Evolutionary conserved sequences are required for the insulation of the vertebrate Hoxd complex in neural cells

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SUMMARY

Transcriptional regulation of vertebrate Hox genes involves enhancer sequences located either inside or outside the gene clusters. In the mouse Hoxd complex, for example, series of contiguous genes are coordinately controlled by regulatory sequences located at remote distances. However, in different cellular contexts, Hox genes may have to be insulated from undesirable external regulatory influences to prevent ectopic gene activation, a situation that would likely be detrimental to the developing embryo. We show the presence of an insulator activity, at one extremity of the Hoxd complex, that is composed of at least two distinct DNA elements, one of which is conserved throughout vertebrate species. However, deletion of this element on its own did not detectably affect Hoxd gene expression, unless another DNA fragment located nearby was removed in cis. These results suggest that insulation of this important gene cluster relies, at least in part, upon a sequence-specific mechanism that displays some redundancy.

Key words: Insulator, Gene regulation, Hox complex, Mouse

INTRODUCTION

During vertebrate development, proteins encoded by the Hox gene family are required to properly instruct cells about their morphological fates, subsequently leading to the emergence and organisation of different structures along the body axis. Mammals have 39 Hox genes, clustered at four genomic loci, which provide these organisational cues to a variety of embryonic axial structures and derivatives. Accordingly, the transcription of these genes must be precisely regulated in time and space, in order to ensure harmonious development. This complex task appears to rely partially upon the genomic organisation of the genes, as a correspondence exists between gene order along the clusters and their spatial and temporal sequences of transcriptional activation (reviewed by Krumlauf, 1994). Although the molecular mechanisms that underlie this phenomenon are not yet fully understood, they may involve high order regulation, such as (for example) a transition in chromatin configuration (Deschamps et al., 1999; Kmita et al., 2000b).

Beside this level of transcriptional regulation, many cis-acting control sequences have been characterised by their ability to impose particular expression patterns to nearby located genes. Various enhancer sequences have thus been described, with distinct functional properties. For example, in several cases, gene-specific activation was shown to result from proximal enhancers selectively interacting with a given promoter. Alternatively, enhancer sharing mechanisms were reported to account for the co-expression of neighbouring genes (Sharpe et al., 1998), a situation favoured by the tight clustered organisation of these genes (Bell et al., 2001). Enhancer sharing processes, within Hox gene clusters, were not only shown to involve proximal enhancers, which can control the expression of neighbouring genes in the same tissue, but also more global, distally located enhancers, which are able to impose a particular regulation to series of contiguous genes. Examples of such a large-scale regulation was provided by the co-expression of several Hoxd genes in either the intestinal hernia or the developing digits (Zakany and Duboule, 1999; Kmita et al., 2000a; Spitz et al., 2001). In these latter cases, co-ordinated expression of several genes at the same place was demonstrated to be necessary to properly build up the concerned structure (Zakany et al., 1997a).

However, this particular regulatory strategy implies that other closely linked gene members of the cluster, the function of which may not be relevant in a given structure, are protected against such a global regulatory influence, such as to prevent their mis-expression. Indeed, ectopic transcription of Hox genes was shown be a potential source of severe morphological and/or physiological alterations (Knezevic et al., 1997; McLain et al., 1992; Morgan et al., 1992; Rijli et al., 1994; Yokouchi et al., 1995). Accordingly, boundary or insulator elements must exist to restrain the action of enhancers specifically to those relevant target genes, by isolating them from their neighbours (Sun and Elgin, 1999; Udvardy, 1999). We have previously showed that a DNA segment located between Hoxd12 and Hoxd13 could prevent both genes from responding to a distally located intestinal hernia enhancer (Kmita et al., 2000a). In
much the same way, Hox clusters must themselves be isolated from external regulatory influences to prevent enhancer sequences that are necessary for closely located, non-Hox genes, to interfere with the precise and particular regulation of sequences that are necessary for closely located, non-Hox genes to respond to these CNS enhancers, in the wild-type context; Hoxd13 promoter lies close to the Hoxd13 complex, (D’Esposito et al., 1991; Bastian et al., 1992).

Evx2 indeed displays specific expression features that are not shared by any Hox genes, not even by Hoxd13, whose promoter lies close to that of Evx2. This is best illustrated by discrete cell types of the developing central nervous system, in both spinal cord and more rostral parts of the brain, in various vertebrate species (Bastian et al., 1992; Brulfert et al., 1998; Dollé et al., 1994; Sordino et al., 1996). In the spinal cord, transcripts are localised in the ventrally located V0 interneurones, as well as in a population of dorsal interneurones (Moran-Rivard et al., 2001). In the developing brain, Evx2 expression is detected in the rhombencephalic isthmus area (the metencephalic-mesencephalic transition) and extends into the superficial layer of the entire midbrain. It is also expressed in the developing hindbrain and in part of the future cerebellum (Dollé et al., 1994).

While the enhancer sequences driving Evx2 expression in the CNS have not yet been precisely identified, experiments involving targeted genomic rearrangements around the Evx2 locus have revealed some of their properties. First, targeted deletions have shown that these enhancer sequences are located at a remote position, upstream the Hoxd complex (Kondo and Duboule, 1999). Second, we showed that a Hoxd9/lacZ transgene was able to respond to the Evx2 CNS-specific enhancer sequences, whenever it was relocated upstream the Hoxd complex, 3’ to Evx2 (Kondo and Duboule, 1999). However, the same transgene was unable to respond similarly when placed within the complex, even when positioned immediately next to the Evx2 promoter (van der Hoeven et al., 1996). These results demonstrated that the Evx2 CNS enhancers had a weak specificity for Evx2 itself, i.e. they were able to interact with other promoters. In addition, Hoxd promoters could respond to such regulatory controls provided they were relocated in the proper genomic environment, i.e. in 3’ of the Evx2 transcription unit. These observations raised the question of which mechanism could prevent Hoxd genes to respond to these CNS enhancers, in the wild-type context. In other words, why a promoter able to respond to a given regulatory sequence, when placed outside the cluster, was unable to do so from within the Hoxd complex, even when localised right next to the Evx2 promoter.

In this set of experiments, we looked for potential sequences, located between Evx2 and the Hoxd cluster, that would be able to isolate this latter cluster from the surrounding regulatory influences. We show that an evolutionary conserved DNA stretch participates in the insulation of the cluster, as revealed by novel genomic rearrangements in this locus. However, even though this sequence was sufficient to ensure proper insulation of the cluster, additional sequences, located nearby, were also found to be involved in this process. The requirement for a combined deletion in cis of these sequences in order to bypass the insulation of the cluster, raised the possibility that some functional redundancy exists between these regulatory sequences.

MATERIALS AND METHODS

Targeted deletion of region XII

Targeted deletion of RXII was engineered by homologous recombination in ES cells. A 1.2 kb AvrII DNA fragment containing RXII was deleted from the 9.5 kb NotI fragment that covers the entire Evx2 to Hoxd13 intergenic region. A PGK-neomycin selection cassette, flanked by loxP sites, was inserted at the NotI site, as described previously (Hérault et al., 1996; van der Hoeven et al., 1996). The resulting targeting vector was electroporated into D3 ES cells. Clones in which homologous recombination had occurred were selected, amplified and injected into mouse embryos. After germline transmission, the HoxdRXII-neo line of mice were obtained and further crossed with partners carrying the CMV-Cre transgene in order to produce the HoxdRXII line of mutant mice that lacked the PGK-neomycin selection cassette.

Recombined lines

Besides the HoxdRXII line, all mutant lines analysed in this work were produced via trans-allelic meiotic recombination (TAMERE) (Hérault et al., 1998b; Kmita et al., 2002). Each allele was obtained in the progeny of ‘trans-loxer’ animals, i.e. males hemizygous for the Sycp1-Cre transgene and trans-heterozygous for different Hox alleles carrying a loxP site at given positions within the Hoxd cluster (indicated in Fig. 4) (Kmita et al., 2002). In particular, Hoxd(del(13)) animals were obtained by combining a Hoxd allele carrying a loxP site between Evx2 and Hoxd13 (the EvDGE3 allele) (Hérault et al., 1996), with an allele carrying a loxP site between Hoxd12 and Hoxd11 (HoxdRXII) (Hérault et al., 1998a). Hoxd(del(13-12)) and Hoxd(del(13-11)) animals were obtained in a similar way, although in these latter cases, the EvDGE3 allele was combined either with HoxdRXII in which a loxP site had been inserted between Hoxd12 and Hoxd11 (Beckers et Duboule, 1998), or with HoxdRXII, containing a loxP site between Hoxd11 and Hoxd10 (Gérard et al., 1996). HoxdRXII(del(13-12)) mice were obtained in the progeny of trans-loxer, which were trans-heterozygous for HoxdRXII and HoxdRXII. Finally, HoxdRXII(del(13-12)) mice were produced through trans-loxer animals trans-heterozygous for both HoxdRXII and HoxdRXII alleles. All these novel lines of mice were selected by Southern blot analysis using tail DNA. The frequency of TAMERE was in the range of 5-10%, as reported previously (Hérault et al., 1998b).

Whole-mount in situ hybridisation (WISH) were carried out on 11.5- and 12.5-day-old foetuses, using a standard procedure and previously described probes (Hérault et al., 1996; Kondo et al., 1998).

RESULTS AND DISCUSSION

Targeted deletion of conserved region XII

The Evx2 gene, a mammalian gene orthologous to the Drosophila even skipped gene (eve), is localised about 8 kb upstream of the most posterior gene member of the Hoxd complex; Hoxd13 (Fig. 1) (Bastian et al., 1992). Because its transcriptional orientation is opposite to that of all Hoxd genes, its promoter lies close to the Hoxd13 promoter (Fig. 1; arrows). Even though this homeobox-containing gene does not in the strictest sense belong to the Hox gene family, it shares some important regulatory features with those Hoxd genes located at the ‘posterior’ end of the cluster, such as Hoxd13. During limb development, the timing of Evx2 expression follows that of Hoxd genes and it is eventually co-expressed with 5’-located Hoxd genes in developing digits (Fig. 1A). This shared regulatory feature is dependent upon the action of a remote enhancer sequence located in 5’ of the Hoxd complex (Spitz et
Fig. 1. (A) Insulating activity within the Hoxd13 to Evx2 intergenic region. The posterior extremity of the Hoxd complex is shown, as well as the position of the Evx2 gene. The expression patterns of both Evx2 and Hoxd13 are depicted above to illustrate enhancer sharing in developing digits (right), whereas expression in the central nervous system (CNS) and spinal cord is detected only for Evx2 (left). The transcriptional orientation of this latter gene is opposite to that of all Hoxd genes (arrows). Enhancer sequences driving Evx2 in various domains of the developing CNS are located downstream the gene, i.e. 5' to the Hoxd cluster; hence, an insulating property is expected to lie between the two promoters (red bar). This was further supported by the relocation of a Hoxd9/lacZ transgene at different positions upstream the cluster (B). When relocated between Evx2 and Hoxd13 (right panel) the transgene was expressed in distal limbs but not in CNS. By contrast, when relocated downstream Evx2 (left panel), the transgene was expressed in both distal limbs and CNS, in a way much related to the Evx2 pattern, demonstrating that Hox promoters can indeed respond to these controls, if placed at an appropriate position.

Fig. 2. Identification and targeted deletion of region XII (RXII). (A) Interspecies conservation within Evx2-Hoxd13 intergenic region. Sequence analyses revealed two stretches of significant conservation, referred to as region XII (RXII), which were found to be located within the insulating area (red bar). The position of these two sequences with respect to both Evx2 and Hoxd13 is schematised below for the mouse (m), chicken (ck) and zebrafish (zf). A high sequence similarity was observed between rodents and avian. The sequence conservation with the zebra fish DNA is less obvious, though significant whenever the respective positions of the two stretches are considered. (C) Strategy to delete region XII through targeted mutagenesis. A targeting vector was engineered lacking region XII and was recombined in ES cells to generate the HoxdRXII-neo mice. The selection cassette was further deleted after crossing these mice with a Cre deleter strain, to produce the HoxdRXII mice. In addition to the deletion of RXII, these mice also carried a loxP site at the exact integration site of the transgene shown under A. This loxP site was used for subsequent meiotic recombination approaches, as described in Fig. 4B,C.

expression may have been conserved between these different genomes.

Comparison between Evx2 to Hoxd13 intergenic DNA sequences, obtained from either the murine, the chick or the zebra fish loci, revealed only two stretches of high sequence similarity localised between Evx2 and the Rel0 position (Fig. 2A,B; red bar). In the mouse genome, these two motives are located within a 1.2 kb large fragment, starting about 1 kb upstream from the first exon of Evx2. This region of significant sequence conservation was referred to as region XII (RXII), following previously characterised conserved regions within the Hoxd cluster (Renucci et al., 1992; Beckers and Duboule, 1998; Gérard et al., 1996; Hérault et al., 1998a). While
sequence conservation was high between murine and avian DNAs for both motives (67% identity over 206 nucleotides), it was less conspicuous when compared with the fish DNA, as only short stretches of sequence identity were scored for both motives. In this latter case, however, the core of the second motif was clearly identified in the zebra fish locus and found at the same relative position (Fig. 2A,B). This unambiguously demonstrated the existence, in the zebra fish locus, of at least one of these two blocks of homologies.

In order to assess the function of these two conserved sequences, we deleted them from their native genomic context by homologous recombination in ES cells. We constructed a targeting vector containing the Evx2 to Hoxd13 intergenic region, but in which the 1.2 kb fragment had been deleted (Fig. 2C). After electroporation in ES cells, clones carrying a targeted deletion of RXII were selected and further injected into mouse blastocysts. After germline transmission, the HoxdRXII-neo line of mice was established. In order to prevent regulatory interferences caused by the presence of the PGK-neomycin selection cassette, HoxdRXII-neo animals were crossed with transgenic mice producing the Cre recombinase (CMV-Cre mice) (Dupe et al., 1997) to delete the selection cassette. Therefore, the final genomic configuration of these HoxdRXII mice was a single deletion of the 1.2 kb fragment containing RXII (Fig. 2C), along with the presence of a loxP site. We subsequently obtained HoxdRXII homozygous mice, which were fully viable and fertile.

The expression of several Hoxd genes was examined at various developmental stages, in animals homozygous for the deletion of RXII, but no detectable difference was scored when compared with their wild type or heterozygous littermates. In particular, ectopic expression of Hoxd genes showing an Evx2 related CNS pattern was not observed. This result suggested that the deleted 1.2Kb DNA fragment was not able, on its own, to function as a boundary-like or insulator element, to isolate the Hoxd cluster from the upstream located Evx2 CNS enhancers. Alternatively, the apparent lack of effect of this deletion may illustrate some redundancy in this regulatory process.

**Nested deficiencies of the 5' Hoxd cluster**

Within the 5' part of the Hoxd cluster, several regions of high interspecies conservation were previously identified (Fig. 3) (RVIII to RXI). Each individual region was assayed for potential regulatory function through targeted deletion/mutation (Gérard et al., 1996; Zakany et al., 1997b; Beckers and Duboule, 1998; Hérault et al., 1998a). Although slight variations in Hoxd gene expression were occasionally observed following these targeted modifications, none of them indicated a potential role for these regions, by themselves, to restrict the accessibility of Hoxd promoters to the Evx2 cis-regulatory sequences. In order to look for their possible cooperation in the implementation of an insulating process, we used the targeted meiotic recombination (TAMERE) strategy (Hérault et al., 1998b) to generate novel genomic configurations in vivo through Cre-mediated meiotic recombination between loxP sites carried in trans by homologous chromosomes. In this way, we produced a set of progressive deletions of the 5' end of the Hoxd complex, involving one, two or three gene loci, as well as RXI, RX and RIX/RVIII, respectively (see Kmita et al., 2002).

First, we generated mice containing the SYCP-Cre transgene (Vidal et al., 1998), along with a Hoxd complex carrying, on one chromosome, a loxP site positioned in the middle of the Evx2 to Hoxd13 intergenic region. The other chromosome had a loxP site recombined either upstream Hoxd12, between Hoxd12 and Hoxd11, or upstream Hoxd10. During meiotic prophase, in some male germ cells, recombination occurred between these loxP sites in trans, leading to unequal chromosomal exchanges, thereby producing spermia carrying a deletion of the DNA fragment located in between. In this way, mice were produced which carried different deletions; a 12 kb large DNA fragment covering the Hoxd13 locus (Hoxd(del13) in Fig. 3); a 18 kb large fragment covering both Hoxd13 and Hoxd12 loci (Hoxd(del13-12)), and a 23 kb large fragment encompassing all three Hoxd13, Hoxd12 and Hoxd11 loci (Hoxd(del13-12)) in Fig. 3). The same 5' break point was used to engineer all three deletions, such that increasingly large deletions concomitantly removed either one (RXI), two (RXI and RX) or four (RXI, RX, RIX and RVIII) conserved sequences, respectively (Fig. 3) (Kmita et al., 2002). Homozygous embryos were collected for each configuration and the expression patterns of the remaining 5' Hoxd genes were examined by whole-mount in situ hybridisation. Again, Evx2-like expression in the CNS was not detected in any of these configurations (data not shown). This suggested that sequences responsible, either alone or in combination, for the insulation of the Hoxd cluster were not exclusively located within these 23 kb large DNA fragment containing the Hoxd13 to Hoxd11 loci, if at all present in this fragment.

![Fig. 3. Nested deficiencies of the posterior Hoxd complex, as produced by targeted meiotic recombination (TAMERE).](image-url)
Combining deletions in cis
This set of data demonstrated that none of the engineered deletions that removed unique evolutionary conserved sequences had an effect on the insulation of the Hoxd cluster. It also showed that larger deletions, i.e. those that removed more than one such sequence from the cluster, were equally ineffective in altering this particular mechanism. One remaining possibility that could account for the insulation effect was the presence of an element located between the Rel0 site and Evx2 (Fig. 2; red bar), but outside the 1.2 kb large fragment that contains region XII, as deletion of this fragment had no effect. An alternative explanation is that the combined effect of region XII and other regions included in the series of deletions described above are responsible. We did not favour the first possibility, assuming that such a tight mechanism, present in many vertebrate species, may likely rely upon some sequence specificity. Therefore, we challenged the second possibility by producing multiple deletions in cis.

We used HoxdRXII as a parental allele in targeted meiotic recombination, to engineer novel genetic configurations in which the RXII deletion was combined in cis with larger deletions (Fig. 3). This was made possible by the strategy that was used to delete region XII, which involved the positioning of a selection cassette flanked by loxP sites, within the Rel0 insertion site, i.e. in the middle of the Evx2 to Hoxd13 intergenic region (Fig. 2C). Consequently, mice carrying the deletion of RXII had a loxP site at this position (Fig. 2C; HoxdRXII), as a left over of the Cre-mediated deletion of the PGKneo selection cassette. We first produced males carrying either the HoxdRXII and HoxdRXI alleles (Fig. 4B), or the HoxdRXII and HoxdRX alleles (Fig. 4C), along with the Cre. In the progeny of these trans-loxer males, we isolated both HoxdRXII-del(13) and HoxdRXII-del(13-12) animals, respectively (Fig. 4). Although a strain of HoxdRXII-del(13) homozygous mice could be established, HoxdRXII-del(13-12) animals died at birth. Homozygous embryos of both genotypes could nevertheless be collected to look at the expression of the remaining 5’ Hoxd genes. We first analysed the expression of Hoxd12, Hoxd11 and Hoxd10 in the HoxdRXII-del(13) strain, i.e. mice that lack both region XII and the Hoxd13 locus. In these animals, ectopic activation of Hoxd genes was not detected within the rostral brain or in the spinal cord (not shown), as one would have anticipated from an alteration of the insulating process.

We next looked at the deletion of both RXII and the 18 kb large fragment containing the Hoxd13 and Hoxd12 loci (Fig. 5). In marked contrast to the previous configuration, a robust ectopic expression of both Hoxd11 and Hoxd10 in the anterior CNS was detected in embryos carrying these two deletions in cis. Ectopic expression of Hoxd11 and Hoxd10 was scored in anterior neural tube, in a subset of cells located dorsally (arrow), as well as in the developing hindbrain, an expression pattern clearly reminiscent of that seen for Evx2 (Fig. 5). Hoxd11 and Hoxd10 transcripts were also detected in the isthmus and in specific domains within the mesencephalon, where Evx2 is also normally detected. Although the complete Evx2 neural pattern was not entirely recapitulated by either Hoxd11 or Hoxd10, the observed gain of expression encompassed several domains that were previously defined as specific for Evx2. Ectopic expression was also observed in heterozygous embryos with a weaker staining intensity, as expected if only one copy of each gene has been activated.

From these results, we concluded that the insulation of the Hoxd complex from the Evx2 regulatory influence, in a large subset of CNS cells, was achieved as a result of the presence of two DNA fragments, one of them being RXII, the other(s) lying around the Hoxd12 locus.

The fact that the deletion of both Hoxd13 and Hoxd12 loci did not induce expression of Hoxd11 in the Evx2 CNS domains, indicated that RXII, which is located in the immediate neighbourhood of the Evx2 start site, was able by itself to mediate such an insulation. Interestingly, in Drosophila, a GAGA-dependent enhancer blocking activity was identified within the promoter region of the orthologous gene even-skipped (eve), and this activity was shown to prevent 5’ located genes to respond to 3’ located enhancers (Ohtsuki and Levine, 1998). Thus, in both organisms, an enhancer
blocking activity was found associated with the eve/EvX2 locus. Whether or not this observation has a phylogenetic meaning, rather than being a mere coincidence, remains to be established. In any case, the underlying molecular mechanisms are likely to be distinct, as RXII does not seem to contain any GAGA-binding site.

The morphological effect of expressing Hoxd genes in the developing anterior CNS, and hence the biological relevance of this insulation, was difficult to assess as HoxdRXII;del(13-12) homozygous specimens died at birth. However, this lethality may not be directly associated to the abrogation of insulation, as neonatal death was also observed for HoxdRXII;del(13-12) homozygous animals, i.e. animals that carried a wild-type RXII and, consequently, did not express Hoxd genes in anterior CNS. In this latter configuration, the deletion of both Hoxd11 and Hoxd12 induced the mis-expression of other Hoxd genes in a variety of embryonic structures, which may have caused lethality (data not shown). Consequently, it is as yet unclear whether such an insulator activity is required to prevent one particular gene to be expressed in developing CNS, or alternatively, if all posterior Hoxd genes would be equally detrimental when expressed there. To precisely assess the biological relevance of this insulation mechanism, specific gain of expression of 5′ Hoxd genes, using conventional transgenic approaches, will be necessary.

### Specificity of the insulation

The presence, at one extremity of a Hox gene cluster, of sequences with insulating potential suggests a general requirement for isolating these chromosomal loci from their surrounding genomic contexts. Interestingly, various gene complexes seem to implement different mechanisms to protect themselves from regulatory interferences (Bell et al., 2001). For example, the β-globin gene complex, which shows some analogies with Hox clusters in its functional organisation, is flanked by sequences carrying properties of insulators (Bell et al., 1999; Saitoh et al., 2000). These latter sequences were proposed to prevent crosstalk between β-globin regulation, on the one hand, and unrelated regulatory influences emanating from closely located genes, such as those encoding odorant receptors, on the other (Bulger et al., 1999; Prioleau et al., 1999). This insulating potential was tightly associated with the 5′ HS4 and the 3′ HS DNAase I hypersensitive sites (Bell et al., 1999; Saitoh et al., 2000). These sites were identified in all cell types and tissues examined, suggesting that insulation of this gene complex is a rather generic mechanism with little cell specificity. By contrast, the insulating activity described in this paper, which prevents Hox genes from responding to upstream located CNS enhancers, was ineffective in a different cellular context. Indeed, the same series of genes was able to respond to another remote enhancer sequence, also located upstream the cluster, which controls Hoxd gene expression in developing digits (Spitz et al., 2001). This indicates that insulation of the Hoxd cluster is tissue-specific; it is effective in CNS cells, but not in limb mesenchymal cells (Kmita et al., 2002).

In the *Drosophila* Bithorax complex (BX-C), the gene orthologous to mammalian 5′ Hoxd genes (AbdB) is controlled, in defined parasegments, by a series of regulatory elements (Boulet et al., 1991; Celniker et al., 1990; Sanchez-Herrero, 1991). Such sequences (Fab genes) are often flanked by frontabdominal elements (Fab genes), which display insulating or boundary properties. Fab sequences are essential for proper parasegmental identity as they prevent crosstalk between distinct Fab (Barges et al., 2000; Mihaly et al., 1997; Zhou et al., 1999). Instead, in the vertebrate Hoxd complex, the insulating activity may rather reflect a general, complex-wide protection against anterior CNS regulation, rather than a way to implement properly a regulatory circuitry in space and time, as is the case for *Drosophila*. Therefore, it is unlikely that the mechanisms involved in these two processes serve identical purposes. It is nonetheless possible that RXII, as do Fab8 and the promoter targeting sequences (PTS) identified adjacent to it (Zhou and Levine, 1999), contains both insulating and ‘enhancer positioning’ activities. Indeed, the bipartite RXII element was also shown to be involved in the mechanism that triggers preferential interaction between the digit enhancer and the most 5′ Hoxd gene (Kmita et al., 2002a). Therefore, the digit enhancer may have a ‘positioning activity’, which might help to bypass the RXII blocking activity in limbs, in a way related to the PTS element which was shown to allow distal enhancers to overcome the Fab8 insulating activity (Zhou and Levine, 1999). This capacity of the digit enhancer to overcome the effect of RXII may not be shared by neural enhancers which, as a consequence, would not be capable of bypassing RXII in CNS cells.

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**Fig. 5** Expression of Hoxd11 and Hoxd10 in HoxdRXII;del(13-12) animals. (A,B) Lateral and dorsal views, respectively, of an 11.5 dpc foetus analysed for Evx2 transcripts. A strong expression is detected in the developing digits, in a domain that is identical to the distal expression domain of Hoxd11 (D). Evx2 transcripts are also observed in columns of cells with the developing spinal cord, up to the posterior hindbrain (black arrowhead), as well as in a region encompassing the cerebellar anlage (black arrow) up to the isthmus. More rostrally, transcripts are found in the mesencephalon, (white arrow) (Dollé et al., 1994). (C) Control embryo of the same age hybridised with a probe specific for Hoxd11 RNA. None of this CNS domain is observed. (D-F) Ectopic expression of Hoxd11 (D,E) and Hoxd10 (F) in the CNS. In addition to the expected expression patterns in limbs and developing trunk (D), these latter genes show clear ectopic activation in domains virtually identical to those where Evx2 is expressed (D-F, compare with A and B), indicating that they now are under the control of Evx2 neural enhancers.
Regulatory redundancy

We show that only a combined deletion of both region XII and an 18 kb piece of the cluster would lead to ectopic transcription of both Hoxd11 and Hoxd10 in CNS. This observation suggests that the DNA fragment that is able, along with RXII, to insulate the Hoxd complex lies around the Hoxd12 transcription unit. Two DNA fragments were shown to display significant interspecies sequence conservation within this interval: regions XI and X (Beckers and Duboule, 1998; Hérault et al., 1998a).

A role for RXI in insulation is unlikely as: (1) it has no counterpart in the fish genome (Hérault et al., 1998a); and (2) its deletion together with RXII in Hoxd\textsuperscript{RXII;del(13)} animals had no apparent effect. Therefore, region X appears as the best candidate element to mediate this activity at the Hoxd12 locus. However, in the act of creating deletion, had no detectable effect upon 5’ Hoxd gene regulation, similar to the case of RXII. This unexpected observation was tentatively explained by the existence of redundant regulatory processes (Beckers and Duboule, 1998).

Regulatory redundancy is a difficult concept to accommodate with our current views of gene regulation. However, if we assume that both regions have insulating potentials, we may understand redundancy as a property associated with one particular cellular context. For example, in order to be functional in a given cell type, RXII may require factors partially specific for this cell type, to properly insulate the cluster. Likewise, in another cell type, RX may recruit a different set of factors to insulate the cluster from the influence of a different enhancer. In the case where both sets of factors would be present in CNS cells, both insulation processes would operate, hence only multiple deletions in cis would reveal this mechanism. Accordingly, the evolution and stability of either one of these two regions might have been driven separately, in different contexts, to become redundant in CNS cells. In this scheme, the question nevertheless remains as to why single deletions have no visible effect, at least in the original context wherein a given element is specifically required? Such tissues or organs might simply have been overlooked; they may, for example, involve vertebrate specific functions (rather recent evolutionary features), the alteration of which may have as yet escaped our attention.

The Hoxd cluster has been thoroughly investigated, in vivo, for the functional relevance of evolutionary conserved DNA sequences. In its most posterior part, i.e. between Hoxd10 and Evx2, five stretches of non-coding sequences were found significantly conserved amongst vertebrates. Using targeted approaches in ES cells, all five sequences were either deleted, mutagenised and/or exchanged for an orthologous sequence (Gérard et al., 1996; Beckers and Duboule, 1998; Hérault et al., 1998a) (this work). Interestingly, although in some cases, slight variations in the expression of the neighbouring genes were scored, none of these drastic genetic modifications led to major regulatory alterations, a counter intuitive observation that is at odd with current speculations regarding sequence conservation outside coding sequences. The results presented in this paper may shed some lights on this puzzling issue, as they suggest that such sequences might relate to high order regulatory processes, rather than to gene-specific cis-acting controls.

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