The Neuronal *Microtubule-Associated Protein 1B* Is under Homeoprotein Transcriptional Control

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To identify genes regulated by homeoprotein transcription factors in postnatal neurons, the DNA-binding domain (homeodomain) of Engrailed homeoprotein was internalized into rat cerebellum neurons. The internalized homeodomain (EnHD) acts as a competitive inhibitor of Engrailed and of several homeoproteins (Mainguy et al., 2000). Analysis by differential display revealed that *microtubule-associated protein 1B* (*MAP1B*) mRNA is upregulated by EnHD. This upregulation does not require protein synthesis, suggesting a direct effect of the homeodomain on *MAP1B* transcription. The promoter region of *MAP1B* was cut into several subdomains, and each subdomain was tested for its ability to bind Engrailed and EnHD and to associate with Engrailed-containing cerebellum nuclear extracts. In addition, the activity, and regulation by Engrailed, of each subdomain and of the entire promoter were evaluated *in vivo* by electroporation in the chick embryo neural tube. These experiments demonstrate that *MAP1B* promoter is regulated by Engrailed *in vivo*. Moreover, they show that one promoter domain that contains all ATTA homeoprotein cognate binding sites common to the rat and human genes is an essential element of this regulation. It is thus proposed that MAP1B, a cytoskeleton protein involved in neuronal growth and regeneration, is under homeoprotein transcriptional regulation.

Key words: neuronal morphogenesis; cytoskeleton; MAP1B; transcriptional targets; homeoproteins; engrailed; in vivo *electroporation; differential display*

Homeoproteins are homeogene-coded transcription factors. They are characterized by a highly conserved 60-amino acid DNA-binding domain, the homeodomain (Gehring et al., 1994), and play key roles at all developmental stages. In the mouse, *Engrailed* homeogene expression starts at the one- and five-somite stages for *Engrailed1* (*En1*) and *Engrailed2* (*En2*), respectively. In the brain, *Engrailed* transcripts are localized to the mid-hindbrain region, and although *En2* is expressed later than *En1*, both genes show identical transcription patterns at the eight-somite stage (Davidson et al., 1988; Davis and Joyner, 1988; Davis et al., 1988, 1991; McMahon et al., 1992). In the newborn, *En1* and *En2* (from now on cited collectively as *Engrailed*) are expressed in large domains of the midbrain and cerebellum, including the substantia nigra, locus coeruleus, and raphe (Davis and Joyner, 1988).

En1 targeting (Wurst et al., 1994) deletes the cerebellum and midbrain, and the mice die at birth. In contrast, *En2* mutants are viable and show only a mild phenotype: the cerebellum is reduced in size and shows an abnormal folding pattern (Joyner et al., 1991; Millen et al., 1994). In the nervous system, the *En1* phenotype is rescued by the insertion of the *En2* coding sequence into the *En1* locus (Hanks et al., 1995). This suggests that the diverging phenotypes of both mutants are caused by differences in times and sites of expression and not by differences in the biochemical activities of the proteins.

Although homeoproteins probably regulate the expression of a wide variety of genes, few direct homeoprotein target genes have been identified (Graba et al., 1997; Mannervik, 1999). In vertebrates, identified Engrailed targets are *fibroblast growth factor-8* (*FGF-8*) (Gemel et al., 1999) and *Pax-6* (Araki and Nakamura, 1999). *RAGS* and *ELF-1* genes, encoding ligands for Eph-like receptors, have also been proposed as putative Engrailed targets. Indeed, *En1* gain of function in the chick embryo rostral tectum provokes an abnormal transcription of the two genes at the site of *En1* overexpression (Logan et al., 1996).

Several homeodomains are internalized by cells in culture (for review, see Prochiantz, 2000) and conveyed to their nucleus, where they specifically interfere with transcription (Le Roux et al., 1995). This property has been exploited to antagonize the binding of endogenous homeoproteins to their cognate promoters in physiological conditions. Homeodomain internalization and activity have allowed us to identify *BPAG1* as a direct homeoprotein target gene, in a protocol in which EnHD was used as an inducer in a gene trap library of embryonic stem (ES) cells (Mainguy et al., 1999, 2000). BPAG1 is a cytoskeletal protein of the plakin family (Houseweart and Cleveland, 1999) with epidermal and neuronal isoforms.

The present work aimed to identify homeoprotein target genes not in "epiblast-like" ES cells but in postnatal cerebellar neurons. To that end the transcripts expressed by cerebellum cells incubated with or without EnHD were compared by differential display (Liang and Pardee, 1992). We show that the *microtubule-*

Received Dec. 21, 2000; revised Feb. 27, 2001; accepted Feb. 28, 2001.

This work was supported by the European Community Biotechnology program (BIOT980227) and by the Association Française de lutte contre les Myopathies. M.L.M. is an European Molecular Biology Organization and Fondation pour la Recherche Medicale fellow. We thank E. Ipendey and S. Dupas for excellent technical assistance and Dr. S. Saule for providing us with polyclonal anti-Engrailed antibody. We also thank Drs. C. Goridis and M. Wassef for their critical reading of this manuscript, as well as Drs. F. Nothias, B. Lesaffre, and A. Trembleau for helpful discussions.

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associated protein 1B (*MAP1B*) gene encoding a cytoskeletal protein is a homeoprotein-regulated target.

MATERIALS AND METHODS

Cell cultures. Primary neurons were prepared as follows. Fragments from posterior mesencephalon and cerebellum (rat postnatal day 1) were incubated (5 min, room temperature) in trypsin-EDTA, washed in phosphate buffer plus 33 mM glucose (PBS) and 10% fetal calf serum (FCS), and incubated (10 min, 37°C) with 30 μg/ml DNase I (Sigma, St. Louis, MO). The cells were dissociated mechanically, washed three times with PBS, and plated at a density of 200,000 cells/cm^2 on dishes (35 mm diameter) coated with 1.5 μg/ml D,L-polyornithine and 5 μg/ml laminin (Sigma). Culture medium (MSS) consisted of DMEM/F12 (1:1; Life Technologies, Cergy, France) with 33 mM glucose, 2 mM glutamine, 10 mM HEPES, pH 7.4, 9 mM $NaHCO_3$, 5 U/ml penicillin, 5 μg/ml streptomycin. MSS was complemented with 0.1% ovalbumin, 25 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 60 μM putrescine, and 30 nM selenium. CHP-100 cells (Schlesinger et al., 1976) were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) plus 15% FCS.

Differential display. Differential display was performed essentially as described by Liang and Pardee (1992) with minor modifications. In brief, 500 ng of total RNA (RNeasy kit; Qiagen, Courtaboeuf, France) from cells treated or not with EnHD (300 ng/10^5 cells) in the presence of cycloheximide (1 μM) and DNase I (15 μg/ml) were reverse transcribed using Superscript II (Life Technologies), following the supplier's protocol. Before reverse transcription, RNA was systematically treated with RNase-free DNase I (Promega, Charbonnières, France) to eliminate DNA contaminations and repurified (RNeasy kit). Anchored oligo-dT primers used for reverse transcription were 5′-AAG CTT TTT TTT TTT C-3′ (HTC primer) or 5′-AAG CTT TTT TTT TTT A-3′ (HTA primer). PCR reactions were performed in the presence of ^{35}S-dATP (>1000 Ci/mmol; Amersham Pharmacia Biotech, Orsay, France) with the following cycles: 93°C for 30 sec, 39°C for 1 min, and 72°C for 1 min (3 cycles); 93°C for 30 sec, 43°C for 1 min, and 72°C for 1 min (37 cycles); 72°C for 5 min. PCR products were separated on 6% denaturing sequencing gels that were dried on Whatman 3MM paper under vacuum and subjected to autoradiography. Bands of interest were excised, eluted, reamplified, and cloned in pBluescript (KS) vector (Stratagene, La Jolla, CA). Partial sequencing was performed by standard techniques. Primers leading to PCR amplification of clone 31 were 5′-AAG CTT ATT GGT C-3′ and HTC.

RNA isolation and Northern blot analysis. Total RNA (15–20 μg, RNeasy kit) was run in formaldehyde agarose gels and transferred by capillarity to Hybond-N+ membranes (Amersham Pharmacia Biotech), and hybridizations were performed following manufacturer's instructions. Probes were labeled with α-^{32}P-dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) by random priming (Rediprime II system, Amersham Pharmacia Biotech). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was used as a control for RNA loading. Radioactive signals were quantified on a Fujix BAS 1000 PhosphoImager (Fujifilm Medical Systems, Stamford, CT).

Proteins. Chick En2 homeodomain (amino acids 200–259 of the protein) was produced in *Escherichia coli* and purified by FPLC on HiTrap heparin-Sepharose columns (Amersham Pharmacia Biotech) (Mainguy et al., 1999). A larger version of EnHD (amino acids 1–9 followed by amino acids 186–289) used in some gel shift assays was produced from expression plasmid pTrc9mEn2ΔSP. This plasmid was obtained by the transfer of myc-tagged En-2 ORF deleted between *Sma*I and *Ppu*MI sites into a derivative of pTrcHis2A vector (Invitrogen, Groningen, The Netherlands).

Glutathione *S*-transferase (GST)-En2 and GST proteins were produced in *E. coli* and purified on Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech), following manufacturer's instructions. GST-En2 fusion (gift from Dr. A. Joliot, Ecole Normale Supérieure, Paris) was prepared by inserting a chick En2 coding sequence into a modified form of pGEX1 (Amersham Pharmacia Biotech).

Nuclear extracts from mouse neonatal (P0) cerebellum and posterior mesencephalon were prepared as in Beckmann et al. (1997). Dissected tissues were homogenized in 5–7 ml of homogenization buffer: 20 mM HEPES, pH 7.9, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.7% Nonidet P−40, 0.5 mM dithiothreitol, 10% (w/v) glycerol, and protease inhibitor mixture Complete 1× (Roche Diagnostics, Meylan, France). After centrifugation (10 min, 2000 × *g*) and washing with 10 ml of homogenization buffer, the pellets resuspended in 300–500 μl of high-salt buffer were incubated for 30 min at 4°C on a rocker. High-salt buffer composition was 20 mM HEPES, pH 7.9, 0.5 M KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 25% (w/v) glycerol, Complete 1× (Roche Diagnostics). Nuclear debris were removed by centrifugation at 14,000 × *g* for 30 min at 4°C, and supernatants were stored in aliquots at −80°C. Protein concentration was determined by a modified Bradford assay (Bio-Rad, Ivry, France), using bovine serum albumin (BSA) as a standard.

Gel shift assays. DNA fragments were end labeled by filling with Klenow-fragment polymerase and α-^{32}P-dCTP. Binding reactions were performed in a final volume of 20 μl (15 mM HEPES, pH 8.0, 0.6 mM dithiothreitol, 6 mM $MgCl_2$, 18% glycerol, 1 μg of salmon sperm DNA, and 10 μg of BSA). Salt concentrations varied with conditions: 100 mM KCl (see Fig. 6*A*), 80 mM KCl (see Fig. 6*B*), 135 mM NaCl (see Fig. 5*A*), or 80 mM KCl plus 40 mM NaCl (see Figs. 5*B*, 7). After incubation on ice for 30 min, DNA–protein complexes were analyzed on 5% polyacrylamide gels (acrylamide/bisacrylamide, 80:1) in 0.25× TBE buffer (TBE 1× is 100 mM Trizma base, 95 mM boric acid, and 2 mM EDTA) and 2.5% glycerol. For supershift experiments, probes were first incubated with the nuclear extracts for 30 min on ice and then for an additional 30 min with a polyclonal antibody that recognizes both En1 and En2 proteins (Plaza et al., 1997) (a gift from Dr. S. Saule, Institut Curie, Orsay). In that case DNA–protein complexes were analyzed on 4% polyacrylamide gels (acrylamide/bisacrylamide, 60:1) in 0.25× TBE buffer and 2.5% glycerol. Gels were prerun at 4°C for 45 min at 130 V and run at 4°C for 1.5 hr at 240 V, dried, and subjected to autoradiography.

Cell transfection assays and in ovo *electroporation*. The promoter region of *MAP1B* (Liu and Fischer, 1996) was amplified by PCR using *Vent* polymerase (New England Biolabs, Beverly, MA), genomic rat DNA as template, and oligonucleotides pMAP1 (5′-TTA TTG CAG ACC CCC AGT GTG A-3′) and pMAP2 (5′-CCT GCC GGC TCT GCT AAA GCC T-3′). The 1.7 kb DNA fragment generated (position −1626 to +60) was cloned in the Klenow-filled *Hin*dIII site of pGL2-basic (Promega, Madison, WI) to generate plasmid pMAP-*luc*, or into the Klenow-filled *Hin*dIII site of pβgal-basic (Clontech, Palo Alto, CA) to generate pMAP-*lacZ*.

Plasmid pD-*lacZ* (see Fig. 8) was obtained by insertion of the Klenow-filled *Bsa*HI–*Bss*HII fragment from pMAP-*luc* (containing promoter region D) into the Klenow-filled *Hin*dIII site of pβgal-basic. To generate pE-*lacZ* (see Fig. 8), plasmid pMAP-*luc* was cut with *Hin*dIII and *Bss*HII, treated with Klenow enzyme, and religated, and the resulting plasmid was cut with *Bgl*II and *Nae*I to obtain promoter fragment E, which was cloned between sites *Bgl*II and *Hin*dIII (Klenow-filled) of pβgal-basic. Plasmid pABCD-*lacZ* (see Fig. 8) was obtained by insertion of the Klenow-filled *Hin*dIII–*Bss*HII fragment from pMAP-*luc* (containing promoter regions A, B, C, and D) into the Klenow-filled *Hin*dIII site of pβgal-basic. To obtain pCD-*lacZ* (see Fig. 8), a fragment containing promoter region C was obtained by *Msc*I–*Bsa*HI digestion of pABCD-*lacZ*, Klenow-filled, and cloned in the Klenow-filled *Bgl*II site of plasmid pD-*lacZ*.

Expression plasmids coding for CMV promoter-driven myc-tagged chick En2 (pCL9mEn2) (Mainguy et al., 2000), En2ΔHD1 (pCL9mEn2ΔH1; derived from pTL1mEn2ΔH1) (Joliot et al., 1998), and mouse Hoxa5 (pCL9A5m; derived from pSP9A5m) (Chatelin et al., 1996) were transfected by electroporation. For cell electroporation, a Bio-Rad Gene Pulser II apparatus and 4-mm-gap cuvettes were used (260 V and 1050 μF in 350 μl of culture medium). In Figure 4*A*, 0.5×10^6 cells were transfected with 2 μg of reporter plasmid and the indicated amounts of expression plasmid, plus empty parental vector (pCL9m) to keep the total amount of DNA constant. In Figure 4*B*, 10^6 cells were transfected with 2 μg of reporter and 6 μg of expressing plasmid, in duplicate. Frequency and nuclear localization of transfected homeoproteins were verified by immunocytochemistry with anti-myc 9E10 antibody (Evan et al., 1985). Luciferase activity was measured 24 hr after transfection (Le Roux et al., 1995) in a Lumat luminometer (Berthold, Bald Wilbald, Germany).

Electroporation of chick embryos at stage HH9–11 (Hamburger and Hamilton, 1951) was performed as described (Muramatsu et al., 1997), using a BTX Electrosquareporator T820 apparatus (four pulses of 25 V and 50 msec) (Genetronics, San Diego, CA). The appropriate reporter plasmid and control or En2 expression plasmids were injected into the neural tube with a micropipette. After 24 hr of incubation at 37°C, embryos were fixed and stained for β-galactosidase activity (Hogan et al., 1994).

RESULTS

Isolation of *MAP1B* cDNA by differential display

To find genes controlled by Engrailed we looked for modifications in expression profiles of postnatal rat cerebellum neurons on treatment by EnHD. Postnatal day 1 cerebellums were dissociated, plated for 4 hr, and incubated for another 12 hr with or without EnHD (in duplicate), in the presence of cycloheximide to prevent protein synthesis and regulation of indirect targets (Mainguy et al., 1999). Cycloheximide was not toxic because its removal allowed all neurons to resume differentiation (data not shown). In these conditions EnHD was internalized by the cells and could be visualized in the nucleus by immunocytochemistry as reported earlier (Mainguy et al., 1999). Expression profiles were then analyzed by differential display (Liang and Pardee, 1992).

Total RNA samples were reverse transcribed in duplicate with one of two different anchored oligo-dT primers (HTA or HTC primers). The subsets of mRNAs defined by each of these primers were amplified by PCR using 12 different 13-mers with arbitrary sequences in combination with the corresponding oligo-dT primer. Samples derived from EnHD-treated and control cells showed a small number of differentially expressed bands (Fig. 1*A*, *asterisks*). One cDNA, more abundant in treated than in control cells (Fig. 1*A*), was reamplified by PCR, cloned, and partially sequenced (Fig. 2*B*). Clone 31 contains a 1.1 kb sequence present in the 3′ untranslated region (nucleotide positions 1838–2961; GenBank accession number: AF115776) of *MAP1B* (Meixner et al., 1999) (Fig. 2). Differential expression of *MAP1B* was confirmed by Northern blot analysis using RNA isolated from rat cerebellum neurons, incubated or not with EnHD in the presence of cycloheximide. A representative Northern blot showing that EnHD increases the expression level of *MAP1B* by twofold is presented in Figure 1*B*.

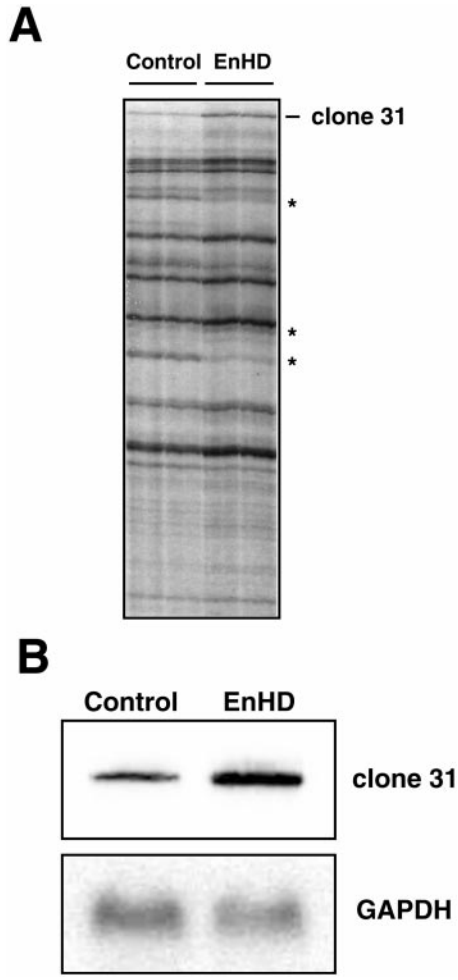

Figure 1. Identification of clone 31 (*MAP1B* gene) by differential display. *A*, Duplicate RT and PCR reactions were performed and run in adjacent gel lanes. The positions of some differentially expressed bands are indicated with *asterisks*. The band corresponding to clone 31 is also indicated. *B*, Northern blot of total RNA from control or EnHD-treated cells probed with clone 31 or *GAPDH* cDNA fragments.

Regulation of *MAP1B* promoter activity by homeoproteins

The promoter region of the rat *MAP1B* gene (Liu and Fischer, 1996, 1997) contains two independent TATA boxes and several regulatory motifs, including two cAMP-responsive elements, an Sp1 site, a "neuronal motif," and a TCC repeat motif (Fig. 3*A*). The *MAP1B* promoter region (nucleotide positions −1626 to +60) was cloned in front of a luciferase reporter gene (pGL2bas promoter-less vector). This construct (pMAP-*luc*) was tested by transient transfection of a human neuroepithelial cell line (CHP-100) (Mainguy et al., 1999). Figure 4*A* illustrates that transfecting increasing amounts of a plasmid expressing En2 leads to a dose-dependent activation of the *MAP1B* promoter.

The En2-mediated activation of *MAP1B* promoter was not observed when an En2ΔHD1-expressing plasmid was used (Fig. 4*B*). En2ΔHD1 (Joliot et al., 1998) is an En2 mutant lacking amino acids 36–46 in the homeodomain; it is still addressed to the nucleus but does not bind DNA (data not shown). To test whether other homeoproteins were also active, pMAP-*luc* and *Hoxa5* coding plasmid were cotransfected in CHP-100 cells. Figure 4*B* shows that Hoxa5 also regulates *MAP1B* promoter activity *ex vivo*.

Identification of Engrailed-binding sites within the *MAP1B* promoter region

The *MAP1B* promoter contains several putative homeoprotein binding sites (ATTA or TAAT sites) (Fig. 3). Five DNA fragments (from A to E) covering the entire *MAP1B* promoter region were generated (Fig. 3*A*), and each fragment was used in gel-shift experiments. Fragments A and E (417 and 138 bp, respectively) contain one ATTA site (overlapping the TATA-2 box in the case of fragment E); fragments B (421 bp) and C (547 bp) contain 10 and 7 ATTA sites, respectively; fragment D (398 bp) contains no ATTA sequence. Purified EnHD (Fig. 5*A*) and GST-En2 protein (Fig. 5*B*) retarded probes A, B, C, and D (the latter to a lesser extent) but failed to retard probe E even when a sevenfold excess of GST-En2 protein was used in the binding reaction (data not shown).

Gel-shift assays were then performed with nuclear extracts from P0 mouse cerebella. Probes A, B, C, and D (although to a lesser degree), but not E, were retarded (Fig. 6*A*), indicating the existence, in the nuclear extract, of one or more factors that specifically recognize the DNA fragments. That Engrailed is one of these factors was confirmed by a supershift experiment using a polyclonal anti-Engrailed antibody (Fig. 6*B*). Finally, to test whether Engrailed forms an active complex with factors in the nuclear extract, a supershift experiment was achieved by adding GST-En2. Figure 7 illustrates that GST-En2 (Fig. 7*A*), but not GST alone (Fig. 7*B*), interacts with one or several factors. Taken

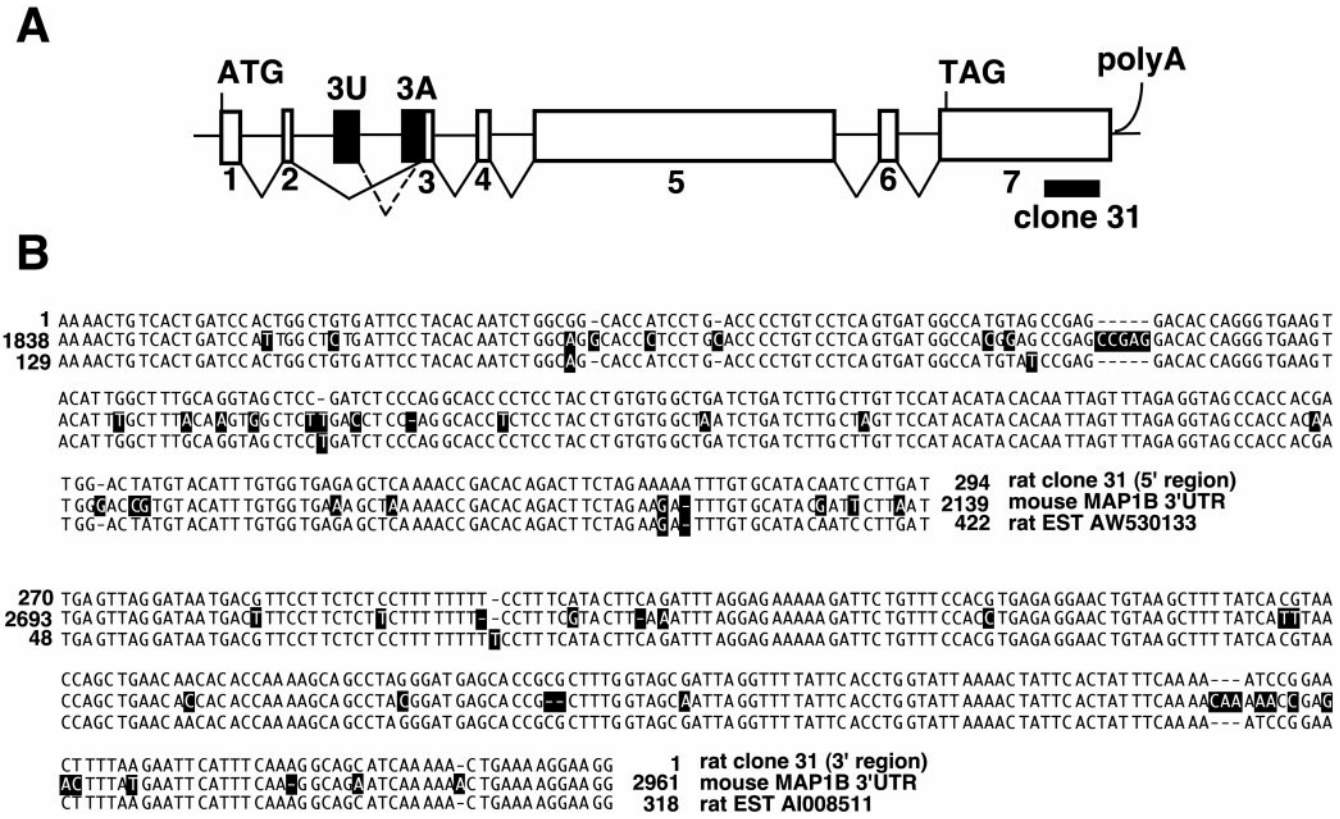

Figure 2. Localization of clone 31 in the *MAP1B* genomic locus. *A*, Schematic representation of the rat *MAP1B* locus [modified from Kutschera et al. (1998) and Meixner et al. (1999)]. The most frequent *MAP1B* transcripts (90% of total *MAP1B* mRNA) are encoded by exons 1–7 (*solid lines* indicate splicing). Alternative *MAP1B* transcripts are encoded by exon 3U/3–7 (splicing indicated by a *dashed line*) and by exon 3A/3–7. Clone 31 localization is shown. *B*, Sequence alignments of clone 31 5′ (*top*) and 3′ (*bottom*) regions with mouse *MAP1B* 3′ untranslated region (*UTR*) and rat expressed-sequence tags (*EST*) AW530133 and AI008511. Residues that differ from clone 31 are shaded in *black*.

together, these results demonstrate that Engrailed is present in the extracts and recognizes different fragments of the *MAP1B* promoter in an appropriate molecular context.

Repression of *MAP1B* promoter activity by Engrailed in the neural tube of chick embryos

The results described above demonstrate that Engrailed binds different fragments in the *MAP1B* promoter and regulates the expression of this promoter in transfected neuroepithelial cells. To better sustain the idea that *MAP1B* is regulated by Engrailed and to identify which domains in the promoter are involved, several *lacZ*-reporter constructions (Fig. 8) were expressed in the neural tube of chick embryos at stage HH9–11 (Hamburger and Hamilton, 1951), and their expression was analyzed 1 d later.

Full-length *MAP1B* promoter is expressed *in vivo* and repressed by Engrailed (Fig. 9*A*). On the basis of cell transfection assays, Liu and Fischer (1996) have proposed that the two *MAP1B* TATA boxes, in association with their adjacent regulatory *cis*-elements, function independently and confer neuron-specific expression. To test whether each TATA box is repressed by Engrailed in the developing chick neural tube, pD-*lacZ* and pE-*lacZ* were electroporated with or without Engrailed. Plasmid pD-*lacZ* contains TATA box1 (TATA-1) and associated upstream motifs (Fig. 3*A*), and plasmid pE-*lacZ* contains TATA box2 (TATA-2) and the upstream Sp1 motif (Fig. 3*A*). As illustrated in Figure 9, only pD-*lacZ* is active (Fig. 9*B*), whereas pE-*lacZ* shows no basal neural activity (Fig. 9*C*) and neither construct expression is regulated by Engrailed. Because some activity was observed in adjacent non-neural tissue in several embryos, it is likely that TATA-2 can drive *lacZ* expression but lacks the motifs necessary for neural expression.

Fragment D contains no ATTA sequence and is not repressed by Engrailed. We thus tested whether fragment ABC, which contains all ATTA sequences, confers regulation. Figure 9*D* illustrates the expression of pABCD-*lacZ* in the neural tube and its repression by En2. Little difference was observed between pABCD-*lacZ* and the full-length promoter (Fig. 9, compare *A* and *D*). Aligning human and rat sequences highlights two homologous *MAP1B* promoter regions and, in particular, shows that only ATTA sites at positions 9, 10, 12, and 13 of the rat promoter (Fig. 3*B*) are conserved between rat and human. Because fragment C contains all of these conserved sites (Fig. 3), pCD-*lacZ* was electroporated in the chick neural tube with or without Engrailed. Figure 9*E* confirms that motifs in fragment C mediate the regulation of *MAP1B* promoter by Engrailed.

DISCUSSION

This study demonstrates that *MAP1B* is a homeoprotein target and, most probably, an Engrailed target. This conclusion is based on the following series of experiments. First, EnHD internalization in primary cerebellum neurons provokes an accumulation of *MAP1B* mRNA in the presence of cycloheximide. Second, cerebellum nuclear extracts retard several fragments of the *MAP1B* promoter, and Engrailed is present in the retarded complex. Finally, the promoter is active and regulated by Engrailed *in vivo*.

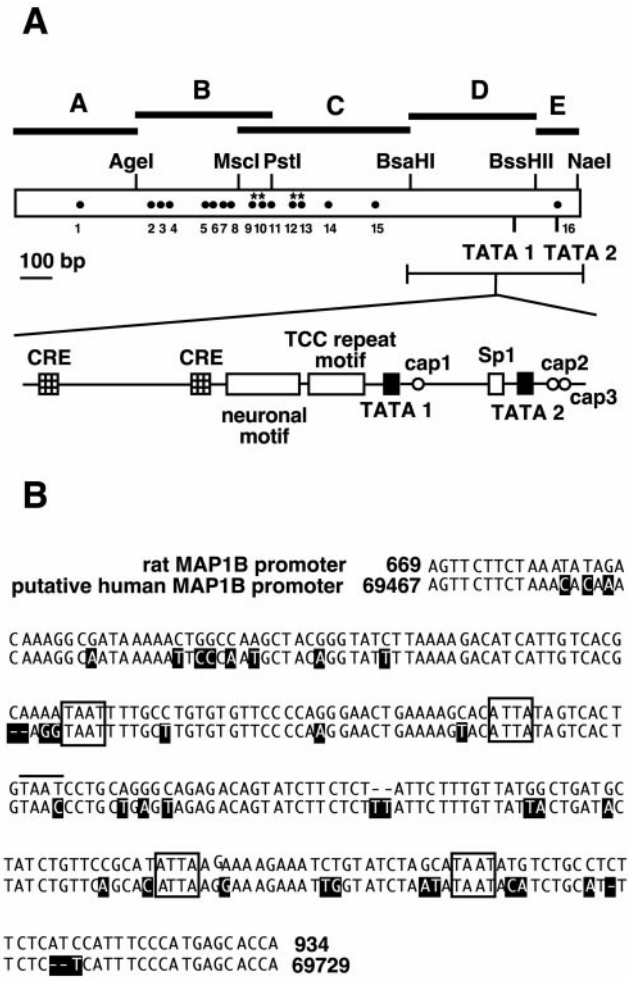

Figure 3. *MAP1B* promoter region. *A*, Promoter of the rat *MAP1B* gene: the two TATA boxes, 5′ capping sites, and putative regulatory motifs described previously (Liu and Fischer, 1996, 1997) are shown. The positions of ATTA or TAAT sites are indicated by *filled circles* and numbered *1-16*. *Asterisks* indicate ATTA sites conserved in the putative human *MAP1B* promoter (GenBank accession number: AC021318). Positions of fragments *A*, *B*, *C*, *D*, and *E* used in gel-shift assays and restriction sites used for their isolation are also indicated. *B*, Sequence comparison of rat and human *MAP1B* promoters showing one of the two conserved regions. A BLAST search using the rat *MAP1B* promoter sequence (GenBank accession number: U55276) was made against the unfinished human genome. Conserved ATTA/TAAT sites are *boxed*; the nonconserved TAAT site is indicated by a *solid line*. Residues that differ from rat sequence are shaded in *black*.

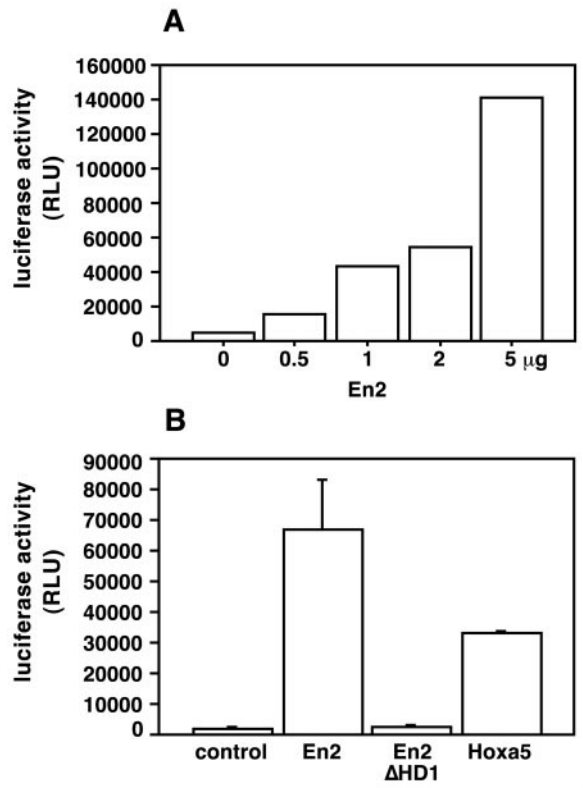

Figure 4. Regulation of *MAP1B* promoter activity in a neuroepithelial cell line. *A*, Cotransfection of CHP-100 cells with the *MAP1B*-luciferase reporter plasmid pMAP-*luc* and increasing amounts of En2-expressing plasmid (DNA was kept constant by adding the empty plasmid). *B*, Cotransfection of CHP-100 cells with pMAP-*luc* and empty vector (*control*) or with a plasmid expressing full-length En2 protein (*En2*), En2 deleted in the homeodomain (*En2ΔHD1*), or full-length Hoxa5 protein (*Hoxa5*). Luciferase activity of cell extracts after 24 hr is indicated in relative light units (*RLU*).

Technology

A differential display approach was associated with the internalization of EnHD by cerebellum primary neurons. The molecular basis of homeodomain internalization has been well established, and it is now clear that specific properties of the third helix are involved (Prochiantz, 1996; Derossi et al., 1998). The absence of chiral receptors and the translocation across artificial lipid layers (Thoren et al., 2000) explain why homeodomains gain direct access to the cytoplasm and thereafter, through the nuclear pores, to the nucleus.

Internalized homeodomains compete with endogenous homeoproteins for cognate binding sites in the genome. This was shown by the use of the homeodomain of Antennapedia (AntpHD) and by its mutated version where glutamine in position 50 has been replaced by an alanine (AntpHD-50A). Indeed, AntpHD-50A is internalized and conveyed to the nucleus but does not bind DNA with high affinity (Le Roux et al., 1993) and, contrary to AntpHD, does not regulate gene expression (Le Roux et al., 1995; Mainguy et al., 1999).

Advantageously, homeodomains internalized by all cells in culture only bind accessible sites in an unmodified chromatin context. Moreover, cycloheximide can be added to identify direct targets. Another interesting aspect of this approach is its relative absence of specificity: wild-type homeodomains with a glutamine in position 50 will recognize a large number of sequences accessible to various homeoproteins of the Q50 family. This is why homeodomain internalization will reveal targets shared by several homeoproteins. Target induction by homeodomains is thus a first step in the identification process as illustrated by this study and by previous work in which *BPAG1* was identified as a homeoprotein target (Mainguy et al., 2000).

In vitro promoter analysis

The *MAP1B* promoter region (Liu and Fischer, 1996) shows 16 putative homeoprotein binding sites. Gel-shift experiments demonstrating Engrailed binding to four promoter fragments (fragments A, B, C, and D) support a direct regulation of *MAP1B* expression by Engrailed. In fact, the use of a protein synthesis inhibitor (cycloheximide) in the differential display protocols

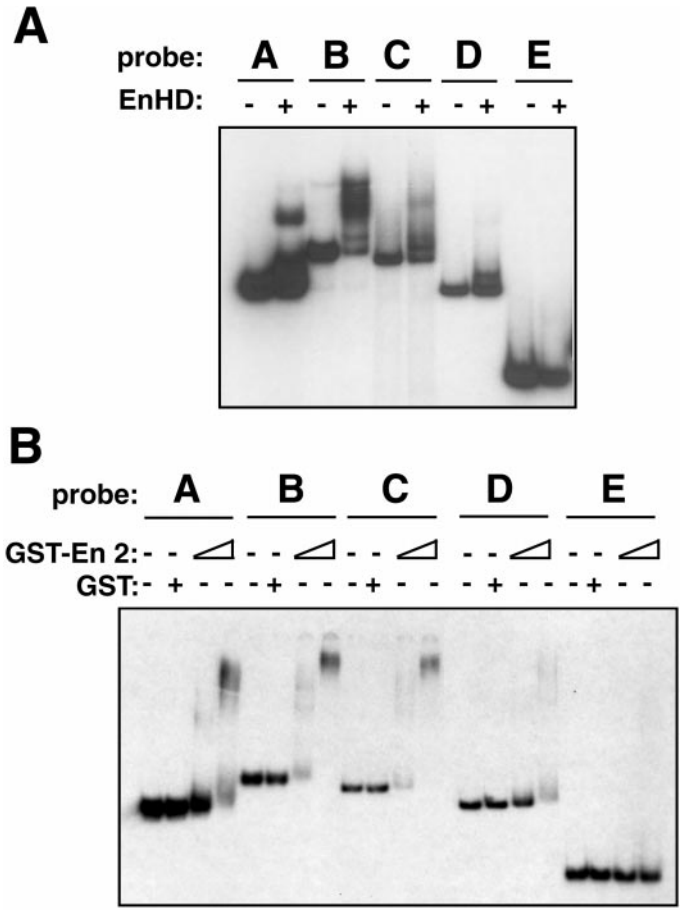

Figure 5. EnHD and full-length En2 bind to the *MAP1B* promoter. *A*, Purified EnHD (−, no protein; +, 50 ng of EnHD) was incubated with 1 ng of the indicated *MAP1B* promoter fragment (from *A* to *E*). *B*, Two different amounts (625 ng or 1.25 μg) of full-length En2 (*GST-En 2*) protein or 5 μg of GST protein were incubated with 0.4 ng of the indicated *MAP1B* promoter fragment (from *A* to *E*). Designation of *MAP1B* probes refers to promoter regions indicated in Figure 3*A*. Note that the five probes are of different sizes and thus migrate with different velocities.

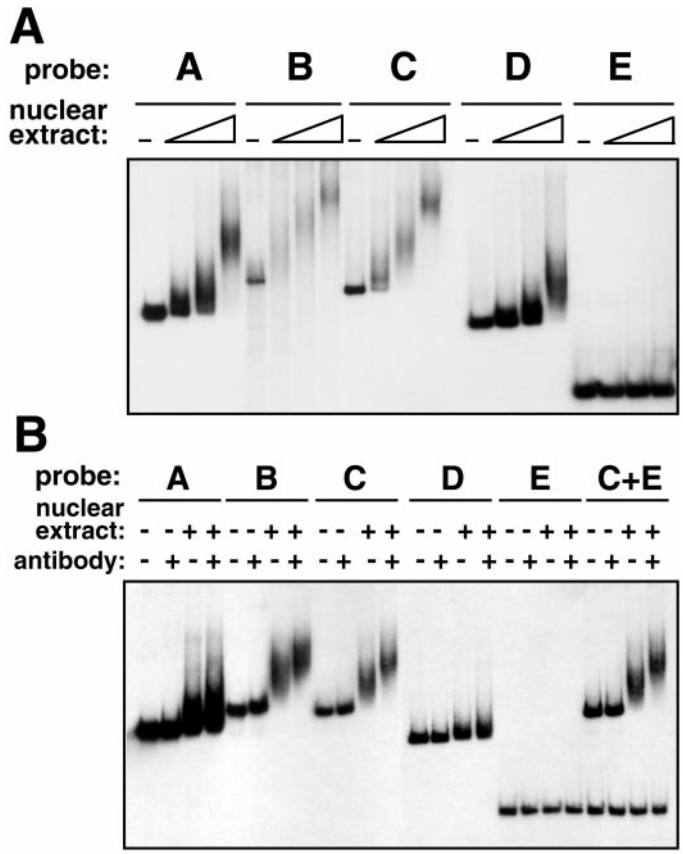

Figure 6. Cerebellum nuclear extracts bind the *MAP1B* promoter, and the binding complex contains Engrailed. *A*, Binding of increasing amounts (0, 0.5, 1, or 2 μg) of nuclear extract from P0 mice cerebellum to fragments *A*, *B*, *C*, *D*, and *E* (0.4 ng). *B*, Supershift assay of fragments *A*, *B*, *C*, *D*, *E*, and *C+E* (0.5 ng each) using an anti-Engrailed antibody (2.5 μl of a dilution 1:10) and 1 μg of nuclear extract. Designation of *MAP1B* probes refers to promoter regions indicated in Figure 3*A*. Note that the five probes are of different sizes and thus migrate with different velocities.

prevents cascade effects and favors the isolation of targets directly regulated by EnHD.

The ATTA motif present in fragment E (which is not retarded) overlaps the second TATA box of the *MAP1B* promoter (Liu and Fischer, 1996) and probably does not represent a physiological homeoprotein-binding site. In contrast, fragment D does not contain any ATTA site but forms a complex with EnHD or En2. However, a comparison of bound versus free probe in gel-shift experiments (same amounts of probe and protein for all fragments) suggests that the relative affinity of Engrailed for the probes is C≥B>A>D (Fig. 5*B*) and that the affinity between En2 and D is low.

Gel-shift experiments gave similar results when purified EnHD, GST-En2, or cerebellum nuclear extracts were used. Engrailed is abundant in the cerebellum at P0 and in the nuclear extracts (data not shown). As demonstrated by the supershift experiments, Engrailed is part of the retarded complexes formed between the probes and proteins in the nuclear extract. Although in these retarded complexes Engrailed might not bind directly to the promoter, the fact that purified En2 binds directly and that nuclear extracts and purified En2 show the same order of relative affinities for the five fragments is very much in favor of a direct binding.

Enhanced retardation of *MAP1B* promoter probes when En2 is added to cerebellum nuclear extracts (Fig. 7) suggests the presence of cofactors. Groucho/TLE and Exd/Pbx are recognized cofactors of Engrailed in invertebrates and vertebrates. Groucho/TLE proteins do not bind DNA but are co-repressors of several transcription factors, including Hairy-related, Runt domain, and Engrailed proteins (for review, see Fisher and Caudy, 1998; Chen and Courey, 2000). In contrast, Pbx homeoproteins have the ability to modulate the binding activity of Hox and Engrailed through cooperative DNA binding (Chang et al., 1995; van Dijk et al., 1995). In rat embryos, *Pbx1* and *Engrailed* show overlapping expression patterns (Roberts et al., 1995), and Pbx1 is detected by Western blot in cerebellum nuclear extracts (data not shown). However, the possibility that added purified En2 binds Pbx1 or other cofactors present in the extract has not been investigated.

Ex vivo and *in ovo* promoter activity

Transfections in the neuroepithelial cell line CHP-100 provided some interesting information. First, Engrailed regulates *MAP1B* promoter activity, and this regulation is lost when 11 amino acids are deleted in the homeodomain. It was indeed verified that the mutated protein is synthesized and accumulates in the nucleus. Second, Hoxa5, another member of the Q50 homeoprotein family, also regulates *MAP1B* promoter expression. This supports the

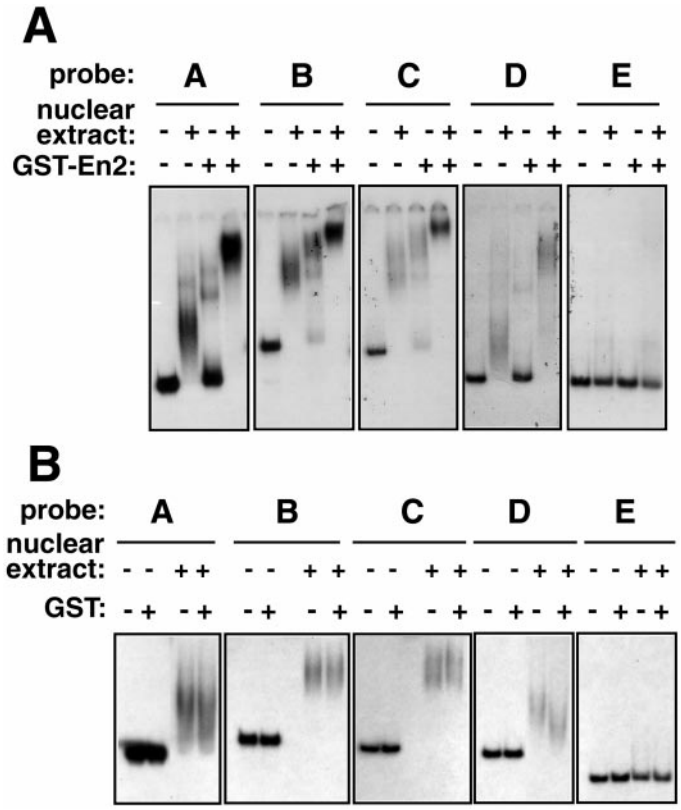

Figure 7. Enhanced retardation of *MAP1B* promoter by GST-En2 and cerebellum nuclear extract. *A*, Band-shift assays with *MAP1B* probes *A* to *E* (0.4 ng) with (+) or without (−) 2 μg of cerebellum nuclear extract and/or 1.25 μg of GST-En2. *B*, Band-shift assays with fragments *A* to *E* (0.4 ng) with (+) or without (−) 2 μg of cerebellum nuclear extract and/or 10 μg of GST. Designation of *MAP1B* probes refers to promoter regions indicated in Figure 3*A*. Note that the five probes are of different sizes and thus migrate with different velocities.

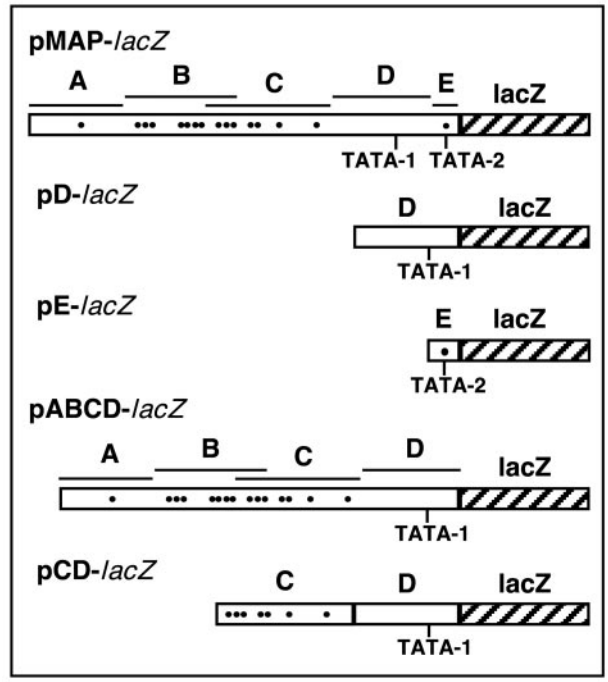

Figure 8. *MAP1B* reporter plasmids for *in ovo* electroporations. The indicated regions of the *MAP1B* promoter were fused to a *lacZ*-reporter gene. ATTA sites are indicated with *filled circles*. *Letters* refer to regions indicated in Figure 3*A*.

idea that different Q50 homeoproteins may regulate the same target genes (Biggin and McGinnis, 1997), including *MAP1B*. This is not a surprise because homeoproteins are region-specific transcription factors, whereas *MAP1B* is expressed in all neurons and not specifically in the mid-hindbrain.

En2 represses the *MAP1B* promoter after electroporation in the chick neural tube, a gain of function protocol that permits the study of gene activity *in vivo* (*in ovo*, rather), thus in a physiological context. This repression is in agreement with the differential display results because internalized EnHD antagonizes Engrailed activity (Mainguy et al., 2000) and leads to an upregulation of the *MAP1B* transcript in cultured midbrain and cerebellum neurons. The *in ovo* experiments show that overexpressed Engrailed completely downregulates *MAP1B* promoter activity. This does not mean that Engrailed-expressing neurons do not express *MAP1B* but that Engrailed acts in conjunction with other transcription factors to regulate the appropriate levels of the protein. In fact, it must be kept in mind that the activity of a transcription factor is both dose and context dependent. For example, in *Drosophila*, Engrailed either activates or represses *polyhomeotic*, and the two opposite effects depend both on Engrailed concentration (high concentrations are inhibitory) and on the presence of Exd as a cofactor (necessary for activation) (Serrano and Maschat, 1998). Another example is given in this study because Engrailed is a repressor of *MAP1B* promoter activity in neuronal cells and an activator in CHP-100 cells.

Expression in the chick embryo of the different reporter constructs demonstrates that only TATA box1 (and not TATA box2, in fragment E) is active in the nervous system. These experiments also show that fragment D is not regulated by Engrailed *in vivo* and suggest that the lower *in vitro* affinity of Engrailed for fragment D (in comparison with the other fragments) is physiologically relevant. This has allowed us to use the TATA box of fragment D to test C domain activity by electroporating pCD-*lacZ* and to show that this domain, which contains all ATTA sequences conserved between rat and human, permits regulation by Engrailed. pMAP-*lacZ*, pABCD-*lacZ*, and pCD-*lacZ* constructions are active in the mid-hindbrain region (Fig. 9) where *Engrailed* is normally expressed, and repression follows Engrailed electroporation. As already discussed in the case of *polyhomeotic*, this illustrates that *MAP1B* regulation by Engrailed is probably dose dependent and that the presence of physiological amounts of Engrailed is compatible with *MAP1B* expression.

In *Drosophila*, *Engrailed,* and *Futsch* (a *MAP1B*-like gene) (Hummel et al., 2000; Roos et al., 2000) are ectopically expressed in *Tramtrack* mutants (Xiong and Montell, 1993; Giesen et al., 1997). Because Tramtrack (a zinc-finger transcription factor) binds the *Engrailed* promoter (Read and Manley, 1992), an appealing possibility is that a direct genetic interaction between *Engrailed* and *MAP1B* has been conserved in several species, including human, rat, chick, and possibly *Drosophila*.

Physiological significance

MAP1B, a major component of the neuronal cytoskeleton (Bloom et al., 1984, 1985), is the earliest MAP expressed during brain development (Riederer et al., 1986), reaching highest expression levels 2–3 d after birth. In the adult, *MAP1B* is downregulated (Safaei and Fischer, 1989; Garner et al., 1990), except in regions that retain high levels of axonal growth and synaptic

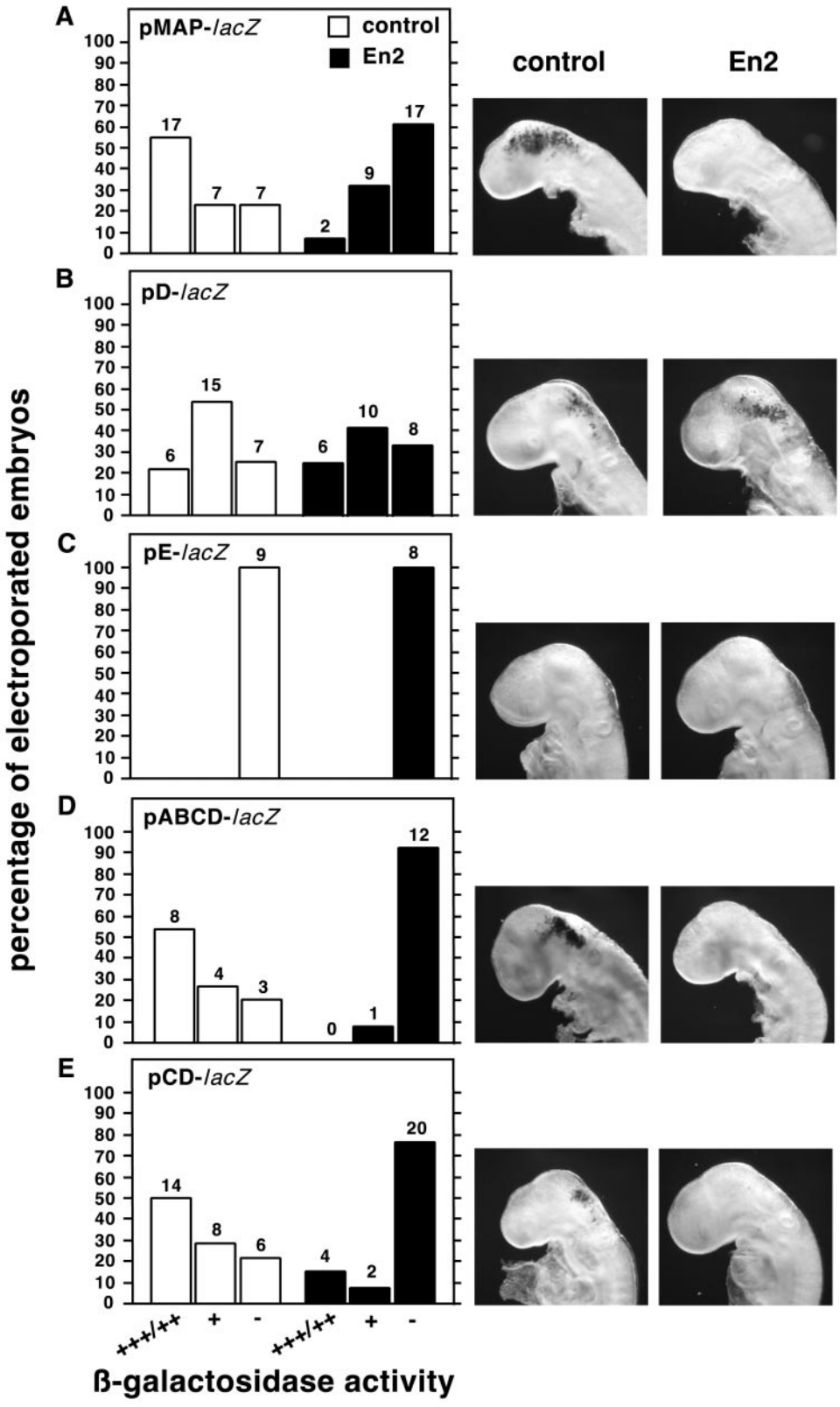

Figure 9. Activity of *MAP1B* promoter fragments in the developing chick neural tube. Chick embryos (HH9–11) were co-electroporated with empty (*control*) or En2-expressing (*En2*) plasmids, together with the following *lacZ*-reporter plasmids: *A*, pMAP-*lacZ*; *B*, pD-*lacZ*; *C*, pE-*lacZ*; *D*, pABCD-*lacZ*; *E,* pCD-*lacZ*. Embryos were stained for β-galactosidase activity 24 hr after electroporation. Histograms show the percentage of electroporated embryos for each condition showing strong (+++/++), weak (+), or no (−) β-galactosidase activity. The *number* of embryos for each condition is indicated on the *top* of the corresponding *bar*. Examples of β-galactosidase expression in each experimental condition are shown on the *right side* of the Figure.

plasticity, for example, the olfactory bulb, the hippocampus, and Purkinje cells in the cerebellum (Sato-Yoshitake et al., 1989; Schoenfeld et al., 1989). Although the precise function of MAP1B remains unclear, the phenotype of *MAP1B* knock-out mice suggests a role in neuronal differentiation (Edelmann et al., 1996; Takei et al., 1997; Gonzalez-Billault et al., 2000). This view is confirmed by antisense experiments showing that *MAP1B* downregulation in PC12 cells or cultured cerebellum macroneurons reduces neurite outgrowth (Brugg et al., 1993; DiTella et al., 1996).

In conclusion, this report adds *MAP1B* to the list of established cytoskeleton genes that interact with one or several homeogenes. This list contains *β3-tubulin* (Serrano et al., 1997), *centrosomin* (Li and Kaufman, 1996), *MAP2* (Ding et al., 1997), *calponin* (Morgan et al., 1999), *NF68* (Biagioni et al., 2000), and *BPAG1* (Mainguy et al., 1999, 2000). The fact that homeoprotein transcription factors regulate the expression of several components of the cytoskeleton, and probably of adhesion molecules (Edelman and Jones, 1993), provides a molecular basis for their well established functions in tissue and cell morphogenesis (Joliot et al., 1991; Bloch-Gallego et al., 1993; Le Roux et al., 1993).

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