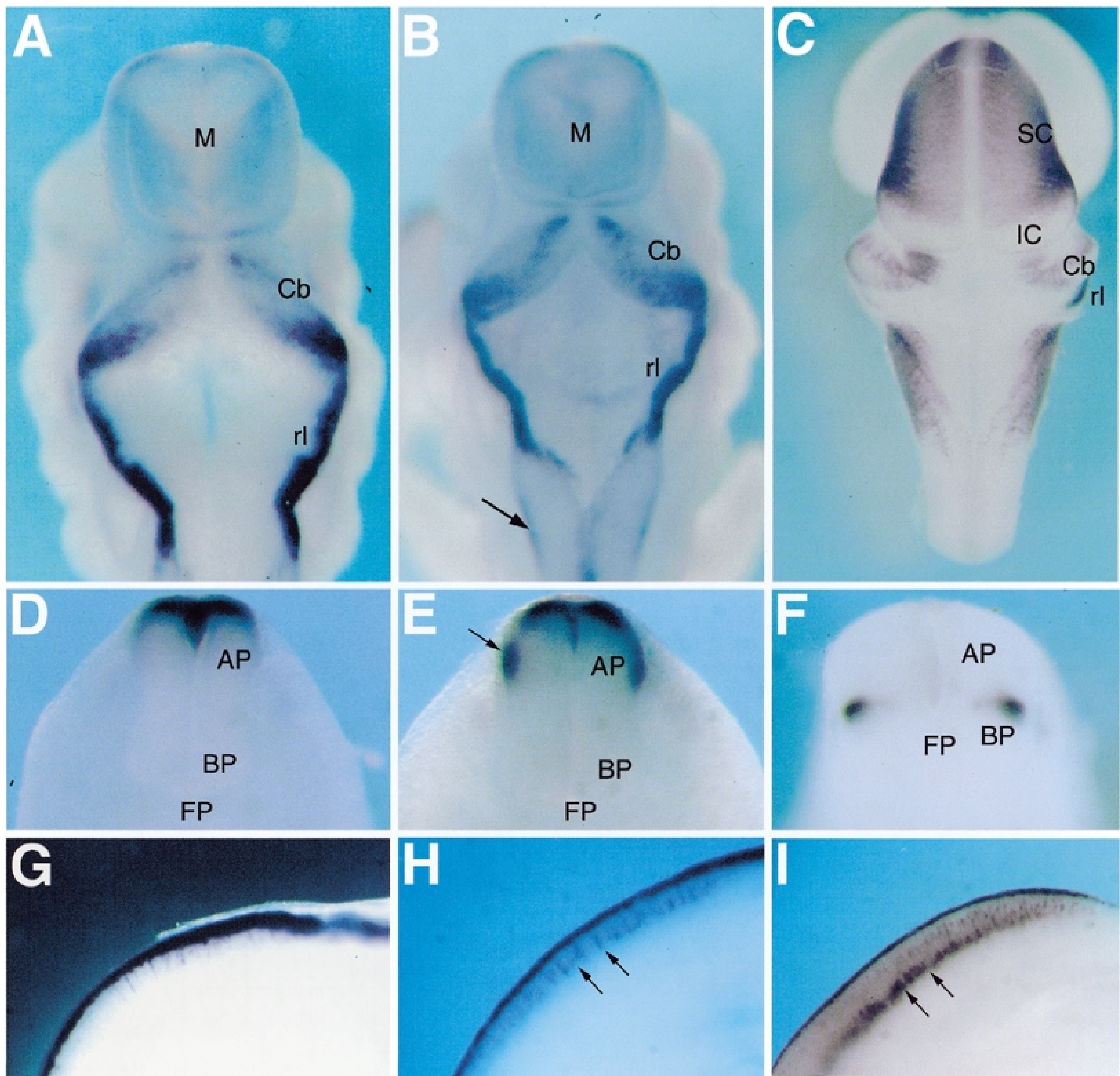

Figure 4. Expression of *Barhl1* in migrating neurons, detected by *in situ* hybridization in whole-mount embryos at E10.5 (**A**, **D** and **G**), E11.5 (**B**, **E** and **H**) and E12.5 (**C**, **F** and **I**). The expression is visualized in a view from posterior for the rhombencephalon (A–C), and in transverse (D–F) and lateral views (G–I) for the spinal cord. At these stages, *Barhl1* is expressed in a domain extending from the midbrain/isthmic boundary, through the rhombencephalon, to the entire extent of the spinal cord. In these structures, this gene is expressed at E10.5 as a longitudinal stripe in the mantle zone along the most dorsal area of the alar plate (A, D and G). From E11.5 the expression domain in the caudal rhombencephalon and of spinal cord expands laterally following the ventral migrating cells from the dorsal alar plate [see arrows in (B), (E) and (H)]. By E12.5, in the spinal cord, *Barhl1* is a expressed almost exclusively in two columns localized in the mantle zone between the alar and the basal plates (F and I). At this stage *Barhl1* is intensively expressed in the entire superior colliculi of the mesencephalon (C). AP, alar plate; BP, basal plate; Cb, cerebellar plate; FP, floor plate; IC, inferior colliculi; M, mesencephalon; rl, rhombic lip; SC, superior colliculi.

The expression pattern of *Barhl1* was also examined in postnatal brain. Expression was observed in brain regions that derived from sites of earlier expression. In particular, *Barhl1* is expressed at postnatal days 4 and 7 (P4 and P7) in the superficial layers of the superior colliculus, in the entire inferior colliculus, in the external cerebellar granular layer, in the pontine nucleus and in a restricted hypothalamic area (Fig. 5D and E, and data not shown).

DISCUSSION

In this paper, we describe the isolation and characterization of a novel mammalian gene, *Barhl1*, which represents a new member of the *Bar* subgroup of homeobox genes, characterized by the presence of a tyrosine residue at position 49 of the homeodomain instead of the more common phenylalanine (Fig. 1B). In particular, *Barhl1* is highly related in terms of

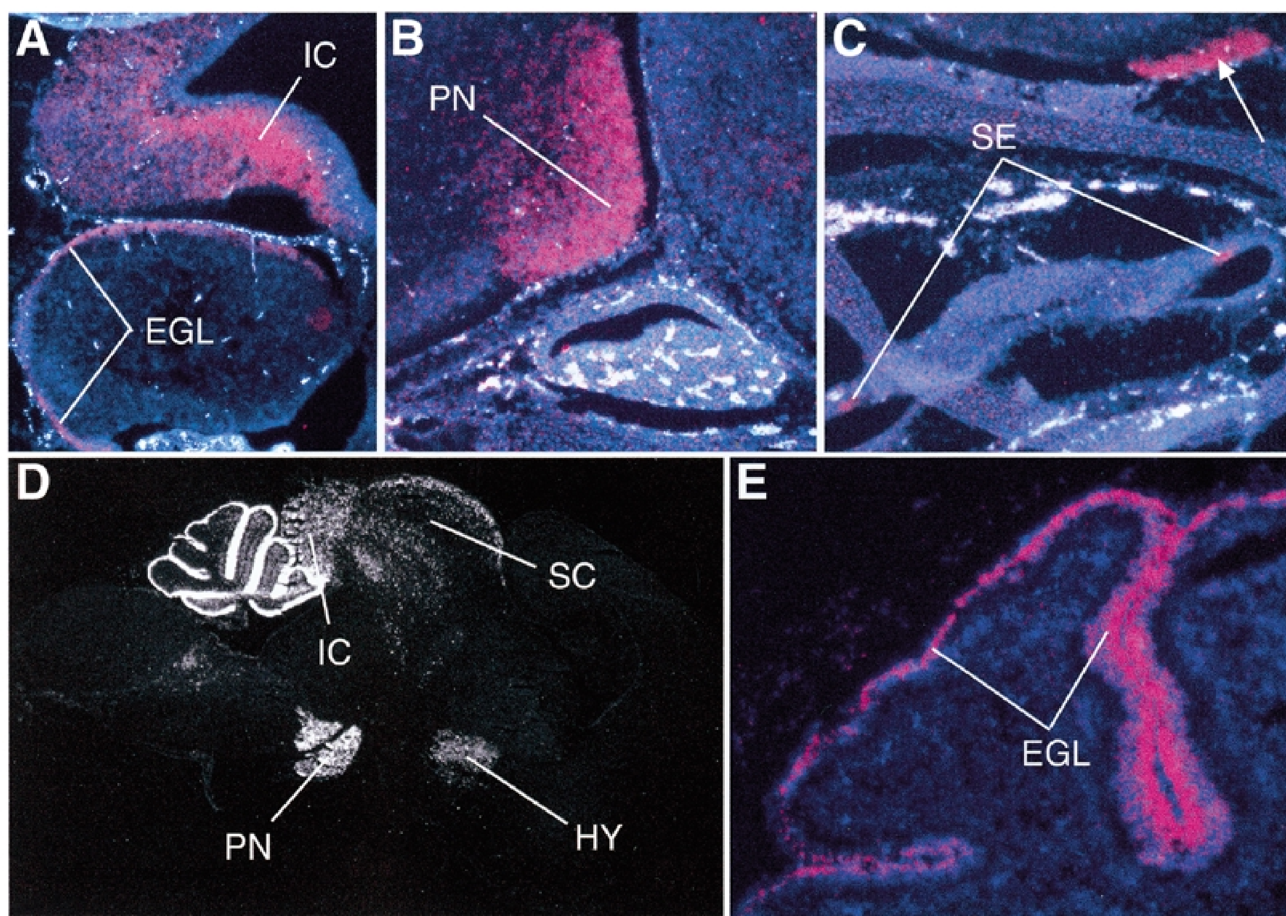

Figure 5. Late embryonic (E16.5) (A–C) and postnatal (P4) (D and E) expression of *Barhl1*. At E16.5 mesencephalic expression is prevalent in the caudal colliculus anlage with a distinct caudo–rostral gradient, while in the rhombencephalon it is expressed in the EGL of the cerebellar anlage (**A**) and in the pontine nucleus [(**B**) and arrow in (C)]. Another site of expression is the sensory epithelium of the inner ear (**C**). At P4 *Barhl1* is selectively expressed in the superior and inferior colliculi of the mesencephalon (D), in the most external part of the EGL (**D** and **E**), in the pontine nucleus (D) as well as in mammillary nuclei of the hypothalamus (D).

sequence similarity to another homeobox-containing gene, *mBH1*, recently described in rat by Saito *et al.* (16). The predicted proteins encoded by the mouse *Barhl1* and the rat *mBH1* cDNAs show very significant sequence similarity particularly in a central portion of 107 amino acids spanning the homeodomain, while the N- and C-termini show much lower homology (Fig. 1A). To rule out the possibility that the *mBH1* gene could be the rat homolog of *Barhl1*, we isolated two partial cDNA fragments belonging to the murine and human counterparts of *mBH1* and verified that they are different from *Barhl1*. Also, the expression patterns of *mBH1* (*Barhl2)* and *Barhl1* are different, since *mBH1* has been reported to be expressed in retina and in the dorsal thalamus (16), sites where *Barhl1* was never detected in our experiments. Therefore, *Barhl1* and *mBH1* (now renamed *Barhl2*, HGNC-approved symbol) represent two members of a new subfamily of *Bar* homeobox genes. *Barhl1* and *Barhl2* are the mammalian genes showing the highest sequence similarity to the two *Bar Drosophila* genes *BarH1* and *BarH2*. In fact, the murine proteins BARX1 and BARX2, which are also characterized by the presence of a Bar-like homeodomain and have previously been reported to be closely related to the Bar *Drosophila* proteins (19,20), show a much lower sequence similarity (restricted to the homeodomain) to the BARH1 and BARH2 proteins (Fig. 1B). These data indicate that the *Barhl* genes represent the mammalian transcripts most closely related to the *Bar Drosophila* genes.

Barhl1 expression and neural development

Barhl1 is exclusively expressed in restricted domains of the developing CNS. The regionalization of gene expression in the embryonic CNS is believed to provide positional information that regulates the generation of defined populations of neurons and appropriate guidance of their axons. On the basis of the analysis of its expression pattern, we argue that *Barhl1* can be involved in such processes at different levels of the neural axis.

Barhl1 is first detected at E9.5 in the basal and caudal regions of the prosencephalon (p1 and p2) that correspond to the anlage of the diencephalon, a structure that will differentiate into the pretectum and the thalamus. From E10.5, expression is detected in the basal zone of the secondary prosencephalon and diencephalon. In the pretectum, *Barhl1* is expressed in the most ventral part of the basal zone (basal p1) and, separately, in the alar domain (alar p1). In the latter region, three other homeobox genes are expressed: *Pax6*, *Lim1* and *Gsh1* (27). Unlike *Pax6*, which begins expression at an earlier stage, *Barhl1*, *Lim1* and

Gsh1 expression in p1 initiate at E10.5. It is therefore reasonable to hypothesize that these genes are involved in establishing the identity of p1 cells as different from mesencephalic cells, and that the timing of *Lim1*, *Gsh1* and *Barhl1* expression suggests that these genes may be downstream of *Pax6*.

At E11.5, *Barhl1* expression extends into the basal plate of the caudal hypothalamus, stopping abruptly at the borders between the mammillary and infundibulary region, the limit between the prosomeres 4 and 5 (Fig. 3). More caudally, basal expression in P2 and P3 extends dorsally into the ZL between the ventral and dorsal thalamus. Expression in the hypothalamus also persists postnatally: *Barh1l* is clearly detected at P4 in the anterior and lateral mammillary nuclei and, at lower levels, in the retromammillary domains (Fig. 5). The extensive expression in the above mentioned embryonic structures, such as the thalamus and the mammillary region, suggests that *Barhl1* may have a role in the development of these central processing stations. In adult brain, the thalamus connects rostral brain centers with the brainstem and the spinal cord, while the mammillary region is selectively involved in some aspects of learning and memory.

In the mesencephalon, *Barhl1* starts to be expressed in the basal plate in a region corresponding to the periaqueductal grey at E11.5. By E12.5, strong expression is detected in the mantle of the tectum. At this stage, *Barhl1* is first expressed in the entire superior colliculi, whereas by E16.5, its expression in the tectum is confined to the caudal colliculus anlage, with a clear caudo–rostral gradient. The inferior colliculi, functionally related to the auditory system, and the superior colliculi, layered structures which are part of the visual system, are generated in the alar plate region of the mesencephalon through a complex neuroblast migration process.

Barhl1 is a novel marker of cerebellar migrating neurons

The post-mitotic neurons generated in the alar rhombencephalon and, in particular, in the region of incomplete closure of the dorsal neural tube named rhombic lip, migrate to various destinations, giving rise to cerebellar granule cells and the precerebellar nuclei. Here, we show that *Barhl1* is strongly expressed in the rhombic lip as well as in the structures deriving from it. The cerebellar granule cell precursors originate from the rostral lip, while the caudal lip will give rise to the ventrally migrating neurons generating the precerebellar nuclei of the medulla and of the pons. Within this migrating stream from the lower lip, *Barhl1* is expressed only in neurons migrating to the pontine nuclei and not in the neurons migrating to the medulla. Interestingly, the formation of these nuclei is selectively disrupted in several knock-out mice, such as the mouse mutants for *Pax6* (26), *Math1* (28), *Netrin1* (25,29) and its receptor *Deleted in colorectal cancer* (*Dcc*) (30). Cells at the dorsal margin of the rostral rhombic lip give rise to granule neuron progenitors (31,32). These progenitors, specified to become granule cell progenitors, express the transcription factor genes *Zic1*, *Zic2* (33,34), *Zipro1* (35) and the basic HLH gene *Math1* (36,37). Recently, it has been shown that *Math1* function is required for the generation of granule cell progenitors (38). Nevertheless, the molecular steps involved in the specification of granule cell fate remain largely unknown.

Recent studies in the development of the cerebellar primordium suggest the involvement of patterning signals, such as FGF and Wnt proteins, that control regional identity at the boundary between the mesencephalon and metencephalon (mes/met boundary) (39), and inductive factors, such as the BMP proteins, which have an important role in the specification of the granule cell precursors (40). The first specified granule neuron progenitors appear in the rhombic lip laterally to the roof plate, a source of several BMPs, at around E10 and they seem to express both the *Math1* (36,37,41) and *Barhl1* genes. The ability of several BMPs (BMP6 and 7, and GDF7) to induce the expression of *Math1* and other dorsal markers (*Zic1*, *Zic2* and *Wnt3a*) has been proved recently (40), suggesting that the induction of the granule precursors may depend on BMP signaling with a mechanism similar to the induction of dorsal interneurons of the spinal cord (42). Once the specification of the granule progenitors has occurred, the putative coexpression of the *Math1* and *Barhl1* genes is maintained. The co-expression is also maintained in the subsequent events characterized by migration to the external granular layer (EGL), expansion of the progenitor population and final differentiation and migration into the internal granular layer (IGL) (36,37; data not shown). Therefore, it is reasonable to hypothesize that the generation of the EGL may depend on a BMP-triggered transduction pathway involving not only *Math1* (38) but also *Barhl1* which has a similar spatial and temporal pattern of expression.

Barhl1 expression and development of dorsal sensory interneurons of the spinal cord

The spinal cord is composed of different cell types which are generated and occupy different positions along the dorso–ventral axis of the neural tube (43). The motor neurons are generated laterally to the floor plate, which represents the ventral midline of the neural tube. Dorsally, after the migration of the neural crest cells and the closure of the neural tube, the roof plate is formed along the dorsal midline and cells lateral to it differentiate into different types of dorsal sensory interneurons. The specification of ventral and dorsal cell types, as well as of individual neuronal subtypes, seems to be regulated by inductive signaling factors, provided by the notochord and floor plate ventrally, and by the epidermal ectoderm and roof plate dorsally. Recent evidence provides strong support for a role of the BMP subclass of TGFβ-related proteins in the control of the differentiation of dorsal neurons (42,44). In particular, it has been demonstrated that *GDF7*, a BMP family member expressed selectively by roof plate cells, is able to induce the differentiation of dorsal commissural interneurons (42). These neurons are generated close to the roof plate, and can be divided in D1A and D1B neurons (45). The *Math1* gene is specifically expressed by the progenitor cells adjacent to the roof plate which generate both subclasses of the D1 neurons (36,37,41,42,44). At E10.5, the *Barhl1* and *Math1* domains of expression seem to overlap entirely in this region. However, at E12.5, *Barhl1* expression becomes restricted to a more ventral domain corresponding to the region where the D1B subclass of neurons migrate (45), while *Math1* expression is confined to the D1A neurons which, for the most part, remain close to the roof plate (42). Therefore, both D1A and D1B neurons seem to originate from *Math1*+ and *Barhl1*+ progenitors, but they subsequently reside in different sites of the dorsal spinal cord with D1A neurons maintaining the *Math1* expression and D1B

neurons maintaining the expression of *Barhl1*. It is worth noting that in GDF7 mutants, only the late *Math1*+ cells, corresponding to the D1A neurons, are lost, whereas the D1B differentiate normally (42). Thus, there might be different TGFβ-related proteins from the roof plate regulating the formation and identity of the specific subtypes of dorsal interneurons.

The expression pattern of *Barhl1* in the developing CNS strongly suggests that this gene, similar to its *Drosophila* counterparts *BarH1* and *BarH2*, may play a crucial role in cell fate determination of neural structures and may therefore be involved in the pathogenesis of CNS developmental anomalies. Interestingly, the human *BARHL1* gene maps to chromosome 9q34, in a region overlapping the critical interval for one form of Joubert syndrome described recently by Saar *et al.* (22). Joubert syndrome, is a rare syndrome with autosomal recessive inheritance characterized by aplasia/hypoplasia of the cerebellar vermis with abnormal eye movements, ataxia and mental retardation. Both the mapping assignment and its remarkable expression in the primordia of the cerebellum and the brainstem, render *BARHL1* an attractive candidate gene for Joubert syndrome. Mutation analysis in patients as well as the generation of a *Barhl1* knockout mouse will be necessary to determine whether the *Barhl1* gene plays a causative role in Joubert syndrome or in other developmental defects of the mammalian nervous system.

MATERIALS AND METHODS

cDNA identification

To identify the murine *Barhl1* full-length transcript, two murine cDNA libraries were used: an E11 embryo (Clontech ML3003a) and an embryonic carcinoma (P19 cell line; Stratagene 937317) cDNA library. For the isolation of the *BARHL1* gene, we used a fibroblast genomic library (Stratagene 946204). Plating, hybridization and washing conditions were as described previously (46).

To isolate the human and murine *BARHL2* genes, we carried out PCR analysis on human genomic DNA using the oligonucleotide primers TO-9557 and TO-9558 designed on the sequence of the rat *mBH1* cDNA (16). Oligonucleotide sequences were as follows: TO-9557, 5'-GTGGAAGCGGCA-GACTGCAG-3' and TO-9558, 5'-CAGGCCGTGGATGAG-CACGC-3'. PCR conditions were 35 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 45 s.

cDNA sequence analysis

cDNA sequence analysis, and nucleotide and protein database searches were performed as described previously (14). Data on similarity/identity were obtained using the Bestfit program of the GCG software package, version 8.1. The multiple alignment analyses were generated using the PileUp program of the Wisconsin GCG software package, version 8.1.

Genomic mapping in human and mouse

For the RH mapping assignment of the human *BARHL1* and *BARHL2*, we used a previously described procedure (14). PCR analysis on the GeneBridge4 radiation hybrid panel (21) was performed using the following two primers designed in the *BARHL1* genomic region, 2 kb downstream of the beginning of the third exon: TO-5571, 5'-TTCTCAACTTGCCCCAGTCC-3' and TO-5572, 5'-CCTCATAAACGCCGACTCAAC-3'. For the mapping of the *BARHL2* gene, we used oligonucleotide primers TO-9840, 5'-CCGAGGCAGGGAACTACTCGG-3' and TO-9841, 5'-CCGCTGCAGCTGGGGATGG-3'. PCR conditions were 35 cycles of 94°C for 45 s, 52°C for 45 s and 72°C for 45 s for both *BARHL1* and *BARHL2*.

Genetic mapping was achieved using a (C57BL/6j×SPRET/Ei)F1×SPRET/Ei (BSS) backcross generated and distributed by the Jackson Laboratory (47). An *Eco*RI RFLP was identified by hybridization of C57BL/6j and SPRET/Ei parental DNAs (cut with each of the following six restriction enzymes: *Eco*RI, *Eco*RV, *Kpn*I, *Msp*I, *Taq*I and *Xba*I) with a *Barhl1* cDNA probe corresponding to the insert of the IMAGE clone 335997. The polymorphism consisted of a single SPRET/Ei-specific *Eco*RI band, and of two C57BL/6JEi-specific *Eco*RI fragments. Four Southern panels containing *Eco*RI-cut parental DNAs and N2 progeny (n = 94) DNAs were hybridized with a *Barhl1* cDNA probe. The analysis of the strain distribution pattern observed in the N2 progeny (n = 92) of the BSS backcross (47) was analyzed with the Map Manager 2.6 program (48) and permitted the localization of *Barhl1* to chromosome 2.

Expression studies

Mouse embryo tissue sections were prepared and RNA *in situ* hybridization experiments were performed as described (49). The IMAGE EST clone 335997 containing part of the *Barhl1* transcript was linearized with appropriate restriction enzymes to transcribe either sense or antisense ^{35}S-labeled riboprobes. Slides were exposed for 10 days. Micrographs are double exposures: red color represents the *in situ* hybridization signal, and the blue shows the nuclei stained with Hoechst 33258 dye.

ACKNOWLEDGEMENTS

We thank E. Roncoroni and the TIGEM sequencing staff for technical support, and M. Smith for preparation of the manuscript. This work was supported by the Italian Telethon Foundation, by the Merck Genome Research Institute (grant no. 37), and by the Armenise Harvard Foundation. S.M. is supported by grants from the EC (BIO4-CT98-0303, DGICYT PM98-0056) and from Fundacion la Caixa 97/101.

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