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Mmot1, 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 *Mmot1*, a new gene encoding a DNA-binding protein strikingly similar to the helix-loop-helix factor Ebf/Olf1. Throughout midgestation embryogenesis, *Mmot1* 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 Mmot1 and Ebf/Olf1, possibly accounting for the reported lack of olfactory or neural defects in *Ebf*$^{-/-}$ knockout mutants. The isolation of *Mmot1* 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)[1] of a PCR-based[2] 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, *Mmot1* (metencephalon-mesencephalon-olfactory 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

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U71189.

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[1] G. G. Consalez, A. Cabibbo, A. Corradi, C. Alli, M. Sardella, R. Sitia, and R. Fesce, submitted for publication.

[2] The abbreviations used are: PCR, polymerase chain reaction; HLH, helix-loop-helix; RT, reverse transcription; PCNA, proliferating cell nuclear antigen; contig, group of overlapping clones; BSS, (C57BL/6j × SPRET/Ei)F_1 × SPRET/Ei; nt, nucleotide(s); EST, expressed sequence tag.

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 *Olf1* (*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 α 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/Olf1-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 Dyedeoxyterminators 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 mM NaCl, 7% SDS, 10% polyethylene glycol. Filters were washed at 65 °C to final stringencies of 0.2 × SSC (1 × SSC: 150 mM NaCl, 15 mM 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 through 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[1] 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/μg of total RNA. Radioactive PCR reactions were performed in duplicate from 1 μl of each RT reaction in a 50-μl final volume with an arbitrary 12-mer (DR34, sequence 5′-GACGAGGCTGGA) (final concentration, 4 μM). PCR conditions were 3 min at 94 °C, 2 min at 80 °C in which *Taq* polymerase was added (hot start), followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 30 s at 72 °C, with a final elongation step of 5 min at 72 °C. 0.1 μl of [α-^{32}P]dCTP was added to each reaction. Amplified products were separated on a 5% denaturing acrylamide gel and visualized by autoradiography. Differentially displayed bands were cut from the gel and electroeluted. The bands were reamplified using the same 12-mer primers and blunt-end cloned into pBluescript II SK+ (Stratagene) as described (11).

Sequence Analysis—Data bank searches (GenBank™, GenEmbl, SwissProt, and Protein Identification Resource) were run through the BLAST server (23). Additional sequence analysis and contig assembly was done using the MacVector program (Oxford Molecular Group) and the Sequencher program (Gene Code Corp.), respectively. The nucleotide sequence of the gene was deposited into the GenBank™ data base with accession number U71189.

Genetic Mapping—Genetic mapping was done on 96 DNAs corresponding to the parentals and 94 N_2 progeny of a (C57BL/6j × SPRET/Ei)F_1 × SPRET/Ei (BSS) backcross generated and distributed by The Jackson Laboratory (Bar Harbor, ME) (24). An *Msp*I restriction fragment length polymorphism was identified in an intronic sequence and amplified with primers p1 and p2 (sequences 5′-GGTTGGCCATAGGAACATT and 5′-TCTTTCCAGCTCCCCAGC) as described under "Results." Its segregation was followed, and linkage analysis was performed with the MapManager 2.6 program (25).

Quantitative RT-PCR—Total RNAs from mouse preparations at E12.5, P4, and adult brain and liver were used in quantitative PCR reactions designed to work within the linear (exponential) range of amplification. Total RNA was reverse-transcribed using a random hexamer primer (Life Technologies, Inc.), and the quantity of cDNA synthesized in each RT reaction was first normalized by means of PCR amplifications with mouse glyceraldehyde-3-phosphate dehydrogenase primers (sequences 5′-CGCATCTTCTTGTGCAGTG and 5′-GTTCAGCTCTGGGATGAC). Each reaction was conducted incorporating 0.1 μl of [α-^{32}P]dCTP in the mix. Amplification products were separated through polyacrylamide gels and quantitated by densitometry (Image Quant, Molecular Dynamics). At each stage examined, identical amounts of cDNA from each RT reaction were then used in two parallel PCR amplifications using *Mmot1*-specific primers (p1 and p2, see above). A touchdown PCR (26) was conducted using 3 min at 94 °C, 2 min at 80 °C in which *Taq* polymerase (Perkin-Elmer) was added; 2 cycles of 1 min at 94 °C, 1 min at 62 °C, 1 min at 72 °C; 2 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C; 2 cycles of 1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C; 21 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C; and a final elongation of 5 min at 72 °C.

In Situ Hybridization and Immunohistochemical Analysis of Mouse Tissue Sections—Radioactive and nonradioactive *in situ* hybridization (27) was carried out as follows. 7-μm paraffin serial sections from a single embryo were displaced in 4–6 adjacent series; two alternative series were used for each probe. Four embryos from at least two litters were studied at 12.5 and 13.5 days of embryonic development. Slides were deparaffinated in xylene, hydrated through an alcohol series, treated with paraformaldehyde and proteinase K, acetylated, and dehydrated through an ethanol series. For radioactive *in situ* studies, 1 μl (3×10^6 cpm) of *Mmot1* riboprobe labeled with ^{35}S-UTP (Amersham) in the hybridization mix was added to each slide. For nonradioactive *in situ* studies, 1 μg of *Mmot1*-linearized plasmid was transcribed *in vitro* in the presence of 0.8 μl of 10 mM digoxigenin-11-UTP (Boehringer Mannheim). Both sense and antisense probes were used. Hybridization was carried out overnight at 65 °C. Slides were washed under stringent conditions (65 °C, 2 × SSC, 50% formamide) and treated with RNase A. Autoradiography was performed with Kodak NT/B2 emulsion. Exposure times were 15 days. Sections were examined and photographed on dark and bright fields using a Zeiss SV11 microscope. Nonradioactive signal was revealed through an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) as recommended by the manufacturer. Immunohistochemical analysis was conducted with a monoclonal anti-PCNA antibody (clone PC10, Boehringer Mannheim). Sections were incubated with a peroxidase-conjugated secondary antibody, and signal was revealed with the Vectastain ABC kit (Vector Laboratories, Inc.) as recommended.

RNase Protection Assay—A *Mmot1*-specific 379-nt sequence was cloned into pBluescript and used to synthesize a riboprobe by *in vitro* transcription with T7 RNA polymerase and incorporation of [α-^{32}P]UTP (800 Ci/mmol) (Amersham Life Science, Inc.). 50 μg of RNA from each of 11 different adult mouse tissues was hybridized, treated, and polyacrylamide gel electrophoresis-separated as described (28). Normalization was achieved through a mouse β-actin riboprobe.

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 μM $ZnSO_4$. The efficiency of *in vitro* translation was assayed by running parallel translation reactions performed in the presence of [^{35}S]methionine (Amersham). The double-stranded synthetic DNA fragment carrying the binding site for the Ebf/Olf1 protein (5′-ACCCATGCTCTGGTCCCCAAGGAGCCTGTC) (29) and a control DNA fragment where the binding site had been mutated (5′-ACCCATGCTCTGGTCAGCAAGGAGCCTGTC) were end-labeled with [γ-^{32}P]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 cpm); 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)[1] to the analysis of differential gene expression in the embryonic and postnatal mouse brain. By RNA fingerprinting, we com-

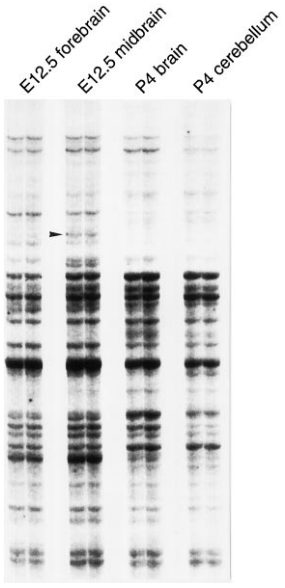

FIG. 1. **RNA fingerprinting experiment comparing cDNAs from anterior and posterior head territories on the 13th day of prenatal development (*E12.5*) and on the 5th day of postnatal development (*P4*).** RNA fingerprinting RT-PCR reactions were conducted in duplicate at each stage examined. *Arrowhead,* band 203 corresponding to the *Mmot1* transcript.

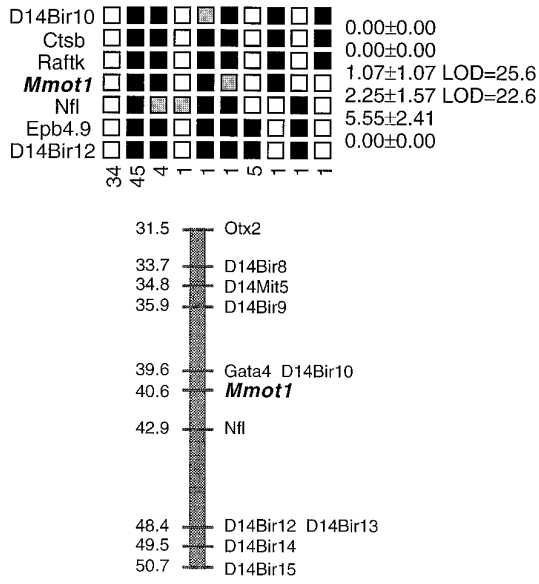

FIG. 2. **Mapping of *Mmot1* in the mouse genome**. *Above*, haplotype and linkage analysis of *Mmot1* and flanking loci on mouse chromosome 14 through the analysis of the BSS backcross (The Jackson Laboratory). *Empty squares* indicate the *Mus spretus* allele; *solid squares* indicate the C57BL/6J allele. *Shaded squares*, genotype not determined. *Numbers* to the *right* indicate recombination fractions ± S.E. and lod scores. *Columns* represent different haplotypes observed on chromosome 14. *Numbers below columns* define the number of individuals sharing each haplotype. *Below,* position of *Mmot1* on chromosome 14 with respect to nearby markers independently mapped by others on the BSS backcross. *Numbers* on the *left* represent approximate genetic distances from the most centromeric chromosome 14 marker in this cross.

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 nt level with a mouse gene named *Ebf* for *early B-cell factor* (18) encoding a helix-loop-helix transcription factor. *Ebf* 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 *Sac*II and *Hin*fI 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

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/6JEi, B6, and SPRET/Ei *spretus*). Automated sequencing of the product ends confirmed them as part of the *Mmot1* gene. The PCR product was digested with frequent cutters (*Rsa*I, *Sau*3AI, *Taq*I, and *Msp*I). An *Msp*I 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 N_2 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 *Raftk* (33) (lod score 25.6) and 2.3 centimorgan proximal to *Nfl*[3] (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 6×10^5 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 band detected by Northern analysis and not shown). By adopting a strategy involving both shotgun cloning and primer walking, we obtained the double strand sequence of the cDNA. The sequence contains a 1659-base pair open reading frame preceded by an in-frame stop codon (TAA, −66).

[3] R. Turner and J. Nadeau, unpublished data.

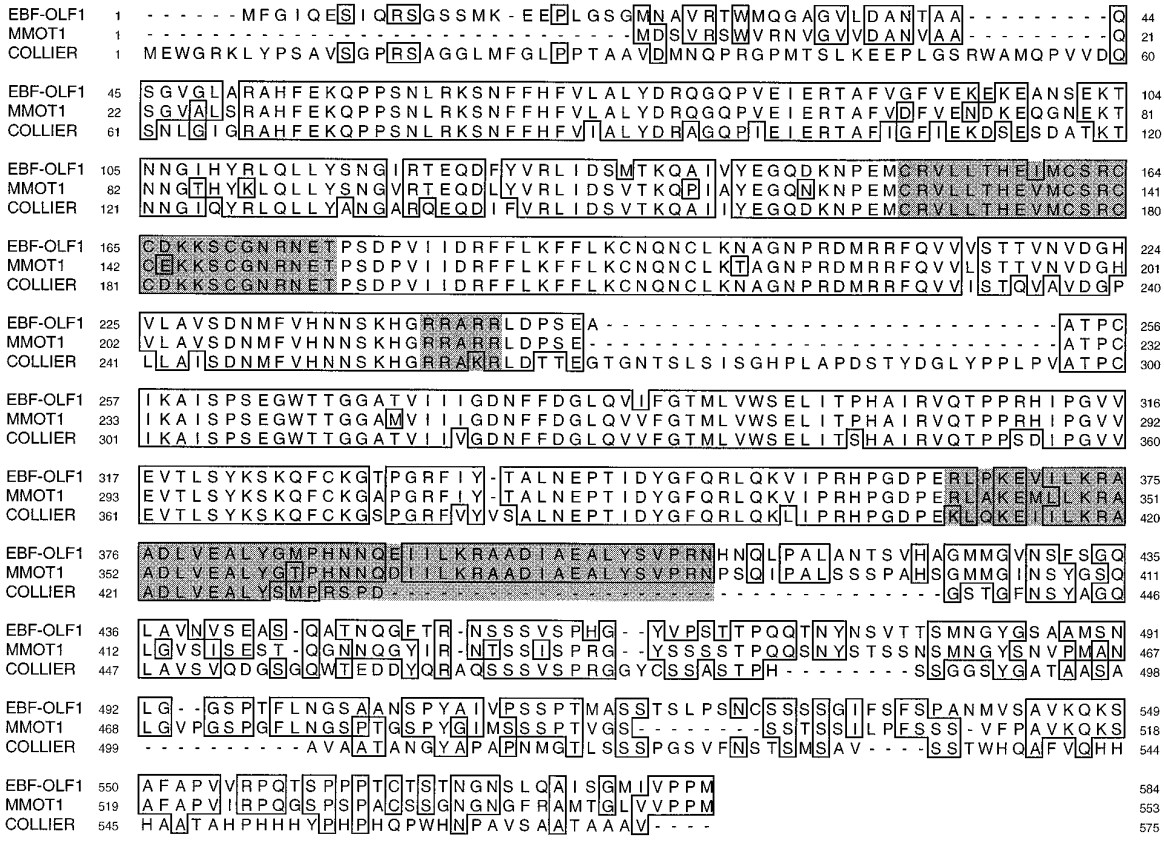

FIG. 3. **Sequence alignment of Ebf/Olf1, Mmot1, and the *Drosophila melanogaster* protein Collier.** *Boxed residues* are identical in different proteins, from N terminus to C terminus. *Shaded residues* represent the zinc finger element, nuclear targeting domain, and helix-loop-helix domain, respectively.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

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). The deduced peptide sequence of *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 mammalians, 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

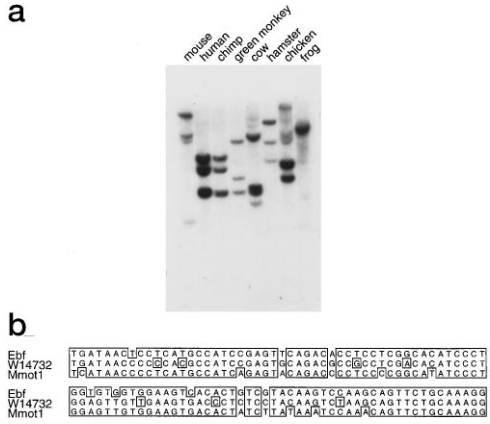

FIG. 4. **Phylogenetic conservation.** *a,* high stringency Southern (zoo blot) analysis of 10 μg of DNA from each of eight mammalian and nonmammalian species. The filter was hybridized with an *Mmot1*-specific probe displaying >40% divergence from the corresponding region of the *Ebf* transcript. For 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. W14732 is an adult mouse brain EST, clearly distinct from *Ebf* and *Mmot1*. *Boxed nucleotides* are identical in at least two sequences. The region shown corresponds to nt 956–1305 of the *Ebf* gene and 1038–1387 of the *Mmot1* gene, spanning the segment encoding the HLH domain.

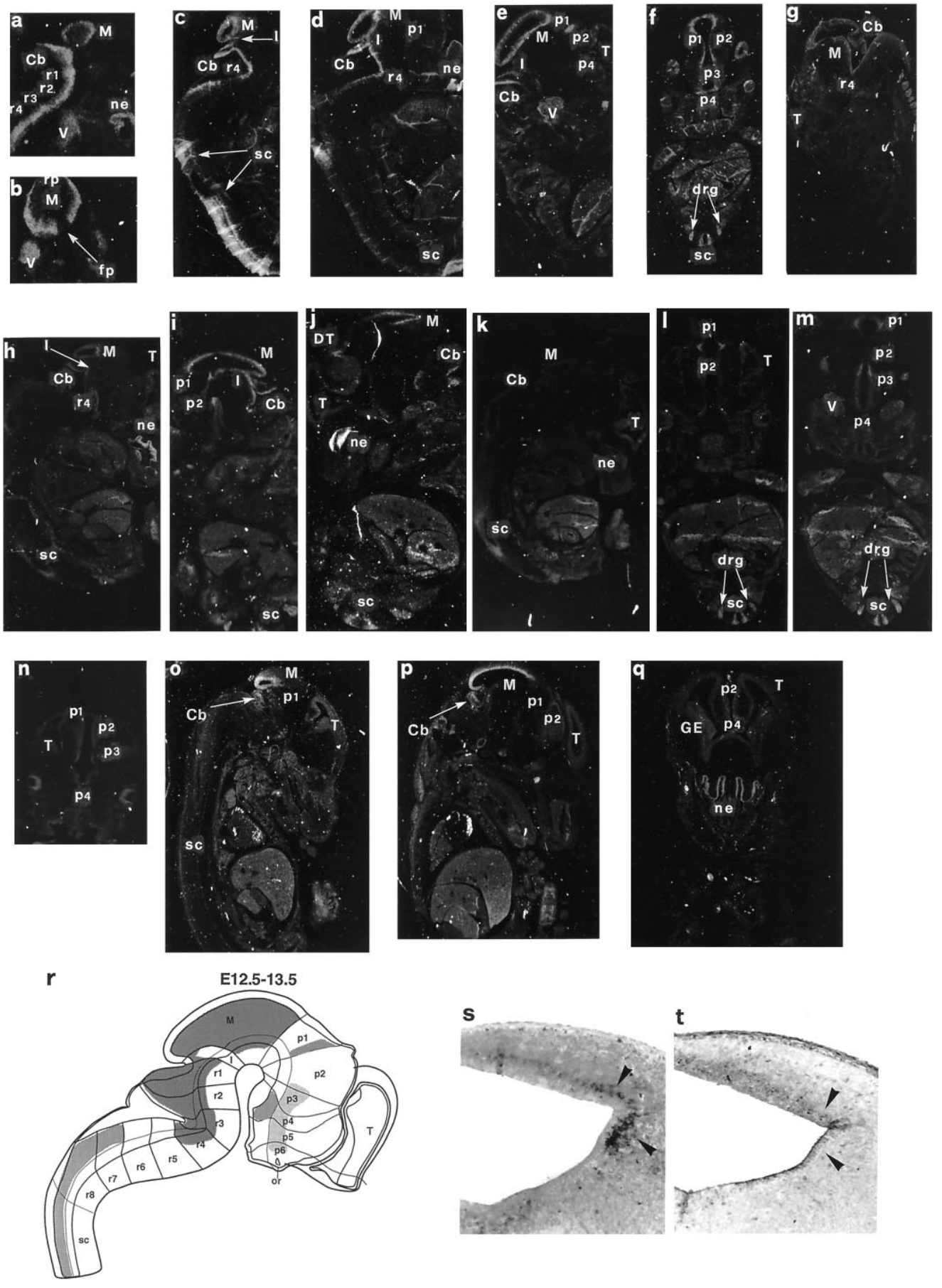

FIG. 5. mRNA *in situ* hybridization of embryonic tissue sections at 10.5 (*a* and *b*), 12.5 (*c–g*), 13.5 (*h–n* and *s*), and 14.5 (*o–q*) days postcoitus. *b*, *f*, *l*, *m*, *n*, *q*, and *s* are coronal sections; all others are sagittal sections. *g*, *k*, and *n* are negative controls, hybridized with a sense riboprobe. *r*,

protein as a query sequence and revealed the existence of at least one other member of the same family, represented in the murine (W14732) (Fig. 4*b*) and human (W21838) EST collections. Murine EST W14732 and human EST W21838 are probably orthologous to each other and distinct from *Ebf/Olf1* and *Mmot1*. At the nucleotide level, *Mmot1* and W14732 are 77.1% identical. As mentioned, a search of GenBank™ revealed a homolog of *Ebf/Olf1* and *Mmot1* in *Drosophila melanogaster* (*collier*, accession number X97803). At the nucleotide level, *Mmot1* and *collier* are 70.9% identical. One additional member of the same gene family was identified in *Caenorhabditis elegans* (accession number C13312). Finally, a dedicated analysis of the complete yeast genome through the TBLASTN program identified no high score homologs in that unicellular eukaryote.

Expression of Mmot1 in the Midgestation Embryo and Adult

To confirm the expression data obtained by RNA fingerprinting (Fig. 1) and quantitative RT-PCR (not shown) and to finely characterize the distribution of our transcript in the embryo, we performed *in situ* hybridization of mouse tissue sections at embryonic days 10.5–14.5 (Fig. 5). As a riboprobe, we employed a 377-nt fragment (position 1340–1717) displaying 66.3% identity to *Ebf*.

E10.5—Mmot1 expression (Fig. 5, *a* and *b*) is localized to the whole rhombencephalon (*r1–r4*) and spinal cord caudally and to the mesencephalon (*M*) and pretectal anlage (prosomere 1) rostrally. Both alar and basal plate cells are positive, unlike the floor plate (*fp*) and roof plate (*rp*). In the peripheral nervous system, the gene is expressed in the trigeminal ganglion (maxillary mesenchyme) and in the dorsal root ganglia. *Mmot1* expression was also detected in the dorsal maxillary epithelium.

E12.5—At this stage (Fig. 5, *c–f*, negative control in *g*), the expression of *Mmot1* includes a rostral domain (metencephalic-mesencephalic territory, excluding the basal portion of the fossa isthmica), a caudal domain (spinal cord), and the anlage of the nasal epithelium. Rostrally, expression stops between mesencephalon and *p1*, to resume weakly in prosomere 1 and, more strongly, between p1 and the dorsal thalamus (*p2*), caudal to the retroflex or habenula-interpeduncular tract; the epithalamus is clearly negative. Moreover, low level signal is observed in the preoptic area and mamillary region. In the r1–r4 interval, expression spans the cerebellar plate and pontine nuclei. Caudally, the expression domain stops abruptly at the r4–r5 interrhombomeric boundary, to resume from r7 all the way to the tail, and is restricted to the alar plate neuroepithelium. The fossa isthmus does not present labeling in its ventral midline, whereas the trigeminal and dorsal root ganglia are still positive.

E13.5–14.5—At these stages (E13.5: Fig. 5, *h–n*, negative controls in *k* and *n*; E14.5: Fig. 5, *o–q*) central nervous system areas expressing the gene at high levels are virtually restricted to the mesencephalon and metencephalon, whereas the cerebellar plate appears less intense in its ventricular zone. Very weak labeling is also observed in the alar plate of the rostral diencephalon and telencephalon. While the alar spinal cord expresses *Mmot1* at E13.5, this expression disappears at E14.5. The distribution of *Mmot1* in the context of the prosomeric model (3) is schematically summarized in Fig. 5*r*. 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.

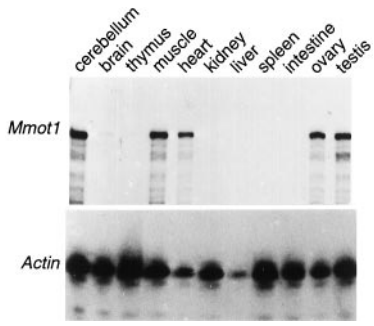

FIG. 6. **Expression of *Mmot1* in 11 adult mouse tissues tested by RNase protection assay with a 379-nt *Mmot1*-specific antisense riboprobe featuring 36% divergence from the corresponding region of the *Ebf* cDNA.** Persistent expression in the adult cerebellum and, at very low levels, in the adult brain is revealed by this assay.

High resolution, nonradioactive *in situ* analysis was conducted on E13.5 sections with an *Mmot1* cRNA probe (Fig. 5*s*). In parallel, immunohistochemical analysis was carried out on adjacent sections with an anti-PCNA monoclonal antibody (Fig. 5*t*) 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).

DISCUSSION

The present paper reports the successful application of a PCR-based, internally primed RNA fingerprinting technique (13, 39)[1] to the isolation of a gene displaying a restricted pattern of expression in the midgestation embryonic mouse

schematic summary of transcript distribution at E12.5–13.5. *s*, nonradioactive *in situ* hybridization of an E13.5 mesencephalic coronal section. *t*, histochemical analysis (anti-PCNA monoclonal antibody) of an adjacent section. *Cb*, cerebellum; *drg*, dorsal root ganglia; *fp*, floor plate; *DT*, dorsal thalamus; *GE*, ganglionic eminence; *I*, isthmus; *M*, mesencephalon; *ne*, nasal epithelium; *p1–p4*, prosomeres; *r1–r4*, rhombomeres; *rp*, roof plate; *sc*, spinal cord; *T*, telencephalon; *V*, trigeminal ganglion; *or*, optic recess.

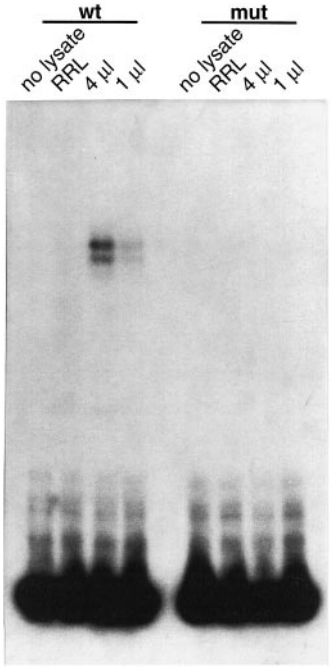

FIG. 7. **Gel mobility shift assay revealing specific affinity of the Mmot1 protein for the Olf1 nucleotide binding site described in Ref. 29.** *Lanes* contain the following: *no lysate*, γ-^{32}P end-labeled double-stranded oligonucleotide; *RRL*, same as *no lysate* but with the addition of rabbit reticulocyte lysate; *4 μl* and *1 μl*, *in vitro* synthesized, unlabeled protein after incubation with 4 and 1 μl γ-^{32}P end-labeled double-stranded oligonucleotide, respectively. A band shift (*doublet*) is observed after incubation of Mmot1 with the wild type (*wt*) nucleotide site but not with a mutant (*mut*) nucleotide site featuring two substitutions that disrupt the palindrome (see "Experimental Procedures" for sequences of wild type and mutant sites).

brain. Among other potentially significant results,[4] this approach has led to the identification of Mmot1, a new helix-loop-helix-type DNA-binding protein homologous to Ebf/Olf1 (19). To date, this family included three known members: a rat gene named *Olf1* (19), a virtually identical mouse gene named *Ebf* (32), and the *Drosophila* gene *collier* (20).

collier appears to be involved in anterior head patterning in *Drosophila*. Its expression is dependent on a gene expression program 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 antisense construct, has displayed down-regulation of the *En* gene in the intercalary segment but not in the mandibular one (20). In *Drosophila*, *collier* is only expressed in actively proliferating territories of neuroectodermal origin (20). In a comparison with Mmot1 and Ebf/Olf1, the *Drosophila* protein appears to lack the second helix of the HLH domain featured in its mammalian counterparts (Fig. 3). This suggests that Collier might be unable to assemble into dimers or bind a palindromic sequence and thus might be involved in the transcriptional regulation of a nonoverlapping developmental pathway with respect to its mammalian homologs.

Although the mouse gene *Ebf* and the rat gene *Olf1* are reportedly translated from different start sites, they most likely represent orthologous sequences and encode virtually identical proteins (18, 19), with well characterized regulatory functions in B-cell and olfactory development. In the present paper, we provide genetic evidence that *Mmot1* represents a separate, phylogenetically conserved member of the same expanding gene family. Moreover, through the analysis of the EST data base (37, 40), we report the identification of one high quality homologous sequence (accession number W14732) corresponding to an adult mouse brain cDNA, suggesting the existence of an additional, as yet uncharacterized, *Ebf/Olf1*-like transcription factor gene in mammalian genomes.

At all stages tested by *in situ* hybridization, the *Mmot1* transcript is found in a finely delimited marginal stratum of the neural tube. The distribution of *Mmot1* compared with that of a proliferation marker (PCNA) (38) suggests that our gene is expressed precociously in a nascent postmitotic, subventricular layer, possibly involved in the genesis and/or differentiation of specific neural cell types.

The *Mmot1* transcript is expressed from the mesencephalon to the tail at 10.5 days of embryonic development but becomes restricted to the metencephalon-mesencephalon and nasal neuroepithelium at E14.5, after which it remains expressed in the adult cerebellum. Between E12.5 and E13.5, the embryonic distribution of the *Mmot1* transcript presents the uncommon feature of a sharp anterior expression boundary within the embryonic diencephalon (between prosomere 1 and 2), with low level expression in prosomeres 3–5 and undetectable levels in the telencephalon. This property may contribute to the definition of factors involved in the fine subdivision of the embryonic forebrain, providing a posterior boundary (between *p1* and *p2*) in the context of the prosomeric model reviewed in Refs. 3 and 41.

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 redundance 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 neural transcription factors whose analysis 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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[4] A. Corradi and G. G. Consalez, unpublished data.

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REFERENCES

1. McGinnis, W., and Krumlauf, R. (1992) *Cell* **68,** 283–302
2. Finkelstein, R., and Boncinelli, E. (1994) *Trends Genet.* **10,** 310–315
3. Rubenstein, J. L., Martinez, S., Shimamura, K., and Puelles, L. (1994) *Science* **266,** 578–580
4. Joyner, A. L., Kornberg, T., Coleman, K. G., Cox, D. R., and Martin, G. R. (1985) *Cell* **43,** 29–37
5. Joyner, A. L., Herrup, K., Auerbach, B. A., Davis, C. A., and Rossant, J. (1991) *Science* **251,** 1239–1243
6. Millen, K., Wurst, W., Herrup, K., and Joyner, A. (1994) *Development* **120,** 695–706
7. Wurst, W., Auerbach, A., and Joyner, A. (1994) *Development* **120,** 2065–2075
8. Joyner, A. (1996) *Trends Genet.* **12,** 15–20
9. Urbánek, P., Wang, Z.-Q., Fetka, I., Wagner, E., and Busslinger, M. (1994) *Cell* **79,** 901–912
10. Crossley, P. H., Martinez, S., and Martin, G. R. (1996) *Nature* **380,** 66–68
11. Consalez, G., Corradi, A., Ciarmatori, S., Bossolasco, M., Malgaretti, N., and Stayton, C. (1996) *Trends Genet.* **12,** 455–456
12. McClelland, M., and Welsh, J. (1994) *PCR Methods Applications* **4,** S66–S81
13. Welsh, J., Chada, K., Dalal, S., Cheng, R., Ralph, D., and McClelland, M. (1992) *Nucleic Acids Res.* **20,** 4965–4970
14. Murre, C., Bain, G., van-Dijk, M., Engel, I., Furnari, B., Massari, M., Matthews, J., Quong, M., Rivera, R., and Stuiver, M. (1994) *Biochim. Biophys. Acta* **1218,** 129–135
15. Jan, Y., and Jan, L. (1993) *Cell* **75,** 827–830
16. Lee, J., Hollenberg, S., Snider, L., Turner, D., Lipnick, N., and Weintraub, H. (1995) *Science* **268,** 836–844
17. Ma, Q., Kintner, C., and Anderson, D. (1996) *Cell* **87,** 43–52
18. Hagman, J., Belanger, C., Travis, A., Turck, C., and Grosschedl, R. (1993) *Genes Dev.* **7,** 760–773
19. Wang, M. M., and Reed, R. R. (1993) *Nature* **364,** 121–126
20. Crozatier, M., Valle, D., Dubois, L., Ibnsouda, S., and Vincent, A. (1996) *Curr. Biol.* **6,** 707–718
21. Hagman, J., Gutch, M. J., Lin, H., and Grosschedl, R. (1995) *EMBO J.* **14,** 2907–2916
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D. (1990) *J. Mol. Biol.* **215,** 403–415
24. Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S. H., Thurston, S. J., and Birkenmeier, E. H. (1994) *Mamm. Genome* **5,** 253–274
25. Manly, K. F., and Elliott, R. W. (1991) *Mamm. Genome* **1,** 123–126
26. Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., and Mattick, J. S. (1991) *Nucleic Acids Res.* **19,** 4008
27. Wilkinson, D. G. (1992) *In Situ Hybridization—A Practical Approach*, IRL Press at Oxford University Press, Oxford
28. Ausubel, F. M., Brent, R., Kingstone, R. E., Moore, D. D., Smith, J. A., and Struhl, K. (1995) *Current Protocols in Molecular Biology*, pp. 4.7.1–4.7.8, John Wiley & Sons, Inc., New York
29. Kudrycki, K., Stein-Izsak, C., Behn, C., Grillo, M., Akeson, R., Margolis, F. L., Wang, M. M., Tsai, R. Y., Schrader, K. A., and Reed, R. R. (1993) *Mol. Cell. Biol.* **13,** 3002–3014
30. Hagman, J., Travis, A., and Grosschedl, R. (1991) *EMBO J.* **10,** 3409–3417
31. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395
32. Milatovich, A., Qiu, R. G., Grosschedl, R., and Francke, U. (1994) *Mamm. Genome* **5,** 211–215
33. Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L. M., White, R. A., Groopman, J. E., and Avraham, H. (1995) *J. Biol. Chem.* **270,** 27742–27751
34. Levy, E., Liem, R. K., D'Eustachio, P., and Cowan, N. J. (1987) *Eur. J. Biochem.* **166,** 71–77
35. Hurst, J., Flavell, D., Julien, J. P., Meijer, D., Mushynski, W., and Grosveld, F. (1987) *Cytogenet. Cell Genet.* **45,** 30–32
36. Somerville, M. J., McLachlan, D. R., and Percy, M. E. (1988) *Genome* **30,** 499–500
37. Boguski, M. S., Lowe, T. M., and Tolstoshev, C. M. (1993) *Nat. Genet.* **4,** 332–333
38. Fedtsova, N., and Turner, E. (1995) *Mech. Dev.* **53,** 291–304
39. Welsh, J., Rampino, N., McClelland, M., and Perucho, M. (1995) *Mutat. Res.* **338,** 215–229
40. Adams, M. D., Kerlavage, A. R., Fleischmann, R. D., Fuldner, R. A., Bult, C. J., Lee, N. H., Kirkness, E. F., Weinstock, K. G., Gocayne, J. D., White, O., *et al.* (1995) *Nature* **377,** (suppl.) 3–174
41. Lumsden, A., and Krumlauf, R. (1996) *Science* **274,** 1109–1114
42. Lin, H., and Grosschedl, R. (1995) *Nature* **376,** 263–267

Mmot1, a New Helix-Loop-Helix Transcription Factor Gene Displaying a Sharp Expression Boundary in the Embryonic Mouse Brain

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