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

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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 *Hoxc8* 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

mutants, *Hoxd11* is activated earlier while *Hoxd4* is activated by retinoic acid (RA) prematurely (Bel-Vialar et al., 2000). 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., 1997; 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 (Bayarsaihan 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 gene 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 *Sph*I-*Spe*I 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 neoflox-8 (gift of K. Rajewsky) and inserted into the *Spe*I-*Hin*dIII sites of 5TA to give 5NlTA. A 3.4 kb fragment containing the 2.2 kb 5′ genomic fragment and the loxP flanked neo cassette is then excised from 5NlTA and inserted into the *Sal*I-*Cla*I sites of a vector containing two copies of the HSV-tk cassettes (gift of T. Williams) to generate 5NeolT. Finally, a 1.8 kb genomic fragment homologous to the 3′ region flanking the EE is cloned into the *Not*I-*Cla*I sites of 5NeolT to generate the targeting vector EENeolxTK (Fig. 1B). For gene targeting, 10^7 CJ-7 embryonic stem (ES) cells (gift of T. Williams) are electroporated with 25 μg *Apa*LI 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 5ee1 (5′-CTG CGT TCT CTT CCC CAG CGC AAC TG-3′) and 5neo2 (5′-CAG CGA TCG CCT TCT ATC GCC TTC-3′); and 3neo1 (5′-CCT CTT GCA AAA CCA CAC TGC TCG AC-3′) and 3sb3r (5′-ACC CCA CAT CCT GAG GTT TGC 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, 10^7 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′-ACA CAC AGC TGG GGA GAG AAA TGA AAG C-3′, and 3neo2 5′-GCA CAG TTT ATT TCC GCT GCT GCC TGC-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

Germline 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 postcoitum (dpc) embryos. Subsequent genotyping is determined by PCR. Primer pair 5neo1 and 3neo2 is used in PCR reaction. The combination of 5neo1 and 3neo2 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 96% ethanol 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/matsearch.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 1. Penetrance (%) of skeletal defects in EEneo and EElox mutant mice

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

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.

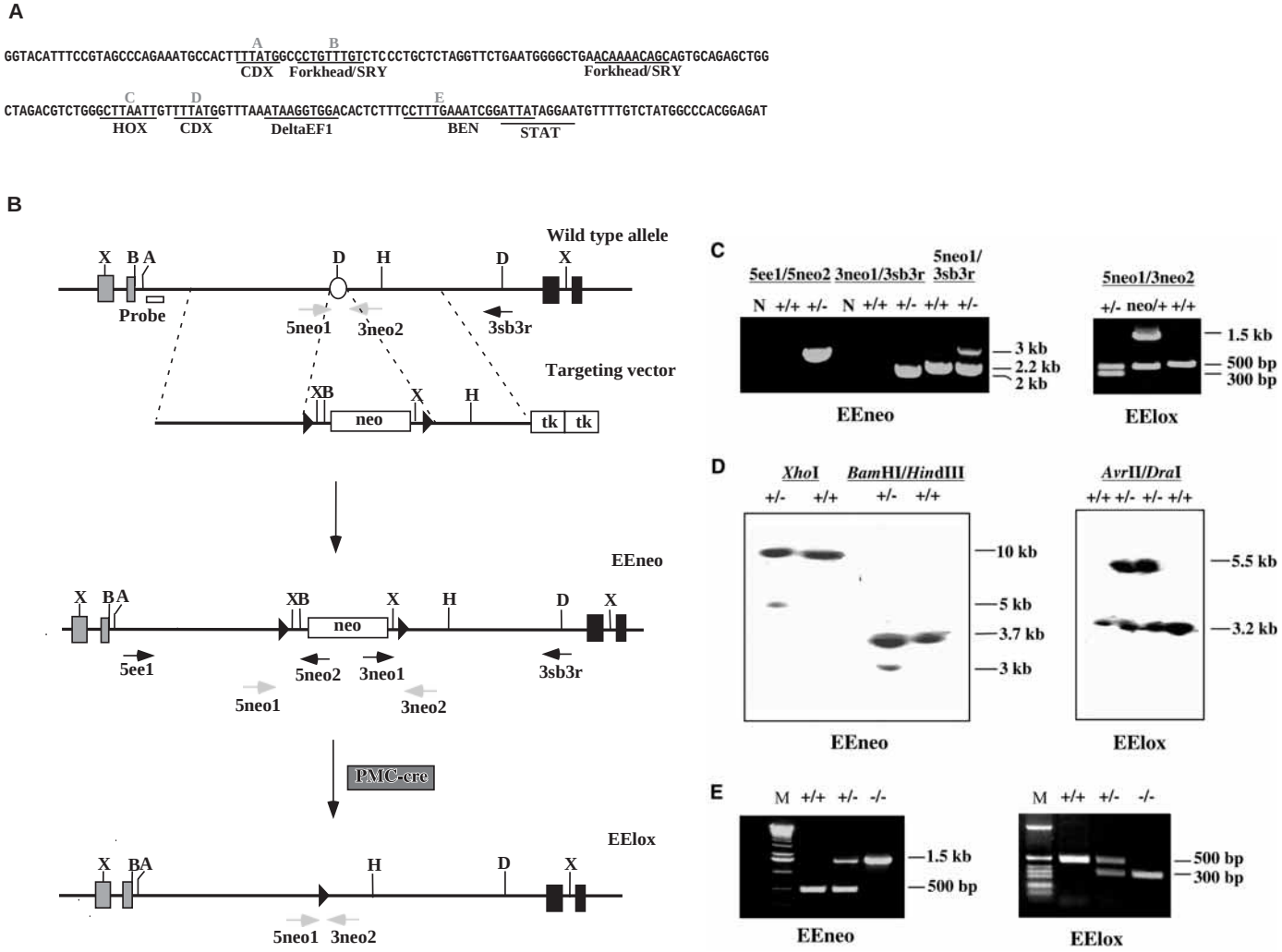

Fig. 1. *Hoxc8* early enhancer sequence and targeted disruption/deletion of *Hoxc8* early enhancer locus. (A) The 200 bp region of the EE. Sequences underlined represent putative transcription factor binding sites. Cis-acting elements A-E are identified by mutational analysis of reporter genes (Shashikant 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, *Avr*II; B, *Bam*HI; D, *Dra*I; H, *Hin*dIII, X, *Xho*I) 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). *Xho*I digestion gives a 5 kb fragment for EEneo allele, and a 10 kb fragment for wild-type allele. *Bam*HI and *Hin*dIII digestion gives a 3 kb fragment for EEneo allele, and a 3.7 kb fragment for wild-type allele. *Avr*II and *Dra*I 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 a 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).

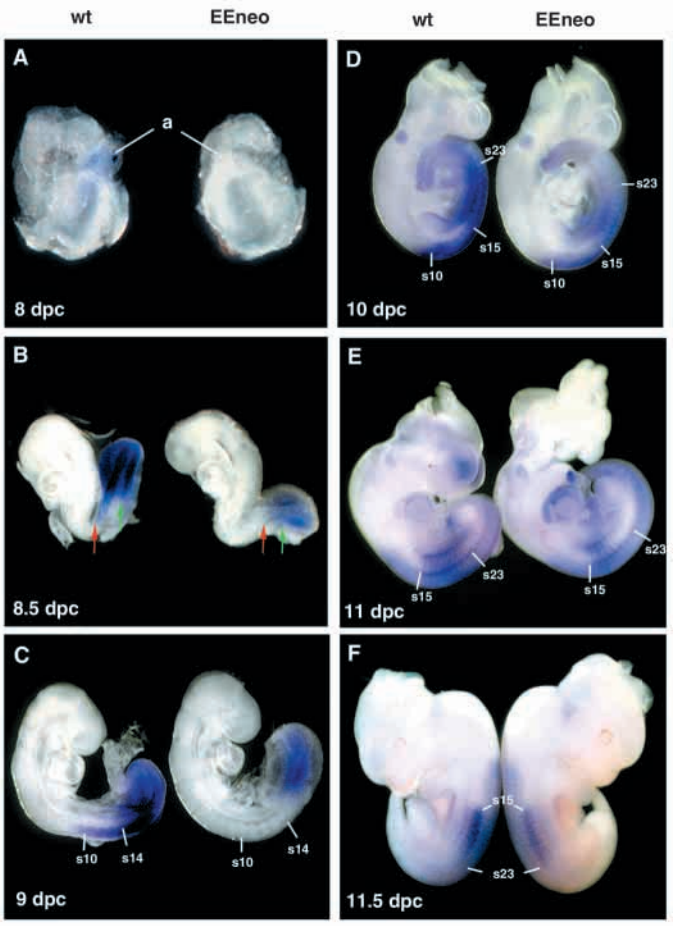

Fig. 2. *Hoxc8* expression in the wild-type and EEneo mouse embryos at different developmental stages by whole-mount in situ hybridization. (A) 8.0 dpc embryos. Expression of *Hoxc8* is detected in the allantois (a) and in the posterior region of the wild-type (wt) embryo. No *Hoxc8* transcript is detected in EEneo embryo. (B) 8.5 dpc embryos with ~10-12 somites. EEneo embryo shows delay of *Hoxc8* expression in both neural tube (red arrow) and paraxial mesoderm (green arrow) when compared with the wild-type embryo. (C) Embryos (9.0 dpc) with ~15-17 somites. In the wild-type embryo, the anterior boundaries of *Hoxc8* expression in the neural tube and somite are established in the level of the 10th and 14th somites (s10 and s14), respectively. In EEneo embryo, expression of *Hoxc8* is still restricted to the paraxial mesoderm. Note that although the anterior limit of *Hoxc8* expression domain in the neural tube is established correctly in EEneo embryo, the expression level is very low and can only be seen under the microscope. (D) 10.0 dpc embryos with ~25-27 somites. *Hoxc8* expression domain within the somites is from s15 to s23 in the wild-type embryo. In EEneo embryo, although the anterior boundaries of *Hoxc8* expression domains are established in both neural tube and somite, the posterior boundary is anteriorized to s21. (E) The same somite expression pattern is observed in 11.0 dpc EEneo embryo. (F) At 11.5 dpc, the expression domains of *Hoxc8* within both neural tube and somite are comparable in the wild-type and EEneo embryos. Note that the expression level at s22 and s23 is stronger in EEneo embryo.

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).

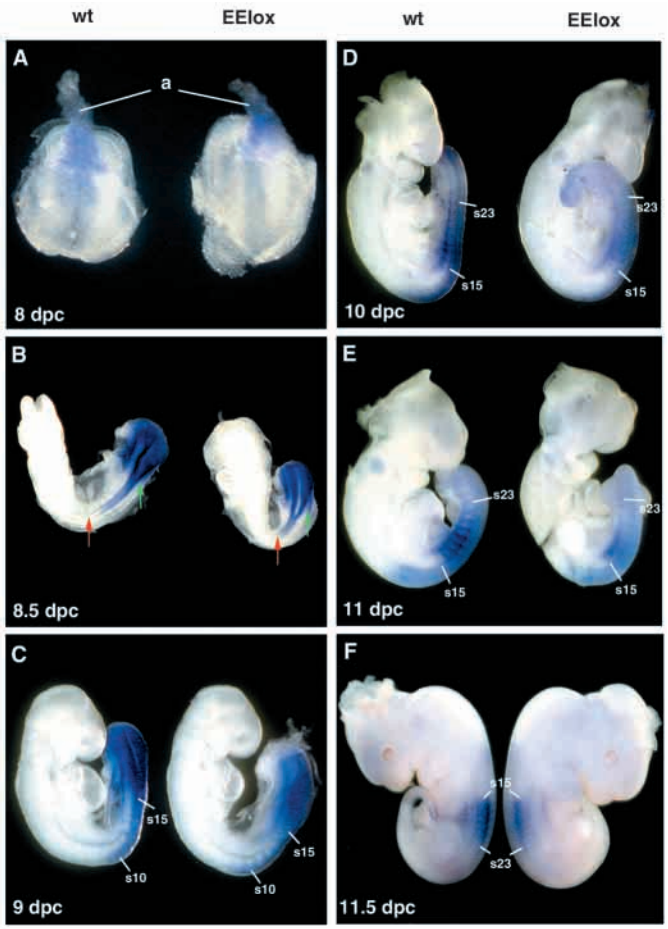

Fig. 3. *Hoxc8* expression in the wild-type and EElox mouse embryos at different developmental stages visualized by whole-mount in situ hybridization. (A) Embryos at 8.0 dpc. Expression of *Hoxc8* is detected in the allantois (a) and in the posterior region of the wild-type and EElox embryos. Note that the expression domain in EElox embryo does not spread forward to more anterior regions when compared with the wild-type embryo. (B) Embryos with ~10-12 somites. The expression patterns of *Hoxc8* in both neural tube (red arrow) and paraxial mesoderm (green arrow) are identical in the wild-type and EElox embryo. (C) 9.0 dpc embryos with approximate 16-18 somites. In both wild-type and EElox embryos, the anterior boundaries of *Hoxc8* expression in the neural tube and somite are established at s10 and s14, respectively. (D) 10.0 dpc embryos with ~24-26 somites. The anterior boundaries of *Hoxc8* expression are identical in both neural tube and somite in the wild-type and EElox embryos. Nevertheless, the posterior boundary of the somite expression domain is anteriorized to s21 in EElox embryo. (E) The same somite expression pattern is observed in 11 dpc EElox embryo. (F) At 11.5 dpc, the expression domains of *Hoxc8* within both neural tube and somite are comparable in the wild-type and EElox embryos.

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

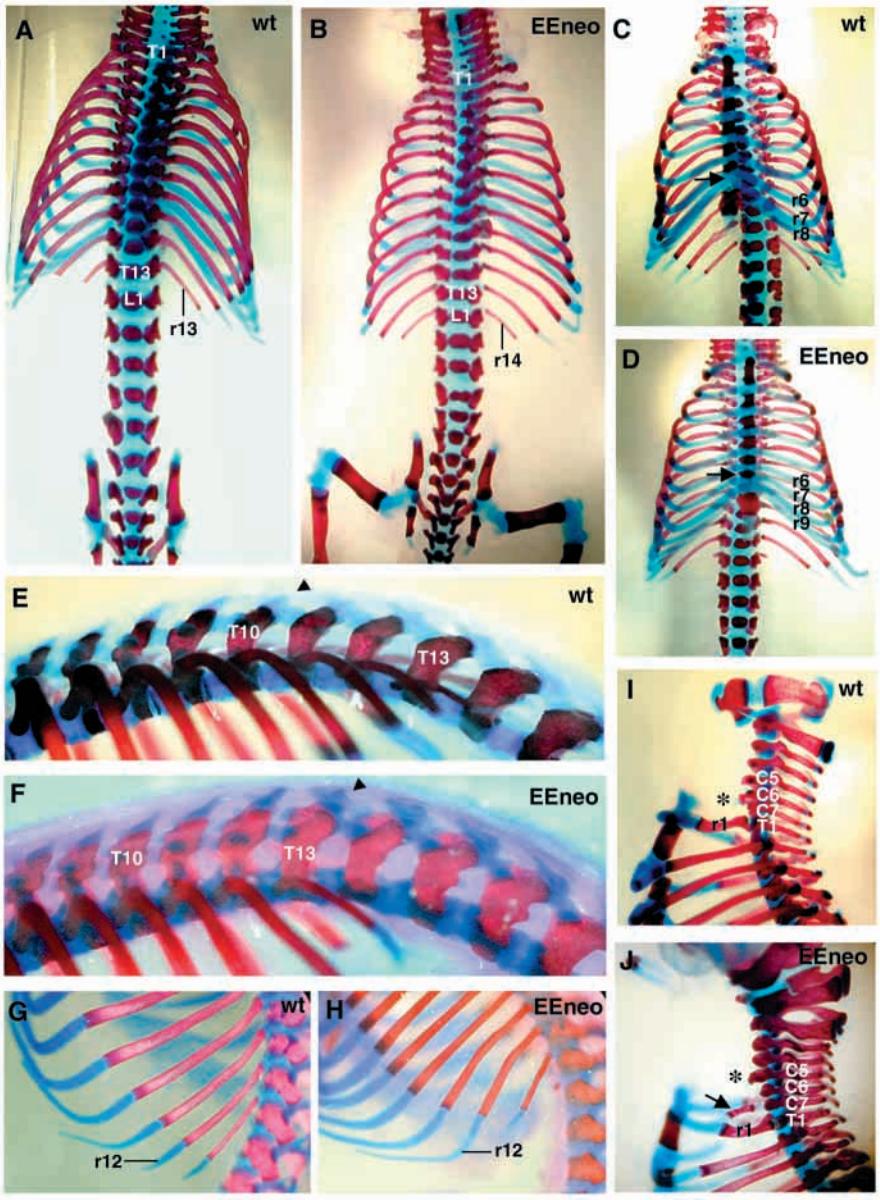

Fig. 4. Morphological alteration of the axial skeletons in newborn EEneo mice. (A,B) Dorsal view of the thoraco-lumbar regions. (A) Wild-type animal showing 13 thoracic vertebrae and 6 lumbar vertebrae. (B) EEneo mouse showing a fully developed extra pair of ribs on L1. (C,D) Ventral view of the thorax. (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).

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 Mouellic 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

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 Mouellic 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.

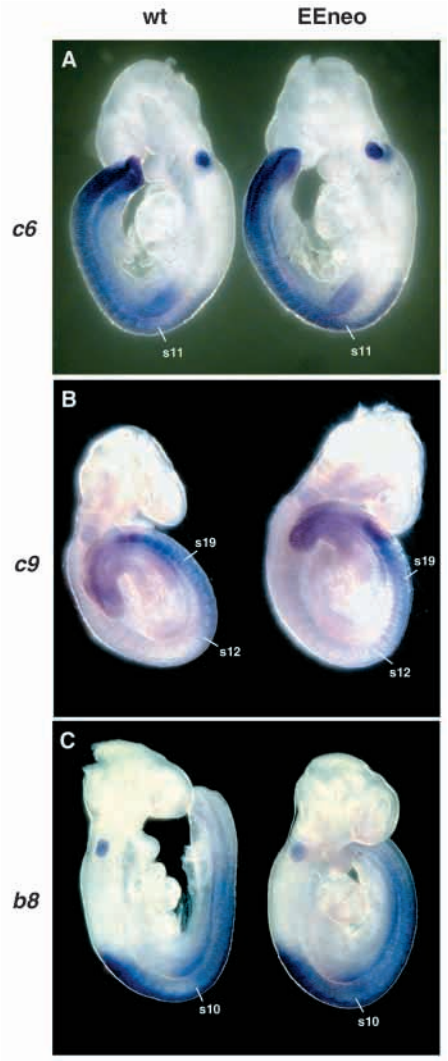

Fig. 5. (A-C) Comparison of the expression pattern of *Hoxc6* (A), *Hoxc9* (B) and *Hoxb8* (C) in 9.5 dpc wild-type and EEneo embryos by whole-mount in situ hybridization. (A,C) The expression patterns of *Hoxc6* and *Hoxb8* are identical in the wild-type and EEneo embryos. (B) The anterior boundary of *Hoxc9* expression in the somites is at the level of s20, one somite posterior when compared with the anterior level of *Hoxc9* in the wild-type embryo.

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 enchancers 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*.

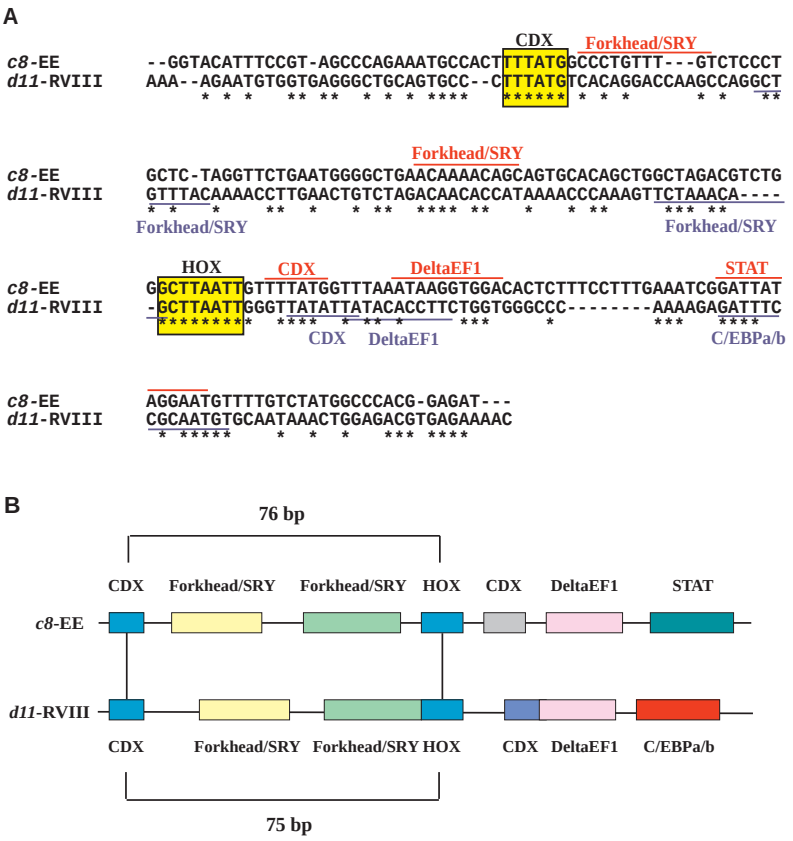

Fig. 6. Sequence similarity between the EE and *Hoxd11* RVIII. (A) Nucleotide sequence comparison of 200 bp *Hoxc8* EE and 276 bp *Hoxd11* RVIII region. The yellow shaded boxes represent 100% identical sequences of CDX and HOX binding motifs within the two enhancers. Red and blue underlines indicate the putative protein binding motifs within the EE and *Hoxd11* RVIII, respectively. Stars indicate conserved nucleotides. (B) Schematic comparison of the EE and *Hoxd11* in relationship to the order of the putative transcription factor binding motifs. Blue boxes indicate identical sequences of CDX and HOX-binding motifs within the two enhancers. Note that the spacing between these two binding motifs in the EE and *Hoxd11* RVIII differs by only one base pair out of a total of 76. The yellow and green boxes represent the first and second Forkhead/Sry within the two enhancers, respectively. These two Forkhead/SRY pairs within each enhancer also show high sequence similarities and differ by only a few base pairs.

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 EEneo, but not for EElox.: 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

conservation that may also be critical functional control elements and serve to buffer effects produced by EElox and EEneo (Belting et al., 1998). Thus, in the case of EEneo, we posit that both the loss of EE and the adjacent presence of neo distorts the chromatin configuration maximally, and this results in the increased penetrance of *Hoxc8* null phenocopies and in addition spreads to more distant regions to allow the induction of non-*Hoxc8* null phenocopies. We believe this hypothesis is supported by and consistent with our previous findings that the Hox complexes are devoid of lines and sines that are reported to perturb chromatin structure and which are highly prevalent elsewhere in the genome (Kim et al., 2000).

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

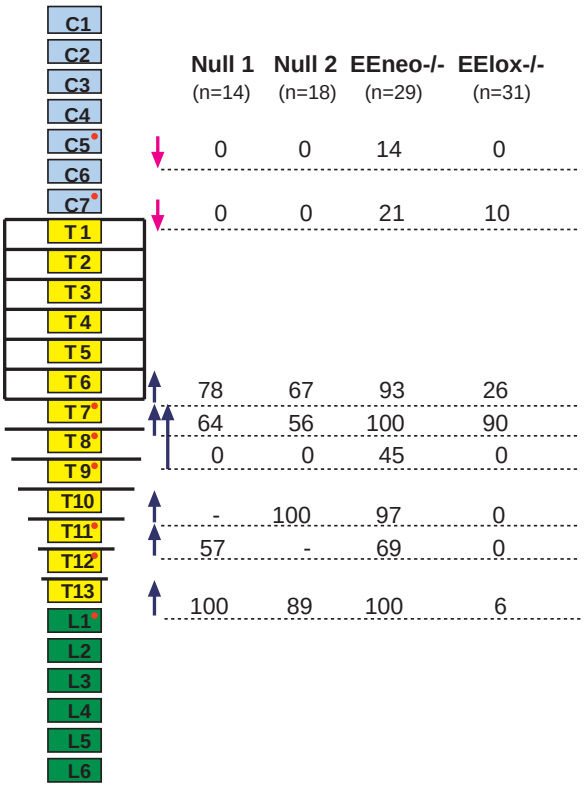

Fig. 7. Comparisons of the skeletal phenotypes between *Hoxc8* null, EEneo and EElox mutations. Cervical, thoracic and lumbar vertebrae are shown as blue, yellow and green blocks, respectively. The transformed vertebrae are indicated by red dots. Posterior and anterior transformation are represented by pink and blue arrows. Table represents penetrance (%) of vertebral column defects in *Hoxc8* null, EEneo and EElox animals. Data of null 1 and null 2 were obtained from Le Mouellic et al. (Le Mouellic et al., 1992) and van den Akker et al. (van den Akker et al., 2001), respectively.

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 (8-8.5 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 (Dubrulle 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 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 evince 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).

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