Regulation of a Purkinje Cell–Specific Promoter by Homeodomain Proteins: Repression by Engrailed-2 vs. Synergistic Activation by Hoxa5 and Hoxb7

Salih Sanlioglu, Xulun Zhang, Stephan L. Baader, John Oberdick

Department of Cell Biology, Neurobiology, and Anatomy/Neuroscience Division and the Neurobiotechnology Center, The Ohio State University, 190 Rightmire Hall, 1060 Carmack Rd., Columbus, Ohio 43210

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ABSTRACT: We have previously demonstrated that a short sequence element (L7ATE) within the proximal promoter of a Purkinje cell–specific gene, *pcp-2* (*L7*), is required for the normal pattern of expression of the gene in the cerebellum of transgenic mice. The presence of a series of TAAT sequence motifs in this element suggested its interaction with homeodomain proteins. To extend these observations, degenerate oligonucleotides were used to clone by reverse-trascriptase polymerase chain reaction members of the mouse Hox gene family expressed in neonatal cerebellum but not forebrain. Two of these, HoxB7 and HoxA5, are continuously expressed from the neonatal period into adult stages in cerebellar Purkinje cells. These Hox proteins are shown to synergistically activate the L7 promoter by cotransfection assay *in vitro*. In contrast, another homeodomain protein that is normally expressed in Purkinje cells only during the embryonic period, En-2, has a negative effect on L7 gene expression. These data suggest a biphasic, combinatorial control mechanism for the Purkinje cell–specific expression of the *pcp-2 (L7)* gene. J Neurobiol 36:559–571, 1998

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Homeodomain proteins are key regulators of early embryonic development, and some select homeodomain proteins play a critical role in the specification of discrete regions of the central nervous system. The mammalian En genes, for example, are required for normal early development of the junction region of the mid- and hindbrain which gives rise to the cerebellum (Wurst et al., 1994; Millen et al., 1994; Kuemerle et al., 1997). Likewise, the largest distinct family of vertebrate homeodomain proteins, those encoded within the four Hox gene clusters which are each functionally and structurally analogous to the HOM-C cluster in *Drosophila,* play a role in the determination of segmental aspects of both spinal cord–associated mesoderm and rhomboncephalic neuralectoderm (Keynes and Krumlauf, 1994). One oft-neglected aspect of expression of these homeobox genes, however, is their continued expression in adult tissues—brain in particular. For example, En-2 and other mammalian homologs of *Drosophila* segment-polarity genes continue to be expressed in the mature nervous system, suggesting a role beyond embryonic patterning (Davis and Joyner, 1988; Baader et al., 1998). The same is true of various members of the Hox gene family (e.g., Odenwald et al., 1987). Furthermore, ectopic expression of homeodomain proteins in adult neurons can perturb specific developmental and functional properties of those neurons suggesting possi-

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ble target genes (Baader et al., 1998; Aisemberg et al., 1997); knowledge of the gene targets of homeoproteins that are either normally or ectopically late-expressed could facilitate understanding the global function of homeobox genes.

A strategy for *in vivo* analysis of a Purkinje cell–specific promoter has been previously described (Oberdick et al., 1993). By introduction into mice of truncated and mutated versions of a fusion transgene between the *pcp-2*(*L7*) gene (Oberdick et al., 1988; Vandaele et al., 1991) and the coding portion of bacterial *lacZ*, positive and negative *cis*-acting control elements were broadly mapped in the promoter. One such element in particular, lying roughly 60 bp upstream of the TATA box, was demonstrated to serve a role in directing some aspects of the L7 expression pattern, mainly oriented along the cerebellar rostrocaudal axis. This element was reported to be AT-rich, bearing three repeats of a hallmark TAAT motif for homeodomain protein binding, and was immediately adjacent to a perfect consensus E-box. The entire AT-rich plus E-box region, the so-called L7ATE, was shown to be protected from DNase by nuclear extracts prepared from neuronal and nonneuronal tissues alike, and the AT-rich portion alone can be protected by virtually any homeodomain protein (Oberdick et al., 1993; Sanlioglu-Crisman and Oberdick, 1997). As homeodomain proteins typically show nondiscriminatory binding behavior to AT-rich sites, more functional approaches, either *in vivo* or in culture, are required to further elucidate the role of homeobox genes in regulation of the *pcp-2*(*L7*) gene.

Here, we extend these studies by examining the regulation of *pcp-2*(*L7*) gene expression by homeodomain proteins normally expressed in cerebellar Purkinje cells. En-2, normally expressed in embryonic Purkinje cells but not mature ones, has a negative effect on the *pcp*-2(L7) gene *in vivo*. The latter was demonstrated by measuring L7 and L7-β-gal levels in transgenic mice in which En-2 was ectopically expressed in Purkinje cells (Baader et al., 1998). In contrast, two Hox proteins (HoxA5 and HoxB7) that are not normally detected in embryonic cerebellum, but which are shown to be enriched in *adult* Purkinje cells, activate the L7 promoter in a synergistic fashion in nonneuronal cell culture. En-2 has a weak negative effect on this activation. These data, in conjunction with the L7 promoter truncation data described previously (Oberdick et al., 1993), suggest a biphasic mechanism of transcriptional control in which negative regulation by En genes predominates during the embryonic period succeeded by the postnatal up-regulation of combinatorially-acting positive regulators.

MATERIALS AND METHODS

Mice and Cell Lines

Construction and analysis of the L7En-2 mice (Baader et al., 1998) and the L7-β-gal mice (Oberdick et al., 1993) have been previously described. The background mouse strain for all experiments described herein is B6C3F1 hybrid from Jackson Labs (Bar Harbor, ME).

National Institutes of Health (NIH)/3T3 (contact-inhibited NIH Swiss mouse embryo), HeLa (human cervical epitheloid carcinoma), and 293 (transformed primary human embryonal kidney) cells were obtained from the American Type Culture Collection (ATCC).

Brain Anatomy and Biochemistry

Procedures for *in situ* hybridization (Bian et al., 1996) and immunocytochemistry (Baader et al., 1998) have been previously described. The pHoxB7–5′ vector (see below) was digested with *HindIII*, and antisense RNA probe was synthesized using SP6 RNA polymerase. This probe was used for *in situ* hybridization experments. HoxA5 (Cat. no. PRB-285C) and HoxB7 (Cat. no. PRB-205C) rabbit polyclonal antisera were obtained from Babco. The peptide used to produce the HoxA5 antiserum was CDSHHGGKNSLGNSSG, which is 40 amino acids C-terminal to the peptide used to generate the antiserum described previously (DLSVGRSGSGHFGSGERARS) (Odenwald et al., 1987). Results reported here are identical to the earlier report. The peptide used to produce the HoxB7 antiserum was CAKAAGAKEQRDSDLA. Antibodies to Zebrin II were provided by Dr. Richard Hawkes (Brochu et al., 1990).

Methods for preparation and use of brain extracts for β-gal quantification have been previously described (Oberdick et al., 1993). Protein concentrations for β-gal assays were determined by the method of Bradford (Bradford, 1976). Expression of each of the two constructs, L7βG1 and L7βG3, was assessed in two wild-type versus L7En-2 transgenic littermate pairs [$n = 2$ for each β-gal measurement in Fig. 5(B)]. Western blots were prepared by previously described methods (Bian et al., 1996) using a chemiluminescence detection system (Renaissance; New England Nuclear).

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) with Degenerate Primers

P0 total cerebellar RNA was prepared using the TRIzol reagent method (Gibco BRL). One microgram of total RNA was used in an RT reaction consisting of 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 1 m*M* dithiothreitol (DTT), 2.5 m*M* $MgCl_2$, 0.5 m*M* dNTP, 2.5 μM random hexamers, and 100 U of M-MLV RT (Gibco BRL) in a 10-μL final volume. The reaction was carried out at 37°C for 30 min; then the RT was inactivated by incubation at 68°C for 5 min. The PCR step was performed in a 50-

μL final volume and consisted of 1× PCR buffer, 200 μM dNTP, 2.5 mM $MgCl_2$, 1 μM of Hox 5′ degenerate primer (gcggatccGA G/A CTGGAGAA G/A GA G/A TT C/T C), 1 μM of Hox 3′ degenerate primer (gcggatccTC C/T TT C/T TT G/C T/C ACTTCAT), and 2.5 U of Taq DNA polymerase (Gibco BRL). The PCR conditions were 95°C for 5 s, 55°C for 5 s, and 72°C for 30 s; this cycle was repeated 35 times on a Perkin Elmer Gene Amp PCR System 9600. Reaction product was digested with *BamHI* and cloned into pGEM-7Zf for sequence analysis following standard procedures (Sambrook et al., 1989).

Clones and Probes

A 370-bp fragment of HoxB7 cDNA was prepared by RT-PCR from P0 cerebellum RNA using the primers 5′: gcggatccTGAGTTCATTGTATTATGCGAAT and 3′: gcggatccAAGTTACTCTCGGCCGCCAAGT. This fragment was digested with *BamHI* and cloned into the *BamHI* site of pGEM-7Zf to produce the pHoxB7−5′ vector. This vector was used to make probe for *in situ* hybridizations. A 653-bp coding fragment of HoxB7 was produced by RT-PCR from P0 cerebellum RNA using the primers 5′: gctctagaATGAGTTCATTGTATTATGCG and 3′: gcggatccTTCTCTGTCCCTCACTCTTCC. An 813-bp coding fragment of HoxA5 was produced by RT-PCR using the primers 5′: gctctagaATGAGCTCTTATTTTGTAAACTC and 3′: gcggatccAGTACTTTGGCCGCTCAGATGC. Each of these fragments was digested with *XbaI* and *BamHI* and cloned into the pCGN eukaryotic expression vector carrying the CMV promoter (Tanaka and Herr, 1990). In addition, an HoxA5 expression plasmid (in pCDM8; Invitrogen) was obtained from Dr. Ward Odenwald and produced results identical to those using our clone. A 980-bp En-2 coding fragment was excised with *BamHI* from the L7En-2 vector (Baader et al., 1998) and transferred into the pcDNA3.1 expression vector (CMV promoter; Invitrogen). The ME2 expression vector was provided by Dr. Toomas Neuman (in pRcCMV; Invitrogen) (Chiaramello et al., 1995).

A fragment corresponding to the 200-bp most proximal region of the L7 promoter was produced by PCR using the L7ΔAUG vector as template and the primers 5′: gcggatccGCTCAGACCTTCTAGACAAGG and 3′: gcggatccCTCAGAAGTGCCCCTAAAT. This fragment was cloned into the *BamHI* site of pXP1 (ATCC) and into the *BglII* site of pGL3 Basic Vector (Promega), both luciferase detection vectors. The 2xL7ATE-pGL3SV40 vector was made by annealling the primers GGCACCTGTAATTGACAAGATTAATTCATTTATAGGGCATCTAATTAGC and GCTAATTAGATGCCCTATAAATGAATTAATCTTGTCAATTACAGGTGCC to form a blunt-end fragment which was self-ligated; then, concatemers were ligated into the *SmaI* site of pGL3-Promoter (SV40) Vector (Promega). The number and orientation of L7ATE units were determined by DNA sequence analysis.

Probes used for RNase protection analysis included β-gal, L7, and glyceraldehyde phosphate dehydrogenase (GADPH). A 230-bp β-gal fragment was gel-purified after digestion of pSP73-β-gal (Ed Giniger, Fred Hutchinson Cancer Research Center, Seattle, WA) with *HincII*. This fragment was cloned into the *HincII* site of pBluescript to generate pβ-galBS. The pL7GEM3 vector is a version of a previously reported L7 cDNA (Oberdick et al., 1988) that was truncated at both ends and cloned into the *BamHI* site of pGEM3 (Bian et al., 1996). The GAPDH plasmid was provided by Dr. Mike Ostrowski (Department of Molecular Genetics, Ohio State University).

Cotransfection Analysis of L7 Promoter Constructs

All cell lines were grown under recommended culture conditions (ATCC) on 60-mm tissue culture dishes and transfected at 50% confluency by the calcium phosphate coprecipitation method. Each plate typically received the same amount (usually 1.5 μg) of luciferase-based promoter test vector and varying combinations and amounts of expression vectors encoding transcription factors. The total amount of DNA added to each plate was normalized both with base expression vector DNA (e.g., pcDNA3.1, pCGN, etc.) without the transcription factor-encoding fragment in experiments where amounts of transcription factors were varied, and with pGEM7Zf as carrier to bring the total DNA in each case to 7−9 μg. The DNAs used in transfection experiments were purified using Qiagen columns (Qiagen Inc.). Cells were harvested 48 h after the transfection and lysed in 1× reporter lysis buffer and assayed for luciferase using the Luciferase Assay System (Promega). All experiments were normalized to total protein in cellular extracts as determined by Bradford assay (Bradford, 1976), but the first set of experiments in HeLa cells [see Fig. 3(A)] were additionally normalized to β-galactosidase levels by cotransfection with pRSV-βgal (MacGregor et al., 1987; Norton and Coffin, 1985). In every case, two cultures were used for each transfection condition ($n = 2$), except for the HeLa experiment [see Fig. 3(A)], in which three cultures were used per condition ($n = 3$). In addition, the latter experiment was repeated a second time ($n = 2$ cultures for each condition), with identical results. Luciferase levels were detected using a Lumat Model LB9501 luminometer (Berthold).

RNase Protection Assay

The RPAII Ribonuclease Protection Assay Kit (Ambion) was used to quantitate changes in mRNA levels. Cerebellar mRNAs were prepared using the TRIzol reagent method (Gibco BRL). Probes used for RNase protection analysis included β-gal, L7 and GAPDH. β-Gal antisense RNA was generated by linearizing the pβ-galBS vector with *XhoI* followed by riboprobe synthesis using T3 polymerase. The pL7GEM3 vector was linearized with *HindIII* before riboprobe synthesis with T7 polymerase. The GAPDH plasmid was linearized with *HindIII* and riboprobe synthesized using T3 polymerase. Data were quantitated using a PhosphorImager and associated software (Molecular Dynamics).

Table 1 A Degenerate RT-PCR Analysis Using P0 Cerebellar Total RNA and P0 Forebrain Total RNA

HOX genes	Cerebellum (P0)	Forebrain (P0)
Hox A4 (Hox 1.4)	6	5
Hox A5 (Hox 1.3)	14	0
Hox B3 (Hox 2.7)	8	12
Hox B4 (Hox 2.6)	0	1
Hox B5 (Hox 2.1)	6	0
Hox B6 (Hox 2.2)	1	0
Hox B7 (Hox 2.3)	5	0
Hox B8 (Hox 2.4)	1	0
Hox C5 (Hox 3.4)	1	0
Hox C8 (Hox 3.1)	0	1
Hox D3 (Hox 4.1)	6	7

Both old and new Hox gene nomenclature are used for clarity. Numbers beneath the tissue type represent the number of times that a particular homeobox gene was isolated through the RT-PCR procedure.

Results

Identification of Hox Genes Expressed in Cerebellar Purkinje Cells

Because known homeodomain proteins have been demonstrated to bind to the L7ATE (Oberdick et al., 1993), and because Hox proteins are members of the largest homeobox gene family (Keynes and Krumlauf, 1994) and are known to be expressed in developing and mature brain (Odenwald et al., 1987), we set out to identify Hox proteins expressed in cerebellar Purkinje cells. For this purpose, we created a panel of degenerate oligonucleotides with selectivity for the homeobox region of the Hox gene family. One primer pair was used to generate RT-PCR fragments from either cerebellum or forebrain, and these were cloned and sequenced. The distribution of identified Hox gene fragments is described in Table 1. In this table, the number of times a particular Hox gene is represented is presumed to be related to its mRNA abundance. As a general rule, those genes that were identified in both forebrain and cerebellum (HoxA4, HoxB3, and Hox D3) have a more rostral boundary of expression during embryogenesis (Hunt et al., 1991; Bass and Baker, 1997) than those genes found to be restricted to cerebellum (HoxA5, HoxB5, and HoxB7).

Two of the most highly represented sequences in the clone pool generated from cerebellum RNA, HoxA5 and HoxB7, were never identified in forebrain RNA. Since the complete cDNA sequences of these genes are known, RT-PCR was again used to isolate unique probes (nonhomeobox) and complete coding sequence fragments. HoxA5 has been previously demonstrated to be expressed at moderate levels in cerebellar Purkinje cells (Odenwald et al., 1987). Using an antibody to a different portion of the HoxA5 protein, this observation was confirmed [Fig. 1(A,B)]; likewise, antibodies to HoxB7 revealed a very similar distribution [Fig. 1(C,D)]. Since the expression of HoxB7 in Purkinje cells was relatively weak compared to HoxA5, HoxB7 mRNA distribution was examined by *in situ* hybridization [Fig. 1(E,F)]. In confirmation of the protein distribution, the mRNA was clearly detectable in cerebellar Purkinje cells. However, neither HoxA5 nor HoxB7 mRNAs were detectable in cerebellum of embryonic day 15 (E15) mice, at which time the rostral-most boundary of expression (of HoxA5) was restricted to the boundary of the caudal brain stem and spinal cord (formerly rhombomere 7/8) (not shown). Since both cDNA clones were isolated from postnatal day 0 (P0) cerebellar RNA, their expression appears to be activated perinatally, continuing into adulthood. This overlaps with the time course of expression of the *pcp-2*(*L7*) gene, which is first activated around E15, but continues its expression into adulthood (Oberdick et al., 1993).

Thus, of the three Hox genes detected only in cerebellum, HoxA5 and HoxB7 have an expression pattern consistent with a possible role in *pcp-2*(*L7*) gene regulation. However, no definitive evidence of the expression of HoxB5 in Purkinje cells has yet been obtained; therefore, to date, this clone has not been further characterized.

Synergistic Activation of the L7 Promoter by HoxA5 and HoxB7

To determine whether HoxA5 and HoxB7 proteins could functionally regulate the L7 gene, we performed cotransfection experiments in cell culture. In the first set of experiments, the 200-bp most proximal part of the L7 promoter (including the TATA box and the L7ATE) [Fig. 2(A)] was cloned into the pXP1 promoter-test vector (Nordeen, 1988) and cotransfected into HeLa cells along with HoxB7 and/or HoxA5 expression plasmids. Relative activation of the promoter fragment was determined by measuring the level of luciferase activity (encoded by the pXP1 vector), normalized both to β-galactosidase (introduced by cotransfection with pRSV-βgal) (MacGregor et al., 1987) and total protein in cell extracts. As shown in Figure 3(A, upper part), the activity of HoxA5 and HoxB7 in combination was four times higher (20.7-fold) than the sum activities of each acting alone (4.8 + 1.0 = 5.8-fold). Although the pXP1 base vector itself was able to direct low levels of activity with either Hox protein,

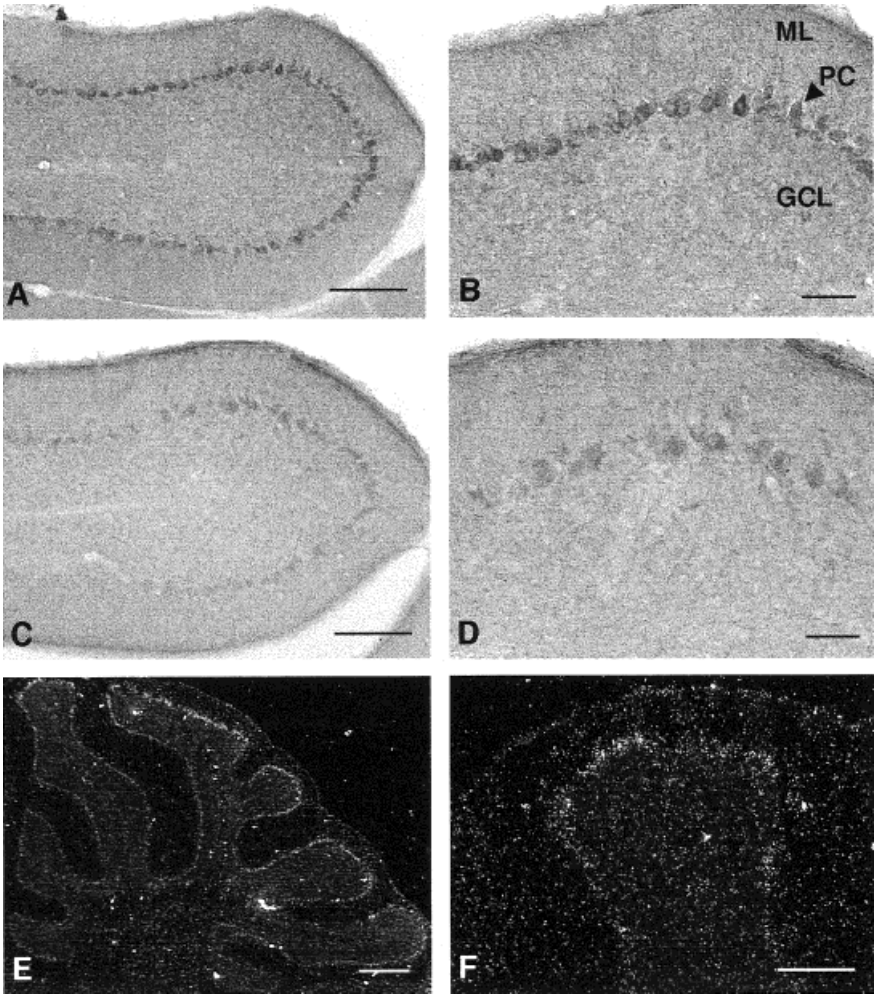

Figure 1 Expression of HoxA5 and HoxB7 in cerebellar Purkinje cells. (A–D) Immunocytochemistry was performed on 40-μm thick sagittal sections of mouse cerebellum using antisera to HoxA5 (A, B) and HoxB7 (C, D). Both proteins are relatively enriched in Purkinje cells, although cells scattered in both the GCL (probably Golgi neurons) and ML (probably basket or stellate cells) appear to be stained with antiserum against HoxA5, as was previously described (Odenwald et al., 1987). Although both proteins are distributed in the nuclei of Purkinje cells, they do not appear to be restricted there, since cytoplasm and proximal dendrites are stained (especially with HoxA5 antiserum). (E, F) To confirm the distribution of HoxB7 which was relatively weak by immunocytochemistry, *in situ* hybridization was performed. Specific signal was moderately detectable in Purkinje cells. Scale bars = 250 μm in (A, C, F); 100 μm in (B, D); 500 μm in (E). ML = molecular layer; PC = Purkinje cell layer; GCL = granule cell layer.

the combined effect in this case was strictly additive [Fig. 3(A), lower part]. Similarly, doubling the concentration of either Hox protein alone resulted in a simple doubling of promoter activity in the case of HoxB7 but little or no increase in the case of HoxA5 [Fig. 3(A), upper part], illustrating the heterotypic nature of the synergistic interaction between the two Hox proteins.

In an attempt both to eliminate the cryptic promoter activity of the base vector and to validate the synergistic effect of the two Hox proteins, the above experiment was repeated, this time in 293 cells using the pGL3 Basic vector. As shown in Figure 3(B), the base vector with and without the promoter was capable of moderate activation with the HoxB7 protein (but not HoxA5). However, only the vector with the 200-bp L7 promoter fragment was capable of sustaining synergistic activation. It should be noted that despite the fact that synergy is typically defined as a greater than additive effect (Lin et al.,

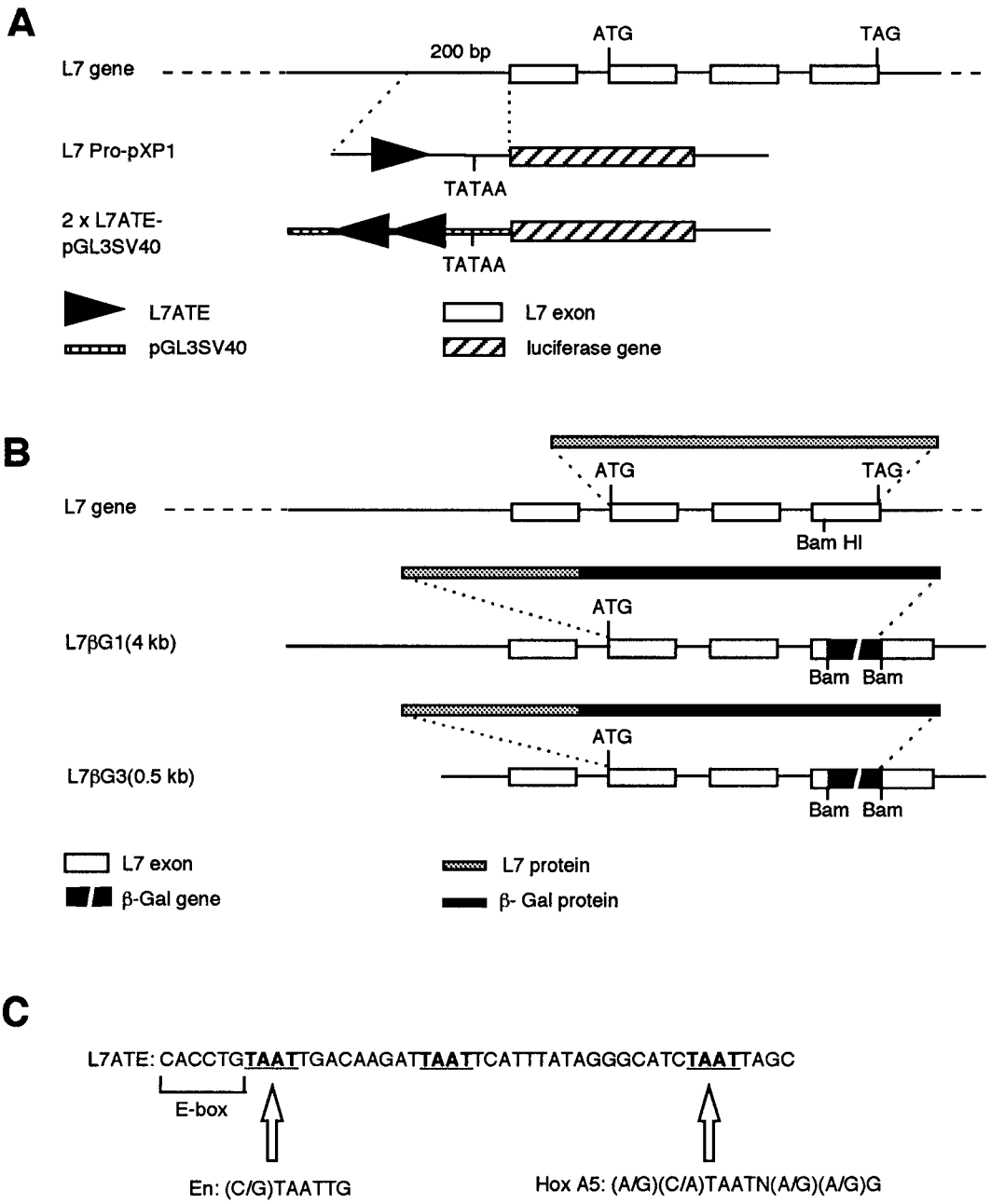

Figure 2 Structures of promoter test vectors. (A) The structure of the endogenous *pcp-2*(*L7*) gene is illustrated at the top. The 200-bp most proximal region of the promoter was cloned into the luciferase reporter vectors pXP1 and pGL3 Basic, as illustrated in the middle panel. This promoter fragment has one L7ATE unit lying just upstream of the TATA box. To test whether the L7ATE could function in the context of an exogenous promoter, two L7ATE units were linked in head-to-tail fashion (but in inverted orientation) to the SV40 promoter of pGL3 promoter. This is illustrated in the bottom panel. (B) Structures of the endogenous L7 gene (top), the ''full-length'' or 4-kb promoter L7-β-gal encoding vector (middle), and the truncated or 0.5-kb promoter L7-β-gal encoding vector (bottom) are recapitulated from Oberdick et al. (1993). The formation of an L7-β-gal fusion protein and the position of the endogenous L7 start codon (ATG) are illustrated. The only difference between the two β-gal–encoding constructs is the length of the promoter region. (C) The sequence of the basic L7ATE unit is recapitulated from Oberdick et al. (1993). The positions of perfect En and near perfect HoxA5 binding sites are indicated, although all of the TAAT core elements would probably serve as reasonable binding sites for any homeo domain proteins.

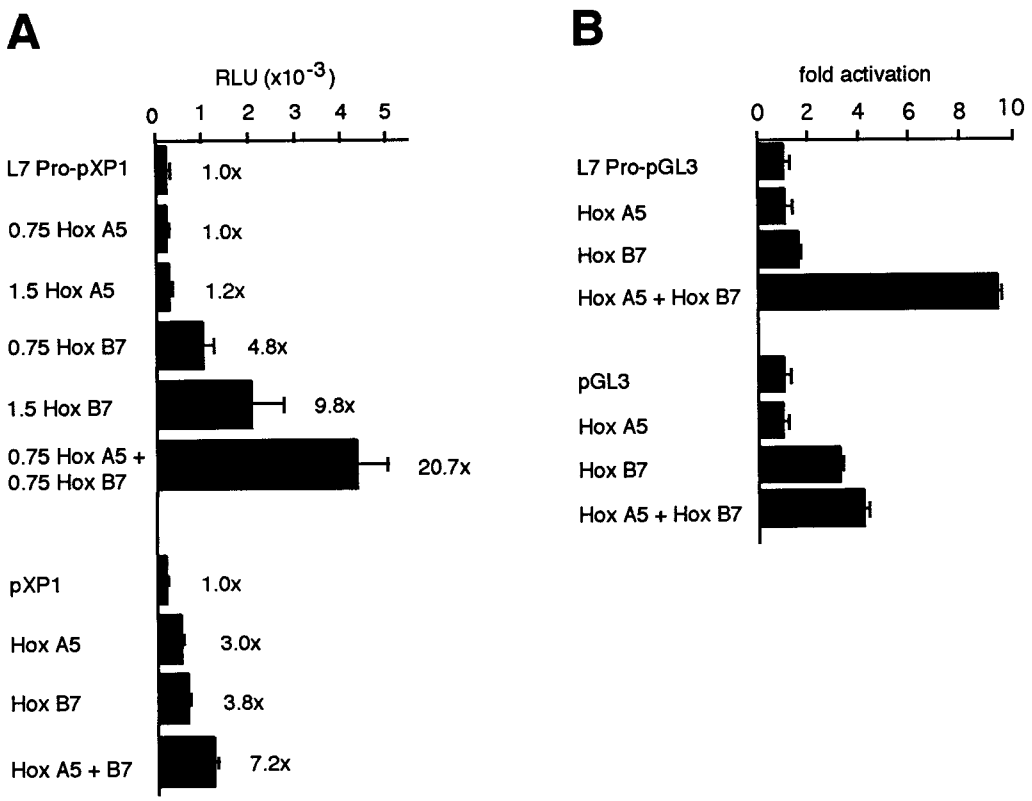

Figure 3 Synergistic activation of the L7 promoter by HoxA5 and HoxB7. (A) A 200-bp promoter fragment (L7 Pro) of the L7 gene was cloned into the pXP1 luciferase detection vector; 1.75 μg of this detection vector was transfected alone into HeLa cells (L7 Pro-pXP1), cotransfected along with 0.75 or 1.5 μg of expression plasmids encoding HoxA5 or HoxB7, or cotransfected with 0.75 μg each of HoxA5 and HoxB7. In this experiment, all cultures were additionally cotransfected with 1.5 μg of RSV-βgal vector to normalize the transfection efficiency. The absolute activity values are presented in relative light units (RLU) normalized to β-gal levels and micrograms of transfected DNA, and each value is accompanied by a fold activation. The pCGN base vector was used as a balancer so that all cultures received 1.5 μg of expression plasmid, and pGEM7Zf was used as a carrier such that all cultures received 7.5 μg of total DNA. Combined activity of HoxA5 and HoxB7 on the base pXP1 vector (no promoter) is strictly additive (bottom). In the presence of the L7 promoter, the combined activity (0.75 HoxA5 + 0.75 HoxB7 = 20.7-fold activation) is much greater than additive (top)(n = 3 for each value). (B) The experiment in (A) was repeated in 293 cells using the pGL3 Basic vector carrying the 200-bp promoter. Again, luciferase activity driven from the base vector (no promoter) was strictly additive (bottom), whereas the presence of the promoter fragment produced a much greater than additive boost in expression when HoxA5 and HoxB7 were combined (top). Data are shown as fold activation (n = 2 for each value).

1990; Carey et al., 1990), the synergistic effect reported throughout the current work is also greater than the multiple of activities of each factor acting alone.

Synergistic Activation by Hox Proteins Is Mediated by the L7ATE

A homeodomain protein-interacting site (L7ATE) is contained within the 200-bp promoter fragment that was tested in culture as described above [Fig. 2(A)]. The L7ATE has been demonstrated to be strongly ''footprinted'' by several purified homeodomain proteins and by crude nuclear extracts from multiple tissues (Oberdick et al., 1993; Sanlioglu-Crisman and Oberdick, 1997), and the L7ATE contains perfect or near-perfect consensus binding sequences for well-characterized homeodomain proteins, including En (Catron et al., 1993) and HoxA5 (Odenwald et al., 1989) [Fig. 2(C)]. To determine

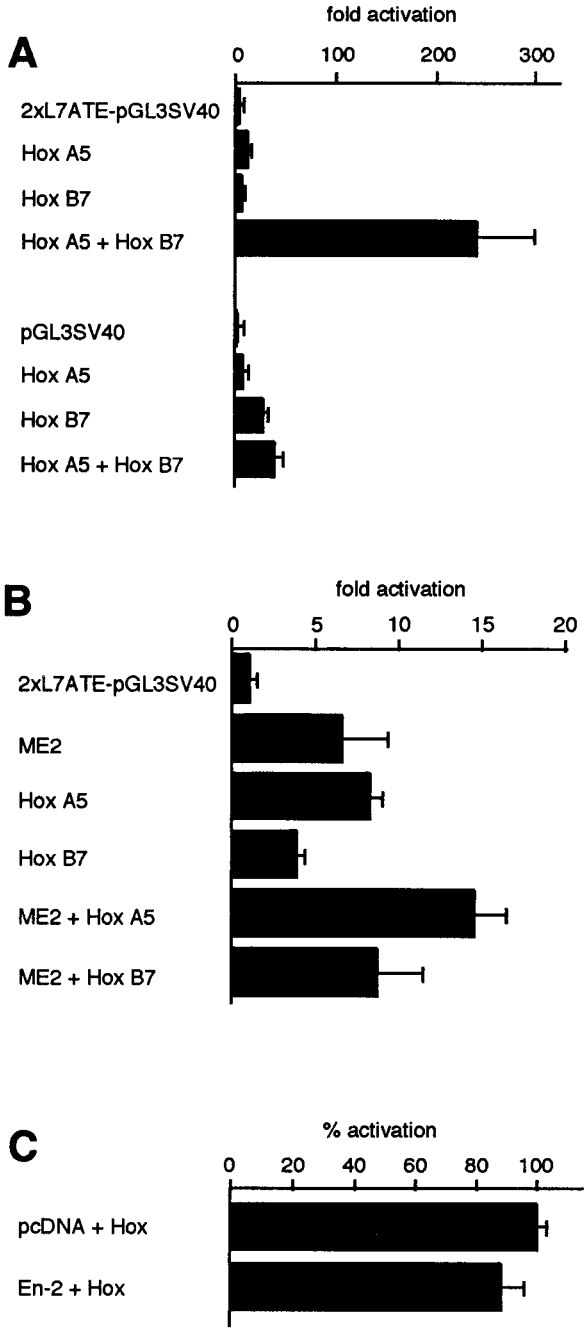

Figure 4 The synergistic effect mediated by the L7ATE is transferable to an exogenous promoter. (A) Two L7ATE units were linked head-to-tail (in reverse orientation) to the SV40 promoter of pGL3 promoter vector. One microgram of this vector was transfected alone into NIH/3T3 cells (2xL7ATE-pGL3SV40), cotransfected with 4 μg of HoxA5 or HoxB7 alone, or 4 μg of each in combination (HoxA5 + HoxB7). Transcription factor plasmid levels were balanced using pCGN base vector (4 μg) without insert. Activities in Hox cotransfection experiments using the base detection vector with no L7ATEs (pGL3SV40) were strictly additive (bottom), whereas inclusion of the L7ATE resulted in a sharp synergistic response when HoxA5 and HoxB7 were combined (top). (B) The experiment in (A) was repeated, but 4 μg of HoxA5 or HoxB7 expression plasmid was tested in combination with 4 μg of ME2 expression plasmid. The responses were strictly additive. (C) One microgram of the 2xL7ATE-pGLSV40 reporter vector, 4 μg of HoxA5, and 4 μg of HoxB7 were cotransfected with 1, 2, or 4 μg of En-2 expression plasmid. The relative activation is presented as an average for all three En-2 conditions. For this experiment, two cultures were tested for each condition ($n = 6$).

whether the L7ATE was able to confer the synergistic activateability to an exogenous promoter, a multimer of the L7ATE was linked to the SV40 promoter (in pGL3-SV40) and this expression vector was cotransfected with HoxA5 and/or HoxB7 into NIH/3T3 cells. Again, both the base plasmid and the base plasmid plus L7ATE enhancers were capable of moderate activation by either HoxA5 or HoxB7. Only the vector with the L7ATE enhancers, however, revealed synergistic activation by a combination of the two Hox proteins (242-fold activation with HoxA5 plus HoxB7, vs. 11.9-fold and 6.5-fold with either HoxA5 or HoxB7 acting alone, respectively) [Fig. 4(A)]. To test the selectivity of this synergistic effect, the brain-specific HLH transcription factor ME2 (Chiaramello et al., 1995) was cotransfected with either HoxA5 or HoxB7 in the same paradigm. ME2 was previously demonstrated to bind to the E-box of the L7ATE as well as activate transcription from the L7ATE-SV40 composite promoter (Sanlioglu-Crisman and Oberdick, 1997). Unlike the combined Hox proteins, the activity of ME2 in combination with either Hox protein was strictly additive [Fig. 4(B)].

Repression of L7 Gene Expression by Engrailed-2

Another homeodomain protein, Engrailed (En), has been demonstrated to act as a transcriptional repressor (Jaynes and O'Farrell, 1991; Ohkuma et al., 1990), and one mechanism of this repression is by attenuation of other synergistically acting homeodomain proteins by interaction at the same site (Han et al., 1989). Mammalian homologs of the *en* gene appear to play a key role in cerebellar development and patterning, and are expressed in Purkinje cell progenitors (Millen et al., 1994, 1995; Wurst et al., 1994). Hence, we set out to determine whether one of these proteins, En-2, could repress transcription from the L7 promoter. There was no effect of En-

2 on basal reporter gene expression in transient transfection assays (not shown). To test whether En-2 protein could interfere with the synergistic activation described above, an En-2 expression vector (in pcDNA3.1) was cotransfected with the HoxA5 plus HoxB7 combination in the L7ATE-SV40 promoter paradigm. Only a small decrease (≈10%) in activity was observed [Fig. 4(C)].

If En-2 was capable of negatively controlling the L7 promoter, there are several reasons why this effect might be masked in culture. For example, the nonneuronal cell lines that were used may lack a cofactor required for Engrailed function. To further explore this possibility, L7 gene expression was examined in transgenic mice (L7En-2 mice) in which En-2 protein was selectively expressed in cerebellar Purkinje cells (Baader et al., 1998). In these animals, roughly 40% of all Purkinje cells are lost and there are subtle effects on the pattern of lobulation. Those cerebellar cells that remain, however, appear to be normal with respect to both morphology and arrangement. Surviving Purkinje cells appear to be biochemically normal, since a panel of Purkinje cell-specific protein markers (calbindin: Jande et al., 1981; zebrin II: Doré et al., 1990; L7: Oberdick et al., 1988) all show an equivalent drop of ≈30% relative to wild-type mice [Fig. 5(A)], which matches the decrease in the corresponding mRNAs [Fig. 5(C)]; this is assumed to reflect a small relative decrease in Purkinje cell number compared to other cerebellar cell types. Therefore, similar to the observation made by cotransfection, En-2 appears to have little effect on L7 gene expression.

To further characterize the biochemistry of the transgenic Purkinje cells, two additional transgenic mouse lines were separately crossed into one L7En-2 line (line 39). The additional transgenes were previously described fusions between the L7 gene and bacterial *lacZ* in which the encoded β-galactosidase was expressed as a fusion protein with L7 (Oberdick et al., 1993). The two constructs differed only in the length of the 5′ flanking region and carried in addition all exons and introns of the L7 gene; the L7βG1 construct carried ≈4 kb. of the L7 promoter, whereas L7βG3 carried only ≈0.5 kb. of the promoter [Fig. 2(B)]. These crosses were initially performed to examine effects on cerebellar patterning as could be revealed by β-gal staining patterns; however, only very subtle effects were observed (as described in Baader et al., in press). In contrast, as shown in Figure 5(B), there is a substantial decrease in β-gal enzyme levels in L7En-2 mice compared to wild type. Compared to the basal decrease of endogenous L7 protein (by 26%), cerebella from L7βG1 mice showed a 70% decrease, and those from L7βG3 mice showed a 94% decrease in β-gal activity in L7En-2 compared to wild type. That this effect is at the level of transcription was confirmed by RNAse protection [Fig. 5(C,D)]. Differential mRNA stability can be excluded as an explanation for the increased repression of the truncated *lacZ* construct, since both transgenes encode an identical transcript [Fig. 2(B)] (Oberdick et al., 1993). Thus, both *lacZ* transgenes were more sensitive to En-2 expression than the endogenous L7 gene, and this sensitivity was increased by promoter truncation.

DISCUSSION

The data presented here suggest that a complex regulatory hierarchy of homeobox genes is involved in controlling some aspects of the maturation of cerebellar Purkinje cells. First, by forcing persistent postnatal expression of En-2 in cerebellar Purkinje cells of transgenic mice, we demonstrate that this homeodomain protein can negatively regulate the *pcp-2*(*L7*) gene. This had been previously suggested by observation of complementary patterns of expression of the two endogenous genes during late embryogenesis in the cerebellum (Baader et al., 1998). However, negative regulation of *pcp-2*(*L7*) by En-2 in the transgenic assay reported here is complex. The endogenous L7 gene is largely unresponsive, while L7-based transgenes respond in a promoter-dependent fashion; repression by En-2 is enhanced by promoter truncation. The data do not allow for the discernment of a direct role for En-2 in regulating the L7 gene. The discrepancy between the *in vivo* effect of transgenically expressed En-2 and the much smaller *in vitro* effect may be explained by an indirect role for En-2 via other transcription factors. Alternatively, cofactors may be required that are not present in the cell lines that were tested here, but which are present in Purkinje cells. Nevertheless, the observation of negative regulation of the L7 promoter by En-2 in the transgenic assay is in keeping with previous observations that *Drosophila* En can also act in a negative fashion (Jaynes and O'Farrell, 1991; Han et al., 1989; Ohkuma et al., 1990). In this context, it should be noted that the 0.5-kb promoter of the L7βG3 vector carries the L7ATE within the 200-bp most proximal region (the same region as was tested in the cotransfection studies); by sequence analysis of the 0.5-kb region, the L7ATE is the only consensus site for homeobox protein binding (Fig. 2(C)]. Therefore, if the effect of En-2 on the truncated L7 promoter is direct, it is most likely exerted through the L7ATE.

The second major observation is that HoxA5 and

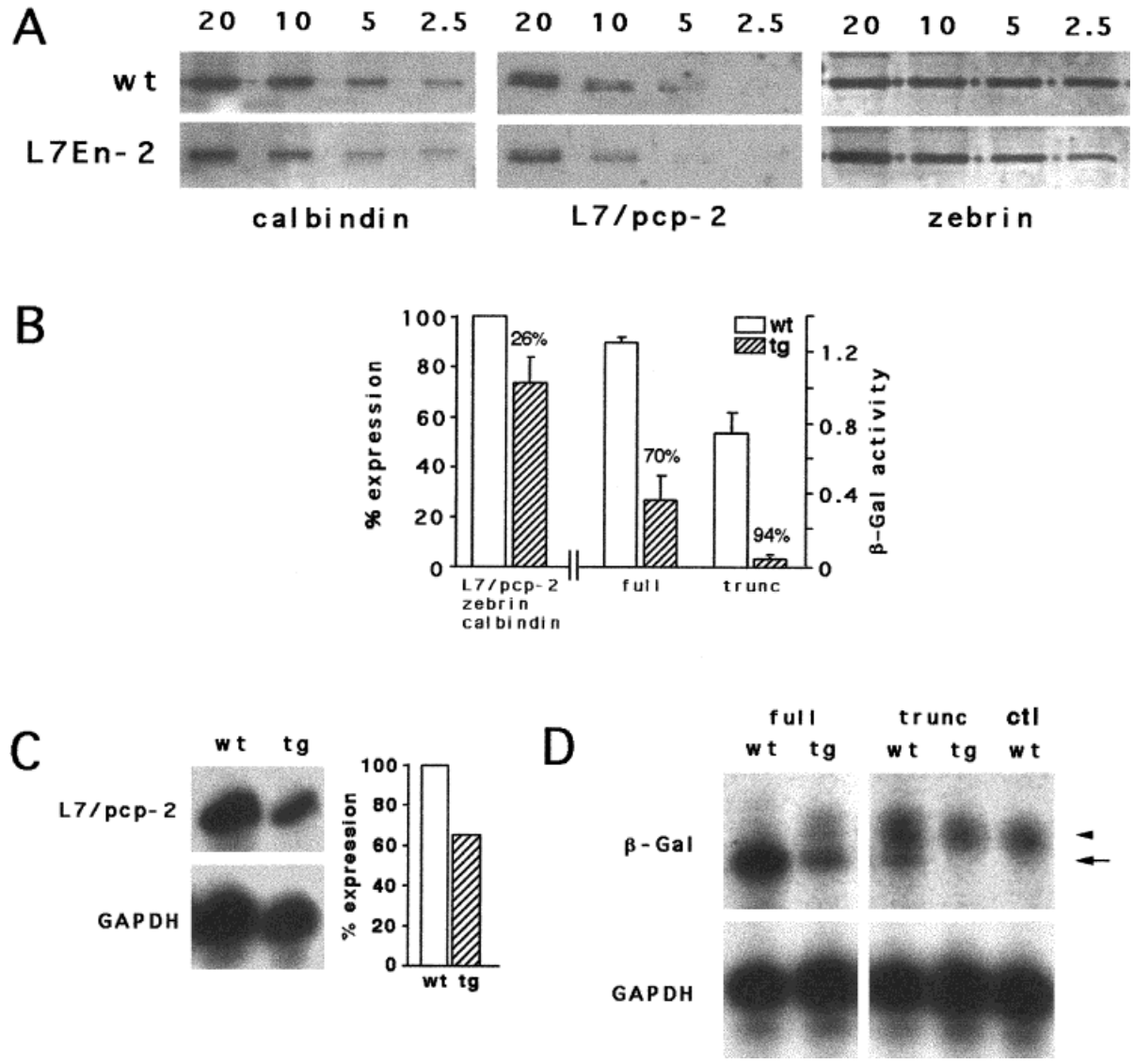

Figure 5 Negative transcriptional effect of En-2 expressed in Purkinje cells *in vivo*. (A) The levels of three marker proteins in wild-type mouse cerebellum were compared to levels in L7En-2 transgenic mice. 20, 10, 5, and 2.5 mg of total protein from cerebellar extracts were analyzed in each case. Each of these marker proteins showed an ≈30% drop in the trangenic mouse background compared to wild type [the quantitation is shown in (B)]. (B) Two L7-β-gal transgenic mouse lines were crossed into wild-type (wt) and L7En-2 transgenic mutant (tg) backgrounds and the level of β-gal enzyme was quantitated. The effects on transgene expression (full = 4 kb L7-β-gal promoter construct; trunc = truncated or 0.5 kb L7-β-gal promoter construct) were compared to the effect on endogenous gene expression. The latter was determined by quantitative scan of the Western data described in (A) averaged for all three markers (left side; % expression) , and the former are presented as relative β-gal levels determined by enzymatic assay of tissue extracts (middle and right; β-Gal activity). The percent decrease is indicated for each tg versus wt set. (C) The effect on endogenous mRNAs was found to parallel the decrease in protein levels described in (A,B). RNase protection data are shown for the endogenous L7 mRNA only. An ≈30% decrease of this mRNA was observed in an L7En-2 mutant (tg) compared to a wild-type (wt) background when values were normalized to GAPDH levels (n = 3). (D) The effect on β-gal transgene mRNAs was found to parallel the effect on enzyme levels described in (B). RNase protection was performed to detect expression of either L7βG1 or L7βG3 transgenes which produce mRNAs of identical structure. When normalized to GAPDH levels a 66% average decrease (n = 11) in L7βG1 transgene mRNA was observed in L7En-2 (tg) mice compared to wild-type (wt) mice (full).

HoxB7 can synergistically activate the L7 promoter *in vitro,* and this activation appears to be exerted through the L7ATE, since it is transferable when this element is linked to an exogenous (SV40) promoter. Synergistic activation of putative target genes by homeodomain proteins has been reported both in *Drosophila* (Han et al., 1989) and in mice (Jones et al., 1993). In addition, En protein has been proposed to repress transcription in multiple ways: It can repress synergistic activation by other homeodomain proteins apparently at the same site (Han et al., 1989); it can repress transcription by competing for binding with TFIID at the TATA box (Ohkuma et al., 1990); and it can act as an ''active'' repressor by interacting with activators at distinct sites (Jaynes and O'Farrell, 1991). The current observations provide yet another example of hierarchical interactions between homeodomain proteins at the level of a putative target gene which is brain specific.

That HoxA5 and HoxB7 proteins can synergistically activate *pcp-2*(*L7*) gene expression in culture while En-2 can have a negative effect *in vivo* and weakly *in vitro* argues for a cascade of gene regulation initially involving a negative influence of En-2 during mid- to late embryonic cerebellar development followed by a postnatal growth period in which combinatorial action of Hox proteins activates Purkinje cell-specific genes such as L7. In normal animals, these overlapping stages are defined by the down-regulation of En genes in Purkinje cells during late embryogenesis and the up-regulation of Hox genes during the postnatal period. The observation of selective action of En-2 on truncated versions of the L7 gene suggests that this negative effect, despite its ''artificial'' generation by a gain-of-function transgenic approach, is masked in the longer versions by enhancers that lie upstream of the En-2 response region.

In the context of earlier work, the current observations are somewhat puzzling. On the one hand, we have previously shown that promoter truncation *in vivo* results in up-regulation of L7 during embryonic stages, suggesting the presence of repressor elements in these upstream regions (perhaps interacting with En-2 or En-2 facilitators). On the other hand, as shown here, the same truncations result in the apparent removal of activating sequences when placed in an adult context, allowing a negative effect of En-2 to be revealed. This may be related to our previous studies in which, in addition to the up-regulation during embryonic stages by L7 promoter truncation, a down-regulation of expression was observed postnatally (Oberdick et al., 1993). The latter suggested the removal of positive control elements, which is further supported by the current work. That the negative and positive effects are temporally segregated, and that En-2 can aggravate the negative effect of promoter truncation that occurs postnatally, supports a biphasic model of L7 gene regulation in which repressive mechanisms dominate embryonically (terminated by the down-regulation of En-1 and En-2 before birth) and activating mechanisms dominate postnatally (marked by the up-regulation of Hox proteins). Owing to the nature of homeodomain protein DNA recognition sequences, these opposite effects could be mediated through the same elements within the L7 promoter, such as the L7ATE.

What role the L7ATE might play in the context of these more distal control elements is unclear. Truncation of the L7 promoter down to ≈200 bp (similar to the promoter fragment used here in the *in vitro* studies), in which the L7ATE is retained, maintains Purkinje cell specificity *in vivo* but at much reduced levels of expression (Oberdick et al., 1993). Thus, even if HoxA5 and HoxB7 interactions at the L7ATE are physiologically relevant for Purkinje cells, interaction of these or other factors with more upstream elements is required to achieve normal levels of expression *in vivo*. Nevertheless, these studies reveal the L7ATE as a powerful facilitator of homeodomain protein interaction, and suggest it as a focal point for interaction of multiple competing factors regulating Purkinje cell-specific L7 expression.

In addition to providing some clues concerning mechanisms of neuron-specific gene expression, these data suggest several possibilities concerning the functional role of late-expressed Hox proteins in the nervous system. First, it is perhaps more than just of passing interest that there is a rough correlation between position of Hox gene expression in the brain and position of expression in the embryonic nervous system. For example, those clones frequently identified in *both* forebrain and cerebellum (HoxA4, HoxB3, and Hox D3) (Table 1) have a

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The percent decrease in L7βG3 transgene mRNA is impossible to calculate, since it is undetectable in the L7En-2 mutant (tg) background, but it is >65% which was the limit of detection of this molecule in the mutant (trunc). The arrow indicates the protected β-gal band; the arrowhead indicates the undigested β-gal probe. ctl = RNA prepared from cerebellum of a control mouse carrying no β-gal transgene.

more rostral boundary of expression in the embryonic CNS (Hunt et al., 1991) than do clones frequently identified *only* in the cerebellum (HoxA5, HoxB5, and HoxB7) (Table 1). This observation may have relevance not only in terms of mechanims of Hox gene regulation, but also in terms of phylogenetic and embryological relationships involved in wiring complex motor circuits (Bass and Baker, 1997). Second, since these proteins play a major role in fate decisions during embryogenesis and early patterning in the nervous system (see Keynes and Krumlauf, 1994, for review), events which are largely concluded by the time frame considered in the present study, one possibility is that there is a more direct functional relevance of these proteins in the brain with respect to cellular or synaptic plasticity. In fact, the Hox proteins described here, HoxA5 and HoxB7, are two of a group of four that have been described to have oncogenic potential (Maulbecker and Gruss, 1993). Thus, it will be important to unravel the relationship between early cellular growth control (and differentiation) mediated by these proteins and events related to cell growth that are important for adult brain function.

Finally, as a putative target gene for these two Hox proteins, it will be important to determine the functional role of the *pcp-2*(*L7*) gene product. Animals carrying functionally null alleles of the latter gene have been described, but no developmental or behavioral abnormalities were observed (Vassileva et al., 1997; Mohn et al., 1997). Nevertheless, the L7 mRNA is abundantly transported into the very distal dendrites of Purkinje cells (Bian et al., 1996; Wanner et al., 1997), and synthesis of the L7 protein in culture is dependent upon factors that influence the electrical exciteability of Purkinje cells (Wanner, Oberdick, and Schilling, unpublished observations). Coupled with the observations that the HoxA5 homeodomain can be taken up by cells and transported to nuclei (Chatelin et al., 1996), that homeodomain proteins can recognize and bind to RNA sequences (Dubnau and Struhl, 1996), and that En proteins, in addition to their nuclear localization, can be found in vesicles that are anterogradely transported into neuronal processes (Joliot et al., 1997), all of these observations suggest a possible signaling role for homeodomain proteins in the mature brain. This may involve both transcriptional control of signaling genes and direct transduction of signals from the periphery.

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