Development 129, 5521-5528

doi:10.1242/dev.00151

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

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Accepted 20 June 2002

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 this gene family. This requirement for a context-dependent insulation is best exemplified by the presence of the *Evx2* gene in the immediate 5′ neighbourhood of the *Hoxd* cluster (D'Esposito et al., 1991; Bastian et al., 1992).

Evx2 indeed displays specific expression features that are not shared by any *Hoxd* 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 would be 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 *Not*I fragment that covers the entire *Evx2* to *Hoxd13* intergenic region. A *PGK-neomycin* selection cassette, flanked by *loxP* sites, was inserted at the *Ns*iI 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 $Hoxd^{RXII\text{-}neo}$ line of mice were obtained and further crossed with partners carrying the *CMV-Cre* transgene in order to produce the $Hoxd^{RXII}$ line of mutant mice that lacked the PGK-*neomycin* selection cassette.

Recombined lines

Besides the $Hoxd^{RXII}$ 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 *Hoxd* 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 EvD^{GE3} allele) (Hérault et al., 1996), with an allele carrying a *loxP* site between *Hoxd13* and *Hoxd12* ($Hoxd^{RXI}$) (Hérault et al., 1998a). $Hoxd^{del(13\text{-}12)}$ and $Hoxd^{del(13\text{-}11)}$ animals were obtained in a similar way, although in these latter cases, the EvD^{GE3} allele was combined either with $Hoxd^{RX}$, in which a *loxP* site had been inserted between *Hoxd12* and *Hoxd11* (Beckers et Duboule, 1998), or with $Hoxd^{RIX}$, containing a *loxP* site between *Hoxd11* and *Hoxd10* (Gérard et al., 1996). $Hoxd^{RXII\text{-}del(13)}$ mice were obtained in the progeny of *trans*-loxer, which were *trans*-heterozygous for $Hoxd^{RXII}$ and $Hoxd^{RXI}$. Finally, $HoxdRXII^{(del(13\text{-}12)}$ were produced trough trans-loxer animals *trans*-heterozygous for both $Hoxd^{RXII}$ and $Hoxd^{RX}$ 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

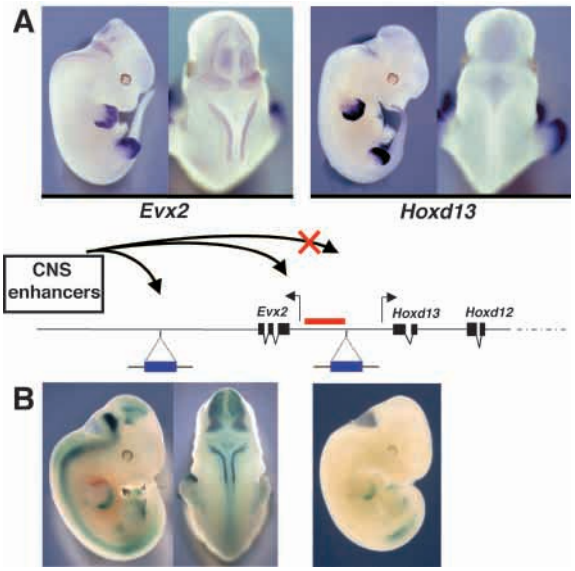

Fig. 1. (A) Insulating activity within the *Hoxd13* to *Evx2* intergenic region. The posterior extremity of the *Hoxd* complex is shown, as well as the position of the *Evx2* gene. The expression patterns of both *Evx2* and *Hoxd13* are depicted above to illustrate enhancer sharing in developing digits (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 relocatd 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.

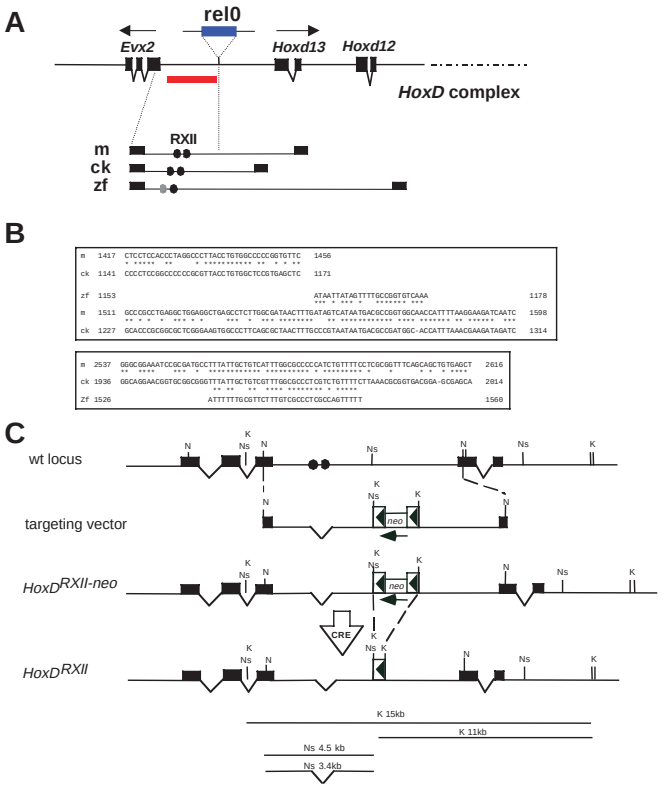

Fig. 2. Identification and targeted deletion of region XII (RXII). (A) Interspecies conservation within *Evx2-Hoxd13* intergenic region. Sequence analyses revealed two stretches of significant conservation, referred to as region XII (RXII), which were found to be located within the insulating area (red 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). (B) Sequence alignment of region XII from mouse (m), chicken (ck) and zebrafish (zf) DNA. 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 *Hoxd*$^{RXII\text{-}neo}$ mice. The selection cassette was further deleted after crossing these mice with a Cre deleter strain, to produce the *Hoxd*^{RXII} 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.

al., 2001; van der Hoeven et al., 1996). Unlike *Hoxd* genes, however, *Evx2* was shown to be transcribed in subset of cells within the central nervous system (Fig. 1A) (Dollé et al., 1994), in response to regulatory sequences that are also located upstream the cluster, as revealed by engineered targeted deletions (Kondo and Duboule, 1999).

These differences in regulation between *Evx2* and *Hoxd13* could hardly be accounted for by the specificity of enhancer/promoter interactions, because a *Hoxd9/lacZ* transgene was able to respond to these neural enhancers when placed 3′ to *Evx2* (Fig. 1B; RelII). This transgene, however, behaved as a proper *Hox* gene when placed between *Evx2* and *Hoxd13*, a position at which it failed to show expression in rostral parts of the brain and in spinal cord (Fig. 1B). These results indicated that the capacity of a Hox promoter to respond to *Evx2* CNS enhancers was abrogated when this promoter was positioned within the cluster, suggesting that a potential insulating element was present between the Rel0 insertion site and *Evx2* (Fig. 1; red bar). Because in birds, fish and mammals *Evx2* lies at the same relative position with respect to *Hoxd13* (Sordino et al., 1996), we anticipated that a DNA sequences that would prevent the *Evx2* neural enhancers from affecting Hox gene 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 $Hoxd^{RXII\text{-}neo}$ line of mice was established. In order to prevent regulatory interferences caused by the presence of the PGK-*neomycine* selection cassette, $Hoxd^{RXII\text{-}neo}$ 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 $Hoxd^{RXII}$ 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 $Hoxd^{RXII}$ 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 sperms 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 ($Hoxd^{del(13)}$ in Fig. 3); a 18 kb large fragment covering both *Hoxd13* and *Hoxd12* loci ($Hoxd^{del(13\text{-}12)}$), and a 23 kb large fragment encompassing all three *Hoxd13*, *Hoxd12* and *Hoxd11* loci ($Hoxd^{del(13\text{-}11)}$ 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.

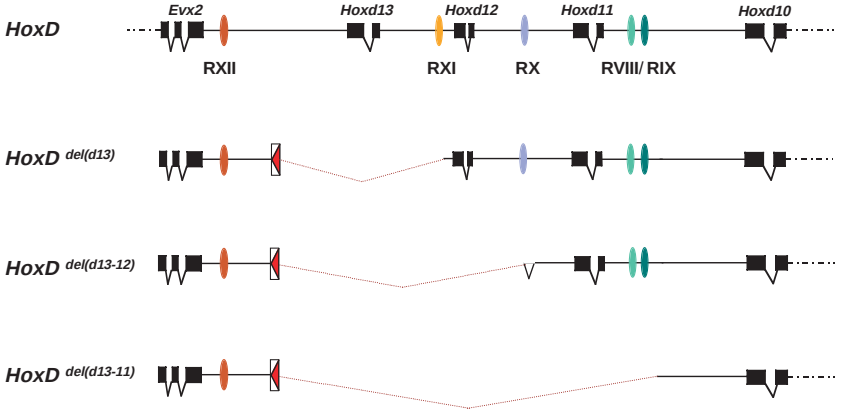

Fig. 3. Nested deficiencies of the posterior *Hoxd* complex, as produced by targeted meiotic recombination (TAMERE). At the top, the positions of the four regions (RIX to RXII) of high interspecies sequence conservation are show. The three deletions considered in this work are schematised below: $Hoxd^{del(13)}$, a deletion of the *Hoxd13* locus including RXI; $Hoxd^{del(13\text{-}12)}$, a deletion of both *Hoxd13* and *Hoxd12* loci, including RXI and RX; and $Hoxd^{del(13\text{-}11)}$, a deletion of all three *Hoxd13* to *Hoxd11* loci, including all conserved sequences but RXII. In each case, a *loxP* site (red triangle) is left upstream RXII, at the position of the 5′ breakpoint of the deletions.

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 $Hoxd^{RXII}$ 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; $Hoxd^{RXII}$), as a left over of the Cre-mediated deletion of the PGK*neo* selection cassette. We first produced males carrying either the $Hoxd^{RXII}$ and $Hoxd^{RXI}$ alleles (Fig. 4B), or the $Hoxd^{RXII}$ and $Hoxd^{RX}$ alleles (Fig. 4C), along with the *Cre*. In the progeny of these *trans*-loxer males, we isolated both $Hoxd^{RXII\text{-}del(13)}$ and $Hoxd^{RXII\text{-}del(13\text{-}12)}$ animals, respectively (Fig. 4). Although a strain of $Hoxd^{RXII\text{-}del(13)}$ homozygous mice could be established, $Hoxd^{RXII\text{-}del(13\text{-}12)}$ 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 $Hoxd^{RXII\text{-}del(13)}$ 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.

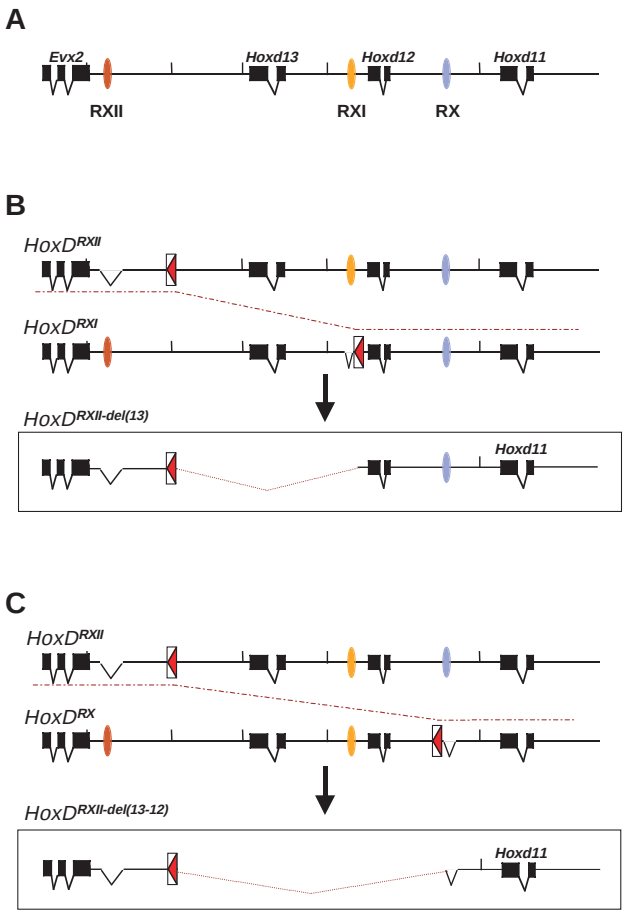

Fig. 4. Combined deletions in *cis*. (A) Scheme of the posterior *Hoxd* complex, with the location of conserved regions X to XII. (B) The first combined deletion in *cis* was produced by meiotic recombination between the $Hoxd^{RXII}$ and $Hoxd^{RXI}$ alleles. Recombination (broken line) between the two *loxP* sites present in these alleles generated the $Hoxd^{RXII\text{-}del(13)}$ allele (boxed), which carries a deletion of RXII as well as of the *Hoxd13* locus containing RXI. (C) The second combined deletion in *cis* was produced by meiotic recombination between the $Hoxd^{RXII}$ and $Hoxd^{RX}$ alleles. Recombination (broken line) between the *loxP* sites present in these alleles generated the $Hoxd^{RXII\text{-}del(13\text{-}12)}$ allele (boxed), which carries a deletion of RXII, as well as of both *Hoxd13* and *Hoxd12* loci, which contain both RXI and RX.

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

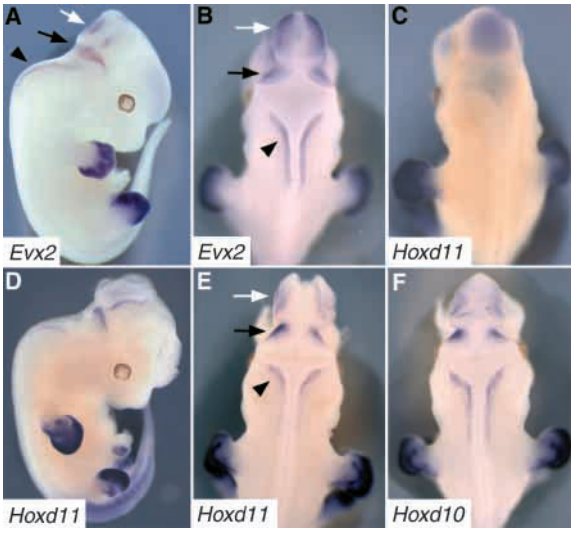

Fig. 5. Expression of *Hoxd11* and *Hoxd10* in *Hoxd*$^{RXII\text{-}del(13\text{-}12)}$ animals. (A,B) Lateral and dorsal views, respectively, of an 11.5 dpc foetus analysed for *Evx2* transcripts. A strong expression is detected in the developing digits, in a domain that is identical to the distal expression 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.

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 *Hoxd*$^{RXII;del(13\text{-}12)}$ 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 *Hoxd*$^{del(13\text{-}12)}$ 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 *Hoxd13* 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 DNAse 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 (Iab 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 Iab (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* insulation 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.

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 $Hoxd^{RXII;del(13)}$ animals had no apparent effect. Therefore, region X appears as the best candidate element to mediate this activity at the *Hoxd12* locus. However, its inactivation in vivo, through targeted 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.

We thank Nadine Fraudeau and Marianne Friedli for technical assistance, as well as Takashi Kondo for sharing data, and colleagues from the Duboule laboratory for discussions and comments on the manuscript. This work was supported by funds from the Canton de Genève, the NCCR ‘Frontiers in Genetics’, the Swiss National Research Fund, the Claraz, Latsis, Cloetta and Louis-Jeantet foundations.

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