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Expression of the murine *Hoxa4* gene requires both autoregulation and a conserved retinoic acid response element

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SUMMARY

Analysis of the regulatory regions of the *Hox* genes has revealed a complex array of positive and negative *cis*-acting elements that control the spatial and temporal pattern of expression of these genes during embryogenesis. In this study we show that normal expression of the murine *Hoxa4* gene during development requires both autoregulatory and retinoic acid-dependent modes of regulation. When introduced into a *Hoxa4* null background, expression of a *lacZ* reporter gene driven by the *Hoxa4* regulatory region (*Hoxa4/lacZ*) is either abolished or significantly reduced in all tissues at E10.5-E12.5. Thus, the observed autoregulation of the *Drosophila Deformed* gene is conserved in a mouse homolog in vivo, and is reflected in a widespread requirement for positive feedback to maintain *Hoxa4* expression. We also identify three potential retinoic acid response elements in the *Hoxa4* 5′ flanking region, one of which is identical to a well-characterized element flanking the *Hoxd4* gene. Administration of retinoic acid to *Hoxa4/lacZ* transgenic embryos resulted in stage-dependent ectopic expression of the reporter gene in the neural tube and hindbrain. When administered to *Hoxa4* null embryos, however, persistent ectopic expression was not observed, suggesting that autoregulation is required for maintenance of the retinoic acid-induced expression. Finally, mutation of the consensus retinoic acid response element eliminated the response of the reporter gene to exogenous retinoic acid, and abolished all embryonic expression in untreated embryos, with the exception of the neural tube and prevertebrae. These data add to the evidence that *Hox* gene expression is regulated, in part, by endogenous retinoids and autoregulatory loops.

Key words: *Hoxa4*, Retinoic acid, Retinoic acid response element, Autoregulation, Transgenic mice

INTRODUCTION

In mammals there are 39 *Hox* genes organized into four clusters homologous to the homeotic genes of *Drosophila melanogaster* (for reviews see McGinnis and Krumlauf, 1992; Krumlauf, 1994; Maconochie et al., 1996). These genes encode a family of transcription factors essential for axial and appendicular patterning (for review, see Krumlauf, 1993; Maconochie et al., 1996) and organogenesis (Manley and Capecchi, 1995; Davis et al., 1995; Hsieh-Li et al., 1995). It is well established that *Hox* genes exhibit specific temporal and spatial patterns of expression during embryogenesis, with genes at the 3′ end of each cluster being expressed earlier and in more anterior parts of the embryo than genes at the 5′ end (for a review see Duboule and Morata, 1994). As mice carrying targeted mutations in particular *Hox* genes have been generated, it has become clear that these precise expression patterns have important consequences for *Hox* gene function. The deletion of a *Hox* gene generally results in malformations in structures located at that gene's anterior boundary of expression, suggesting that the establishment of the axial pattern of *Hox* gene expression in a partially overlapping array is critical for normal development (for a review see Manak and Scott, 1994).

Given the evolutionary conservation and important functional role of *Hox* genes, there has been intense interest in identifying upstream factors that regulate *Hox* gene expression. For a few *Hox* genes, sequences have been identified that promote reporter gene expression in transgenic mice in patterns that approximate those of the endogenous genes (Puschel et al., 1991; Whiting et al., 1991; Gerard et al., 1993; Shashikant and Ruddle, 1996). In previous work we identified a regulatory region for the murine *Hoxa4* gene (Behringer et al., 1993) which contains at least four potential binding sites for the HOXA4 protein (Wu and Wolgemuth, 1993), raising the possibility that HOXA4 itself is required to maintain its own expression. An autoregulatory component to *Hox* gene regulation was initially identified in studies of the *Drosophila Deformed* (*Dfd*) gene, which requires both epidermal (Bergson and McGinnis, 1990) and neural (Lou et al., 1995) autoregulatory enhancers for persistent embryonic expression. That this mode of regulation might be conserved among the murine *Dfd* homologs was suggested by the finding that the *Dfd* epidermal autoregulatory element could direct reporter gene expression to a specific region in the postotic

mouse hindbrain with the correct anterior boundary (Awgulewitsch and Jacobs, 1992). Moreover, a human *HOXB4* regulatory element was found to promote spatially localized expression in the head region of *Drosophila* embryos (Malicki et al., 1992), again suggesting the presence of an evolutionarily conserved autoregulatory circuit. Finally, a putative autoregulatory element of the murine *Hoxd4* gene has been identified in vitro by transient expression assays in embryonal carcinoma cells (Popperl and Featherstone, 1992).

Apart from auto- and cross-regulation of *Hox* gene expression, a significant body of evidence now suggests that *Hox* genes are also major targets of endogenous retinoids during embryogenesis (for a review see Means and Gudas, 1995). Mice lacking retinoic acid receptors α and γ exhibit transformations of cervical vertebrae similar to those observed in mice lacking one or more *Hox* genes (Lohnes et al., 1994). In addition, administration of all-*trans* retinoic acid to pregnant mice results in a rostral expansion of *Hox* gene expression domains in embryos (Kessel and Gruss, 1991; Conlon and Rossant, 1992; Frasch et al., 1995; Morrison et al., 1996). In at least a few cases this effect is likely to be a direct one, as retinoic acid response elements (RAREs) have been identified in regions flanking the *Hoxd4* (Popperl and Featherstone, 1993), *Hoxa1* (Langston and Gudas, 1992; Frasch et al., 1995), and *Hoxb1* (Marshall et al., 1994; Langston et al., 1997) genes. A direct effect has been confirmed for the *Hoxa1* gene. In transgenic mice carrying a *lacZ* reporter gene driven by a *Hoxa1* enhancer containing a mutated version of the RARE, expression is lost in the neuroepithelium caudal to rhombomere 4 (Frasch et al., 1995). Strikingly, when this RARE is deleted by a gene targeting strategy, the result is a partial phenocopy of the *Hoxa1* total knockout, with the mice exhibiting rhombomere and cranial nerve abnormalities (Dupé et al., 1997).

Given the precedents for both autoregulation and retinoic acid-dependent regulation of *Hox* genes, we asked in the present study whether these modes of regulation are involved in specifying *Hoxa4* expression in vivo. To examine *Hoxa4* autoregulation we crossed two previously characterized lines of mice: one a transgenic line that carries a *lacZ* reporter gene driven by the *Hoxa4* regulatory region (Behringer et al., 1993), the other a mutant for *Hoxa4* (Horan et al., 1994), in order to examine the expression of the transgene in a *Hoxa4* null background. Our results indicate that autoregulation is essential for maintaining *Hoxa4* expression in vivo in various parts of the embryo. In addition, sequencing of the *Hoxa4* 5′ regulatory region revealed three potential RAREs, one of which is identical to the *Hoxd4* RARE. Administration of retinoic acid to pregnant transgenic females resulted in a stage-dependent, *Hoxa4*-dependent anterior shift in reporter gene expression, suggesting that retinoids and autoregulatory loops cooperate to activate and maintain *Hoxa4* expression. Finally, mutagenesis of the best consensus RARE eliminated both ectopic reporter gene expression in response to exogenous retinoic acid and reporter gene expression in the gut, lung, metanephros and mesonephros in untreated embryos.

MATERIALS AND METHODS

Mice

Details of the generation of the *Hoxa4/lacZ* transgenic and *Hoxa4* mutant lines have been described (Behringer et al., 1993; Horan et al., 1994). For timed matings, the day of the detection of the vaginal plug was considered embryonic day (E) 0.5, and the developmental stage was determined according to Theiler (1972). Genotyping of *Hoxa4* mutant embryos was carried out by Southern analysis using a *Hoxa4* digoxigenin-labeled probe (Horan et al., 1994). For detection of reporter gene expression, embryos were fixed, stained with X-gal (Bachem), and processed for histology as described previously (Behringer et al., 1993).

Retinoic acid treatment

All-*trans* retinoic acid (Sigma) was dissolved in dimethyl sulfoxide at a concentration of 25 mg/ml and stored in the dark at –20°C. Immediately before use, a sample was thawed and diluted in sesame oil in order to deliver 20 mg/kg body mass in a volume of 200 μl by oral gavage. Control mice were treated with an equal volume of sesame oil alone.

Sequencing

Sanger dideoxy sequencing with fluorescent dyes was carried out by the DNA Synthesis and Sequencing Facility of the Comprehensive Cancer Center of Columbia University using an Applied Biosystems 373A DNA sequencer.

Mutagenesis

Each of the 12 nucleotides in the direct repeats of the –2.9 RARE was mutated by PCR site-directed mutagenesis (Fig. 1A) (Weiner and Costa, 1996). Two oligonucleotide primers were synthesized by the Protein Chemistry Core Facility of Columbia University. The first primer (5′-CTAGTCACTTCCTCGAGGCGCGTCTTACAAAT-3′) carries the mutated RARE, with a diagnostic *Xho*I site (underlined) replacing the second direct repeat. Also included in the PCR was a complementary, non-mutagenic primer (5′-CGGCCTTGGACTTTG-3′). The template in the PCR was pE-1, which consists of a p2484-derived (see Fig. 1) 1.8 kb *Kpn*I-*Xba*I fragment (containing the consensus RARE) inserted into Bluescript KS(+) (Stratagene). 30 cycles of the PCR were carried out, with each cycle at 94°C for 1 minute, 52°C for 1 minute and 72°C for 3 minutes. The methylated parental plasmid was then digested with *Dpn*I, and the unmethylated *Dpn*I-resistant PCR product was blunt-ended with Vent DNA polymerase (New England BioLabs) and religated. A 1.1 kb *Xba*I fragment from p2484 was replaced by the same fragment from the pE-1 PCR product in order to regenerate p2484 containing the mutant –2.9 RARE. Transgenic mice carrying this construct were generated as described (Behringer et al., 1993).

RESULTS

Autoregulation of *Hoxa4* in vivo

The *Hoxa4* regulatory region under study consists of approximately 3.8 kb of 5′ flanking sequence, the first exon of the coding region, the intron, and the second exon, excluding sequences 3′ of the *Eco*RI site in the homeobox (Fig. 1A). This sequence, when linked to a *lacZ* reporter gene, has been shown to be sufficient to recapitulate the expected pattern of *Hoxa4* expression in transgenic embryos (Behringer et al., 1993). To ask whether *Hoxa4* is subject to autoregulation in vivo, we introduced the *Hoxa4/lacZ* transgene into a *Hoxa4* null background, using our previously generated line of mice in which the *Hoxa4* gene had been deleted by gene targeting (Horan et al., 1994).

At E10.5, the pattern of reporter gene expression in the *Hoxa4* null embryo (Fig. 2B) appeared to be grossly similar to that seen in a *Hoxa4* heterozygous embryo (Fig. 2A). Prominent

sites of expression at this time include the neural tube with an anterior boundary about 600 μm caudal to the otic vesicle, the spinal ganglia, the prevertebrae with an anterior boundary at the second cervical vertebra, the gut and the mesonephros. When the embryos were sectioned, however, it was clear that there was significantly less expression of the *Hoxa4/lacZ* transgene in the gut and mesonephros of the *Hoxa4* null embryo (Fig. 2C,D), suggesting that HOXA4 protein is required to maintain its own transcription at these sites. The anterior boundaries of neuroectodermal and mesodermal expression appeared to be normal. At E11.5, the difference in *Hoxa4*/*lacZ* expression was more dramatic, with the *Hoxa4* null embryo exhibiting no expression in the spinal ganglia, prevertebrae, somites, gut, lung and mesonephros, and less intense expression in the neural tube (Fig. 2E,F). At E12.5, we observed a loss of expression in the peripheral nerves between the precursors of the ribs, as well as in all of the sites that were affected at E11.5 with the exception of the prevertebrae, where some expression was restored (Fig. 2G-J). These results indicate that autoregulation is essential for maintaining normal levels of *Hoxa4* expression in the embryo.

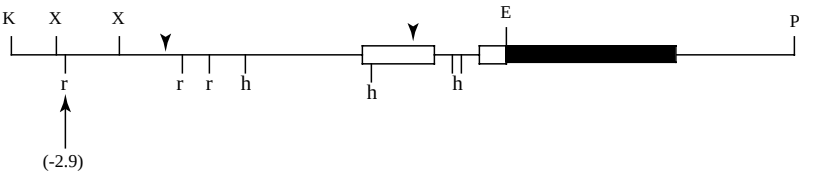
Retinoic acid-induced ectopic expression of *Hoxa4* in the neural tube and hindbrain

Examination of the sequence of the 5′ flanking region, in an attempt to identify other regulators of *Hoxa4*, revealed three putative RAREs (Fig. 1A,B) at 2.9, 1.8 and 1.4 kb upstream of the translation start site. The two most proximal RAREs (not shown) exhibit the least identity with the RAREs flanking the *Hoxa1* (Frasch et al., 1995), *Hoxb1* (Studer et al., 1994), and *Hoxd4* (Popperl and Featherstone, 1993) genes. In contrast, the direct repeats of the distal RARE at –2.9 are identical to those in the *Hoxd4* RARE (Fig. 1B).

To test whether there is a role for retinoic acid in regulating *Hoxa4* expression in vivo, we administered all-*trans* retinoic acid (ATRA) by oral gavage to pregnant *Hoxa4/lacZ* transgenic females at various times during gestation and examined the expression of the reporter gene. Initially, ATRA was administered to pregnant transgenic females at E8.5, and the embryos were isolated 18 hours later, approximately at E9.25. In order to be precise about the developmental timing of *Hoxa4* sensitivity to retinoic acid, we staged all of the embryos by morphological criteria according to Theiler (1972), as well as by gestational age. An embryo treated at E8.5 and examined approximately at E9.25, Theiler stage 15 (T15), exhibited no significant alteration of reporter gene expression, except for a rather diffuse anterior boundary in the neural tube, suggesting that *Hoxa4* might be weakly sensitive to ATRA at this stage (Fig. 3A).

In contrast, a series of embryos treated with ATRA approximately at E9.5 and examined 18 hours later, approximately at E10.25 (T16-T17) exhibited a clear rostral shift in *Hoxa4/lacZ* expression, the extent of which varied according to the developmental stage. At T17, an untreated embryo has an anterior boundary of reporter gene expression caudal to the otic vesicle (Fig. 3B). At T16, an ATRA-treated embryo exhibited an anterior boundary at the base of the otic vesicle (Fig. 3C), rostral to its normal position. An ATRA-treated embryo that had reached a slightly more advanced stage of development (T17) after 18 hours exhibited reporter gene expression up to and including the otic vesicle, with an additional stripe of expression in a more rostral region of the hindbrain (Fig. 3D). While the normal boundary of *Hoxa4* expression in the hindbrain is at the rhombomere 6/7 (r6/7) junction and is not reached at least until E10.5-E11 (T17-T18) (Galliot et al., 1989; Behringer et al., 1993), coronal sections through the hindbrain of the ATRA-treated embryo at T17 revealed ectopic expression of the reporter gene in r6, r4, and in the ventral region of r5 (Fig. 3F,G). Ectopic expression in r4 was observed at T19 as well (Fig. 3E).

A



B

***Hox* gene retinoic acid response elements**

AGGTCA G T	N	AGGTCA G T	consensus (N=1, 2, or 5)	
AGGTGA	ACTTC	AGGTCA	(11/12)	Hoxa4 (-2.9)
AGGTGA	ACTTC	AGGTCA	(11/12)	HOXA4 (-3.1)
AGGTGA	AATGC	AGGTCA	(11/12)	Hoxd4 (-2.5)
GGTTCA	CCGAA	AGGTCA	(12/12)	Hoxa1
GGTTCA	TAGAG	AGTTCA	(11/12)	Hoxb1 (3' DR5)
AGGTGA	AA	AGGTCA	(11/12)	Hoxb1 (3' DR2)

C

AGGTGA	ACTTC	AGGTCA	(11/12)	Hoxa4 (-2.9)
CTAGTC	ACTTC	CTCGAG	(0/12)	Hoxa4 mutation

Fig. 1. (A) The construct containing the *Hoxa4* regulatory region linked to a *lacZ* reporter gene (p2484), previously described by Behringer et al. (1993). The open area indicates the *Hoxa4* exons and the closed area indicates the location of the *lacZ* gene. Also indicated are four potential *Hoxa4* binding sites (h) (Wu and Wolgemuth, 1993) and three newly identified potential RAREs (r). The boundaries of the region demonstrated to confer retinoic acid inducibility to a reporter gene in F9 cells (Galliot et al., 1989) are indicated by arrowheads. Enzyme designations are as follows: K, *Kpn*I; X, *Xba*I; E, *Eco*RI; P, *Pst*I. (B) The sequences of RAREs flanking the mouse *Hoxa4* (this study), human HOXA4 (Doerksen et al., 1996), mouse *Hoxd4* (Popperl and Featherstone, 1993) mouse *Hoxa1* (Langston and Gudas, 1992) and mouse *Hoxb1* (Marshall et al., 1994; Langston et al., 1997) genes. Also indicated are the numbers of nucleotides in the RARE direct repeats that match the consensus. (C) The sequence of the *Hoxa4* –2.9 RARE followed by the sequence of the mutation used to test the role of this element in *Hoxa4/lacZ* transgene expression.

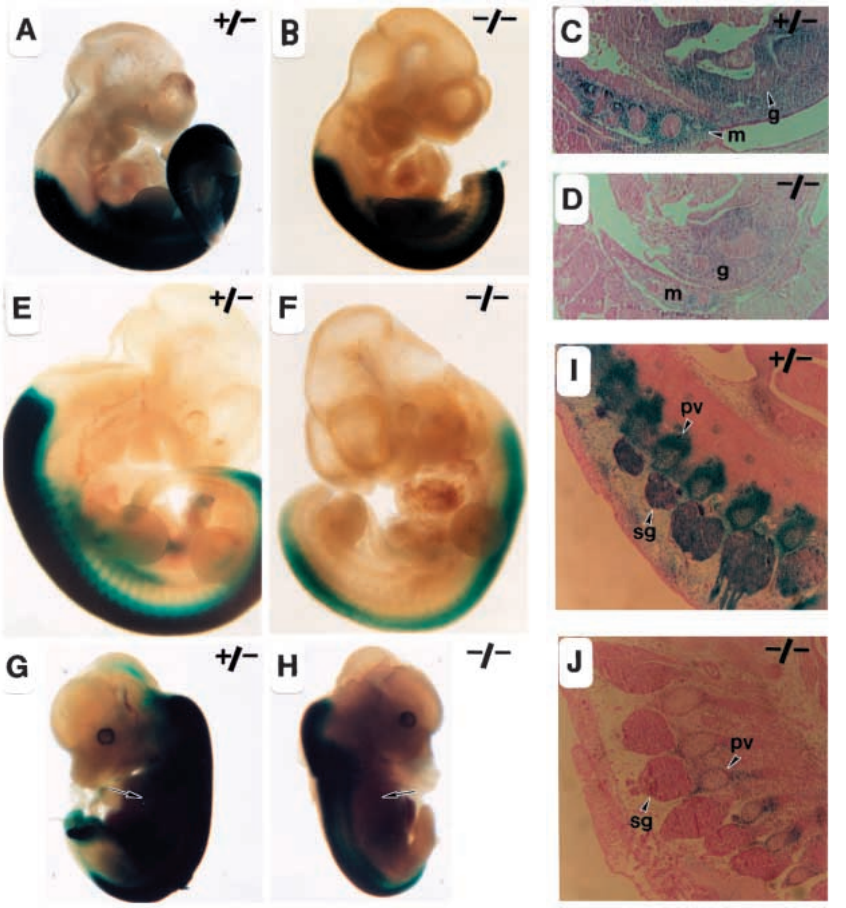


Fig. 2. Embryos derived from the mating of two mice heterozygous for both *Hoxa4* and the *Hoxa4/lacZ* transgene reveal an autoregulatory requirement for *Hoxa4* expression. The *Hoxa4* genotype of each embryo is indicated in the upper right of each panel. (A,C) *Hoxa4* +/– and (B,D) *Hoxa4* null embryos at E10.5 (T17) carrying the *Hoxa4/lacZ* transgene and stained with X-gal. Note the less intense expression in the gut (g) and mesonephros (m) of the *Hoxa4* null embryo (D). Reduced expression in the *Hoxa4* null embryo (F) (T18) is also observed at E11.5 when compared to a *Hoxa4* +/– embryo (E) (T19). At E12.5 (T20-21) the *Hoxa4* null embryo (H) exhibits no expression in the peripheral nerves between the precursors of the ribs (arrows), which were stained in the *Hoxa4* +/– embryo (G). The *Hoxa4* null embryo (J) also lacked expression in the gut and mesonephros (not shown) and spinal ganglia (sg). Some prevertebral (pv) expression was restored, but it was still at a reduced level compared to that found in the *Hoxa4* +/– embryo (I). Two embryos of each genotype from two different litters were analyzed at each time point, each exhibiting the same pattern of reporter gene expression.

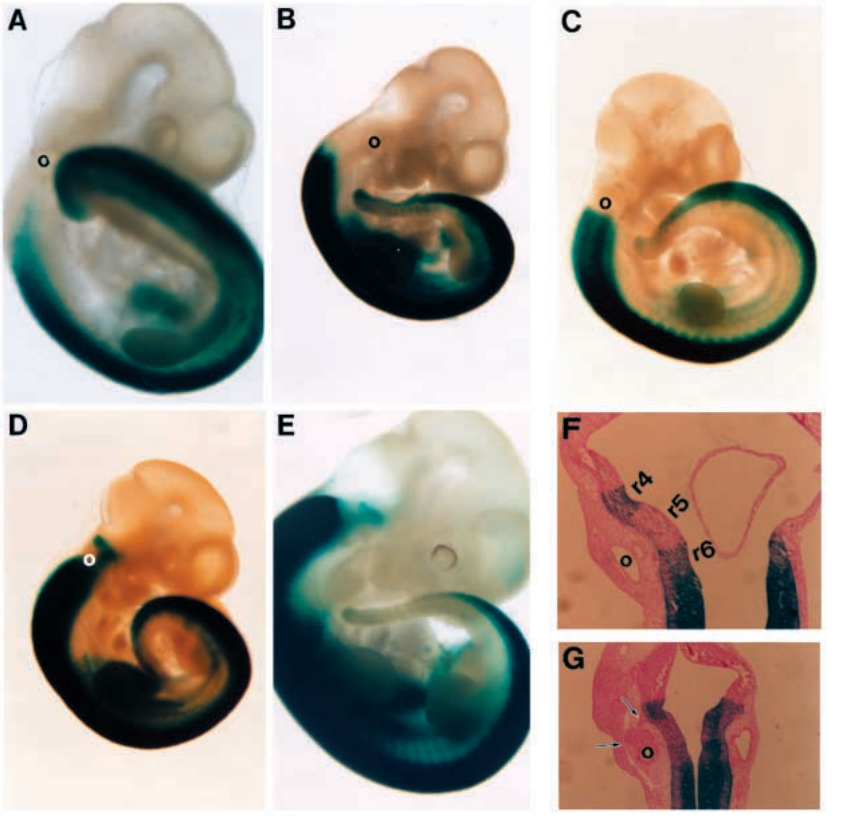


Fig. 3. Treatment of *Hoxa4/lacZ* transgenic embryos with ATRA. (A) An embryo at approximately E9.25, Theiler stage 15 (T15), following 18 hours of exposure to ATRA, beginning at approximately E8.5. (B) An untreated embryo at E10.5 (T17) exhibits an anterior boundary of expression caudal to the otic vesicle (o). (C) An embryo at E10.25 (T16), following 18 hours of exposure to ATRA beginning at approximately E9.5, was found to have an anterior boundary of expression at the base of the otic vesicle, which is shifted rostrally from the normal anterior boundary. (D) A T17 embryo following 18 hours of exposure to ATRA. Note the ectopic reporter gene expression in rhombomeres 4 and 6 and in the ventral half of rhombomere 5. (E) Ectopic expression in r4 in an E11.5 embryo (T19) following 18 hours of exposure to ATRA. Coronal sections through the hindbrain of the ATRA-treated embryo at T17 also reveal the ectopic expression in r4 and r6 (F) and in the ventral half of r5 (G). The seventh and eighth cranial sensory ganglia (arrows in G) can be seen, confirming the anterior boundary at the r3/4 junction. Retinoic acid treatments were repeated at least three times at each time point.

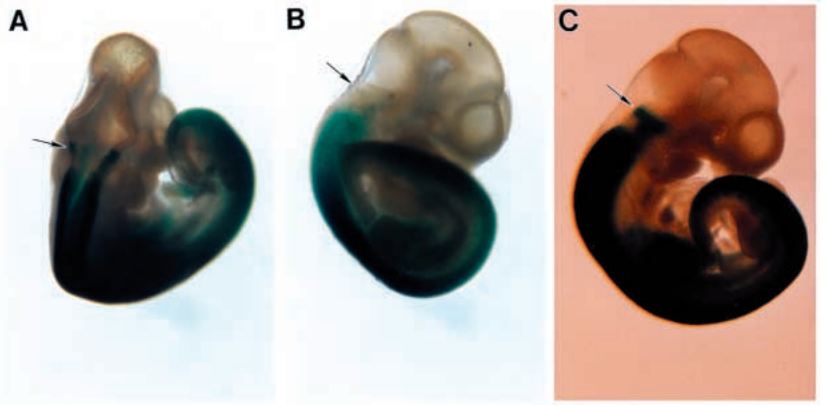


Fig. 4. The response to ATRA at T17 is *Hoxa4*-dependent. A *Hoxa4* heterozygous embryo treated with ATRA beginning at E9.5 for 18 hours exhibited the expected ectopic expression of the *Hoxa4/lacZ* transgene in r4 (A), but a *Hoxa4* null embryo from the same litter did not (B). Note the very faint stripe of expression in r4 in (B). (C) A Hoxa4 null embryo treated with ATRA beginning at E10 for 4 hours exhibited strong ectopic expression in r4. Arrows indicate the position of r4 in each embryo.

Persistent retinoic acid-induced ectopic expression requires *Hoxa4*

Since it is likely that the ATRA administered by oral gavage was metabolized within a few hours (Satre and Kochhar, 1989), we hypothesized that another factor was maintaining the observed ectopic expression in the absence of exogenous retinoic acid. Given the autoregulatory requirement for *Hoxa4* expression, we asked whether retinoic acid could still promote ectopic expression in the hindbrain in the absence of *Hoxa4*. Using the aforementioned line of mice in which the endogenous *Hoxa4* gene had been deleted by gene targeting (Horan et al., 1994), we introduced the *Hoxa4/lacZ* transgene into a *Hoxa4* heterozygous background to generate mice carrying one wild-type copy of *Hoxa4* and one copy of the *Hoxa4/lacZ* transgene. After crossing two such mice, the oral gavage experiment was repeated on pregnant transgenic females carrying *Hoxa4/lacZ* transgenic embryos that were either genotypically wild-type, heterozygous or null for *Hoxa4*. *Hoxa4* heterozygotes treated with ATRA and examined 18 hours later exhibited the expected ectopic expression in r4 (Fig. 4A), but *Hoxa4* null littermates exhibited only weak ectopic expression in r6 and a very faint stripe of expression in r4 (Fig. 4B). As this result suggested that direct activation of *Hoxa4* by ATRA was transient, and that persistent expression requires an autoregulatory loop, we also examined reporter gene expression in *Hoxa4* null embryos after only 4 hours of exposure to ATRA at E10-E10.25. In this case, strong ectopic expression in r4 was observed (Fig. 4C), confirming that HOXA4 is required for maintenance, but not for activation, of the ATRA-induced expression.

The consensus RARE at –2.9 kb is required for wild-type expression and retinoic acid-induced ectopic expression of *Hoxa4*

To test the role of the RARE at –2.9 kb in specifying the normal pattern of *Hoxa4* expression, a series of point mutations were introduced into the direct repeats of this element (Fig. 1C), in the context of the entire regulatory region. This construct was then used to generate a line of mice carrying this mutated transgene. When reporter gene expression in these transgenic embryos was examined at E12.5 we observed normal expression in the neural tube and prevertebrae, but no expression in peripheral nerves (Fig. 5B), unlike the strong expression in the peripheral nerves of embryos carrying the unmutated transgene (Fig. 5A). To test whether the –2.9 RARE was required for the response of *Hoxa4* to exogenous retinoic acid, pregnant transgenic females carrying the mutated transgene were adminstered ATRA at E9.5 in an identical fashion to mice carrying the unmutated transgene, and embryonic reporter gene expression was examined 18 hours later. As seen in Fig. 5D, the anterior boundary of neural tube expression in the transgenic embryo carrying the mutated RARE is significantly caudal to the otic vesicle, as opposed to the shifted boundary in the ATRA-treated transgenic embryo carrying the wild-type RARE (Fig. 5C). This result suggests that the response to exogenous ATRA requires an intact RARE at –2.9 kb.

To assess the role of the –2.9 RARE in driving *Hoxa4* expression in visceral organs, embryos at E14.5 were dissected

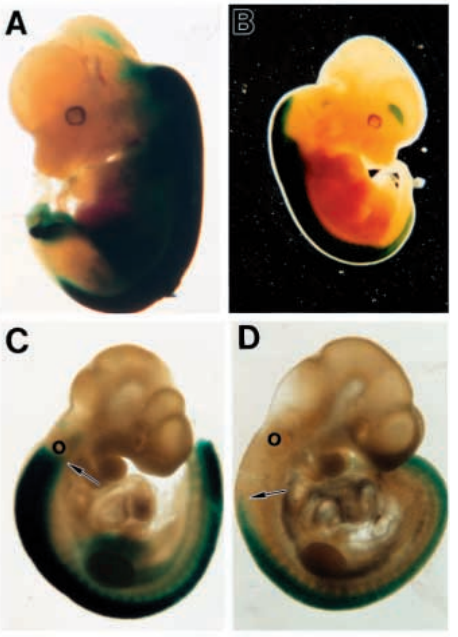


Fig. 5. (A) A transgenic E12.5 embryo carrying the unmutated *Hoxa4/lacZ* transgene. Expression is seen in the neural tube, prevertebrae, peripheral nerves and gut. (B) A transgenic E12.5 embryo carrying the *Hoxa4/lacZ* transgene with the mutated RARE exhibits normal expression in the neural tube and prevertebrae, but not in the peripheral nerves and gut. (C) Administration of ATRA to E9.5 embryos carrying the unmutated transgene results in ectopic expression in the neural tube after 18 hours, but this shift is not observed in embryos carrying the mutated transgene (D). Arrows indicate the anterior boundary of expression, caudal to the otic vesicle (o). Also note the lack of expression in the gut. Identical patterns of reporter gene expression were observed in three embryos each from two independent lines carrying the mutated transgene, with and without retinoic acid treatment.

to allow staining of tissues outside of the nervous system. Embryos carrying the mutated transgene exhibited no expression in the lung, metanephros and gut (Fig. 6D-F), revealing a requirement for retinoids in specifying *Hoxa4* expression in these organs during their growth and differentiation. In addition, no expression was observed in the gut in either the E10.25 ATRA-treated embryo (Fig. 5D) or in the untreated E12.5 embryo (Fig. 5B), suggesting that transgene expression is never initiated in the gut. Tissues from embryos carrying the unmutated transgene are presented to show the extent of normal expression at E14.5 (Fig. 6A-C).

DISCUSSION

Our results indicate that an autoregulatory loop is required to maintain normal levels of *Hoxa4* at each site of embryonic expression. In addition to providing further support for the evolutionary conservation of autoregulation between the *Drosophila Dfd* gene and its homologs in mice, the observed *Hoxa4* autoregulation reveals a strikingly widespread requirement for this positive feedback loop. Previous studies of *Hox* gene autoregulation in mice had identified autoregulatory enhancers involved in localized *Hox* gene expression, such as the specification of the anterior boundary of expression of *Hoxb4* (Gould et al., 1997) and the maintenance of *Hoxb1* expression in rhombomere 4 (Popperl et al., 1995). Our results suggest that most or all aspects of the expression patterns of the group 4 *Hox* genes (and possibly other *Hox* genes as well) may be under autoregulatory control. Several individual autoregulatory enhancers for each *Hox* gene might be required to maintain expression throughout embryogenesis. The extent to which persistent *Hox* gene expression is required for wild-type function, however, is not yet clear. The identification and deletion of specific autoregulatory elements for *Hoxa4* and for other *Hox* genes should allow the generation of embryos in which particular *Hox* genes are expressed only briefly during development.

Although the lack of expression in the gut, mesonephros and spinal ganglia of the *Hoxa4* null embryos persisted at least until E12.5, expression in the prevertebrae was partially restored between E11.5 and E12.5. This compensation may reflect the activity of other upstream factors such as *Cdx1*, an activator of *Hox* gene expression in somitic derivatives (Subramanian et al., 1995), or cross-regulation by other *Hox* genes. Indeed, an evolutionarily conserved enhancer shared by *Hoxb3* and *Hoxb4* has been identified that mediates the proper specification of their anterior boundary of expression by other *Hox* genes, including 'para-regulation' of *Hoxb4* by *Hoxd4* (Gould et al., 1997). These examples of para-regulation likely underlie the increasing severity of the phenotypes of mice lacking two or three paralogs (Horan et al., 1995a,b). The recent identification of a conserved regulatory element in the *Hoxa4* intron that contains three potential homeodomain binding sites suggests that cross-regulation by other *Hox* genes is likely to be involved in *Hoxa4* regulation as well (Keegan et al., 1997). The fact that the neural tube is the least affected site of *Hoxa4* expression may reflect the greater potential for cross-regulation in this tissue, as opposed to sites, gut and mesonephros for example, where only a subset of *Hox* genes is expressed

Our studies of the retinoic acid-dependent regulation of *Hoxa4* indicate that a consensus RARE at −2.9 kb is required for activation of embryonic expression, with the exception of the dorsal neural tube and prevertebrae. Further mutational analysis will be required to determine the functional significance of the relatively poorly conserved potential RAREs at −1.8 and −1.4 kb. Although a construct encompassing these two potential RAREs, but not the RARE at −2.9 kb, did not promote reporter gene expression in transgenic mice (Behringer et al., 1993), a similar construct was shown to be sensitive to retinoic acid in vitro (Galliot et al., 1989), raising the possibility that they are functional elements.

The −2.9 RARE also mediates the response to exogenous retinoic acid, promoting ectopic expression in the neural tube at one stage (T16) and in the hindbrain at a slightly later stage (T17). While this manuscript was in preparation, it was reported that ATRA could promote ectopic expression of the endogenous *Hoxa4* gene at E9.5 in a pattern similar to that which we observed at T16 (Morrison et al., 1997). Although ATRA-induced expression of *Hoxa4* in r6 and r4 was not reported (Morrison et al., 1997), it is possible that embryos treated slightly later in development would have exhibited such a response, as was observed in our *Hoxa4/lacZ* embryos. The partly discontinuous pattern of ectopic expression detected in the hindbrain at T17 in this study is of particular interest, since it is reminiscent of that observed for the ATRA-induced ectopic expression of *Hoxd4/lacZ* (Zhang et al., 1997) and *retinoic acid receptor β 2/lacZ* (*RARβ 2)* transgenes (Mendelsohn et al., 1994a). Normal *RARβ 2* expression is roughly coincident with *Hoxa4* in the anterior spinal cord and hindbrain, and upon ATRA treatment *RARβ 2* is induced in r6 and r4, raising the possibility that it mediates the effect of ATRA on *Hoxa4* expression, as was suggested for *Hoxd4* (Zhang et al., 1997).

In addition to this spatial restriction of ATRA-induced

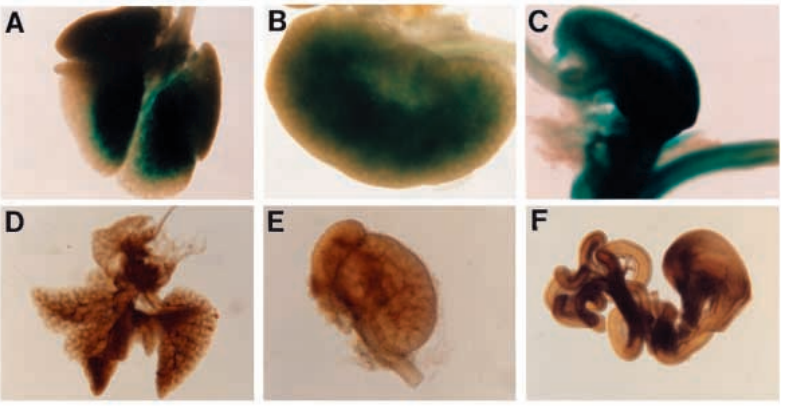


Fig. 6. (A-C) Tissues dissected from E14.5 embryos carrying the unmutated *Hoxa4/lacZ* transgene prior to X-gal staining. Reporter gene expression is observed in the lung (A), metanephros (B) and gut (C). (D-F) Tissues dissected from E14.5 embryos carrying the transgene with the mutated RARE prior to X-gal staining. Reporter gene expression is completely abolished in lung (D), metanephros (E) and gut (F). In each case, positively stained remnants of the neural tube were observed when treated in parallel with the dissected tissues. Four embryos each from two lines of transgenic mice were examined.

expression, we also observed that the sensitivity of *Hoxa4* to exogenous ATRA was temporally restricted, with inducible expression limited to E9.5 and later. Although the group 1 *Hox* genes are sensitive to exogenous ATRA as early as E7.5, our results are quite similar to the timing of sensitivity of a human *HOXD4* retinoid-responsive enhancer (Morrison et al., 1996) and the endogenous murine *Hoxa4* gene (Morrison et al., 1997). The authors of the *HOXD4* study proposed that one or more cofactors might be required for the retinoid-inducibility of particular *Hox* genes (Morrison et al., 1996). One such factor might be the retinoid-inducible transcription factor AP-2, which is required for normal neural tube and skeletal development (Zhang et al., 1996), and has been shown to bind to a specific site in the human *HOXA4* promoter which interacts with a nearby RARE (Doerksen et al., 1996). As the murine *Hoxa4* regulatory region contains an AP-2 site adjacent to the −2.9 RARE (D. A. C., A. I. P. and D. J. W., unpublished observations), such an interaction may be conserved in the mouse, and may restrict the spatiotemporal response of *Hoxa4* to retinoids.

The loss of reporter gene expression upon mutation of the −2.9 RARE is distinct from that seen when the *Hoxa1* (Frasch et al., 1995) and *Hoxb1* (Marshall et al., 1994) RAREs were mutated. The most obvious difference is that mutation of the *Hoxa1/lacZ* and *Hoxb1/lacZ* transgene RAREs eliminated most or all of the neuroectodermal expression, while mutation of the *Hoxa4/lacZ* transgene RARE had no effect on normal expression in the neural tube. This difference may reflect the fact that the *Hoxa1* and *Hoxb1* studies examined the effect of a RARE mutation on expression driven by a single enhancer, whereas our RARE mutation was made in the context of the entire regulatory region, possibly allowing for compensation by other *cis*-acting elements. In fact, a truncated *Hoxa4/lacZ* transgene containing only 2.5 kb of 5′ flanking sequence is sufficient to drive expression in the neural tube (D. A. C., A. I. P. and D. J. W., unpublished observations), suggesting that there is a distinct 5′ *Hoxa4* neural enhancer in a region that does not include the RARE. Although the RARE is dispensible for normal neuroectodermal expression, it is clearly required for the anterior shift of expression observed in the neural tube when exogenous ATRA is delivered to the embryo. It should also be noted that a partially retinoid-responsive neural enhancer was recently identified in the *Hoxa4* 3′ flanking region (Morrison et al., 1997), raising the possibility that there is some functional overlap between 5′ and 3′ retinoid-responsive elements.

Outside of the neuroectoderm, we observed that gut expression was lost in both the *Hoxa4* and *Hoxa1* RARE mutants, suggesting that this is one tissue where retinoids might be essential for *Hox* gene activation. In addition, we observed a loss of expression in the lung and metanephros in the −2.9 RARE mutant mice. Although most of the work on RARE mutations and their effect on *Hox* gene expression has focused on the nervous system at E7.5-E9.5, it is clear from this study that retinoids can mediate *Hox* gene expression in tissues of mesodermal origin at E12.5-E14.5. Retinoic acid has been shown to have dramatic effects on the development of lungs (Schuger et al., 1993) and kidneys (Vilar et al., 1996) in vitro, and ATRA appears to promote *Hoxa4* expression in cultured embryonic lungs and metanephroi at E14.5 (A. I. P., L. A. Ambrozewicz and D. J. W., unpublished observations). Since compound null mutants of the *RAR* genes exhibit hypoplasia of the lungs and kidneys (Mendelsohn et al., 1994b), retinoid-dependent *Hox* gene expression may prove to be involved in the morphogenesis of visceral organs in addition to its well-characterized role in patterning the nervous system and vertebral column.

Our results also suggest that retinoic acid-dependent expression of *Hoxa4* is transient and that autoregulation is required for maintenance. Such an interaction had been suggested from in vitro studies on the human *HOXB1* gene, where cotransfection of a *HOXB1* expression vector with a RARE-driven reporter gene resulted in a potentiation of retinoid-dependent reporter gene expression (Ogura and Evans, 1995). Of note in the present study is the fact that the pattern of *Hoxa4/lacZ* expression in the E11.5-E12.5 *Hoxa4* null embryos is quite similar to that seen in the embryos carrying the mutated −2.9 RARE. This implies that the activation and maintenance of much of the *Hoxa4* expression in the embryo may require the cooperative action of retinoic acid and HOXA4 protein.

Finally, the potential for the conservation of regulatory elements between homologous and paralogous *Hox* genes has been widely discussed (Maconochie et al., 1996; Morrison et al., 1997; Zhang et al., 1997), and the present findings lend support to this idea, particularly in regard to the murine *Hoxa4* and *Hoxd4* genes. Their similar timing of ATRA sensitivity, the identical direct repeats in their RAREs (Fig. 1B), the conserved elements in their introns (Keegan et al., 1997), and the potential for autoregulation of both genes all suggest that the expression of these two paralogs is controlled by similar, if not identical, regulatory mechanisms. The subtle differences in their patterns of expression, particularly at the anterior limit of expression in the neuroectoderm and paraxial mesoderm, probably reflects the influence of paralog-specific regulatory elements whose identification will require further analysis.

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