In vertebrates, the axial skeleton derives from the somites, segmental units organized in pairs on both sides of the developing neural tube (Hirsinger et al. 2000). Somites are formed in a rostro-caudal sequence by the epithelialization of mesenchymal cells at the rostral end of the presomitic mesoderm (Hirsinger et al. 2000). Although somites look morphologically similar, the skeletal elements they form are specific for the axial level at which they are positioned, eventually generating a vertebral formula characteristic for each species. In the mouse, this formula consists of seven cervical, 13 thoracic (which have ribs attached), six lumbar, four sacral, and a variable number of caudal vertebrae (12-20) (Burke et al. 1995).

Hox genes are among the major players in the specification of the morphological identity of the vertebrae (Krumlauf 1994). In addition, it has recently been described that some of the Hox genes play a global patterning role in vertebral development (Wellik and Capècechi 2003). When and how Hox genes determine somitic segmental identity is still an unresolved question. It is generally accepted that a specific combination of Hox genes expressed at a particular somitic level determines the axial identity of the resulting structures (Kessel and Gruss 1991; Krumlauf 1994). However, an association between Hox somitic expression and mutant phenotypes is not always easy to establish. For instance, axial phenotypes were observed in embryos that recovered appropriate Hox expression domains after retarded activation or transient expression in the presomitic mesoderm (Zakany et al. 1997, Kondo and Duboule 1999). Also, for some Hox genes, the rostral expression boundaries in the somites seem to lie posterior to their functional domains of activity, an apparent paradox. Hox group 10 genes are a good example of such a situation. Recent genetic studies revealed that these genes are functionally relevant up to the thoracic/lumbar transition [Wellik and Capècechi 2003], but published expression patterns for the three Hox group 10 genes [Hoxa10, Hoxc10, and Hoxd10] rarely extend to the corresponding somitic level, which in mice corresponds to somite 25 (Burke et al. 1995), and seem to differ according to the embryonic stage analyzed [Kessel and Gruss 1991, Burke et al. 1995; Favier et al. 1996, Zakany et al. 1997]. In this study we show that the Hox10 group expression domain, indeed, corresponds to the genetically defined functional domain but only at the stage at which the somites that correspond to the thoracic/lumbar transition are being formed in the presomitic mesoderm, which suggested that the activity of these genes is functionally relevant at this stage of somite development. We evaluated this hypothesis by comparing the activity of a Hox10 group and a Hox11 group gene in the presomitic versus the somitic paraxial mesoderm in transgenic mice. Our results reveal that the relevant function of Hox genes is provided in the presomitic mesoderm. We further sustain this conclusion with the finding that Gbx2, another homeobox-containing gene expressed in the presomitic and not in the somitic mesoderm, is required for proper patterning of the axial skeleton.

**Results and Discussion**

When we re-evaluated expression of the three Hox10 group genes at various embryonic stages [Supplemental Material], we found that the anterior expression border of all three Hox10 group genes is compatible with their reported functional domain [i.e., the thoracic/lumbar transition] only at the stage at which the corresponding somites are being formed. However, as development proceeds, this border recedes to more caudal somites located within the lumbar or sacral areas, or even almost disappears from the somites [Supplemental Material]. Considering that all members of the paralog group 10 seem to be functionally equivalent and functionally relevant up to the thoracic/lumbar transition [Wellik and Capècechi 2003], these results could indicate that Hox10 activity is essential at the stage of somitic formation or shortly thereafter, but it becomes dispensable at later stages of somitic development.

**Hoxa10 activity in the presomitic versus somitic mesoderm**

To test this idea, we used a transgenic approach in which Hoxa10 was expressed under the control of the Dll1 pro-
Hox10 activity in the presomitic mesoderm

Figure 1. Patterning activity of Hoxa10 expressed with the Dll1 promoter. Skeletal staining of wild-type (A–C) and Dll1–Hoxa10 transgenic (D–F) newborns. (A) A global view of the animal, after removing the forelimbs for clarity. (D) In the transgenic, the ribs are missing from the area labeled with an asterisk. (B,E) An anterior view of the sternum with the associated cartilaginous part of the ribcage (mostly missing in the transgenic animal). (C,F) The sacral area. The arrow points to the lateral fusion between sacral vertebrae, missing in the transgenic embryo. (G,H) An in situ hybridization analysis of Hoxa10 expression in a Dll1–Hoxa10 transgenic embryo. (G) A dorsal view of the presomitic mesoderm. (H) A lateral view of an embryonic day 9.0 (E9.0) embryo.

Figure 2. Patterning activity of Hoxa10 expressed in the somites. (A) Analysis of the expression activity of the sm promoter. The bacterial tetR gene was used as a reporter, and its expression was detected by in situ hybridization. The arrows indicate some somites, and the arrowhead indicates the presomitic mesoderm. (B) High-power view of the tailbud region of the embryo shown in A. The arrow indicates the last formed somite. (C) Ventral view of the thoracic area of a wild-type newborn. The sternum and the cartilaginous area of the ribcage were removed for clarity. (D) Lateral view of the thoracic area of an sm-Hoxa10 transgenic embryo, oriented rostral to the left and ventral at the bottom. The sternum (S), ribs (R), and vertebrae (V) are indicated. (E) Ventral view of the thoracic area of an sm-Hoxa10 transgenic embryo, oriented rostral at the top. The ossified area is strongly malformed. The sternum and the cartilaginous area of the ribcage were removed for clarity and are shown in F.

motor [Beckers et al. 2000]. This promoter has been shown to be active in the paraxial mesoderm at presomitic stage and in the most recently formed somites, but not at later stages of somite development [Beckers et al. 2000; Cordes et al. 2004]. Skeletal analysis of these transgenic embryos revealed striking phenotypes in the axial skeleton (six/six transgenics examined), which varied in intensity with the transgene copy number. In four of these transgenics, a total absence of ribs was evident in the thoracic area [Fig. 1D], consistent with the known activity of Hox10 group genes in suppressing rib formation [Wellik and Capecchi 2003]. The absence of ribs was associated with the absence of sternebrae on the sternum, which appeared as a flat ossified structure [Fig. 1E]. In addition, the cervical vertebrae were bigger than those in normal animals, and no cartilaginous fusions between vertebrae at the theoretical sacral level were found [Fig. 1F]. In the remaining two transgenic embryos, a fainter effect was found, restricted to the absence of ribs from vertebrae T1, T12, and T13 (Supplemental Material; data not shown). These results indicate that expression of Hoxa10 under the control of the Dll1 promoter was able to activate a dominant Hox10 patterning program in somites at all axial levels. If, as reported, the activity of this promoter is restricted to the presomitic mesoderm and newly formed somites [Beckers et al. 2000; Cordes et al. 2004], these data would imply that Hox10 activity in the forming somite is sufficient to determine the Hox10-dependent morphogenetic program to the somite.

In situ hybridization analysis of Dll1–Hoxa10 transgenics revealed that in some embryos Hoxa10 expression was not restricted to the presomitic and recently formed somitic paraxial mesoderm, but extended to more rostral somites [Fig. 1G,H], thus opening the possibility that the effect we observed was due to Hoxa10 expression in the already formed somite. To test this possibility, we generated a new set of transgenic animals (sm-Hoxa10) in which Hoxa10 was expressed under the control of a promoter that is not active in the presomitic mesoderm and becomes activated in the somites after they bud off the already formed somite. (Beckers et al. 2000; Cordes et al. 2004). Skeletal analysis of these transgenics, extending further dorsally to attach the vestiges of an embryonic day 9.0 (E9.0) embryo. [Image 151x197 to 299x451]
of the ossified areas (cf. Figs. 1B and 2F). We do not have an explanation for this phenotype, which is, however, more consistent with abnormal skeletogenesis than with the global patterning defects expected for Hox group 10 genes (Wellik and Capecchi 2003). Nevertheless, rib formation was not suppressed in sm-Hoxa10 transgenic animals. Direct comparison of somitic Hoxa10 expression levels by real-time RT-PCR indicates that higher Hoxa10 somitic expression in Dll1–Hoxa11 embryos cannot account for the qualitative differences in the phenotypes observed in the Dll1–Hoxa10 and sm-Hoxa10 transgenics [Supplemental Material]. This is also supported by the clear phenotypic differences between the most affected sm-Hoxa10 and the less affected Dll1–Hoxa10 transgenics [Fig. 2, Supplemental Material]. Altogether, these results indicate that the strong patterning effects observed in Dll1–Hoxa10 transgenics did not result from Hoxa10 expression in the somites but from its expression in the unsegmented paraxial mesoderm. Thus, Hoxa10 confers specific patterning instructions to the somites in the presomitic mesoderm.

Hoxa11 activity in the presomitic versus somitic mesoderm

To investigate if this effect can be extended to other Hox genes, we performed a similar experiment with Hoxa11. We selected a gene of the Hox11 group because, as for the Hox10 group, the effects of its overexpression can be predicted; the Hox11 group is essential for the genesis of sacral and caudal vertebrae (Wellik and Capecchi 2003), and thus, their overexpression is expected to produce signs of sacralization or caudalization at other levels of the axial skeleton. Ectopic expression of Hoxa11 in the presomitic mesoderm [Dll1–Hoxa11 transgenics] produced two main phenotypes in affected transgenics [five out of nine]. First, at the thoracic level they all had multiple fusions between adjacent ribs [Fig. 3D,E]. Since fusion of their lateral processes is a characteristic of sacral vertebrae, the alterations observed in the thoracic region of Dll1–Hoxa11 transgenics can, indeed, be scored as partial sacralization. This is reminiscent of the sacral region of Hox10-null mutants, which show small ribs fused at their lateral margins, which has been interpreted as Hox11 gene activity in the absence of Hox10 genes (Wellik and Capecchi 2003). Thus, thoracic rib fusion is apparently a sign of Hox11 activity in a Hox10 negative area. The second general characteristic was an anteriorized position of the sacrum. In wild-type animals, the first sacral vertebra [S1] is vertebra number 27. In Dll1–Hoxa11 transgenics, S1 was vertebra number 26 [one case], 25 [three cases], or 24 [one case] [Fig. 3B,C]. In addition, cartilaginous lateral fusions characteristic of sacral vertebrae were observed between adjacent lumbar vertebrae [Fig. 3C] [n = 2], between adjacent caudal vertebrae [observed in the most affected transgenic] [Fig. 3F], and between adjacent vertebrae in the cervical area [two transgenics] [Fig. 3H]. Finally, in two of the affected transgenics, anteriorly projecting protuberances resembling those observed in posterior sacral and caudal vertebrae emerged from the anterior lumbar vertebrae and replaced the rib in T13 [Fig. 3B].

To elucidate the contribution of the Hoxa11 somitic activity to the Dll1–Hoxa11 phenotype, we generated sm-Hoxa11 transgenics, which express Hoxa11 in the somites but not in presomitic mesoderm. None of the affected transgenic animals [four out of nine] showed the fusion between adjacent ribs that was observed in Dll1–Hoxa11 transgenics, and their thoracic region conserved the general rib pattern. Instead, a clear and reproducible phenotype in the axial skeleton was observed in affected transgenic animals. In the lumbar area, all vertebrae contained anteriorly projecting lateral protuberances [Fig. 3J,K]. As these protuberances were not fused, they can be considered a caudal rather than a sacral characteristic [Fig. 3I]. Interestingly, the ribs also contained anteriorly projecting cartilaginous nodules close to their dorsal extremities [Fig. 3K], resembling the ectopic structures observed in the lumbar area, but inserted on the ribs. In addition, there was a clear tendency toward a shortening of the ossified area at the expense of the cartilaginous area, similar to that described for the sm-Hoxa10 transgenics [Fig. 3K, data not shown]. S1 was located at the appropriate axial level [vertebra 27] in three of the four affected transgenics. In the fourth, the sacrum was anteriorized unilaterally by one segment [Fig. 3L]. Finally, no...
caudal vertebra acquired sacral characteristics in sm-Hoxa11 transgenics. Taken together, the above results indicate that the sacralization observed in Dll1–Hoxa11 transgenics derived from Hoxa11 expression in the presomitic mesoderm, and that the anteriorly projecting protuberance seen in T13 and the anterior lumbar area in two of these transgenics was probably due to residual Hoxa11 expression in the somites. We conclude that, similar to what we found for the Hox group 10 genes, somites can acquire a Hox group 11 program when a gene of this group is expressed while somites are being formed.

**Homeotic transformation in Gbx2 mutant embryos**

In an independent study aimed at the identification of downstream targets of Hoxa2 in the second branchial arch (Bobola et al. 2003; Kutejova et al. 2005), we identified Gbx2 as a gene that is repressed by Hoxa2 activity (Supplemental Material). When we analyzed the skeletal phenotype of Gbx2 mutant embryos (Wasserman et al. 1997), we found that these embryos presented Hox-like homeotic transformation in the axial skeleton. In particular, they had 14 rib pairs (12 of 18 embryos) [Fig. 4C,D], indicating that L1 was transformed into a T14; eight ribs, instead of seven, were attached to the sternum (seven of 18 embryos), which indicates that T8 acquired T7 identity [Fig. 4E,F], and the transition vertebra was T11 instead of T10 [10 of 18 embryos] (Supplemental Material), another anterior transformation in the thoracic region. This result was completely unexpected because Gbx2 is not expressed in the somites at any developmental stage (Fig. 4A,B; Bouillet et al. 1995; Wasserman et al. 1997, Supplemental Material). Interestingly, it is expressed in the presomitic mesoderm (Fig. 4A,B; Supplemental Material). Gbx2 expression is broad in the posterior presomitic mesoderm with a diffuse anterior limit caudal to somitomere S0. The anterior expression border seems to be slightly more caudal at later developmental times when compared with younger embryos [Fig. 4B; Supplemental Material]. Analysis of many embryos did not show evidence of a cycling behavior in the Gbx2 expression in the presomitic mesoderm.

One possibility for the axial phenotype of Gbx2−/− embryos is that the absence of Gbx2 resulted in the alteration of the anterior borders of Hox gene expression, similar to what has been previously described for mutants in the Cdx genes (Subramanian et al. 1995; Chawengsaksophak et al. 1997; van den Akker et al. 2002). Considering the strong similarity between the Gbx2 and Hoxc8 mutant phenotypes (Le Mouellic et al. 1992; van den Akker et al. 2001), we first examined Hoxc8 expression to find no obvious alteration in its spatial-temporal expression in Gbx2 mutant embryos (Supplemental Material). Phenotypes of mice containing specific mutations in the Hox9 and 10 groups also shared some characteristics with the Gbx2 mutant phenotype, particularly extra ribs in the lumbar area (Fromental-Ramain et al. 1996; Chen and Capecchi 1997; Wellik and Capecchi 2003). As for Hoxc8, we found no alterations in their anterior borders of somitic expression in Gbx2 mutant embryos (Supplemental Material). These results indicate that other homeobox-containing genes (Gbx2 also contains a homeobox) not belonging to the Hox clusters can provide segmental identity to the axial skeleton without affecting the anterior borders of somitic expression of genes within the Hox clusters. In addition, and most relevantly to the present study, the results support the finding that under physiological conditions, the activity of a homeobox-containing gene in the presomitic mesoderm is sufficient to provide patterning instructions to the resulting somites.

From our data it is not clear how Gbx2 patterns the axial skeleton. As discussed above, it is highly unlikely that it does it by modulating Hox gene expression. Considering that Gbx2 is a target of a Hox gene in the branchial area (Supplemental Material), one possibility is that it acts in the presomitic mesoderm downstream of another Hox gene. A good candidate is Hoxc8, as their mutant phenotypes in the axial skeleton are very similar. We are currently analyzing this possibility. An alternative hypothesis is that Gbx2 does not function upstream or downstream of the Hox genes, but just modulates similar cellular/molecular processes as these genes. In fact, it also contains a homeobox.

**Where do Hox genes pattern the somites?**

The above transgenic experiments show that the patterning programs provided by Hox genes to the paraxial mesoderm may differ when they act during somite formation or in the already formed somites. For the Hox10 group, the most relevant contribution to the morphogenesis of the axial skeleton seems to be already provided at the presomitic stage and not in the differentiating somites. The physiological role of expression of these

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**Figure 4.** Axial skeletal phenotype of Gbx2 mutant embryos. [A,B] Expression of Gbx2 in E10.5 embryos by in situ hybridization. [B] A close-up look at the tail tip. It is expressed in the presomitic mesoderm [arrow] but not in the somites [arrowheads point to some of them]. [C,D] Ventral view of the lower thoracic, lumbar, and sacral areas of a wild-type [C] and a Gbx2 mutant [D] newborn. The sternum and the cartilaginous area of the ribcage were removed for clarity. The 21st vertebra, normally the first lumbar (L1), has an extra rib [arrow] in the Gbx2 mutants. [E,F] Cartilaginous area of the ribcage of a wild-type [E] and a Gbx2 mutant [F] newborn to show that the eighth rib [r8] is attached to the sternum in the Gbx2 mutants but not in the wild-type embryos.
genes at later stages of somitic development has not been elucidated by our experiments. Our data do suggest that patterning by the Hox11 group requires a combination of instructions given in the segmental plate and later in the somites. While formation of sacral structures is apparently instructed by the expression of Hox group 11 genes in the presomitic mesoderm, caudal vertebrae seem to require the activity of these genes in the somites. Interestingly, both areas are affected when all six Hox group 11 alleles are inactivated (Welylik and Capecci 2003).

It is very likely that the prominent role of Hox gene expression in the presomitic mesoderm that we have observed in our transgenic animals is also relevant for the physiological Hox gene activity during axial patterning. This is supported by the finding that the expression of the Hox group 10 genes closely matches their functional domains at the stage when the relevant somites are being formed rather than at later stages, when somites start their differentiation programs. Also, at late developmental stages Hox group 11 genes seem to be active in somites fated to form caudal rather than sacral vertebrae (Burke et al. 1995). Our finding that inactivation of the Gbx2 gene produced a typical Hox mutant phenotype in the axial skeleton without any apparent effect on Hox gene expression gives further support to the view that homeotic genes can provide patterning instructions in the presomitic mesoderm, as expression of this gene within the paraxial mesoderm is restricted to the segmental plate without any somitic contribution.

An important consequence from our findings is that understanding the patterning of the axial skeleton by Hox genes will require focusing the search for their target genes and respective mechanisms of activity to specific areas of the paraxial mesoderm. Interestingly, it has been shown that anomalous activity of the Notch, Wnt, and Fgf signaling pathways in the presomitic mesoderm also produces Hox-like transformations in the axial skeleton (Partanen et al. 1998; Ikeya and Takada 2001; Cordes et al. 2004). It has been suggested that the segmentation clock operating in the presomitic mesoderm could be linked to specific temporal activation of Hox genes, thus determining its appropriate anterior expression limit (Zakany et al. 2001). Our finding that Hox gene expression seems to commit the presomitic mesoderm opens an interesting, yet not exclusive, alternative. Thus, it could be hypothesized that Hox genes operate by modulating the response of the presomitic mesoderm to specific signaling inputs. In each segmentation cycle (Dale and Pourquié 2000), the segmentation signals operate on presomitic mesoderm expressing a particular combination of Hox genes, which would define specific patterns of response to the same signals, resulting in the formation of somites already containing specific patterning programs. In support of this, grafting experiments in chicken embryos have shown that presomitic mesoderm corresponding to a specific axial level transplanted to replace the presomitic mesoderm of a different level produces structures consistent with the position of the donor tissue (Kiency et al. 1972). Moreover, the ability of Hox genes to modulate the response of mesenchymal cells to Fgf signals has already been described in the craniofacial area (Bobola et al. 2003), and it could thus also be operative in the paraxial mesoderm. In light of this, it will be interesting to observe if stage-specific variations in the molecular cascades triggered by Notch, Fgf, and Wnt signals exist in the presomitic mesoderm and if they are affected by mutations in specific Hox genes.

Materials and methods

Transgenic and mutant animals

Transgenic constructs were generated using standard molecular biological techniques (Sambrook et al. 1989). The Dll1 msd promoter (Beckers et al. 2000), the corresponding CDNAs, and the polyadenylation signal from SV40. The mb constructs contained a 2.5-kb BamHI fragment of the Hoxa2 gene that includes the enhancer for rhombomere 2 (Frasch et al. 1995), the corresponding CDNAs, and the polyadenylation signal of SV40. The activity of the mb promoter was evaluated using the bacterial tetR gene as a reporter, whose expression was detected by whole-mount in situ hybridization using a tetR-specific probe (Mallo et al. 2003). The Hoxa10 CDNA (mouse) was obtained from IMAGE clone 6511608. The Hoxa11 CDNA (human) was obtained from IMAGE clone 5587615. Constructs were liberated from bacterial vector sequences, gel-purified, and used to generate transgenic embryos and animals by pronuclear injection according to standard protocols (Hogan et al. 1994).

The Gbx2 mutant mice have been described before (Wassarman et al. 1997).

Molecular and phenotypic analyses

Whole-mount in situ hybridization was performed as described in Kanzler et al. (1998). The Hoxa10 and Hoxa10 probes were obtained from IMAGE clones 6511608 and 6516538, respectively. The Hoxc10 probe was a 1091-bp fragment extending from position 29 to position 1119 of the mouse mRNA cloned by RT-PCR. The Gbx2 probe was a 1.5-kb SmaI/Xbal fragment of the Gbx2 CDNA.

Skeletal analyses were performed using the alcian blue/alizarin red staining method as described in Mallo and Brandin (1997).

For transcript quantification, total RNA was isolated from dissected somites using TRI Reagent (Roche) and first-strand cDNA was synthesized using random hexamer-primed reverse transcription. Hoxa10 transcripts were then quantitated with the LightCycler (Roche) using the SYBR green PCR kit (IQIgen) and primers 5′-AGCGAGTCTCTGAGCTCCACCAGC-3′ and 5′-GTCGGTGGATCCGACCCCTG-3′. Gapdh transcripts, amplified with primers 5′-ACACAGTCATCCACGATC-3′ and 5′-TCCACACCCCTGTTGGCTGA-3′, were used for normalization.

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References


Chawengsaksophak, K., James, R., Hammond, V.E., Koenig, F., and Beck, F. 1997. Homeosis and intestinal tumours in Cdx2 mutant....
Hox activity in the presomitic mesoderm


Wasserman, K.M., Lewandoski, M., Campbell, K., Joyner, A.L., Rubenstein, J.L., Martinez, S., and Martin, G.R. 1997. Specification of the anterior hindbrain and establishment of a normal mid/hindbrain or...