Region-specific enhancers near two mammalian homeo box genes define adjacent rostrocaudal domains in the central nervous system

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To gain insight to the mechanisms underlying region-specific gene expression in mammalian development, we investigated the regulatory DNA associated with the proximal promoter of two homeo box genes, murine Hox-1.3 and human Hox-5.1. Using lacZ gene fusions in transgenic mice, we identified regulatory elements in the 5'-flanking sequences of the Hox-1.3 and the Hox-5.1 genes that specifically direct β-galactosidase expression to the brachial and the upper cervical regions (respectively) of the central nervous system (CNS). These two elements act at the transcriptional level, are active in either orientation, and confer region-specific expression to unrelated promoters, satisfying the criteria for enhancer elements. The two spatial domains defined by these enhancers are directly adjoining, extend along the rostrocaudal axis for the same span of 6–7 metameres, and represent specific subsets of the overall CNS regions expressing all endogenous Hox-1.3 or Hox-5.1 transcripts. The adjacent domains in the developing murine CNS that express Hox-1.3 and Hox-5.1 gene fusions are strikingly reminiscent of the adjacent stripes of expression in Drosophila embryos seen with Sex combs reduced and Deformed, the two Drosophila homeotic genes most homologous to Hox-1.3 and Hox-5.1, respectively. These findings represent the first demonstration of region-specific mammalian enhancers and raise the possibility that the mammalian CNS may be subdivided into a series of rostrocaudal domains on the basis of the activity of enhancers near homeo box genes.

[Key Words: region-specific gene expression; homeo box genes Hox-1.3 and Hox-5.1; mammalian CNS]

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The homeo box, a 180-bp DNA segment that encodes a protein domain with specific DNA binding properties, has been found in several Drosophila genes implicated in the control of development (McGinnis et al. 1984a; Scott and Weiner 1984; for review, see Gehring 1987). Products of these genes may activate or repress transcription of specific target genes during embryogenesis (Hiromi and Gehring 1987; Jaynes and O'Farrell 1988). Vertebrate homeo box-containing genes have also been identified and isolated (Carrasco et al. 1984; McGinnis et al. 1984b; McGinnis et al. 1984c; for review of murine genes, see Fienberg et al. 1987) and have been found to be specific DNA-binding proteins (Fainsod et al. 1986; Odenwald et al. 1989) as well as regulators of transcription (Mangalam et al. 1989). A role for these genes in mammalian developmental processes has not yet been defined; however, ectopic expression of Hox-1.1 in craniofacial structures (Balling et al. 1989) or overexpression of Hox-1.4 in the colon (Wolgemuth et al. 1989) produces developmental abnormalities in transgenic mice. Evidence of homeotic transformation, namely the alteration of regionally identifiable structures (as exhibited by Drosophila homeotic mutants), was not observed. Therefore, it is still uncertain whether the mammalian homeo box-containing genes are functional homologs to those in Drosophila. The expression of mammalian homeo box genes of the Antennapedia class (Hox genes) in specific rostrocaudal regions of the neuroectoderm and mesoderm, however, indicates that these genes may play an important role in the specification of the body plan (Holland and Hogan 1988). This hypothesis is significantly reinforced by the observation that, in striking similarity to the situation in Drosophila, Hox genes are also organized in clusters, and the order of the genes in the clusters corresponds to the order of their anterior limits of expression along the anterior–posterior axis (Boncinelli et al. 1988; Gaunt et al.

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was observed in metamere C3 or more anterior metatween the fourth cervical (C4) and second thoracic (T2) gion of the spinal cord, that is, to the CNS region be-cause of the expression of a defined 912-bp regulatory region of mouse embryos (Zakany et al. 1988). These studies de-note that the observed β-galactosidase pattern specifically reflects the spatial expression of the proximal promoter located within the 912-bp fragment. In this context, we note that the reported in situ RNA pattern (Dony and Gruss 1987) was obtained with a common probe that detected both proximal and distal transcripts. Alternatively, the β-galactosidase pattern may accurately reflect the Hox-1.3 proximal protein pattern, which may differ from the proximal Hox-1.3 RNA pattern as a result of translational control mechanisms. This latter situation is possible because transgene also contains Hox-1.3 5'-untranslated mRNA sequences which could contribute to translational control. Finally, it is also pos-sible that the expression pattern observed in the brachial spinal cord is artifically created as a result of the insert-ion of lacZ sequences.

We conducted experiments that argue against the role of translational control or artificially created regulatory elements and that demonstrate the importance of tran-scriptional control in the observed spatial expression of Hox-1.3/lacZ genes. We extended our analysis to the human homeo box gene Hox-5.1 (Mavilio et al. 1986; Featherstone et al. 1988) and showed that the regulatory mechanisms and the spatial and temporal expression patterns found with Hox-1.3 are not unique but can be observed with another homeo box gene. Here, we report our analysis of the spatial and temporal expression of Hox-1.3 and Hox-5.1 gene fusions in transgenic mice. Inter-estingly, this analysis has led to the identification of two region-specific enhancers near these genes and to the definition of two adjacent rostrocaudal domains in the developing CNS.

Results

Hox-1.3 regulatory elements are separable into a proximal promoter element and an orientation-independent element(s) required for region-specific expression in the embryonic spinal cord

A 912-bp DNA fragment located immediately 5' to the Hox-1.3 protein-coding region fused to the lacZ gene (construct B/1.3/lacZ in Fig. 1) was sufficient to direct β-galactosidase expression to dorsal cells in the brachial region of the embryonic spinal cord, that is the region between the fourth cervical (C4) and second thoracic (T2) metameres (Zakany et al. 1988). However, a lacZ fusion with only the 3' 308 bp of this DNA fragment, which contains the proximal Hox-1.3 promoter and 5'-untranslated mRNA sequences (termed fragment C, construct 1.3/lacZ in Fig. 1), was inactive, indicating that the deleted 604-bp fragment (termed fragment B) is necessary for region-specific expression. Because the only promoter elements in the 912-bp fragment have been mapped to fragment C (Murphy et al. 1988; Zakany et al. 1988; Garbern et al. 1989), it follows that no pro-moter elements and only regulatory elements are present on fragment B. To determine if these regulatory elements have the enhancer property of functioning in either direction, and to demonstrate that the β-galacto-
Figure 1. Structure and expression of homeo box fusion genes in transgenic mice. (A) Structure of the Hox-1.3 genomic region and proximal transcript. Exons are shown in boxes, translated region in close parallel lines, and the homeobox in black. (B) Structure and expression of transgenes. All schematic representations are to scale except for the lacZ gene and fragment D. DNA fragments B and Hox-5.1, which contain enhancer elements, are depicted as large arrows, whereas other DNA fragments are depicted as boxes. Small arrows above fragment C indicate start of transcription from the Hox-1.3 proximal promoter. Expression is listed as the number of expressing embryos (lines) over the total number of transgenic mice obtained per construct. *Data from Zakany et al. 1988. †Floorplate cells in mesencephalon also express lacZ as observed previously (Zakany et al. 1988). ‡Additional site of expression is the brachial peripheral nervous system, which was observed in three embryos (data not shown). Two of five expressors are F0 embryos, the other three of five are expressing transgenic lines. (C) Structure of the human Hox-5.1 genomic region. Symbols used are as in A.

Figure 2. In situ detection of β-galactosidase activity in Bflip/1.3/lacZ and B/hsp68/lacZ transgenic embryos. (A) Whole-mount lateral view of day 12.5 embryo showing expression of Bflip/1.3/lacZ gene fusion in the brachial spinal cord region. (B) Dorsal view of embryo in A showing two columns of expressing cells. (C) Histological cross section of embryo in A shows β-galactosidase activity is observed only in the dorsal region of the spinal cord. [Magnification, 20×.] (D) Whole-mount lateral view of day 12.5 embryo expressing B/hsp68/lacZ gene fusion in the brachial region of the spinal cord. There is also a low level of expression in the sacral region of the spinal cord. (E) Dorsal view of embryo in D, showing mosaic expression of β-galactosidase within the brachial region. (F) Histological cross section of embryo in D shows expression of β-galactosidase is in the dorsolateral cells of the spinal cord exclusively. [Magnification, 20×.] Line in A and D is drawn to show plane of section in C and F, respectively. (SC) spinal cord.
β-galactosidase activity can be observed in the dorsal cells of the brachial spinal cord, as well as in the mesencephalon floorplate cells. This expression pattern is identical to that observed for the B/1.3/lacZ construct (Zakany et al. 1988) and indicates that regulatory elements within fragment B act in an orientation-independent manner. This result argues that fragment B contains enhancer-like elements that act in conjunction with fragment C to produce the region-specific pattern observed. However, this result does not define whether region specificity is mediated by the enhancer elements within fragment B, by the promoter elements, potential regulatory elements (Odenwald et al. 1989), the 5′-untranslated mRNA sequences on fragment C, or by a combination thereof. The next experiment was undertaken to address this issue.

Region-specific expression in the CNS of Hox-1.3/lacZ transgenic mice is controlled at the transcriptional level by an enhancer(s) located within a 604-bp fragment

The Hox-1.3 protein, when synthesized in the baculovirus system, binds at least one specific site within fragment C (Odenwald et al. 1989). This indicates that fragment C, in conjunction with Hox-1.3 protein, may be important in the regulation of the Hox-1.3 gene. Therefore, it was of interest to determine whether fragment C, while insufficient, is necessary for expression in the brachial spinal cord, or whether fragment B, while necessary, is also sufficient for the expression pattern observed. To determine whether region specificity can be directed by fragment B only, we replaced fragment C in B/1.3/lacZ gene with the promoter and 5′-untranslated sequences from the mouse hsp68 gene. These hsp68 sequences, when fused to the lacZ gene, are silent in mouse embryos in the absence of stress or nearby regulatory elements (Kothary et al. 1988); therefore, any β-galactosidase activity in transgenic embryos carrying the B/hsp68/lacZ gene should be attributable to regulatory elements on fragment B. Fragment B was placed upstream of the hsp68–lacZ fusion (construct B/hsp68/lacZ in Fig. 1), and transgenic mice were generated and analyzed as above. Of the eleven transgenic embryos obtained, five showed β-galactosidase activity at day 12.5 of gestation. Expression of the transgene in a representative embryo can be observed in the dorsolateral cells of the brachial spinal cord (Fig. 2D–F), mimicking the results observed when the Hox-1.3 promoter is regulated by the B fragment enhancer (constructs B/1.3/lacZ and Bflip/1.3/lacZ). This result indicated that 1) the Hox-1.3 promoter and 5′-untranslated sequences on fragment C are dispensable, 2) the region-specific expression of lacZ is produced at the transcriptional level as no Hox-1.3 5′-untranslated sequences are present in the hsp/lacZ RNA encoding β-galactosidase, and 3) the enhancer elements on fragment B are sufficient to confer region-specific expression onto an unrelated hsp68 promoter. With the B/hsp68/lacZ fusion gene, expression was not observed in the mesencephalon, an ectopic site observed in B/1.3/lacZ embryos. There is also a low level of β-galactosidase expression in the sacral region of the spinal cord. However, expression in this region was observed in only two B/hsp68/lacZ embryos out of five expressors and was observed occasionally in other Hox-1.3–lacZ transgenic mice (C.K. Tuggle, J. Zakany, and M.C. Nguyen-Huu, unpubl.), thus it is difficult to judge the significance of expression in this region.

Regulatory sequences near human Hox-5.1 direct lacZ expression to the upper cervical CNS of transgenic mice

To explore how general is the phenomenon of region-specific enhancers operating near homeo box genes, we searched for regulatory elements near other homeo box genes. The homeo box gene Hox-5.1 is located in a different complex, Hox-5. Like Hox-1.3, Hox-5.1 is expressed at the RNA level in the murine embryonic CNS, but the rostral boundaries of expression of the two genes are different [Featherstone et al. 1988; Gaunt et al. 1988]. The transcription pattern of both Hox-1.3 and Hox-5.1 is characterized by multiple mRNAs derived from more than one promoter [Mavilio et al. 1986; Zakany et al. 1988; Graham et al. 1989; L. Cianetti and C. Peschle, unpubl.]. We considered the possibility that sequences near the Hox-5.1 proximal promoter (i.e., the one proximal to the initiation codon) may function in an analogous manner to the identified enhancer region at the Hox-1.3 proximal promoter. Therefore we fused a 2832-bp HincII fragment located just 5′ to the proximal promoter (L. Cianetti and C. Peschle, unpubl.) and protein-coding region of the human Hox-5.1 gene (previously called c13, Mavilio 1986) upstream of the 1.3/lacZ fusion gene, which has been shown to be inactive in midgestational embryos [Zakany et al. 1988, construct 1.3/lacZ in Fig. 1]. This gene fusion (construct 5.1/1.3/lacZ in Fig. 1) was used to generate both transgenic embryos [which were analyzed 12 days after DNA microinjection] and transgenic mouse lines for analysis of transgene expression during gestation. Figure 3 shows whole mounts and a cross section of a representative embryo carrying this 5.1/1.3/lacZ fusion gene. Similar to B/1.3/lacZ, the 5.1/1.3/lacZ fusion gene shows tissue-specific expression in dorsolateral cells of the spinal cord. However, the interesting difference is that expression of 5.1/1.3/lacZ is found in the upper cervical region of the spinal cord (from the caudal myelencephalon to metamere C3) rather than in the brachial region (from metamere C4 to metamere T2). Expression in this upper cervical region was observed in five out of six independent transgenic embryos and lines (see table in Fig. 1), and must be attributable to the presence of Hox-5.1 regulatory elements on the transgene. As has been already observed with Hox-1.3/lacZ gene fusions, expression of Hox-5.1/1.3/lacZ in the upper cervical spinal cord does not recapitulate the entire Hox-5.1 RNA expression pattern as determined by in situ hybridization analysis, using probes that detect all forms of Hox-5.1 mRNA, expression has been detected in the myelencephalon, throughout the spinal cord and in several prevertebrate
Region-specific expression of Hox-5.1/1.3/lacZ hybrid gene in the upper cervical spinal cord of transgenic mice. [A] Whole-mount lateral view of day 12.5 embryo shows β-galactosidase expression exclusively in the upper cervical region of the spinal cord. [B] Dorsal view of the embryo in A showing two columns of expressing cells in the spinal cord. [C] Histological cross section of embryo in A shows β-galactosidase expression is limited to the dorsolateral cells of the spinal cord. (Magnification, 35 x.)

Expression of 5.1/1.3/lacZ is controlled at the transcriptional level by an enhancer element

It is likely that regulatory elements on the Hox-5.1 fragment control lacZ transcription from the heterologous Hox-1.3 promoter. Alternatively, Hox-5.1 promoter element(s) could be involved in transcription of lacZ in the upper cervical CNS. To distinguish between these possibilities, the Hox-5.1 fragment was reversed in orientation. Of six independent embryos carrying this construct (5.1flip/1.3/lacZ in Fig. 1), three were found to express the transgene in the same pattern as seen with the previous construct [data not shown]. This indicates that transcription of lacZ occurs from the Hox-1.3 proximal promoter and that regulatory elements on the Hox-5.1 fragment act independent of orientation. Because the Hox-1.3 promoter can be instructed by either the Hox-1.3 enhancer or the Hox-5.1 enhancer to become active in two different rostrocaudal regions of the CNS (Figs. 2 and 3), it is likely that the region specificity observed with Hox-5.1/1.3/lacZ gene fusions is encoded by the Hox-5.1 enhancer rather than by the Hox-1.3 promoter. On the other hand, it is not clear whether the
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tissue specificity observed is encoded solely by the Hox-5.1 enhancer or may result from interactions between the Hox-5.1 enhancer and the Hox-1.3 promoter. Gene fusions between the Hox-5.1 enhancer and the hsp promoter would help to answer this question.

Activity of the 5.1/1.3/lacZ transgene is temporally controlled with expression detectable from embryonic day 10.5 to day 14.5

The Hox-1.3/lacZ gene has been shown to be temporally regulated during gestation, such that expression of β-galactosidase begins on day 11.5, increases to maximal levels on day 12.5, persists on day 13.5, and is undetectable by day 14.5 (Zakany et al. 1988). To discover whether a similar temporal regulation is occurring with the Hox 5.1/1.3/lacZ gene, four transgenic lines were established and three lines showed expression of β-galactosidase in the upper cervical CNS of day 12.5 embryos. Expression of β-galactosidase during development for these lines was then investigated. Analysis of transgenic embryos at each day of gestation from day 9.5 to 15.5 indicates a temporal expression pattern similar (if somewhat more extensive) to that seen for the Hox-1.3/lacZ gene [Fig. 4]. Expression is first observed in day 10.5 embryos, continues through to day 14.5 embryos, with maximal expression on day 12.5 and 13.5. Expression is undetectable by day 15.5. This result is consistent with the finding that maximal expression of Hox-5.1 RNA was found at the earliest day of gestation examined, day 11, and decreased to low levels by day 15 (Featherstone et al. 1988). β-Galactosidase activity outside the upper cervical spinal cord in day 13.5 and older embryos is attributable to endogenous enzyme activity, because this activity is reproducibly observed in both transgenic and nontransgenic embryos.

Discussion

Region-specific enhancers in the developmental regulation of mammalian Hox genes

Region-specific expression is one prerequisite of genes controlling pattern formation during development. All mammalian homeo box genes investigated thus far show spatially restricted expression in the embryonic central nervous system and often in the prevertebrae (Holland and Hogan 1988). How are these region-specific patterns generated? Our results support a simple regulatory model that would place region-specific enhancer elements near homeo box genes to direct their expression to discrete body regions. The Hox-1.3 and Hox-5.1 regulatory regions identified here clearly direct transgene activity to discrete rostrocaudal domains of the developing mouse CNS and have properties characteristic of transcriptional enhancers: they behave as cis-acting elements to regulate gene expression in a positive manner, they act in an orientation-independent fashion, and they confer specificity onto unrelated promoters. We propose that a likely function of these enhancer elements is to activate transcription from the Hox-1.3 and Hox-5.1 proximal promoters within the specific CNS domains that have been defined here. Further analysis of the elements controlling the region-specific expression of other homeo box genes (Zakany et al. 1989) will be necessary to assess the generality of this model, but it is encouraging that our analysis has identified region-specific enhancers in the first two mammalian homeo box genes investigated. Whereas many mammalian tissue-specific enhancers have been identified and characterized (Maniatis et al. 1987; Atchison 1988), the mammalian enhancers reported here are the first to show region-specific activity. Tissue-specific control of transcription is often mediated by enhancers, which are believed to be composed of several smaller units or modules [Schaffner et al. 1988; Dynan 1989] whose effects are then combined and interpreted by the cell to produce specificity of expression. Thus, it will be of interest to dissect the enhancers defined here that have the ability to interpret positional information, as well as tissue-specific activity, into simpler components for comparative analysis. Deletion mapping and a comparison of the Hox-1.3 and Hox-5.1 enhancer regions will indicate DNA sequences important for temporal and tissue-specific CNS expression, and will also uncover sequences mediating region-specific expression within the CNS. Because the two enhancer regions identified here have been completely sequenced (Hox-1.3—Zakany et al. 1988, Hox-5.1—L. Cianetti and C. Peschle, unpubl.), we compared their primary structure to determine whether regions of similar sequence exist that could contribute to common or similar characteristics of Hox-1.3 and Hox-5.1 expression. Using the sequence analysis program of the GCG group [University of Wisconsin], the most similar region was TGCCCTCTGCTGTGCTATTGCA (15 of 18 in common, superscript nucleotides are untranslated sequences. In addition, the Hox-1.3 promoter and 5′-untranslated sequences. In addition, the Hox-1.3 promoter can be redirected to express β-galactosidase in the upper cervical spinal cord by regulatory elements near...
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Hox-5.1. These results argue strongly against the possibility of cloning artifacts and the role of translational control in generating the regional expression patterns observed. Our data are most consistent with the possibility that the β-galactosidase patterns observed may specifically reflect the domains of CNS expression of the proximal promoters of Hox-1.3 and Hox-5.1, and that RNA expression detected in the other CNS regions by in situ studies may reflect the activity of the distal promoters. This possibility is supported by several other considerations. First, it has already been demonstrated that the multiple Hox-1.3 transcripts are not regulated in parallel in the CNS; the ratio of distal to proximal transcripts changes dramatically between midgestation and birth (Zakany et al. 1988; Garbern et al. 1989). Second, because all sequences on the proximal transcript are present on the distal transcript(s), in situ RNA analysis cannot distinguish between regions expressing the proximal transcript alone and regions expressing both the distal and proximal transcripts. However, in situ analysis of transgene expression in Hox-1.3/lacZ embryos shows that lacZ RNA is present only in the brachial region of the spinal cord (A. Perez-Castro, C.K. Tuggle and M.C. Nguyen-Huu, in prep.). This result indicates that the β-galactosidase pattern is controlled at the RNA level, and may reflect the pattern of the proximal transcript. Finally, and most importantly, we believe that the patterns of expression observed with the Hox-1.3/lacZ and Hox-5.1/lacZ transgenes are biologically consistent with the postulated homology between the Drosophila homeotic gene complexes and the different mammalian Hox gene complexes, as outlined below.

For homeo box genes in the Drosophila ANT-C/BX-C and the mammalian Hox complexes, a striking correlation exists between the anterior limits of expression of an individual gene and the order of that gene within the complex (Boncinelli et al. 1988; Gaunt et al. 1988; Graham et al. 1989; Duboule and Dolle 1989; Giampaolo et al. 1989; Wright et al. 1989). Genes at the 5' end of the complexes are expressed more posteriorly, whereas genes at the 3' end of the complexes are expressed more anteriorly. On the basis of conservation of both gene structures and expression patterns, it has been suggested that the Hox complexes and the ANT-C/BX-C complexes share a common ancestry and may be truly homologous chromosomal regions (for review, see Akam 1989, Wright et al. 1989). Thus, we might expect an individual Hox gene to exhibit an anterior–posterior domain of expression similar to that of the Drosophila homeotic gene to which it is most similar in structure and position along the complex. For Hox-1.3 this is Sex combs reduced (Scr), while for Hox-5.1 this is Deformed (Dfd) (Graham et al. 1989; Duboule and Dolle 1989). Scr is expressed in a narrow region, including part of the posterior head and anterior thoracic region (Kuroiwa et al. 1985; Martinez-Arias et al. 1987). Interestingly, the β-galactosidase expression pattern in transgenic mouse embryos delineated by the Hox-1.3 enhancer spans the border between the lower cervical and anterior thoracic CNS regions. Dfd, the 3' neighbor to Scr in the ANT-C complex, is expressed in the posterior head, just anterior to the region expressing Scr (Martinez-Arias et al. 1987; Chadwick and McGinnis 1987). Similarly, the Hox-5.1/lacZ expression pattern is found entirely within the upper cervical CNS region, just anterior to the region expressing Hox-5.1/lacZ. Thus the adjacent expression in the murine CNS of the Hox-5.1/lacZ and Hox-1.3/lacZ fusion genes is strikingly reminiscent of the adjacent expression in the Drosophila embryo of two neighboring homeotic genes, Dfd and Scr.

Whereas Dfd and Scr are neighboring genes in the ANT-C complex, Hox-1.3 and Hox-5.1 are on two different complexes in the mouse. However, Hox-5.1 is the homolog of Hox-1.4, the 3' neighbor to Hox-1.3 (Duboule and Dolle 1989; Graham et al. 1989). Therefore, it is interesting to note that the CNS region expressing the Hox-5.1/lacZ transgene is identical to the CNS region expressing endogenous Hox-1.4 RNA (Toth et al. 1987). Moreover, it may not be coincidental that the CNS region expressing the Hox-1.4 RNA and Hox-5.1/lacZ transgenes is directly anterior to the CNS region expressing the Hox-1.3/lacZ transgene, considering that 3' Hox genes are expressed more anteriorly than 5' Hox genes.

Specification of regional domains along the rostrocaudal axis of the mammalian CNS

The mouse is thought to be a segmented animal, primarily because of the obvious metamerism in the somites and the subsequent vertebral column (Holland and Hogan 1988). There was little data to support similar ideas about segmentation in the central nervous system, although transient periodic swellings, called neuromeres (Lumsden and Keynes 1989) had been observed in the neuroepithelium in numerous vertebrates. Recently, however, axonal growth and cranial nerve development have been shown to follow the segmental pattern of neuromeres in the chick brain (Lumsden and Keynes 1989). In addition, the zinc finger gene Krox-20 is expressed in two specific alternating neuromeres [r3 and r5] of day 9.5 mouse embryos (Wilkinson et al. 1989), indicating that segmentation at the molecular level may exist in the hindbrain at this stage.

In this paper we show that region-specific enhancers associated with Hox genes can be used to define specific domains in the developing mammalian CNS. Whereas the CNS domains expressing Krox-20 comprise two alternating single neuromeres, the two CNS domains defined here span 6–7 metameres. We note that murine CNS domains expressing the different Xlhbox 1 gene products span 7–8 metameres (Oliver et al. 1988; Wright et al. 1989), and we suggest that the developing CNS may be subdivided into a series of 6–8 metamere long domains that are distinguished by having different combinations of homeo box proteins. The establishment of a series of such domains within the developing vertebrate CNS may be used to specify position along the rostrocaudal axis, in analogy to the patterning system
found in *Drosophila* in which segment identity is defined by interaction of homeo box gene products with target genes. Using these established transgenic mouse lines, uncovering biochemical and functional differences between the cells in the upper cervical and the brachial CNS regions may now be possible. For example, fluorescence-activated cell sorting (FACS), in conjunction with a fluorescent β-galactosidase substrate [Nolan et al. 1988], can be used to purify specific "brachial" or "upper cervical" populations of spinal cord cells for further study.

**Methods**

**DNA cloning and manipulation**

The Bflip/I.3/lacZ gene was constructed by BglII and XhoI digestion of pHox-I.3/lacZ/SV40 [Zakany et al. 1988], filling in with Klenow enzyme, and religation to flip the orientation of the BglII–XhoI region. This generated BglII sites at both ends of the flipped region, so a partial BglII and complete BamHI digest and subcloning into the BamHI site of pGEM2 was performed to allow isolation of large amounts of the 5800-bp fragment via digestion with SalI and BamHI for microinjection. All further gene fusions were constructed within a new plasmid specifically designed to facilitate excision of large DNA fragments for microinjection, a step which may be problematic because unique restriction sites are required on both ends of the desired construct. NotI linkers were inserted at both the PvuII and EcoRV sites of pSP73 [Promega Biotec] to create pCKT15-11. This plasmid can be used to clone DNA constructs by the use of any of the 12 unique restriction sites between the pCKT15-11. This plasmid can be used to clone DNA constructs thus 16-fold less likely to cut within the desired DNA fragment with NotI, an enzyme recognizing an 8-base sequence and deleted the AccI-HindIII Hox-l.3/lacZ.

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**Mouse strains, breeding, and production of transgenic mice**

Mice were obtained from Jackson Labs except where noted. Fertilized eggs for microinjection were obtained from matings between superovulated C57BL/6/DBA prepubescent females and C57BL/6/DBA adult males. Pseudopregnant females for embryo transfer were produced by matings between C57BL/6/DBA adult females and vasectomized CD-1 males [Charles River]. Microinjection and oviduct transfer of injected embryos was performed as described [Hogan et al. 1986]. Resulting pregnant females were either sacrificed at day 12.5 of gestation for F0 embryo analysis or allowed to bring fetuses to term for generation of transgenic lines. DNA isolated from placentas of embryos or the tails of live mice was used to ascertain transgenicity. Embryos were analyzed for lacZ activity as described previously [Zakany et al. 1988], except that 0.25% glutaraldehyde in phosphate-buffered saline was used to fix whole embryos prior to lacZ staining. The temporal pattern of expression of the 5.1/1.3/lacZ transgene was analyzed by breeding founder males with CD-1 females [Charles River]. The day on which vaginal plugs were observed was designated day 0.5 of gestation. Pregnant females were then sacrificed on specified days of gestation and embryos were analyzed as above.

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Note added in proof

To visualize directly the upper cervical and brachial CNS domains within the same mouse embryo, we crossed \textit{Hox-3.1 enhancer/lacZ} transgenic mice with \textit{Hox-5.1 enhancer/lacZ} transgenic mice. Progeny of this cross include embryos that contain and express both gene fusions (see cover picture).
Region-specific enhancers near two mammalian homeo box genes define adjacent rostrocaudal domains in the central nervous system.

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