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26. We thank E. Amento for the BT4 cell line, J. Kaplan for review, R. Byington, A. Durno, and T. Paradis for technical assistance, and D. Riley for manuscript preparation. This study was supported by PHS grants CA 09382-04, CA 27569, and CA 12464 from the National Cancer Institute, a Massachusetts Department of Public Health Contract and the Mashud A. Mezerhane B. Fund. M.V. is supported by the Swiss National Science Foundation.

28 November 1986; accepted 2 February 1987

Region-Specific Expression of Two Mouse Homeo Box Genes

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Mammalian homeo box genes have been identified on the basis of sequence homology to *Drosophila* homeotic and segmentation genes. These studies examine the distribution of transcripts from two mouse homeo box genes, Hox-2.1 and Hox-3.1, throughout the latter third of prenatal development. Transcripts from these genes are regionally localized along the rostro-caudal axis of the developing central nervous system, yielding expression patterns very similar to patterns of *Drosophila* homeotic gene expression.

MANY LABORATORIES ARE CURrently pursuing three general strategies that should help to define specific genes that regulate morphogenesis and cellular differentiation during vertebrate development. The first strategy is the isolation of region-, stage-, or tissue-specific embryonic gene products (RNAs or proteins), with the anticipation that these localized factors are involved in specific determinative events (*1*). The second uses insertional mutagenesis to induce developmental mutations that can be molecularly characterized after using the inserted sequences as a tag for the cloning of the disrupted genes (*2*). The third approach, and the one that we are pursuing, is the analysis of vertebrate genes containing sequences homologous to morphogenetic loci of the fruit fly, *Drosophila melanogaster* (*3*).

We and others (*4–11*) have previously reported the isolation of several murine genes containing sequences homologous to the "homeo box sequences" found in many of the homeotic and segmentation genes of *Drosophila* (*12–14*). On the basis of sequence conservation, homeo boxes are currently divided into two classes: the Antennapedia class and the Engrailed class.

In *Drosophila*, Antennapedia class homeo boxes have been empirically defined as highly conserved, 180-bp, open reading frames found in various genes of the Antennapedia and Bithorax gene complexes (*13*). Each of the homeotic genes of these complexes appears to be required for the proper morphogenesis of a distinct region along the anteroposterior axis of the fruit fly (*14, 15*). Mutant alleles of these genes can cause cells from one region of the embryo to form structures normally found elsewhere in the body. The region-specific functions of these genes are reflected by region-specific patterns of expression. To a first approximation, the expression of any one of these genes during embryonic and larval development appears to be spatially restricted to those cells that will give rise to the body segments most disrupted by mutations in that particular gene (*13, 16–19*).

We have previously proposed that mammalian homeo box genes of the Antennapedia class might perform similar region-specific determinative functions during mammalian development (*4, 10*). This hypothesis is supported by the spatially restricted expression of a murine homeo box gene, Hox-3.1, within the newborn and adult central nervous system (CNS) (*10*). Another possibility that has received some experimental support is that mammalian homeo box genes are involved in the differentiation of specific cell or tissue types (*5, 6, 8, 10, 11*). Although not mutually exclusive, these two hypotheses lead to different expected patterns of mammalian homeo box gene expression: the first to patterns that are region-specific, irrespective of cell or tissue type; and the second to patterns that are cell type–specific.

We recently reported that Hox-3.1 expression within the newborn mouse CNS is spatially restricted to cells posterior to the third cervical vertebra (*10*). We have now examined the distribution of Hox-2.1 transcripts within the CNS of the newborn mouse. The Hox-2 complex on chromosome 11 contains at least four homeo box sequences. The Hox-2.1 and Hox-3.1 probes used for Northern blot and in situ hybridization analysis are shown in Fig. 1a. The Hox-2.1 probe used was a 265-bp Hae III fragment that contains all but the first 10 bp of the homeo box. To date, only one homeo box sequence, Hox-3.1, has been identified in the Hox-3 locus on mouse chromosome 15 (*10*). The Hox-3.1 probe used was a 320-bp Hae III fragment that contains the entire homeo box sequence and 140 bp of flanking sequences (*10*). Hox-2.1 transcripts of between 1.7 and 1.9 kb were detected by Northern blot analysis with RNA samples from the brain (Fig. 1b, lane 1) and the cervical and thoracic regions of the spinal column (lanes 2 and 3, respectively). No transcripts were detectable in the RNA sample from the lumbar spinal column (lane 4). Figure 1c shows the results of a control hybridization of the same filter with a β_2-microglobulin probe, demonstrating comparable amounts of RNA in each track. When this same filter was hybridized with a probe from the Hox-3.1 gene (*10*), tran-

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scripts were detected in the cervical, thoracic, and lumbar spinal column; but no transcripts were detected in the brain.

To more precisely determine the differences in the spatial distributions of transcripts from these two homeo box genes, we have performed in situ hybridization to serial sections of newborn brain and spinal column with Hox-2.1 and Hox-3.1 probes in parallel (Fig. 2). Within the newborn mouse brain, the Hox-2.1 probe specifically labeled only the medulla, the most caudal region of the hindbrain (Fig. 2, a/a′). More rostral regions showed only weak labeling comparable to the nonspecific labeling we observed with the Hox-3.1 probe (Fig. 2, d/d′) and a Hox-2.1 control probe of the opposite orientation. In addition, Hox-2.1 transcripts were detectable in the cervical spinal cord (Fig. 2, b/b′) but not in the thoracic spinal cord (Fig. 2, c/c′). As expected on the basis of our previous experiments (*10*), no Hox-3.1 transcripts were detected in the brain or the anterior part of the cervical spinal cord (Fig. 2, d/d′ and e/e′), but they were easily detected in the anterior region of the thoracic spinal cord (Fig. 2, f/f′).

Thus, transcripts encoded by the Hox-2.1 and Hox-3.1 genes display different spatial localizations within the CNS of the newborn mouse. We have also carried out experiments to determine whether similar patterns of expression are found during earlier stages of development. Figure 3 illustrates the results of an in situ hybridization experiment performed with serial sagittal sections of an embryo 13.5 days post coitum (pc). At this stage, Hox-2.1 transcripts were again detected in the medulla and spinal cord, but not in more rostral regions of the CNS (Fig. 3, a/a′ and b/b′). Hox-3.1 expression within the CNS again appeared caudal to the third cervical vertebra (Fig. 3, c/c′ and d/d′). Thus, the regional distribution of transcripts from these two genes within the CNS may remain constant throughout the final week of prenatal development. Although not readily apparent in the figure, weak labeling of several thoracic vertebrae of the 13.5-pc embryo was also observed with the Hox-3.1 probe (Fig. 3, c/c′). Identical results have been obtained at least three times for each probe. In the two experiments in which control probes of the opposite orientation were used, no tissue or organ of the embryo was labeled above background by the control probes. The use of the Hox-2.1 and Hox-3.1 probes in parallel serves as an additional control for the specificity of the hybridization signals, since their homeo box sequences are 64% homologous at the DNA level.

The observed patterns of transcript accumulation have been confirmed by performing in situ hybridization to serial cross sections of the 13.5-pc embryo (Fig. 4). Hox-2.1 transcripts were abundant in the medulla (Fig. 4, a/a′) but not in the thoracic spinal cord (Fig. 4, b/b′). In contrast, Hox-3.1 transcripts were not detected in cross sections at the level of the hindbrain (Fig. 4, c/c′) but were abundant in the anterior thoracic region of the spinal cord (Fig. 4, d/d′). At this stage, Hox-3.1 transcripts within the spinal cord also displayed an asymmetric distribution along the dorso-ventral axis. Hybridization was observed predominantly over the ventral two-thirds of the spinal cord (Fig. 4, d/d′). This type of dorso-ventral localization was not observed in the newborn spinal cord (*10*) (Fig. 2, f/f′).

This report demonstrates the regionally localized expression of two mouse homeo box genes, Hox-2.1 and Hox-3.1, along the rostro-caudal axis of the CNS of the developing mouse. Each gene displayed a unique anterior boundary of expression within the CNS, and these boundaries persisted throughout the latter third of prenatal development. Thus, within the CNS of both the 13.5-pc embryo and the newborn mouse, Hox-3.1 expression appears restricted to the spinal cord caudal to the third cervical vertebra, with the highest levels of expression occurring just caudal to this vertebra. At these same stages, Hox-2.1 expression within the CNS appears restricted to the medulla and spinal cord, with the highest levels of expression observed in the medulla. This

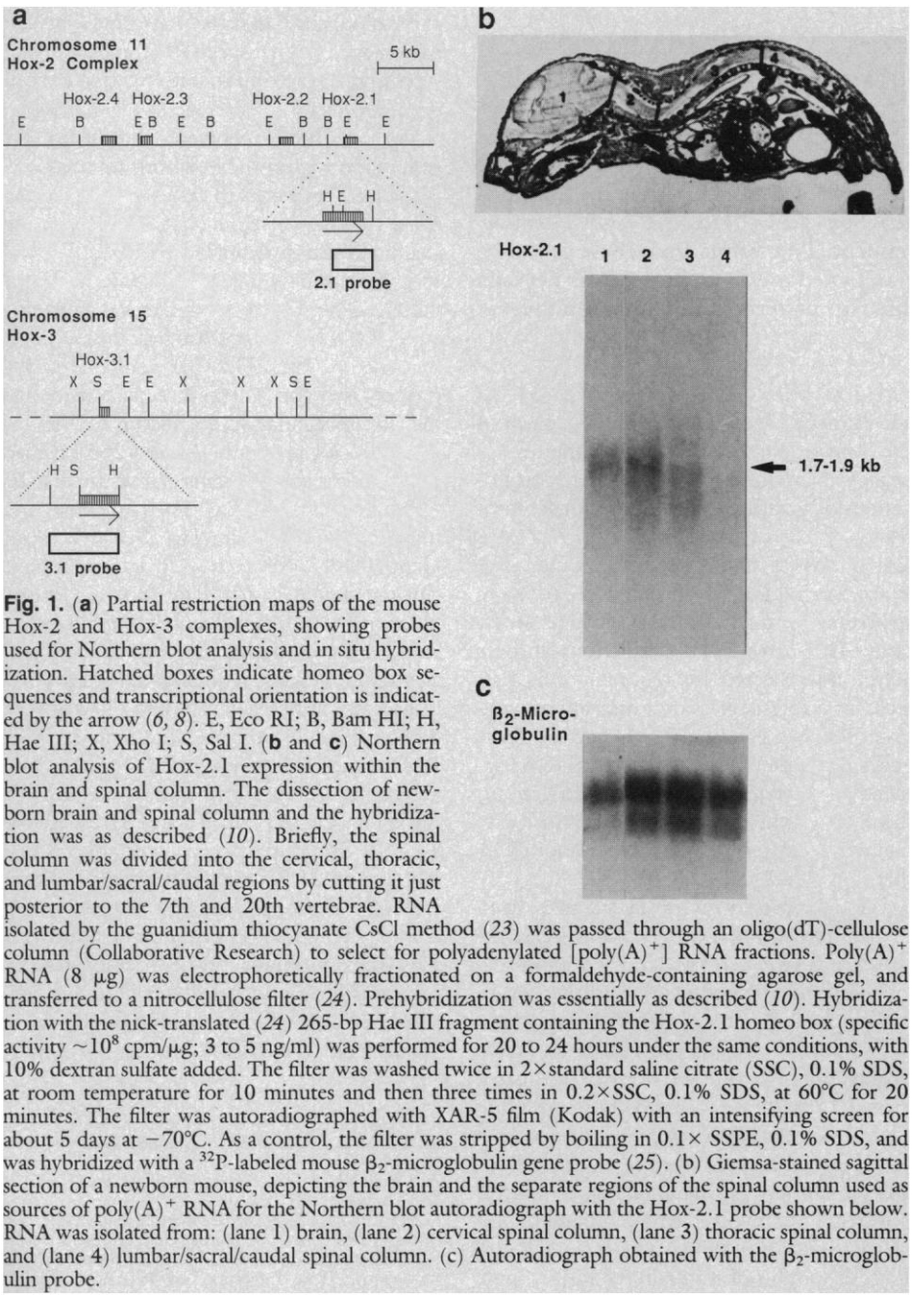

Fig. 1. (**a**) Partial restriction maps of the mouse Hox-2 and Hox-3 complexes, showing probes used for Northern blot analysis and in situ hybridization. Hatched boxes indicate homeo box sequences and transcriptional orientation is indicated by the arrow (*6*, *8*). E, Eco RI; B, Bam HI; H, Hae III; X, Xho I; S, Sal I. (**b** and **c**) Northern blot analysis of Hox-2.1 expression within the brain and spinal column. The dissection of newborn brain and spinal column and the hybridization was as described (*10*). Briefly, the spinal column was divided into the cervical, thoracic, and lumbar/sacral/caudal regions by cutting it just posterior to the 7th and 20th vertebrae. RNA isolated by the guanidium thiocyanate CsCl method (*23*) was passed through an oligo(dT)-cellulose column (Collaborative Research) to select for polyadenylated [poly(A)$^+$] RNA fractions. Poly(A)$^+$ RNA (8 μg) was electrophoretically fractionated on a formaldehyde-containing agarose gel, and transferred to a nitrocellulose filter (*24*). Prehybridization was essentially as described (*10*). Hybridization with the nick-translated (*24*) 265-bp Hae III fragment containing the Hox-2.1 homeo box (specific activity ~10^8 cpm/μg; 3 to 5 ng/ml) was performed for 20 to 24 hours under the same conditions, with 10% dextran sulfate added. The filter was washed twice in 2×standard saline citrate (SSC), 0.1% SDS, at room temperature for 10 minutes and then three times in 0.2×SSC, 0.1% SDS, at 60°C for 20 minutes. The filter was autoradiographed with XAR-5 film (Kodak) with an intensifying screen for about 5 days at −70°C. As a control, the filter was stripped by boiling in 0.1× SSPE, 0.1% SDS, and was hybridized with a ^{32}P-labeled mouse β_2-microglobulin gene probe (*25*). (b) Giemsa-stained sagittal section of a newborn mouse, depicting the brain and the separate regions of the spinal column used as sources of poly(A)$^+$ RNA for the Northern blot autoradiograph with the Hox-2.1 probe shown below. RNA was isolated from: (lane 1) brain, (lane 2) cervical spinal column, (lane 3) thoracic spinal column, and (lane 4) lumbar/sacral/caudal spinal column. (c) Autoradiograph obtained with the β_2-microglobulin probe.

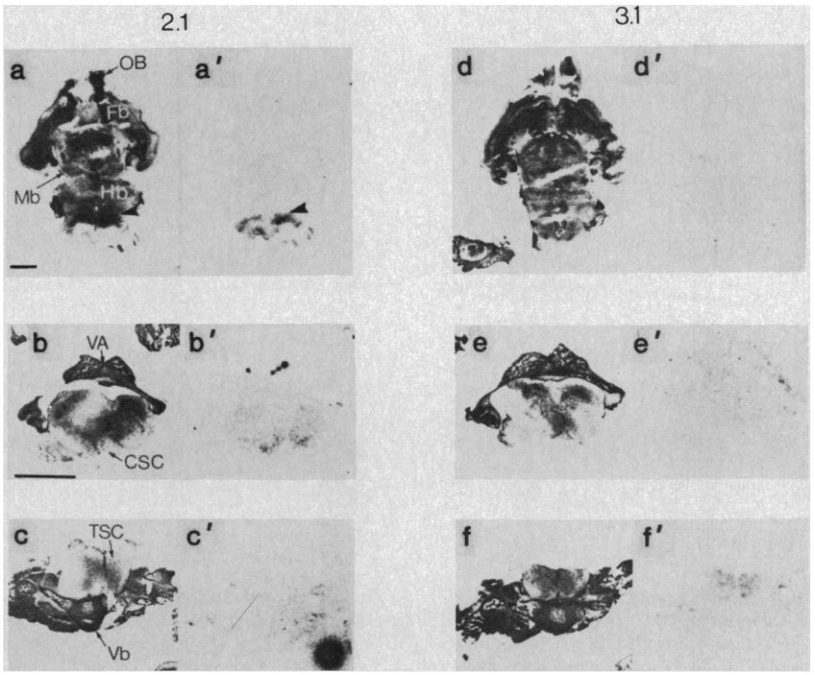

Fig. 2. In situ hybridization analysis of Hox-2.1 expression within the CNS of the newborn mouse. Hae III fragments (Fig. 1a) were cloned into the Gemini vector system (Promega Biotec), and ^{35}S-labeled, single-stranded RNA probes (specific activity $\approx 2 \times 10^8$ cpm/μg) were prepared. Probes were hybridized in parallel to serial cryostat sections of the newborn brain and spinal column (*10*). As an internal control, the brain and several regions of the spinal column from the same animal were embedded in one block so that sections from various levels of the CNS could be processed on the same microscope slide. Hybridization was carried out overnight at 50°C, and the final wash conditions were as follows: 2×SSC, 50% formamide, 0.1% β-mercaptoethanol for 30 minutes at ~58°C, followed by 0.1× SSC, 0.1% β-mercaptoethanol for 30 minutes at ~58°C. After hybridization and washing, the sections were exposed to Kodak XRP-1 film for 10 days and stained with cresyl violet acetate. (**a–c**) Photographs of stained sections after hybridization with the Hox-2.1 probe. (**a′–c′**) Autoradiographs obtained from sections in a–c. (**a/a′**) Horizontal section through newborn brain. Arrowheads indicate the specific labeling of the medulla. OB, olfactory bulb; Fb, forebrain; Mb, midbrain; Hb, hindbrain. (**b/b′**) Cross section through newborn spinal column at the level of the first cervical vertebra. VA, vertebral arch; CSC, cervical spinal cord; (**c/c′**) Cross section through newborn spinal column at the level of the first thoracic vertebra. No structures are labeled significantly above background. TSC, thoracic spinal cord; Vb, vertebra. (**d–f/d′–f′**) Sections after hybridization with the Hox-3.1 probe. (**d/d′**) Horizontal section through newborn brain. No structures are labeled significantly above background. (**e/e′**) Cross section through newborn spinal column at the level of the first cervical vertebra. No structures are labeled above background. (**f/f′**) Cross section through newborn spinal column at the level of the first thoracic vertebra. Bars ≈ 1 mm. Panels (a/a′) and (d/d′) are at the same magnification; (b/b′), (c/c′), (e/e′), and (f/f′) are at the same magnification.

distribution of Hox-2.1 transcripts is consistent with the results of Jackson *et al.* (*6*) who detected Hox-2.1 transcripts within the CNS of 12.5-pc embryos by means of a ribonuclease protection assay.

Drosophila homeotic selector genes display similar region-specific patterns of expression along the antero-posterior axis of the CNS of the developing fly embryo and larva (*13, 16–19*). The expression of each of these genes is localized to distinct but overlapping regions of the CNS. These localized patterns of expression reflect the region-specific determinative functions of these genes. The patterns are consistent with previous proposals that the expression of homeotic selector genes serves as a cellular memory of determinative decisions made early in development (*20*). These decisions would confer distinct segmental identities to cells along the antero-posterior axis.

The distinct, spatially restricted patterns of expression we observed for the Hox-2.1 and Hox-3.1 genes on the 13th and 20th day of mouse development are consistent with a patterning function similar to that performed by the fly genes. Thus Hox-2.1 expression may serve to determine the fate of cells in the region of the medulla and anterior spinal cord, whereas Hox-3.1 may perform determinative functions posterior to the third cervical vertebra. Although our results do not rule out that Hox-2.1 and

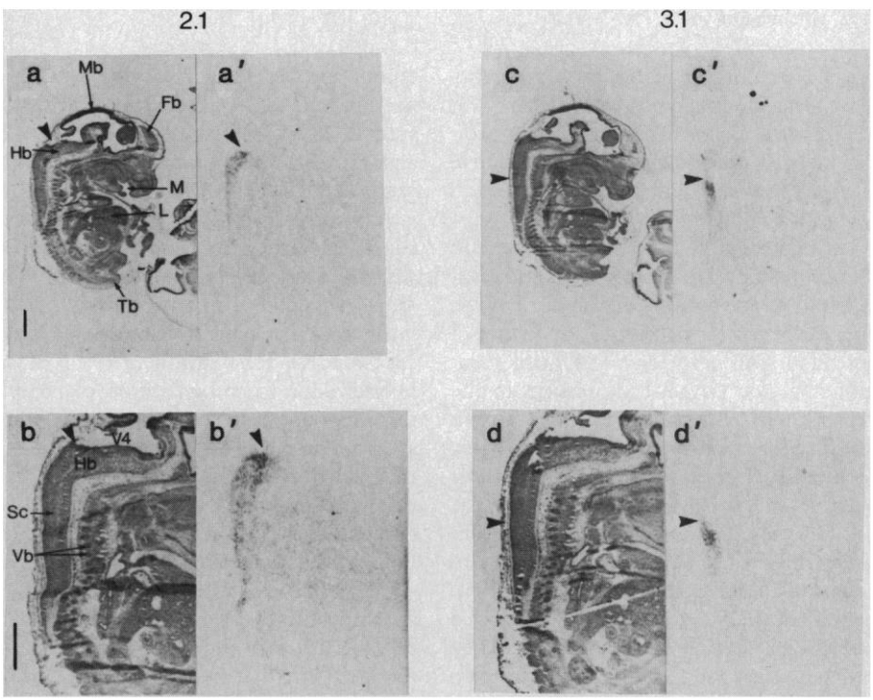

Fig. 3. In situ hybridization analysis (as in Fig. 2) of Hox-2.1 and Hox-3.1 expression in serial sagittal sections of the 13.5-pc mouse embryo. Sections were exposed to Kodak XRP-1 film for 14 days and stained with Giemsa. (**a–c**) Photographs of stained sections; (**a′–c′**) corresponding autoradiographs. Arrowheads indicate the rostral boundary of specific labeling. (**a/a′**) Hybridization with the Hox-2.1 probe. Fb, forebrain; Mb, midbrain; Hb, hindbrain; M, mouth; L, liver; Tb, tailbud. (**b/b′**) Higher magnification view of the hindbrain and spinal cord region after hybridization with the Hox-2.1 probe. V4, fourth ventricle; Hb, hindbrain; Sc, spinal cord; Vb, vertebrae. (**c/c′**) Hybridization with the Hox-3.1 probe. (**d/d′**) Higher magnification view of the hindbrain and spinal cord region after hybridization with the Hox-3.1 probe. Bars ≈ 1 mm. Panels (a/a′) and (c/c′) are at the same magnification; (b/b′) and (d/d′) are at the same magnification.

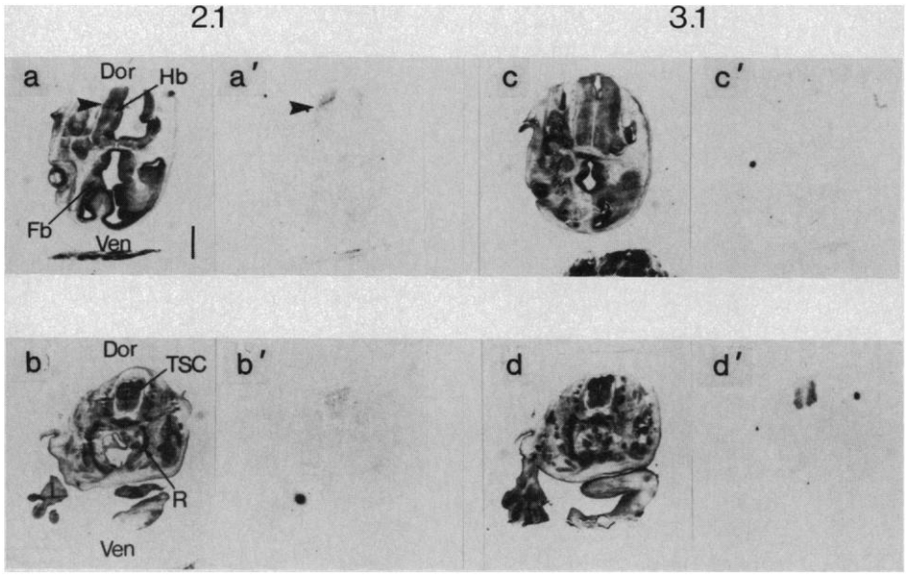

Fig. 4. In situ hybridization analysis of Hox-3.1 and Hox-2.1 expression in serial cross sections of the 13.5-pc mouse embryo. Probes were hybridized in parallel to cross sections. Hybridization time was 5 hours, and final wash conditions were 1×SSC, 50% formamide, 0.1% β-mercaptoethanol for 30 minutes at 50°C. Sections were then exposed to Kodak XRP-1 film for 6 days, dipped in Kodak NTB-2 emulsion, developed after 1 month (*10*), and stained with Giemsa. (**a', b'**) Photographs of stained sections after hybridization with the Hox-2.1 probe. (**a', b'**) XRP-1 autoradiographs obtained from the sections in a and b. (**a'/a'**) Cross section at the level of the hindbrain. Arrowhead indicates the rostral boundary of specific labeling. Dor, dorsal; Ven, ventral; Fb, forebrain; Hb, hindbrain. (**b', b'**) Cross section at the level of the first thoracic vertebra. The spinal cord at this level is labeled only weakly above background. Dor, dorsal; Ven, ventral; TSC, thoracic spinal cord; L, limb; R, rib. (**c, d**) Photographs of stained sections after hybridization with the Hox-3.1 probe. (**c', d'**) XRP-1 autoradiographs obtained from the section in c and d. (**c/c'**) Cross section at the level of the hindbrain. No structures are labeled above background. (**d/d'**) Cross section at the level of the first thoracic vertebra. Bar, ≈1 mm. All at same magnification.

Hox-3.1 are important for the differentiation of specific cell types within the CNS, the observed spatial restrictions of expression suggest that such cytodifferentiative functions, if any, would have to be confined to specific regions of the CNS.

It is important to note that our data and its interpretation are subject to several caveats. First, we have discussed the patterns we observed in terms of absolute boundaries of expression. Our best evidence for this point of view is for Hox-3.1 expression in the newborn CNS. In this case, the in situ hybridization evidence was corroborated by Northern analysis in which we were unable to detect any Hox-3.1 transcripts within the brain. However, since no tissue sample containing only nervous tissue located rostral to the medulla has been subjected to Northern analysis, the expression boundary observed for Hox-2.1 has only been demonstrated by the less sensitive in situ hybridization technique. It thus remains possible that a low level of Hox-2.1 expression, below the limit of detection by in situ hybridization, could be demonstrated rostral to the medulla by a more sensitive assay. The same qualification applies to the expression boundaries observed in the 13.5-pc CNS. However, even if they separate quantitative and not absolute differences in the level of expression, the abrupt and highly reproducible boundaries we observe still indicate notable region-specific differences in the level of Hox-2.1 and Hox-3.1 expression along the rostrocaudal axis of the CNS.

A second caveat concerns the apparent constancy of the expression boundaries. Our data are consistent with the expression patterns remaining constant throughout the final week of prenatal development. However, since we have not performed in situ hybridization to embryos from each day of this developmental period, we have not shown that the boundaries are the same on each day between 13.5 pc and birth. It remains a possibility that the expression boundaries established in the 13.5-pc embryo are altered in more advanced embryos and then return to the 13.5-pc position by the time of birth. We consider this possibility less likely than the simpler interpretation that the patterns remain constant.

With the Hox-3.1 probe we also observe weak labeling of several thoracic vertebrae of the 13.5-pc embryo. Although again region-specific, the anterior boundary of Hox-3.1 expression within the vertebrae is more posterior than that of spinal cord expression. This is not unprecedented as a pattern of expression of a homeo box gene, as *Ultrabithorax* neural and mesoderm expression boundaries are out of register in late stage *Drosophila* embryos (*17*).

Note added in proof: The expression patterns described here for the mouse Hox-2.1 gene are consistent with Northern analysis of human Hox-2.1 expression by Simeone *et al.* (*21*). We also note that position-specific expression of the mouse homeo box gene Hox-1.5 (Mo-10) has recently been demonstrated by Gaunt *et al.* (*22*).

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25. We gratefully acknowledge P. Leder for the β_2-microglobulin probe, K. Gordon for her participation in the early phases of this project, N. McGinnis and V. Salerno for excellent technical assistance, S. Pafka for photographic expertise. A. Fainsod and L. Bogarad for help with in situ hybridization, and M. Constantine-Paton for helpful comments and the use of equipment. M.F.U. is the recipient of a Life and Health Insurance Medical Research Fund Scholarship. Supported in part by grants from NIH (GM09966) to F.H.R. and from NSF and the Searle Scholar Program to W.M.

15 October 1986; accepted 30 December 1986