Mмот1, a New Helix-Loop-Helix Transcription Factor Gene Displaying a Sharp Expression Boundary in the Embryonic Mouse Brain*

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Several genetic factors have been proven to contribute to the specification of the metencephalic-mesencephalic territory, a process that sets the developmental foundation for prospective morphogenesis of the cerebellum and mesencephalon. However, evidence stemming from genetic and developmental studies performed in man and various model organisms suggests the contribution of many additional factors in determining the fine subdivision and differentiation of these central nervous system regions. In man, the cerebellar ataxias/aplasias represent a large and heterogeneous family of genetic disorders.

Here, we describe the identification by differential screening and the characterization of Mмот1, a new gene encoding a DNA-binding protein strikingly similar to the helix-loop-helix factor Ebf/Olf1. Throughout midgestation embryogenesis, Mмот1 is expressed at high levels in the metencephalon, mesencephalon, and sensory neurons of the nasal cavity. In vitro DNA binding data suggest some functional equivalence of Mмот1 and Ebf/Olf1, possibly accounting for the reported lack of olfactory or neural defects in Ebf<sup>−/−</sup> knockout mutants. The isolation of Mмот1 and of an additional homolog in the mouse genome defines a novel, phylogenetically conserved mammalian family of transcription factor genes of potential relevance in studies of neural development and its aberrations.

A number of transcription factor genes regulate cell identity in specific body regions, both in invertebrates and vertebrates (1). In vertebrates, the Hox genes control identity along the body axis and provide positional cues for the developing neural tube, particularly the rhombencephalon and spinal cord from the branchial area to the tail. Conversely, development of the anteriormost body domain, including the metencephalic, mesencephalic, and prosencephalic territories (2), has remained relatively obscure in invertebrates and vertebrates alike. Indeed, the molecular specification of compartments or subdivisions in the vertebrate forebrain is still a matter of debate (3).

Specifically, regarding morphogenesis of the metencephalon-mesencephalon boundary, a recent breakthrough came with the identification of the mouse En-1 and En-2 genes, which were cloned based on their homology to the Drosophila segment polarity gene engrailed (4). En-2 homozygous mutant mice created by homologous recombination in embryonic stem cells are viable and exhibit a patterning defect in the cerebellum (5, 6). In contrast, En-1 homozygous mutant mice die at birth and show a deletion of most of the colliculi and cerebellum (7). Likewise, the Wnt and Pax families of genes have been implicated in cerebellar patterning by means of genetic or neurobiological studies (8, 9), whereas the Fgf-8 gene has been shown to play a critical role in the induction of the isthmic organizing center (10).

Despite these and other relevant advances, mostly based on developmental mechanisms conserved from Drosophila to vertebrates, our knowledge of rostral central nervous system differentiation in general and of metencephalic-mesencephalic specification in particular remains fragmentary to date, and many other as yet unidentified regulatory genes may at different times play a role in various cell fate specification or terminal differentiation processes.

To help elucidate some of the molecular mechanisms underlying the fine subdivision and differentiation of primary brain structures during midgestation brain development, our group set out to screen for developmentally regulated genes restricted in their spatial and temporal expression domains within the embryonic head. This was achieved through a modification (11) of a PCR-based<sup>2</sup> differential screening technique named RNA fingerprinting (12, 13).

Among other embryonic central nervous system genes of regulatory significance found in this way, we have isolated a new helix-loop-helix (HLH) transcription factor gene, Mмот1 (metencephalon-mesencephalon-gliafactory transcription factor 1), differentially expressed along the anteroposterior axis, displaying a sharp anterior expression boundary within the diencephalon as well as a high level specific expression in the sensory portion of the olfactory epithelium.

HLH transcription factors are nuclear proteins that bind DNA...
as homo- or heterodimers. HLH transcription factors have been subdivided into various subfamilies (14), and their role has been recognized in *Drosophila* neurogenesis and sex differentiation as well as vertebrate myogenesis (15) and neurogenesis (16, 17). The newest subclass of HLH proteins identified so far includes two virtually identical, independently cloned genes: a mouse gene named *Ebf* (early B-cell factor) (18) and a rat gene named *Ofi1* (olfactory-neuronal transcription factor) (19) as well as their *Drosophila* homolog (collier) (20). A specific feature of this subfamily is that its members lack the basic domain found upstream of the first a helix in basic HLH transcription factors, which mediates DNA binding. In Ebf, the establishment of DNA-protein interactions is mediated by an N-terminal domain, inclusive of a zinc finger element, whereas the HLH domain appears exclusively involved in dimerization (21).

The present paper describes the isolation, genetic characterization, in situ expression studies, and in vitro DNA binding properties of *Mmot1*, a new member of the Ebf/Ofi-like subclass of HLH transcription factors.

### EXPERIMENTAL PROCEDURES

#### General Methods—Standard molecular techniques including nucleic acid purification, restriction analysis, gel electrophoresis, DNA ligation, cloning, subcloning, dideoxy sequencing, probe radiolabeling, Northern and Southern analysis, RNase protection assays, and library screening were performed according to established protocols (22). Automated sequencing with DyeDeoxy primers or DyeDeoxynucleotidemers was performed on an ABI 373 machine. Hybridizations of Northern, Southern, and zoo blot filters (Pall) were performed at 65 °C in 125 mM sodium phosphate (pH 7.2), 250 μm NaCl, 7% SDS, 10% polyethylene glycol. Filters were washed at 65 °C for final stringencies of 0.2 × SSC (1 × SSC: 150 μm NaCl, 15 μm sodium citrate, pH 7) for 10 min. Phage plaque hybridizations and subsequent washes were carried out under comparable stringency conditions.

#### Tissue Preparation and RNA Extraction—Preparations of E12.5 embryonic central nervous system samples were done as follows. Under a dissection microscope, brain tissue was separated from surrounding mesoderm and ectoderm. Neuroectodermal tissue spanning fourth ventricle to midbrain was separated from prosencephalic territories. Fresh tissue preparations from two CD1 litters were pooled and lysed in guanidine isothiocyanate. RNA extraction was carried out on a cesium chloride gradient (22).

#### RNA Fingerprinting—Clone 203 was derived through a modification of the RNA fingerprinting protocol (13) comparing mRNAs of mouse E12.5 mesencephalon, E12.5 prosencephalon, and postnatal mouse brain and cerebellum. RNA fingerprinting was conducted as follows. A reverse transcription reaction was carried out using a (dT)16 primer on total RNAs extracted by the cesium chloride method (22) and digested with 4 IU of DNase I/total RNAs extracted by the cesium chloride method (22). Neuroectodermal tissue spanning fourth ventricle to midbrain was separated from prosencephalic territories. Fresh tissue preparations from two CD1 litters were pooled and lysed in guanidine isothiocyanate. RNA extraction was carried out on a cesium chloride gradient (22).

#### Gel Mobility Shift Assay—In vitro transcription, transcript purification, and translation using rabbit reticulocyte lysate (Promega) were done according to manufacturer recommendations, with the addition of 10 μl ZnSO4. The efficiency of in vitro translation was assayed by running parallel translation reactions performed in the presence of [35S]methionine (Amersham). The double-stranded synthetic DNA fragment carrying the binding site for the Ebf/Ofi protein (5'-ACCATG-CTCGTGCTCCAGAGGACCCTGTC) (29) and a control DNA fragment where the binding site had been mutated (5'-ACCATGCTCCTGTGCA-GCAAGGACCCTGTC) were end-labeled with [γ-32P]ATP (Amersham) using 20 units of T4 polynucleotide kinase (Ambion Inc.) according to established protocols (28). In vitro DNA binding and electrophoretic mobility shift assays were performed as described (30), except that 2 μg of poly(dI-dC) were used as a nonspecific competitor. In each binding reaction (20 μl) we employed 0.15 pmol of labeled double-stranded DNA (about 40,000 copies); 10 μl were then applied to a nondenaturing 6% polyacrylamide gel.

#### RESULTS

**Cloning of *Mmot1* by PCR-based Differential Screening**

We applied a modified RNA fingerprinting protocol (11) to the analysis of differential gene expression in the embryonic and postnatal mouse brain. By RNA fingerprinting, we com-
pared the following stages and districts: E12.5, mesencephalon + cerebellar primordium; E12.5, prosencephalon; P4, brainstem + cerebellum; P4, forebrain. As primers we employed a panel of arbitrary 12-mers, some of which were carrying a partially degenerate position at their 3′ ends obtained through computer simulations of PCR experiments run on a nonredundant mouse nucleotide data base.1 At embryonic day 12.5, band 203 (778 nt, nucleotides 939–1717 of the full-length transcript), obtained with primer DR34, amplified almost exclusively in the posterior region (metencephalon-mesencephalon), whereas it failed to show an obvious band at postnatal day 4 (P4) either in the anterior or posterior head (Fig. 1). The band was gel-excised, reamplified, and cloned into pBluescript II SK+ (Stratagene). Clones were screened as described (11), and plasmid 203.14 was manually sequenced. A data base search was run with BLASTN and BLASTX using the Genetics Computer Group interface (31). The search revealed 72% identity at the nucleotide level with a mouse gene named Mmot1 for early B-cell factor (18) encoding a helix-loop-helix transcription factor. Mmot1 is virtually identical to an independently cloned rat gene named Olf1 (olfactory transcription factor 1) (19). To confirm that Mmot1 and Ebf are indeed different genes, we analyzed the Mmot1 cDNA by restriction mapping, identifying SacII and HinfI restriction sites absent from Ebf (or Olf1) (positions 1529 and 1472 of the Mmot1 transcript, respectively) as predicted from sequence analysis.

Genetic Linkage Analysis Defines Mmot1 as a New Member of the Ebf/Olf1-like Gene Family

Based on the above evidence, we set out to isolate clones spanning the entire coding sequence of Mmot1. To this end, we plated out 6 × 10⁶ plaque-forming units from an embryonic day 11.5 whole-embryo cDNA library (CLONTECH, ML1027). Again, as a probe, we utilized a region of Mmot1 displaying low degree similarity to Ebf. After high stringency hybridization and washes, we isolated five positives, one of which spanned the full-length transcript (5.4 kilobases, corresponding to the coding portion of the gene. The experiment was conducted on the parental strain DNAs of the BSS backcross (C57BL/6J × Mus spretus). Automated sequencing of the product ends confirmed them as part of the Mmot1 gene. The PCR product was digested with frequent cutters (RsaI, Sau3AI, TaqI, and MspI). An MspI polymorphism was identified consisting of 2,450- and 250-base pair fragments in B6 DNA and a 2,700-base pair fragment in spretus DNA. This polymorphism was employed to type the 94 individual N2 progeny of the BSS backcross by PCR and restriction fragment length polymorphism analysis. 93 out of 94 progeny were typed successfully. Linkage analysis performed with MapManager 2.6 unequivocally localized Mmot1 to mouse chromosome 14, 1.1 centimorgan distal to Rafk (33) (lod score 25.6) and 2.3 centimorgan proximal to Nfl (34) (lod score 22.6). The data are summarized in Fig. 2. The human homolog of Nfl has been mapped to chromosome 8p21 (35, 36).

Cloning of the Full Coding Sequence

Based on the above evidence, we set out to isolate clones spanning the entire coding sequence of Mmot1. To this end, we plated out 6 × 10⁶ plaque-forming units from an embryonic day 11.5 whole-embryo cDNA library (CLONTECH, ML1027). Again, as a probe, we utilized a region of Mmot1 displaying low degree similarity to Ebf. After high stringency hybridization and washes, we isolated five positives, one of which spanned the full-length transcript (5.4 kilobases, corresponding to the coding portion of the gene. The experiment was conducted on the parental strain DNAs of the BSS backcross (C57BL/6J × Mus spretus). Automated sequencing of the product ends confirmed them as part of the Mmot1 gene. The PCR product was digested with frequent cutters (RsaI, Sau3AI, TaqI, and MspI). An MspI polymorphism was identified consisting of 2,450- and 250-base pair fragments in B6 DNA and a 2,700-base pair fragment in spretus DNA. This polymorphism was employed to type the 94 individual N2 progeny of the BSS backcross by PCR and restriction fragment length polymorphism analysis. 93 out of 94 progeny were typed successfully. Linkage analysis performed with MapManager 2.6 unequivocally localized Mmot1 to mouse chromosome 14, 1.1 centimorgan distal to Rafk (33) (lod score 25.6) and 2.3 centimorgan proximal to Nfl (34) (lod score 22.6). The data are summarized in Fig. 2. The human homolog of Nfl has been mapped to chromosome 8p21 (35, 36).

Genetic evidence obtained by other authors (32) had assigned Ebf to proximal mouse chromosome 11. To strengthen our evidence defining Mmot1 and Ebf as distinct genes, we set out to localize Mmot1 in the mouse genome by linkage analysis in the BSS backcross generated and maintained at The Jackson Laboratory (24). Using a primer pair (p1 and p2) from a region of low degree homology with Ebf, we amplified a 2.7-kilobase genomic fragment spanning an intronic sequence in the coding portion of the gene. The experiment was conducted on the parental strain DNAs of the BSS backcross (C57BL/6J × Mus spretus). Automated sequencing of the product ends confirmed them as part of the Mmot1 gene. The PCR product was digested with frequent cutters (RsaI, Sau3AI, TaqI, and MspI). An MspI polymorphism was identified consisting of 2,450- and 250-base pair fragments in B6 DNA and a 2,700-base pair fragment in spretus DNA. This polymorphism was employed to type the 94 individual N2 progeny of the BSS backcross by PCR and restriction fragment length polymorphism analysis. 93 out of 94 progeny were typed successfully. Linkage analysis performed with MapManager 2.6 unequivocally localized Mmot1 to mouse chromosome 14, 1.1 centimorgan distal to Rafk (33) (lod score 25.6) and 2.3 centimorgan proximal to Nfl (34) (lod score 22.6). The data are summarized in Fig. 2. The human homolog of Nfl has been mapped to chromosome 8p21 (35, 36).

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Protein Sequence Analysis

The deduced peptide sequence (553 residues) was analyzed with a variety of local and on-line programs. The primary sequence is 80.6% identical to the Ebf protein, with more conserved regions clustered around a putative zinc finger domain (residues 134–147, sequence HEVMCSRCCEKKSC) and a helix-loop-helix domain (residues 344–387, sequence KEMLL...VPRNP). Also perfectly conserved is a putative nuclear targeting domain (residues 219–223, sequence RRARR). A Drosophila melanogaster gene named collier (accession number X97803) was also found to encode a highly similar protein (20).

Mmot1 is illustrated in Fig. 3 and compared with the two other known members of the subfamily (Ebf/Olf1 and Collier).

Mmot1 Belongs to an Expanding, Phylogenetically Conserved Gene Family

Conservation of the Mmot1 gene was assessed experimentally by zoo blot analysis. A Southern blot containing DNAs of six mammals, a frog, and chicken was hybridized with a fragment of the Mmot1 coding sequence (positions 1340–2340) that shows the highest degree of divergence from Ebf. Both hybridization and washes were carried out at high stringency conditions (see “Experimental Procedures”). The experiment suggested the existence of strongly conserved homologs of Mmot1 in all organisms tested, including chicken and Xenopus laevis (Fig. 4 a).

Because the isolation of Mmot1 in mouse defines a new family of closely related mammalian HLH proteins, we looked to identify possible new homologs of our gene and Ebf/Olf1 in the Expressed Sequence Tag (EST) data base (37). The search was conducted with the BLAST programs using the Mmot1

![Figure 3: Sequence alignment of Ebf/Olf1, Mmot1, and the Drosophila melanogaster protein Collier. Boxed residues are identical in different proteins, from N terminus to C terminus. Shaded residues represent the zinc finger element, nuclear targeting domain, and helix-loop-helix domain, respectively. The second helix of the conserved HLH domain is not present in the Collier protein.](image1)

![Figure 4: Phylogenetic conservation. a, high stringency Southern (zoo blot) analysis of 10 μg of DNA from each of eight mammalian and nonmammalian species. The filter was hybridized with an Mmot1-specific probe displaying >40% divergence from the corresponding region of the Ebf transcript. For details on hybridization and washing conditions, see “Experimental Procedures.” b, the analysis of the EST data base revealed the existence of several possible new members of the Ebf family of HLH factors in the human and mouse genomes.](image2)
FIG. 5. mRNA in situ hybridization of embryonic tissue sections at 10.5 (a and b), 12.5 (c–g), 13.5 (h–n and s), and 14.5 (o–q) days postcoitus. 
b, f, l, m, n, q, and s are coronal sections; all others are sagittal sections. 
g, k, and n are negative controls, hybridized with a sense riboprobe. r,
The distribution of Mmot1 model (3) is schematically summarized in Fig. 5. Extraneural labeling for Mmot1 can be detected in the limb bud. At all stages examined, very strong signal is present in the olfactory portion of the nasal cavity, with a sharp demarcation at the boundary with the respiratory epithelium, which does not express the gene. No other major expression sites could be seen at the embryonic stages examined.

High resolution, nonradioactive in situ analysis was conducted on E13.5 sections with an Mmot1 cRNA probe (Fig. 5a). In parallel, immunohistochemical analysis was carried out on adjacent sections with an anti-PCNA monoclonal antibody (Fig. 5c) used as a marker of proliferating neuroblasts (38). The comparison reveals that Mmot1 is expressed in a thin postmitotic stratum of the ventricular wall, apical to the interkinetic migration range of neuroepithelial cells.

To extend the characterization of Mmot1 expression beyond embryonic development, we determined the distribution of the transcript in 11 adult mouse tissues by RNase protection assay (Fig. 6). In the adult mouse, Mmot1 is specifically expressed in the cerebellum, muscle, heart, ovary, and testis. Lower expression levels are shown in the adult brain. Very low level or absent signal is observed in the thymus, kidney, liver, spleen, and intestine.

Mmot1 Binds the Nucleotide Target Site of Olf1 in Vitro

To characterize the DNA binding properties of Mmot1, in a comparison with its close cognate Ebf/Olf1, we synthesized the protein by in vitro transcription and translation of a full coding Mmot1 cDNA subclone (ab1). The translation product (60 kDa) matched the size of our deduced amino acid sequence. The DNA binding domains of Mmot1 and Ebf/Olf1 are extremely similar, and we therefore tested whether Mmot1 could recognize the same sequence bound by Ebf/Olf1. Labeled double-stranded oligonucleotides corresponding to the wild type and mutant Olf1 recognition sites (29) were employed in a gel mobility shift assay. A band shift was observed after incubation of the Mmot1 protein produced by in vitro translation with the wild type double-stranded oligonucleotide but not with a mutant double-stranded oligonucleotide in which the Olf1 target site was disrupted (Fig. 7).
A New HLH Factor in Developing Metencephalon-Mesencephalon

In a comparison with Mmot1, the Ebf/Olf1 nucleotide site features two substitutions that disrupt the palindrome (see "Experimental Procedures" for sequences of wild type and mutant sites).

In addition to displaying high expression levels in the cerebellar primordium, midbrain, and dorsal thalamus, Mmot1 is transcribed at remarkable levels in the olfactory neurons of the nasal cavity and vomeronasal organ. Olf1, a closely related gene, was found by other authors to drive the expression of several olfactory-specific proteins (19). However, mice homozygous for induced mutations of Ebf, the mouse ortholog of Olf1, display no alterations in the morphogenesis of the olfactory area or in the expression of olfactory proteins (42). Likewise, they present with no obvious abnormalities in midbrain or hindbrain development. Evidence of strong similarities in the expression of Ebf/Olf1 (42) and Mmot1 (present paper) at many sites in the developing brain and nasal neuroepithelium provides a possible explanation for the lack of neurodevelopmental and olfactory defects in Ebf−/− knockout mutant mice.

The notion of genetic redundancy in the pathway involving Ebf/Olf1 and Mmot1 is strengthened by in vitro functional evidence presented in this paper, which proves that Ebf/Olf1 and Mmot1 share DNA binding affinity and specificity (29) as expected based on the marked similarities in their dimerization and DNA binding domains. Moreover, similarities in the HLH domains of the two proteins suggest the possibility that they might assemble as heterodimers in those territories where the corresponding genes are coexpressed. In this scenario, the generation of Mmot1 lack- or gain-of-function mutants and the analysis of double knockout mutants for Mmot1 and Ebf will assist in the genetic dissection of functional pathways involving the two genes while clarifying their role in midbrain/hindbrain subdivision and olfactory development.

In summary, Mmot1 is a new, developmentally regulated, restrictedly expressed member of a novel, expanding family of transcription factor genes in mammalian genomes. Its expression is dependent on a gene expression regulation involving cytoplasmic polarity genes (bicoid) and gap rule genes (buttonhead, in particular) (20). A phenocopy of the collier mutant, produced by transgenic insertion of an anti-rule genes (32), and the Drosophila bicoid program involving cytoplasmic polarity genes (33) provides a possible explanation for the lack of neurodevelopmental and olfactory defects in Ebf−/− knockout mutant mice. The notion of genetic redundancy in the pathway involving Ebf/Olf1 and Mmot1 is strengthened by in vitro functional evidence presented in this paper, which proves that Ebf/Olf1 and Mmot1 share DNA binding affinity and specificity (29) as expected based on the marked similarities in their dimerization and DNA binding domains. Moreover, similarities in the HLH domains of the two proteins suggest the possibility that they might assemble as heterodimers in those territories where the corresponding genes are coexpressed. In this scenario, the generation of Mmot1 lack- or gain-of-function mutants and the analysis of double knockout mutants for Mmot1 and Ebf will assist in the genetic dissection of functional pathways involving the two genes while clarifying their role in midbrain/hindbrain subdivision and olfactory development.

In summary, Mmot1 is a new, developmentally regulated, restrictedly expressed member of a novel, expanding family of transcription factor genes whose expression may have considerable impact on the study of midgestation neural development in general and the mechanisms of normal and aberrant cerebellar ontogeny in particular.

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