Hoxd10 induction and regionalization in the developing lumbosacral spinal cord

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SUMMARY

We have used Hoxd10 expression as a primary marker of the lumbosacral region to examine the early programming of regional characteristics within the posterior spinal cord of the chick embryo. Hoxd10 is uniquely expressed at a high level in the lumbosacral cord, from the earliest stages of motor column formation through stages of motoneuron axon outgrowth. To define the time period when this gene pattern is determined, we assessed Hoxd10 expression after transposition of lumbosacral and thoracic segments at early neural tube stages. We present evidence that there is an early prepattern for Hoxd10 expression in the lumbosacral neural tube; a prepattern that is established at or before stages of neural tube closure. Cells within more posterior lumbosacral segments have a greater ability to develop high level Hoxd10 expression than the most anterior lumbosacral segments or thoracic segments. During subsequent neural tube stages, this prepattern is amplified and stabilized by environmental signals such that all lumbosacral segments acquire the ability to develop high levels of Hoxd10, independent of their axial environment. Results from experiments in which posterior neural segments and/or paraxial mesoderm segments were placed at different axial levels suggest that signals setting Hoxd10 expression form a decreasing posterior-to-anterior gradient. Our experiments do not, however, implicate adjacent paraxial mesoderm as the only source of graded signals. We suggest, instead, that signals from more posterior embryonic regions influence Hoxd10 expression after the early establishment of a regional prepattern. Concurrent analyses of patterns of LIM proteins and motor column organization after experimental surgeries suggest that the programming of these characteristics follows similar rules.

Key words: Chick embryo, Spinal cord, Axial patterning, Hox genes, Motoneuron, Paraxial mesoderm

INTRODUCTION

How the vertebrate nervous system becomes patterned along the anteroposterior (AP) axis is a central question in developmental neurobiology. The embryonic hindbrain and spinal cord provide intriguing models for the study of this process. Several morphological and molecular characteristics are repeated along the AP axis but with variations that permit the identification of specific axial levels. Throughout the hindbrain and spinal cord, motoneurons that project to specific targets are located in spatially discrete nuclei. Nuclei of a single broad type (i.e. somatic or visceral targets, limb or axial muscle targets) share similarities of position within the transverse plane and common sites of axon exit. Each hindbrain segment or rhombomere can be identified by its unique complement of nuclei, axon exit sites, and peripheral axon trajectories. Within the spinal cord, motoneurons are arranged in multi-segment columns that reflect broad target type. For example, motoneurons innervating limb muscles are located in the lateral motor column (LMC) while motoneurons innervating axial and body wall muscles are located within the medial motor column (MMC). Different AP regions of the spinal cord (i.e. brachial vs. thoracic) can then be defined by the complement of motor columns that they contain (for reviews see Pfaff and Kintner, 1998; Eisen, 1999).

Members of the LIM family of transcription factors are expressed in subsets of motoneurons in a combinatorial manner. A specific LIM homeodomain profile distinguishes somatic from visceral motor nuclei, motoneurons with different sites of exit from the neural tube, and motoneurons innervating different broad classes of skeletal muscle targets (Tsuchida et al., 1994; Varela-Echavarria et al., 1996; Sharma et al., 1998). Unique expression patterns of members of the Eph receptor family may also distinguish broad classes of somatic motoneurons (Kilpatrick et al., 1996; Ohta et al., 1996; Iwamasa et al., 1999; Olivieri and Miescher, 1999; Yue et al., 1999; Kury et al., 2000). As such, the differential expression of LIM and Eph receptor genes facilitates the identification of AP identity by providing molecular markers for the unique complement of motoneurons at a specific axial level. Further, experimental studies implicate members of the LIM (Kania et al., 2000; Sharma et al., 1998; Sharma et al., 2000) and the Eph receptor families (Donoghue et al., 1996; Ohta et al., 1997; Wang and Anderson, 1997; Yue et al., 1999; Wang et al., 1999;
Feng et al., 2000; Helmbacher et al., 2000) in the specification of motor nuclei position and axon outgrowth patterns.

Members of the Hox family of transcription factors are expressed in discrete axial regions delineating hindbrain segments (Lumsden and Keynes, 1989) and morphologically defined spinal regions (Gaunt, 1994; Burke et al., 1995). Four linkage groups of Hox genes are recognized (Hox A, B, C, and D). Within each group, the expression of a single Hox gene along the body axis is colinear with its genomic position, such that a 3’ Hox gene is expressed in a more anterior region than its 5’ neighbor (see Kessel and Gruss, 1990; Krumlauf, 1994). Gene misexpression studies implicate 3’ Hox genes in the patterning of hindbrain segments and their motor components (see Lumsden and Krumlauf, 1996; Capecci, 1997; Bell et al., 1999), whereas more 5’ Hox genes have been implicated in motor patterning in spinal regions (Le Mouellic et al., 1992; Rijli et al., 1995; Carpenter et al., 1997; Tietz et al., 1998; de la Cruz et al., 1999).

Hox gene expression patterns have been assessed after alterations in the environment of developing avian hindbrain or spinal segments to define the timing and nature of AP regionalization. Segmental differences within the avian hindbrain appear to be programmed gradually. Following posterior-to-anterior transpositions of rhombomeres at neural plate and early neural tube stages, motoneuron organization and Hox patterns within individual segments develop in accord with origin (Guthrie et al., 1992; Kuratani and Eichele, 1993; Simon et al., 1995). In contrast, after anterior-to-posterior transpositions at similar stages, rhombomeres acquire a more posterior character (Grapin-Botton et al., 1995; Grapin-Botton et al., 1997; Itasaki et al., 1996). A posteriorization of segmental character can also be induced by exposure to posterior paraxial mesoderm or neural tissue (Grapin-Botton et al., 1995; Grapin-Botton et al., 1997; Itasaki et al., 1996). These observations suggest that an early segmental prepattern exists in the hindbrain at neural plate stages but that it is refined or stabilized by posteriorizing signals well into neural tube stages.

In the anterior spinal cord, features that identify cervical, brachial and thoracic regions are firmly programmed at neural tube stages (O’Brien and Oppenheim, 1990; O’Brien et al., 1990; Yaginuma et al., 1996; Ensini et al., 1998). For example, following transposition of brachial (B) and thoracic (T) neural tube segments in both anterior and posterior directions, patterns of motoneuron organization, LIM expression, and Hox expression develop in accord with axial origin prior to surgery (Ensini et al., 1998). A short period of plasticity exists during the earliest neural tube stages, but there is little evidence of a prepattern established at neural plate stages. When transposed just after neural tube closure, B and T spinal segments take on virtually all characteristics of their new axial position. Furthermore, a switching of regional fate can be initiated by exposure of B or T segments to foreign paraxial mesoderm at the earliest neural tube stages (Ensini et al., 1998). These findings suggest that the process of anterior spinal regionalization differs from that of the hindbrain in beginning relatively later and ending more abruptly.

Extant studies of regionalization in the posterior spinal cord present a somewhat different picture. Analyses of motor projections after reversals of lumbosacral (LS) segments suggest that motor pool identity is encoded within local LS regions at neural tube stages (Lance-Jones and Landmesser, 1980; Matise and Lance-Jones, 1996). In contrast, analyses of Eph receptor distribution in surgically displaced LS segments suggest an earlier determination. LS neural tissue transplanted into a T mesodermal region develops distinct LS-like patterns of EphA4 even when transplanted at neural plate stages (Fukishima et al., 1996; Tanaka et al., 1997).

Is regionalization at posterior spinal levels a more gradual process than that at anterior spinal levels or are we simply seeing the programming of different features at different times? To address this question, we assessed the emergence of regional characteristics after transposition of posterior spinal segments or paraxial mesoderm at selected early stages. We focused first on the encoding of a Hox gene pattern in neural tissue, as no prior studies have examined the determination of Hox gene patterns in the posterior spinal cord. We specifically chose Hoxd10 because prior studies in mice indicated high expression in lumbar and sacral regions of the neural tube (Dolle and Duboule, 1989; Duboule and Dolle, 1989; Gerard et al., 1996). Further, targeted disruptions of Hoxd10 implicate this gene in the positioning of the LS LMC and the development of motor projections in the limb (Carpenter et al., 1997; de la Cruz et al., 1999). Second, we characterized the development of posterior motor column morphology and LIM gene patterns after early surgeries to determine if the programming of these features was coincident with that of Hoxd10. Preliminary reports of this work were published in abstract form (Sharma and Lance-Jones, 1996).

MATERIALS AND METHODS

Animals and embryonic surgery

White Leghorn chicken and Japanese quail eggs (SPAFAS) were incubated in a forced-draft incubator at 38°C, 50-70% relative humidity. Embryos to be used for analyses of normal patterns of Hoxd10 expression were incubated until embryonic days (E)2-10, removed into saline, and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Eggs for in ovo surgeries were windowed at E2-E2.5, stained with a 0.5-1% Neutral Red/saline solution and staged. All young embryos were staged according to somite (s) number and placed into Hamburger and Hamilton stages in the following manner: stage 11, 12-15s; stage 12, 16-18s; stage 13, 19-21s; stage 14, 22-23s; stage 15, 24-27s.

Regionalization in the posterior spinal cord was assessed after two basic types of surgery. To examine the timing and role of axial position in regionalization, T and anterior LS spinal segments were displaced along the AP axis at selected neural tube stages. These transplants were carried out with and without the underlying notochord to evaluate the dependence of AP regionalization on notochord signals. To examine the role of paraxial mesoderm in regionalization, we replaced paraxial mesoderm adjacent to T or anterior LS spinal segments with foreign paraxial mesoderm and combined neural transposition with transposition of adjacent mesoderm. The earliest surgeries were performed immediately after neural tube closure (stage 11 for T regions, stage 13 for anterior LS regions). The latest surgeries were performed at stage 15, before the onset of neuronal births in the LS region (Hollyday and Hamburger, 1977). Most surgeries were performed using chick embryos only. Paraxial mesoderm transplants, however, included the placement of quail donor tissue into host chick embryos to trace the fate of transplanted mesodermal cells. Schematics and details of individual experimental manipulations are given in the Results section.

General surgical techniques were those of Matise and Lance-Jones (1996). Neural and mesodermal tissues were usually separated from surrounding tissues with flame-sharpened tungsten needles. In a few cases, enzymatic digestion (0.1% dispase or pancreatin) was used. No
differences in outcome were obtained with and without enzyme treatment. Sham operations in which neural tube (n=6) or paraxial mesoderm segments (n=6) were simply removed and replaced at the same axial level were carried out without effect on the development of regional characteristics.

**Determination of transplant levels**

At the time of surgery, axial level in older embryos (stages 14-15) was determined by counting somites or somite-equivalent lengths of unsegmented paraxial mesoderm. Prior experiments (Lance-Jones, 1988; Matise and Lance-Jones, 1996) indicated that at stages 14-15, prospective T segments were adjacent to somite levels 19-25±1 whereas prospective anterior LS segments were adjacent to somite levels 26-29±1. In younger embryos (stages 11-13), transplant levels were determined in preliminary experiments in which small morphological disruptions indicated surgery site (see below). At stages 11-12, T levels generally corresponded to the posterior half of the closed neural tube adjacent to unsegmented paraxial mesoderm. At stage 13, T levels were located just anterior and posterior to the most recently formed somite. At stage 13, anterior LS levels corresponded to the posterior half of the closed neural tube adjacent to unsegmented paraxial mesoderm.

Experimental embryos were sacrificed at stage 26-35, with the majority being at stage 28-29. At the time of sacrifice, transplant regions could be identified by slight bends or constrictions in the spinal cord or slight disruptions in the adjacent mesodermal derivatives and spinal nerves. Transplant boundaries were similarly recognized in tissue sections. For some paraxial mesoderm transplants, the use of quail embryos as a source of donor tissue provided additional evidence of transplant position. In about half of the T-LS exchange embryos, the specific identity of transplanted segments was verified by comparing the position of the transplants in the pair. In the remaining cases, where one member of the original pair had died prior to sacrifice, segment identity was approximated by comparison with embryos operated on at a similar stage and axial level.

**In situ hybridization and histochemistry**

A pBluescript plasmid with a Hoxd10 insert (1.7 kb) was kindly provided by C. Tabin. Sense and antisense digoxigenin-labeled riboprobes were synthesized according to supplier protocol (Boehringer Mannheim). The method used for whole-mount in situ hybridization was that of Nieto et al. (Nieto et al., 1996) with the exception that hybridization and post-hybridization washes were carried out at 68-70°C. In situ hybridization was carried out on frozen or paraffin sections as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993).

Older (stage 21-36) embryos to be processed for whole-mount in situ hybridization were quickly decapitated, evicerated and a ventral laminectomy performed to expose the ventral surface of the cord. A laminectomy was generally not performed on younger embryos or embryos to be processed for section in situ hybridization. Control and chick/chick transplant embryos were fixed in 4% paraformaldehyde and then taken through a methanol series to 100% for whole-mount preparations, or prepared as 14-16 μm serial frozen sections. Quail/chick chimeras were fixed in modified Carnoy’s (Chaerrier et al., 1999) and prepared as 10 μm serial paraffin sections. Patterns of Hoxd10 expression were found to be equivalent in embryos processed by whole-mount and section in situ protocols. Sense probes were tested on sections and showed no staining.

Selected frozen sections from control and experimental embryos were processed immunocytochemically to define region-specific patterns of LIM gene expression as well as motor column morphology. Monoclonal antibodies, 4D5 and 4F2, were used to detect islet 1/islet 2 (Isl1/Isl2) and Lim1/Lim2, respectively (Tsuchida et al., 1994; Ensini et al., 1998). These antibodies were obtained from the Developmental Studies Hybridoma Bank and through the generosity of T. Jessell. A Vectastain ABC system (mouse IgG, peroxidase) was used according to the manufacturer’s instructions. Alternate paraffin sections from quail/chick chimeras were stained with Feulgen to visualize distinctive quail nuclei.

**RESULTS**

**The normal development of neural Hoxd10 expression**

Prior studies defined Hoxd10 expression in mesodermal (Burke et al., 1995; Nelson et al., 1996) and endodermal (Roberts et al., 1995) tissues in the chick embryo, but not in neural tissues. We, therefore, began by characterizing neural expression patterns in normal embryos with a specific focus on motor regions of the spinal cord. For each of the stages described below, 3-6 embryos were examined after either whole-mount or section in situ hybridization. The earliest stages chosen (stages 13-16) correspond to a time period when the future LS neural tube is forming (see Schoenwolf and Smith, 1990). At stage 13, the neural tube is closed through prospective anterior LS levels (LS1-3). As noted earlier (Roberts et al., 1995) slight expression is detected in the stage 13 tailbud, hindgut, and adjacent lateral mesoderm, but not in the neural tube (data not shown). At stages 15-16, when a full LS neural tube is present (LS1-8), a diffuse and low level of Hoxd10 expression is evident in extreme posterior regions; in the neural tube, the lateral plate mesoderm, and the tailbud (Fig. 1A,B). Expression in the neural tube corresponds to prospective middle and posterior LS segments (30+ somite level). By stage 17-18, this expression has spread anteriorly to include prospective anterior LS neural tube segments (27+ somite level, data not shown).

LS motoneurons are born, begin to migrate laterally to form the motor columns, and initiate axon outgrowth between stages 17 and 21 (Hollyday and Hamburger, 1977; Tosney and Landmesser, 1985). High Hoxd10 expression is first seen at stages 19-21 (Fig. 1C,D). It is restricted to the ventral LS spinal cord where motoneurons have begun to leave the ventricular zone. Diffuse expression of Hoxd10 is still evident at the posterior end of the neural tube and in the tailbud, although this expressing region is now separated from the LS region by a non-expressing axial region (see Fig. 1C). At this and later stages, Hoxd10 is also detected in the limb buds and LS somites (see Fig. 1D), in accord with prior descriptions (see Dolle and Duboule, 1989; Burke et al., 1995; Nelson et al., 1996).

Between stages 21 and 26, the LS motor columns expand in size and motor axons enter the limb proper (see Tosney and Landmesser, 1985; Tsuchida et al., 1994). In a stage 25 embryo, Hoxd10 is expressed throughout the full axial extent of the LS neural tube but not in more anterior and posterior spinal segments (Fig. 1E). As viewed in transverse section, virtually all postmitotic cells in LS segments appear to express high levels of Hoxd10, including cells within dorsal and intermediate regions (Fig. 1F). Expression is also evident along the ventral roots; presumably in future peripheral glial cells (Fig. 1F).

Stages 27-29 represents a time period when there is an adult-like arrangement of motor columns and functional connections have been established with specific limb muscles (Landmesser, 1978a; Tsuchida et al., 1994). At these stages, neural Hoxd10 expression is high throughout the LS cord and tapers off in segments posterior to the LS region (Fig. 2A). Low level
expression is visible in the last T segment (T7), but not in more anterior T segments (Fig. 2A,B). *Hoxd10* expression is also visible in LS sensory ganglia and along LS ventral roots and spinal nerves (Fig. 2A). In whole-mount ventral views, *Hoxd10* expression appears as two expanding strips in LS1-3 (Fig. 2A). In transverse section (Fig. 2C-E), this pattern can be seen to reflect heterogeneity in *Hoxd10* expression within motor column regions. In LS1-2, *Hoxd10* expression is high in extreme lateral LMC regions but low in medial LMC and MMC regions (Fig. 2C,D). In LS3, expression is high in all but dorsomedial LMC regions (arrow, Fig. 2E). A similar expression pattern was evident at stage 36, after the peak period of cell death (Hamburger and Oppenheim, 1982), with the exception that motor column regions in LS1-3 appeared to show even lower levels of expression (normal data not shown, but see experimental embryo in Fig. 3C).

The above observations indicate that the embryonic nervous system shows two phases of *Hoxd10* expression. During LS neural tube formation, a pattern of diffuse, low level *Hoxd10* expression spreads from the tailbud and future posterior LS neural regions to anterior LS neural segments. This early phase of expression is similar to that noted for other Hox genes (see Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994; Belting et al., 1998). As cells in posterior spinal regions begin to withdraw from the cell cycle, a new phase is initiated; a phase in which high expression is evident and restricted virtually exclusively to LS spinal segments. The restriction of *Hoxd10* expression to LS segments continues through peak periods of motoneuron outgrowth and naturally occurring cell death (see Tosney and Landmesser, 1985 and Hamburger and Oppenheim, 1982). During this time period, however, the pattern within individual anterior LS segments is modified. At early stages of LS motor column formation most cells appear to express high levels of *Hoxd10*. With time, cells within more medial motor column regions show lower levels of expression than cells within extreme lateral regions. As motoneurons are generated in mediolateral sequences (see Hollyday and Hamburger, 1997; Whitelaw and Hollyday, 1983), it is possible that some motor column cells express *Hoxd10* for only a short period following withdrawal from the cell cycle (see also Graham et al., 1991). Nevertheless, expression of *Hoxd10* is highest in motor column regions at
stages when a memory of positional identity might be used to form unique motor column associations and establish specific projections in the periphery.

Evidence of differences in Hoxd10 determination between stages 13 and 15

Previous neural tube reversal experiments indicate that the period between stage 13 and stage 15 is a critical one for the determination of specific motoneuron projections from segments LS1-3 (Lance-Jones and Landmesser, 1980; Matise and Lance-Jones, 1996). We, therefore, first investigated whether the ability to express high levels of Hoxd10 is also programmed into LS1-3 during this time period. At stages 13 and 15, the positions of future posterior T and anterior LS segments were altered by reversing neural tube segments T5/6-LS2/3 about the AP axis (Fig. 3). Hoxd10 expression was assessed by whole-mount in situ hybridization at stages 27-34.

In virtually all embryos operated on at stage 15 (n=10/11), we found evidence of a reversal of the normal patterns of Hoxd10 expression in the reversal region. The displaced LS segments showed high levels of Hoxd10 and a preservation of characteristic expression patterns in individual LS segments (compare Fig. 3A-C). The displaced T segments showed little or no expression; a pattern matching that seen in normal embryos. No consistent differences were noted between embryos in which the reversal extended through LS3 (n=6) and those where it ended at LS2 (n=5).

Embryos operated on at stage 13 showed mixed results. In those embryos in which the reversal extended through LS3 (T6-LS3; n=2), we found evidence of a pattern reversal like that described above. The original LS2 shows Hoxd10 expression above that found in a normal mid-T segment. st, stage. Bars, 250 µm.
between Hoxd10 expression levels and the exclusion or inclusion of the underlying notochord at the time of surgery. These results suggest, first, that there are differences between T and LS segments as early as stage 13. LS segments can develop normal high levels of Hoxd10 expression when placed in T regions at stage 13, while T segments never develop high level expression when placed in LS regions at the same stage. These findings suggest that there is a prepattern for the adult phenotype in the stage 13 posterior neural tube. This prepattern is, however, less complete than that present at stage 15. At stage 15, all anterior LS segments (LS1-3) develop high levels of Hoxd10 expression following reversal into T regions. Following reversals at stage 13, LS3 develops high levels of Hoxd10 expression. However, LS1 and LS2 only develop high Hoxd10 levels when transplanted with LS3. This observation suggests that, at stage 13, there is an increasing anterior-to-posterior gradient of competence to express Hoxd10 and/or to respond to local inducing signals. Why do LS1-2 develop high Hoxd10 levels after reversals of T6-LS3 but not after reversals of T5/6-LS2? Reversals of T5/6-LS2 place LS1-2 in a more anterior position than reversals of T6-LS3. If signals inducing Hoxd10 are graded and stronger in more posterior axial regions, then reversals of T5/6-LS2 may have exposed LS1-2 to less inductive signal than reversals of T6-LS3. Alternatively, planar signals originating from LS3 may normally initiate a program for high Hoxd10 expression in more anterior LS segments between stages 13 and 15.

Evidence for graded signals from non-neural tissue

Prior studies provide substantial evidence that the development of regional characteristics within hindbrain tissue is dependent on signals from local mesoderm that are graded on the AP axis (see Itasaki et al., 1996; Grapin-Botton et al., 1997). Is tissue outside the LS neural tube at stages 13-15 a source of signals for the establishment of a stable pattern for Hoxd10 expression? Are these signals graded with the anterior boundary of the signal gradient being dependent on developmental age? To address these questions, we challenged stage 13-15 LS segments with one of two environments; the anterior T region, far from the source of a potential signal, and the posterior T region, close to the source of such a signal. Two-4 anterior LS segments (±T7) were transplanted from stage 13-15 donor embryos into the T regions of stage 11-15 host embryos (see Fig. 4). The anterior T transplant group included those embryos where the transplant had a posterior boundary at or before T4. The posterior transplant group included those embryos with posterior transplant boundaries at T5-T7.

To compare the level of Hoxd10 expression in different experimental groups, we used a qualitative scoring system. Individual donor and host segments within whole-mount processed embryos were given a score from 0 to 4 depending on the level and extent of staining on the ventral cord surface. A score of 0 was given when no staining above background was evident (equivalent to a normal mid-T segment at stages 27-30). A score of 1 was given when staining was low and diffuse (usually equivalent to a normal T7); a score of 2 when half or less than half of the ventral surface showed high expression (usually equivalent to a normal LS1); a score of 3 when the majority of the ventral surface showed high expression (usually equivalent to a normal LS2); and a score of 4 when the full ventral surface showed high expression (usually equivalent to a normal LS3/4 segment). For each experimental embryo, the total segmental score in the transplant was compared to that of its host’s corresponding segments and expressed as a percentage. A mean percentage or Hox level value was then determined for different transplant groups. Standard errors were used as a coarse barometer of variability. Given the qualitative nature of our scoring system, we did not determine significance. Overall, this procedure allowed a correction for differences in expression levels due to variations in the processing of embryos or differences related to age of sacrifice.

In all embryos with stage 14-15 donor tissue (n=19), Hoxd10 expression was high in transplanted LS segments and similar in level and spatial pattern to that in the equivalent LS segments of the host (Fig. 4A). Mean Hox expression values were virtually identical for transplants occupying posterior and anterior T positions (Fig. 5). In contrast, a striking difference was noted among the embryos with stage 13 donor tissue (n=29). Transplants occupying a posterior T position (n=11) showed high levels of Hoxd10 expression; the mean value being very similar to that of the equivalent stage 14-15 transplant group (Figs 4C, 5). Transplants occupying an anterior T position (n=18) showed a very low mean Hoxd10 expression value; a value approximately 1/3 of that for the stage 14-15 anterior transplant group (Figs 4B, 5). These findings suggest that the programming of Hoxd10 patterns...
within stage 13 LS neural segments can be influenced by non-neural signals and that such signals are graded on the AP axis. Further, our data suggest that signals exist not only within LS non-neural regions, but also within T regions.

While stage 13 transplants placed into anterior T regions showed low Hoxd10 expression, this expression was usually above background and present in a gradient matching the original orientation of the donor. Transplants placed into hosts in normal AP orientation showed the highest expression in posterior transplant regions (see Fig. 4B). Transplants placed into hosts in reversed AP orientation (n=3/18) showed the highest expression in anterior transplant regions. These observations reinforce the conclusion drawn from neural tube reversals that there is a prepattern for Hoxd10 expression present at stage 13.

In neural tube reversal experiments we found low levels of Hoxd10 expression in LS1-2 after stage 13 reversals of T5/6-LS2. This observation appears, at first, to conflict with our finding of high Hoxd10 expression after direct placement of stage 13 LS segments into posterior T regions. It should be noted, however, that all of our posterior T transplants involved displacements of LS1-3, not just LS1-2. We suggested earlier that LS3 may be more competent to express Hoxd10 than LS1-2 at stage 13 and that Hoxd10 inducing signals may spread in a planar manner from LS3 to LS1-2. Our anterior T transplant group did include transplants w/LS3; allowing us to determine if different levels of Hoxd10 expression were found depending on whether or not LS3 was present in the transplant. We included only those embryo in which the donor embryo survived (n=7); permitting a precise identification of transplant level of origin. Although Hoxd10 levels were quite variable, transplants that included LS3 showed a mean value that was approximately twice that of transplants ending at LS2 (Fig. 5). This finding supports the idea of a gradient of competence and, possibly, planar signaling.

Three additional variables were assessed in the stage 13 anterior T transplant group and found to have no effect or smaller effects on mean expression values. No striking differences were found when transplants placed in reversed and non-reversed AP orientation were compared, suggesting that a marked incompatibility in maturation gradients did not affect outcome. Nor did we find a consistent difference between transplants that did and did not include the underlying notochord. This observation is compatible with those of prior studies (Fukushima et al., 1996; Matise and Lance-Jones, 1996; Tanaka et al., 1997), in suggesting that the notochord is not a source of local AP regionalization signals. A small gradual increase in mean expression value was seen with increasing host age (mean values: stage 11 hosts, 24±17, n=3; stage 12 hosts, 32±7, n=5; stage 13 hosts, 36±9, n=10). This age-dependent change hints at the existence of transient, local signals affecting axial regionalization. For example, these observations could be related to the proximity of the transplant to the primitive node, a potential source of signals that may specifically promote T rather than LS character at the earliest transplant stages. In a stage 11 host, the transplant was placed just anterior to the primitive node. With increasing host age, the distance between transplant and node increases, possibly attenuating effects of the early node. It is important to point out that even in the subsets of anterior T transplants + LS3 or the subset transplanted into stage 13 hosts, Hoxd10 mean expression values were much

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**Fig. 6. Hoxd10 and LIM patterns following transplantation of LS segments to T regions.** (A-C) Adjacent sections from the LS2/3 region of a stage 28 control embryo. Hoxd10 expression (A) is high throughout the transverse plane of the spinal cord except in the ventricular zone and in a medial strip of motor column cells. Isl1/2+ staining (B) identifies all motoneurons and facilitates the identification of a large, laterally directed motor column region characteristic of the normal LS region. Lim1/2+ staining (C) identifies motoneurons unique to the normal LS region, the LMCI (arrow). (D-L) Adjacent sections from mid-T regions of the same control embryo. Hoxd10 expression is virtually absent in the cord (J). Isl1/2+ staining (K) shows a motor column region that is much smaller than that in LS sections. In addition, Isl1/2+ cells of the Column of Terni are visible in intermediate regions, along the edge of the ventricular zone (arrowhead). (D-I) Transverse sections through the transplant regions of stage 28-30 experimental embryos. (D-F) Sections taken from an embryo in which stage 13 LS segments had been transplanted into posterior T region of a stage 12 host embryo. Hoxd10 expression (D), Isl1/2 staining (E), and Lim1/2 staining (F) patterns are LS-like in character. (G-I) Sections taken from an embryo in which stage 13 LS segments had been transplanted into anterior T regions of a stage 13 host embryo. Here, Hoxd10 expression (G) is present only at low levels. Isl1/2 (H) and Lim1/2 staining (I) patterns are mid-T-like in character. Bars, 250 μm.
lower than mean level values for posterior T transplant groups. Clearly, LS segments can be influenced by their non-neural environment at stage 13; an influence that ultimately affects their level of Hoxd10 expression.

LS motor column morphology and LIM patterns change in concert with Hoxd10 patterns

To investigate whether the determination of other LS features follows the same rules as those for Hoxd10, we examined LIM patterns and motor column morphology in conjunction with Hoxd10 expression in a separate set of embryos with LS-to-T transplants. Representative sections from stage 28-30 control and experimental embryos are shown in Fig. 6. Sections from transplants placed into posterior T regions at stage 13 (n=5) showed patterns of Hoxd10 that were very similar to those seen in control LS sections (compare Fig. 6A,D). Expression is high throughout most of the transverse plane of the cord. In both normal LS and transplant sections, the LMC shows a characteristic striped pattern; Hoxd10 expression being less intense in the dorsomedial LMC than in lateral and ventromedial LMC regions. In contrast, sections from transplants placed in anterior T regions at stage 13 (n=3) show little Hoxd10 expression (compare Fig. 6G,J).

LIM patterns and motor column morphology appeared to change in concert with Hoxd10 expression after surgery. At the embryonic stages examined, all spinal motoneurons normally express Isl1/2 (mAb 4D5), including somatic motoneurons in the ventral motor columns (both the LMC and the MMC) and visceral motoneurons in the intermediate Column of Terni (Tsuchida et al., 1994). Sections from transplants placed in posterior T regions show LS-like patterns of Isl1/2 expression (compare Fig. 6B,E) that parallel their LS-like patterns of Hoxd10 expression. Isl1/2-positive somatic motoneurons form laterally expanded clusters within the ventral horns and visceral motor columns are absent. Sections from transplants placed in anterior T regions show T-like patterns of Isl1/2 expression (compare Fig. 6H,K) that parallel their T-like patterns of Hoxd10 expression. Somatic motor columns are smaller than in LS segments and visceral motor columns (CT) appear to be present. Lim1/2 expression (mAb 4F2) seems to follow similar rules. Lim1/2 is expressed in a subset of lateral LMC (LMCl) motoneurons unique to the LS region. These motoneurons project to dorsal limb muscles (see Fig. 6C,L and Tsuchida et al., 1994). In experimental embryos, Lim1/2-positive motoneurons can be seen in sections from posterior T transplants (Fig. 6F) but not in sections from anterior T transplants (Fig. 6I). LS-like patterns of Hoxd10, Isl1/2 and Lim1/2 staining were also found in sections from stage 15 transplants (n=2).

T regional characteristics are largely determined at early neural tube stages but can be modified by signals from middle LS regions

We next investigated whether patterns within T segments could be modified by exposure to posterior signals. T regionalization was assessed following placement of T segments into LS regions (n=42); the embryos being the companions to the LS-to-T transplant series. In no case, even with stage 11 T transplants (n=12), did we find a complete induction of Hoxd10. In most embryos we found either no expression (n=21) or low expression, equivalent to a normal T7 (n=14) in posterior transplant regions (Fig. 7A). T7-like expression was not due simply to the inclusion of T7 as it was found in transplants from both anterior and posterior T regions. In fact, in the T-LS transplant group as a whole, no correlation was found between level of origin of the transplant and degree of Hoxd10 expression. In 7/42 embryos, we found levels of Hoxd10 expression that approached LS levels in some segments of the transplant. These embryos shared three characteristics. First, all had transplants of stage 11-13 T
segments rather than stage 14-15 T segments. Second, all of these transplants extended into LS4-5, as opposed to ending at the posterior border of LS3. Finally, high Hoxd10 expression was found only in posterior segments of these transplants. Fig. 7B-G show in situ hybridization of sections from a representative embryo. Little expression is present in anterior regions of the transplant (Fig. 7B), while LS-like expression is present in posterior transplant regions (Fig. 7E). These findings are in agreement with the results of reversal experiments in suggesting that T segments are largely refractory to signals.

Fig. 8. The development of regional character in LS neural segments bordered by T paraxial mesoderm. (Top left) A schematic of the surgery. Paraxial mesoderm on one side of the anterior LS neural tube of a stage 13 chick embryo was replaced with stage 11-13 anterior T paraxial mesoderm from a chick or quail embryo. (A) Ventral view of the LS cord from one stage 29 embryo. Hoxd10 expression is high in LS neural tissue adjacent to the transplant region, although, the lateral strip of high Hoxd10-expressing cells is reduced. (B-D) Sections through the anterior LS region of a stage 29 embryo. (B) The experimental side (arrow) shows lower Hoxd10 expression in the mesoderm than the control side. In neural tissue, Hoxd10 expression (B) as well as Isl1/2 (C) and Lim1/2 (D) protein patterns are normal on the experimental side with the exception of a decrease in motor column size. (E-G) Sections through the anterior LS region in a stage 29 quail/chick chimera. The experimental side is marked with an arrow. Hoxd10 expression is equivalently high on both sides of the LS cord (E), despite the fact that the control side is bordered by chick myotomal cells of LS origin (F) and the experimental side is bordered by quail myotomal cells of T origin (G). Bars, (A-E) 250 μm; (F-G) 25 μm.

Fig. 9. The development of Hoxd10 expression in T segments bordered by LS paraxial mesoderm and LS segments transplanted to T regions with adjacent paraxial mesoderm. On the left are schematics of the surgical procedures. Whole-mount in situ hybridization was done at stages 28-29. On the right are ventral views of T and LS spinal regions. (A) Hoxd10 is not induced in the T spinal cord after placement of stage 13 anterior LS paraxial mesoderm into the T region of a stage 11 embryo. (B) Stage 13 LS neural tissue + its adjacent paraxial mesoderm was transposed to anterior T regions in a stage 11 host. Hoxd10 expression is low in transplanted neural segments as it is following transposition of LS neural tissue alone (see Fig. 4B). Only the most posterior transplant regions show expression above that in adjacent host regions. (C) No expression is induced in anterior T neural segments despite transplantation of stage 14 posterior LS paraxial mesoderm into anterior T regions at stage 11. Hoxd10 expression is visible in the transplanted mesoderm (see bracket). (D) Hoxd10 expression is also not induced in posterior T neural segments following an equivalent transplant of posterior LS paraxial mesoderm into posterior T regions. Again expression is visible in transplanted mesoderm. Bars, 250 μm.
setting *Hoxd10* expression. They also indicate that *Hoxd10* expression can be induced when T segments are placed in a sufficiently posterior LS region.

As with the LS-to-T transplant series, LIM patterns in T-to-LS transplant embryos appeared to change in parallel with *Hoxd10* patterns. More anterior segments of T transplants showed T-like LIM patterns (Fig. 7C,D). More posterior segments showed more LS-like LIM patterns (Fig. 7F,G). It should be noted that *Hoxd10* and LIM patterns in posterior T transplant regions occasionally appeared hybrid in nature. For example, in the embryo shown in Fig. 7E-G, the right side of the transplant region is largely, but not completely, LS-like.

**Paraxial mesoderm adjacent to the early LS neural tube is neither necessary nor sufficient to induce *Hoxd10* expression**

Grafting experiments in the hindbrain (Itasaki et al., 1996; Grapin-Botton et al., 1997) and the anterior spinal cord (Ensini et al., 1998) as well as explant culture studies (Gould et al., 1998; Muhr et al., 1997) suggest that paraxial mesoderm signals play a role in setting *Hox* patterns at multiple developmental stages. To address the role of paraxial mesoderm in the determination of *Hoxd10* patterns we examined fist if anterior LS paraxial mesoderm was specifically required at stage 13 for the development of high *Hoxd10* expression in LS1-3. Paraxial mesoderm adjacent to the LS1-3 neural tube was removed from a stage 13 host embryo and replaced with anterior T paraxial mesoderm from a stage 11-13 donor chick or quail embryo (Fig. 8A). These surgeries were carried out unilaterally as prior studies had indicated that regionalization at neural plate/neural tube stage occurs independently on each side of the embryo (see Grapin-Botton et al., 1995; Ensini et al., 1998).

Examinations of experimental embryos were made at stages 28-30 (n=17). In the transplant region, mesodermal *Hoxd10* expression was reduced or absent in accord with its T origin (see Fig. 8B, arrow). That the transplanted T mesoderm persisted after surgery was verified by analyses of chick embryos that had received a quail mesodermal transplant (n=3/17). Myotomal and sclerotomal tissues at the level of LS1-3 were largely of quail origin on the experimental side but of chick origin on the control side (Fig. 8E-G). Despite the presence of T paraxial mesoderm, all embryos showed high *Hoxd10* expression in LS1-3 and normal LS-like patterns of LIM protein and motor column organization (Fig. 8A-E). The LS spinal cord on the experimental side often (n=10/17) showed a slight reduction in the size of the LMC (Fig. 8A,B). This reduction was most evident as a depletion of the lateral strip of high *Hoxd10*-expressing cells (Fig. 8A); a strip likely to represent the last cells to contribute to the LMC (see Whitelaw and Hollyday, 1983; Leber and Sanes, 1995). A decrease in LMC size was not noted in sharn operated embryos, where LS paraxial mesoderm had simply been removed and replaced (see Materials and Methods). These observations implicate distinct signals for different aspects of LS regionalization. Stage 13 anterior LS paraxial mesoderm does not appear to be a required signaling source for programming *Hoxd10* and LIM expression patterns or the overall organization of the motor columns. In contrast, signals from this tissue may influence the extent of proliferation or cellular contribution to the LMC.

Results from three additional experiments support our conclusion that stage 13 anterior LS paraxial mesoderm is not a prime signal source. *Hoxd10* expression was not induced in either anterior T (n=3) or posterior T (n=6) neural tissues following transplantation of stage 13 anterior LS paraxial mesoderm into stage 11 host embryos (Fig. 9A). Further, high levels of *Hoxd10* expression were not found in anterior LS segments transplanted with adjacent paraxial mesoderm to anterior T regions (n=8; Fig. 9B). In fact, the mean level value for *Hoxd10* expression after such transplants was very similar to that after transplantation of the anterior LS neural tube alone (see Fig. 5).

While anterior LS paraxial mesoderm may not be a prime source of signals, two pieces of evidence indicated that more posterior LS paraxial mesoderm should be considered as a source. First, our neural tube transposition experiments suggest that environmental signals are present in an increasing anterior-to-posterior gradient. Such signals may normally spread from posterior to anterior LS paraxial mesoderm, after stage 13. Second, fate mapping studies of posterior regions in the avian embryo indicate that mesodermal and neural tissues undergo axial displacements during early neural tube stages (Schoenwolf, 1977; Schoenwolf et al., 1992; Catala et al., 1995; Catala et al., 1996). Mesodermal tissues originating from any one level of the primitive streak or tailbud are displaced anteriorly, while neural tissue from the same level elongates posteriorly. Our analyses of quail/chick chimeras indicated that the mesoderm adjacent to anterior LS neural segments contained a significant complement of quail cells following replacement of chick anterior LS paraxial mesoderm with quail T paraxial mesoderm. However, we cannot rule out the possibility that some mesodermal cells from posterior LS regions contribute to anterior LS mesoderm after surgery, cells that might be a source of *Hoxd10*-inducing signals. To assess the role of posterior LS paraxial mesoderm, we transplanted stage 13-14 posterior LS paraxial mesoderm in place of either anterior T (n=8) or posterior T (n=15) paraxial mesoderm in stage 11-12 host embryos. No *Hoxd10* expression was induced in anterior T neural segments bordered by posterior LS mesoderm (Fig. 9C). Slight *Hoxd10* expression was detected in posterior T segments bordered by posterior LS mesoderm in a minority of embryos (n=4/15). However, most of these embryos showed no expression above normal in posterior T segments (Fig. 9D). Overall, the results of these paraxial mesoderm transplants suggest that signals from stage 13 LS paraxial mesoderm are neither necessary nor sufficient to induce high *Hoxd10* expression and a conspicuous LS character.

**DISCUSSION**

Our studies have focused on AP regionalization at LS axial levels and on early events in this process. We have used tissue transposition and assessments of subsequent development to provide pictures of two developmental time points and of events that are likely to occur in the intervening period. At the first time point, shortly after neural tube closure, we present evidence that the future LS region differs from the adjacent T region. Specific characteristics are determined or programmed within LS regions at this early neural tube stage. However, our results also suggest that the determination of these characteristics is incomplete. We therefore refer to the existence of a LS prepattern at this time point, rather than a
fully determined pattern. At a second time point, a late neural tube stage, we present evidence of a complete programming of specific LS features. This complete programming appears to be accomplished, in the intervening period, through the actions of graded signals. We also present evidence that such signals are unlikely to arise solely from paraxial mesoderm adjacent to the early LS neural tube. Rather, signals may arise from more posterior environmental tissues and/or be relayed to the neural tube via paraxial mesoderm at later stages.

High level Hoxd10 expression is a unique feature of the LS region and the pattern of Hoxd10 expression was therefore used as a primary marker of regional character. We also assessed patterns of motor column morphology and LIM protein distribution. In agreement with studies of determination at more anterior CNS levels, we found that these phenotypic characteristics developed in concert with Hox gene patterns (see also Grapin-Botton et al., 1995; Ensini et al., 1998). For brevity, we will use Hoxd10 patterns in the following discussions to illustrate aspects of the regionalization process.

A prepattern for unique T and LS characteristics is present at the earliest neural tube stages

A central conclusion to be drawn from our experiments is that the specification of regional characteristics within prospective T and LS segments begins before or at the time of neural tube closure (stage 11 for T segments, stage 13 for anterior LS segments). Stage 13 LS neural tube segments show two distinctive characteristics. First, anterior LS segments (LS1-3) are capable of developing high levels of Hoxd10 even when placed in posterior T regions at stage 13. No such high level expression ever develops in posterior T segments. Second, posterior LS2-LS3 (but not LS1-anterior LS2) can develop at least some level of Hoxd10 expression when placed in anterior T regions at stage 13. As in the normal LS cord, a gradient of Hoxd10 expression is evident in displaced LS segments with the originally most-posterior segment showing the highest level of expression. In marked contrast, stage 11-13 T segments are distinguished by their inability to develop high levels of Hoxd10 expression after displacement to LS regions. Only when placed in middle to posterior LS regions, did stage 11-13 T segments occasionally express LS-like levels of Hoxd10.

These observations parallel findings made in the avian hindbrain where segment-specific patterns of Hox gene expression and motor nuclei have been assessed after AP segment transposition. Hindbrain segments develop in accord with their origin following posterior to anterior transposition both before and after neural tube closure (Grapin-Botton et al., 1995; Grapin-Botton et al., 1997; Itasaki et al., 1996; for review see Guthrie, 1996). Hindbrain segments do not develop in accord with their origin, following anterior to posterior transpositions (see below), however, they clearly show a gradient of competence to respond to their new environment (see Grapin-Botton et al., 1997). The interpretation given is that there is a prepattern within hindbrain segments at neural plate and neural tube stages, but that it may be refined by environmental signals.

Our observations differ from those made for brachial (B) and thoracic (T) segments (Ensini et al., 1998). When T and B segments are exchanged shortly after neural tube closure, they develop patterns of LIM gene expression, motor column morphology, and Hoxc8 expression that are in accord with their new position. No prepatterns of T or B regional characteristics are observed. A possible explanation for the differences may rest with the fact that Hoxc8 and Hoxd10 belong to two different classes of Hox genes; those related to the Drosophila Abdominal A and B genes (Schubert et al., 1993). Hoxc8 is normally expressed in both B and T segments, but in different spatial patterns; Hoxd10 is normally highly expressed only in LS segments. The prepattern we have identified may reflect an early division of the neural plate into regions that are capable of expressing one class of Hox genes but not another. Evidence supporting this idea is provided by the observation that only certain classes of Hox genes can be induced in hindbrain segments after posterior transplantation (Grapin-Botton et al., 1997). We initially asked if regionalization in posterior spinal regions was more gradual than that in anterior spinal regions or if different features are programmed at different times. We would suggest that the latter is true; that a finer division of spinal regions follows a coarse division into Hox class regions.

Our experiments do not tell us when an LS prepattern is first established. LS neural plates transplanted into T mesoderm regions of a host embryo develop distinct LS-like patterns of EphA4 and low-affinity NGF receptors (Fukushima et al., 1996; Tanaka et al., 1997). In light of these findings, it would be particularly interesting to assess Hoxd10 expression after equivalent transplantsations.

Hoxd10 expression patterns are determined and stable 6-10 hours after neural tube closure

In virtually all cases where LS segments are transposed to T regions at stages 14-15, they subsequently develop high levels of Hoxd10 expression in accord with their origin. No difference was noted between transplants placed at anterior or posterior T levels. Information specifying patterns of Hoxd10 expression appears to be completely programmed within LS segments at stage 14-15, 6-10 hours after neural tube closure and just

Fig. 10. Schematics of a possible mode of Hoxd10 induction. At stage 13, a prepattern for Hoxd10 expression is present in the anterior LS neural tube. The most anterior LS segments (LS1-2) have less ability to develop high levels of Hoxd10 expression than more posterior segments (LS3+). This gradient of competence may reflect exposure to a very early gradient of inducing signals, in extreme posterior embryonic regions. At stage 13+, inducing signals may spread anteriorly, as far as posterior T regions, stabilizing and amplifying the LS prepattern. Associated with this process is an anterior extension of non-axial mesoderm and a posterior elongation of the LS neural tube (arrows, after Catala et al., 1995). The asterisks denote displacement of mesoderm at one axial level.
before the first LS neural cells begin to withdraw from the mitotic cycle (Hollyday and Hamburger, 1977). Motoneuron target identity is also firmly encoded within LS segments at stage 15 (Lance-Jones and Landmesser, 1980; Matise and Lance-Jones, 1996). These observations suggest that multiple and perhaps linked specification events are occurring within LS segments at the same time. At stages 14–15, T segments appear to be completely refractory to signals that might initiate Hoxd10 expression. These findings are compatible with those of Ensini et al. (Ensini et al., 1998) in indicating an encoding of T regional character several hours after neural tube closure.

While the ability to express high levels of Hoxd10 may be encoded within anterior LS segments at stage 14-15, we did not detect expression in these segments in normal embryos until stage 17-18. This observation suggests that Hoxd10 expression is programmed within anterior LS segments before the spread of expression has reached that level. In turn, this conclusion is in agreement with studies of the programming of Hoxd4 expression where it has been observed that forward spreading and development of normal Hoxd4 patterns can occur despite the placement of barriers between early posterior-expressing regions and more anterior regions (Gaunt and Strachan, 1994).

At intermediate neural tube stages, T and LS regional characteristics can be changed by graded non-neural signals

A second major conclusion to be drawn from our experiments is that between early and late neural tube stages, graded non-neural signals have an impact on the development of regional character. Evidence in support of this statement comes first from the observation that stage 13 LS segments transplanted to different AP levels within the T region subsequently develop very different levels of Hoxd10 expression. LS segments placed in anterior T regions develop low levels of Hoxd10 expression while LS segments placed in posterior T regions developed high levels. We also found a difference in the induction of Hoxd10 in T segments after transplantation to different AP levels within LS regions. Stage 11-13 T segments did not develop LS-like levels of Hoxd10 expression when positioned at anterior LS levels but did occasionally when positioned slightly further posteriorly.

These findings suggest that non-neural signals capable of altering Hoxd10 expression are present in a gradient within posterior embryonic regions. Such signals could be inhibitory in nature and distributed in a gradient that is highest in anterior T regions and decreases through LS regions. Inhibitory or repressive signals have been implicated in the CNS regionalization (see Kondo et al., 1998; Rowan et al., 1999). However, a substantial body of data suggests that inducing signals play a major role in the early specification of posterior CNS regions and that such signals are distributed in a decreasing posterior to anterior gradient (see Doniach, 1993; Muhr et al., 1999). Most strikingly, studies in which anterior hindbrain segments have been transplanted into posterior hindbrain regions indicate that posterior tissues can impose a posterior identity at neural tube stages (Grabin-Botton et al., 1995; Grabin-Botton et al., 1997; Grabin-Botton et al., 1999; Itasaki et al., 1996).

We would suggest that signals inducing Hoxd10 expression are distributed in a decreasing posterior to anterior gradient, that they, in fact, extend into posterior T regions, and that LS segments require exposure to them or after stage 13 in order to develop high levels of expression. If Hoxd10 inducing signals are present in both LS and T regions, how then does Hoxd10 expression become restricted to LS segments during normal development? The answer may lie with the existence of an early prepattern. This prepattern might be viewed as a competence gradient with individual T and LS segments showing a level of competence to respond to posteriorizing signals and to develop high Hoxd10 that parallels their relative AP order (see also Grapin-Botton et al., 1997). The programming of Hoxd10 during normal development may, thus, be limited to the prospective LS region because it is only in this region that high signals and high competence normally coincide. A temporal dimension to these gradients is also likely to be present. Our reversal experiments suggest that a stage 13 LS3 is more firmly programmed to express Hoxd10 than a stage 13 LS1 or LS2. At stage 15, both LS1-2 and LS3 appear to be fully programmed. These conclusions, in turn, suggest that the anterior boundary of proposed gradients changes with developmental age. Schematics of possible competency and signal gradients are shown in Fig. 10.

LS paraxial mesoderm is not the sole source of regionalization signals between stage 13-15

We were led to examine the role of local paraxial mesoderm signals by studies carried out in hindbrain and more anterior spinal regions; studies indicating that AP differences in paraxial mesoderm signaling at neural tube stages can influence the subsequent attainment of regional character (Itasaki et al., 1996; Grapin-Botton et al., 1997; Ensini et al., 1998). Surprisingly, we found no evidence that LS paraxial mesoderm is a required source of signals setting Hoxd10 expression at early neural tube stages. Replacement of anterior LS paraxial mesoderm with that from anterior T regions at stage 13 had a slight effect on LMC size but no effect on the development of Hoxd10 expression, LIM protein patterns or general motor column organization. Nor was high Hoxd10 expression induced in stage 11-12 T neural segments when bordered by either anterior or posterior LS paraxial mesoderm. Finally, neural Hoxd10 expression was equivalently low in transplants of LS neural segments to T regions and transplants of LS neural+ LS paraxial mesoderm segments to T regions.

Why did we obtain such different results from those of prior studies, especially given the fact that our experiment approach was very similar to that used previously? We think it is unlikely that entirely separate mesodermal tissues (i.e. lateral vs. paraxial) provide regionalization signals at different CNS levels. In fact, in preliminary experiments, where early LS neural segments + all adjacent mesoderm (paraxial + intermediate + lateral) are transplanted to anterior T regions, high Hoxd10 expression is still not obtained in the transplanted LS segments. Rather, we would suggest that regionalization in LS segments takes place on a different time scale with more players involved than at more anterior CNS levels. The LS region is unique. At anterior LS levels, the neural tube forms by primary neurulation; at posterior LS levels, by secondary neurulation (see Schoenwolf and Smith, 1990). From the site of secondary neurulation, posteriorizing signals may be capable of overriding any signals produced by transplanted mesoderm. Such signals may remain for a longer
developmental time period than they do at more anterior axial levels. Inductive signals or the cells that produce them might also spread further anteriorly at relatively later stages (see Catala et al., 1995). Finally, signals might spread to anterior LS neural segments not only via lateral tissues but also via planar signals from posterior to anterior neural regions (Grapin-Botton et al., 1995; Grapin-Botton et al., 1997). Our neural transposition experiments provide hints that Hoxd10 inductive signals spread from LS3 to LS1-2. When LS3 was included in a transplant or reversal into T regions, Hoxd10 levels were higher in LS1-2 than when LS3 was not included. It is important to point out, however, that a more extensive experimental series, including the transplantation of posterior LS neural segments and the use of quail donor tissue, is needed to definitively address the question of planar signaling.

Our data also indicate that there is a prepattern for Hoxd10 expression at the earliest neural tube stages and we suggest that the ability to express broad classes of Hox genes may be programmed before the ability to express Hox gene members of a single class (see also Grapin-Botton et al., 1997). At early neural tube stages, transplants of LS paraxial mesoderm may be incapable of inducing Hoxd10 expression in T neural tissues because T neural tissues are largely refractory to signals programming this member of a posterior Hox gene class. Only placement of T segments very posteriorly, near the site of secondary neurulation, can change neural fate. In LS regions, alterations in adjacent paraxial mesoderm may have little influence on subsequent regional development because of the existence of an early prepattern and a spread of posteriorizing signals at late neural tube stages.

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