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Expression pattern of a murine homeobox gene, *Dbx*, displays extreme spatial restriction in embryonic forebrain and spinal cord

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ABSTRACT **Homeobox genes specify regional identity during development. A homeobox sequence that we have named *Dbx* was isolated from 13.5-day embryonic mouse telencephalon cDNA. The *Dbx* homeodomain shows highest sequence homology to *Drosophila H2.0* and chicken *CHox E*. We report here the expression pattern of *Dbx* during mouse embryogenesis. *In situ* hybridization analyses indicate that *Dbx* is expressed exclusively within the embryonic central nervous system in a highly restricted manner. *Dbx* transcripts are detected within a region of the prospective cerebral cortex of the midgestation telencephalon. *Dbx* is also expressed in the diencephalon as well as in two thin continuous columns of neuroblasts within the hindbrain and spinal cord. This expression is limited to regions of active mitosis. *Dbx* may act to specify subsets of neuroblasts during the development of the central nervous system.**

In both vertebrates and invertebrates homeobox genes encode transcriptional regulators that act to specify spatial domains within the developing embryo. More than 20 mammalian Antennapedia (1)-class (class I) homeobox genes are expressed in the developing central nervous system (CNS). Their expression patterns are spatially restricted along both the anteroposterior and dorsoventral axes. The spatial restrictions within the dorsoventral axis of the CNS are limited and often extend over many cell layers. These genes generally have different anterior boundaries of expression that are never anterior to the hindbrain (see ref. 2 for review). Expression patterns of several divergent homeobox genes have fundamental differences from those of the class I genes. *En-1* (3) and *Evx-1* (4), the murine homologs of the *Drosophila* engrailed and evenskipped genes respectively, show a much greater localization of transcripts along the dorsoventral axis. *En-1*, *En-2* (3), *Dlx* (5), *Tes-1* (6), and some members of the POU gene family (7) are expressed more anteriorly than the hindbrain. Among these *Dlx*, *Tes-1*, and the POU homeobox genes *Brn-1*, *Brn-2*, and *Tst-1* are detected in the telencephalon.

Recently, we reported (8) a strategy to detect homeobox sequences by polymerase chain reaction (PCR). Degenerate primers of helix 1 and helix 3 of homeoboxes were used to amplify homeobox regions. With this approach, several new homeobox sequences were isolated from the developing mouse telencephalon (8). The telencephalon is an evolutionarily unique structure in chordates and is of great interest neurologically. Although it has been actively investigated for decades, little is known about the molecular mechanism underlying the regulation of telencephalon development. For this reason we decided to further study *Dbx* (*d*eveloping *b*rain, homeob*x* gene), which was formerly termed Mmox C (8). Here we report the expression pattern of *Dbx*, which is extremely restricted within regions of the embryonic CNS. *Dbx* transcripts were detected in the telencephalon, diencephalon, and mesencephalon as well as in two continuous neural columns that extended from the mesencephalon through the entire length of the brainstem and spinal cord. *Dbx* is also expressed within a region in the 12.5-day embryonic mouse telencephalon that forms part of the cerebral cortex.

MATERIALS AND METHODS

***Dbx* cDNA Isolation and Characterization.** A 10.5-day mouse embryo cDNA library (3×10^5 plaques; gift of Gail Martin, University of California, San Francisco) was screened with the Mmox C probe (8) labeled by random priming (Stratagene). Hybridization was conducted at high stringency [6× standard saline citrate (SSC)/10× Denhardt's solution/0.1% SDS with salmon sperm DNA (20 mg/ml) at 65°C]. The nitrocellulose filters were sequentially washed in 2× SSC/0.1% SDS (30 min at room temperature, 30 min at 65°C) and 0.2× SSC/0.1% SDS (30 min twice at 65°C). Screening was repeated twice until single plaques were obtained. Inserts of positive plaques were transferred to a pGEM (Promega)-derived plasmid and characterized by restriction enzyme mapping and sequencing with the dideoxy chain-termination method (T7 DNA polymerase, Pharmacia). A 574-base-pair *Sma* I–*Hin*dIII fragment that is 3′ to the *Dbx* homeobox was subcloned into a pGEM-derived vector (S.L., unpublished data). The insert of this plasmid SAH2aSV was used as *Dbx* probe in the studies reported in this paper.

RNA Purification and Northern Analysis. Total cellular RNA was purified as described (9). Poly(A)$^+$ RNA was purified with oligo(dT)-cellulose (Pharmacia). Six milligrams of poly(A)$^+$ RNA was fractioned in formaldehyde/agarose gel and transferred to nitrocellulose. The SAH2aSV probe was labeled by random priming (Stratagene). Northern analysis was performed as described (10). Hybridization signals were detected by autoradiography (Kodak XAR-5 film).

Preparation of Mouse Embryo Sections. Embryos were obtained by natural matings of CD-1 mice. The morning that vaginal plugs were detected was designated as 0.5 day. Pregnant female mice were sacrificed at the desired day by cervical dislocation and the embryos were fixed and embedded in paraffin (11). The embedded embryos were sectioned at 4–6 μm and the sections were put on 3-aminopropyltriethoxysilane-coated microscope slides (12).

***In Situ* Hybridization.** ^{35}S-labeled single-stranded RNA probes of both sense and antisense strands were prepared from plasmid SAH2aSV by using [α-[^{35}S]thio]UTP (Amersham) and a Riboprobe kit (Promega). Unincorporated nucleotides were removed by Sephadex G-50 (Sigma) spin column. *In situ* hybridizations were performed as described (11). Sense (negative control) and antisense probes were applied to adjacent sections. After hybridization and washing, sections were exposed to NTB-2 emulsion (Kodak) for 4–5 weeks at 4°C. The sections were developed in D19

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Abbreviation: CNS, central nervous system.

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developing solution (Kodak), fixed in rapid fixer (Kodak), and stained briefly in Giemsa reagent (Sigma). Microphotography was performed on a Wild 400 macroscope with T160 slide film (Kodak).

RESULTS

Isolation of *Dbx*. *Dbx* cDNAs were isolated from a 10.5-day mouse embryo cDNA library by using the Mmox C probe (8). The *Dbx* homeodomain shares 87% amino acid sequence homology with chicken *CHox E* (13), 66% with *Drosophila H2.0* (14), 69% with mouse *Hlx* (15), and 38% with *Drosophila* Antennapedia (1) (Fig. 1). *Dbx* has been mapped to mouse chromosome 7 by interspecific backcross methods (unpublished work). Northern analysis using the *Dbx*-specific probe SAH2aSV detected a major transcript of 2.1 kilobases (kb) and two minor transcripts of 4.5 kb and ≈13 kb in 13.5-day embryonic mouse head poly(A)$^+$ RNA (Fig. 2).

Expression of *Dbx* During Mouse Embryogenesis. *Days 9.5 and 10.5. In situ* hybridization analysis was performed to determine the *Dbx* expression pattern during mouse embryogenesis. Both antisense and sense (negative control) probes were used and no specific hybridization signal was observed with the control probe in any experiment. Therefore, only hybridizations with the antisense probe will be described here.

Mouse embryos were analyzed from embryonic days 9.5 through 12.5. This developmental period is marked by the onset of cytodifferentiation, cell migration, and dramatic morphogenesis within the CNS. *Dbx* transcripts were detected at all stages analyzed. At 9.5 days the brain and spinal cord consist of only undifferentiated neuroblasts. *Dbx* expression is regionally localized within the ventral prosencephalon (Fig. 3 *A* and *B*). More posteriorly, two bilaterally symmetrical stripes of *Dbx* expression (Fig. 3*B*) run throughout the entire length of the spinal cord. These stripes consist of only four to six cell layers within the spinal cord along the lateral axis. *Dbx* expression within this limited group of cells may act as a signal to commit these cells to a particular neuronal lineage.

During day 10 of embryogenesis, cytodifferentiation begins within the CNS. The brain grows in size and complexity as the prosencephalon expands to form the telencephalon and diencephalon. Three ventricles are derived from the former prosencephalon and the walls of these ventricles thicken due to active mitosis in the ependymal layer (16). At this stage, *Dbx* expression is detected within the posterior diencephalon (Fig. 3 *C* and *E*), which later gives rise to the hypothalamus. *Dbx* transcripts are also detected in specific regions of the dorsal mesencephalon, ventral metencephalon, and myelencephalon (Fig. 3 *C–E*). *Dbx* expression within the mesencephalon and the myelencephalon, including all of the neuromeres, is continuous with the two stripes of expression detected in the spinal cord (Fig. 3 *D* and *F*).

Days 11.5 and 12.5. The neuronal content of the central nervous system of 11.5- and 12.5-day embryos greatly increases as differentiation continues. During this period *Dbx* transcripts are detected in localized regions of the diencephalon, mesencephalon, metencephalon, myelencephalon, and spinal cord (Fig. 4). Furthermore, *Dbx* is also expressed in the telencephalon at 12.5 days (see below). In the diencephalon of 11.5- and 12.5-day embryos, *Dbx* is detected in a band of cells that spans the diencephalon. This is demonstrated by serial coronal sections in which labeling is first detected in the anterior wall of the diencephalon at the level of cranial flexure (Fig. 4 *C* and *I*) and then in the posterior wall, the anlage of the hypothalamus (Fig. 4 *D, K,* and *L*). *Dbx* is also expressed in the dorsolateral wall of the mesencephalon (Fig. 4 *B* and *I*), which gives rise to the superior and inferior colliculi. This labeling is continuous through the dorsal metencephalon (Fig. 4 *C* and *J*). Posterior to the rhombencephalic isthmus, the expression switches from the dorsal metencephalon to the ventral side (Fig. 4 *J* and *K*). These signals continue through the ventral myelencephalon (Fig. 4 *C–E* and *J–L*) and emerge as two thin stripes that run through the entire length of the spinal cord (Fig. 4 *N* and *O*). The position of the stripes is immediately dorsal to the ventral horn and ventral to the sulcus limitans (Fig. 4 *E, N,* and *O*). Chicken *Chox E* has a similar expression pattern in the hindbrain and spinal cord. However, *Chox E* has an anterior boundary of expression at the rhombencephalic isthmus (13), whereas *Dbx* is expressed in more anterior regions. It is noteworthy that *Dbx* expression is slightly different in the anterior and posterior spinal cord (Fig. 4 *N* and *O*). Although the positions of the signals are the same, a much higher percentage of cells express *Dbx* in the posterior spinal cord.

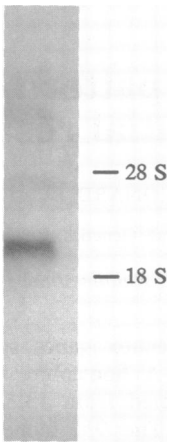

FIG. 2. Northern hybridization of 13.5-day embryonic mouse head poly(A)$^+$ RNA with SAH2aSV probe. Positions of 18S and 28S rRNA are indicated.

The expression patterns change significantly in the forebrain between 11.5 and 12.5 days. At 12.5 days *Dbx* transcripts are detected as two dorsoventrally oriented stripes in the lateral walls of the telencephalon (Fig. 4 *L* and *M*) whereas no expression is detected in the telencephalon at 11.5 days. The *Dbx*-positive regions of the telencephalon will give rise to part of the basal ganglia and cortical neurons at the lateral cerebral walls. This labeling is less intense than in the diencephalon (Fig. 4*L*). The anteroposterior width of

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Dbx      GMLRRAVFSDVQRKALEKTFQKQKYISKPDRKKLASKLGLKDSQVKIWFQNRRMKWRNSKE

CHox E   -I-------ED-------M---------T------IN----E------------------

dH2.0    RSWS-----NL---G--IQ--Q----T----R---AR-N-T-A---V----------HTR-

Hlx      RSWS-----NL---G---R-EI---VT-----Q--AM---T-A---V----------H---

Antp     RKRG-QTYTRY-TLE---E-HFNR-LTRRR-IEI-HA-C-TER-I----------KKENK
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FIG. 1. Comparison of the amino acid sequence of the *Dbx* homeodomain with those of other homeodomains. Dashes indicate positions where amino acids are identical with those in the *Dbx* sequence. *dH2.0, Drosophila* H2.0; *Antp*, Antennapedia.

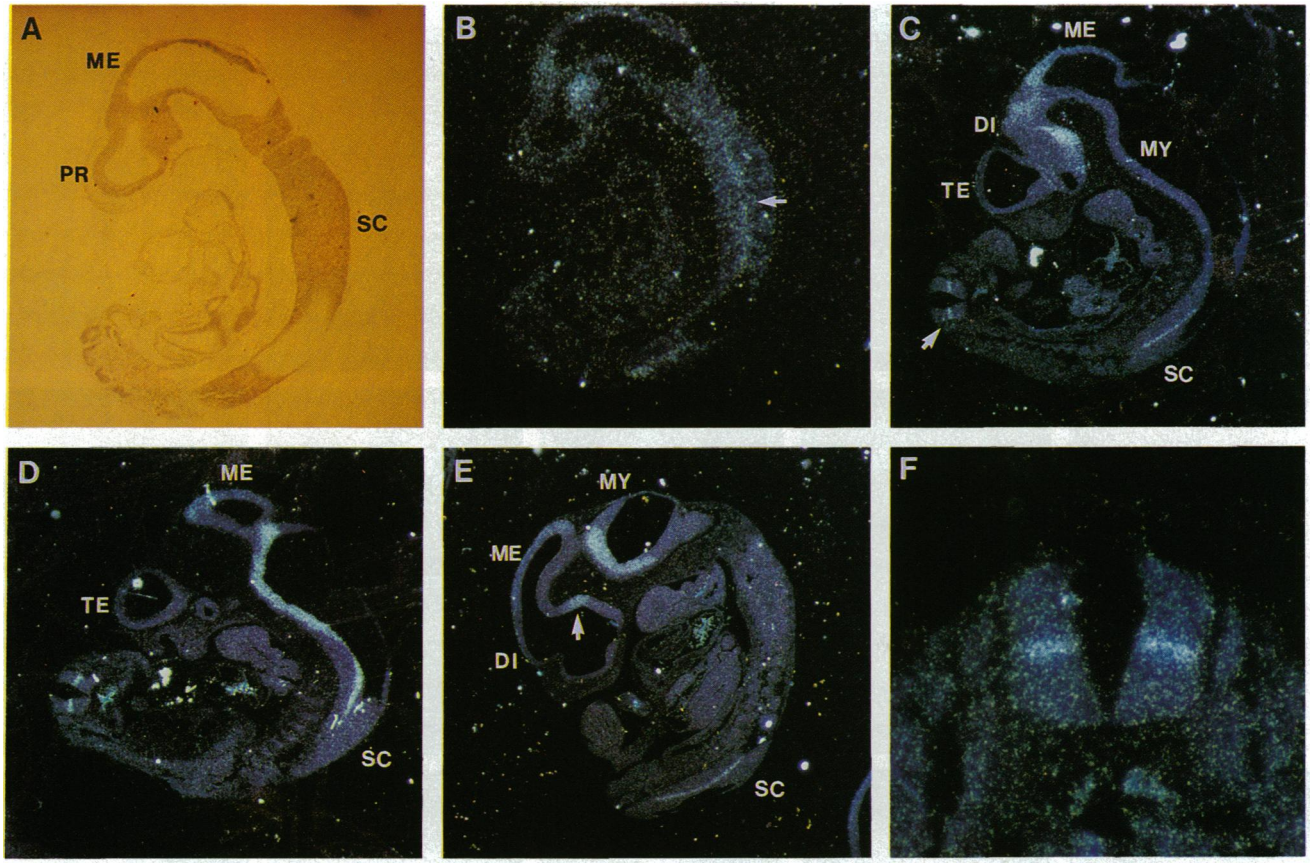

FIG. 3. *In situ* hybridization analysis of *Dbx* expression in 9.5-day and 10.5-day mouse embryos. (*A* and *B*) Light-field and dark-field photomicrographs, respectively, of sagittal sections of a 9.5-day embryo. Arrow points to the hybridization signal in the spinal cord. (*C–E*) Sagittal sections of a 10.5-day embryo. Arrow in *E* points to the posterior diencephalon. (*F*) Transverse section of the posterior spinal cord of a 10.5-day embryo. DI, diencephalon; ME, mesencephalon; MY, myelencephalon; PR, prosencephalon; SC, spinal cord; TE, telencephalon.

these stripes is <50 μm and they were detected in only several adjacent coronal sections. This expression of *Dbx* is very restricted and may serve to specify a limited number of cells within the cerebral cortex.

DISCUSSION

Murine homeobox genes, like their *Drosophila* homologs, act to determine the regional and cellular identities during embryogenesis (17–21). The region-specific expression patterns of homeobox genes determine their possible domains of function. Our results show that *Dbx* is expressed in an extremely restricted manner in many regions of the embryonic CNS. This suggests that *Dbx* specifies a small group of neural cells during development.

The telencephalon has been much investigated because it is the control center of the higher brain functions. In the telencephalon of 12.5-day embryos, *Dbx* is expressed in a small group of cells. It is localized in the postmitotic cells of the basal ganglia and cerebral cortex, unlike the *Tes-1* gene, which is expressed in the proliferating ganglionic eminence. Some of the *Dbx*-expressing cells may be prospective neocortex that differentiates early during embryogenesis (22). It is significant that regional expression in the cortical plates occurs at 12.5 days, prior to the arrival of thalamic input, suggesting a beginning of intrinsic specification as suggested by Rakic (23).

Within the hindbrain and spinal cord, *Dbx* labeling is limited to cells within the mitotically active ventricular zone (Fig. 4 *E*, *N*, and *O*). Transcripts detected in the posterior diencephalon and metencephalon are also restricted to regions next to the lumen (Fig. 4 *K* and *L*). This suggests that *Dbx* expression may be limited to only mitotically active progenitor cells in these regions. This is supported by the differences in expression patterns between anterior and posterior spinal cord. Since the maturation of neuroblasts in the spinal cord follows an anterior-to-posterior gradient, the posterior spinal cord contains more progenitor cells. The posterior spinal cord also contains a higher percentage of *Dbx*-expressing cells than the anterior spinal cord.

In the mammalian CNS, neuronal cell bodies sharing similar functions are often organized into columns. We have demonstrated that *Dbx* is expressed within the spinal cord and hindbrain as two thin columns of neuroblasts. This expression is continuous through the mesencephalon and is also found more anteriorly in the diencephalon and telencephalon. Thus, the *Dbx* gene is expressed in many regions of the CNS; however, its expression is highly localized within each region. This suggests that *Dbx*-positive cells may give rise to neurons with specific functions. It is postulated that neuroblasts in the hindbrain and spinal cord migrate radially as they differentiate. If the migration of *Dbx*-positive cells follow radially aligned routes, they will give rise to the preganglionic neurons of the visceral motor system (24). This suggests that *Dbx* may participate in the development of the autonomic motor system. Expression of *Dbx* in the anlage of the hypothalamus, which is the control center of the autonomic system, supports such a view. This raises the possibility of the functional specialization of divergent homeobox genes within particular subsets of cell types. However, it is also possible that, after differentiation, the *Dbx*-positive cells migrate to unrelated regions of the CNS, where they have functions yet to be determined.

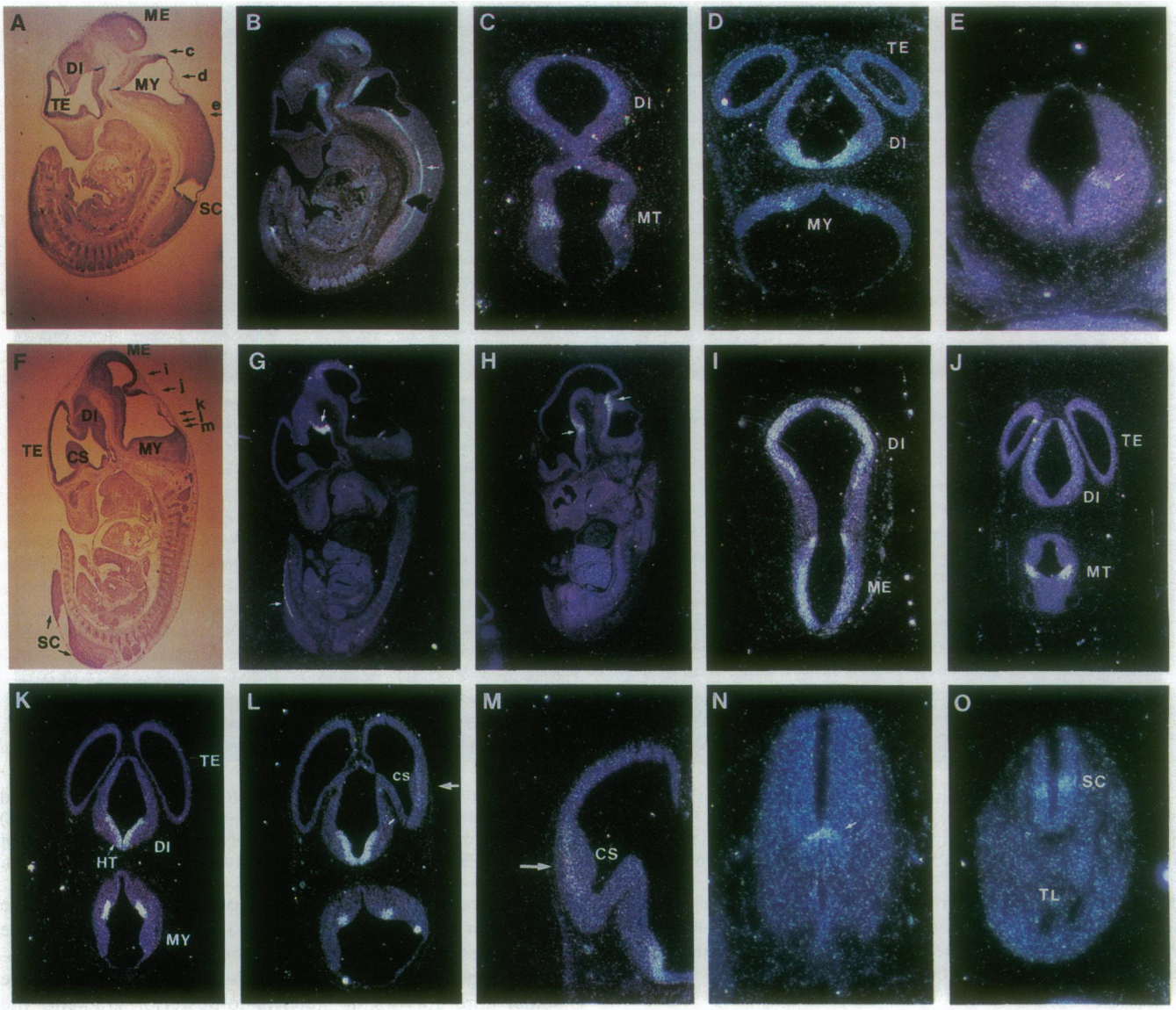

FIG. 4. *In situ* hybridization analysis of *Dbx* expression in 11.5- and 12.5-day mouse embryos. (*A* and *B*) Light- and dark-field photomicrographs of a sagittal section of an 11.5-day embryo. Arrow in *B* points to the hybridization signal in the spinal cord. (*C–E*) Coronal sections of an 11.5-day embryo. Levels of sectioning are from cranial flexure (*C*) to the hindbrain (*E*) as indicated by the corresponding arrows c–e in *A*. Arrow in *E* points to the lateral limit of the hybridization signal in the hindbrain. (*F–H*) Sagittal sections of 12.5-day embryos: light-field photomicrograph (*F*), dark-field photomicrograph of a section more medial than that in *F* (*G*), and a more medial section of another 12.5-day embryo with arrows pointing to the labeling in diencephalon and metencephalon (*H*). (*I–M*) Coronal serial sections of a 12.5-day embryo. Levels of the sections in *I–M* are progressively more posterior as shown by corresponding arrows i–m in *F*. *I* is a rather anterior section in which only two brain vesicles are shown. *J–L* show the transition from metencephalon to myelencephalon in which the hybridization signal is continuous. Arrows in *L* and *M* point to the signal in the telencephalon. Note that the levels of sectioning are slightly off the coronal level and that hybridization signals appear in only one of the telencephalon lobes in each section. (*N* and *O*) Transverse sections of thoracic spinal cord and tail showing the sacral spinal cord. CS, corpus striatum; DI, diencephalon; HT, hypothalamus; ME, mesencephalon; MT, metencephalon; MY, myelencephalon; SC, spinal cord; TE, telencephalon; TL, tail.

Several families of mammalian developmental regulatory genes have been shown to be expressed in spatially restricted patterns within the CNS. Homeobox genes, PAX genes, and POU genes are examples (2, 25, 26). They specify regions along the anteroposterior and dorsoventral axes of the embryo. The expression patterns of some *Hox*, Pax (paired-box), and POU genes overlap with that of *Dbx*, which introduces the potential for cross-regulatory interactions among these genes that may further delineate specific regions or classes of cells within the CNS.

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