

Position-specific activity of the *Hox1.1* promoter in transgenic mice

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Summary

During development, positional values have to be assigned to groups of cells. The murine *Hox* genes are a class of genes that are predicted to be involved at some stage in this process. During embryogenesis they are expressed in distinct overlapping region- and stage-specific patterns and therefore must be regulated in response to positional information. In this study, we have analysed the activity of *Hox1.1* promoter sequences in transgenic mice. The use of *lacZ* as a marker allows a detailed analysis of expression at the single cell level during early embryonic development. We show that 3.6 kbp of promoter and 1.7 kbp of 3' sequences provide sufficient regulatory information to express a transgene in a spatial and temporal manner indistinguishable from the endogenous *Hox1.1* gene during the period of development when *Hox1.1* expression is established. The

activation occurs in a strict order in specific ectodermal and mesodermal domains. Within each of these domains the transgene is activated over a period of four hours apparently randomly in single cells. In a following second period, *Hox1.1* and transgene expression patterns diverge. In this period, transgene expression persists in many mesodermally derived cells that do not express *Hox1.1* indicating the absence of a negative regulatory element in the transgene. The anterior boundary of transgene expression is identical to that of *Hox1.1*. However, no posterior boundary of transgene expression is set, suggesting that a separate element absent from the transgene specifies this boundary.

Key words: gastrulation, homeo-box, *lacZ*, pattern formation, transgenic mice.

Introduction

Trying to understand how an organism develops is to ask how patterns are established and elaborated during development. It is generally assumed that the physical basis for pattern formation is the precise spatial and temporal regulation of gene expression.

The fruitfly *Drosophila melanogaster* is, at the moment, the best characterised multicellular organism with respect to the genetic and molecular basis of pattern formation (Akam, 1987; Ingham, 1988). Isolation and analyses of developmental genes from *Drosophila* led to the identification of several conserved sequence elements coding for distinct protein domains (for reviews see: Gehring and Hiromi, 1986; Dressler and Gruss, 1988). One of these is the homeo-box, which is found in many genes in *Drosophila* involved in the specification of positional information. Subsequent to their identification in the fly, homeo-boxes have been found in many organisms as diverse as *Caenorhabditis elegans* (Way and Chalfie, 1988; Costa *et al.* 1988) and *Homo sapiens* (Boncinelli *et al.* 1988 and references therein). At least 30 different homeo-box containing genes (*Hox* genes) have been isolated from the mouse genome (for reviews see: Martin *et al.* 1987; Dressler and Gruss, 1988; Dressler, 1989). The *Hox* genes are expressed in complex overlapping region- and stage-

specific patterns during embryonal development (Holland and Hogan, 1988). Based on their homology to *Drosophila*, developmental genes, such as *Antennapedia* or *Ultrabithorax*, and transcription factors, like Oct1 and Oct2 (Herr *et al.* 1988, and references therein), it is currently assumed that the *Hox* genes are sequence-specific DNA-binding transcription factors (for reviews see: Levine and Hoey, 1988; Wright *et al.* 1989). Ectopic expression of *Hox1.1* in transgenic mice (Balling *et al.* 1989) or overexpression of *Xhox3* by injection of synthetic RNA into *Xenopus* embryos (Ruiz i Altaba and Melton, 1989) leads to specific developmental abnormalities. All evidence accumulated so far is consistent with the hypothesis that the *Hox* genes have a function in pattern formation or positional specification as transcriptional regulators. However, their precise role in this process remains to be determined.

Hox1.1 is expressed from day 7.5 to day 14 of murine embryogenesis (Colberg-Poley *et al.* 1985). Expression is initially detectable only in the allantois (day 7.5) (Mahon *et al.* 1988). Subsequently the transcript appears in a specific region of the neural plate (day 8) and in the mesoderm (day 8.5). During organogenesis (day 9.5 to 11.5) expression is maintained in specific mesodermal structures and progressively turned off in others (Mahon *et al.* 1988; Dressler and Gruss, 1989). At day

12 of development *Hox1.1* is expressed in two overlapping domains in neural tube, spinal ganglia and prevertebrae.

As regulation of *Hox1.1* expression is dependent on positional information, a study of the regulatory elements defining the domain of expression relative to the anteroposterior axis will help to elucidate the molecular mechanism specifying positional information during murine embryogenesis. Putative *Hox1.1* promoter sequences were linked to the *lacZ* gene and transgenic mice were generated. As a first step to unravel the mechanisms regulating pattern formation during gastrulation in the mouse, we report the partial reproduction of *Hox1.1* regulation during embryogenesis in transgenic mice. The transgene was correctly regulated early in development (day 7.5–8.5) but its expression pattern diverged from that of the endogenous gene after this period. The correct expression early in development allowed a detailed analysis of the establishment of position-specific expression of a Hox gene.

Materials and methods

Recombinant DNA

A 1.7 kbp *PvuII*–*EcoRI* fragment isolated from pm6-12 (Colberg-Poley *et al.* 1985) was ligated into pSPT19 cut with *Bgl*II, which was blunt-ended by Klenow polymerase treatment, and *EcoRI*. A *Hind*III linker (Biolabs, 8mer) was inserted into the *EcoRI* site resulting in plasmid pm6 Δ 3'. The 5.3 kbp *Sal*I–*Bgl*II fragment isolated from λ m6 was cloned into the pSPT19 vector cut with *Sal*I and *Bam*HI (pm6 Δ 5'). To obtain pm6 Δ this fragment was isolated after *Sal*I and *Kpn*I digestion and cloned into pm6 Δ 3'. This procedure resulted in the deletion of a 110 bp *Bgl*II–*Pvu*II fragment from the genomic DNA. To construct pm6lacZ1 the following fragments were linked in the indicated order: a 3.7 kbp *Sal*I–*Sac*I fragment from pm6 Δ , a 1 kbp *Hind*III–*Cla*I fragment from pCH110 (Pharmacia), a 2 kbp *Cla*I–*Bam*HI fragment from pMC1871 (Pharmacia) and a 1.7 kbp *Kpn*I–*EcoRI* fragment from pm6 Δ 3'. The vector is based on pm6 Δ 3'. The *lacZ* coding sequences were fused to 120 bp coding *Hox1.1* DNA that are not translated in m6lacZ1 because of a stop codon in the *lacZ* fragment and 1.18 kbp 3' nontranslated sequences. The fusion gene was separated from the vector for embryo injections by *Hind*III digestion. Fragments were separated on agarose gels and isolated by electroelution. The fragment was further purified by phenol:chloroform extraction, chloroform extraction, ethanol precipitation, gel filtration over a P30-column (Biorad) and filtration through a 0.22 μ m filter (Schleicher and Schuell). To generate RNA probes for RNase protection assays pSPTm6Ex2 was constructed by cloning a *EcoRI*–*Pst*I fragment from the second *Hox1.1* exon into pSPT19 (see Fig. 1). pSPTm6AP was constructed to generate ³⁵S-labelled RNA probes for *in situ* hybridisations. A 308 bp *Alu*I–*Pst*I fragment from the first *Hox1.1* exon (Kessel *et al.* 1987) was cloned into pSPT19 to generate this plasmid.

Generation of transgenic mice

NMRI outbred mice and C57Bl/6/DBA/F1 mice were purchased from the Zentralinstitut für Versuchstierzucht, Hannover. Transgenic mice were produced essentially as described by Hogan *et al.* (1986). Six week old NMRI female

mice were superovulated by injecting intraperitoneally (i.p.) 5 i.u. of gonadotropin from pregnant mare serum 48 h prior to injecting 5 i.u. of human chorionic gonadotropin. Female mice were then mated with C57Bl/6/DBA/F1 male mice. The next day, one-cell stage embryos were flushed from the oviducts of the mated female mice with M2 medium (Hogan *et al.* 1986). The eggs were freed of cumulus cells by hyaluronidase treatment. The male pronuclei of the fertilized eggs were microinjected with approximately 2 picoliters of DNA at a concentration of 2 ng μ l⁻¹. The injected, fertilized eggs were then transferred to pseudopregnant recipient NMRI female mice. Transgenic mice were identified by Southern blot analysis of DNA extracted from mouse tail biopsies as described by Hogan *et al.* (1986).

RNA analysis

RNase protection assays were done essentially as described (Balling *et al.* 1989). 50 μ g of total embryonic RNA was hybridized overnight with 600 000 cts min⁻¹ ³²P-labelled RNA transcribed from a *Oxa*NI-linearized pSPTm6Ex2 template at 65°C in 50 % formamide, 400 mM NaCl, 40 mM Pipes (pH 6.4), and 1 mM EDTA. The hybridisation mixture was digested for 90 min after addition of 350 μ l 10 mM Tris (pH 7.5), 5 mM EDTA, 250 mM NaCl, 50 μ g ml⁻¹ RNase A, and 150 000 u ml⁻¹ RNase T1 at 37°C.

In situ hybridisation

In situ hybridisations were performed essentially as described previously (Dony and Gruss, 1987). pSPTm6AP was used to transcribe a ³⁵S-labelled RNA probe specific for *Hox1.1*. The transcription reaction and purification of the probe was done as described for the RNase protection assays.

X-Gal staining and histology

Embryos between day 7 and 13 (the day of detection of the vaginal plug was day zero) were fixed in 1 % formaldehyde, 0.2 % glutaraldehyde, 0.02 % NP40, 1×PBS for 30 min at 4°C, followed by two washes in 1×PBS for 20 min at room temperature. Embryos were stained at 30°C in 1 mg ml⁻¹ X-Gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 1×PBS overnight. For sectioning, stained embryos were fixed overnight in 4 % formaldehyde in PBS, dehydrated and embedded in paraffin. Cryosections were air-dried, fixed at 4°C for 10 min like whole embryos and stained overnight as described above. Staining of cryosections gave a better representation of β -galactosidase activities than staining of whole embryos. Neural tube and some parts of the prevertebrae were poorly stained in whole-mount stainings but show high β -galactosidase levels in stained cryosections. The assay is linear only over a short range of activities. Sections were incubated for different times to obtain a complete representation over the whole range of transgene activity in different tissues.

Results

Identification of *Hox1.1* promoter sequences

In a first series of experiments, 7.1 kbp of genomic DNA from the *Hox1* cluster were used to generate transgenic mice (Fig. 1A). This DNA contains both exons of *Hox1.1*, the intron, 3.6 kbp of 5' and 0.3 kbp of 3' nontranscribed sequences. A 110 bp deletion was introduced into the second exon of *Hox1.1* to allow detection of transgenic RNA by a RNase protection assay. This construct (m6 Δ) was sufficient to direct

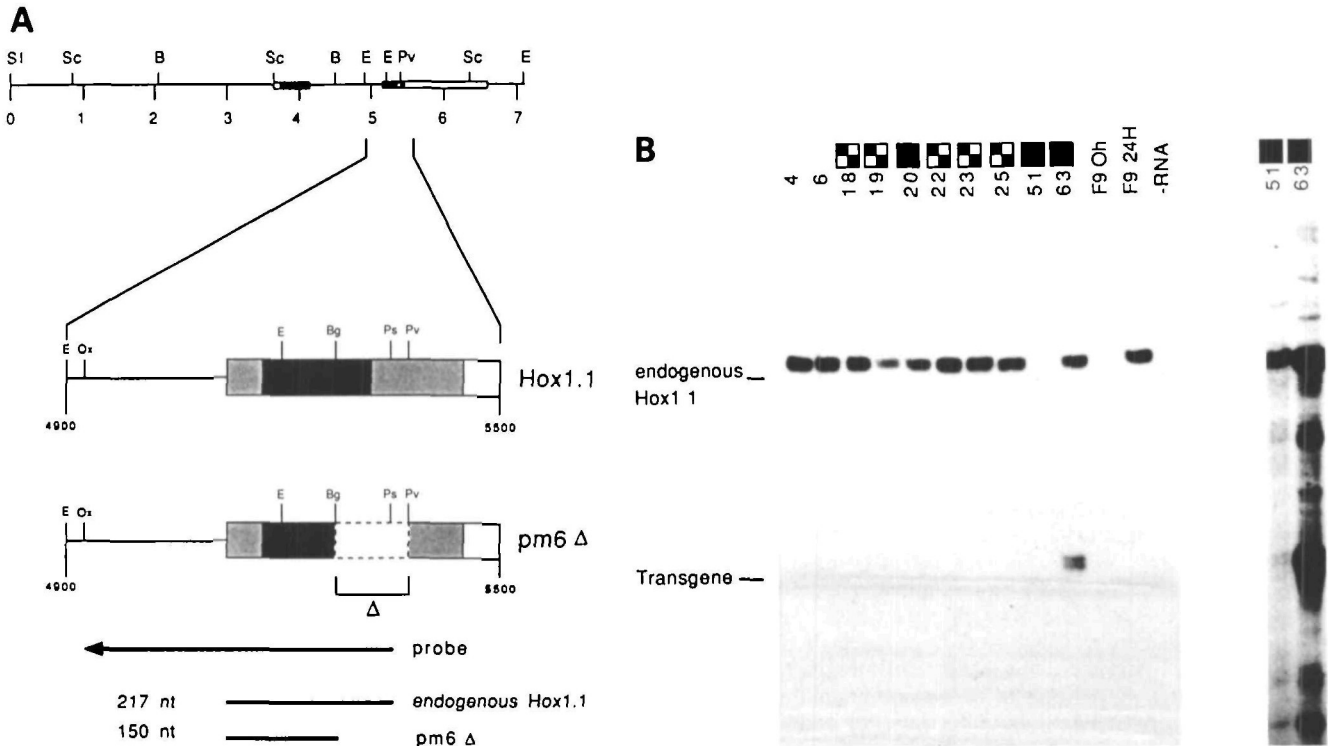


Fig. 1. Identification of *Hox1.1* promoter sequences. (A) Structure of *Hox1.1* and *m6Δ*. In the top half a restriction map of 7.1 kbp from the *Hox1* locus used to generate *m6Δ* is shown. Open boxes represent exons, stippled boxes coding sequences and black boxes the homeobox. Sizes are indicated in kbp. From this DNA a 110 bp *Bgl*II–*Pvu*II fragment was deleted and replaced by pSPT19-polylinker sequences (see Materials and methods) as indicated in the bottom half. Broken lines represent the deletion. An *Eco*RI–*Pst*I fragment cloned into pSPT19 (pSPTm6Ex2) was used to generate an antisense RNA-probe (arrow) for RNase-mapping. Black bars indicate sizes of the protected RNAs (217 nucleotides for the endogenous *Hox1.1* RNA and 150 nucleotides for RNA transcribed from *m6Δ*). The abbreviations for restriction enzyme sites are: B for *Bam*HI, Bg for *Bgl*II, E for *Eco*RI, Ox for *Oxa*NI, Ps for *Pst*I, Pv for *Pvu*II, Sc for *Sac*I and SI for *Sal*I. (B) RNase-mapping of RNA from transgenic embryos. Fertilized oocytes were injected with *m6Δ* and retransferred to pseudopregnant foster mothers. Embryos were recovered on day 12 of gestation. Transgenic embryos were identified by Southern blot analysis of placenta DNA. Total RNA was prepared from embryos and analysed by a RNase-protection assay using a probe transcribed from pSPTm6Ex2 (see Materials and methods). Numbers indicate the numbers of the individual embryo. As a control RNA from undifferentiated (F9 0h), differentiated F9 cells (F9 24h, differentiated with retinoic acid and cyclic AMP for 24 hours) and no RNA (–RNA) were included. Boxes indicate RNAs from transgenic embryos and the copy number relative to the endogenous *Hox1.1* gene (black box: one or more copies per haploid genome, mosaic box: less than one copy per haploid genome). Protected fragments corresponding to the endogenous and the transgenic RNA are indicated. On the left hand side, an overnight exposure is shown, on the right hand side, a four day exposure of the same gel is shown. Only low amounts of RNA were recovered from embryo 51.

expression after transfection into F9 cells (A.W.P. unpublished results), which express *Hox1.1* mRNA upon differentiation with retinoic acid and cyclic AMP.

Fertilized oocytes were injected and retransferred to pseudopregnant foster mothers. Embryos were analysed 12 days after retransfer (corresponding to day 12.5 of development). Of the 70 embryos obtained, 8 embryos were found to be transgenic by Southern blot analysis of placenta DNA. RNA extracted from 8 transgenic and 2 nontransgenic embryos was analysed by RNase-protection assays (Fig. 1B). Expression of *m6Δ* was found in two transgenic embryos. One embryo (embryo 63) showed high, a second (embryo 51) only low levels of *m6Δ* transcripts. Thus 2 out of 8 transgenic embryos expressed transgenic RNA. 5 of the 8 transgenic embryos contained less than one copy of the transgene per haploid genome and were probably

mosaic. This might account for the lack of expression in these embryos, since none of the mosaic embryos contained detectable amounts of transgenic RNA. As the transgene was active in two out of three embryos containing one or more transgene copies per genome, we conclude that the transgene contains sufficient information for expression during embryogenesis.

Generation of transgenic mice containing a *Hox1.1*–*lacZ* fusion gene

The 7.1 kbp of the *Hox1* locus contain at least some *cis*-acting elements that allow expression during embryogenesis. To analyse the spatial expression pattern, 3.6 kbp of *Hox1.1* promoter sequences were fused to the *E. coli lacZ* gene as a readily assayable marker (Fig. 2A). A poly(A) addition signal was provided by a 1.7 kbp DNA which contains most of the second exon

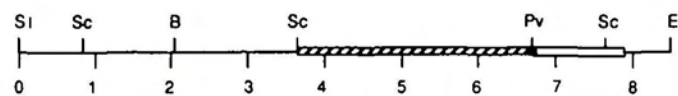
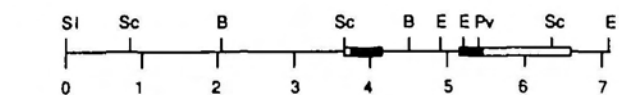
Fig. 2. Transgenic mice expressing a *Hox1.1 lacZ* fusion gene. (B) Structure of *Hox1.1* and m6lacZ1. In the top line the structure of the fragment from the *Hox1* locus used to generate m6lacZ1 is shown. See Fig. 1 for explanations. Sequences between the *SacI* site upstream of the *Hox1.1* ATG and the *PvuII* site in the second exon were replaced by *lacZ* coding sequences (crossed bar; see Materials and methods). (B–D) Transgenic embryos recovered on day 12 of gestation. (B,C) Fertilized oocytes were injected with m6lacZ1 and retransferred to pseudopregnant foster mothers. Embryos were recovered on day 12 of gestation, fixed and incubated with X-Gal (see Materials and methods). Embryos E4 (B) and E49 (C), which show a comparable expression pattern, are shown. (D,E) Embryos recovered from transgenic line L4 on day 12 of gestation. Transgenic lines carrying m6lacZ1 as a transgene were established. Transgenic males were mated to NMRI mice and embryos recovered on day 12 of gestation, fixed and incubated with X-Gal. Embryos stained overnight at 30°C (D) or 4°C (E) are shown. Incubation at 4°C results in lower overall expression but gives a better representation of different levels of expression. (F) Summary of transgenic embryos (E4, E49 and E98) and transgenic lines (L1–L5) obtained. Mosaic embryos with very low levels of β -galactosidase activity were not included. Embryos with completely different patterns of expression compared to the prototypic pattern of L4 embryos (ectopic) or additional, ectopic, sites of expression (add. ectopic) are indicated. **Fig. 3.** Expression of *Hox1.1* and m6lacZ1 at day 12 of gestation. Expression was analysed by *in situ* hybridisation of RNA probes to cryosections (B,D,F), staining of cryosections of transgenic embryos (A,C,E,G) or staining of whole embryos, which were afterwards embedded in paraffin and sectioned (H,I). Comparable levels of sections are shown. Except in A, left is caudal and right is rostral.

All sections are from day 12 embryos obtained from line L4. (A) Sagittal section stained with X-Gal. (B,D,F) Parasagittal sections hybridized with a *Hox1.1* specific probe. (B) *Hox1.1* is expressed in spinal ganglia. Expression above background levels is first detectable in the 5th cervical ganglion (C5). (D,F) *Hox1.1* is expressed in neural tube (nt) and prevertebrae (pv) T3 (third thoracic prevertebra) to T13. No *Hox1.1* RNA is detectable in chondrification centres. (C,E,G) Parasagittal section stained with X-Gal. (C) m6lacZ1 is expressed in spinal ganglia. The first ganglion with detectable amounts of stain is the 5th cervical ganglion (C5; the second cervical ganglion is indicated as C2). (E) The transgene is expressed in neural tube (nt) and prevertebrae (pv). Low level of β -galactosidase activity can be found in the third thoracic prevertebra (T3). The level in T3 is too low to be represented on the picture. Like in spinal ganglia expression in the most rostral expressing prevertebrae is not uniform. Single cells show higher levels of activity than most others. (G) The transgene is not expressed in chondrification centres. Transgene expression is more extensive than that of *Hox1.1*. (H) Frontal section. A whole day 12 embryo was incubated in staining solution, paraffin-embedded and sectioned. Due to the procedure, staining is lower than in stained cryosections. Neural tube (nt) is stained poorly. Expression in neural tube and spinal ganglia (sg) is detectable starting at the level of the fifth cervical ganglion. (I) Sagittal section of a whole mount stain. Expression levels in the rostral and caudal half are different in the C7 ganglion. A similar pattern is seen in the C6 ganglion in C. Due to the lower extent of staining in I compared to C, this pattern is seen in the C7 ganglion in I but not in C. Shorter incubation of cryosection in staining solution, however, shows a similar division in C7 (data not shown).

and 0.47 kbp 3' nontranscribed sequence. The 5' and 3' ends of m6lacZ1 were identical to m6 Δ . This plasmid (m6lacZ1) showed transcriptional activity in F9 cells (A.W.P. unpublished results). For production of transgenic mice, vector sequences were removed. Fertilized oocytes were injected with m6lacZ1 and retransferred to pseudopregnant foster mothers. Embryos were recovered on day 12 of gestation by laparotomy and analysed for β -galactosidase (β -gal) activity by incubation with 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal). Transgene expression was analysed by external inspection and in serial sections of paraffin-embedded embryos. This allowed a quick test if the *Hox1.1* transgene was active in transgenic embryos and if the *Hox1.1* expression pattern could be fully or partially reproduced. Embryos were analysed at day 12 of gestation as at this stage *Hox1.1* transcript levels peak and the expression domain is most clearly defined (Mahon *et al.* 1988; Dressler and Gruss, 1989; this paper). Out of a total of 118 embryos, 15 embryos showed only a few cells positive for transgene activity. Three embryos (E4, E49, E98) were obtained that strongly expressed the transgene (Fig. 2F). Since m6lacZ1 was active in transgenic embryos and the pattern was similar to that of *Hox1.1* (see below), transgenic lines containing m6lacZ1 were established for a detailed analyses. 5 out of 70 live offspring were

identified as transgenic by Southern blot analysis (lines L1–L5; Fig. 2F). These founder animals were crossed to NMRI mice to establish transgenic lines and produce transgenic embryos for histochemical analysis. As shown in Fig. 2F, 2 out of the 5 transgenic lines did not express the transgene (L1, L2), whereas strong β -gal activity was detected in 3 lines (L3, L4, L5). Embryos from line L4 and transgenic embryos E4 and E49 had an identical expression pattern with the exception of some ectopic expression unique to each integration site in E4 and E49 (Fig. 2B–E). The pattern of β -gal activity in L4 embryos was identical within a litter and between different litters derived from different transgenic males. Expression in day 12 embryos from lines L3 and L5 differed from the L4 pattern (data not shown) although analyses of earlier stages showed expression identical to that of L4 embryos (see below). Embryo E98 showed a completely different pattern of expression (data not shown). As the patterns of ectopic expression in E4, E49, E98 and later stages of L3 were unique to each embryo or line they probably represent influences of the integration site. Similar observations have been made for other *Hox1.1 lacZ* transgenes (A.W.P., R.B. and P.G., unpublished results). Transgenic embryos showing ectopic expression were not analysed in detail here and are subject of a separate report (A.W.P., R.B. and P.G., manuscript in preparation). Embryos derived

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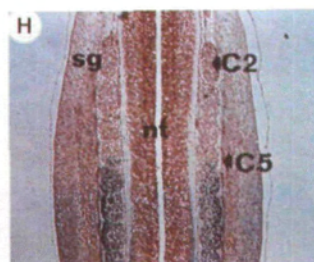
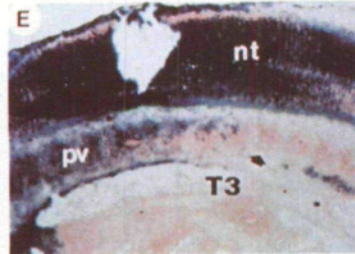
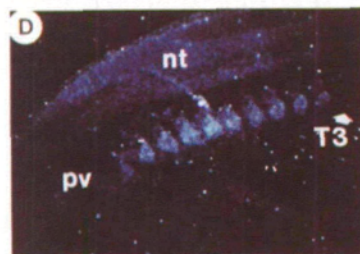
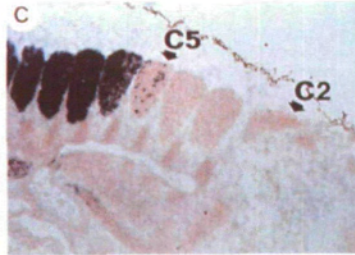
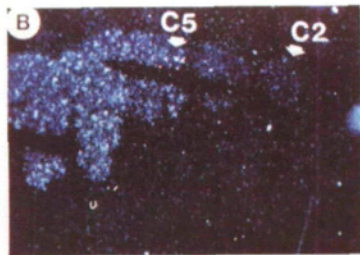
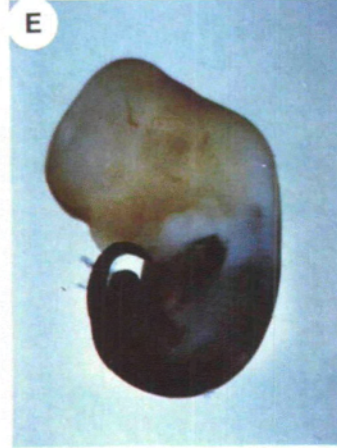


Hox1.1

m6lacZ1

F

	Expression	Transmission	Characteristics
E4	+	n.d.	add ectopic
E49	+	nd	add ectopic
E98	+	nd	ectopic
L1	-	+	
L2	-	-	
L3	+	+	ectopic
L4	+	+	
L5	+	+	patchy



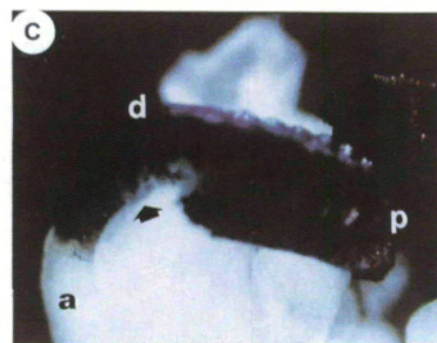
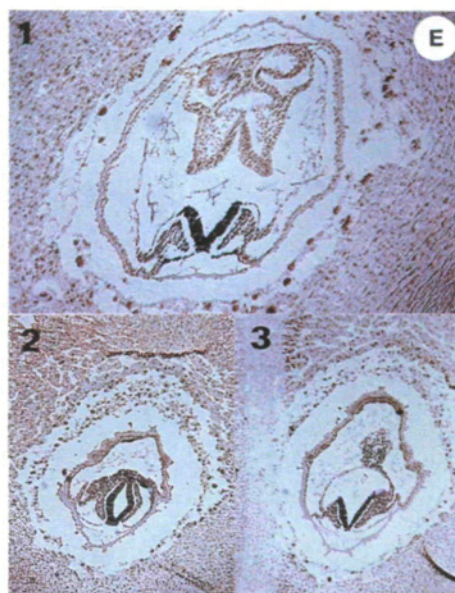
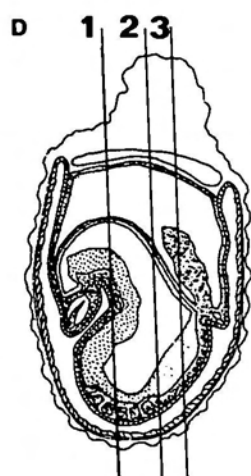
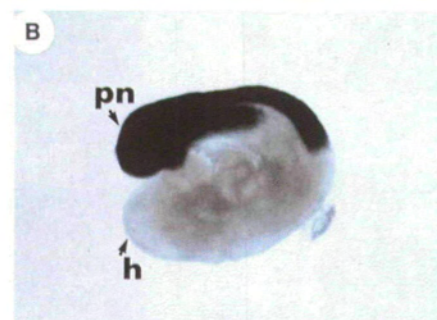
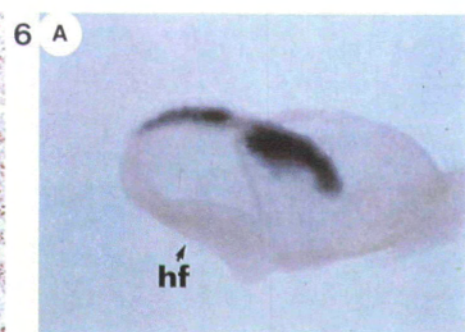
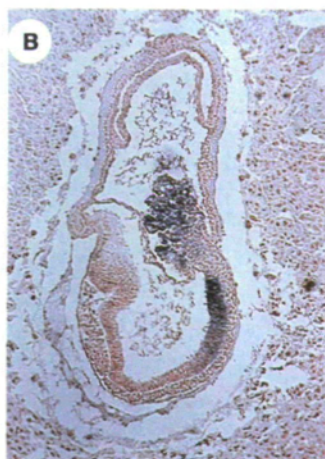
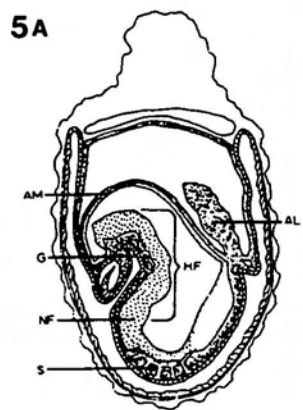
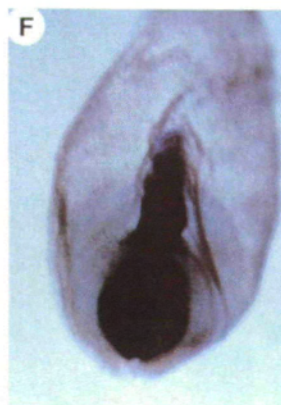
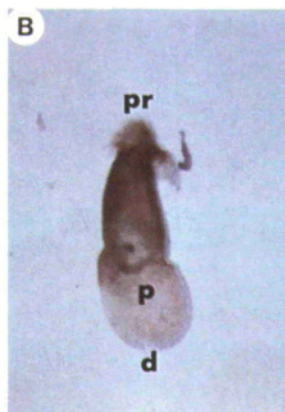
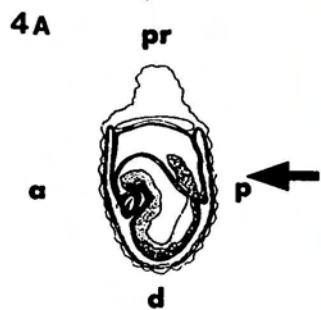


Fig. 4. Activation of transgene expression between day 7.5 and 8.5. Transgenic males were mated to NMRI mice in two hour intervals and embryos recovered at the indicated days of gestation and processed as described in Fig. 2.

(A) Schematic representation of a day 8.5 embryo. The arrow indicates the direction of view in all pictures. Abbreviations are: a, anterior; p, posterior; pr, proximal; d, distal. Embryos were recovered at the following approximate times after detection of the vaginal plug; 7 days and 16 hours (B), 7 days and 18 hours (C), 7 days and 20 hours (D), 7 days and 22 hours (E), 8 days (F), 8.5 days (G).

Fig. 5. Expression of m6lacZ1 in transgenic day 8 embryos. Embryos derived from line L4 were recovered on day 8.0 (B) or day 8.5 (C,E) of gestation including the decidua, fixed, stained with X-Gal, embedded in paraffin and sectioned. (A) Schematic representation of a day 8.5 embryo. Abbreviations are: AL, allantois; AM, amnion; G, foregut; HF, head fold; NF, neural fold; S, somite. (B) Sagittal section of a day 8.0 embryo. High amounts of stain are seen in allantois and neural plate. (C) Sagittal section of a day 8.5 embryo. High levels of transgene activity are detectable in allantois and neural plate, low levels in unsegmented mesoderm in the posteriormost quarter. A similar pattern was observed in stained cryosections. (D) Schematic representation of a day 8.5 embryo. Levels of sections in (E) are indicated (1,2,3). (E) Different levels of cross section through a day 8.5 embryo. Transgene activity can be detected in all three germ layers.

Fig. 6. Transgene expression between day 8 and 9. (A) Lateral view of a day 8.0 L4 embryo. (B) Lateral view of a day 9.5 L4 embryo. (C) Lateral view of a day 9.5 L4 embryo. The arrow points at presumptive migrating neural crest cells visible in somites not expressing m6lacZ1. Abbreviations are: a, anterior; d, dorsal; h, head; hf, headfold; p, posterior; pn, posterior neuropore.

from line L5 showed a patchy expression on day 12 (data not shown). The reason for this is unclear.

Hox1.1 and transgene have identical anterior boundaries of expression

The *Hox1.1* expression pattern in day 12 embryos was determined by *in situ* hybridisation of cryosections with RNA probes and compared to sections of stained transgenic embryos. In addition, embryos from line L4 were analysed by staining of serial cryosections. Hybridisation with a *Hox1.1*-specific probe showed high RNA levels extending from the 3rd to the 13th thoracic prevertebrae (Fig. 3D,F). Somewhat lower expression was seen in neural tube (Fig. 3D) and spinal ganglia posterior to the fourth cervical ganglion (Fig. 3B). Expression within this domain was not uniform. It increased gradually over prevertebrae T3 and T4 and decreased over T11 to T13. Expression in ganglia was first detectable above background levels in the fifth cervical ganglion and increased to maximal levels in the sixth cervical ganglion (Fig. 3B).

Analysis of serial sections from all transgenic embryos showed that the anterior boundary of transgene expression was the same as that of *Hox1.1* (Fig. 3C,E,G,H). The intensity of staining was not uniform along the first three ganglia. Similar to *Hox1.1* a

gradient of increasing transgene activity could be seen (Fig. 3B). Within the ganglia C5 and C6, few cells showed high levels of β -gal activity in a background of cells with low or no detectable staining. A similar observation was made for the endogenous *Hox1.1* gene (Fig. 3B). There was a marked difference in intensity of staining between the caudal and the rostral halves of the C6 and C7 ganglion (Fig. 3C,I, and data not shown). This difference probably reflects the origins of the neural crest cells that gave rise to these ganglia (see Discussion). A gradient of increasing transgene activity was also seen in prevertebrae (Fig. 3E,G). In prevertebrae and rib primordia, both *Hox1.1* and the transgene were not active in the centres of chondrification (Fig. 3F,G). Many characteristics of *Hox1.1* expression therefore were reproduced by the transgene.

On day 12 the domain of transgene expression is extended relative to Hox1.1

In marked contrast to *Hox1.1*, there was no posterior limit of transgene expression. Staining extended uniformly to the posterior end of the day 12 embryo (Fig. 3A). Within this domain transgene expression was not limited to prevertebrae, ganglia and neural tube. High levels of stain could be found in epidermis, connective tissue and some myotome derivatives (Fig. 3A,G). These tissues do not contain detectable levels of endogenous *Hox1.1* RNA (Fig. 3F; Mahon *et al.* 1988; Dressler and Gruss, 1989) and therefore represent ectopic sites of transgene expression.

In summary, the transgene was expressed during embryogenesis in a specific region of the body. The expression domain of m6lacZ1 was colinear at the anterior boundary with *Hox1.1* but not restricted at the posterior end. Expression was extended not only posteriorly but also to many mesodermally derived cells. *Hox1.1* is initially expressed in a broad region of the embryo and expression is progressively shut off in most mesodermal tissues (Mahon *et al.* 1988). This process might not take place for m6lacZ1 resulting in widespread transgene expression. To test this possibility transgene expression in early stages of development was analysed.

Transgene expression is activated and expressed identical to Hox1.1 at early stages of development

Line L4 was chosen for an extensive analysis of transgene expression at various stages of development. NMRI mice were mated to transgenic males and embryos dissected out at different stages of development, fixed and incubated with a staining solution containing X-Gal. Timed matings yielded 100 transgenic embryos at approximately two hour intervals between day 7.5 p.c. and day 8.5 p.c. Embryos younger than 7.5 days were not analysed.

Transgene expression was activated in a strict temporal and spatial order in embryonal tissues. Expression was first detectable on day 7.5 to 7.75 (Fig. 4B) in the allantois. Expressing cells appeared at the tip of the allantoic bud and subsequently increased continuously in number in the following two to four hours

exclusively in the allantois (Fig. 4C,D). The first stained cells in the embryo appeared in the neuroectoderm when already all cells in the allantois expressed m6lacZ1 (day 7.8) (Fig. 4E).

During the following approximately 2 to 4 hours of development, the number of stained cells in the neuroectoderm increased continuously (Figs 4E,F, 5B,C,E). Activation within this region apparently occurs randomly in individual cells. No obvious regular pattern of activation within a germ layer is detectable during this period. Cells positive for *lacZ* were confined to an area in the posterior part of the embryo (Fig. 5C). Serial sections showed a higher amount of stain in the posterior part of the neural plate (Fig. 5B,C). Thus, as has been observed for the *Hox1.1* and *Hox1.3* genes (Dressler and Gruss, 1989), expression at early stages was strongest at the caudal end of the embryo and decreases cranially. At day 8.0 p.c. all cells within a defined region of the neural plate expressed the transgene (Figs 4F, 6A). Starting at day 8.0, the transgene was activated in mesodermal cells (day 8.0 to 8.5) (Fig. 4G). As observed in allantois and neuroectoderm activity spread from posterior to anterior, the anterior border of expression initially being diffuse in contrast to the very defined border from day 9.5 on (Figs 4G, 6B). The apparent anteroposterior movement of transgene expression reflects the growth of the mesoderm by addition of cells to the presomitic mesoderm. Serial sections showed that all three germ layers, ecto- meso- and endoderm, were positive for *lacZ* expression at day 8.5 (Fig. 5C). The pattern and time course of transgene activation coincides with that of *Hox1.1* as far as it has been described (Mahon *et al.* 1988; Dressler and Gruss, 1989). The early pattern of *lacZ* expression described for L4 was identical to that found in embryos of the lines L3 and L5 (data not shown). Therefore, the early developmental regulation of m6lacZ1 in the embryos described was due to the *cis*-acting sequences present in the transgene and not the result of the integration sites.

After day 8.5, Hox1.1 and transgene expression diverge

At day 8.5 m6lacZ1 expression was established in a specific region of the embryonic body. In the following 5 days of development expression was maintained in most tissues derived from mesodermal and ectodermal cells within this region.

Starting at day 8.5 p.c. expression in the allantois was diminishing (Fig. 4G). After closure of the anterior neuropore, expression was detectable at day 9.5 in neural tube, epidermal ectoderm and all mesodermal tissues in a region extending from the level about 6 to 7 somites posterior to the anterior border of m6lacZ1 expression in neural tube to the posterior end of the embryo (Fig. 6B). In the region of this offset of expression between neural tube and somites, some stained cells are visible within or between adjacent somites (Fig. 6C). Presumably these are migrating neural crest cells that will give rise to the spinal and sympathetic ganglia developing at these positions.

Between day 10.5 p.c. and day 12.5 p.c. the pattern

and the boundaries of m6lacZ1 activity do not change (data not shown).

Discussion

Region-specific expression during embryogenesis is the most conspicuous feature of mammalian Hox genes and, together with their homology to developmental genes in *Drosophila*, is evidence for a potential function in the specification of positional information along the anterior-posterior axis. Independent of a functional characterisation, these genes permit analysis of gene regulation depending on positional information.

To identify sequences responsive to positional cues, *Hox1.1* promoter sequences were linked to a marker gene, the *E. coli lacZ* gene, and its spatiotemporal expression pattern analysed in transgenic mice. Using 3.6 kbp *Hox1.1* promoter sequences and 1.7 kbp *Hox1.1* 3' sequences, we obtained transgenic mice that showed *lacZ* expression in a specific region of the embryo. The ease and high resolution of β -galactosidase detection allowed a more precise analysis than previously possible by *in situ* hybridisation. As the expression pattern of the transgene was identical to that of the endogenous gene early in development it was possible to analyse the establishment of the pattern in detail.

Regulation of Hox1.1 expression occurs in two phases

The activation of m6lacZ1 parallels the endogenous *Hox1.1* gene in a strict temporal and spatial manner early during development. Beginning at day 7.5 *lacZ* expression is first detectable in the allantois and subsequently in neural ectoderm. Activation in embryonic mesoderm is both delayed and more posterior along the body axis. Expression in presomitic mesoderm is first detectable when the cells corresponding to the first somites expressing *Hox1.1* are added to the presomitic mesoderm. The subsequent expansion of expressing mesodermal cells reflects the growth of the mesoderm by recruitment of cells first from the primitive streak and later from the tail bud. A different observation has been made for one other Hox-gene. The *Xenopus XIHbox1* gene is initially expressed in a narrow band in ectoderm and mesoderm sharing the same anterior and posterior boundaries (De Robertis *et al.* 1989). Subsequent morphogenetic movements, however, lead to an offset of expression between mesoderm and neural tube. Based on the early expression pattern of *XIHbox1*, homeogenetic induction across germ layer boundaries (De Robertis *et al.* 1989) was proposed as a mechanism to account for the precise alignment of expression in different germ layers. Although *Hox1.1* is not a paralog of *XIHbox1*, it is possible to conclude that the proposed mechanism can not be a general one. At least, our data exclude this model for the murine *Hox1.1* gene.

m6lacZ1 and *Hox1.1* are regulated in parallel early in development during the period when expression is established (day 7.5 to 8.5). Expression patterns di-

verge, however, when within this region *Hox1.1* expression becomes restricted. Although the anterior boundary of m6lacZ1 expression (5th cervical ganglion and 3rd thoracic prevertebrae) is identical to that of *Hox1.1*, there is, in contrast to *Hox1.1*, no posterior boundary. In addition, in marked contrast to *Hox1.1*, expression is not restricted to a subset of mesodermally derived tissues, the sclerotomes. Thus the second regulatory process is not executed by m6lacZ1. Some aspects of this later regulatory process, however, are executed, since the transgene, like *Hox1.1*, is not expressed in the chondrification centres of prevertebrae. The *Hox1.1* sequences present in m6lacZ1 are not sufficient to specify all aspects of *Hox1.1* expression. Sequences further 5' or 3' or internal sequences like the intron removed to construct m6lacZ1 could contain this information. Peak levels of expression in each germ layer are initially found in the posterior part of the expressing domain (days 8.5 to 9.5) but shift anteriorly later during development (day 12.5).

A highly specific activity has also been reported for *Hox1.3* promoter sequences (Zakany *et al.* 1988). However the spatial and temporal profile of this pattern does not correlate with the described distribution of *Hox1.3* mRNA (Dony and Gruss, 1987; Dressler and Gruss, 1989). The *Hox1.3/lacZ* transgene is expressed in only a small subset of cells expressing *Hox1.3*. In addition transgene activity is first detectable at day 11, about four days later than *Hox1.3* is first expressed. It has still to be determined if this difference reflects different mechanisms of position-specific regulation or if the *Hox1.3* element regulates just one aspect of the late expression pattern of *Hox1.3* (days 11 to 13).

Hox1.1 expression as a positional marker in neuroectoderm

Hox1.1 and the transgene m6lacZ1 are expressed at day 8.5 in a specific region of the neural plate. The neural crest cells originating from this region will form spinal ganglia expressing *Hox1.1* (Mahon *et al.* 1988) and m6lacZ1 later during development. In day 9.5 embryos these neural crest cells are readily detectable by X-Gal staining. The differential expression of m6lacZ1 in neural crest cells along the anteroposterior axis indicates that the migrating neural crest cells must have acquired position-dependent properties. It is not clear, however, if these properties are stable or if they can be changed by interacting with a different cellular environment. Once spinal ganglia are formed, some ganglia (C6 and C7) show nonuniform transgene activity in anterior and posterior halves. In the chick, a single spinal ganglion arises from the neural crest cells originating from two somitic levels (Teillet *et al.* 1987). Thus different populations of neural crest cells seem to contribute to the halves of the ganglion also in the mouse.

The limits of *Hox1.1* expression in Fig. 3 differ slightly (by one segment) from a previous report (Mahon *et al.* 1988) which sets the anterior boundary at the 4th cervical ganglion. We do not know the reason for this difference. One possibility is that the C2

ganglion has been mistaken for the C1 ganglion, which degenerates at day 11 of development (Dawes, 1930). This agrees with our observations as we have not detected a ganglion between the C2 ganglion and Frioriep's ganglion in three serial sectioned day 12 embryos (Fig. 3H and data not shown).

Conclusions

The identification of position-responsive promoter sequences not only enables dissection of the responsible *cis*-acting elements but also, by the use of *lacZ* as a marker, allows detailed analyses of the establishment and development of the specified pattern. Analysis of the development of the m6lacZ1 expression pattern compared to *Hox1.1* suggests that at least two phases of regulation can be distinguished. In the period from day 7.5 to 8.5, the initial pattern is established. Modifying this pattern, the final distribution of the gene product is obtained by selective persistence of expression in specific subpopulations of cells. The identification of regulatory elements necessary for this process will uncover the molecular mechanisms defining regions of *Hox1.1* expression along the anterior-posterior axis.

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