Enhancer timing of Hox gene expression: deletion of the endogenous Hoxc8 early enhancer

Aster H. Juan and Frank H. Ruddle*

Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520, USA

*Author for correspondence (e-mail: frank.ruddle@yale.edu)

Accepted 13 June 2003

Development 130, 4823-4834
© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00672

Summary
The proper expression of Hox genes is necessary for the accurate patterning of the body plan. The elucidation of the developmental genetic basis of transcriptional regulation of Hox genes by the study of their cis-regulatory elements provides crucial information regarding the establishment of axial specification. In this report, we investigate the role of the early enhancer (EE) of the murine Hoxc8 gene to better understand its role in pattern formation. Previous reports show that knockouts of the endogenous Hoxc8 coding region result in a combination of neural, behavioral and skeletal phenotypes. In this report, we limit ourselves to a consideration of the skeletal abnormalities. Early reports from our laboratory based on exogenous transgenic reporter constructs implicate a 200 bp non-coding element 3 kb upstream of the Hoxc8 promoter as a crucial enhancer that regulates the transcription of Hoxc8. In the present work, we have deleted this regulatory region from the endogenous genome using embryonic stem cell technology. Our results show that the deletion of the EE results in a significant delay in the temporal expression of Hoxc8. We also show that the deletion of the EE does not eliminate the expression of the Hoxc8 protein, but delays the attainment of control levels of expression and anterior and posterior boundaries of expression on the AP axis. The temporal delay in Hoxc8 expression is sufficient to produce phenocopies of many of the axial skeletal defects associated with the complete absence of Hoxc8 gene product as previously reported for the Hoxc8-null mutation. Our results are consistent with emerging evidence that the precise temporal expression of Hox genes is crucial for the establishment of regional identities. The fact that the EE deletion does not eliminate Hoxc8 expression indicates the existence of a Hox8 transcriptional regulatory apparatus independent to some degree of the Hoxc8 EE. In a comparison of our results with those reported previously by others investigating temporal control of Hox gene expression, we have discovered a structural similarity between the Hoxc8 EE reported here and a transcriptional control element located in the Hoxd11 region. We speculate that a distributed system of expression timing control may exist that is similar the one we propose for Hoxc8. Last, our data is consistent with the position that disparate regulatory pathways are responsible for the expression of Hoxc8 in the organogenesis of somites, neural tube and limb bud.

Key words: Enhancer, Hoxc8, Gene regulation, Mouse

Introduction
The proper temporal expression of the Hox genes during embryogenesis is essential for normal development, yet the control mechanisms involved are poorly understood. It has been shown that Hox gene expression can be resolved into three phases: activation, establishment and maintenance (Deschamps et al., 1999). Activation occurs when the genes are initially expressed in the tail bud in conjunction with primitive streak formation. Establishment is defined by the extension of expression anteriorly and the establishment of an anterior limit of expression. During this phase, some but not all genes may show a decrease in posterior expression with the concomitant formation of a posterior boundary. Maintenance is characterized by the perpetuation of these expression patterns throughout organogenesis. The individual Hox genes are expressed in strict temporal sequence determined by their position in the Hox gene cluster (temporal colinearity), the more 3’ genes first and subsequently the more 5’. Obviously, these patterns of expression are highly regulated, and hypotheses have been advanced recently to provide a explanation for temporal control of Hox gene expression.

A two-step mechanism for precise temporal control of Hox gene expression in the initiation and establishment phases has been advanced (Gaunt and Strachan, 1996; Kondo et al., 1998; Kondo and Duboule et al., 1999; Zákány et al., 2001). In the first step, progressive chromatin modification of the clusters extending from 3’ to 5’ potentiates gene activity. Isolation of a cis-element that mediates temporal activation of Hox genes is consistent with this model. This element is located outside of the 5’ terminus of the Hoxd cluster. In vivo deletion of the element prevents the premature activation of 5’ Hox genes (Kondo et al., 1998; Kondo and Duboule et al., 1999). Studies on the Polycomb-group (Pc-G) family further enhances the importance of chromatin status regarding temporal regulation of Hox expression. The Pc-G family is involved in transcriptional regulation of Hox genes through modification of chromatin structure (Gould, 1997; Gebuhr et al., 2000; Simon and Tamkun, 2002). In the Pc-G family M33 null
These studies suggest that in the initiation phase, a repressive regulatory mechanism is involved in regulating the correct temporal activation of Hox genes by modulating chromatin structure. Therefore, Hox genes must release sequentially in a 3' to 5' direction in order to be transcribed in the proper temporal sequence.

In the second step, the expression of Hox genes is regulated by a specific response to transcription signals. Recent studies showed that after chromatin opening, transcription of Hox genes are increased in part by the segmentation clock pathway (Dubrulle et al., 2001; Zákány et al., 2001). A ‘segmentation stripe enhancer’ has been proposed on the basis of deletion analyses and postulated to control the temporal expression of several Hoxd genes possibly through segmentation signals (Zákány et al., 2001). Other transcriptional inducers such as RA or the Caudal (cdx) gene family of transcription factors may also be involved in temporal regulation of Hox expression following the initial activation stage. Several cis-regulatory elements possessing binding motifs of candidate transcription factors have been identified. In vivo modification of these enhancers leads to transient expression delay of Hox genes in early mouse embryos. Although the correct expression patterns of the corresponding Hox genes are re-established later, the adult mice display morphological modifications (Dupé et al., 1995; Zákány et al., 1997). These data highlight the importance of exact expression timing of Hox genes in early development and suggest that regulation of the spatial and temporal expression of Hox genes is mediated through diverse cis-regulatory pathways.

We consider these hypotheses in the light of our deletion of the Hoxc8 early enhancer. The EE extends over 200 bp and is located 3 kb upstream of the Hoxc8 promoter. The EE is highly conserved (95%) on the basis of nucleotide sequence comparison between human and mouse. Reporter gene analysis shows that this element is necessary and sufficient to reconstitute the endogenous pattern of Hoxc8 expression in ectodermal and mesodermal derivatives (Shashikant et al., 1995). A minimum of seven putative transcription factor binding motifs including two CDX-binding sites have been identified within the EE by means of sequence analysis and mutational studies involving reporter constructs in transgenic mice (Shashikant et al., 1995; Shashikant and Ruddle, 1996). In addition, recent data show that the EE may be involved in chromatin remodeling through histone deacetylase 3 (Bayarsaikhan and Ruddle, 2000; Tussie-Luna et al., 2002). Therefore, the function and structure of the EE indicate that it is not only important for setting up the spatial expression domain of Hoxc8, but also crucial for regulating proper Hoxc8 temporal activation.

In order to define the timing functions of the EE more precisely, we have deleted it endogenously using stem cell targeting technologies. Homozygous knockout animals exhibit a number of mutant phenotypes that both resemble and differ those reported for Hoxc8-coding region knockouts. Our findings show that the EE contributes to the temporal control of Hoxc8 in the context of the endogenous genome, but is not necessary for its expression, contrary to expectations based on our earlier transgene experiments. We show that the deletion of the EE delays the expression of Hoxc8 early in development at a time coincident with somitogenesis and possibly at a time when chromatin modifications are taking place within the Hox clusters. We also show that a second domain similar to the EE in both structure and function resides in the Hoxd cluster, indicating the possible existence of a distributed system regulating the temporal expression of the Hox genes.

Materials and methods

Gene targeting

Genomic Hoxc8 sequences are isolated from a P1 clone obtained from a mouse 129/Sv genomic library (Genome Systems). To construct the targeting vector, a 2.2 kb SplI-Spel genomic fragment corresponding to the 5' region flanking the 200 bp EE is subcloned in pCR 2.1-TOPO (Invitrogen) to generate the 5TA vector. A 1.2 kb neo selection cassette flanked by loxP sites is excised from neofox-8 (gift of K. Rajewsky) and inserted into the SpeI-HindIII sites of 5TA to give 5NITA. A 3.4 kb fragment containing the 2.2 kb 5' genomic fragment and the loxP flanked neo cassette is then excised from 5NITA and inserted into the SplI-ClaI sites of a vector containing two copies of the HSV-κk cassettes (gift of T. Williams) to generate 5NeoIT. Finally, a 1.8 kb genomic fragment homologous to the 3' region flanking the EE is cloned into the NotI-ClaI sites of 5NeoIT to generate the targeting vector EENeolXTK (Fig. 1B). For gene targeting, 10⁷ CJ-7 embryonic stem (ES) cells (gift of T. Williams) are electroporated with 25 μg ApaLI linearized EENeolXTK targeting vector using a GenePulser (Biorad) set to 250 μF and 320V, and selected in 0.3 mg/ml G418 and 2 μM ganciclovir for 9 days. Homologous recombinants are screened first by PCR using the primer pairs See1 (5'-CTG CTG TCT CCC CAG CGC AAC TG-3') and See2 (5'-CAG GGA TCG CCT TCT ACC GCA AAC TG-3'); and 3neo1 (5'-CCT TCC GCA AAA CCA TCG TCG AC-3') and 3neo2 (5'-ACC CCA CAT CCT GAG GTT TCG AGG TTA GG-3') (Fig. 1B,C). Correctly targeted clone (EEneo) is expanded and confirmed by Southern analysis using two different enzyme and probe combinations (Fig. 1B,D). To delete the neo cassette, 10⁷ targeted ES cells are transfected with 50 μg Cre expression plasmid (gift of K. Rajewsky) by electroporation and selected in medium containing 2 μM ganciclovir. ES cell clones (EElox), having undergone Cre-mediated deletion events, are identified by PCR and Southern hybridization. The following primers are used for the PCR: 5neo1 5'-CAG GGA TCG CCT TCT ACC GCA AAC TG-3', and 3neo1 5'-CCT TCC GCA AAA CCA TCG TCG AC-3'; and 3neo2 5'-ACC CCA CAT CCT GAG GTT TCG AGG TTA GG-3' (Fig. 1B,C). The strategy of Southern hybridization is shown in Fig. 1. ES cell clones carrying the mutated enhancer region (EEneo and EElox) are injected into C57BL/6j blastocysts by standard procedures.

Mouse breeding and genotype analysis

Germine male chimeras are crossed with B6129F1 females and the resulting heterozygous offspring are interbred to generate homozygous mice. DNA is isolated from tail biopsy samples from newborns and adult mice and from yolk sacs of 7.5-11.5 days postcoitus (dpc) embryos. Subsequent genotyping is determined by PCR. Primer pair 5neo1 and 3neo1 is used in PCR reaction. The combination of 5neo1 and 3neo1 makes it possible to identify the wild-type (472 bp product) and mutated (1472 bp product of EEneo colony; 306 bp product of EElox colony) Hoxc8 early enhancer loci (Fig. 1E).

Skeletal preparations

Skeleton of newborn mice are stained with Alcian Blue 8GX and Alizarin Red S as previously described (van den Akker et al., 2001). Briefly, Newborns are skinned, eviscerated and fixed in 4% paraformaldehyde in PBS overnight followed by cartilage staining with Alcian Blue (0.5 mg/ml Alcian Blue in 80% ethanol/20% acetic acid) overnight. Skeletons are rinsed twice for 1 hour in 96% ethanol and cleared in 1.5% KOH for
5 hours. The bone is then stained overnight in 0.5% KOH and 0.15 mg/ml Alizarin Red S. Stained skeletons are cleared in 0.5% KOH/20% glycerol for 3 days or longer and stored in 20% ethanol/20% glycerol.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed on genotyped embryos at 7.5-11.5 dpc following standard procedures. The probes used for hybridization are mouse genomic fragments containing part of exon I of mouse Hoxb8 (211 bp), Hoxc6 (342 bp), Hoxc8 (248 bp) and Hoxc9 (291 bp). All probes are labeled with digoxigenin using standard procedures.

DNA sequence analysis

Regions of nucleotide sequence similarity between the EE and Hoxd11 RVIII (Zákány et al., 1997) are detected by importing the sequences to MacVector (IBI-Kodak) and aligned manually. The putative transcription factor binding motifs within the two enhancers are identified by TFSEARCH V1.3 (Yutaka Akiyama, Kyoto University, 1995, http://www.cbrc.jp/research/db/TFSEARCH.html) and MatInspector V2.2 (http://transfac.gbf.de/cgi-bin/matSearch/matssearch.pl).

Results

Generation of mice lacking the Hoxc8 early enhancer region

The Hoxc8 early enhancer (EE) is located 3 kb upstream from the Hoxc8-coding region. This 200 bp region contains several putative transcription factor binding sites, including CDX, Forkhead/ SRY, HOX and STAT motifs (Fig. 1A). Previous EE reporter analysis in transgenic mice showed that the EE is essential for the activation of Hoxc8 expression (Shashikant et al., 1995; Shashikant and Ruddle, 1996). We deleted the Hoxc8 EE by means of ES targeting in order to evaluate its functional role in an endogenous context. A targeting vector replaces the 200 bp region with a loxP flanked neomycin selection cassette via homologous recombination (Fig. 1B). PCR and Southern analysis confirm a single correctly targeted ES cell clone, termed EEneo, among 640 ES clones (Fig. 1C,D). To prevent potential transcriptional interference, the neo cassette is removed from the EEneo locus by transfecting a Cre expression plasmid into EEneo ES cells (Fig. 1B). Positive ES cell clones are identified by PCR and confirmed by Southern hybridization (Fig. 1C,D). ES cells carrying both versions of mutated alleles of the EE are used to generate chimeric mice, and heterozygous and homozygous animals for the EE deletion are produced.

It should be noted, unless otherwise stated, that we refer only to the homozygous EE deleted embryos, as the heterozygous animals are in most respects identical phenotypically to wild-type embryos (Table 1). The EE homozygotes are of two types: those carrying the neo cassette and those in which the cassette has been deleted. We refer to these animals as EEneo and EElox, respectively.

Hoxc8 expression in EEneo and EElox embryos

EEneo embryos

Significantly, Hoxc8 mRNA expression is not eliminated in homozygously EE deleted mice. Instead, Hoxc8 expression levels and timing of expression are modified. The temporal modifications in Hoxc8 result in a significant number of phenotypic modifications in body plan. These can be compared with phenotypes previously reported that result from Hoxc8 knockout loss-of-function mutations, and Hoxc8 gain-of-function mutations. Thus, the EE deletion provides a new parameter with which to investigate the regulation of Hoxc8 and its role in shaping morphogenesis.

Whole-mount in situ hybridization was performed on 7.5 to 11.5 dpc embryos in order to compare the spatiotemporal pattern of Hoxc8 expression in wild-type and EEneo embryos. Hoxc8 activity is not observed in any embryonic or extra-embryonic tissues in both wild-type and EEneo embryos at 7.5 dpc. Expression of Hoxc8 is first detected in the tail bud region, including the allantois at 8 dpc in wild-type embryos. However, expression of Hoxc8 is not detected in EEneo embryos (Fig. 2A). At 8.5 dpc, Hoxc8 is expressed in both neural tube and paraxial mesoderm in wild-type embryos. Although the strongest expression is in the posterior region of the embryos, the expression domains in both tissues are extended to more anterior regions (Fig. 2B). In EEneo embryos, expression of Hoxc8 is detected in both neural tube and paraxial mesoderm. However, the expression domains in both tissues are restricted to the caudal region and are relatively lower in intensity when compared with wild-type embryos (Fig. 2B).

The anterior boundaries of Hoxc8 expression in the neural tube and mesoderm are formed at 9 dpc in wild-type embryos. The anterior limit of peak Hoxc8 expression in the neural tube is at the level of the tenth somite, while the anterior limit in the paraxial mesoderm is at the level of the 15th somite (Fig. 2C). By contrast, in EEneo embryos, the expression of Hoxc8 in the paraxial mesoderm is still weak and restricted to the unsegmented tail bud region. In the neural tube, although the anterior boundary of Hoxc8 expression in EEneo embryos is extended to the same somite level as in wild-type embryos, the expression level is significantly reduced (Fig. 2C). The anterior limit is not well demarcated in Fig. 2C, but it can be clearly discerned under the microscope by direct examination.

The expression domains of Hoxc8 in the neural tube and paraxial mesoderm are well established in wild-type embryos at 10 dpc. Hoxc8 is expressed in the neural tube between somites 9 and 15 and in the paraxial mesoderm between

<table>
<thead>
<tr>
<th>Type of transformation</th>
<th>C5 to C6</th>
<th>C7 to T1</th>
<th>T7 to T6</th>
<th>T8 to T7</th>
<th>T9 to T7</th>
<th>T12 to T10</th>
<th>T11 to T10</th>
<th>T12 to T11</th>
<th>L1 to T13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n=22)</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EEneo (+/−) (n=35)</td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>71</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EEneo (−/−) (n=29)</td>
<td>14</td>
<td>21</td>
<td>93</td>
<td>100</td>
<td>45</td>
<td>97</td>
<td>69</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Wild type (n=20)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EElox (+/−) (n=33)</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EElox (−/−) (n=31)</td>
<td>0</td>
<td>10</td>
<td>26</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1. Penetration (%) of skeletal defects in EEneo and EElox mutant mice
somites 15 and 23. This expression profile is maintained until 11.5 dpc, while the expression intensity of the paraxial mesoderm in somite 22 and 23 becomes significantly weaker at 11.5 dpc (Fig. 2D-F). In EEneo embryos, the expression level of Hoxc8 within the neural tube is normal at 10 dpc. In the paraxial mesoderm, although the anterior boundary of Hoxc8 expression is established normally at somite 15, the posterior boundary of the Hoxc8 expression domain is anteriorized to somite 21 with the strongest expression between somites 16 to 19 (Fig. 2D). This failure to form a normal posterior boundary in the paraxial mesoderm continues through 11 dpc (Fig. 2E). However, expression of Hoxc8 in the paraxial mesoderm is fully recovered at 11.5 dpc. The expression intensity within somite 22 and 23 is even stronger when compared with wild-type embryos (Fig. 2F). Overall, deletion of the Hoxc8 early enhancer leads to temporal delay of Hoxc8 expression and expression domain alteration at certain developmental stages.

Fig. 1. Hoxc8 early enhancer sequence and targeted disruption/deletion of Hoxc8 early enhancer locus. (A) The 200 bp region of the EE. Sequences underlined represent putative transcription factor binding sites. Cis-acting elements A-E are identified by mutational analysis of reporter genes (Shashikant et al., 1995; Shashikant and Ruddle, 1996). (B) Schematic representation of the EE (oval), targeting vector, disrupted EE locus (EEneo) and deleted EE locus (EElox). Exons of Hoxc8 (black boxes) and Hoxc9 (gray boxes), and restriction enzyme sites (A, AvrII; B, BamHI; D, DraI; H, HindIII, X, Xhol) are shown. A PGKneo cassette (neo) flanked by loxP sites (arrowheads) and two copies of the HSV thymidine kinase gene (tk) are used for positive and negative selection, respectively. Arrows indicate different primer sets used for PCR screening. The position of the 5′ probe used for Southern hybridization is also indicated. Broken lines represent the homologous recombination regions. (C) PCR analysis of genomic DNA isolated from ES cells using different primer pairs. 5ee1 and 5neo2, 3neo1 and 3sb3r and 5neo1 and 3sb3r amplify 2.5 kb, 2 kb and 3 kb product for EEneo allele. 5neo1 and 3sb3r amplify 2.2 kb product for wild-type allele. 5neo1 and 3neo2 amplify 300 bp product for EElox allele, 500 bp product for wild-type allele and 1.5 kb product for EEneo allele. N, negative control without DNA. (D) Southern hybridization of ES cell genomic DNA using an 5′ external probe (box labeled as probe in B). Xhol digestion gives a 5 kb fragment for EEneo allele, and a 10 kb fragment for wild-type allele. BamHI and HindIII digestion gives a 3 kb fragment for EEneo allele, and a 3.7 kb fragment for wild-type allele. AvrII and DraI digestion gives a 5.5 kb fragment for EElox allele, and a 3.2 kb fragment for wild-type allele. (E) PCR analysis of mouse tail DNA using primer set 5neo1 and 3neo2. Amplification of wild-type alleles produces a 472 bp fragment, while amplification of mutated alleles gives a 1472 bp fragment (EEneo) or 306 bp fragment (EElox).
EElox embryos

We removed the neo cassette from EEneo ES cells to generate an EElox mouse line using the Cre/loxP system in order to evaluate the effect of the neo cassette on Hoxc8 expression in EE deleted embryos. Whole-mount in situ hybridization in EElox embryos at 7.5 dpc does not show any Hoxc8 activity (data not shown). At 8.0 dpc, while expression of Hoxc8 is detected in the allantois in EElox embryos, the expression domain has not spread from the allantois to more anterior regions, as in wild-type embryos, suggesting a temporal delay of Hoxc8 expression at an early developmental stage in EElox embryos (Fig. 3A). However, there is no spatial or temporal modification of the Hoxc8 expression pattern in EElox embryos at 8.5 dpc and 9.0 dpc, showing full recovery of normal expression (Fig. 3B,C).

The normal expression domains of Hoxc8 in the neural tube and paraxial mesoderm are established in both wild-type and EElox embryos at 10 dpc, with the exception that the overall expression level of Hoxc8 in EElox embryos appears lower as judged by comparisons between EElox and wild-type embryos at day 10 by whole-mount staining intensity. Furthermore, the expression intensity within somite 22 and 23 is reduced (Fig. 3D). At 10.5 dpc, the posterior boundary of the paraxial mesoderm expression domain is anteriorized to somite 20 with the strongest expression between somite 16 to 18 (Fig. 3E). This alteration of expression pattern in the paraxial mesoderm is similar to the retarded expression pattern in the paraxial mesoderm observed in EEneo embryos around 10 and 11 dpc (Fig. 2D,E). However, at 11.5 dpc, expression of Hoxc8 in the paraxial mesoderm is fully recovered in EElox embryos, although the expression intensity within the somites is relatively lower when compared with wild-type embryos (Fig. 3F).

Growth rate and behavioral phenotypes in postnatal EEneo and EElox mice

Adult mice heterozygous and homozygous for the EE deletion (EEneo and EElox) are obtained in the expected Mendelian ratio and are viable, healthy and fertile. However, double homozygous crosses of our EEneo colony result in lower fecundity when compared with wild-type or heterozygous crosses. In addition, about 11% of the EEneo heterozygous and 16% of the EEneo homozygous mice show stunted growth. They are smaller than their littermates at birth, and do not attain normal weight as adults.

Approximately 12% of the heterozygous and 30% of the homozygous adult mice of EEneo and EElox colonies show an abnormal contraction and clasping reflex of both the fore- and hindlimbs upon tail suspension. Adult wild-type mice extend their limbs when suspended by their tails. Although some of the heterozygous and homozygous mice can extend their limbs when first suspended, within a few seconds they hug their bodies with the fore- and hindlimbs. This phenotype resembles the neurological defect observed in cyclin D1 (Sicinski et al., 1995), Mf3 (Labosky et al., 1997) and Hoxb8 (van den Akker et al., 1999) null mice.

Skeletal phenotypes in EEneo and EElox embryos

EEneo embryos

Expression of Hoxc8 in mesodermal derivatives of the thoracic region suggest that Hoxc8 is involved in specifying positional identities in this region. Indeed, ablation of Hoxc8 has been shown to induce skeletal transformations in the trunk (Le Mouellic et al., 1992; van den Akker et al., 2001). EEneo mice show a modification in temporal display of Hoxc8 expression that also results in skeletal modifications in the thoracic region.
in some respects similar to those induced by the complete loss of Hoxc8 gene expression. In the upper lumbar region, all the EEneo mice show either a rudimentary rib or a fully developed pair of ribs on L1, suggesting L1 is transformed anteriorly into T13 (Fig. 4A,B). Anterior transformations in the thoracic vertebral column are also observed. In the rib cage, about 93% of the EEneo mice develop an extra sternebra between T6 and T7 (Fig. 4D). In addition, eight pairs of ribs attach to the sternum instead of seven in 71% of the EEneo heterozygotes and all of the EEneo mice (Fig. 4C,D). In about half of the EEneo mice, the ninth ribs also attach to the sternum contralaterally (Fig. 4D). These abnormalities indicate minimally that T7, T8 and T9 are transformed anteriorly in EEneo mice. In wild-type animals, T10 is called the transitional vertebra as the dorsal process in the thoracic vertebrae are normally pointed posteriorly from T3 to T9, and anteriorly from T11 to more caudal region (Fig. 4E). In almost all the EEneo mice, however, T13 becomes the transitional vertebra (Fig. 4F), suggesting that T10, T11 and T12 are transformed anteriorly. Furthermore, the 12th rib is transformed anteriorly to the identity of the 11th rib in about 69% of EEneo mice. In wild-type or heterozygous animals, the total length of the 12th rib is about half of the length of the 11th rib, and the length of the cartilage portion of the 12th rib is about half the length of the bony part. However, in EEneo mice, the length of the 12th rib is almost equal to the length of the 11th rib, and the cartilage and the bony part of the 12th rib are of equal length, similar to the appearance of the 11th rib (Fig. 4G,H). In addition, skeletal transformations are observed in the cervico-thoracic region in EEneo mice with low penetrance. The anterior tuberculum (AT) that is normally attached to the 6th cervical vertebra is attached to the 5th cervical vertebra in 14% of the EEneo mice, and there is no AT on C6, suggesting that C5 and C6 are transformed posteriorly to the identity of C6 and C7 (Fig. 4I,J). Furthermore, 21% of the EEneo mice developed extra ribs on C7, and these ectopic ribs are often fused with the first rib attached to the first thoracic vertebra, suggesting a posterior transformation of C7 into T1 (Fig. 4I,J).

Table 1 shows an overview of the axial skeletal defects found in EEneo mice. The penetrance and the types of the defects in the lower thoracic and upper lumbar region in EEneo mice are similar to those found in Hoxc8 gene knockout mice (Le Mouellic et al., 1992; van den Akker et al., 2001). However, in the mid-thoracic region, the penetrance of skeletal defects in EEneo mice is actually enhanced when compared with Hoxc8 gene knockout mice. In addition, the phenotype of the ninth rib transformation found in EEneo mice has not been reported for Hoxc8 gene knockout mice. Instead, this phenotype was reported in Hoxc9 knockout mice (Suemori et al., 1995). Furthermore, EEneo mice display posterior transformation in the cervico-thoracic region and these phenotypes also have not been reported for Hoxc8 gene knockout mice. These results show that the replacement of the Hoxc8 early enhancer with the neo cassette not only enhances the severity of skeletal transformations, but also produce novel phenotypes.

EElox embryos
Several types of skeletal defects are observed in EElox mice. These defects include low penetrance of posterior transformation of C7 to T1, and anterior transformation of T7 to T6 and L1 to T13 (Table 1). The most prominent skeletal phenotype in EElox mice is the anterior transformation of T8 to T7, with 90% penetrance (Table 1).

Expression patterns of Hoxc6, Hoxc9, and Hoxb8 in EEneo and EElox embryos
Several phenotypes are observed in EEneo and EElox mice that have not been reported for Hoxc8 gene knockout mice as reported above. These results suggest that the expression of
other Hox genes paralogous to *Hoxc8* might also be modified. To investigate this possibility, we examined mRNA expression pattern of *Hoxc6* and *Hoxc9*, the immediate neighboring genes flanking *Hoxc8*, and *Hoxb8*, a *Hoxc8* paralog in the *Hoxb* cluster. The expression pattern of *Hoxc6* in EEneo and EElox embryos at 8.5 and 9.5 dpc is unchanged with respect to controls in both the neural tube and paraxial mesoderm (Fig. 5A, data not shown). However, although the expression pattern of *Hoxc9* in the neural tube is not altered, the anterior expression boundary in paraxial mesoderm is shifted one somite posteriorly in two out of three 9.5 dpc EEneo embryos (Fig. 5B). We do not find any modifications of *Hoxc9* expression in 9.5 dpc EElox embryos (data not shown). The alteration of *Hoxc9* expression in paraxial mesoderm in EEneo embryos correlates with the ninth thoracic vertebra transformation observed in EEneo mice, suggesting that the neo cassette may interfere with the regulation of *Hoxc9* transcription. We next examined the expression pattern of *Hoxb8* in 7.5, 8.5 and 9.5 dpc EEneo and EElox embryos. No departure from baseline expression was detected (Fig. 5C, data not shown).

**Discussion**

**Hoxc8 expression delay and thoracic anteriorization**

Our whole-mount in situ data of *Hoxc8* transcripts in wild-type mouse embryos at different developmental stages confirm previously reported studies (Gaunt, 1988; Le Meoullie et al., 1988). Transcription initiates at the allantois at early 8 dpc and then the expression domain gradually moves anteriorly to all the tissues from 8 dpc to 9.5 dpc. At 10 dpc, the anterior and posterior boundaries are determined, establishing the definitive expression domains within the paraxial mesoderm and neural tube. The correlation between *Hoxc8* expression and regional morphology suggests that *Hoxc8* plays a role in the specification of mid- and lower thoracic identities within the

---

**Fig. 4.** Morphological alteration of the axial skeletons in newborn EEneo mice. (A,B) Dorsal view of the thoraco-lumbar regions. (A) Wild-type animal showing 13 thoracic vertebrae and 6 lumbar vertebrae. (B) EEneo mouse showing a fully developed extra pair of ribs on L1. (C,D) Ventral view of the thorax. (C) In the wild-type mouse, there are seven pairs of ribs attached to the sternum. The sixth and seventh ribs attach to the same point of the sternum without sternebra separation (arrow). (D) EEneo mouse with nine pairs of ribs attached to the sternum, extra sternebra developed between the sixth and the seventh ribs (arrow). (E-H) Lateral view of lower thoracic/upper lumbar regions. (E) Arrowhead indicates that the position of the transitional vertebra is in T10 in the wild-type mouse. (F) The transitional vertebra (arrowhead) shifted from T10 to T13 in EEneo mouse. (G,H) Anteriorization of the 12th rib in EEneo mouse is revealed by the total length and the length of the cartilage. (G) Total length of the 12th rib in the wild-type animal is about two thirds of the length of the 11th rib and the cartilage part of the 12th rib is half the length of the bony part. (H) The length of the 12th rib is almost equal to the length of the 11th rib and the length of the cartilage segment is equal to the bony part of the 12th rib in EEneo mouse. (I,J) Lateral view of the cervicothoracic transition. (I) Wild-type mouse showing the anterior tuberculum (AT) is on C6 (asterisk) and the first rib is attached to T1. (J) EEneo mouse showing the AT is on C5 (asterisk) instead of C6 and an ectopic rib is developed on C7 and fused to the first rib (arrow).
paraxial mesoderm. This is supported by *Hoxc8* knockout studies in which mice that lack *Hoxc8* show anterior transformations from the seventh thoracic vertebrae through the first lumbar vertebra (Le Mouelllic et al., 1992; van den Akker et al., 2001).

It has naturally been assumed that the underlying cause of these phenotypes has been the absence of the *Hoxc8* protein. However, this cannot be the complete explanation, because in the case of the EEneo and EElox mice the identical phenotypic modifications are found in the face of essentially normal expression levels and limits of anterior expression of *Hoxc8* in 10.0 dpc embryos. We propose that the delay of activation and forward spreading of *Hoxc8* at early developmental stages in the mutant embryos account for the morphological aberrations in the vertebral column. The importance of strict control of transcriptional timing of Hox gene expression has been reported previously (Castelli-Gair and Akam, 1995; Gérard et al., 1997; Zákány et al., 1997). In the case of transcriptional regulation of *Hoxd11*, expression of *Hoxd11* for a few hours early or late leads to anterior or posterior transformation in the lumbosacral region, respectively. This suggests that the function of the gene was required prior to morphogenesis (Gérard et al., 1997; Zákány et al., 1997). The correlation between the temporal delay of *Hoxc8* expression and the skeletal phenotypes in *Hoxc8* early enhancer knockout mice support this view. In EEneo embryos, the onset of *Hoxc8* expression is delayed for at least 12 hours, and the forward spreading of the expression domain as well as the establishment of an anterior boundary in the paraxial mesoderm is delayed by 1 day. The temporal delay of *Hoxc8* expression is associated with anterior transformations along the trunk region with high penetrance, similar to that found in *Hoxc8* gene knockout mice. EElox embryos show activation of *Hoxc8* expression at 8 dpc and their expression domain is restricted to the most posterior region of the embryos without forward extension. However, at 8.5 dpc the expected expression pattern is re-established. Even this slight temporal delay is sufficient to induce anterior transformations of the seventh and the eighth thoracic vertebrae. This indicates that the morphological identity of T7 and T8 are determined around 8-8.5 dpc. As the chondrification of ribs and thoracic vertebrae starts around 13 dpc (Rugh, 1990), our data are in agreement with previous findings that the functional expression of Hox genes is required 5 days before vertebra formation (Zákány et al., 1997). Our results also show that even though the correct anterior boundary of *Hoxc8* is established later, it could not rescue the aberrant regional identities induced by an *Hoxc8* deficit in the presomitic mesoderm. Taken together, our data indicate that transcriptional activation of *Hoxc8* at precise times prior to somite condensation are crucial for establishing thoracic identities.

Our results are similar to those reported by Duboule et al. in which an enhancer (RVIII) located between *Hoxd10* and *Hoxd11* was deleted by stem cell targeting (Zákány et al., 1997). Similarities include (1) an enhancer of several hundred bp highly conserved in mammals, birds, and fishes, (2) reduction in the activation of the *Hoxd* genes in early embryos, (3) restoration of normal *Hoxd* expression both in terms of level and anterior limits at later stages of embryogenesis, (4) phenotypic modification in the axial skeleton similar to those reported for *Hoxd10* and *Hoxd11* knockouts, and (5) increased penetrance of expression in animals carrying a neo cassette compared with lox animals.

The similarity in the properties of the RVIII and EE enhancers prompted us to make sequence comparisons between the two. Sequence analysis shows similarities within an upstream region of about 200 bp (Fig. 6A). There are two known protein-binding motifs (CDX and HOX) that show complete sequence identity, two Forkhead/SRY pairs that differ by only a few base pairs, congruence in serial order of the four protein-binding sites, and highly similar spacing relationships differing by only one base pair out of a total of 76 (Fig. 6A,B). This upstream region is also highly conserved for both RVIII and EE between mammals, birds and fish. The degree of expression and sequence similarity between RVIII and EE suggests the existence of a common, highly conserved and primitive mechanism of Hox gene regulation operating in
different Hox clusters and paralogy groups, and possibly reflective of a broadly distributed mechanism associated with other Hox genes. We consider it likely that there is a conserved system of signals that serve to deploy the Hox genes in their proper temporal and spatial patterns. It would be sensible if signals were to activate the early expression phase of Hox gene expression. The presence of CDX elements in RVIII and EE enhancers is moreover consistent with early activation, as CDX is activated early, prior to Hox expression in a gradient fashion with highest activity in the tailbud and lowest activity cranially.

Another example of the temporal delay of Hox expression in early embryos was reported in in vivo deletion of a retinoic acid response element (RARE) 3’ of Hoxa-1 (Dupé et al., 1997). Hoxa1 3’ RARE knockout mice shows (1) a delay of forward spreading of Hoxa1 expression domain in early stages, (2) re-establishment of anterior boundary and downregulation of posterior expression domain of Hoxa1 at later stages, and (3) mild hindbrain and cranial nerve defects when compared with the Hoxa1 gene knockout mice. These results are similar to those observed in EE deletion mice, suggesting that RA is also important for setting the correct timing of Hox activation. Bel-Vialar et al. (Bel-Vialar et al., 2002) demonstrated that in the chicken neural tube, initiating the expression of 3’ Hox genes rely on RA, but not FGF/CDX signaling pathway, whereas 5’ Hox genes rely on FGF/CDX, but not RA signaling pathway. This indicates that the initiation of the expression of different subgroups of Hox genes may depend on a common cis-regulatory mechanism via different trans-factors such as RA and FGF/CDX. In a recent report, Gilthorpe et al. (Gilthorpe et al., 2002) make a similar argument for a distributed system regulating Hox early expression and point out a similarity between control elements in Hoxb4 and Hoxc8.

Comparison of EElox and EEneo mutations
It is of interest to compare the EElox and EEneo mutations in terms of their similarities and differences as tabulated in Table 1 and Figs 2-5. First, both mutations induce a number of identical phenocopies: C7 to T1, T7 to T6, T8 to T7 and L1 to T13. However, the penetrance of expression is significantly greater for EEneo than EElox in all instances and especially so for L1 to T13. In five cases, segmental transitions are found for EElox, but not for EEneo: C5 to C6, T9 to T7, T12 to T10, T11 to T10 and T12 to T11. It should be noted that transitions observed for EEneo, but not for EElox, are novel in that they do not correspond to phenocopies for Hoxc8 null. Moreover, we show that the EEneo mutation exerts a greater temporal delay than does EElox.

How might these similarities and differences be explained? The neo gene is fully functional transcriptionally with necessary coding and non-coding control elements. Thus, one explanation might be a competition between the neo gene and neighboring gene(s) for transcription factors (Olson et al., 1996; van der Hoeven et al., 1996). We think this is unlikely for the following reasons: (1) although Hoxc8 expression is retarded, it ultimately expresses at control levels; and (2) some phenotypes are observed that do not correspond to Hoxc8 null phenocopies. Although not yet proven, we speculate that the observed phenomena can most likely be explained in terms of chromatin configuration modifications in the Hoxc8 and surrounding genomic domains. This interpretation argues that EElox imparts a minimal chromatin distortion that is then corrected within a short period of time by unperturbed chromatin elements in the immediate vicinity. We have shown previously that the EE is highly conserved at a 95% nucleotide sequence level when compared with representative species of the orders of mammals, indicating its critical functionality (Shashikant et al., 1998). We have also shown that throughout the upstream 5’ region there are numerous elements that show lower, but still significant levels of nucleotide sequence
Comparisons between Hoxc8 null, EEneo, and EElox mutations

It is of interest to compare our EE deletion mutations with Hoxc8 null mutants reported previously by others (Fig. 7). Mid-thoracic anterior homeotic transitions (T7 to T6 and T8 to T7) are associated with all three mutant types, suggesting that the expression of Hoxc8 at an early time is required for the support of normal patterning. More posterior thoracic transformations (T9 to L1) are associated with Hoxc8 null and EEneo deletion mutations, and only minimally so with EElox deletions. We posit that normal patterning in this region is also dependent on Hoxc8 expression, but at a later time point. It is unlikely that Hoxc8 by itself can mediate normal patterning of both mid- and posterior thoracic somites. Rather, we believe, an interaction of Hoxc8 with factors specific for mid and posterior thoracic somites is essential. This implies that the mid-thoracic factor (protein interaction/gene activation or suppression, etc.) is available only for interaction with Hoxc8 during an early time period, while the postulated posterior thoracic factor is crucially available at a later time.

The cervical anterior transitions are especially interesting, as Hoxc8 is not normally expressed in this region. This fact, together with the observation that Hoxc8 null mutations are not associated with cervical transitions, suggests that Hoxc8 expression itself may not be causal in these transitions. Alternatively, these transitions may be attributed directly to the loss of the EE, possibly through long range chromatin effects. The T9 to T7 transition might also be explained in this way, particularly so, as it is associated with the posteriorization of the Hoxc9 boundary by one somite.

In summary, we conclude the following: (1) normal patterning of mid and posterior thoracic vertebrae is dependent on the early expression of Hoxc8 at appropriate times and boundary positions; (2) there may exist two distinct critical time points early and somewhat later that govern normal patterning in mid- and posterior thoracic vertebrae, respectively; and (3) expression of Hoxc8 at later time points is crucial for neural, but less so for axial developmental, as EEneo and EElox mutations do not seriously affect neural development, save for the limb clasping phenotype, whereas Hoxc8 null mutations are associated with serious neural defects (Le Mouellic et al., 1992; Tiret et al., 1998).

Cis- and trans-regulation of Hoxc8

We have shown that the EE is responsible for setting up the correct expression patterns of Hoxc8 in both initiation and maintenance phases. In the initiation phase, the EE is required for regulating Hoxc8 activation. Belting et al. (Belting et al., 1998) proposed that in the initiation phase of Hoxc8 expression (8.5 dpc), the EE responds to an inductive signal emanating from the posterior tip of the embryo in a planar fashion that drives the forward spreading of Hoxc8. Recent studies on the coordination of segmentation clocks and Hox gene activation shed light on the possible mechanisms of Hoxc8 regulation. These studies suggest a two-step mechanism for precise control of Hox gene expression in the somites. In the first step, there is an increase in Hox cluster accessibility for transcriptional activation in presomitic cells during gastrulation. In the second step, strong bursts of Hox expression are activated by the segmentation clock in the presomitic mesoderm cells about to transit to somitic cells. The activation of Hox genes in the presomitic cells thus establishes the anterior boundaries of Hox expression domains and marks the morphological fate of each somite (Dubruille et al., 2001; Tabin and Johnson, 2001; Zákány et al., 2001). Given the fact that the EE is important for the appropriate temporal control of Hoxc8 activation, it is likely that it responds to clock signals mediating the timing of Hoxc8 expression in the presomitic mesoderm.

Alternatively, the EE may regulate Hoxc8 activation through a FGF/CDX signaling pathway. Studies in Xenopus and chick show that FGFs directly mediate early Hox expression, and that the effect of FGFs on Hox regulation is mediated by CDX.
protein (Pownall et al., 1996; Isaacs et al., 1998; Bel-Vialar et al., 2002). As we stated in the previous discussion, the presence of CDX binding motifs in both Hoxd11 RVIII and Hoxc8 EE enhancers suggest CDX may direct the regulation of Hox expression via their cis-regulatory elements. Indeed, the ability of the EE to drive a reporter gene expression in mesoderm and neural tube is completely negated when mutations are introduced at both CDX-binding sites within the EE. (Shashikant and Ruddle, 1996). These results are consistent and neural tube is completely negated when mutations are introduced at both CDX-binding sites within the EE. (Shashikant and Ruddle, 1996). These results are consistent with the observation that in the EE deleted embryos, the expression of Hoxc8 is delayed for a certain period of time. Taken together, these data suggest that CDX transduces FGF signaling by directly interacting with the EE thus regulating Hoxc8 activation and expression.

Finally, the EE may be also important for opening and maintaining a chromatin configuration that is active for transcription by directly binding with chromosomal remodeling factors. In previous studies, we have identified proteins that bind to specific sequences in the 5’ region of the EE using the yeast one hybrid methodology. One of these proteins, BEN (binding to early enhancer) contains six helix-turn-helix domains located in separate exons, a leucine zipper motif and a nuclear localization signal (Bayarsaihan and Ruddle, 2000). The structural properties of BEN are consistent with its role as a transcription factor. BEN is a member of the TFII-I gene family. BEN and TFII-I map side by side within the genome (human chromosome 7q 11.23) and have undoubtedly arisen from a precursor gene by unequal crossing over and mimic a high level of functional and structural similarities. In a recent report, we have shown that TFII-I interacts functionally with histone deacetylase 3 and has the capability of modifying chromatin structure thus enabling access by control factors to enhancers and promoters (Tussie-Luna et al., 2002). We have also shown that BEN is dynamically regulated during early development and is expressed from early cleavage stages through fetal development in a broad spectrum of tissues with both cytoplasmic and nuclear localization (Bayarsaihan et al., 2003). The fact that BEN and TFII-I have the capability to bind to the EE and modify its chromatin structure lends credence to the concept that the activation of the Hoxc8 gene may proceed through distinct stages that relate to the previously described early activation and late maintenance phases of Hox gene regulation (Deschamps and Wijgerde, 1993; Deschamps et al., 1999).

We thank K. Rajewsky and T. Williams for their kind gifts of plasmids and ES cells. We are grateful to J. Deschamps, S. Holley and K. Sumiyama for carefully reading the manuscript and providing helpful comments. This work was supported by NIH grant GM09966

References


