Sequences 5' of the homeobox of the *Hox-1.4* gene direct tissue-specific expression of *lacZ* during mouse development

Richard R. Behringer[1],*, David A. Crotty[2], Virginia M. Tennyson[3,4], Ralph L. Brinster[1], Richard D. Palmiter[5] and Debra J. Wolgemuth[2,6,7],†

[1]Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
[2]Department of Genetics and Development, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032, USA
[3]Anatomy and Cell Biology, and [4]Pathology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032, USA
[5]Department of Biochemistry, Howard Hughes Medical Institute, University of Washington, SL-15, Seattle, WA 98195, USA
[6]Department of Obstetrics and Gynecology, and [7]The Center for Reproductive Sciences, Columbia University College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032, USA

*Present address: Department of Molecular Genetics, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA
†Author for correspondence at address 2

SUMMARY

The murine homeobox-containing gene *Hox-1.4* is expressed in restricted patterns during embryogenesis and in male germ cells. To begin identification of the *cis*-acting elements regulating this expression, transgenic mice were generated carrying a chimeric construct that contained approx. 4 kb of 5' flanking sequence and approx. 1 kb of structural gene, fused in frame to the *E. coli lacZ* gene. This construct directed expression of the resulting Hox-1.4,β-galactosidase fusion protein in a pattern that reproduced virtually the complete embryonic and adult sites of expression of the endogenous gene. Embryonic expression of the fusion protein was first detected in mesoderm at day 8.0 of gestation (E 8.0). Between gestational ages E 8.5 to E 12.5, β-gal expression was observed in the somites, the lateral walls of the posterior myelencephalon, the dorsal region and ventral wall of the spinal cord, spinal ganglia and pre-vertebrae and their surrounding mesenchyme, between presumptive ribs, as well as in mesenchymal layers in the lung, kidney and portions of the gut. Expression was also noted in the pancreas and in the supporting cells and sheath around subsets of peripheral nerves, sites that had not been detected previously. Adult expression was observed in testes, specifically in meiotic and post-meiotic male germ cells. In contrast, transgenic mice carrying 5' deletions of the construct which leave approx. 1.2 kb or approx. 2.0 kb of *Hox-1.4* sequence 5' to the embryonic promoter, did not exhibit β-gal staining. These deletion experiments defined at least one *cis*-acting control element necessary for the expression of the *Hox-1.4* gene to a 2 kb region located 2 to 4 kb 5' of the embryonic transcription start site.

Key words: homeobox gene, *cis*-acting elements, *lacZ*, mouse

INTRODUCTION

The homeobox, a highly conserved 183 bp sequence found in several classes of development-regulating genes in *Drosophila*, is also conserved in the genome of organisms as evolutionarily diverse as sea urchins and mammals (reviewed by Gehring, 1987; Scott et al., 1989). Genes containing this domain are known to function as transcription factors, regulating the expression of target genes. The sequence similarity in the homeodomain has provided a means for isolating potential development-regulating genes from the complex genomes of higher organisms. Mouse genes with a homeodomain most similar to that of the *Drosophila Antennapedia* gene are designated *Hox* genes (Martin et al., 1987). More than thirty-five *Hox* genes have

been identified and the patterns of expression of several of them have been characterized in embryos and adult tissues. Like their *Drosophila* counterparts, the mammalian homeobox genes exhibit temporal, spatial and cellular specificity in their expression both during embryogenesis and in adult tissues. However, the regulatory mechanisms involved in the establishment of these complex patterns are not well understood.

The mouse gene termed *Hox-1.4* was isolated from a cDNA library prepared from adult mouse testis RNA (Wolgemuth et al., 1986). Like other mouse homeobox genes characterized to date, *Hox-1.4* is expressed during mid-gestational development of the mouse embryo in a temporally and spatially restricted pattern. In day 12.5 embryos (E 12.5), *Hox-1.4* expression has been observed by in situ and

northern blot hybridization analysis in the dorsal region of the spinal cord, the myelencephalon, spinal ganglia, prevertebrae, lung mesenchyme, the mesenchymal tissue between aortic arches and the base of the heart, in the mesenchyme surrounding the base of the larynx and trachea, diaphragm, thymus, stomach and gut mesenchyme, kidney (mesonephros and metanephros), and in areas lining the ribs (Wolgemuth et al., 1987, 1989; Toth et al., 1987; Galliot et al., 1989; Gaunt et al., 1988, 1989). *Hox-1.4* expression in the adult is unique in that it is expressed at high levels in meiotic and postmeiotic male germ cells, and at very low levels (if at all) in other adult tissues (Wolgemuth et al., 1986, 1987; Rubin et al., 1986).

Overexpression of transgenic constructs carrying either 9 kb or 4 kb of upstream flanking sequence and the entire *Hox-1.4* coding region resulted in the first phenotype associated with expression of a mammalian *Hox* gene (Wolgemuth et al., 1989). The phenotype resembles congenital megacolon and appears to involve abnormalities in the enteric nervous system in the caudal region of the colon (V. M. Tennyson et al., unpublished data). In situ hybridization of transgenic embryos at E 12.5 showed that these constructs were able to direct expression of the transgene in a tissue-specific manner similar to that of the endogenous *Hox-1.4*. However, it appeared that regions controlling quantitative regulation might have been lacking, since the transgenic mRNA levels were lower than the endogenous transcripts in the testis and were elevated in the embryo, particularly in the mesenchyme of the embryonic mid-gut (Wolgemuth et al., 1989).

Both general and tissue-specific *cis*-acting regions of the murine homeobox genes *Hox-1.1* (Püschel et al., 1990, 1991), *Hox-1.3* (Zakany et al., 1988; Tuggle et al., 1990), *Hox-2.2* (Schugart et al., 1991), *Hox-2.3* (Kress et al., 1990), *Hox-3.1* (Bieberich et al., 1990) and *Hox-2.6* (Whiting et al., 1991) have been localized by examining the expression of *lacZ* fusion constructs with X-gal staining in transgenic mice. With the exception of the recent studies on *Hox-1.1* (Püschel et al., 1991) and *Hox-2.6* (Whiting et al., 1991), most of the studies have focused on analyzing the effect of 5′ flanking regions and have yielded only a limited recapitulation of the in vivo embryonic expression patterns. Sequences in both the 5′ and 3′ ends of the *Hox-2.6* gene were shown to be important for establishing the appropriate tissue specificity and anterior boundary of expression in the embryo (Whiting et al., 1991) and a role for the intron in *Hox-1.1* has been reported (Püschel et al., 1991). Except for the study on *Hox-2.3* (Kress et al., 1990), adult expression patterns have not been assessed in detail, especially at the cellular level.

The goal of the present study was to determine which sequences of the mouse *Hox-1.4* gene were needed to drive expression of reporter constructs in transgenic mice in a pattern that would mimic the endogenous expression pattern. These constructs would then serve as a baseline against which to compare changes in the pattern of expression in subsequent deletion analyses to identify temporal and tissue-specific *cis*-acting regulatory elements. In addition, the enhanced resolution and sensitivity of detection with the X-gal-staining method might be expected to reveal in greater detail the pattern of *Hox-1.4* expression. Further-

more, mice carrying reporter constructs conferring the correct *Hox-1.4* expression pattern would provide a readily assayable system for cross-breeding experiments to examine the effect of various mutations and genetic backgrounds on the expression of the *Hox-1.4* gene. Our observations on regions necessary for *Hox-1.4* expression are also compared with recent studies examining the sequences involved in determining the appropriate patterns of expression of the closely related, paralogous gene *Hox-2.6* (Whiting et al., 1991).

MATERIALS AND METHODS

Hox-1.4,lacZ constructs and production of transgenic mice

A 5 kb *Kpn*I-*Eco*RI fragment that extends 5′ of the *Eco*RI site in the homeodomain of *Hox-1.4* was fused to the *Nco*I site at the initiation codon of *lacZ* in the plasmid placF. placF contains about 0.6 kb of mouse *protamine-1* sequences at the 3′ end of *lacZ* to provide an intron and polyadenlyation signal (Mercer et al., 1991). The *Eco*RI and *Nco*I sites were filled in with the Klenow fragment of DNA polymerase before ligation to create the sequence GAA TTC ATG G at the junction and to continue the reading frame of *Hox-1.4* into the *lacZ* gene. An 8.5 kb *Kpn*I-*Pst*I fragment containing Hox-1.4,lacZ was separated from the pUC plasmid vector and injected into fertilized eggs to generate transgenic mice (Brinster et al., 1985). In subsequent experiments, 5′ deletions of this construct were created by converting the *Eco*RV sites, at 2.2 and 3.0 kb 5′ of the *Eco*RI site in the homeobox, to *Sal*I and the *Sal*I-*Pst*I fragment was then isolated.

Detection of fusion protein expression in adult tissues and in embryos

Typically, transgenic males were bred with non-transgenic B6/SJL F_1 hybrid females to generate timed matings. The day of detection of the vaginal plug was considered embryonic day E 0.5. Assessment of the developmental progression of embryonic specimens was performed on selected samples according to Theiler (1972), and designated by both gestational age and Theiler stage. Verification of transgenic animals was performed by Southern and/or dot blot analysis of placental or tail DNA with a *lacZ* specific probe. Non-transgenic littermates or tissues from non-transgenic adults were stained to detect any sites of endogenous β-gal activity.

Whole embryos or tissues were prepared by a 1 hour fixation at room temperature in 0.1 M phosphate buffer, pH 7.3, 0.2% glutaraldehyde, 2% formaldehyde, 5 mM EGTA pH 7.3, and 2 mM $MgCl_2$. Fixation was followed by three 30 minute rinses at room temperature in 0.1 M phosphate buffer, pH 7.3, 0.1% deoxycholic acid (sodium salt), 0.2% Nonidet P40, and 2 mM $MgCl_2$. Specimens were stained overnight at 37°C in 1 mg/ml X-Gal (dissolved at 25 mg/ml in DMSO), 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide made up in the above rinse solution. After staining, tissues were briefly rinsed in 0.1 M phosphate buffer, pH 7.3, and then photographed or prepared for sectioning.

In embryos of gestational ages of E 12.5 or older, we observed that the X-gal stain did not fully penetrate tissues. Therefore, at this stage embryos were fixed and then dissected to allow penetration of the stain, or were fixed, embedded in agar and cut at 50-200 μm with a vibratome, and then stained. Embryos or tissues were prepared for sectioning in either frozen blocks of OCT compound (Tissue-Tek, Baxter Scientific) and stained directly on slides, or stained as whole embryos as described above and sectioned in paraffin blocks at 8 μm or agar blocks (50-200 μm).

For paraffin sections, embryos or tissues were fixed, rinsed and stained as described above. After staining overnight, the samples were rinsed briefly in 0.1 M phosphate buffer. Tissues were then post-fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, at 4°C and processed for paraffin embedding according to standard procedures (Jaffe et al., 1990). 8 μm sections were cut onto TESPA-treated slides. Slides were deparaffinized by 1 wash with xylene for 1 minute, followed by 1 minute in 100% ethanol, 2 minutes in 95% ethanol, and eosin counterstaining. Sections of testes were counterstained with both hematoxylin and eosin.

RESULTS

Generation of Hox-1.4,lacZ transgenic mice

Our previous transgenic experiments suggested that at least in the mid-gestation embryo and adult, constructs consisting of either 9 or 4 kb of 5′ flanking sequence and 1.5 kb of the structural *Hox-1.4* gene itself were both capable of mimicking the endogenous spatial pattern of expression (Wolgemuth et al., 1989; and data not shown). We therefore began these experiments with a construct that contained 4 kb of *Hox-1.4* 5′ flanking sequence and approx. 1 kb of structural gene fused in frame to the *E. coli* gene *lacZ* (Fig. 1). The 3′ end of the construct consisted of sequences from the mouse *protamine-1* (*Prm-1*) gene (Mercer et al., 1991) and contained both an intron and polyadenylation signal. Expression of this construct should generate a Hox-1.4 fusion protein possessing β-gal activity but lacking an intact homeodomain, and hence, with no DNA binding function. Founder (F_o) embryos were examined to establish the generality of the expression patterns. Founder adults expressing this gene construct were produced and were used to establish two lines of transgenic mice, Tg(Hox-1.4,lacZ)Bri142 and Tg(Hox-1.4,lacZ)Bri143, that exhibited identical patterns of X-gal staining and were studied in detail.

Pattern of expression in early mid-gestation embryos

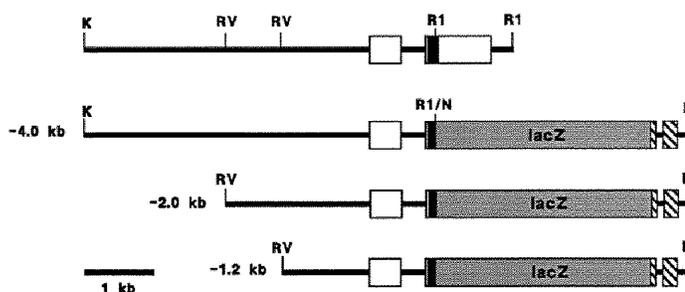The activity of the β-gal fusion protein was monitored in embryos beginning at E 7.5. Expression was first detected

Fig. 1. Diagram of the constructs used to generate the transgenic mice. See Materials and Methods for details on the generation of these constructs. K, *Kpn*I; RV, *Eco*RV; RI, *Eco*RI; N, *Nco*I; P, *Pst*I. The open box indicates *Hox-1.4* coding sequences; the solid box indicates the homeodomain; the shaded box indicates *lacZ* sequences, and the diagonally hatched box indicates *Prm-1* sequences.

at E 8.0 and was restricted to the mesodermal germ layer (Fig. 2A). No activity was apparent in the overlying ectoderm at this stage. At E 8.5 (Theiler stage 14, T14), β-gal activity was observed in somites and in the neural tube (Figs 2B, 3A). Somite staining was observed caudally from the 6th somite, with the most intense staining appearing in the internal surface of the dermamyotome, immediately adjacent to the basal lamina which divides dermamyotome from sclerotome (Fig. 3B). Within the sclerotome, some cells are stained, but there is no apparent difference in the distribution of stained cells between the anterior and posterior regions of the somite. The anterior boundary of expression in the neural tube was at a level just below somite 1 and expression extended caudally into the tail.

In E 9.5 (T16) embryos, the anterior limit of expression in the neural tube was well defined and was located approximately 600 μm caudal to the base of the otic placode (Fig. 2C). In caudal regions, β-gal activity was observed throughout the neural tube as well as in somites (Fig. 4A), which exhibited the same pattern of staining in the epithelium between the dermamyotome and the sclerotome as seen in somites at E 8.5. In more rostral regions of the neural tube, β-gal activity was confined to the dorsal half (Fig. 4B). Spinal ganglia were beginning to form and showed β-gal activity, whereas dermamyotome and sclerotome regions were losing stain (Fig. 4B). β-gal activity was also detected along the length of the nephrogenic cord (Fig. 4A) and within the mesenchyme of the umbilical gut (Fig. 4B).

Expression in mid-gestation embryos

At E 10.5 (T18), β-gal expression was seen along the length of the neural tube (Fig. 2D), with the clearly defined anterior limit of activity at the level of the caudal hindbrain (approx. 1.25 mm rostral to the first cervical ganglia). At this stage, staining throughout the spinal cord exhibited the dorsal positive/ventral negative pattern (Fig. 5A) observed the in rostral spinal cord of E 9.5 (T16) embryos. In addition, positively staining commissural fibers crossing under the floor plate were observed. Rostral to the first cervical ganglia, β-gal activity was no longer detected in the ventral wall of the neural tube, while the lateral walls remained positive. Spinal ganglia that had developed at this stage and mesenchymal regions between adjacent ganglia showed β-gal activity (Figs 3C, 5A). In contrast, the cells of the sclerotome, which have now migrated toward the notochord, as well as the cells of the dermamyotome were now negative for staining. Staining due to β-gal activity was pronounced in most of the gut (Fig. 2D), but was absent in the esophagus and the terminal bowel at the point where it enters the cloaca. The mesonephros, metanephros, and connective tissue between the heart and liver where the diaphragm will develop also showed β-gal staining. Some peripheral nerves, particularly in the region of the brachial plexus, revealed β-gal activity in the sheath surrounding the axon (Fig. 5A).

At E 11.5 (T20), expression was localized within the dorsal portion of the spinal cord, with an anterior restriction in the posterior hindbrain (Fig. 2E,F). Examination of serial sections in the region of the myelencephalon established the anterior limit of the β-gal activity in the neural tube within the base of the 4th ventricle (data not shown).
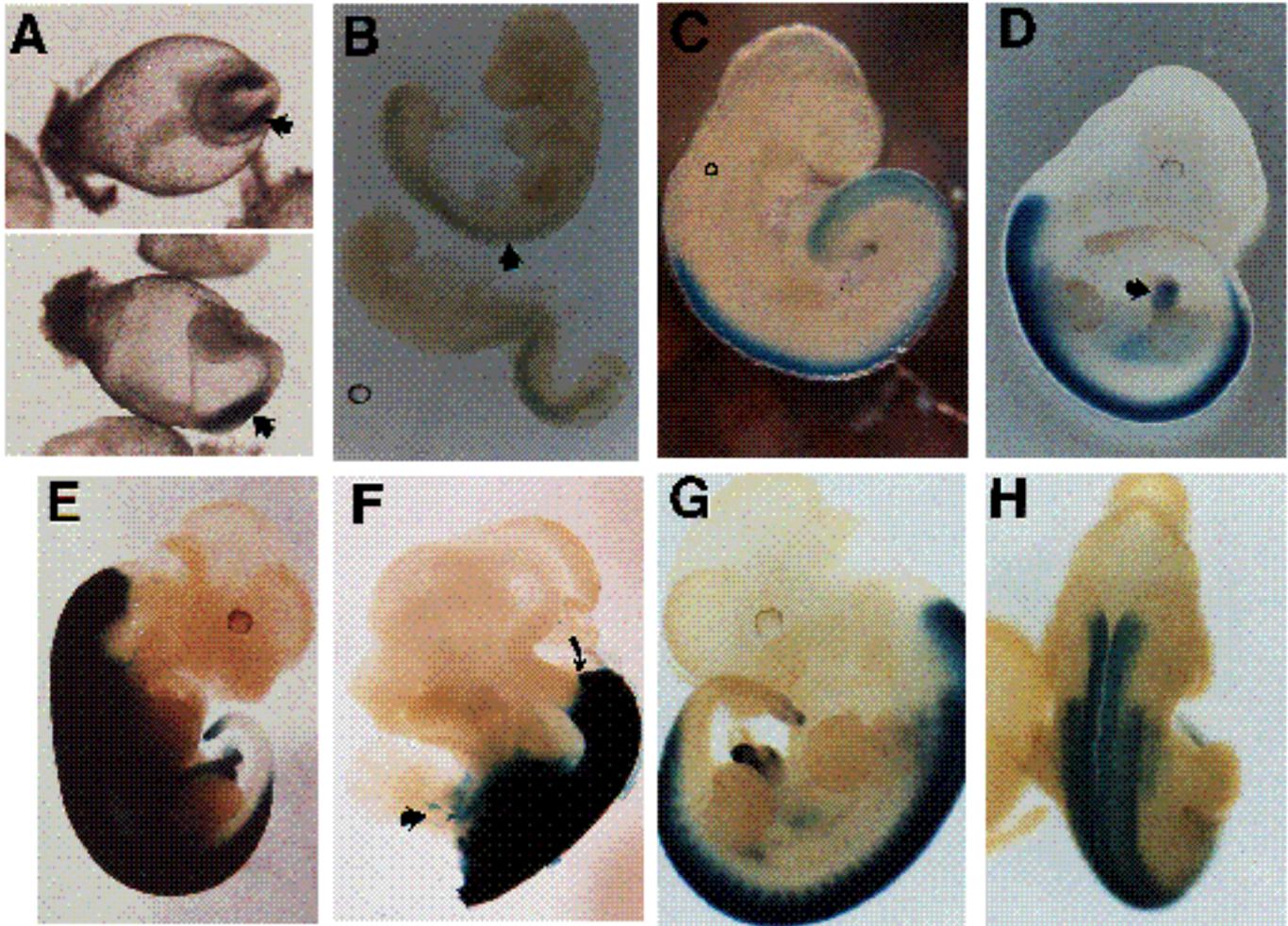
Fig. 2. Developmental time course of -gal staining pattern in Hox-1.4,lacZ transgenic embryos. (A) E 8.0 (arrows indicate mesodermal staining). (B) E 8.5 (T14; arrow indicates somitic staining). (C) E 9.5 (T16; O indicates otic placode). (D) E 10.5 (T18; arrow indicates umbilical gut). (E) E 11.5 (T20). (F) Rostral portion of an E 11.5 (T20) embryo bisected mid-sagitally (short arrow indicates nerve trunk staining within limb; curved arrow indicates anterior boundary of expression in the central nervous system). (G) E 12.5 (T21). (H) E 12.5 (T21), dorsal view.

Expression extended most rostrally in the lateral walls of the hindbrain and slightly more caudally in the ventral wall. The pattern of staining within commissural fibers crossing under the floor plate of the spinal cord was unchanged (Figs 5B, 6A). -gal staining was also localized within spinal ganglia and in mesenchyme between the ganglia. Between E 11-11.5 (T19-20), -gal activity became visible in mesenchymal tissue lateral and ventral to the spinal ganglia and in the region in and around where cells of somitic origin were differentiating.

Diffuse staining of lateral mesenchymal tissue was observed in the region of the brachial and lumbar plexuses (Fig. 2E). A large -gal-positive nerve trunk with branches was observed in the forelimbs of E 11.5 (T20) embryos (Fig. 2F). In addition, -gal positive peripheral nerves, located between the precursors of the ribs, were observed. In all cases, while the staining in whole mounts of embryos suggested the presence of the fusion protein in the nerve, our analysis of transverse sections indicated that the staining appeared to be limited to the sheath around the axons,

rather than within the axons themselves (Fig. 6B). In addition, there was diffuse staining of the mesenchyme in the dorsolateral wall of the thorax. -gal activity was also observed in the apical ectodermal ridge (AER) of the forelimbs and hindlimbs of E 11.5 (T20) fetuses. This pattern first became apparent at E 11.0 (T19). No -gal activity was observed in the AER of non-transgenic fetuses of the same age or in transgenic embryos at E 10.5 (T18) or earlier.

Examination of dissected tissues and histological sections of E 11.5 (T20) embryos revealed -gal staining in most of the mesodermally derived tissues where *Hox-1.4* mRNA expression had previously been observed. Staining was seen in the mesenchymal layer of the developing gut of *Hox-1.4,lacZ* transgenic fetuses (Fig. 6C,F). This activity was readily observed in stomach and umbilical gut, but no activity was observed in esophagus or the distal end of the colon. Staining was also noted in the pancreas (Fig. 6C), a site previously reported to lack *Hox-1.4* transcripts (Gaunt et al., 1989). -gal activity was observed in the lungs, with
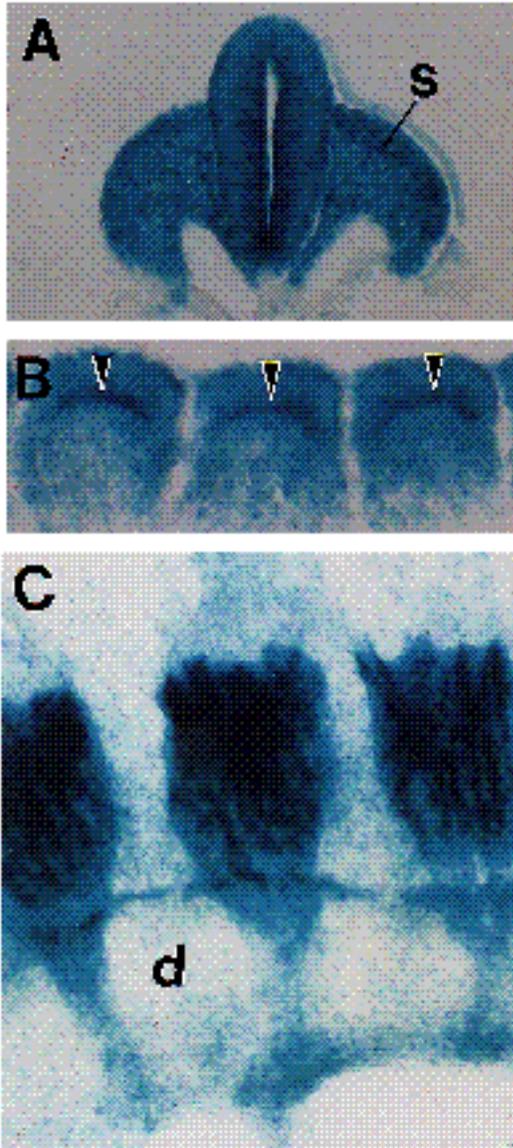
Fig. 3. Vibratome sections tracing transgenic -gal activity in somites and somitic derivatives. (A) Transverse 50 µm section of an E 8.5 (T14) embryo. Staining was visible in the neural tube and somites (s). (B) Sagittal 50 µm section through dissected somites from an E 8.5 (T14) embryo. The arrowheads indicate the region of increased stain in the epithelium dividing the dermamyotome and sclerotome. (C) Spinal ganglia dissected from an E 10.5 (T14) embryo. Staining was present within the ganglia, but was absent from the dermamyotome (d).

more intense staining in the lower lobes (Fig. 6D). Additional sites of staining included the mesonephros and metanephros (Fig. 6E) and developing diaphragm (Fig. 6F).

Fusion protein staining patterns in E 12.5 (T21) embryos were grossly similar to E 11.5 (T20) embryos (Fig. 2G and 2H). However, at this stage the X-gal was unable to penetrate all tissues when stained as whole embryos, particularly within the spinal cord (Fig. 6G and 6H). Examination of sectioned and dissected E 12.5 (T21) embryos revealed a continuation of the dorsal staining of the spinal cord with positive commissural fibers crossing below the floorplate

(Figs 4C, 6G). Staining within spinal ganglia continued and mesenchymal staining was more widespread, particularly in the region of the forelimb. -gal activity was seen dorso-lateral and lateral to the spinal ganglia and around developing skeletal muscle (Fig. 4C), as well as in the perichondrium and differentiating cartilage of the transverse processes of the developing vertebrae (Fig. 6I). The anterior limit of expression in the spinal cord was at the level of the 4th ventricle and at the level of cervical vertebra 2 in the mesoderm, in agreement with previous in situ analyses (Galliot et al., 1989; Gaunt et al., 1989). The pattern of staining of other tissues, including nerve sheaths in limbs and between developing ribs, the AER, the gastrointestinal tract, and other visceral organs, was similar to that observed in E 11.5 embryos. -gal staining continued in E 14.5 and E 15.5 embryos (the latest stages examined in this study), but details are not presented in this report because of inconsistencies due to incomplete penetration of stain.

β-gal activity in postnatal and adult testes

In the adult mouse, *Hox-1.4* expression is restricted to meiotic and postmeiotic male germ cells (Wolgemuth et al., 1987). To determine the temporal and cellular specificity of expression of the Hox-1.4,lacZ transgene during spermatogenesis, testes were examined from transgenic mice from both lines at various times after birth and in adults (Fig. 7). At birth, a group of spermatogonia begin to proliferate and differentiate to generate spermatozoa (Bellve, 1979). At postnatal day 6 (pn d 6), germ cells have not yet entered meiosis. By pn d 16, spermatogenesis has progressed into meiosis and pachytene spermatocytes are present. At pn d 21, meiotic spermatocytes and secondary spermatocytes and the first postmeiotic spermatids begin to appear. No -gal activity was observed in pn d 6 (data not shown) or d 7 transgenic testes (Fig. 7A). -gal activity was first observed in pn d 16 transgenic testes within a subset of regions of the seminiferous tubules (Fig. 7B). Uniform light fusion protein staining was observed in the seminiferous tubules of pn d 21 transgenic testes (Fig. 7C). No activity was observed in interstitial cells of the testes. In the testes from adult transgenics, this same germ cell-specific pattern of -gal activity was observed except that strong staining was observed in interstitial cells. However, this interstitial staining is due to endogenous -gal activity since it was also observed in non-transgenic testes. High levels of an endogenous -gal activity were also observed in the epididymis of transgenic and non-transgenic mice of all ages (data not shown). Histological sections of stained Hox-1.4,lacZ adult testes confirmed that -gal activity was specifically localized to meiotic and postmeiotic spermatogenic cells (Fig. 7D). Of the other adult tissues examined, including lung, colon and kidney, no transgene-specific fusion protein staining was detected.

Expression of β-gal in transgenic mice carrying 5′ deletion constructs

Deletion analysis was carried out to define further the location of *cis*-acting regulatory elements within the 5 flanking region. The first deletion construct analyzed was truncated at the *Eco*RV site located approx. 1.2 kb 5 to the
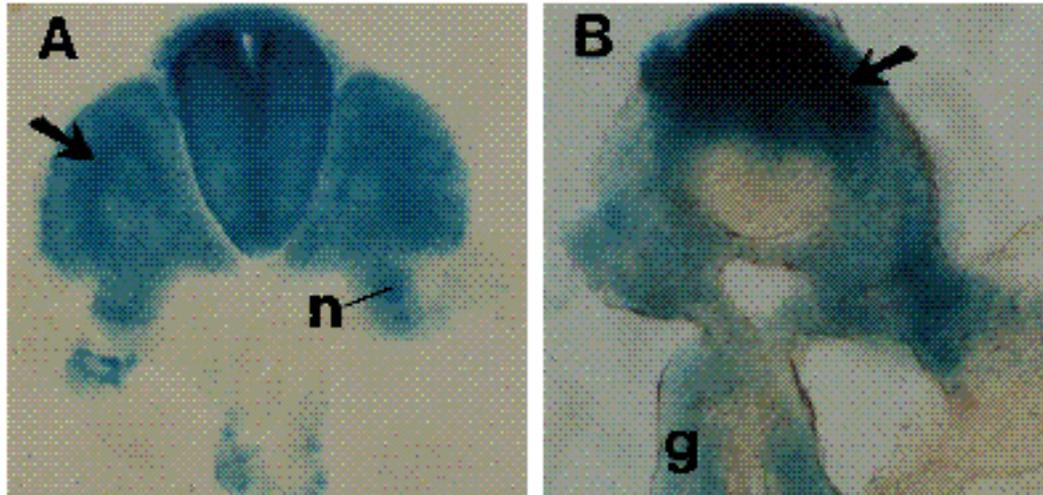
Fig. 4. Vibratome sections of E 9.5 (T16) embryo. (A) 50 μm transverse section through the caudal region of the embryo. Staining was seen in both the dorsal and ventral regions of the neural tube, and in the nephrogenic cord (n) and the somites. Within the somite, staining was concentrated along the epithelium separating dermamyotome and sclerotome (arrow). (B) 100 μm transverse section of the rostral region of the same embryo. Staining was present only in the dorsal half of the spinal cord and was found in the gut mesenchyme (g) and the developing spinal ganglia (arrow). Cells of the dispursing somites were losing β-gal activity.

embryonic promoter (Fig. 1). No adult or embryonic β-gal activity was observed in tissues from 4 established transgenic lines carrying this construct. A second deletion construct, truncated at the *Eco*RV site approx. 2 kb 5′ to the embryonic promoter (Fig. 1) was also analyzed. Seven founder E 12.5 embryos carrying this construct were analyzed and no β-gal activity was observed. Southern blot analysis of DNA revealed that the transgenes had integrated intact (data not shown). Thus, it appears that at least one major regulatory region necessary for *Hox-1.4* expression in both the embryos and adult is located in the 2 kb region

between −4 and −2 kb upstream of the embryonic promoter in the *Hox-1.4* gene (Fig. 1).

DISCUSSION

Identification of sequences capable of conferring the endogenous expression pattern of the *Hox-1.4* gene

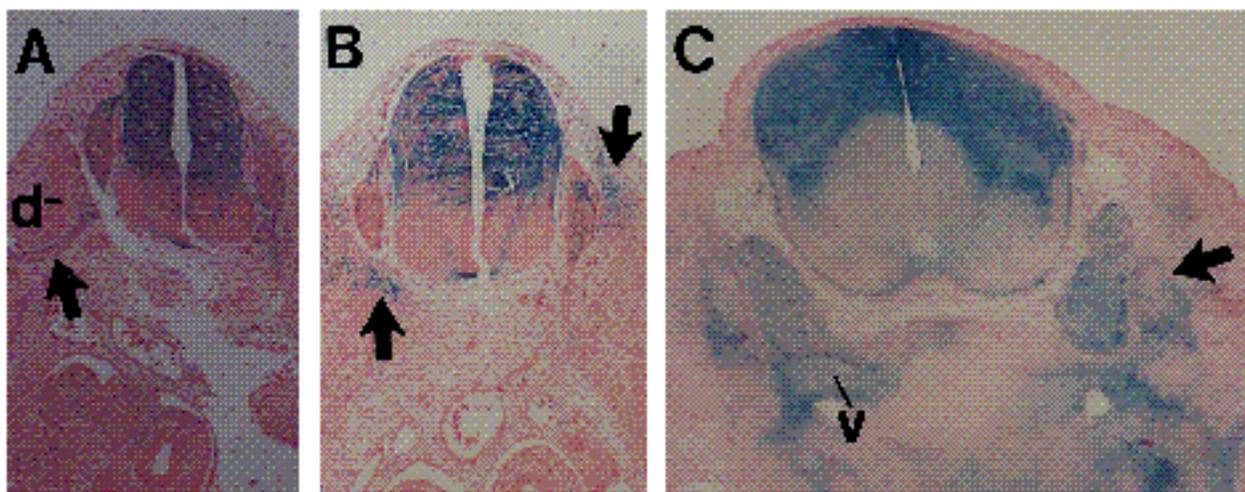We have shown that a construct consisting of 4 kb of 5′ flanking sequence and 1 kb of the coding region was suf-

Fig. 5. Transverse (8 μm) sections of the brachial region of Hox-1.4,lacZ embryos. (A) E 10.5 (T19), staining was observed in the dorsal region of the spinal cord, commissural fibers crossing the floor plate of the spinal cord, spinal ganglia, and the mesenchymal sheath of nerve roots (arrow). Sclerotome and dermamyotome (d) no longer revealed staining. (B) E 11.5 (T20), staining was present in the same regions as in E 10.5 embryos, but with the addition of staining in the mesenchyme lateral to spinal ganglia, as well as between spinal ganglia and dispersing somitic tissue (arrows). (C) E 12.5 (T21), staining continued to be detected in the dorsal spinal cord, commissural fibers crossing under the floor plate and spinal ganglia. Mesenchymal staining was now more widespread, and was visible dorsolateral to the spinal ganglia, around developing skeletal muscle (arrow), and in developing vertebrae (v).
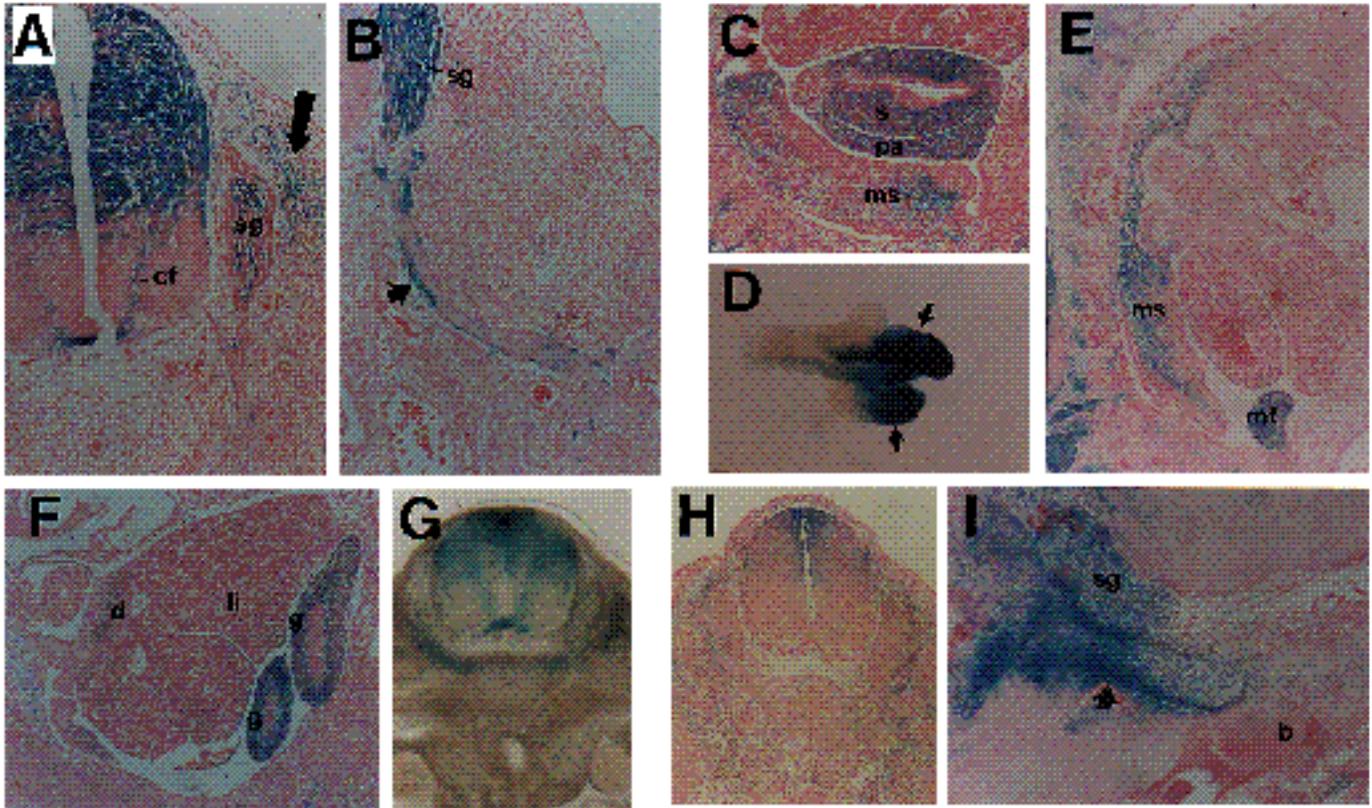
Fig. 6. -gal staining patterns in specific regions of Hox-1.4,lacZ transgenic mice. (A) E 11.5 (T20) spinal cord and ganglia. Staining was present in dorsal regions of the spinal cord, but not in ventral regions with the exception of the commissural fibers (cf) that cross the floor plate. Staining within the spinal ganglia (sg) exhibited a mottled pattern, and mesenchymal staining could be seen lateral to the spinal ganglia (arrow). (B) E 11.5 (T20) peripheral nerve root running to the brachial plexus. Staining was limited to the mesenchymal sheath (arrow) of all peripheral nerves examined (sg indicates spinal ganglia). (C) E 11.5 (T20), stomach (s), pancreas (pa) and mesonephros (ms). Staining was present in the mesoderm of stomach and absent in endoderm. (D) E 11.5 (T20), lung. Staining was more intense in lower lobes (arrows). (E) E 11.5 (T20), mesonephros (ms) and metanephros (mt). (F) E 11.5 (T20), sections through the gut. Staining was visible in mesodermal layers of the intestine (g) and absent in the endoderm. Staining could also be seen in the region of the developing diaphragm (d; li indicates liver). (G, H) Comparison of the staining patterns observed in E 12.5 (T21) embryos processed as 200 μm vibratome sections (G) or as whole embryos (H). (G) -gal activity could be seen in the dorsal half of the spinal cord, commissural fibers crossing the floor plate, spinal ganglia, portions of developing vertebrae and lateral mesenchyme. (H) Due to penetration problems, stain was apparent only in the outer regions of the dorsal spinal cord and in mesenchyme. Staining was not detected in spinal ganglia, much of the dorsal spinal cord, commissural fibers and most of the developing cartilage. (I) E 12.5 (T21), developing vertebrae. Staining was specific to regions of differentiating or newly differentiated cartilage; being most intense in the perichondrium (arrow) and transverse process but almost undectable in the body of the vertebrae (b).
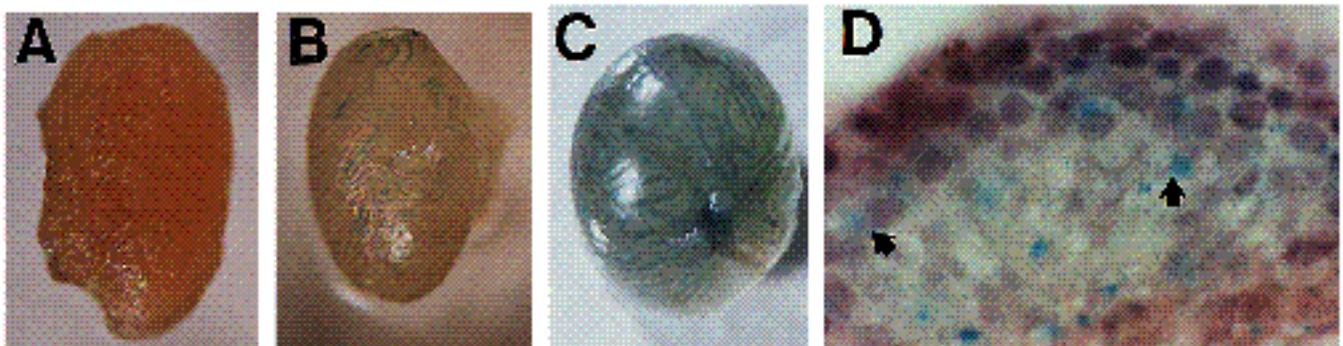

Fig. 7. Postnatal -gal staining in transgenic Hox-1.4,lacZ testes. (A) d 7.0 pn transgenic testis. No staining within tubules was visible. (B) d 16.0 pn transgenic testis. A limited number of seminiferous tubules began to show -gal staining. (C) d 21.0 pn transgenic testis. Staining was widespread throughout the tubules. (D) 30 μm paraffin section of adult (approx. 90 days pn) testis. Staining could be seen in germ cells in both meiotic and postmeiotic stages (arrows).

ficient to reproduce the endogenous pattern of *Hox-1.4* expression in embryonic and adult tissues at stages that have been examined to date. In the embryo, -gal staining was observed, for the most part, in the predicted tissues (tissue regulation) within the correct regions (spatial regulation) at the appropriate time (temporal regulation). In the adult, expression was detected only in the testis, specifically in germ cells that had entered meiosis. Thus, the correct tissue, cellular, and temporal specificity of the adult expression pattern of the endogenous gene was conferred by the transgene. It is difficult to assess quantitative aspects of the expression of *lacZ* relative to endogenous *Hox-1.4* due to differences in mRNA and fusion protein stability.

In addition, we have compiled a detailed analysis of the -gal expression pattern in sequential stages of mid-gestational development. Our analysis consistently revealed several previously undetected sites of expression and permitted finer resolution of localization. It should be noted that these sites may have been missed or less precisely described in previous studies using in situ hybridization analysis. Among the more striking patterns observed were staining in the commissural fibers of the spinal cord and in mesenchyme around various peripheral neurons. We interpreted these patterns as indicative of the endogenous expression of *Hox-1.4* for the following reasons. First, the localization was consistent with previous northern blot hybridization data pattern on the expression of the endogenous gene (Wolgemuth et al., 1987). Second, the distribution of -gal staining in the histological sections was in general agreement with previous in situ hybridization results; however, since the X-gal staining is more sensitive and affords higher resolution at the cellular level than in situ hybridization using radioactive probes and autoradiographic localization, we have defined more precisely the cellular localization. Further, the whole-mount staining data were also in agreement with previous in situ observations, but permitted detection of expression in structures such as nerve sheaths which would not have been detected by in situ analysis of histological sections. This pattern will now provide a blueprint for future studies using antibody localization of the Hox-1.4 protein as well as for deletion studies to identify *cis*-acting elements conferring tissue-specificity of expression.

Apparent differences in tissue distribution were observed between the fusion protein staining and previous in situ localization of mRNAs in the thymus and pancreas (Gaunt et al., 1989; Galliot et al., 1989). We observed -gal activity in the pericardial mesenchyme at the base of the aortic arches, a region that corresponded to the regions designated as 'thymus localization' of *Hox-1.4* transcripts (Galliot et al., 1989). It is also possible that Hox-1.4,lacZ mRNA was produced in these regions but was not translated or translated at such low levels that it was not detected in the staining assay. Expression of -gal was also observed in a region we identified as pancreas. Previous studies at the in situ level revealed *Hox-2.6* expression in a comparable structure, labeled as mesentery (Gaunt et al., 1989), but *Hox-1.4* mRNA localization was not reported. The staining within the lung, a site of in vivo expression of *Hox-1.4*, was not uniform, often with lower lobes staining more intensely than upper regions (Fig. 4D). Hox-2.6,lacZ transgenic con-

structs produced a similar staining pattern in lung (Whiting et al., 1991), a site of endogenous expression of the *Hox-2.6* gene (Graham et al., 1988). We also observed slight but distinct -gal activity in the AER of developing limb bud of embryos from E 11 on, in contrast to the previously reported low level of diffuse distribution of *Hox-1.4* transcripts detected by in situ in E 9.5 limb buds (Galliot et al., 1989). The significance of this staining in the AER is difficult to assess. Although there was no endogenous -gal activity in the AER of non-transgenic embryos, other transgenic experiments with -gal constructs have revealed X-gal staining in the AER as well (although the observations were not noted in the text; e.g. Whiting et al., 1991).

An additional point of interest concerns the variability that may be encountered depending upon the techniques used to localize the -gal staining. That is, the method of detection may influence interpretation, beyond just the issue of sensitivity. Whereas staining appeared to be localized wholly within structures such as peripheral nerves and spinal ganglia (Fig. 2G) upon visualization of whole embryos, examination of transverse sections revealed that, in certain stages, much of the visualized staining was actually in mesenchymal layers around the structures or within specific regions of these structures, such as the sheath of peripheral nerves. This may be important in analyzing the results of previous mRNA localization analyses, most of which were performed on sagittal sections using radioactively labeled probes and detection by autoradiography.

Previous studies (Whiting et al., 1991; Kress et al., 1990) reported problems with penetration of stain in older embryos (E 13.5 and E 14.5 respectively). Our studies have shown that incomplete X-gal penetration occurs earlier, at E 12.5 (T21). Comparison of tissues of animals stained as whole mounts and tissues cut into 200 μm thick sections before staining, revealed discrepancies in -gal activity, particularly within the spinal cord. Although the spinal cord appeared uniformly positively stained when the embryo was viewed as a whole mount, sections of these embryos revealed staining only in the most dorsal region of the spinal cord. In contrast, when embryos were cut into thick (200 μm) sections and then stained, -gal activity was shown to be present throughout the dorsal half of the spinal cord as well as within commissural fibers crossing under the floor plate. Exposed regions of dissected embryos also showed this pattern, while inner, less exposed regions showed the dorsal surface-only pattern. Future studies of E 12.5 (T21) and later embryos should take this phenomenom into account.

Possible role of *Hox-1.4* in the process of differentiation

Our results reveal an apparent inverse correlation between the expression of *Hox-1.4* and extent of differentiation of tissues of somitic origin. This correlation was evident in the developing and differentiating somites. In E 8.5 embryos and caudal regions of E 9.5 embryos, the somites were still intensely stained. However, in the more rostral regions of E 9.5 embryos and in E 10.5 embryos, staining was lost as cells in the dermamyotome and sclerotome began to disperse. Concomitantly, staining was apparent in spinal ganglia as they formed in the E 9.5-10.5 embryos.

Furthermore, as somitically derived cartilage and skeletal muscle began to differentiate, -gal activity was again observed. Staining was apparent in the mesenchyme differentiating into skeletal muscle, but not in the tissue that has already differentiated. In the vertebrae, staining was most obvious in the differentiating perichondrium, was less intense in the more differentiated transverse process, and was not detectable in the differentiated cartilage of the body of the vertebrae. Expression of *Hox-1.4* within these differentiating cells, along with the vertebral transformations seen in mutations and misexpression of homeobox genes (LeMouellic et al., 1992; Lufkin et al., 1992), provides further evidence that *Hox* genes play a major role in the organization and differentiation of the vertebral column.

Another site of staining was the mesenchymal layer of the developing gut, a site of expression that had also been detected by in situ hybridization (Gaunt et al., 1989; Galliot et al., 1989; Wolgemuth et al., 1989). Neural crest cells migrate into this region from the vagal and possibly the sacral neural tube levels and differentiate into the neurons and glia of the enteric nervous system (Gershon, 1987). This was of particular interest in view of the developmental abnormality that we have observed in transgenic mice overexpressing *Hox-1.4* in the gut (Wolgemuth et al., 1989). These animals develop congenital megacolon, which in our preliminary analysis of the distal region of the neonatal and adult colon of one of the transgenic lines, involves hypoganglionosis in the myenteric plexus (V. M. Tennyson et al., unpublished data). In addition, in late fetal life, the distal colon is colonized by some tyrosine hydroxylase immunoreactive neurons not normally found in the myenteric plexus. Thus, expression of *Hox-1.4* may be important in regulating genes providing cues for proper neural crest migration and differentiation. We could not be certain whether neural crest cells themselves exhibited -gal activity, but there was clearly expression of *Hox-1.4* in regions through which the cells are migrating, both in the gut and in the somites, as well as in crest-derived tissue, such as the spinal ganglia.

Expression of the transgenic constructs in the adult testis

The Hox-1.4,lacZ transgene that contained 4 kb of 5 flanking sequence and a portion of the structural *Hox-1.4* gene was active in adult testes of all lines of mice tested. The *Hox-1.4,lac Z* transgene contains sequences from the mouse *Prm-1* gene that supply an intron and polyadenylation signal (Mercer et al., 1991; Fig. 1). The 3 untranslated sequences of *Prm-1* have been shown to delay the translation of a transgene, expressed during early spermatid differentiation until late in spermatid differentiation (Braun et al., 1989). Therefore, the presence of the *Prm-1* sequences in the Hox-1.4,lacZ construct might have influenced the translation of the mRNA generated from this transgene. However, -gal activity was observed at the time of postnatal development when meiotic prophase spermatocytes normally express the endogenous *Hox-1.4* gene as well as in pachytene spermatocytes and later spermatogenic stages in histological sections of transgenic testes. This suggests that the translation of the transgenic mRNA in pachytene spermatocytes was not influenced by the presence of the

Prm-1 sequences. It should be recalled that the endogenous *Prm-1* gene is not expressed during meiotic prophase. Therefore, either the factors responsible for translational control are not present in pachytene spermatocytes or this chimeric mRNA is not responsive to them. It was not possible to determine whether the transgene transcripts present in spermatids were translationally regulated since the -gal produced in the earlier stages of differentiation of the cells would obscure differences in translation in the later stages.

Deletion constructs identify a 2.0 kb upstream region involved in regulating *Hox-1.4* expression

Our results using the deletion constructs indicate that at least one element absolutely necessary for expression in embryonic tissues is located in a 2 kb region between −2 and −4 kb 5 of the embryonic transcription start site (Fig. 1). The shorter constructs were not able to direct expression in any of the expected sites, including the adult testis, nor did they result in unregulated expression throughout the embryo or in adult tissues. This further suggested that cis elements required for germ cell-specific expression of *Hox-1.4* also reside within the 4 kb of *Hox-1.4* sequence involved with establishing the embryonic pattern. Thus, while the shorter constructs were not sufficient to direct expression of *Hox-1.4* in vivo (in mice), it is interesting that there are both *Krox 20* (Chavrier et al., 1989) and *Hox-1.4* homeodomain (K. Wu and D. J. Wolgemuth, unpublished data) binding sites within these constructs and constructs containing this region have been shown to be transcriptionally active in transfection experiments in tissue culture cells (Galliot et al., 1989; K. Wu and D. J. Wolgemuth, unpublished data). It will obviously be of interest to identify those elements within the *Hox-1.4* gene that are involved in directing the embryonic and adult expression to specific tissues and lineages. Deletion experiments are in progress to further delineate which DNA elements are necessary to confer this tissue-specific regulation.

Comparison of the elements necessary for *Hox-1.4* expression versus other Hox genes characterized to date

Data on regions of the *Hox-2.6* gene (the paralogue of *Hox-1.4* in the *Hox-2* cluster) responsible for conferring the endogenous pattern of expression (Whiting et al., 1991) and our observations on *Hox-1.4* concur on several levels. First, the appropriate spatial and tissue-specific patterns of expression in the mid-gestation embryo (*Hox-1.4* and *Hox-2.6*) and in the adult (*Hox-1.4*) could be achieved by portions of the respective genes when physically removed from their *Hox* complexes. This is of interest because it is clear that there has been selective pressure to maintain these genes in clusters and even within specific linear arrays within the clusters (Akam, 1989). This observation also argues against the existence of distant regulatory elements critical for the gross definition of tissue specificity of expression for *Hox-1.4*. However, it is possible that elements elsewhere in the cluster may be important for regulation in vivo, particularly at the quantitative level. Given our previous transgenic results, this may well be the case (Wolgemuth et al., 1989; R. Raz and D.J. Wolgemuth, unpublished data).

An interesting and potentially important difference was noted between our results and the results obtained from the studies on *Hox-2.6*. These two genes exhibit remarkably similar patterns of expression in the embryo, particularly in the spinal cord (Gaunt et al., 1989; Galliot et al., 1989; F. Watrin and D. J. Wolgemuth, unpublished observations). Whereas sequences at the 3 end of the *Hox-2.6* gene were essential for establishing the proper anterior boundary of expression in the mid-gestation spinal cord, the appropriate boundary of *Hox-1.4* expression was observed without the presence of any 3 sequences of *Hox-1.4* in the construct. Furthermore, we have no evidence for gross ectopic expression, even with the most truncated constructs. This is distinct from the observations in studies identifying regulatory elements of *Hox-1.1*.in which truncated Hox-1.1,lacZ fusion constructs resulted in ubiquitous expression of the fusion protein throughout the caudal half of the embryo. Critical negative regulatory elements were necessary to limit the expression to the correct tissues (Püschel et al., 1990, 1991). In contrast to *Hox-1.1* and similar to *Hox-2.6*, the *Hox-1.4* promoter appears to require positive elements for expression.

In summary, the fact that the constructs described here faithfully reflect the normal expression pattern of *Hox-1.4* greatly facilitates future mutational analysis aimed at identifying critical *cis*-acting regulatory elements. These transgenic mice can also be crossed with various mouse mutants to determine how the genetic background influences expression of the *Hox-1.4* gene. This is particularly valuable now that various members of the *Hox* family are being mutated by homologous recombination in embryonic stem cells (e.g. Chisaka and Capecchi, 1991; Lufkin et al., 1991; Le Mouellic et al., 1992). Thus, the effect of the neighboring *Hox-1.5* and *Hox-1.6* genes on *Hox-1.4* expression can be assessed easily by the appropriate breeding of these reporter mice with null mutants for the *Hox* genes. Thus, this study will serve as a valuable starting point for future genetic analysis of the regulation of this complex family of transcription factor genes.

REFERENCES

Akam, M. (1989). Hox and HOM: Homologous gene clusters in insects and vertebrates. *Cell* **57**, 347-349.

Bieberich, C. J., Utset, M. F., Awgulewitsch, A. and Ruddle, F. (1990). Evidence for a positive and negative regulation of the *Hox-3.1* gene. *Proc. Natl. Acad. Sci. USA* **87**, 8462-8466.

Bellve, A. R. (1979). The molecular biology of mammalian spermatogenesis. *Oxford Rev. Reprod. Biol.* **1**, 159-261.

Braun, R. E., Peschon, J. J., Behringer, R. R. and Palmiter, R. D. (1989). Protamine 3 -untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. *Genes Dev.* **3**, 793-802.

Brinster, R. L., Chen, H. Y., Trumbauer, M., Yagle, M. K. and Palmiter, R. D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA* **82**, 4438-4442.

Chavrier, P., Vesque, C., Galliot, B., Vigneron, M., Dolle, P., Dubuole, D. and Charnay, P. (1990). The segment-specific gene *Krox-20* encodes a transcription factor with binding sites in the promoter region of the *Hox-1.4* gene. *EMBO J.* **9**, 1209-1218.

Chisaka, O. and Capecchi, M. R. (1991). Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene *Hox-1.5*. *Nature* **350**, 473-479.

Galliot, B., Dolle, P., Vigneron, M., Featherstone, M. S., Baron, A. and Dubuole, D. (1989). The mouse *Hox-1.4* gene: Primary structure, evidence for promoter activity and expression during development. *Development* **107**, 343-359.

Gaunt, S. J., Sharpe, P. T. and Duboule, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. *Development* **104**, 169-179.

Gaunt, S. J., Krumlauf, R. and Duboule, D. (1989). Mouse homeogenes within a subfamily, *Hox-1.4, -2.6* and *-5.1*, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation. *Development* **107**, 131-137.

Gehring, W. J. (1987). Homeo boxes in the study of development. *Science* **236**, 1245-1252.

Gershon, M. D. (1987). Insights into neural development provided by the bowel. Fidia Research Foundation. Neuroscience Award Lectures 1986, Liviana Press, Padria, Italy pp 108-143.

Graham, A., Papalopulu, N., Lorimer, J., McVey, J. H., Tuddenham, E. G. D. and Krumlauf, R. (1988). Characterization of a murine homeo box gene, *Hox-2.6*, related to the *Drosophila Deformed* gene. *Genes Dev.* **2**, 1424-1438.

Jaffe, L., Jeanotte, L., Bikoff, E. K. and Robertson, E. J. (1990). Analysis of beta2-microglobulin gene expression in the developing mouse embryo and placenta. *J. Immunol.* **145**, 3474-3482.

Kress, C., Vogels, R., De Graaf, W., Bonnerot, C., Meijlink, F., Nicholas, J. F. and Deschamps, J. (1990). *Hox-2.3* upstream sequences mediate lacZ expression in intermediate mesoderm derivatives of transgenic mice. *Development* **109**, 775-786.

Le Mouellic, H., Lallemand, Y. and Brulet, P. (1992). Homeosis in the mouse induced by a null mutation in the *Hox-3. 1* gene. *Cell* **69**, 251-264.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M. and Chambon, P. (1991). Disruption of the *Hox-1.6* homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66**, 1105-1119.

Lufkin, T., Mark, M., Hart, C. P., Dolle, P., LeMeur, M. and Chambon, P. (1992). Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. *Nature* **359**, 835-841.

Martin, G. R., Boncinelli, E., Duboule, D., Gruss, P., Krumlauf, R., Lonai, P., McGinnis, W., Ruddle, F. and Wolgemuth, D. J. (1987). Nomenclature for homeobox-containing genes. *Nature* **325**, 21-22.

Mercer, E. H., Hoyle, G. W., Kapur, R. P., Brinster, R. L. and Palmiter, R. D. (1991). The dopamine -hydroxylase gene promoter directs expression of *E. coli lacZ* to sympathetic and other neurons in adult transgenic mice. *Neuron* **7**, 703-716.

Püschel, A. W., Balling, R. and Gruss, P. (1990). Position-specific activity of the *Hox-1.1* promoter in transgenic mice. *Development* **108**, 435-442.

Püschel, A. W., Balling, R. and Gruss, P. (1991). Separate elements cause lineage restriction and specify boundaries of *Hox-1.1* expression. *Development* **112**, 279-287.

Rubin, M. R., Toth, L. E., Patel, M. D., D'Eustachio, P. and Nguyen-Huu, M. C. (1986). A mouse homeo box gene is expressed in spermatocytes and embryos. *Science* **233**, 663-667.

Schughart, K., Bieberich, C. J., Eid, R. and Ruddle, F. H. (1991). A regulatory region from the mouse *Hox-2.2* promoter directs gene expression into developing limbs. *Development* **112**, 807-811.

Scott, M. P., Tamkun, J. W. and Hartzell, G. W. (1989). The structure and function of the homeodomain. *Biochim. Biophys. Acta* **989**, 25-48.

Theiler, K. (1972). *The House Mouse. Development and Normal Stages from Fertilization to 4 Weeks of Age.* New York: Springer-Verlag.

Toth, L. E., Slawin, K. L., Pintar, J. E. and Nguyen-Huu, M. C. (1987). Region-specific expression of mouse homeobox genes in the embryonic mesoderm and central nervous system. *Proc. Natl. Acad. Sci. USA* **84**, 6790-6794.

Tuggle, C. K., Zakany, J., Cianetti, L., Peschle, C. and Nguyen-Huu, M. C. (1990). Region-specific enhancers near two mammalian homeobox genes define adjacent rostrocaudal domains in the central nervous system. *Genes Dev.* **4**, 180-189.

Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P. W. J., Stott, D. and Allemann, R. K. (1991). Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. *Genes Dev.* **5**, 2048-2059.

Wolgemuth, D. J., Engelmeyer, E., Duggal, R. N., Gizang-Ginsberg, E., Mutter, G. L., Ponzetto, C., Viviano, C. and Zakeri, Z. F. (1986). Isolation of a mouse cDNA coding for a developmentally regulated, testis-specific transcript containing homeo box homology. *EMBO J.* **5**, 1229-1235.

Wolgemuth, D. J., Viviano, C., Ginzang-Ginsberg, E., Frohman, M. A., Joyner, A. L. and Martin, G. R. (1987). Differential expression of the mouse homeobox-containing gene *Hox-1.4* during male germ cell differentiation and embryonic development. *Proc. Natl. Acad. Sci. USA* **84**, 5813-5817.

Wolgemuth, D. J., Behringer, R., Mosteller, M. P., Brinster, R. L. and Palmiter, R. D. (1989). Transgenic mice overexpressing the mouse homeobox-containing gene *Hox-1.4* exhibit abnormal gut development. *Nature* **337**, 464-467.

Zakany, J., Tuggle, C. K., Patel, M. D. and Nguyen-Huu, M. C. (1988). Spatial regulation of homeobox gene fusions in the embryonic central nervous system of transgenic mice. *Neuron* **1**, 679-691.