A regulatory region from the mouse *Hox-2.2* promoter directs gene expression into developing limbs

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### Summary

To characterize cis-acting regulatory elements of the murine homeobox gene, *Hox-2.2*, transgenic mouse lines were generated that contained the *LacZ* reporter gene under the control of different fragments from the presumptive *Hox-2.2* promoter. A promoter region of 3600 base pairs (bp) was identified, which reproducibly directed reporter gene expression into specific regions of developing mouse embryos. At 8.5 days postcoitum (p.c.) reporter gene activity was detected in posterior regions of the lateral mesoderm and, in subsequent developmental stages, expression of the *LacZ* gene was restricted to specific regions of the developing limb buds and the mesenchyme of the ventrolateral body region. This pattern of *Hox-2.2-LacZ* expression was found in all transgenic embryos that have been generated with the 3.6 kb promoter fragment (two founder embryos and embryos from five transgenic lines). In addition, embryos from two transgenic mouse lines expressed the reporter gene at low levels in the developing central nervous system (CNS). Our results are consistent with the idea that in addition to their presumptive role in CNS and vertebrae development, *Hox-2.2* gene products are involved in controlling pattern formation in developing limbs.

Key words: homeobox genes, *LacZ*, β-galactosidase, transgenic mice, gene regulation.

### Introduction

Murine homeobox genes are expressed in stage-, region-, and tissue-specific patterns during embryonic development (for reviews, see Kessel and Gruss, 1990; Sashikant *et al.* 1990). The *Hox-2.2* gene, a member of the *Hox*-2 cluster located on chromosome 11 in the mouse, has been described as being expressed in specific regions of the developing central nervous system (CNS), differentiating vertebrae and spinal ganglia (Schughart *et al.* 1988; Graham *et al.* 1989). The pattern of *Hox-2.2* expression in the CNS is very similar to the expression patterns of other *Hox*-2 genes. Each gene, however, exhibits a specific anterior boundary of expression within the CNS. Most interestingly, for all *Hox*-2 genes, except for the *Hox-2.9* gene, a strict correlation between the anterior boundary of expression and the order of genes along the chromosome has been described (Utset *et al.* 1981; Graham *et al.* 1989; Wilkinson *et al.* 1989; Murphy *et al.* 1989; Frohmann *et al.* 1990).

To understand the molecular mechanisms involved in the regulation of murine homeobox genes, we have analyzed the promoter region of the *Hox-2.2* gene in transgenic mice for the presence of cis-acting regulatory elements. Our studies identified a regulatory element from the *Hox-2.2* promoter, which directs gene expression into specific regions of the developing limbs and the CNS.

### Materials and methods

**Construction of Hox-2.2-LacZ reporter genes and production of transgenic mice**

A 3.6 kb *BssHII* fragment upstream of the *Hox-2.2* coding region (position -3644 to position -16 with respect to the predicted AUG start codon) was cloned by blunt end ligation in front of the *LacZ* reporter gene. The reporter gene was isolated as a *BamH*-HindIII fragment from plasmid pCH110 (Hall *et al.* 1983). This fragment contains a translational start site from the *E. coli gpt* gene, the *LacZ* coding region, and the SV40 polyadenylation signal present in plasmid pCH110 (Hall *et al.* 1983). The 1.2 kb promoter construct was prepared from the 3.6 kb *Hox-2.2-LacZ* clone by deleting the region upstream of an *XbaI* restriction site at position -1186 (in respect to the AUG start codon). DNA fragments were purified from proaryotic vector sequences and injected into pronuclei of fertilized eggs as described by Hogan *et al.* 1986.
Fertilized eggs were collected from CD1 females (Charles River) after mating to B6D2 F1 (Jackson Laboratory) males. Transgenic embryos and mouse lines were characterized by Southern analysis using DNA prepared from placentas or tail biopsies. Genomic DNA was digested with EcoRI and PstI and hybridized to a 226 bp long PstI–StuI Hox-2.2 probe (position −959 to −733 in respect to the AUG start codon).

**RNAase protection assays**

RNA was prepared as described (Chomczynski and Sacchi, 1987). Radioactively labeled riboprobes were generated by SP6 RNA polymerase from a 330 bp PvuII–EcoRI Hox-2.2 cDNA fragment (Schughart et al. 1988). The full-length riboprobes were 408 bp long, the fragment protected by Hox-2.2 mRNA was expected to be 330 bp. 15 µg of total RNA from various tissues and 2.5 × 10^5 cts min⁻¹ of riboprobe were hybridized at 55°C overnight. RNAase protection analysis was performed as described (Gilman, 1989).

**Staining embryos for β-galactosidase activity and tissue sections**

Embryos were prepared as described by Hogan et al. 1986, fixed and incubated in an X-Gal solution at 37°C overnight as described by Zakany et al. (1998). Midday after detection of vaginal plugs was taken as day 0.5 p.c. Developmental stages correspond to Theiler (1972). For tissue sections, embryos were fixed in 4% paraformaldehyde after X-Gal staining, and prepared for paraffin sections as described (Bogard et al. 1989). Sections were not counterstained, and photographs were taken on a Leitz Aristoplan microscope using interference contrast optics. To visualize chondrogenic condensations, sections of limb buds from 13.5 day p.c. embryos were counterstained with Alcian Blue 8GX (McLeod, 1980).

**Results**

**A regulatory element from the Hox-2.2 gene directs gene expression into specific regions of developing limb buds and the CNS**

Gene constructs were generated in which the bacterial LacZ gene was placed under the control of two regions from the presumptive Hox-2.2 promoter (Fig. 1) in order to characterize cis-acting regulatory regions in the Hox-2.2 promoter. The larger construct contains a 3.6 kb fragment starting 16 bp upstream of the Hox-2.2 coding region and including most of the 5' untranslated leader region. The shorter fragment represents a subfragment of the 3.6 kb region containing the proximalmost 1.2 kb (Fig. 1).

Transgenic embryos carrying the 3.6 kb construct exhibited strong expression of the LacZ reporter gene in the menenchyme of the developing limb buds and the ventralateral body region between the limb buds (Figs 2, 3). Most strikingly, all transgenic embryos that have been obtained with the 3.6 kb construct, (two founder embryos and offspring from five transgenic lines) showed the same pattern of expression in the limb and ventralateral body regions. Besides the region-specific mesodermal expression, embryos from two of the transgenic lines exhibited weak β-galactosidase activity in the developing CNS and spinal ganglia (Fig. 4). In four cases, LacZ expression could be detected in additional regions. These additional regions of expression were unique to individual lines or founder embryos and are most likely due to the influence of regulatory regions at the site of integration.

Analysis of the shorter 1.2 kb promoter fragment from the Hox-2.2 gene (Fig. 1) revealed no consistent β-galactosidase expression in eight founder embryos that have been obtained. Six founder embryos showed no LacZ expression at all. One embryo exhibited reporter gene expression in a few cells of the head and the anterior region of the hindlimb. In the second embryo, some cells in the posterodistal region of the forelimb and a few cells in the hindlimb ectoderm showed β-galactosidase staining, but no positive cells were present in the ventralateral body region. Therefore, both expression patterns appeared to be unrelated.

![Diagram](image)

**Fig. 1.** Structure of the Hox-2.2–LacZ fusion genes used to generate transgenic lines and transgenic founder embryos. Two Hox-2.2–LacZ constructs were generated, one containing a 3.6 kb fragment, the other containing a 1.2 kb fragment of the presumptive Hox-2.2 promoter region. The previously characterized Hox-2.2 transcript (Schughart et al. 1988) is indicated by the arrow. Several transcription start sites have been mapped to a region 200 to 30 bp upstream of the Hox-2.2 AUG start codon (Z. Liang, KS and FHR, unpublished results). Boxed regions indicate open reading frames and striped boxes show the location of the Hox-2.2 homeodomain. Restriction site: B, BamHI; BS, BstHI; H, HindIII; E, EcoRI; X, XbaI. (*) Number of LacZ-expressing embryos or lines compared to number of transgenic embryos/lines obtained with this construct.
Fig. 2. Transgenic embryos from line 2Z35–15 stained with X-Gal at various developmental stages. (A) 8.5 day p.c., (B) 9.5 day p.c., (C) 10.5 day p.c., (D) 11.5 day p.c., (E) 12.5 day p.c., (F) 14.5 day p.c. Expression of β-galactosidase in the mesencephalon is not specific for the Hox-2.2–LacZ construct. β-galactosidase activity in the striped pattern along the lateral region is only observed in embryos from line 2Z35–15 at day 11.5 p.c. and has to be considered as ectopic expression due to the site of integration of the transgene in this line.
Fig. 3. Tissue sections of transgenic embryos after X-Gal staining. (A) Cross-section of 8.5 day p.c. embryo (magnification 400×), (B) oblique section of 9.5 day p.c. embryo at the level of forelimb buds (200×), (C) frontal section of 9.5 day p.c. embryo at the level of hindlimb buds (200×), (D) cross-section of hindlimb bud of 11.5 day p.c. embryo (250×). ng, neural groove; lm, lateral mesoderm; nt, neural tube; g, gut; lb, limb bud; e, ectoderm; m, mesenchyme; a, anterior; p, posterior; d, dorsal; v, ventral.

Fig. 4. Expression of LacZ reporter gene in the CNS of embryos from line 2Z35-9. (A) 11.5 day p.c. embryo. (B) Frontal section of 11.5 day p.c. transgenic embryo after X-Gal staining showing LacZ expression in two columns of cells running along the anteroposterior axis (magnification 80×). sc, spinal cord; a, anterior; p, posterior.
to each other and to the patterns described for the 3.6kb promoter fragment.

**Expression of the Hox-2.2-LacZ reporter gene in developing limb buds**

The expression of the LacZ gene in the limb bud mesenchyme and the ventrolateral body region has been analyzed in detail in embryos derived from line 2Z35-15 (Figs 2,3). β-galactosidase expression was first detected in the amnion and the allantois of 7.5 day p.c. embryos. At day 8.5 p.c., reporter gene expression was observed in the lateral mesoderm of the embryo proper (Figs 2A,3A). Mesoderm expression at that stage was restricted to regions posterior to the developing 6th somite. At days 9.5 and 10.5 p.c., the LacZ reporter gene was expressed in the mesenchyme of the emerging limb buds and in the ventrolateral mesenchyme between the limb buds (Figs 2B,2C,3B,3C). In the developing forelimb bud, only the posterior region exhibited β-galactosidase activity whereas the entire hindlimb region strongly expressed the LacZ reporter gene. At these developmental stages, cells in the endoderm and surrounding mesoderm of the developing mid- and hindgut also showed expression of the transgene (Figs 3B,C). The expression pattern in the hindlimb bud changed at day 11.5 p.c. in that reporter gene activity was restricted to two lateral (anterior and posterior) stripes in the mesenchyme of the developing hindlimb (Figs 2D,3D). The expression pattern in the limb regions established at day 11.5 p.c. was maintained throughout development until day 15.5 p.c. (Figs 2E,F). Tissue sections from 13.5 day limb buds revealed that transgene expression was only found in undifferentiated mesenchyme and not in regions that form myogenic and chondrogenic condensations (not shown). At day 14.5 p.c., β-galactosidase activity was limited to small regions in fore- and hindlimbs (Fig. 2F) and was barely detectable at day 15.5 p.c. In extraembryonic tissues, reporter gene expression was found in yolk sac, amnion, allantois and, at later stages, in the umbilical cord.

Reporter gene expression has also been investigated in two more mouse lines (2Z35-5, 2Z35-9) and representative stages (10.5 days p.c. and 11.5 days p.c.) from the other lines (2Z35-7, 2Z35-14). Until day 11.5 p.c. of development, an identical pattern of LacZ expression in the developing limb buds and ventrolateral regions was observed in embryos from all lines. At later stages, embryos from lines 2Z35-7, 2Z35-9, and 2Z35-5 expressed the transgene in the same pattern as line 15, except that embryos from line 9 also showed β-galactosidase activity in the limb ectoderm at day 12.5 p.c. and later. In embryos from line 2Z35-9, the lateral stripes of positive cells in the limb buds were broader and cells in more distal regions of the limb buds also stained positive for reporter gene expression. Embryos from line 14 exhibited the same pattern as line 15 at 10.5 p.c., but at day 11.5 p.c. no LacZ-positive cells could be detected in the forelimb. These slight variations of transgene expression are probably due to the influence of regulatory regions at the sites of integration.

The endogenous Hox-2.2 gene is expressed in developing mouse limb buds

Although in mouse embryos Hox-2.2-specific mRNA has not previously been detected by in situ hybridization in developing fore- and hindlimb buds, the high level and consistency of reporter gene expression in those regions suggested that the endogenous Hox-2.2 gene may be expressed in developing limbs. RNAase protection assays of RNA isolated from different tissues revealed Hox-2.2 expression in fore- and hindlimbs of 11.5 day p.c. embryos and adult kidney but not in adult liver (Fig. 5). These results demonstrate that, in addition to expression in the CNS and prevertebra (Schughart et al. 1988; Graham et al. 1989), the endogenous Hox-2.2 gene is also transcribed in fore- and hindlimbs of developing mouse embryos.

![Fig. 5. RNAase protection assays demonstrating Hox-2.2 expression in developing limb buds. The protection of a 350 bp fragment (arrow) indicates the presence of Hox-2.2 mRNA in the respective tissues. RNA was prepared from: (b) hindlimb buds of 11.5 day p.c. embryos, (c) forelimb buds of 11.5 day p.c. embryos, (d) adult kidney, (e) adult liver, (f) whole 11.5 day p.c. embryos from which limb buds had been removed, (g) tRNA. (a) marker lanes: pBR322 restricted withMspI, (h) undigested probe.](image-url)
Expression of the Hox-2.2–LacZ reporter gene in the developing nervous system

In addition to limb bud expression, two transgenic lines (2Z35–9, 2Z35–14) exhibited weak β-galactosidase activity in the CNS and in spinal ganglia (Fig. 4). LacZ-positive cells in the CNS were found in brain (mesencephalon-metencephalon boundary, myelencephalon) and spinal cord. LacZ activity in the mesencephalon–metencephalon region was detected in all lines and embryos obtained, but expression in hindbrain, spinal cord regions and spinal ganglia was only observed in lines 2Z35–9 and 2Z35–14. Because reporter gene expression in the mesencephalon–metencephalon region has also been reported by other groups using different LacZ constructs (Kress et al. 1990), we would conclude that expression in this region is not specific for the Hox-2.2 promoter. To describe expression patterns in the spinal cord regions in more detail, we have studied embryos from line 9 from day 8.5 to 13.5 p.c. of development. Expression of the reporter gene in the spinal cord started at day 10.5 p.c. and was maintained throughout later stages (Fig. 4, 11.5 day p.c. embryo). LacZ-positive cells were restricted to two longitudinal columns of cells running parallel to the central canal of the entire spinal cord (Fig. 4). In cross-sections, those positive cells could be located in the ventrolateral regions of the spinal cord. The anterior boundary of expression is located in the hindbrain (myelencephalon). Therefore, the expression of the reporter gene from the 3.6 kb promoter region seems to be restricted to a subset of cells in the developing spinal cord in which Hox-2.2 transcripts have been previously detected (Schughart et al. 1988; Graham et al. 1989; M. Utset and FHR, pers. communication). These results suggest that the 3.6 kb promoter region contains only some of the regulatory elements that direct the expression of the Hox-2.2 gene (Schughart et al. 1988; Graham et al. 1989). It is evident, however, that only a subset of cells in the CNS in which Hox-2.2 transcripts have been detected by in situ hybridization expressed the LacZ reporter gene. For example, at day 10.5 and 11.5 p.c. Hox-2.2 transcripts are found throughout the dorsoventral axis of the spinal cord and are not restricted to ventrolateral regions (Schughart et al. 1988; M. Utset and FHR, pers. communication). These results suggest that the 3.6 kb promoter region contains only some of the regulatory elements that direct the expression of the Hox-2.2 gene into neural tissues. To date, the presence of neurogenic elements in homeobox gene promoters has been described for the murine Hox-1.1, -1.3, -2.3, -3.1 genes, and the human Hox-5.1 gene (Püschel et al. 1990; Zakany et al. 1989; Kress et al. 1990; Bieberich et al. 1990; Tuggle et al. 1990; respectively). To identify additional cis-acting regulatory elements required for the correct and high level of Hox-2.2 expression in the CNS, it will be necessary to test larger genomic fragments.

In the future, individual protein-binding sites within the Hox-2.2 promoter region and transcription factors recognizing these sites will have to be identified to test this hypothesis. Recently, the Hox-2.3 gene, which is located immediately upstream of the Hox-2.2 gene in the Hox-2 cluster, has been found to be expressed only in the posterior half of the forelimb region (Vogels et al. 1990).

In addition to expression in the limb regions weak expression of the transgene could be detected in the CNS of embryos from two transgenic lines containing the 3.6 kb promoter fragment. The anterior boundary of expression within the hindbrain appears to correspond to the expression boundary described for the Hox-2.2 gene (Schughart et al. 1988; Graham et al. 1989). It is evident, however, that only a subset of cells in the CNS in which Hox-2.2 transcripts have been detected by in situ hybridization expressed the LacZ reporter gene. For example, at day 10.5 and 11.5 p.c. Hox-2.2 transcripts are found throughout the dorsoventral axis of the spinal cord and are not restricted to ventrolateral regions (Schughart et al. 1988; M. Utset and FHR, pers. communication). These results suggest that the 3.6 kb promoter region contains only some of the regulatory elements that direct the expression of the Hox-2.2 gene into neural tissues. To date, the presence of neurogenic elements in homeobox gene promoters has been described for the murine Hox-1.1, -1.3, -2.3, -3.1 genes, and the human Hox-5.1 gene (Püschel et al. 1990; Zakany et al. 1989; Kress et al. 1990; Bieberich et al. 1990; Tuggle et al. 1990; respectively). To identify additional cis-acting regulatory elements required for the correct and high level of Hox-2.2 expression in the CNS, it will be necessary to test larger genomic fragments.

The 1.2 kb promoter fragment does not give rise to reproducible expression patterns, and we therefore conclude that limb-specific expression may be regulated by an enhancer element present within a 2.4 kb region, located 1.2 kb upstream of the Hox-2.2 coding region. Our results demonstrate that the genomic region immediately upstream of the Hox-2.2 coding region contains a partial promoter that can function outside of
the Hox-2 cluster. Similar results have been obtained with Hox-1.1, -1.3, -2.3, and -3.1 promoters (Püschel et al. 1990; Zakany et al. 1989; Kress et al. 1990; Bieberich et al. 1990; respectively). These observations suggest that several or perhaps all of the homeobox genes in a cluster contain an independent functional promoter that is able to direct gene expression at least in subsets of the normal expression domains. In contrast, in all studies described to date, even large upstream regions of individual homeobox genes do not contain all necessary regulatory elements (Kress et al. 1990; Bieberich et al. 1990) indicating that cis-acting regulatory regions are missing that may be located in introns or downstream regions, or can be found in more distal regions upstream or downstream of neighboring genes or at the ends of the homeobox gene clusters.

All embryos containing the 3.6 kb Hox-2.2 promoter region expressed the transgene in a reproducible pattern in the developing limb regions. Therefore, the described Hox-2.2 promoter element may represent an extremely valuable tool for directing expression of other developmental genes (i.e. other homeobox genes) into specific patterns in developing limbs. Studies of the phenotypic changes resulting from the ectopic expression of these genes should yield valuable insights into their developmental functions during embryogenesis.

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