Cdx Protein Interaction with *Hoxa5* Regulatory Sequences Contributes to *Hoxa5* Regional Expression along the Axial Skeleton

Sébastien Tabariès,¹† Jérôme Lapointe,¹† Terri Besch,² Marcelle Carter,¹ John Woollard,² Christopher K. Tuggle,² and Lucie Jeannotte^{1*}

Centre de Recherche en Cancérologie de l'Université Laval, Centre Hospitalier Universitaire de Québec, Québec, Canada,¹ and Department of Animal Science, Iowa State University, Ames, Iowa²

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***Hox* gene functions are intimately linked to correct developmental expression of the genes. The identification of *cis*-acting regulatory sequences and their associated *trans*-acting factors constitutes a key step in deciphering the mechanisms underlying the correct positioning of the functional domain of *Hox* genes along the anterior-posterior axis. We have identified DNA elements driving *Hoxa5* regionalized expression in mice, using the 2.1-kb mesodermal enhancer (MES) localized in *Hoxa5* 3' flanking sequences as a starting point. The MES sequence comprises regulatory elements targeting *Hoxa5* expression in the limbs, the urogenital and gastrointestinal tracts, and the cervical-upper thoracic region of the prevertebral column. A 164-bp DNA fragment within the MES caudally restricts *Hoxa5* expression at the level of prevertebra 10, corresponding to the posterior limit of its functional domain. Cdx proteins directly bind to this element in vitro via two conserved sites. Preventing Cdx binding by mutating the sites causes caudal expansion of the transgene expression domain. Of all three murine Cdx proteins that bind this element in vitro, Cdx4 has emerged as a potential regional posterior repressor of *Hoxa5* expression. The restrictive control provided by Cdx interactions with *Hoxa5* regulatory sequences may be one of the critical events in cervicothoracic axial specification.**

The establishment of the morphological diversity of the body plan requires that genetic information be processed to ultimately dictate specific shapes in subsets of domains along the body axes. In this cascade of molecular events, *Hox* genes are key components that define how and where structures will be elaborated. This gene family encodes transcription factors that specify regional identities along the embryonic axes of diverse organisms. The murine genome contains 39 *Hox* genes organized into four clusters, *HoxA* to *HoxD* (35). The organization of *Hox* complexes is fundamental for the precise spatiotemporal regulation and function of each gene. A colinear relationship exists between the relative order of *Hox* genes on the chromosome, their expression domains along the anterior-posterior axis, and their temporal onset: genes located at the 3' ends of the clusters are expressed earlier and more anteriorly in the embryo than those in more 5' positions (16, 31). As a result, *Hox* gene expression domains are both spatially restricted and overlapping, extending from specific anterior boundaries to the caudal end of the embryo. *Hox* developmental functions were first extrapolated from these expression patterns. However, mutational analyses of murine *Hox* genes have shown that their domains of action are mainly restrained to the most anterior regions of their expression domains (4, 56). *Hox* gene functions are also intimately linked to correct developmental regulation of the genes, as illustrated by the homeotic transformations observed when *Hox cis*-acting regulatory elements are mutated (17, 22, 65).

Even though substantial information has been gained regarding *Hox* gene regulation, a better comprehension of the controls governing *Hox* gene expression is needed to decipher the mechanistic basis responsible for *Hox* gene function in axial patterning. *Hox* gene regulation is thought to be achieved by a combination of strategies involving (i) chromatin remodeling (12), (ii) global enhancer sequences located outside the cluster and acting in a relatively non-promoter-specific manner (30, 33, 55), and (iii) an integrated regulation of neighboring genes through the sharing, competition, and/or selective use of defined local *cis*-acting sequences (23, 53; reviewed in reference 31). Extensive studies have been devoted to the identification of control elements, demonstrating that *Hox* gene expression can be recapitulated partly or entirely upon random integration in the genome of transgenes containing DNA regulatory elements (7, 10, 36, 45, 46, 48, 53, 61, 64). The common emerging picture is that the dynamic profile of *Hox* gene expression relies on the interpretation of positional information transduced by transcriptional factors interacting with both positive and repressive DNA sequences to restrict the enhancer activities to defined regional domains within specific expression boundaries (22, 44, 57). Among the upstream factors identified so far, *caudal*-related family members have emerged as key regulators (13, 19, 49, 62). The main evidence for Cdx protein involvement in *Hox* regulation comes from analyses of targeted mutations of these homeodomain-containing proteins in mice, as they result in skeletal defects akin to those previously observed in *Hox* mutant mice (14, 58, 62). In *Cdx1*^{-/-}, *Cdx2*^{+/-}, and *Cdx1*^{-/-} *Cdx2*^{+/-} compound mutants, the posterior shift in *Hox* gene boundaries correlates with the transformations observed along the anterior-posterior axis. Moreover, potential Cdx binding sites are found within the *cis*-regulatory regions of several *Hox* genes (13, 47, 54, 58). Finally, recent data substan-

* Corresponding author. Mailing address: Centre de Recherche de L'Hôtel-Dieu de Québec, 9 rue McMahon, Québec, QC G1R 2J6, Canada. Phone: (418) 525-4444, ext. 15552. Fax: (418) 691-5439. E-mail: lucie.jeannotte@crhdq.ulaval.ca.

† S.T. and J.L. contributed equally to this work.

tiate the view that Cdx proteins may act as integrators of multiple signaling pathways elicited by either retinoic acid, Wnt, or fibroblast growth factors (8, 9, 25, 27, 28, 37, 49, 50). Consequently, Cdx proteins likely convey positional information from these signals to *Hox* genes (38).

To fully understand the regulatory events governing *Hox* gene expression, we are studying the *Hoxa5* gene. This gene provides a good model system for deciphering the regional control of *Hox* regulation, as its loss-of-function mutation affects a well-defined subset of its axial expression domain (2, 29, 36). In the developing embryo, *Hoxa5* expression extends from the caudal end to the posterior myelencephalon in the neural tube and to prevertebra (pv) 3 in the axial skeleton. *Hoxa5* transcripts are also detected in the mesenchymal components of several organs, including the trachea, lungs, stomach, intestine, and kidneys (1, 2, 3, 5, 15, 21). Aside from morphological defects in foregut derivatives (1, 5, 40), targeted disruption of the *Hoxa5* gene perturbs axial skeleton identity in a region confined between pv3 and pv10, the most anterior domain of *Hoxa5* expression in the prevertebral column (2, 29). Several transcripts, of 1.8, 5.0, 9.5, and 11 kb in length, encompassing *Hoxa5* coding sequences originate from the use of different promoters and from alternative splicing (29; Y. Coulombe and L. Jeannotte, unpublished data). As a result, the global *Hoxa5* expression profile derives from a combination of the expression domains of all *Hoxa5* transcriptional units initiating from both the proximal and the distal promoters. The major 1.8-kb transcript, which encodes the Hoxa5 270-amino-acid protein, is specifically expressed at high levels in the axial domain, where the *Hoxa5* mutation exerts its effect (36).

The presence of complex and overlapping transcriptional units at the *Hoxa5* locus implies the presence of dispersed and shared regulatory regions in the cluster. This prompted us to characterize the regulatory elements directing *Hoxa5* developmental expression by using a transgenic approach. *cis*-acting sequences lying within an 11.1-kb genomic fragment can recapitulate the temporal expression and substantially reproduce the spatial pattern of the *Hoxa5* 1.8-kb transcript (36). Deletion analyses have revealed several DNA control elements, including a 604-bp brachial spinal cord enhancer (BSC) in 5' flanking sequences (45, 61) and a 2.1-kb mesodermal enhancer (MES) downstream of *Hoxa5* coding sequences, with the latter being essential for paraxial and lateral plate mesoderm expression in the cervical and upper thoracic regions (36). Additional regulatory sequences involved in *Hoxa5* lung and gut expression have also been identified near the *Hoxa4* gene (42). As a result of pursuing the characterization of the MES sequence by deletion and mutational analyses, we present evidence that the MES contains DNA elements that limit the *Hoxa5* regional specific expression domain along the anterior-posterior axis and that Cdx proteins can directly interact with MES sequences to correctly position the *Hoxa5* expression domain.

MATERIALS AND METHODS

Construction of *Hoxa5/lacZ* transgenes. Construct 1 of this study, also named pLJ133, has been described previously (Fig. 1B) (construct 9 in reference 36). It contains the bacterial *lacZ* gene inserted into the *SacI* site of the first exon of the *Hoxa5* gene, allowing translation of the *lacZ* open reading frame from the *Hoxa5* AUG. Construct 1 was digested with *HindIII* to remove a 2.08-kb fragment from

kb +2.85 to +4.93 and was self-ligated to produce an intermediate construct (pLJ143 [not shown]) containing a continuous *XhoI-HindIII* *Hoxa5* genomic fragment extending from bp -235 to kb +2.85 that does not direct β -galactosidase expression, as previously shown (64). The insertion of a *HindIII-PstI* fragment (from kb +2.85 to +4.60) at the *HindIII* site (kb +2.85) of pLJ143 resulted in construct 2. Constructs 3 to 5 were generated by a series of deletions at the 3' end of construct 1. Construct 1 was partially digested with *Eco47III* to produce construct 3 and with *AvrII* and *XhoI* to generate constructs 4 and 5, respectively. An 892-bp *XhoI/HindIII* filled-in fragment extending from kb +4.04 to +4.93 was inserted either at the *HindIII* site (kb +2.85) or at the *XhoI* site (bp -235) of pLJ143 to generate constructs 6 and 7, respectively. Construct 8 was derived from construct 1 by deleting the *AvrII-Eco47III* sequence, which was thereafter referred to as the *AvEc* 164-bp fragment located between kb +4.28 and +4.44. Construct 9 was obtained by cloning the *AvEc* 164-bp fragment into the *HindIII* (kb +2.85) site of pLJ143. For constructs 10, 11, and 12, nucleotide substitutions in the identified consensus Cdx binding sites were produced by an overlapping PCR strategy using synthetic oligonucleotide primers containing appropriate base changes (63). The modifications introduced are shown in Fig. 4A. The 2.08-kb *HindIII* fragment extending from kb +2.85 to +4.93 was subcloned, and the resulting construct was used as a template for synthetic primers. All introduced mutations were subsequently validated by sequencing. The plasmids containing the desired mutations were digested with *HindIII*, and the fragments were subcloned into the *HindIII* site (kb +2.85) of pLJ143 to generate constructs 10 (mut1), 11 (mut2), and 12 (mut1,2). All of the constructs described in the present study were made in pBluescript II SK(+) (Stratagene) and were purified by cesium chloride centrifugation.

Production and genotyping of transgenic mice. All experiments were performed according to the guidelines of the Canadian Council on Animal Care and were approved by the institutional animal care committee. The *Hoxa5/lacZ* sequences from all constructs were isolated by a *KpnI* and *NotI* digestion to remove vector sequences and were purified on agarose gels. They were then injected into the pronuclei of fertilized eggs derived from C57BL/6 $\times$ C3H F₁ hybrid intercrosses according to standard procedures (24). Transgenic founder embryos were recovered from foster mothers, genotyped by Southern analysis of yolk sac DNAs with a *lacZ*-specific probe to verify the integrity of the microinjected constructs, and analyzed for *lacZ* expression by β -galactosidase staining as previously described (36). Permanent mouse lines for construct 1 were also obtained and genotyped by the use of tail DNA.

The effect of Cdx proteins on *Hoxa5/lacZ* transgene expression was assessed by the use of either a *Cdx1* mutant mouse line originally produced in the laboratory of P. Gruss (58) and provided by D. Lohnes or a *Cdx2* mutant mouse line generated in the laboratory of F. Beck and provided by K. Chawengsaksophak (14). The *Cdx1* and *Cdx2* mutations were maintained in the 129/SvEv and ICR genetic backgrounds, respectively. In addition to the pLJ133-carrying established transgenic line (corresponding to construct 1 in the present study) (Fig. 1B), a permanent mouse line obtained previously for the pLJ57 *Hoxa5/lacZ* construct was tested (construct 2 in reference 36). The pLJ57 transgene contains a *Hoxa5* genomic fragment extending from kb -2.13 to +7.19, with the *lacZ* reporter gene inserted into the first exon of the gene. To generate *Cdx1*^{+/+} pLJ133^{+/+} and *Cdx1*^{+/+} pLJ57^{+/+} animals, we set up matings between *Cdx1*^{-/-} and *Hoxa5/lacZ* mice. *Cdx1*^{+/+} pLJ133^{+/+} and *Cdx1*^{+/+} pLJ57^{+/+} males were then bred with *Cdx1*^{+/+} females to produce animals for all six possible genotypes. In parallel, pLJ133^{+/+} or pLJ57^{+/+} males were bred with *Cdx2*^{+/+} females to generate animals for all four possible genotypes. The early embryonic lethality of *Cdx2*^{-/-} mutants prevented the analysis of *Cdx2*^{-/-} embryos carrying a *Hoxa5/lacZ* transgene (14). We also produced *Cdx1*^{+/+} pLJ57^{+/+} males that were bred with *Cdx1*^{+/+} *Cdx2*^{+/+} females to evaluate the impact of three *Cdx* mutated alleles on *Hoxa5/lacZ* transgene expression (*Cdx1*^{-/-} *Cdx2*^{+/+} pLJ57^{+/+} specimens). The morning of vaginal plug detection was considered embryonic day 0.5 (E0.5). Embryos were obtained at various embryonic ages and stained for β -galactosidase activity. Yolk sac DNAs were used for genotyping of the *lacZ* and *Cdx1* alleles by Southern blot analysis (58). The *Cdx2* genotype was determined by PCR as described previously (62).

EMSA. The expression vectors pSG5-*Cdx1*, pSG5-*Cdx2*, and pSG5-*Cdx4* were provided by J. Deschamps (13). The TnT7 Quick coupled transcription-translation system (Promega Corporation) was used to produce each of the Cdx proteins in vitro, which were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and directly assayed by an electrophoretic mobility shift assay (EMSA). The *AvEc* 164-bp fragment was purified, end labeled with T4 polynucleotide kinase, and tested for Cdx1, Cdx2, and Cdx4 binding by EMSA. A combination of double-stranded end-labeled oligonucleotides covering the following regions were also tested: (i) the 5' half of the *AvEc* 164-bp fragment, from nucleotides 1 to 86 (see Fig. 3B); (ii) the 3' half of the *AvEc* 164-bp

FIG. 1. Characterization of regulatory regions of the *Hoxa5* gene in transgenic mouse embryos. Schematics of reporter gene constructs and sites of expression are shown. (A) Partial restriction map of the *Hoxa5* locus. Black boxes indicate homeoboxes of the *Hoxa5* and *Hoxa6* genes, gray boxes correspond to translated regions, and white boxes indicate transcribed regions. The boxes labeled BSC and MES denote the enhancer domains defined in references 36 and 61, respectively. (B) Diagram of the constructs used to generate transgenic embryos. The asterisks in constructs 10, 11, and 12 correspond to mutations of Cdx binding sites 1 and 2 (see Fig. 4 for the nucleotide sequences of these mutations). (C) Summary of transgenic expression analyses. The first column represents the number of *lacZ*-expressing embryos out of the total number of F0 transgenic embryos generated. The number of positively stained embryos for each structure listed is indicated. post. bound., defined upper thoracic posterior boundary along the prevertebral column; pv, prevertebrae; som, somites; uro. tract, urogenital tract. A, AccI; Av, AvrII; B, BglII; Ec47, Eco47III; H, HindIII; Pst, PstI; RI, EcoRI; RV, EcoRV; S, SacI; Xh, XhoI.

fragment, from nucleotides 67 to 164; and (iii) sequences encompassing Cdx binding sites, including 5'CCGGATTAACTTTAGTCTTTGAAT3', 5'TTGA AAGGGTTAAAGCGCT3', and Oligo1. We focused on two consensus Cdx binding sites, sites 1 and 2 present in Oligo1, that showed Cdx binding activity. EMSAs were performed with double-stranded end-labeled oligonucleotides harboring either the wild-type sequence 5'TGAAGCAATAAACTTTATGGCCG GA3' (Oligo1) or sequences with mutated binding sites, i.e., 5'TGAAGCAAT GCCCTTTATGGCCGGA3' [Oligo1 (mut1)], 5'TGAAGCAATAAACTTTATGGC GGCCGGA3' [Oligo1 (mut2)], and 5'TGAAGCAATGCCCTTTGCGGGCCG GA3' [Oligo1 (mut1,2)] (nucleotide substitutions are underlined). Binding

reactions containing 0.5 to 4 ng of probe (100,000 cpm), 1 μ l of Cdx protein, 1 μ g of poly(dI-dC), and 100 ng of yeast tRNA prepared in a buffer comprising 15 mM HEPES (pH 7.9), 50 mM NaCl, 80 μ M ZnCl₂, 800 μ M dithiothreitol, 0.5% NP-40, 2 mM MgCl₂, and 3% Ficoll were equilibrated for 10 min at 25°C and separated by electrophoresis through a 6% polyacrylamide (29:1) gel containing 0.25 $\times$ Tris-borate-EDTA. The specificity of Cdx binding was assessed by the addition of a 100-fold excess of unlabeled probe prior to the addition of the radiolabeled probe. A 23-bp doubled-stranded DNA fragment of the sucrose-isomaltase promoter, SIF1 (5'GTGCAATAAACTTTATGAGTAA3'), which contains two sequences interacting with the Cdx2 protein, was used as a positive

FIG. 2. Representative transgenic E12.5 embryos stained for β -galactosidase activity showing the effects of different deletion constructs on the expression pattern of the *Hoxa5/lacZ* transgene. (A) Construct 1. (B) Construct 2. (C) Construct 3. (D) Construct 4. (E) Construct 5. (F) Construct 6. (G) Construct 7. (H) Construct 8. (I) Construct 9. (J) Construct 10. (K) Construct 11. (L) Construct 12. Arrowheads indicate the posterior boundary of strong transgene expression in the prevertebral column. Similar patterns of staining in the prevertebrae were observed for constructs 1, 2, 3, 6, 7, 10, and 11, with the presence of a posterior limit of expression at the upper thoracic level. This posterior restriction of expression along the anterior-posterior axis was lost with constructs 4, 5, 8, and 12. No *lacZ* expression was detected in F_0 transgenic embryos with construct 9.

control and as a competitor for some binding assays (59, 60). Two unrelated double-stranded DNA fragments (TB2 and SP1) were also used as competitors. The sequence of the 97-bp double-stranded oligonucleotide TB2 used for this study was 5'TGGCAAACCGACCCCAACCTCTACACAAAGCCAGAGGGGATACAAAGCCGGGGACCCGAAGTTGTTGTACCCGATTCTAAGTCAACACCTCCCCCG3', and the sequence of the 22-bp double-stranded oligonucleotide SP1 was 5'ATTCGATCGGGGCGGGGCGAGC3'.

Supershift assays. E9.5 whole-cell extracts were prepared as described previously (13). Binding reactions containing 0.5 to 4 ng of probe (100,000 cpm), 20 mM HEPES (pH 8), 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, and 1 μ g of poly(dI-dC) were incubated for 15 min at 25°C. The Cdx4 antibody (provided by C. Wright) was then added, and the incubation was continued for an additional 20 min at 25°C. The reaction products were separated by electrophoresis through a 6% polyacrylamide (29:1) gel containing 0.25 $\times$ Tris-borate-EDTA and 2.5% glycerol. The specificity of antibody binding was assessed by the use of a control antibody raised against skeletal muscle calsequestrin obtained from E. Lonergan. Supershift assays were also done with Cdx1 and Cdx2 antibodies obtained from D. Lohnes.

RESULTS

Deletion analysis of *Hoxa5* MES. We previously identified a 5.17-kb DNA region encompassing the *Hoxa5* locus (construct

1) (Fig. 1B) that can specifically direct expression in mesodermal derivatives of the cervical-upper thoracic domain (36). This sequence includes a 2.1-kb HindIII fragment (MES) possessing enhancer properties and localized downstream of *Hoxa5* coding sequences between kb +2.85 and +4.93. In E12.5 F_0 transgenic embryos carrying construct 1, β -galactosidase expression was detected in the prevertebral column between pv3 and pv10/11, with a stronger signal in the pv3-6 region (Fig. 2A) (36). The urogenital tract and the forelimb buds also expressed the transgene, in addition to the hindlimbs in a few specimens (not shown). No transgene expression was detected in the neural tube since the BSC enhancer element was not included in construct 1 (Fig. 1) (61).

Deletions of the MES fragment were made to delineate the DNA elements involved in tissue-specific expression and were tested in E12.5 F_0 transgenic embryos. The removal of 331 bp from the 3' end of the MES resulted in a pattern similar to that observed with construct 1, except that expression in the mesenchymal condensations of the developing limb buds was de-

tected in only one transgenic embryo (construct 2) (Fig. 1C and 2B; not shown). The deletion of an additional 160 bp (construct 3) resulted in an X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) staining pattern comparable to that seen for constructs 1 and 2 (Fig. 2C). Furthermore, staining in the limb buds was recovered in six of eight F₀ β -galactosidase-expressing transgenic embryos (Fig. 1C). In concordance with the delay in the formation of embryonic posterior structures versus more anterior ones, the forelimb buds appeared fully stained, whereas only the distal extremities of hindlimb buds, corresponding to the interdigital region, were positive. Construct 3 was also expressed in the guts of five F₀ β -galactosidase-expressing transgenic embryos and in the urogenital tracts of all β -galactosidase-expressing embryos (Fig. 1C and 2C).

When an extra 164-bp DNA fragment was removed from the 3' terminus of the MES (construct 4), all of the F₀ β -galactosidase-expressing embryos obtained showed strong X-Gal staining in the prevertebral column, from the anterior limit at pv3 to the caudal end of the embryo, indicating that the posterior restriction at pv10 was lost (Fig. 1 and 2D). This 3' deletion also led to the absence of reporter gene expression in the developing limb buds, the urogenital system, and the gut for the majority of F₀ β -galactosidase-expressing embryos, suggesting the presence of tissue-specific regulatory elements in the AvrII-HindIII 655-bp sequence. We also performed an additional 3' deletion up to the XhoI site located at kb +4.04 that generated construct 5 (Fig. 1B). As observed for construct 4, most of the F₀ β -galactosidase-expressing embryos expressed the transgene from pv3 down to the tail of the embryo (Fig. 2E). Furthermore, no transgene expression was detected in the urogenital tract, the limb buds, or the gut (Fig. 1C). Taken together, these results indicated that the 3' half of the MES encompasses several *cis*-acting regulatory elements that are involved in specific regional control activities. The loss of posterior restriction along the prevertebral column also suggested that sequences between the AvrII (kb +4.28) and Eco47III (kb +4.44) restriction sites are involved in the positioning of the posterior boundary of expression at the upper thoracic level.

To further define the contribution of the 892-bp XhoI-HindIII DNA fragment to MES activity, we designed constructs carrying this DNA sequence either at the 3' end (construct 6) (Fig. 1B) or at the 5' end (construct 7) (Fig. 1B) of a *Hoxa5/lacZ* minimal construct. This *Hoxa5/lacZ* minimal vector included 3.09 kb of *Hoxa5* genomic sequence, from bp -235 to kb +2.85, that on its own cannot direct transgene expression (64). Constructs 6 and 7 presented similar patterns of expression in the interdigital region and the mesenchymal condensations along the proximodistal axis of the limb buds and in the gut (Fig. 1C and 2F and G). Only F₀ β -galactosidase-expressing embryos for construct 7 showed X-Gal staining in the urogenital tract (Fig. 1C). Like constructs 1, 2, and 3, constructs 6 and 7 drove β -galactosidase expression in the cervical region of the prevertebral column, with a well-established posterior limit in the upper thoracic region (Fig. 2F and G). The results described above suggested that the XhoI-HindIII sequence was able to direct region-specific expression independent of its position relative to the *Hoxa5* promoter. However, the XhoI-HindIII fragment was unable to drive

transgene expression when tested in front of a heterologous promoter (*hsp68/lacZ*) (not shown), suggesting that the *Hox* genomic context is essential for the regulatory action of the 3' fragment of the MES region. These results also indicated that the genuine enhancer activity of the MES relies on its integrity.

Distinct MES regulatory sequences mediate *Hoxa5/lacZ* regional expression. The 3' sequential deletion of the MES suggested that the AvEc 164-bp fragment contains DNA regulatory elements that restrict *Hoxa5/lacZ* transgene expression to the cervical-upper thoracic region along the axis. We directly tested this hypothesis by removing the 164-bp sequence from construct 1 to generate construct 8 (Fig. 1B). Deletion of the AvEc 164-bp fragment resulted in a loss of posterior restriction along the prevertebral column for three of five F₀ β -galactosidase-expressing transgenic embryos (Fig. 1C). Transgene expression was stronger in the cervical region, but the extent of the loss of posterior restriction varied from embryo to embryo (Fig. 2H). The loss of posterior restriction was the only difference in expression between constructs 1 and 8, as both constructs drove *lacZ* gene expression in the urogenital system and the limb buds but not in the gut (Fig. 1C and 2H). When linked to the *Hoxa5/lacZ* minimal vector (construct 9) (Fig. 1B), the AvEc 164-bp fragment did not direct any reporter gene expression, indicating that it has no enhancer properties (Fig. 1C and 2I). Altogether, these data show that the AvEc 164-bp fragment contains DNA elements that participate in the positioning of the posterior boundary of *Hoxa5* expression at the upper thoracic level along the axial skeleton.

Cdx gene products bind in vitro to the AvrII-Eco47III 164-bp fragment. To gain insight into critical sequences involved in axial regional specification, we investigated whether the AvEc 164-bp fragment contains binding sites for known *trans*-acting factors that may be involved in transducing positional information to *Hox* genes. By sequence comparisons with the TF-search database (<http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>), we found, among several binding sites of putative *Hox* transcriptional regulators, seven potential sites with high homology to the consensus (TTTATA/G) for the *Cdx* gene products (Fig. 3B) (39, 52, 59). Sequence comparisons of the surrounding *Hoxa5* genomic environment revealed that Cdx consensus binding sites were predominantly localized in the MES region and that the majority of these Cdx binding sites were confined to the 3' half of the AvEc 164-bp fragment (Fig. 3B). To establish whether Cdx proteins can bind the AvEc 164-bp fragment, we performed EMSAs with the three murine Cdx proteins, Cdx1, Cdx2, and Cdx4, which we produced in vitro (Fig. 3C). All three Cdx proteins formed binding complexes with the AvEc 164-bp fragment (Fig. 3C, lanes 3, 6, and 9). The specificity of binding was confirmed by competition studies with a 100-fold excess of unlabeled probe, which resulted in a substantial reduction of Cdx protein binding (Fig. 3C, lanes 4, 7, and 10), and by the addition of a 100-fold excess of an unrelated primer, TB2 (lanes 5, 8, and 11). Further characterization of the specific interactions of Cdx proteins with each half of the AvEc 164-bp fragment revealed that the Cdx1, Cdx2, and Cdx4 proteins bound specifically to the 3' half of the fragment. No complex with the 5' half of the AvEc 164-bp fragment was detected, despite the presence of two potential Cdx binding sites (not shown). To identify which sites from the 3' half of the region were involved in binding activity,

FIG. 3. Characterization of the AvrII-Eco47III 164-bp fragment. (A) Restriction map of the 892-bp XhoI/HindIII fragment, extending from kb +4.04 to +4.93 in the 3' half of the MES regulatory region. The box denotes the location of the AvEc 164-bp regulatory region defined by transgenic studies. (B) Complete sequence of the AvEc 164-bp fragment. Arrows indicate the positions and orientations of potential Cdx binding sites, while the bold underlined sequence corresponds to Oligo1 used in EMSA experiments. Oligo1 contains two potential Cdx binding sites. (C) The capacity of the AvEc 164-bp fragment to bind Cdx proteins produced in vitro was tested by EMSA (lanes 3, 6, and 9). Specific binding complexes were competed by a 100-fold excess of unlabeled probe (lanes 4, 7, and 10). The specificity of Cdx protein binding was also assessed by the addition of a 100-fold excess of an unrelated primer, TB2 (lanes 5, 8, and 11). (D) EMSA with Cdx proteins produced in vitro and with Oligo1 (lanes 4, 8, and 12). The specificity of Cdx protein binding to Oligo1 was assessed by the addition of a 100-fold excess of unlabeled Oligo1 probe (lanes 5, 9, and 13), a SIF1 positive control (lanes 6, 10, and 14), or an unrelated SP1 primer (lanes 7, 11, and 15) to the binding reactions. Comp., competitor; SIF1, sucrase-isomaltase factor 1.

we tested synthetic oligonucleotides containing the potential Cdx binding sequences by an EMSA with the three proteins. We identified a 25-bp double-stranded oligonucleotide named Oligo1, which spans two Cdx consensus sites, as the only target for Cdx protein binding (Fig. 3D, lanes 4, 8, and 12). Indeed, the other three consensus sites for *caudal*-related proteins located in the 3' half of the AvEc 164-bp fragment did not form any complexes with Cdx proteins (not shown). Cdx protein binding to Oligo1 was efficiently competed by the addition of a 100-fold excess of unlabeled probe (Fig. 3D, lanes 5, 9, and 13). The specificity of the interaction was further demonstrated by

use of the competitor SIF1, a 23-bp double-stranded DNA fragment of the sucrase-isomaltase promoter which was previously shown to interact with Cdx2 (Fig. 3D, lanes 6, 10, and 14) (59). Finally, additional support for the specificity of binding was provided by the addition of a 100-fold excess of an unrelated SP1 primer (Fig. 3D, lanes 7, 11, and 15). To compare the importance of each site for the formation of Cdx-Oligo1 complexes, we introduced three nucleotide substitutions into Cdx binding sites 1 and 2, either individually or in combination (Fig. 4A). While nucleotide substitutions in site 1 (mut1) or site 2 (mut2) did not alter the binding of the three

FIG. 4. Both Cdx sites are involved in the binding of Cdx proteins to Oligo1. (A) Nucleotide sequences of wild-type and mutant versions of Oligo1. The two potential Cdx binding sites, sites 1 and 2, are underlined, and the introduced mutations are shown with larger letters. (B) EMSA with Cdx proteins produced in vitro and with different versions of Oligo1. The three Cdx proteins were able to bind Oligo1 (lanes 2, 3, and 4), Oligo1 (mut1) (lanes 10, 11, and 12), and Oligo1 (mut2) (lanes 14, 15, and 16), but no binding was observed with Oligo1 (mut1, 2) (lanes 6, 7, and 8).

Cdx proteins to Oligo1 (Fig. 4B, lanes 2 to 4, 10 to 12, and 14 to 16), their combined mutations (mut1,2) in Oligo1 abolished the formation of specific binding complexes (Fig. 4B, lanes 6 to 8).

To further establish the functional significance of the two Cdx binding sites, we produced E12.5 F₀ *Hoxa5/lacZ* transgenic embryos to assess the effect on transgene expression of nucleotide substitutions at sites 1 and 2, either individually or in combination. These mutations were introduced into construct 1 to generate constructs 10, 11, and 12, respectively (Fig. 1B). Transgenic embryos for constructs 10 or 11 carrying individual mutations displayed the same regionally restricted expression pattern of *lacZ* staining as their cognate wild-type construct (Fig. 1C and 2A, J, and K). Expression in the urogenital system, the developing limb buds, and the gut was detected in some F₀ β-galactosidase-expressing specimens (Fig. 1C). In contrast, all F₀ β-galactosidase-expressing embryos carrying construct 12, with nucleotide substitutions in both binding sites, presented a lack of posterior restriction

along the prevertebral column (Fig. 1C and 2L). X-Gal staining was stronger in the cervical region and extended caudally. Altogether, these results indicated that the *Cdx* gene products can interact with the AvEc 164-bp fragment via two Cdx consensus binding sites confined within a 25-bp sequence and that this interaction may participate in vivo in the positioning of a posterior boundary of expression in the upper thoracic region.

***Hoxa5/lacZ* transgene expression in *Cdx* mutant backgrounds.** Experimental data have shown that the overexpression or loss of function of *Cdx* genes can affect *Hox/lacZ* transgene activity and directly regulates endogenous *Hox* gene expression upon interaction with Cdx binding sites in the flanking regions of *Hox* genes (13, 58, 62). To test the in vivo requirement of *Cdx1* or *Cdx2* gene function in the correct positioning of the *Hoxa5* expression domain, we verified whether the expression of construct 1 could be altered in *Cdx1* or *Cdx2* mutant embryos. To do so, we introduced the pLJ133 *Hox/lacZ* transgene (corresponding to construct 1) into the *Cdx1* or *Cdx2* mutant background by successive matings. E12.5

FIG. 5. Effect of *Cdx1* and *Cdx2* mutations on *Hoxa5/lacZ* transgene expression patterns. (A to C) pLJ57 transgene expression at E8.5. (D to F) pLJ57 transgene expression at E9.5. (H to J) pLJ57 transgene expression at E10.5. (K to M) pLJ133 (construct 1) expression at E12.5. The pLJ57 transgene reconstitutes *Hoxa5* endogenous temporal expression. No major change in expression profile was observed between wild-type (wt), *Cdx1*^{-/-}, and *Cdx2*^{+/-} embryos at E8.5, E9.5, and E10.5. At E12.5, the pLJ133 expression profile for wild-type embryos (K) was identical to that observed for *Cdx1*^{-/-} (L) and *Cdx2*^{+/-} (M) embryos. pLJ57 transgene expression was also analyzed in a *Cdx1*^{-/-} *Cdx2*^{+/-} compound background. No change in the expression profile was detected at E9.5 (G).

transgenic embryos carrying the *Hoxa5/lacZ* transgene and either the *Cdx1* or *Cdx2* mutant allele were generated and stained for β -galactosidase activity. In *Cdx1*^{+/-} pLJ133^{+/-} transgenic embryos, X-Gal staining was detected in the forelimb buds, the urogenital tract, and the cervical region of the prevertebral column, with a posterior limit at the upper thoracic level, similar to the staining obtained for construct 1 (Fig. 2A and 5K). The loss of *Cdx1* function did not affect the expression pattern of the transgene, and no posterior shift of the caudal boundary along the prevertebral column could be

observed for the majority of the specimens analyzed in the cohort (*Cdx1*^{-/-} pLJ133^{+/-}) (Fig. 5L). Although early embryonic lethality precluded the analysis of *Cdx2*^{-/-} mutants, the loss of one *Cdx2* allele causes alterations in vertebral identity at the anterior thoracic and posterior cervical levels (14). *Cdx2*^{+/-} pLJ133^{+/-} embryos displayed a *lacZ* staining profile similar to that of control and *Cdx1*^{-/-} pLJ133^{+/-} specimens (Fig. 5M), suggesting that the lack of both *Cdx1* alleles or one *Cdx2* allele does not affect the regional expression of construct 1.

Construct 1 can direct β -galactosidase activity in a narrow

window of time, from E11 to E13 (J. Lapointe and L. Jeanotte, unpublished data). To assess the effect of the *Cdx1* and *Cdx2* mutations on the expression pattern of the *Hoxa5/lacZ* transgene at earlier stages of embryogenesis, we introduced the pLJ57 transgene into both *Cdx* mutant backgrounds. The pLJ57 construct allows *lacZ* expression from E8.0 to E8.25 and onward, reproducing the endogenous temporal expression of the *Hoxa5* gene (36). E8.5, E9.5, and E10.5 transgenic embryos carrying both the pLJ57 transgene and the *Cdx1* or *Cdx2* mutant allele were analyzed. As reported for *Cdx* mutant embryos carrying the pLJ133 transgene, we did not detect noticeable variations in pLJ57 regional expression in the cohorts of *Cdx1*^{-/-} pLJ57^{+/+} and *Cdx2*^{+/-} pLJ57^{+/+} specimens at all embryonic ages analyzed (Fig. 5A to F and H to J). Since functional redundancy between the *Cdx1* and *Cdx2* genes can occur in *Hox* gene regulation (62), we also introduced the pLJ57 transgene into the *Cdx1*^{-/-} *Cdx2*^{+/-} mutant background. As shown for E9.5, no alteration of the transgene expression profile was noticed in *Cdx1*^{-/-} *Cdx2*^{+/-} pLJ57^{+/+} embryos (Fig. 5G). An examination of the *Hoxa5* endogenous expression pattern also failed to reveal any changes in *Cdx1*^{-/-} embryos, thus validating the transgene expression studies (not shown). Taken together, these results indicate that a loss of *Cdx1* function, a *Cdx2* haploinsufficiency, or a combination of both does not perturb the regional expression of the *Hoxa5* gene along the prevertebral column.

Cdx4-containing embryonic protein extracts form complexes with Oligo1. The lack of genetic evidence supporting the involvement of the *Cdx1* and *Cdx2* genes in the control of *Hoxa5* regional expression led us to evaluate whether the third member, *Cdx4*, could be implicated in *Hoxa5* gene regulation. Since no *Cdx4* mutant mouse line has been reported, we used a biochemical approach. First, we tested whether the *Cdx* gene products present in embryos possessed the ability to bind the Oligo1 sequence. We performed EMSAs with E9.5 embryo protein extracts and with Oligo1, Oligo1 (mut1), Oligo1 (mut2), and Oligo1 (mut1,2), used as probes (Fig. 6A). Proteins present in the extract were able to bind Oligo1, Oligo1 (mut1), and Oligo1 (mut2) (Fig. 6A, lanes 2, 6, and 10). In the last case, the different shift pattern suggested that other proteins may bind this mutated form. The specificity of binding was confirmed by competition studies with a 100-fold excess of unlabeled probe that resulted in the abolition of protein binding (Fig. 6A, lanes 3, 7, and 11). Moreover, the addition of a 100-fold excess of unlabeled Oligo1 (mut1,2) was unable to efficiently compete for protein binding (Fig. 6A, lanes 4, 8, and 12), suggesting that proteins from the embryo extract bind Oligo1 specifically via the two Cdx binding sites identified. An EMSA with Oligo1 (mut1,2) produced a specific pattern of bands revealing that different proteins can bind Oligo1 (mut1,2) (Fig. 6A, lane 14). This specificity of binding was confirmed by competition with a 100-fold excess of an unlabeled Oligo1 (mut1,2) probe, demonstrating that these proteins were indeed different from those binding Oligo1 (Fig. 6A, lane 15). The specificity of binding was also assessed by the addition of a 100-fold excess of the SP1 binding site (Fig. 6A, lane 16).

To test if the E9.5 embryo protein complexes that bound the Oligo1 sequence contained Cdx proteins, we performed supershift EMSAs with E9.5 embryo protein extracts, Oligo1, and

Cdx-specific antibodies. The specificity of each anti-Cdx antibody was assessed by EMSAs with the different Cdx proteins produced in vitro, as shown for the anti-Cdx4 antibody (Fig. 6B, lanes 5, 8, and 11). The addition of an anti-Cdx4 antibody to the embryo protein extract-Oligo1 reaction led to a near-complete loss of the most abundant Oligo1-protein complex (Fig. 6C, lanes 2 and 3). This may have been caused either by the inhibition of stable DNA-protein complex formation following antibody binding or by the strong alteration of the electrophoretic mobility of the DNA-protein-antibody complex. Such a result was not observed when an anti-Cdx1, anti-Cdx2, or control antibody (anticalsequestrin) was used (Fig. 6C, lane 4; not shown). Taken together, these results demonstrate that Cdx4 present in E9.5 embryo protein extracts can specifically bind the Oligo1 sequence. Thus, Cdx4 appears as a potential regulator of the correct positioning of the posterior boundary of *Hoxa5* expression in the upper thoracic region along the axial skeleton.

DISCUSSION

MES sequence directs *Hoxa5* expression in several structures. The correct development of structures from the cervical and upper thoracic axial level necessitates the proper control of *Hoxa5* gene expression. To define the mechanisms involved, we have refined our analysis of the MES region. In addition to directing expression in the skeleton at this axial level, the MES sequence encloses DNA elements in its 3' half that are able to target transgene expression in the limb buds and in the urogenital and intestinal tracts (Fig. 1). Although the present work did not focus on the delimitation of these tissue-specific elements, insights can be gained from our deletion analysis. For instance, gut expression seems to result from the equilibrium between positive and negative sequences, located between the AvrII (kb +4.28) and Eco47III (kb +4.44) restriction sites and the Eco47III (kb +4.44) and HindIII (kb +4.93) restriction sites, respectively. Additional *cis*-acting sequences located in the proximity of the *Hoxa4* gene are also required for gut expression, suggesting the coordinated action of several regulatory elements (42). As for the urogenital tract, we can infer that a combination of DNA elements, including the AvEc 164-bp fragment, is required for expression in this organ system. The control of limb bud expression also implicates interspersed elements in the XhoI (kb +4.04) to HindIII (kb +4.93) region. Our analysis clearly demonstrates that coordination between several *cis*-acting regulatory elements is needed to fully reproduce the correct spatial and temporal *Hoxa5* gene profile. Further work will be needed to finely map these additional regulatory sequences.

Positioning of *Hoxa5* posterior boundary depends on MES sequences. The definition of adequate boundaries is indispensable for proper *Hox* gene action. The functional limits of *Hoxa5*, as delineated by the axial skeletal phenotype of *Hoxa5* mutant mice, are restricted to the pv3-10 region (2, 29). Specific expression in this domain can be driven by an 892-bp XhoI-HindIII DNA fragment (constructs 6 and 7) (Fig. 1 and 2). Posterior restriction at the pv10 level is further delimited by the AvEc 164-bp fragment enclosed within the XhoI-HindIII fragment, which acts as a repressor. We have shown that the pv3-10 region corresponds to the specific expression do-

FIG. 6. In vivo detection of Cdx protein binding to Oligo1 sequence. (A) EMSA with E9.5 embryonic protein extract and different versions of Oligo1 (lanes 2, 6, 10, and 14). The specificity of binding to Oligo1 was assessed by the addition of a 100-fold excess of unlabeled Oligo1 probe (lane 3). Similar results were observed for binding to Oligo1 (mut1) and Oligo1 (mut2) when we used unlabeled Oligo1 (mut1) (lane 7) and Oligo1 (mut2) (lane 11), respectively, as competitors. The addition of a 100-fold excess of unlabeled Oligo1 (mut1,2) was unable to efficiently compete for protein binding (lanes 4, 8, and 12). An EMSA with Oligo1 (mut1,2) produced a specific pattern of bands that was competed by an unlabeled Oligo1 (mut1,2) probe (lanes 14 and 15), revealing that the binding proteins differed from those binding Oligo1. The Oligo1 (mut1,2) binding specificity was also assessed by the addition of a 100-fold excess of the SP1 binding site (lane 16). (B) EMSA with in vitro-translated Cdx proteins and the AvEc 164-bp fragment (lanes 4, 7, and 10). In the presence of an anti-Cdx4 antibody, a supershift was only observed with the Cdx4 protein (lanes 5, 8, and 11). No supershift was observed when a control antibody (anti-calsequestrin) was used (lanes 6, 9, and 12). Anti-Cdx1 and anti-Cdx2 antibodies also produced specific supershifts with their respective proteins (not shown). (C) EMSA with an Oligo1 probe and an E9.5 embryonic protein extract (lane 2). A near-complete loss of the most abundant Oligo1-protein complex was observed in the presence of the Cdx4 antibody (lane 3), but not when a control antibody was added (lane 4). Ab, antibody; Comp., competitor.

main of the 1.8-kb transcript (36). This transcript is also the sole *Hoxa5* transcript expressed in the mesenchyme of several structures that are affected by the mutation at this axial level, such as the respiratory tract, the thyroid gland, and the pectoral girdle (1, 2, 6, 40). Since this transcript is likely to carry the functional form of the *Hoxa5* gene, its expression domain must be tightly regulated. The transgenes used for the present study reflect the regulation of the proximal promoter from which the 1.8-kb transcript is produced, as the distal promoter, which resides in the vicinity of the *Hoxa7* gene and is responsible for transcription of the larger forms, was not included in the constructs (Y. Coulombe and L. Jeannotte, unpublished data). Consequently, this approach provides us with a useful means to elucidate the regulatory mechanisms governing *Hoxa5* regional expression in its functional domain.

As proposed by Kmita and Duboule (31), axial patterning relies upon a given partitioning of *Hox* functional domains along the developing anterior-posterior axis. The concept of posterior prevalence states that posteriorly expressed *Hox* genes functionally prevail over more anterior genes. Hence, the functional readout of the *Hox* overlapping transcription domains may reflect the prominent function of the most caudal *Hox* protein produced in a particular region (16). Several strategies may be envisaged to explain the suppressive mechanism by which a posterior *Hox* gene imposes its functional dominance (31). In the present case, a transcription-based partitioning of *Hoxa5* gene function may account for the restriction of the functional domain to the pv3-10 area. Thus, the control provided by the AvEc 164-bp fragment may be an important regulatory event in cervicothoracic axial specification through the constraint of the *Hoxa5* expression domain. The *Hoxb5* gene also displays a pattern of expression that represents the sum of multiple *Hoxb5* transcripts (34). The *Hoxb5* mutation exerts its effect in the most anterior expression domain of the gene, a region where the *Hoxb5* protein is specifically found (51, 53). One may speculate that *Hoxb5* gene expression is transcriptionally regulated similarly to its paralog *Hoxa5* and that the *Hoxb5* protein expression domain reflects the distribution of the *Hoxb5* major transcript. On the other hand, posttranscriptional events whereby a *Hox* transcript is selectively destabilized in its posterior domain may be used to confine *Hox* gene expression, as demonstrated for the *Hoxb4* gene (11). These strategies may coexist, but we do not have evidence that posttranscriptional control occurs in *Hoxa5* spatial regulation (S. Tabariès and L. Jeannotte, unpublished data). While much emphasis has been given to the regulation of the establishment and maintenance of the anterior boundary of expression (e.g., see references 17, 22, 32, and 46), our present work underscores the importance of defining posterior boundaries as well.

Cdx gene products participate in restriction of *Hox* gene expression. *caudal*-related genes are involved in anterior-posterior patterning by regulating members of the *Hox* gene family in various species (13, 18, 26, 28, 41, 58). Our demonstration that Cdx proteins physically interact with *Hoxa5* regulatory sequences and that they likely participate in the definition of the *Hoxa5* functional domain further confirms the importance of Cdx genes in axial patterning via *Hox* gene regulation. While Cdx proteins are mainly recognized for their role as positive regulators of *Hox* gene expression (13, 58, 62), our data show

that murine Cdx proteins may also act to restrict *Hox* gene expression. This was also shown to be the case for *Drosophila*, in which *caudal* acts as a repressor of the *Abd-B* homeotic gene during analia structure formation (43). Moreover, the fact that *Xcad* overexpression in *Xenopus* embryos leads to the repression of anterior *Hox* genes while posterior *Hox* genes exhibit more anterior expression substantiates the notion of a repressive function for Cdx proteins (19, 28). Taken together, these data suggest that *caudal*-related gene products possess dual functions of posterior-promoting and anterior-suppressing activities toward *Hox* gene expression, as proposed by Isaacs and colleagues (28). This dual role would allow Cdx proteins to differentially modulate the expression of subsets of *Hox* genes, resulting in the definition of specific *Hox* functional domains that in turn would set up the correct posterior development of the organism. For *Hoxa5*, the binding of Cdx proteins to the AvEc 164-bp fragment would preclude its action posterior to the pv10 limit. However, *Hoxa5* posterior restriction also depends on other regulators since the mutation of both Cdx binding sites did not result in a gain of posterior expression of the transgene that was as strong and as distal as that obtained for some of the deletions tested (construct 12 versus construct 4, 5, or 8) (Fig. 2D, E, H, and L). Recent data also support the notion that additional sequences located in the 3' extremity of the MES region may cooperate with the AvEc 164-bp fragment for *Hoxa5* regional expression (Tabariès and Jeannotte, unpublished data). Finally, since Cdx proteins may act as intermediates of retinoic acid, Wnt, and fibroblast growth factors (38), it remains to be defined if these signaling molecules impinge on the correct setting of *Hoxa5* functional boundaries via Cdx transduction.

Despite the fact that the three murine Cdx proteins possess the ability to bind in vitro the *Hoxa5* AvEc 164-bp fragment, Cdx4 emerged as the potential in vivo *trans*-acting factor for *Hoxa5* regulation. The Cdx4 protein was also shown to regulate *Hoxb8* expression (13). The Cdx1 and Cdx2 proteins can likely be excluded from *Hoxa5* regulation based on biochemical and genetic evidence. The absence of a modification of the *Hoxa5* expression pattern along the prevertebral column of single or compound *Cdx1* and *Cdx2* mutants was unanticipated considering that the homeotic transformations observed in *Cdx* mutants encompassed the functional domain of *Hoxa5*. However, although similarities exist between some of the skeletal transformations seen in *Hoxa5* and *Cdx* mutants, most of the vertebrae affected do not display the same type of transformation. For instance, the predominant homeotic defect observed in *Hoxa5* mutant mice is the posteriorization of the 7th cervical vertebra (C7) into a thoracic one. In contrast, in *Cdx* mutants, C7 adopts a C6 identity (2, 14, 29, 58, 62). Moreover, for *Cdx2* heterozygous specimens, we could not exclude the possibility that enough Cdx2 protein remained to ensure proper *Hoxa5* regulation. Unfortunately, to our knowledge, no *Cdx4* mutant mouse line is available to genetically address the importance of *Cdx4* in *Hoxa5* gene regulation.

The three Cdx proteins are expressed in the embryo according to a gradient with a posterior maximum (38). Of these, Cdx4 is the most posteriorly expressed, and its expression ceases at E10.5 (20). Construct 1 and its derivatives did not reproduce the temporal expression pattern of the *Hoxa5* endogenous gene, as they directed expression in a narrow time

frame, from E11 to E13 (64; Lapointe and Jeannotte, unpublished data). Therefore, the X-Gal staining pattern observed in E12.5 F₀ transgenic embryos was likely due to an earlier regulatory event directed by Cdx4. Indeed, *in vivo* detection of Cdx4 protein binding occurred with E9.5 embryo extracts but not with specimens from older embryos. Altogether, these data suggest that Cdx4 may be involved in the establishment of the correct *Hoxa5* posterior boundary at the upper thoracic level.

In summary, our work gives insights into the mechanisms by which Cdx proteins, most likely Cdx4, directly participate in the posterior delimitation of the *Hoxa5* functional domain. The data favor the view that a transcription-based partitioning of *Hox* gene function is a strategy for the establishment of posterior prevalence in the specification of the cervical-upper thoracic axial level.

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REFERENCES

- Aubin, J., M. Lemieux, M. Tremblay, J. Bérard, and L. Jeannotte. 1997. Early postnatal lethality in *Hoxa-5* mutant mice is attributable to respiratory tract defects. *Dev. Biol.* **192**:432–445.
- Aubin, J., M. Lemieux, M. Tremblay, R. R. Behringer, and L. Jeannotte. 1998. Transcriptional interferences at the *Hoxa4/Hoxa5* locus: importance of correct *Hoxa5* expression for the proper specification of the axial skeleton. *Dev. Dyn.* **212**:141–156.
- Aubin, J., P. Chailier, D. Ménard, and L. Jeannotte. 1999. Loss of *Hoxa5* gene function in mice perturbs intestinal maturation. *Am. J. Physiol.* **277**:C965–C973.
- Aubin, J., and L. Jeannotte. 2001. Implication des gènes *Hox* dans les processus d'organogenèse chez les mammifères. *Médecine/Science* **17**:54–62.
- Aubin, J., U. Déry, M. Lemieux, P. Chailier, and L. Jeannotte. 2002. Stomach regional specification requires *Hoxa5*-driven mesenchymal-epithelial signaling. *Development* **129**:4075–4087.
- Aubin, J., M. Lemieux, J. Moreau, J. Lapointe, and L. Jeannotte. 2002. Cooperation of *Hoxa5* and *Pax1* genes during formation of the pectoral girdle. *Dev. Biol.* **244**:96–113.
- Behringer, R. R., D. A. Crotty, V. M. Tennyson, R. L. Brinster, R. D. Palmiter, and D. J. Wolgemuth. 1993. Sequences 5' of the homeobox of the *Hox-1.4* gene direct tissue-specific expression of *lacZ* during mouse development. *Development* **117**:823–833.
- Béland, M., N. Pilon, M. Houle, K. Oh, J.-R. Sylvestre, P. Prinos, and D. Lohnes. 2004. *Cdx1* autoregulation is governed by a novel Cdx1-LEF1 transcription complex. *Mol. Cell. Biol.* **24**:5028–5038.
- Bel-Vialar, S., N. Itasaki, and R. Krumlauf. 2002. Initiating *Hox* gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the *HoxB* genes in two distinct groups. *Development* **129**:5103–5115.
- Bradshaw, M. S., C. S. Shashikant, H.-G. Belting, J. A. Bollekens, and F. H. Ruddle. 1996. A long-range regulatory element of *Hoxa8* identified by using pCluster vector. *Proc. Natl. Acad. Sci. USA* **93**:2426–2430.
- Brend, T., J. Gilthorpe, D. Summerbell, and P. W. Rigby. 2003. Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of *Hoxb4* expression. *Development* **130**:2717–2728.
- Chambeyron, S., and W. A. Bickmore. 2004. Chromatin decondensation and nuclear reorganization of the *HoxB* locus upon induction of transcription. *Genes Dev.* **18**:1119–1130.
- Charité, J., W. de Graaff, D. Consten, M. J. Reijnen, J. Korving, and J. Deschamps. 1998. Transducing positional information to the *Hox* genes: critical interaction of *cdx* gene products with position-sensitive regulatory elements. *Development* **125**:4349–4358.
- Chawengsaksophak, K., R. James, V. E. Hammond, F. Kontgen, and F. Beck. 1997. Homeosis and intestinal tumours in *Cdx2* mutant mice. *Nature* **386**:84–87.
- Dony, C., and P. Gruss. 1987. Specific expression of the *Hox1.3* homeobox gene in murine embryonic structures originating from or induced by the mesoderm. *EMBO J.* **6**:2965–2975.
- Duboule, D., and G. Morata. 1994. Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.* **10**:358–364.
- Dupé, V., M. Davenne, J. Brocard, P. Dollé, M. Mark, A. Dierich, P. Chambon, and F. M. Rijli. 1997. *In vivo* functional analysis of the *Hoxa-1* 3' retinoic acid response element (3'RARE). *Development* **124**:399–410.
- Ehran, L. A., and K. E. Yutzey. 2001. Anterior expression of the *caudal* homologue *cCdx-B* activates a posterior genetic program in avian embryos. *Dev. Dyn.* **221**:412–421.
- Epstein, M., G. Pillemer, R. Yelin, J. K. Yisraeli, and A. Fainsod. 1997. Patterning of the embryo along the anterior-posterior axis: the role of the *caudal* genes. *Development* **124**:3805–3814.
- Gamer, L. W., and C. V. Wright. 1993. Murine *Cdx-4* bears striking similarities to the *Drosophila caudal* gene in its homeodomain sequence and early expression pattern. *Mech. Dev.* **43**:71–81.
- Gaunt, S. J., P. L. Coletta, D. Pravtcheva, and P. T. Sharpe. 1990. Mouse *Hox-3.4*: homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. *Development* **109**:329–339.
- Gérard, M., J. Y. Chen, H. Gronemeyer, P. Chambon, D. Duboule, and J. Zakany. 1996. *In vivo* targeted mutagenesis of a regulatory element required for positioning the *Hoxd-11* and *Hoxd-10* expression boundaries. *Genes Dev.* **10**:2326–2334.
- Gould, A., A. Morrison, G. Sproat, R. A. White, and R. Krumlauf. 1997. Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping *Hox* expression patterns. *Genes Dev.* **11**:900–913.
- Hogan, B., R. S. Beddington, F. Constantini, and E. Lacy. 1994. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Houle, M., J. R. Sylvestre, and D. Lohnes. 2003. Retinoic acid regulates a subset of *Cdx1* function *in vivo*. *Development* **130**:6555–6567.
- Hunter, C. P., J. M. Harris, J. N. Maloof, and C. Kenyon. 1999. *Hox* gene expression in a single *Caenorhabditis elegans* cell is regulated by a caudal homolog and intercellular signals that inhibit wnt signaling. *Development* **126**:805–814.
- Ikeya, M., and S. Takada. 2001. Wnt-3a is required for somite specification along the anteroposterior axis of the mouse embryo and for regulation of *cdx-1* expression. *Mech. Dev.* **193**:27–33.
- Isaacs, H. V., M. E. Pownall, and J. M. Slack. 1998. Regulation of *Hox* gene expression and posterior development by the *Xenopus* caudal homologue *Xcad3*. *EMBO J.* **17**:3413–3427.
- Jeannotte, L., M. Lemieux, J. Charron, F. Poirier, and E. J. Robertson. 1993. Specification of axial identity in the mouse: role of the *Hoxa-5* (*Hox1.3*) gene. *Genes Dev.* **7**:2085–2096.
- Kmita, M., N. Fraudeau, Y. Héroult, and D. Duboule. 2002. Serial deletions and duplications suggest a mechanism for the collinearity of *Hoxd* genes in limbs. *Nature* **420**:145–150.
- Kmita, M., and D. Duboule. 2003. Organizing axes in time and space; 25 years of colinear tinkering. *Science* **301**:331–333.
- Knittel, T., M. Kessel, M. H. Kim, and P. Gruss. 1995. A conserved enhancer of the human and murine *Hoxa-7* genes specifies the anterior boundary of expression during embryonal development. *Development* **121**:1077–1088.
- Kondo, T., and D. Duboule. 1999. Breaking colinearity in the mouse *HoxD* complex. *Cell* **97**:407–417.
- Krumlauf, R., P. W. H. Holland, J. H. McVey, and B. L. M. Hogan. 1987. Developmental and spatial patterns of expression of the mouse homeobox gene *Hox2.1*. *Development* **99**:603–617.
- Krumlauf, R. 1994. *Hox* genes in vertebrate development. *Cell* **78**:191–201.
- Larochelle, C., M. Tremblay, D. Bernier, J. Aubin, and L. Jeannotte. 1999. Multiple *cis*-acting regulatory regions are required for restricted spatiotemporal *Hoxa5* gene expression. *Dev. Dyn.* **214**:127–140.
- Lickert, H., C. Domon, G. Huls, C. Wehrle, I. Duluc, H. Clevers, B. I. Meyer, J. N. Freund, and R. Kemler. 2000. Wnt/(beta)-catenin signaling regulates the expression of the homeobox gene *Cdx1* in embryonic intestine. *Development* **127**:3805–3813.
- Lohnes, D. 2003. The Cdx1 homeodomain protein: an integrator of posterior signaling in the mouse. *Bioessays* **25**:971–980.
- Margalit, Y., S. Yarus, E. Shapira, Y. Gruenbaum, and A. Fainsod. 1993. Isolation and characterization of target sequences of the chicken *Cdx4* homeobox gene. *Nucleic Acids Res.* **21**:4915–4922.
- Meunier, D., J. Aubin, and L. Jeannotte. 2003. Perturbed thyroid morphology and transient hypothyroidism symptoms in *Hoxa5* mutant mice. *Dev. Dyn.* **227**:367–378.
- Mlodzik, M., G. Gibson, and W. J. Gehring. 1990. Effects of ectopic expression of *caudal* during *Drosophila* development. *Development* **109**:271–277.
- Moreau, J., and L. Jeannotte. 2002. Sequence analysis of a *Hoxa4-Hoxa5* intergenic region including shared regulatory elements. *DNA Seq.* **13**:203–209.
- Moreno, E., and G. Morata. 1999. Caudal is the *Hox* gene that specifies the most posterior *Drosophila* segment. *Nature* **400**:873–877.
- Morrison, A., L. Ariza-McNaughton, A. Gould, M. Featherstone, and R.

- Krumlauf. 1997. *HOXD4* and regulation of the group 4 paralog genes. *Development* **124**:3135–3146.
45. Nowling, T., W. Zhou, K. E. Krieger, C. Larochelle, M. C. Nguyen-Huu, L. Jeannotte, and C. K. Tuggle. 1999. *Hoxa5* gene regulation: a gradient of binding activity to a brachial spinal cord element. *Dev. Biol.* **208**:134–146.
 46. Oosterveen, T., K. Niederreither, P. Dollé, P. Chambon, F. Meijlink, and J. Deschamps. 2003. Retinoids regulate the anterior expression boundaries of 5' *Hoxb* genes in posterior hindbrain. *EMBO J.* **22**:262–269.
 47. Papenbrock, T., R. L. Peterson, R. S. Lee, T. Hsu, A. Kuroiwa, and A. Awgulewitsch. 1998. Murine *Hoxc-9* gene contains a structurally and functionally conserved enhancer. *Dev. Dyn.* **212**:540–547.
 48. Pöpperl, H., M. Bienz, M. Studer, S. K. Chan, S. Aparicio, S. Brenner, R. S. Mann, and R. Krumlauf. 1995. Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell* **81**:1031–1042.
 49. Pownall, M. E., A. S. Tucker, J. M. Slack, and H. V. Isaacs. 1996. eFGF, *Xcad3* and *Hox* genes form a molecular pathway that establishes the antero-posterior axis in *Xenopus*. *Development* **122**:3881–3892.
 50. Prinos, P., S. Joseph, K. Oh, B. I. Meyer, P. Gruss, and D. Lohnes. 2001. Multiple pathways governing *Cdx1* expression during murine development. *Dev. Biol.* **239**:257–269.
 51. Rancourt, D. E., T. Tsuzuki, and M. R. Capecchi. 1995. Genetic interaction between *hoxb-5* and *hoxb-6* is revealed by nonallelic noncomplementation. *Genes Dev.* **9**:108–122.
 52. Rivera-Pomar, R., X. Lu, N. Perrimon, H. Taubert, and H. Jackle. 1995. Activation of posterior gap gene expression in the *Drosophila* blastoderm. *Nature* **376**:253–256.
 53. Sharpe, J., S. Nonchev, A. Gould, J. Whiting, and R. Krumlauf. 1998. Selectivity, sharing and competitive interactions in the regulation of *Hoxb* genes. *EMBO J.* **17**:1788–1798.
 54. Shashikant, C. S., C. J. Bieberich, H. G. Belting, J. C. Wang, M. A. Borbely, and F. H. Ruddle. 1995. Regulation of *Hoxc-8* during mouse embryonic development: identification and characterization of critical elements involved in early neural tube expression. *Development* **121**:4339–4347.
 55. Spitz, F., F. Gonzalez, and D. Duboule. 2003. A global control region defines a chromosomal regulatory landscape containing the *HoxD* cluster. *Cell* **113**:405–417.
 56. Stein, S., R. Fritsch, L. Lemaire, and M. Kessel. 1996. Checklist: vertebrate homeobox genes. *Mech. Dev.* **55**:91–108.
 57. Studer, M., H. Pöpperl, H. Marshall, A. Kuroiwa, and R. Krumlauf. 1994. Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265**:1728–1732.
 58. Subramanian, V., B. I. Meyer, and P. Gruss. 1995. Disruption of the murine homeobox gene *Cdx1* affects axial skeletal identities by altering the mesodermal expression domains of *Hox* genes. *Cell* **83**:641–653.
 59. Suh, E., L. Chen, J. Taylor, and P. G. Traber. 1994. A homeodomain protein related to caudal regulates intestine-specific gene transcription. *Mol. Cell. Biol.* **14**:7340–7351.
 60. Taylor, J. K., T. Levy, E. R. Suh, and P. G. Traber. 1997. Activation of enhancer elements by the homeobox gene *Cdx2* is cell line specific. *Nucleic Acids Res.* **25**:2293–2300.
 61. Tuggle, C. K., J. Zakany, L. Cianetti, C. Peschle, and M. C. Nguyen-Huu. 1990. Region-specific enhancers near two mammalian homeobox genes define adjacent rostrocaudal domains in the central nervous system. *Genes Dev.* **4**:180–189.
 62. van den Akker, A. E., S. Forlani, K. Chawengsaksophak, W. de Graaff, F. Beck, B. I. Meyer, and J. Deschamps. 2002. *Cdx1* and *Cdx2* have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* **129**:2181–2193.
 63. Wang, W., and B. A. Malcolm. 1999. Two-stage PCR protocol allowing introductions of multiple mutation deletions and insertions using quick-change site-directed mutagenesis. *BioTechniques* **26**:680–682.
 64. Zakany, J., C. K. Tuggle, M. D. Patel, and M. C. Nguyen-Huu. 1988. Spatial regulation of homeobox gene fusions in the embryonic central nervous system of transgenic mice. *Neuron* **1**:679–691.
 65. Zakany, J., M. Gérard, B. Favier, and D. Duboule. 1997. Deletion of a *HoxD* enhancer induces transcriptional heterochrony leading to transposition of the sacrum. *EMBO J.* **16**:4393–4402.