Functional specificity of the Hoxa13 homeobox

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SUMMARY

To better define Abd-B type homeodomain function, to test models that predict functional equivalence of all Hox genes and to initiate a search for the downstream targets of Hoxa13, we have performed a homeobox swap by replacing the homeobox of the Hoxa11 gene with that of the Hoxa13 gene. The Hoxa11 and Hoxa13 genes are contiguous Abd-B type genes located at the 5' end of the HoxA cluster. The modified Hoxa11 allele (A1113hd) showed near wild-type function in the development of the kidneys, axial skeleton and male reproductive tract, consistent with functional equivalence models. In the limbs and female reproductive tract, however, the A1113hd allele appeared to assume dominant Hoxa13 function. The uterus, in particular, showed a striking homeotic transformation towards cervix/vagina, where Hoxa13 is normally expressed. Gene chips were used to create a molecular portrait of this tissue conversion and revealed over 100 diagnostic gene expression changes. This work identifies candidate downstream targets of the Hoxa13 gene and demonstrates that even contiguous Abd-B homeoboxes have functional specificity.

Key words: Hox, Hoxa13, Hoxa11, Homeobox, Functional specificity, Abd-B, Downstream targets, Mouse

INTRODUCTION

There remain important questions concerning the functional specificity of the mammalian Hox gene encoded proteins. What are their functional relationships? Do they regulate the same, overlapping, or completely distinct sets of downstream targets? The clustered homeobox (Hox) genes encode transcription factors with the DNA binding homeodomain. In Drosophila, mutations of these genes can result in remarkable homeotic transformations of body parts, suggesting distinct patterning functions. In mammals, however, Hox mutations generally result in more-subtle phenotypes, with reduced or absent structures. This has prompted some to propose a difference in Hox gene function in mammals and flies. It has been stated that 'even a wing to haltere transformation cannot be explained by solely differential proliferation of the same cell types. Conversely, the majority of transformations observed (so far) in mutant mice are unlikely to derive from the selection of alternative developmental programs; they may not even engage the regulation of qualitatively different target genes.' (Duboule, 1995), and 'a “horizontal” scheme may be at work, in which developmental decisions are taken by integrating quantitative inputs rather than by relying on genes’ individuality' (Duboule, 2000). In other words, quantity of mammalian Hox gene expression may be more important than quality. In its extreme form, this model states that all mammalian Hox genes regulate identical or functionally equivalent downstream cell proliferation target genes. According to this model, the apparent axial segment homeotic transformations sometimes observed in Hox mutant mice are interpreted as resulting from changes in the sculpted shapes of the vertebral bones caused by altered cell proliferation rates (Duboule, 1995). They are viewed as the result of perturbations of cell proliferation patterns, rather than changes in segment identity. The functional equivalence model is consistent with the observed common DNA recognition sequences of homeodomain proteins identified by in vitro DNA-binding assays (Desplan et al., 1988; Hoey and Levine, 1988), and with the ability of Hox genes to sometimes act as oncogenes (Blatt et al., 1988; Blatt and Sachs, 1988; Perkins and Cory, 1993; Raza-Egilmez et al., 1998; Care et al., 1999). Furthermore, in several cases, Hox downstream targets have now been shown to be involved in the regulation of cell proliferation (Care et al., 1996; Bromleigh and Freedman, 2000; Raman et al., 2000).

The evidence arguing for functional equivalence of paralogous mammalian Hox genes is particularly strong. Paralogs reside at equivalent positions within different Hox clusters and are derived by evolutionary gene duplication from a single ancestral Hox gene. They generally encode very similar homeodomains, and show overlapping gene expression patterns. Gene targeting experiments have shown striking functional redundancy for paralogs. For example, mice mutant for either Hoxa11 or Hoxd11 show no kidney defects and only mild limb defects (Small and Potter, 1993; Davis and Capecchi, 1994). But mice mutant for both these paralogs show absent or rudimentary kidneys, and have the ulna and radius of the forearm reduced to less than one tenth of normal size (Davis et al., 1995). In addition, a Hoxa11-expressing transgene has been shown to be able to rescue Hoxa11 loss of function (Zakany et al., 1996). Furthermore, complete coding sequence exchanges between the Hoxa3 and Hoxd3 paralogs have indicated that their proteins carry out identical biological
functions (Greer et al., 2000). These results have been interpreted to support the functional equivalence model (Duboule, 2000).

Although it has been argued by some that the *Drosophila* paradigm may not apply to mammalian *Hox* gene function, it is nevertheless useful to consider what has been learned from that system. In *Drosophila* there is one homotic complex (HomC) of genes, which represents a single split cluster. Ectopic expression experiments have shown that different HomC genes can induce very distinct developmental destinies. For example, misexpression of *Antennapedia* can cause imaginal disc cells that would normally form antennae to instead give rise to legs, protruding from the head (Schneuwly et al., 1987). And misexpression of *Ultrabithorax* can result in the homeotic transformation of wing into haltere (Lewis, 1982). The combinatorial code expression of HomC genes has been proposed to determine segment identity (Lewis, 1978). The apparently distinct functional specificities of different HomC genes have several sources. First, the encoded homeodomains are not functionally equivalent. Homeobox swap experiments in *Drosophila* show that developmental function often tracks with the homeobox, suggesting the presence of in vivo target sequence binding specificity not detected by in vitro DNA-binding assays (Kuziora and McGinnis, 1989; Gibson et al., 1990; Mann and Hogness, 1990). Second, target specificity is influenced by co-factor interactions. Different Exd-HomC protein heterodimers, for example, have distinct DNA-binding specificities, even when measured in vitro (Chan et al., 1994; Mann and Chan, 1996), and there is evidence for similar gains in specificity in mammalian systems (Popperl et al., 1995; Chan et al., 1997). Third, different HomC proteins may possess activation or repression domains, which can determine developmental function by their distinct impacts on the same target gene expression. Fourth, according to the activity regulation model, binding sites for specific combinations of co-factors in a given promoter would confer activation or repression effects (Li et al., 1999). This is consistent with the observation that individual HomC proteins are often able to repress some target genes and activate others, as measured by both genetic and transfection assays (Vachon et al., 1992; Capovilla et al., 1994; Saffman and Krasnow, 1994). In summary, a number of mechanisms exist in *Drosophila* for providing individual HomC proteins with functional specificity. It would seem reasonable to suppose that similar molecular processes might be used in mammals.

Nevertheless, even in *Drosophila* there is considerable evidence for functional overlap of HomC genes. For example, misexpression of *Ubx*, *Abd-A* or *Abd-B* can cause cells that would normally form wing to form haltere instead (Casares et al., 1996). This is particularly informative, as *Abd-A* and *Abd-B* are normally only involved in development of the abdomen, which has no wings or halteres. It has also been shown that a hybrid *Ubx*-VP16, with enhanced transcription activation function, mimics *Antp* in developmental specificity, presumably by regulating the same set of downstream targets (Li and McGinnis, 1999). Furthermore, several promoter analysis studies suggest that the HomC genes regulate overlapping sets of downstream target genes (Manak et al., 1994; Mastick et al., 1995). These observations suggest that the differences in function between the *Drosophila* HomC and mammalian Hox genes are less pronounced than proposed by the mammalian Hox functional equivalence model. Perhaps the *Drosophila* HomC genes are not each functionally unique, and perhaps the mammalian Hox genes are not all functionally the same.

To address multiple issues of homeodomain function, including the question of functional specificity, we performed a mammalian homeobox swap experiment. The homeobox of the *Hoxa11* gene was precisely replaced with that of the *Hoxa13* gene. The *Hoxa11* and *Hoxa13* genes are closely related *Abd-B* type genes. In a broad sense they can be considered paralogs, as they are derived from a common ancestral *Abd-B* Hox gene. The functional equivalence model proposes that these two genes have identical or functionally equivalent downstream targets, and therefore predicts that the swapped allele would have wild-type function. This was indeed observed in the developing kidneys, male reproductve tract and axial skeleton. Striking mutant phenotypes were seen, however, in the limbs and female reproductive tract. Of particular note, in mice with the swapped allele, the uterus underwent a homeotic transformation towards cervix/vagina, as determined by both histology and gene chip analysis of gene expression profiles. This homeotic transformation indicates a patterning function for Hox genes. The altered gene expression profiles identify candidate *Hoxa13* downstream targets. The results indicate that the *Hoxa11* and *Hoxa13*-encoded homeodomains are not functionally equivalent, and that in some developing tissues the *Hoxa11* allele with a swapped *Hoxa13* homeobox assumed a *Hoxa13* developmental function.

**MATERIALS AND METHODS**

**Targeting construct**

An 11 kb *SpeI-*SpeI111 genomic fragment subcloned in pBS SK was cut with *Nhel*, releasing a 9 kb segment with both *Al1* exons. The original clone, with the 9 kb *Nhel* fragment removed, was ligated to re-circularize, and *loxP-PGKNeo-loxP* was subcloned into the unique *Fse* site 1.1 kb 3′ of the second *Al1* exon and herpes simplex virus thymidine kinase (*mc1HSV-4K*) was subcloned into the unique *SalI* sequence in the multiple cloning site, giving construct I. The released 9 kb *Nhel* segment was subcloned into a modified pBS, with the multiple cloning site *XhoI* and *BstXI* sites removed, giving construct II. This clone had unique *XhoI* and *BstXI* sites flanking the *Al1* second exon with the homeobox. This *XhoI*-BstXI segment was subcloned into pBS, giving the A11(XhoI-BstXI) vector.

Two new restriction sites, *HincII* and *PstI* were introduced at the junction regions of the *Al1* homeobox of the *XhoI*-BstXI segment by *PCR* mutagenesis (Bi and Stambrook, 1998). The primers used were: XhoI primer 5′ to 3′, TCCTCTGCCACCTCCCCACctcgagAGAGC-TGG; HincII primer 5′ to 3′, TCCTTCCATTAGGTTgcaacGACC-C-GAAAA:  

(BstXI+PstI) primer 5′ to 3′, CCAATTCagtagctggAGCCTTACAGAAGTGGATTAGCTAGTActgcaAGCCGTCTC- (TGT,)....

Lowercase letters represent restriction enzyme sites, underlines indicate silent mutation sites, bold letters represent homeobox region and parenthesized letters represent sequence not included in the primer sequence.

The *Al1* homeobox was PCR amplified from strain 129 DNA using the following primers.
$A13\ HincII$ primer: ACCCGAGCTCCTgtcaacGGGGGAGAAAG-A;
$A13\ PsI$ primer: CCATTACTAGTcatcgacCTGGTCCTGATGACTTTTCTCT.

The A13 homeobox PCR product was digested with $HincII$ and $PsI$ and subcloned into the $A11/\text{Xhol-BSTXI}$ vector, also cut with $HincII$ and $PsI$, replacing the A11 homeobox. This Xhol-BSTXI segment was then subcloned into Xhol, BSTXI cleaved construct II, and the 9 kb $Nhel$ segment of the modified construct II was then subcloned back into $Nhel$ cut construct I, making the final targeting construct. The construct was confirmed by DNA sequencing.

**Gene targeting**

The targeting constructs were introduced into ES cell lines E14.1 and R1 (Hooper et al., 1987; Nagy et al., 1993). Targeted clones were enriched by positive-negative selection and identified by Southern blot and PCR analysis. DNA sequencing confirmed the precise homeobox swap. Four out of 100 R1 clones and 16 out of 300 E14 clones were enriched by positive-negative selection and identified by Southern blot and PCR analysis. DNA sequencing confirmed the precise homeobox swap. Four out of 100 R1 clones and 16 out of 300 E14 clones were

**RESULTS**

**Targeting construct**

We performed a homebox swap experiment to examine the functional specificity of $Abd-B$ type homeboxes, in order to better define their normal developmental function and to search for candidate downstream targets. The Abd-B type $Hoxa11\ (A11)$ and $Hoxa13\ (A13)$ genes are located at the 5’ end of the HoxA cluster and encode homeodomains identical at 36 of 60 amino acids (Fig. 1A,B). The homebox swap was made with a replacement targeting construct consisting of the $A11$ gene with a precise $A13$ homebox replacement. A $Neo$ gene flanked by $loxP$ sequences was placed 1.1 kb 3’ of the $A11$ gene (Fig. 1C). After electroporation into ES cells, correct targeting was confirmed by Southern blot, PCR and sequencing (Fig. 2). The $Neo$ gene was removed by breeding to Cre-expressing mice, leaving the replaced homebox and the 34 bp $loxP$ sequence 3’ of the $A11$ gene. To test for possible effects of the $loxP$ insertion, we also made an $A11$ allele with the $loxP$ flanked $Neo$ inserted at the same 3’ position, but without the homebox swap. Of interest, the presence of $Neo$, even at this 3’ position, inactivated the $A11$ gene. Cre mediated removal of $Neo$, leaving only $loxP$, restored full wild-type A11 function (data not shown).

$Hoxd11^{+/−}\ (D11^{+/−})$ mice provided the most sensitive measure of A11 function. The paralogous A11 and D11 genes are functionally redundant in the development of the axial skeleton, limbs, kidneys and reproductive tracts (Davis et al., 1995). Mice with $A11^{+/−}$ or $D11^{+/−}$ mutations, for example, have fairly normal development of the kidney and forelimb zeugopod (ulna and radius), but in $A11^{+/−}\ D11^{+/−}$ double homozygous mutants the kidneys are absent or severely reduced in size, and the zeugopod of the forelimb is almost entirely missing.

The A11 gene with an A13 homebox provided near wild type function in the development of the kidneys, male reproductive tract and axial skeleton $A11^{+/−}\ D11^{+/−}$ double mutant mice commonly suffer perinatal death from kidney failure (Davis et al., 1995). The presence of one wild-type allele ($A11^{+/−}\ D11^{+/−}$ or $A11^{−}/D11^{−}−$) restores

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**Histology**

Tissues were fixed in 4% paraformaldehyde overnight, dehydrated and then embedded in paraffin. Kidneys were sectioned frontally (5 μm) and stained using a Periodic Acid Schiff (PAS) kit (Sigma). Testes were stained with Hematoxylin and Eosin. Female reproductive tracts were sectioned and also stained with Hematoxylin and Eosin.

**Alizarin staining of adult skeletons**

Skeletals of 4-week-old animals were prepared and stained as previously described (Selby, 1987; Small and Potter, 1993).

**Affymetrix gene chip analysis**

Uterus tissue was from the uterus horn above the uterus horn joint junction and below the uterus-oviduct junction, and cervix tissue was collected from the region below the uterus corpus and above the vaginal hymen. RNA was prepared using RNAlol reagent (Tel-test). Preparation of biotinylated RNA, hybridization, washing, staining and scanning of Affymetrix GeneChip probe arrays were carried out according to Affymetrix protocols. Data was analyzed with Affymetrix software.

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The homeobox swapped $A11^{13hd}$ allele also provided wild-type function in the development of the male reproductive tract. $A11^{-/-}$ $D11^{+/+}$ males are generally sterile with undescended testes and a ductus deferens that is anteriorized to resemble the epidydimis (Hsieh-Li et al., 1995). $A11^{13hd/13hd}$ $D11^{+/+}$ and $A11^{13hd/-}D11^{++}$ males were fertile, with descended testes and mature sperm in seminiferous tubules (Fig. 4A), and their ductus deferens lacked the tortuosity typical of the anteriorized ductus deferens of the $A11^{-/-}D11^{+/+}$ mouse (Hsieh-Li et al., 1995; Fig. 4B).

$A11^{-/-}D11^{+/+}$ mutants showed posteriorization of the 13th thoracic segment into the first lumbar, and anteriorization of the first sacral segment to a sixth lumbar (Small and Potter, 1993). Interestingly, in all six $A11^{13hd/13hd}$ $D11^{+/+}$ mice examined the axial skeleton was normal, without anteriorization or posteriorization (data not shown).

In summary, the $A11^{13hd}$ allele provided apparent wild-type function in the development of the kidney, male reproductive tract and axial skeleton, confirming that the targeted allele produces functional mRNA and protein. These observations are consistent with models predicting that all Hox-encoded proteins bind identical or functionally equivalent downstream targets. However, in examining the limbs and female reproductive tracts, quite different results were obtained.

The $A11^{13hd}$ allele provided antagonizing function in the development of the limbs

In the developing hindlimb, the $A11^{13hd}$ allele antagonized normal $A11$, $D11$ function. The $A11$ gene is expressed in the zeugopod (tibia and fibula in the hindlimb), while the $A13$ gene is expressed more distally, in the autopod (paw; Yokouchi et al., 1991; Haack and Gruss, 1993; Small and Potter, 1993). At least five mice were examined for each of the following genotypes: $A11^{13hd+/+}$, $A11^{13hd+}D11^{+/+}$, $A11^{13hd+}D11^{-/-}$, $A11^{13hd+}D11^{++}$, $A11^{13hd+}D11^{+/+}$, $A11^{13hd+}D11^{+/-}$ and $A11^{13hd+}D11^{++}$. The $A11^{13hd}$ allele gave more severe phenotypes than $A11^{-/-}$. The $A11^{-/-}D11^{+/+}$ hindlimb was the same as wild type on the genetic background used in these studies. In the zeugopod region of the hindlimb, the $A11^{13hd+/+}$ $D11^{+/+}$ mutants showed a distinct separation of the distal tibia and fibula, similar to that seen in the $A11^{-/-}D11^{+/+}$ mice, and the $A11^{13hd+}D11^{+/+}$ hindlimbs showed an even more pronounced separation (Fig. 5A). In additional allele combination genotypes, a substitution of the $A11^{-/-}$ allele with the $A11^{13hd}$ allele consistently resulted in more severe separation of the distal tibia and fibula (Fig. 5B and data not shown). In the autopod of the hindlimb, the talus and calcaneous bones of $A11^{-/-}D11^{+/+}$ mice appeared normal, whereas for $A11^{13hd+/+}$ $D11^{+/+}$ mice the talus was malformed (not shown) and the calcaneous truncated to the length of the talus (Fig. 5C). All allele combinations with at least one $A11^{13hd}$ showed calcaneous truncation, whereas in the absence of $A11^{13hd}$ the calcaneous appeared normal in all allele combinations, except for $A11^{-/-}D11^{-/-}$, in which the calcaneous was shortened and also fused with the fibula (data not shown). The penetrance of the truncated calcaneous phenotype increased with increasing dosage of $A11^{13hd}$ and to a lesser extent, $A11^{-/-}$ and $D11^{-/-}$ alleles. For example, the truncated calcaneous was seen in 1/14 (7%) of $A11^{13hd+/+}$ $D11^{+/+}$, in 4/12 (33%) of $A11^{13hd+/+}$ $D11^{+/+}$, in 8/11 (72%) of $A11^{13hd+/+}$ $D11^{-/-}$ and in 10/10 (100%) of $A11^{13hd+}$...
The A11 -wild type. C, cortex; M, medulla. severely reduced in size and disrupted by cysts (asterisk) when compared with the (A) Gross appearances of wild type, A11+/+ D11+/+ and A1113hd/D11+/+ kidneys. The A1113hd/D11+/+ kidneys shown are among the least affected, coming from one of the two mice that survived to P30. One kidney was severely reduced in size, with the other more normal. The A1113hd/D11+/+ kidneys appeared grossly normal except for a reproducible indentation on the left kidney (arrow). (B) Kidney histology. Top and middle panels: the A1113hd/D11+/+ kidneys appeared relatively normal compared with the A11+/+ D11+/+ kidneys, which showed many occluded proximal tubule (P) lumens (long arrows) and severely dilated distal (D) tubules (arrowheads). Short arrows point to glomeruli. Bottom panels: the medulla layer of both the A11+/+ D11+/+ kidney and the A1113hd/D11+/+ kidney (not shown) was severely reduced in size and disrupted by cysts (asterisk) when compared with the wild type. C, cortex; M, medulla.

**Fig. 3.** The A1113hd allele provided near normal function in kidney development. (A) Gross appearances of wild type, A11+/+ D11+/+ and A1113hd+/+ D11+/+ kidneys. The A11+/+ D11+/+ kidneys shown are among the least affected, coming from one of the two mice that survived to P30. One kidney was severely reduced in size, with the other more normal. The A1113hd+/+ D11+/+ kidneys appeared grossly normal except for a reproducible indentation on the left kidney (arrow). (B) Kidney histology. Top and middle panels: the A1113hd+/+ D11+/+ kidneys appeared relatively normal compared with the A11+/+ D11+/+ kidneys, which showed many occluded proximal tubule (P) lumens (long arrows) and severely dilated distal (D) tubules (arrowheads). Short arrows point to glomeruli. Bottom panels: the medulla layer of both the A11+/+ D11+/+ kidney and the A1113hd+/+ D11+/+ kidney (not shown) was severely reduced in size and disrupted by cysts (asterisk) when compared with the wild type. C, cortex; M, medulla.

The A1113hd allele assumes A13 function in development of the female reproductive tract

The A1113hd allele caused a partial homeotic transformation of the uterus to the more posterior cervix/vagina. The A11 gene is expressed in the developing uterus and cervix, while expression of A13 is more posteriorly restricted, to the cervix and vagina (Taylor et al., 1997; Post and Innis, 1999). Mice examined were 4.5 weeks of age, and estrous cycle matched (Laboratory, 1966). The lining of the wild-type uterus consists of a single layer of columnar epithelial cells, while the lining of the wild-type cervix is many cells thick, making a squamous epithelium. In contrast to A11+/+ D11+/+ mice, in the A1113hd+/+ D11+/+ mutants the uterine lining resembled that of the wild-type cervix (Fig. 7, arrows). This transformation extended throughout most of the uterus, with the columnar to squamous transition normally present at the

D11−/− mice. All mice with two A1113hd alleles showed the truncated calcaneus phenotype.

The A1113hd allele also had antagonizing effects on forelimb development. Although the A1113hd+/+ D11+/+ forelimb was normal, the A1113hd+/+ D11+/+ forelimb was more severely malformed than the A11+/+ D11+/+ forelimb. In the zeugopod region, the ulna and radius were about one half of normal length, somewhat resembling the three allele null mutant A11−/− D11−/−, although with distinctive shapes (Fig. 6A). The styloid apophyses, which were only very mildly affected in A11−/− D11+/+ mutants were reduced and/or fused to the ulna and radius in A1113hd/D11+/+ mice (Fig. 6B, small arrows), approaching the severity of the A1113hd−/− D11−/− or A11−/− D11−/− mutants (Fig. 6B, large arrows). In addition, similar to the hindlimb zeugopod, a substitution of the A11− allele with the A1113hd allele in other allele combinations generally resulted in more severely shortened and malformed forelimb zeugopod (data not shown), with the exception of the A1113hd−/− D11−/− mice, which had relatively more normal forelimb development than the A11−/− D11−/− mice (Fig. 6A). This exception could reflect some normal limb development function of the A1113hd allele, which remained hidden in the presence of wild-type A11 or D11 alleles. Alternatively, the null A11 mutation, with a deletion and an insertion of a PGK-Neo, may have produced subtle alterations in the expression patterns of flanking Hox genes not present with the A1113hd allele during limb development. It is interesting to note that ectopic expression of Hoxa13 or Hoxd13 in the chick zeugopod (Yokouchi et al., 1995; Goff and Tabin, 1997), or ectopic expression of Hoxd13 in the mouse zeugopod (van der Hoeven et al., 1996; Herault et al., 1997; Peichel et al., 1997) also results in shortening of this limb element, similar to the effect observed for A1113hd.

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uterus-cervix junction in wild-type mice shifted anterior to near the uterus-oviduct junction in \( A11^{13hd/13hd} D11^{+/+} \) mutants. The stromal layer of the mutant uterus also approximated that of the wild-type cervix, with lower cell density and more fibrous tissue (Fig. 7, asterisks). The \( A11^{13hd/13hd} D11^{+/+} \) mutants also lacked uterine glands and were sterile. While \( A11^{-/-} D11^{+/+} \) mice are fertile, females with even a single \( A11^{13hd} \) allele were missing uterine glands and sterile. It was therefore necessary to carry the homeobox swapped \( A11 \) gene with the Neo insertion, which gave a null recessive phenotype, and then to remove the Neo by germline Cre activity in the last step of breeding. Because of \( A11^{13hd/-} \) female infertility, it was extremely difficult to make mice with the \( A11^{13hd/13hd} D11^{-/-} \) genotype.

The homeotic transformation of the \( A11^{13hd/13hd} D11^{+/+} \) uterus was confirmed at the molecular level with Affymetrix.

**Fig. 4.** Wild-type function of \( A11^{13hd} \) in the male reproductive tract. (A) Sperm were present in the lumens of wild-type and \( A11^{13hd/13hd} D11^{+/+} \) seminiferous tubules (arrows), but not in \( A11^{-/-} D11^{+/+} \) testis. (B) The \( A11^{13hd/13hd} D11^{+/+} \) ductus deferens showed a wild-type morphology, while \( A11^{-/-} D11^{+/+} \) mutants showed a coiled configuration resulting from an anteriorization towards epididymis (arrows). E, epididymis; T, testis; V, vas deferens.

**Fig. 5.** \( A11^{13hd} \) hindlimb phenotype. (A) The arrowhead points to the normal tibia and fibula fusion point and broad arrow points to the calcaneus bone in wild-type hindlimb. Mice of \( A11^{13hd/+} D11^{+/+} \) or \( A11^{-/-} D11^{+/+} \) genotypes showed similar more distal separation of tibia and fibula, while \( A11^{13hd/13hd} D11^{+/+} \) mice showed more extreme tibia, fibula separation (thin arrows) when compared with wild type. (B) Substitution of the \( A11^{-/-} \) allele by an \( A11^{13hd} \) allele in either \( A11^{+/+} D11^{+/+} \) or \( A11^{13hd/-} D11^{+/+} \) mutants resulted in greater separation of tibia and fibula (arrows). (C) Ventral views of isolated autopods of hindlimbs. \( A11^{13hd/13hd} D11^{+/+} \) mutants had a severely truncated calcaneus compared with wild type (arrows). \( A11^{-/-} D11^{+/+} \) mutants were normal. c, calcaneus; F, fibula; T, tibia.
The Hoxa13 homeobox gene chip probe arrays. Murine genome U74A gene chips, with approximately 12,000 genes, were used to measure gene expression levels in the 4.5-week-old wild-type uterus, wild-type cervix and A113hd/13hdD11+/+ uterus. The resulting molecular fingerprints were then used to determine if the A113hd/13hd D11+/+ uterus was shifted towards the cervix in character. The transcript profile of the mutant uterus created a detailed molecular portrait showing clear posteriorization. Over 30 genes normally expressed in the cervix but not the uterus were found transcribed in the A113hd/13hd D11+/+ uterus. This list contained several keratin genes associated with squamous epithelium, including K16, K6α, K6β, K14 and, notably, K13, a marker of the ectocervical epithelium in the female reproductive tract (Gorodeski et al., 1990), and several other genes of interest (see Appendix). Other genes, normally expressed in the wild-type uterus and absent in cervix, were inactive in the mutant uterus, again consistent with posteriorization to cervix. This list included KAP and calbindin-28, both of which show estrogen responsive expression in the normal uterus (Meseguer et al., 1989; Gill and Christakos, 1995; Runic et al., 1996), and the decysin gene, which encodes a metalloprotease (Mueller et al., 1997). In addition to these qualitative on/off differences there were many quantitative changes in the mutant uterus gene expression levels diagnostic of posteriorization (Appendix). Hoxa13 expression was not detected in either the mutant uterus or wild-type uterus. In total, comparison of mutant uterus versus wild-type uterus gave 108 genes with over tenfold difference. The two lists of differently expressed genes shared 54 genes, or about half, consistent
with the incomplete homeotic transformation of mutant uterus to cervix observed at the level of histology.

DISCUSSION

In this study, we precisely replaced the homeobox of the Hoxa11 gene with the homeobox from the Hoxa13 gene. The major conclusion is that even for these two closely related ABD-B type genes, the homeoboxes were not functionally equivalent.

In the kidney, male reproductive tract and axial skeleton, the A11\textsuperscript{13hd} allele provided near wild-type function, consistent with models predicting identical or functionally equivalent downstream target genes for all Hox encoded proteins. In the developing limbs, however, the homeobox swapped A11 gene gave antagonizing function. This could result from a dominant negative effect, with the A11\textsuperscript{13hd} encoded protein binding to the same downstream gene targets as for A11/D11, but with opposite effect (e.g. repression versus activation). Alternatively, the A11\textsuperscript{13hd} protein could bind to different targets, perhaps those of A13, leading to distinct developmental outcome. Of interest, according to the ‘posterior prevalence’ rule in Drosophila, Hox genes located at more 5’ positions in the Hox clusters, and expressed in more posterior domains, are dominant over more 3’ genes. This rule predicts that the more 5’A13 gene, or a homeobox swapped A11 gene with A13 function, would be dominant over a wild-type A11 gene. It has been shown that ectopic expression of Hoxa13 or Hoxd13 (but not Hoxa4) in the zeugopod region in the chick resulted in zeugopod truncation (Yokouchi et al., 1995; Goff and Tabin, 1997). In addition, ectopic expression of Hoxd13 in the developing zeugopod in mice also resulted in reduction of this limb element (van der Hoeven et al., 1996; Herault et al., 1997; Peichel et al., 1997), similar to what we observed in mutants with the swapped A11 gene. This suggests that the A11\textsuperscript{13hd} allele assumed Hoxa13 function in the limb and antagonized function of the group 11 Hox genes, probably using the same mechanism that controls the posterior prevalence phenomenon in normal development. It is notable that severe zeugopod truncation was observed in the forelimbs but not in the hindlimbs in our mutants. This could be due to a quantitative insufficiency of antagonizing activity in the hindlimb, as an additional group 11 Hox gene, Hoxc11, is normally expressed in the hindlimb, but not forelimb (Peterson et al., 1994; Hostikka and Capecchi, 1998).

It is interesting to note that possible antagonizing interactions between the paralogous group 13 and 11 Hox genes were also previously observed in kidney development. Insertion of a Hoxd9lacZ construct into the 5’ region of the HoxD complex causes ectopic expression of Hoxd13, resulting in kidney agenesis that resembles the agenesis found in mice without A11 and D11 function (Kmita et al., 2000). This result contrasts with our observation that the A11\textsuperscript{13hd} allele drives near normal kidney development. The most likely explanation is that the induced misexpression of Hoxd13 does not properly recapitulate normal Hox group 11 expression in terms of cell type, timing and expression levels, therefore perturbing rather than promoting kidney development. It is also possible that expression of the entire Hoxd13 protein gives different developmental consequences in the kidney than expression of the A11 protein with a group 13 swapped homeodomain.

The homeotic transformation of uterus towards cervix/vagina clearly indicated that in this tissue the A11\textsuperscript{13hd} allele assumed A13 function. The Hox code model of Lewis (Lewis, 1978) predicts that Hox null mutations will result in anteriorizations and Hox ectopic expression will drive posteriorizations. Null mutations of A11 and A10 have been previously reported to anteriorize the uterus towards oviduct (Satokata et al., 1995; Gendron et al., 1997), and mutation of A13 anteriorizes the cervix/vagina towards uterus (Post and Innis, 1999). The A11\textsuperscript{13hd} allele appeared to effectively give ectopic expression of A13 function in the uterus, causing it to posteriorize to cervix/vagina, where the A13 gene is normally expressed. These results suggest patterning function for Hox genes in the development of the female reproductive tract.

Distinct segment identity functions have also been defined for Hox genes in the developing rhombomeres of the mammalian hindbrain. Both misexpression (Alexandre et al., 1996; Bell et al., 1999) and targeted mutation (Chisaka et al., 1992; Carpenter et al., 1993; Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1997; Gavalas et al., 1998; Rossel and Capecchi, 1999) studies support a Hox code model. For example, ectopic expression of Hoxb1 results in the homeotic transformation of rhombomere 2 to rhombomere 4 (Bell et al., 1999). These results are consistent with those described in this report, and are again difficult to reconcile with models predicting that Hox gene function is restricted to the regulation of cell proliferation.

The genes altered in expression in the mutant uterus are candidate downstream targets of the A13 gene. The single initial difference between wild type and mutant developing uteri was the presence of the A11\textsuperscript{13hd} allele. Expression of this swapped homeobox gene dramatically shifted the gene expression profile of the mutant uterus towards that of the cervix/vagina. The differently expressed genes therefore represent the combination of direct and indirect targets of the A11\textsuperscript{13hd} allele. Moreover, as the identity of the mutant uterus is shifted towards the cervix/vagina, where A13 is normally expressed and as the A11\textsuperscript{13hd} and A13 alleles encode identical homeodomains, these genes are also excellent downstream target candidates for A13 itself. Many of the target genes appear to have functions not related to the regulation of cell proliferation.

It is interesting to note that mutation of Hoxc13, a paralog of A13, gives a hairless mouse (Godwin and Capecchi, 1998). It has been suggested that ‘Hoxc13 could directly control transcription of hair keratin genes’ (Godwin and Capecchi, 1998). The A13 and Hoxc13 genes encode homeodomains identical in 55 out of 60 amino acids. The observed increased expression of a number of keratin genes in the A11\textsuperscript{13hd/13hd} uterus further indicates that the Hox genes of the 13 paralogous group can regulate keratin genes.

It has previously been reported that the Hoxa3 and Hoxd3 encoded proteins are functionally equivalent (Greer et al., 2000). This added to evidence indicating strong functional redundancy between Hox paralogs (Condie and Capecchi,
1994; Davis et al., 1995; Horan et al., 1995; Fromental-Ramain et al., 1996a; Fromental-Ramain et al., 1996b). In this report, however, we show that even the homeoboxes of two contiguous Abd-b type Hox genes are not functionally interchangeable in all developing tissues.

APPENDIX

Proteins present in A113hd/13hd D11+/+ uterus and wild-type cervix, but absent from wild-type uterus

Keratins
K1.13, K2.6a, K2.6β, K1.14, K1.16, K2.1,K2.4

Cornified cell envelope components
SPRR1b protein, SPRP 3, Repetin, Loricrin

Transcription factors
Sine oculis-related homeobox 1 (six1)
HNF-3/forkhead homolog 1 like (HFH-II)
E74-like factor 5 (Elf5)

Desmosomal adhering junction proteins
Plakophilin 1, Desmocollin 2

Others
p73H (p53 related protein)
Maspin (tumor suppressor)
Apoptosis signal-regulating kinase 2 (ASK2)
Neuropsin (extracellular protease)
Maspin (tumor suppressor)
p73H (p53 related protein)

Accession numbers
AA727291 +37-fold +28-fold
M32486 +6-fold +14-fold
AW228162 +15-fold +13-fold
AA854029 +17-fold +11-fold
AI844853 Absent
AI813446 +10-fold +8-fold
AA85344 +4-fold +7-fold

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REFERENCES


Care, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani,


Peterson, R. L., Papenbrook, T., Davda, M. M. and Awgulewitsch, A.


