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In vitro and transgenic analysis of a human *HOXD4* retinoid-responsive enhancer

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SUMMARY

Expression of vertebrate *Hox* genes is regulated by retinoids in cell culture and in early embryonic development. We have identified a 185-bp retinoid-responsive transcriptional enhancer 5′ of the human *HOXD4* gene, which regulates inducibility of the gene in embryonal carcinoma cells through a pattern of DNA-protein interaction on at least two distinct elements. One of these elements contains a direct repeat mediating ligand-dependent interaction with retinoic acid receptors, and is necessary though not sufficient for the enhancer function. The *HOXD4* enhancer directs expression of a *lacZ* reporter gene in the neural tube of transgenic mouse embryos in a time-regulated and regionally restricted fashion, reproducing part of the anterior neuroectodermal expression pattern of the endogenous *Hoxd-4* gene. Administration of retinoic acid to developing embryos causes alterations in the spatial restriction of the transgene expression domain, indicating that the *HOXD4* enhancer is also a retinoid-responsive element in vivo. The timing of the retinoic acid response differs from that seen with more 3′ *Hox* genes, in that it occurs much later. This shows that the temporal window of competence in the ability to respond to retinoic acid differs between *Hox* genes and can be linked to specific enhancers. Mutations in the direct repeat or in a second element in the enhancer affect both retinoid response in culture and developmental regulation in embryos, suggesting that co-operative interactions between different factors mediate the enhancer activity. These data provide further support for a role of endogenous retinoids in regulation and spatial restriction of *Hox* gene expression in the central nervous system.

Key words: developmental regulation, enhancer, homeobox, neural tube, retinoic acid, human, mouse

INTRODUCTION

Hox genes encode a family of transcription factors containing a highly conserved DNA binding homeodomain (Gehring et al., 1990), and play a key role in the specification of regional identities along the anteroposterior (AP) axis in embryonic development (reviewed by Krumlauf, 1993; McGinnis and Krumlauf, 1992). In vertebrates, *Hox* genes are organised in four homologous clusters, and are expressed according to characteristic, spatially restricted patterns (reviewed by Duboule, 1992; Krumlauf, 1993; McGinnis and Krumlauf, 1992). In all four *Hox* clusters, there is a linear relationship between the order of genes along the chromosome, the timing of their activation in development, and the position of their expression domains along the embryonic AP axis, according to a 3′-early-anterior/5′-late-posterior colinearity rule (Duboule and Dollé, 1989; Graham et al., 1989).

Reconstruction of *Hox* expression patterns in transgenic mice using DNA sequences from within the clusters fused to a *lacZ* reporter gene has provided a convenient functional assay for identifying *cis*-regulatory elements and their associated factors. In some cases, *Hox*/*lacZ* transgenes with adjacent flanking regions retain most of the spatial and temporal expression patterns of the original genes, despite the fact that they are integrated outside their normal chromosomal locations (Behringer et al., 1993; Frasch et al., 1995; Gerard et al., 1993; Marshall et al., 1992, 1994; Püschel et al., 1990, 1991; Sham et al., 1992; Studer et al., 1994; Whiting et al., 1991). However, in other cases, the transgenes generate only limited subsets of the complete expression patterns, suggesting that for their normal regulation, multiple elements and long-range interactions are essential (Eid et al., 1993; Kress et al., 1990; Vogels et al., 1993).

Hox genes are specifically regulated, both in vivo and in cell culture by retinoids, vitamin A derivatives with pleiotropic effects on development and cell differentiation (Blomhoff et al., 1990; Saurat, 1990; Summerbell and Maden, 1990; Tabin, 1991). Retinoids are present at active concentrations in embryonic structures with a proven role in pattern formation, such as the zone of polarising activity (Thaller and Eichele,

1987, 1990), Hensen's node or its mammalian equivalent (Chen et al., 1992; Hogan et al., 1992), and the floor plate of the neural tube (Horton and Maden, 1995; Wagner et al., 1992, 1990). Systemic or local administration of excess retinoic acid (RA) to developing vertebrate embryos has profound effects on axial patterning and specification of regional identities in a number of structures, including the CNS, axial skeleton and limbs (reviewed by McGinnis and Krumlauf, 1992; Summerbell and Maden, 1990; Tabin, 1991). This is invariably accompanied by re-patterning of *Hox* gene expression domains (Conlon and Rossant, 1992; Izpisúa-Belmonte et al., 1991; Kessel, 1992; Kessel and Gruss, 1991; Marshall et al., 1992; Nohno et al., 1991; Oliver et al., 1990; Simeone et al., 1995), suggesting a specific role for *Hox* gene products as molecular transducers of the morphogenetic (positional?) signals provided, or at least mimicked, by retinoids (McGinnis and Krumlauf, 1992; Tabin, 1991 and references therein).

In human and mouse embryonal carcinoma (EC) cells, *Hox* genes are activated by RA in a dose-dependent, 3′→5′ sequential pattern similar to that observed in embryonic development (Papalopulu et al., 1991; Simeone et al., 1990, 1991), providing an in vitro model to study the molecular mechanisms of *Hox* gene regulation. Studies carried out both in vitro and in vivo allowed the identification of RA-responsive elements at the 3′ end of the mouse or human *Hoxa* and *Hoxb* clusters, which confer to a *lacZ* reporter RA inducibility in EC cells (Langston and Gudas, 1992; Ogura and Evans, 1995a,b) or RA inducibility and regulated expression in transgenic mouse embryos (Frasch et al., 1995; Marshall et al., 1994; Studer et al., 1994). Retinoid-responsive elements are therefore likely to participate in the activation of *Hox* clusters, and are part of the regulatory mechanisms underlying temporal and spatial regulation of *Hox* gene expression in development. However, it is unknown if the colinear RA response is mediated by a single region at the 3′ end of the complexes, or involves multiple RAREs spread throughout the clusters.

The human *HOXD4* gene is expressed in multiple transcripts, developmentally regulated in embryonic development (Cianetti et al., 1990; Mavilio et al., 1986), and induced in the human EC cell line NT2/D1 by exposure to retinoids in the nM to μM concentration range (Simeone et al., 1990). *Hoxd-4* is activated early in mouse embryogenesis, and is expressed in 12.5-day embryos mainly in the limbs, paraxial mesoderm and CNS, with an anterior restriction at the level of rhombomere (r) 6/7 boundary (Gaunt et al., 1989; Hunt et al., 1991). We and others have previously reported the characterisation of a 185-bp transcriptional enhancer, upstream of the human and mouse *Hoxd-4* genes. This enhancer regulates gene inducibility by all-*trans*- and 9-*cis*-RA in culture, through a complex pattern of DNA-protein interactions on at least four distinct

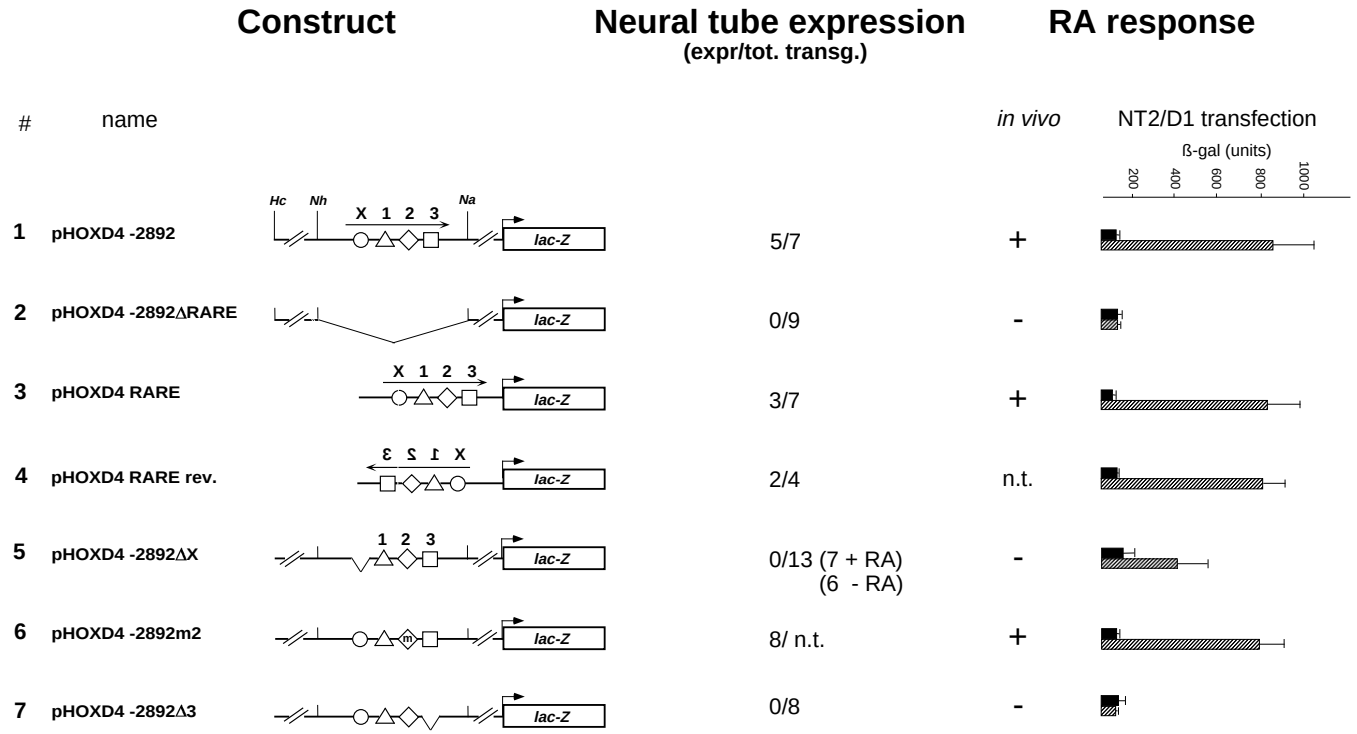

Fig. 1. Left: map of the *HOXD4-lac-Z* reporter constructs. The symbols represent the footprinted elements X, 1, 2 and 3 of the 185-bp RA responsive enhancer (see Fig. 4C). Deletion or mutation (m) of an element is indicated. The horizontal arrow indicates the orientation of the enhancer with respect to the promoter. The short arrow represents the proximal promoter of the *HOXD4* gene (Moroni et al., 1993) in the constructs used for cell transfection, or the murine *Hoxb-4* promoter in the constructs used to generate transgenic mice. Constructs no. 3 and 4 contained only the *Hoxb-4* promoter. Hc, *Hin*cII; Nh, *Nhe*I; Na, *Nar*I. Middle: generation of neural tube-specific *lacZ* expression pattern in transient transgenic mouse embryos or lines, as number of positively expressing/total transgenic embryos. In the case of construct no. 6, yolk sac DNA sample were lost, so it was impossible to determine the total number of transgenic embryos (n.t., not tested). Right: response of the constructs to RA induction in vivo (+, anterior shift in the neural tube expression domain; –, no change; n.t., not tested) and in vitro (transfection in NT2/D1 cells). Activity of the *lacZ* reporter in transfected cells is expressed as β-gal arbitrary units (mean ± s.e.m. of at least three separate, duplicate experiments), after normalisation for the luciferase activity derived from a co-transfected pT81luc plasmid (see methods).

elements (Moroni et al., 1993; Pöpperl and Featherstone, 1993). Although ligand-dependent activity of RA receptors (RARs) upon one of these elements has a key role in the enhancer function, RARs represent only a fraction of the transcription factors interacting with the RA-responsive enhancer, which appears to be a complex element requiring specific combinations of nuclear factors for its proper function (Moroni et al., 1993).

In this study, we show that the *HOXD4* enhancer is able to mediate expression of a *lacZ* reporter gene in the neural tube of transgenic mice according to a time-regulated, regionally restricted pattern, which reproduces part of the expression pattern of the endogenous *Hoxd-4* gene. Mutations in two of the four elements of the enhancer affect both RA-inducibility in EC cells and developmentally regulated expression in transgenic mice, establishing direct correlations between specific RA-dependent functions identified in vitro and their role in gene regulation in vivo.

MATERIALS AND METHODS

Plasmids

A partial *Hinc*II/*Ase*I digest spanning positions −2892 to +21 from the P2 proximal transcription start site in the *HOXD4* upstream genomic sequence, was subcloned from the λ13G phage (Mavilio et al., 1986) in pBluescript SK (Stratagene). The fragment was recovered as a *Sal*I/*Xba*I fragment and cloned in the *Eco*RI/*Xho*I sites of the pNASSβ vector (Clontech), to generate the p*HOXD4*-2892-*lacZ* vector (Fig. 1, construct no. 1). Before injection, plasmid vector sequences were removed by *Eco*RI/*Sal*I digestion. The same fragment was subcloned in the *Bam*HI site of plasmid pB4-*lacZ* (Whiting et al., 1991), in which the *lacZ* reporter gene is driven by the mouse *Hoxb-4* promoter. Fragments for injection were isolated after digestion with *Not*I to remove vector sequences. The p*HOXD4*-2892Δ RARE vector (constructs no. 2) was generated by internal deletion of the *Nhe*I/*Nar*I fragment, containing the entire RA-responsive enhancer (from position −2711 to −2333). The p*HOXD4* RARE and p*HOXD4* RARE rev. vectors (constructs nos. 3 and 4) were generated by cloning the *Nhe*I/*Nar*I fragment in both orientations in the pB4-*lacZ* vector. Deletions of the X (p*HOXD4*-2892ΔX, construct no. 5) and 3 (p*HOXD4*-2892Δ3, construct no. 7) elements, and mutation of the element 2 (p*HOXD4*-2892m2, construct no. 6), were obtained by PCR-mediated splicing by overlap extension on the *Hin*dIII/*Xho*I fragment (−2720 to −2109), subcloned in pBSK as template, using SK and T7 as external primers, and specific internal primers for element X (direct: 5′-CGTTAAATATTCACTCCCGCTCTAGAGTCTCTAGGGCTTTTTGTGC-3′, reverse: 5′-GCACAAAAAGCCCTAGAGACTCTAGAGCGGGAGGTGAATATTTAACG-3′), element 3 (direct: 5′-CCTGTCTGAACAAGTCGAGCGATCGTCTAACAAATATGAAAATGTG-3′, reverse: 5′-CCTGTCTGAACAAGTCGAGCGATCGTCTAACAAATATGAAAATGTG-3′) and element 2 (direct: 5′-TCAATAACTCGTTGGCTTGGGCCCGTCTAGACCAGTCGAGCAATAAGGTG-3′, reverse: 5′-CACCTTATTGCTCGACTGGTCTAGACGGGCCCAAGCC AACGAGTTATTGA-3′). Each mutant was then recovered from the template plasmid as *Nhe*I/*Nar*I fragment, and used to replace the wild-type *Nhe*I/*Nar*I fragment into the p*HOXD4*-2892 constructs.

The pT81luc expression vector (Nordeen, 1988), containing the fruitfly luciferase gene under the control of the enhancerless HSV-*TK* promoter, was used as internal control in all transfection experiments.

Cell culture and transfection

The human embryonal carcinoma cell line NT2/D1 (Andrews et al.,

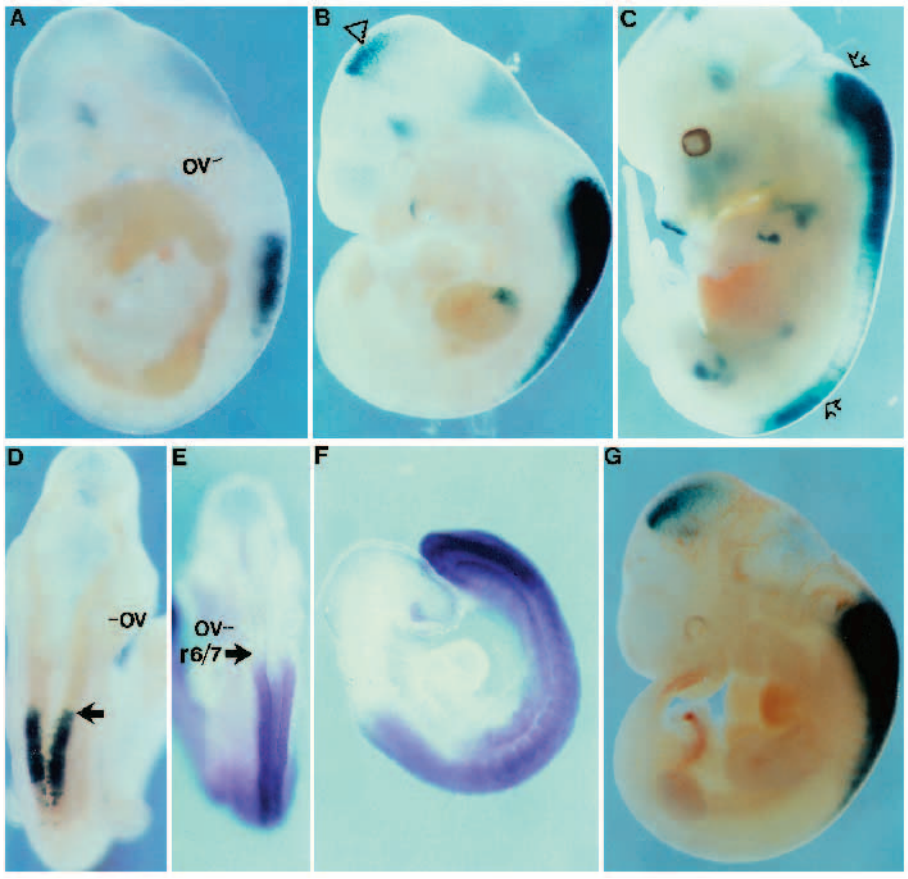

Fig. 2. Time course of β-galactosidase expression from a transgenic line (AL15) containing construct no. 1. (A) 9.5 d.p.c., (B) 10.5 d.p.c. and (C) 12.5 d.p.c. (D) Dorsal view of embryo in A, showing that the anterior limit of *lacZ* expression lies at a position more caudal to the otic vesicle (OV) than the limit of endogenous *Hoxd-4* expression, at the r6/7 boundary of a 9.5 d.p.c. embryo (dorsal view (E) and lateral view (F)), as detected by in situ hybridisation. The open triangle in B indicates the midbrain staining mediated by the basal pB4-*lacZ* vector in approx. 50% of integration sites. (G) The expression at 11.5 d.p.c. of a second independent transgenic line (AL12) carrying construct no. 1. Expression extends posteriorly along the neural tube, eventually producing a biphasic distribution of *lacZ* staining which is clearly evident by 12.5 d.p.c., indicated in C by open arrows.

1984) was cultured in high-glucose Dulbecco's medium (DMEM) supplemented with 10% fetal calf serum and 20 mM Hepes buffer (all from GIBCO) in 5% CO_2 in air humidified atmosphere. For transfection, 5×10^5 cells were seeded in 60-mm dishes, induced for 24 hours with 10 μM all-*trans*-retinoic acid (Sigma) as previously described (Simeone et al., 1990), and then transfected in calcium phosphate/Hepes-buffered saline solution (Sambrook et al., 1989) with a total of 10 μg of plasmid DNA (9 μg reporter + 1 μg pT81luc), always in the presence of RA. Total protein extracts were prepared after 36 additional hours of culture by three cycles of freezing and thawing, and assayed for luciferase or β-galactosidase activity as described (de Wet et al., 1987; Sambrook et al., 1989). Transfection of non-induced cells was carried out by the same protocol, without adding RA.

DNase I footprinting analysis

Crude nuclear extracts were prepared as described by Dignam et al. (1983) from 10^7-10^8 uninduced or RA-induced NT2/D1 cells, aliquoted and stored in liquid nitrogen. A 171-bp subcloned fragment corresponding to the –2720 to –2449 *HOXD4* upstream region was 3′ end-labeled, purified on polyacrylamide gel, and incubated with 10 to 60 μg of NT2/D1 nuclear extracts and 1 μg of poly(dI-dC) for 10 minutes at room temperature (RT) in a binding reaction containing 50 mM $MgCl_2$, 20 mM Tris-HCl pH 7.9, 1 mM DTT and 25% glycerol. 300 ng of DNase I (Boehringer) were added to the mixture and the incubation continued for 90 seconds at RT. Reaction was stopped with 0.2 M Tris-HCl pH 7.5, 0.2 M NaCl, 1% (w/v) SDS, 30 mM EDTA and 20 μg yeast tRNA, extracted with phenol-chloroform and precipitated in ethanol. Pellets were resuspended in 8 μl of standard 80% formamide dye, denatured for 5 minutes at 95°C and run on a 6% urea-polyacrylamide gel. Gels were dried and exposed overnight at –70°C to Kodak X-AR5 films.

Whole-mount in situ hybridisation

Mouse embryos at 9.5-12.5 d.p.c. were fixed in 4% paraformaldehyde and then hybridised using a modification of the method of Wilkinson (1992). The digoxigenin-labeled RNA probe corresponded to the full-length *Hoxd-4* cDNA (Featherstone et al., 1988).

Transgenic mice

Transgenic mice were produced using intercrosses of (CBA × C57BL10) F_1 mice. Pronuclear injection was performed as described by Hogan et al. (1986). In timed pregnancies, the day of appearance of the vaginal plug was taken as 0.5 d.p.c. β-galactosidase staining was performed as previously described (Whiting et al., 1991). Embryos for sectioning underwent a further fixation in 4% paraformaldehyde overnight at 4°C, and following standard processing of wax embedded sections (6 μm) were counterstained with eosin. All-*trans*-retinoic acid (Sigma; 25 mg/ml in dimethylsulphoxide) was diluted 1/10 in sesame seed oil and approximately 200 μl of this given to pregnant mothers by gavage at 7.5, 8.5 or 9.5 d.p.c. for a final dose of 25 mg/kg as previously described (Conlon and Rossant, 1992; Marshall et al., 1992). Total number of transgenic embryos was determined by PCR analysis on yolk sac DNA from embryos harvested for staining, except in the case of construct no. 6, where DNA samples were not taken and efficiency could not be measured. The line ML19, containing the *Hoxb-2* r3/5 *lacZ* transgene (Sham et al., 1993) was mated with lines carrying construct no. 1 to determine the relative position of the *HOXD4* enhancers anterior boundary of expression with respect to rhombomere 5.

RESULTS

The *HOXD4* upstream genomic region directs neural-specific expression in transgenic mice

We first tested the activity of the 2.9 kb *Sal*I-*Xba*I *HOXD4* genomic fragment spanning the –2892/+21 region of the human *HOXD4* gene in transgenic mice to assay for its functional properties in vivo. This fragment, which contains a previously identified retinoid-responsive enhancer (Moroni et al., 1993) and two promoters (Cianetti et al., 1990), was placed in the context of the promoter-less pNASSβ vector, containing a *lacZ* reporter gene. Out of 5 transgenic founder embryos harvested at 9.5 days post coitum (d.p.c.) and identified by PCR, none showed any expression (not shown). The same construct was able to direct RA-inducible *lacZ* expression in human NT2/D1 cells (construct no. 1, Fig. 1) as well as in mouse P19 EC cells (not shown). This suggested that either the regulatory region was non-functional in the transgenic assay, or that the human *HOXD4* promoter(s) used in transfected NT2/D1 cells are different from those used in embryonic tissues. Indeed, it has been shown for a number of *Hox* genes (including *HOXD4*) that in human and mouse EC cells only a subset of the embryonic transcripts are present and induced by RA (Papalopulu et al., 1991; Pöpperl and Featherstone, 1993; Simeone et al., 1990, 1991). Therefore, to assay for regulatory activity within the fragment in transgenic mice we generated constructs which were independent of the endogenous *HOXD4* promoters. We placed the *HOXD4* genomic fragment into a different vector (pB4-*lacZ*), which contains the basal promoter of the mouse *Hoxb-4* gene driving a *lacZ* reporter. This basic mouse vector was originally generated in a transgenic deletion analysis of the *Hoxb-4* gene, where it displayed no reporter activity on its own except for slight staining in the superior colliculi of the midbrain (Whiting et al., 1991). We have routinely used the pB4-*lacZ* vector to test for enhancer activity in transgenic mice (Aparicio et al., 1995; Marshall et al., 1994; Morrison et al., 1995; Sham et al., 1992, 1993; Whiting et al., 1991). Seven independent transgenic animals were obtained with the human *HOXD4* fragment in this construct, and five of these expressed the transgene (two lines and three founder embryos). All five had an identical pattern of expression in the neural tube, restricted to the spinal cord just posterior to the hindbrain (Fig. 2). The expression pattern was consistent with that previously obtained with a *HOXD4* upstream genomic fragment linked to the basal mouse *Hoxa-5* promoter (Tuggle et al., 1990), showing that the *HOXD4* enhancer element drives a consistent expression pattern on at least two different mouse basal promoters. Therefore, all *HOXD4* genomic fragments tested in transgenic mice were in the context of the basal pB4-*lacZ* reporter, whereas those tested in parallel in NT2/D1 cells were in the context of the pNASSβ reporter (human promoters). The parallel constructs are listed in Fig. 1 with the same number and name, regardless of the promoter usage.

The two independent transgenic lines (AL-12 and AL-15) carrying construct no. 1 were used to generate a developmental time course of expression (Fig. 2A-D,G). Expression of the transgene was first detected between 9.0-9.5 d.p.c. in the neural tube in a gradient with a rostral limit in the spinal cord posterior to the otic vesicle (Fig. 2A,D) and posterior expression boundary mapping to the mid thoracic region. This was close to but caudal to the anterior expression limit of the endogenous *Hoxd-4* gene, which lies at the r 6/7 boundary as revealed by in situ hybridisation (Fig. 2E,F). At 10.5 d.p.c., levels of *lacZ* expression had increased and expanded posteriorly along the neural tube. The distance between the rostral limit of expression and the otic vesicle had shortened, suggesting that

the anterior extension domain of expression shifts rostrally with time (compare also Fig. 3A,B with Fig 4A,B). At later stages (11.0 d.p.c. onwards), the anterior boundary of expression becomes fixed at a level which appeared to be posterior to that of the endogenous *Hoxd-4* gene (Gaunt et al., 1989; Hunt et al., 1991). By 12.5 d.p.c., transgene expression could be seen in two distinct domains within the neural tube, one extending from the hindbrain/spinal cord junction into the mid-thoracic region, and the other at the level of the posterior limb buds (Fig. 2C). These results show that the *HOXD4* upstream region directs a subset of the neural-specific expression pattern of the endogenous *Hoxd-4*.

The *HOXD4* enhancer is a retinoid-responsive element in vivo

Since the *HOXD4* upstream region contains an enhancer mediating a response to RA in EC cells (Moroni et al., 1993), we wished to determine its ability to respond to exogenous RA administered in utero. Previously it had been shown that the *Hoxb-1* gene contains a RARE which mediates a response to RA treatment in utero at 7.5-8.0 d.p.c., but not upon exposure in later periods (Conlon and Rossant, 1992; Marshall et al., 1992, 1994), so we initially used the same conditions to test for a response by the *HOXD4* enhancer. Because the anterior boundary of transgene expression generated by the *HOXD4* enhancer shifts slightly over time (Fig. 2), we mated a line carrying construct no. 1 to a second line which contained an r3/r5 specific enhancer of the mouse *Hoxb-2* gene linked to a *lacZ* reporter (Sham et al., 1993) to create an internal standard. The r5 stripe of expression from the *Hoxb-2* enhancer is a valid control, because it has previously been shown that this domain is not altered by RA treatment in utero (Marshall et al., 1992). Therefore, the anterior position of *HOXD4* transgene expression, in both treated and untreated embryos, was always determined by proximity to the reference stripe of r5-specific *lacZ* gene expression. The dorsal and lateral views of control and RA-treated embryos clearly show that the anterior boundary of *HOXD4* expression remains unchanged relative to r5 (Fig. 3A,B,D,E). Therefore, despite the presence of typical anterior phenotypic abnormalities induced by RA treatment at 7.5 d.p.c., there was no effect on *lacZ* expression in the lines carrying the *HOXD4* construct no. 1 (Fig. 3A,B,D,E).

Mammalian *Hox* genes show a colinear, 3′→5′ response to retinoids in culture (Papalopulu et al., 1991; Simeone et al., 1990, 1991), and since *HOXD4* is located in a more 5′ position in its cluster with respect to *Hoxb-1*, higher RA doses or a later period of exposure might be required for its induction in vivo. Higher RA concentrations administered at 7.5 d.p.c. had no effect on the *lacZ* expression driven by construct no. 1(not shown), however we did find stimulation when transgenic embryos were exposed to RA after 9.0 d.p.c. Pregnant females carrying embryos containing construct no. 1 and the *Hoxb-2* r3/r5 *lacZ* transgene, were treated with all-*trans*-RA at 9.5 d.p.c., and embryos were harvested 12-24 hours (10.0-10.5 d.p.c.) after treatment to monitor transgene expression compared to untreated or mock-treated embryos of the same age. At both time points after treatment, construct no. 1 responded by shifting its expression in an anterior direction. At 10.0 d.p.c. *lacZ* expression had clearly moved rostrally compared to that of untreated embryos, being closer to the r5 stripe of expression, with an anterior boundary of expression mapping at the r6/7 junction (se flat mounts Fig. 3C,F,I). We have also checked that the expression boundary of the endogenous *Hoxd-4* gene moved anteriorly as a consequence of RA treatment under the same conditions (not shown), i.e. response to RA at late but not early stages of treatment.

Given that the normal time course of expression from the lines containing construct no. 1 showed a progressive anterior shift in expression up to 10.5-11.0 d.p.c., we wished to determine whether the RA induced changes initiated early (9.5 d.p.c.) persist or were even more extensive in later periods. We treated embryos carrying construct no. 1 and the *Hoxb-2* r3/r5 *lacZ* transgene with RA at 9.5 d.p.c. and analysed the *lacZ* staining patterns at 11.5 d.p.c., 48 hours after treatment (Fig. 4A-D). It is not possible to observe the morphology of rhombomere boundaries in embryos older than 10.5 d.p.c., however using the r5 stripe of expression as a guide, the anterior boundary of expression from construct no. 1 still appears to lie at the r6/7 junction following RA treatment, while the boundary in untreated embryos maps more caudally, at the hindbrain/spinal cord junction (Fig. 4A,B). Therefore, the RA induced shifts in anterior expression initiated at 9.5 d.p.c. are stable and persist to later stages, and appear to be fixed at the r6/7 junction.

Together, these results indicate that the *HOXD4* upstream fragment acts in vivo as a retinoid-responsive region, and is responsible for an anterior shift in the neural tube expression domain observed after exogenous administration of excess RA.

Retinoid response in vivo and in cell culture maps to a 185-bp conserved enhancer

Since the human *HOXD4* upstream region mediates neural-restricted gene expression and RA response in vivo in transgenic mice, as well as RA-dependent induction in NT2/D1 cells (Fig. 1, construct no. 1, and Moroni et al., 1993), we wanted to determine if these activities were related to each other, and under the control of similar factors interacting with similar *cis*-acting regions. It was previously shown that a 185-bp sequence, spanning position −2693 to −2508 from the proximal transcription start site of *HOXD4*, is highly conserved in the mouse *Hoxd-4* upstream regulatory region, and can mediate response to all-*trans*- and 9-*cis*-RA in culture (Moroni et al., 1993; Pöpperl and Featherstone, 1993). Internal deletion of the conserved 185 bp sequence from the 2.9 kb *HOXD4* upstream region (−2711 to −2333, construct no. 2, Fig. 1) completely abolished its activity in cell culture as well as in transgenic mice. Of eight transgenic founder embryos and one line obtained with this construct, none showed any expression in the neural tube. Two of these embryos however, did show some ectopic expression patterns, probably due to position effects at the site of integration, indicating that construct no. 2 was capable of activity in vivo (Fig. 5A).

A construct containing only the conserved 185 bp *HOXD4* region linked to a basal *Hoxb-4* minimal promoter/*lacZ* reporter was able to confer RA inducibility in EC cells in either orientation (construct no. 3 and 4, Fig. 1), at levels comparable to those obtained with the whole −2892/+21 region (Fig. 1). This confirms previous results obtained with larger fragments from either the human or the mouse gene, driving the herpes virus thymidine kinase or the adenovirus major late promoter respectively (Moroni et al., 1993; Pöpperl and Featherstone, 1993). The two minimal enhancer constructs were also

sufficient to direct neural tube-specific *lacZ* expression in transgenic mice (Fig. 5B-E). Three out of seven transgenic founder embryos obtained with construct no. 3, and two out of four with construct no. 4, showed *lacZ* staining in the neural tube in a pattern indistinguishable from that seen with construct no. 1, containing the full-length 2.9-kb fragment. The minimal region contained in construct no. 3 and 4 contains neither of the human *HOXD4* EC cell promoters, nor the region homologous to a putative autoregulatory element previously found in the mouse *Hoxd-4* gene (Pöpperl and Featherstone, 1992). These results show that the conserved 185-bp enhancer is both necessary and sufficient to direct spatially restricted expression in the neural tube in vivo, and RA-inducibility in cell culture.

Molecular dissection of the *HOXD4* enhancer

DNase I footprinting of the *HOXD4* minimal enhancer with nuclear extracts from NT2/D1 cells identified four distinct elements, termed X, 1, 2 and 3, and several DNase I hypersensitive sites (Fig. 6). These footprints become evident after 3-7 days of RA induction, as compared to a wide, less distinct footprint covering the entire region observed in uninduced cells (Fig. 6A). Two of the four footprinted sequences contain direct repeats (DR) matching the PuG[G/T]TCA consensus sequence for nuclear hormone receptors (Leid et al., 1992), i.e., a TGTTCA(N3)AGGTCA (DR3) motif (in reversed orientation) in the footprinted element 2, and a AGGTGA(N5)AGGTCA (DR5) motif in element 3 (Fig. 6C). Furthermore, the footprinted element 1 contains a half-site TGTTCA motif, together with two sequences matching in 11 out of 13 nucleotides the 5′-GTTAATNATTAAC-3′ consensus binding site for the HNF1/LBF1 family of proteins (Courtois et al., 1988; De Simone et al., 1991) (Fig. 6C). Conversely, the element X contains no recognisable binding site for nuclear hormone receptors, nor for other known transcription factors. The factors actually binding element X, 1 and 2 are unknown, whereas the DR5 motif present in element 3 was previously shown to bind protein complexes containing RARs, and to be necessary for the RAR-dependent, RA-inducible activity of the *HOXD4* enhancer in murine and human EC cells (Moroni et al., 1993; Pöpperl and Featherstone, 1993). Fig. 6A shows that the footprint on element 3 is generated by NT2/D1 nuclear extracts several days after RA exposure, indicating that availability of the ligand is not the only factor required for binding of the RAR-containing protein complexes on the *HOXD4* enhancer.

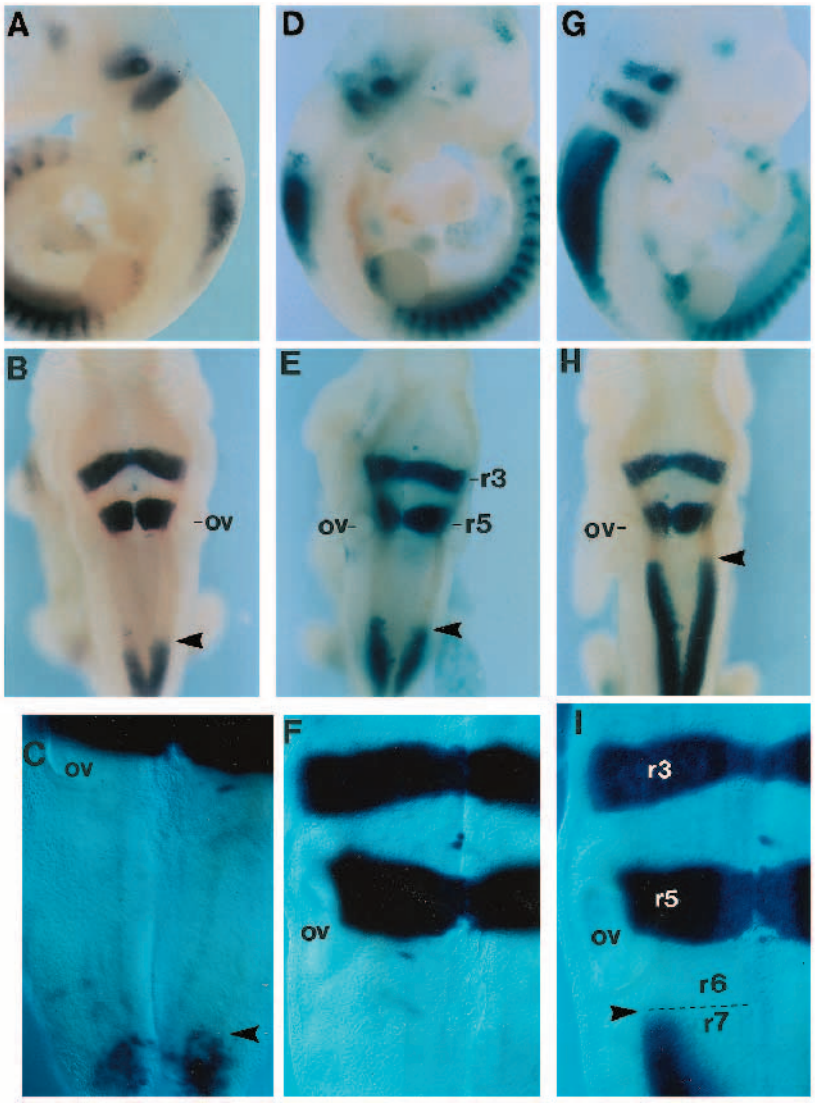

Fig. 3. The effect of RA upon the *lacZ* staining pattern obtained using the line (AL15) containing construct no. 1. The position of the anterior expression boundary of the *HOXD4* transgene is shown relative to r5, marked with a second transgene that uses the *Hoxb-2* r3/5 specific enhancer to drive *lacZ* expression. The r3/5-specific *lacZ* transgene is contained in the line ML19. Embryos containing both transgenes were obtained by interbreeding male and female mice from these two transgenic lines. (A) Expression of the two transgenes in a 10.0 d.p.c. embryo which was not treated with RA. (B,C) Dorsal view and flat mount respectively of the embryo shown in A, illustrating the considerable distance between the anterior boundary of the *HOXD4* transgene expression (arrowhead) and r5. (D) Expression of the two transgenes in a 10.0 d.p.c. embryo which was treated with RA in utero at 7.5 d.p.c. Note that the relative positions of the anterior boundary of the *HOXD4* transgene expression and r5 are unchanged compared with the untreated control shown in A and B. (E) Dorsal view of the embryo shown in D. (F) Flat mount of non-RA-treated embryo shown in A, showing both the r3 and r5 stripes of *lacZ* expression. No expression from the *HOXD4* transgene can be seen in this field of view. (G) Expression of the two transgenes in a 10.0 d.p.c. embryo which was treated with RA in utero at 9.5 d.p.c.. (H) Dorsal view of the embryo shown in G, note that the anterior boundary of the *HOXD4* transgene has shifted rostrally towards r5 and the otic vesicle (OV). (I) Flat mount of embryo shown in G, showing that expression of the *HOXD4* transgene extends up to the r6/7 boundary (dashed line). Compare this with a similar field of view (F) from the untreated control embryo shown in A.

The role of the single footprinted elements in the activity of the *HOXD4* enhancer was first tested by transient transfection in NT2/D1 cells, in the context of the −2892/+21 genomic region with the two native promoters driving the *lacZ* reporter gene. Deletion of element X (−2654 to −2628, see Fig. 6C) had no significant effect on the basal activity of the reporter in uninduced NT2/D1 cells, whereas it caused a 70% reduction of its activity in RA-induced cells (construct no. 5, Fig. 1). Extensive mutation of the DR3 motif in element 2 (from TGACCTGTCTGAACA to **GGGCCC** GTCT**AG**AC**C**, mutations in bold) had virtually no effect on the reporter activity in both uninduced and induced cells (construct no. 6, Fig. 1), while deletion of element 3 (−2556 to −2532, see Fig. 5C), abolished the RA response to the same extent as deletion of the whole enhancer (construct no. 7, Fig. 1). These results confirmed the essential role of the DR5-containing element 3 in mediating the ability of the *HOXD4* enhancer to confer RA response in EC cells, and showed that element X, which contains no obvious RAREs nor binds RARs or RAR-containing complexes (Moroni et al., 1993), is nonetheless crucial for the enhancer function. Deletion of either element X or element 3, as well as mutation of the DR3 in element 2, had no effect on the relative spacing of the remaining elements, and caused no apparent effect on their ability to bind nuclear proteins in 7-day NT2/D1 extracts, as shown by DNase I footprinting (Fig. 6B). This suggests that binding of nuclear factors to the four elements occurs independently, and is not strictly based on co-operative interactions between each element to initiate binding.

We next tested the mutated constructs in transgenic mice. Of 13 transgenic founder embryos containing construct no. 5, deleted in the X element, none showed any expression in the neural tube. Again variable ectopic expression was observed in two embryos showing that the reporter gene was functional (Fig. 7A). Since deletion of the X element did not completely abolish the RA responsiveness of the enhancer in transfected EC cells, we tested whether construct no. 5 could still respond to exogenous RA in vivo. Seven of the 13 embryos obtained

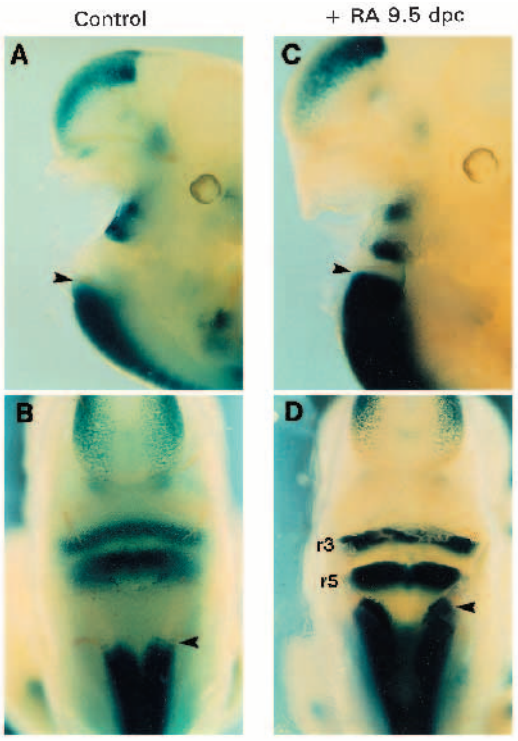

Fig. 4. The expression of the *HOXD4* and *Hoxb-2* r3/5 transgenes in 11.5 d.p.c. embryos after either mock treatment (A) or RA treatment (C) at 9.5 d.p.c.. (B) Dorsal view of embryo shown in A. (D) Dorsal view of RA-treated (9.5 d.p.c.) embryo shown in C. Again the anterior boundary of expression of construct no. 1 in the RA-treated embryo is more rostral than in the mock treated embryos.

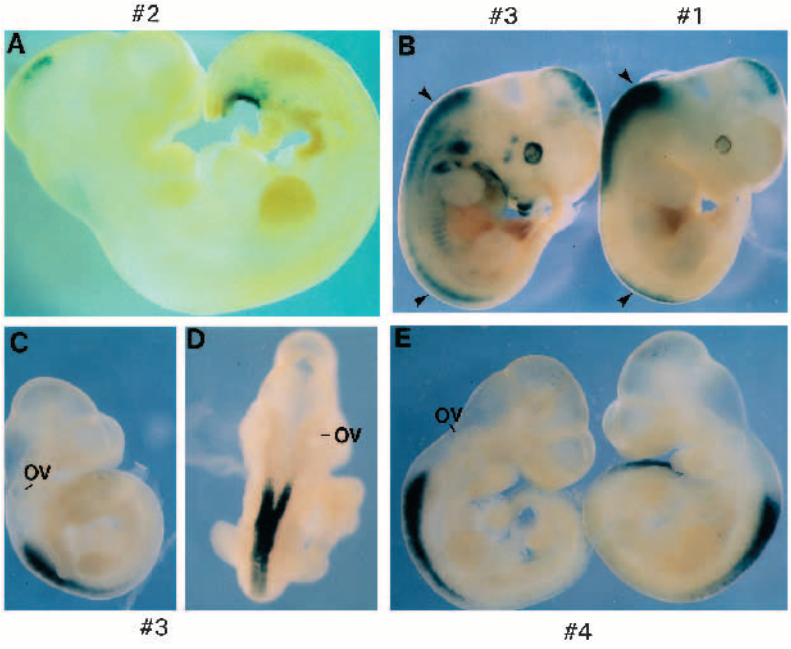

Fig. 5. The conserved 185-bp enhancer element is necessary and sufficient to produce the same expression pattern seen with the full-length *HOXD4* upstream region. (A) A 10.5 d.p.c. transgenic embryo containing construct no. 2. No expression is observed in the neural tube although ectopic expression is seen in the superior colliculi and caudal mesoderm. This staining illustrates that the construct is capable of activity. (B) A comparison of the *lacZ* staining patterns obtained in 11.5 d.p.c. embryos carrying constructs no. 3 and no. 1 (indicated above each embryo). Expression is seen in the neural tube up to the same anterior boundary, again with a more posterior biphasic distribution indicated by the arrowheads. The additional expression in the lateral mesoderm and face of the embryo carrying construct no. 3 was not observed in other examples (see C), suggesting that this is due to position effects at the site of integration for this embryo. (C) Expression of a 10.5 d.p.c. embryo carrying construct no. 3. (D) A dorsal view of the embryo in C showing that the rostral limit to expression in the neural tube is caudal to the r6/7 boundary, which lies immediately behind the otic vesicle (OV). (E) Two 10.5 d.p.c. transgenic embryos carrying construct no. 4, showing an identical staining pattern to that seen with constructs no. 1 and no. 3.

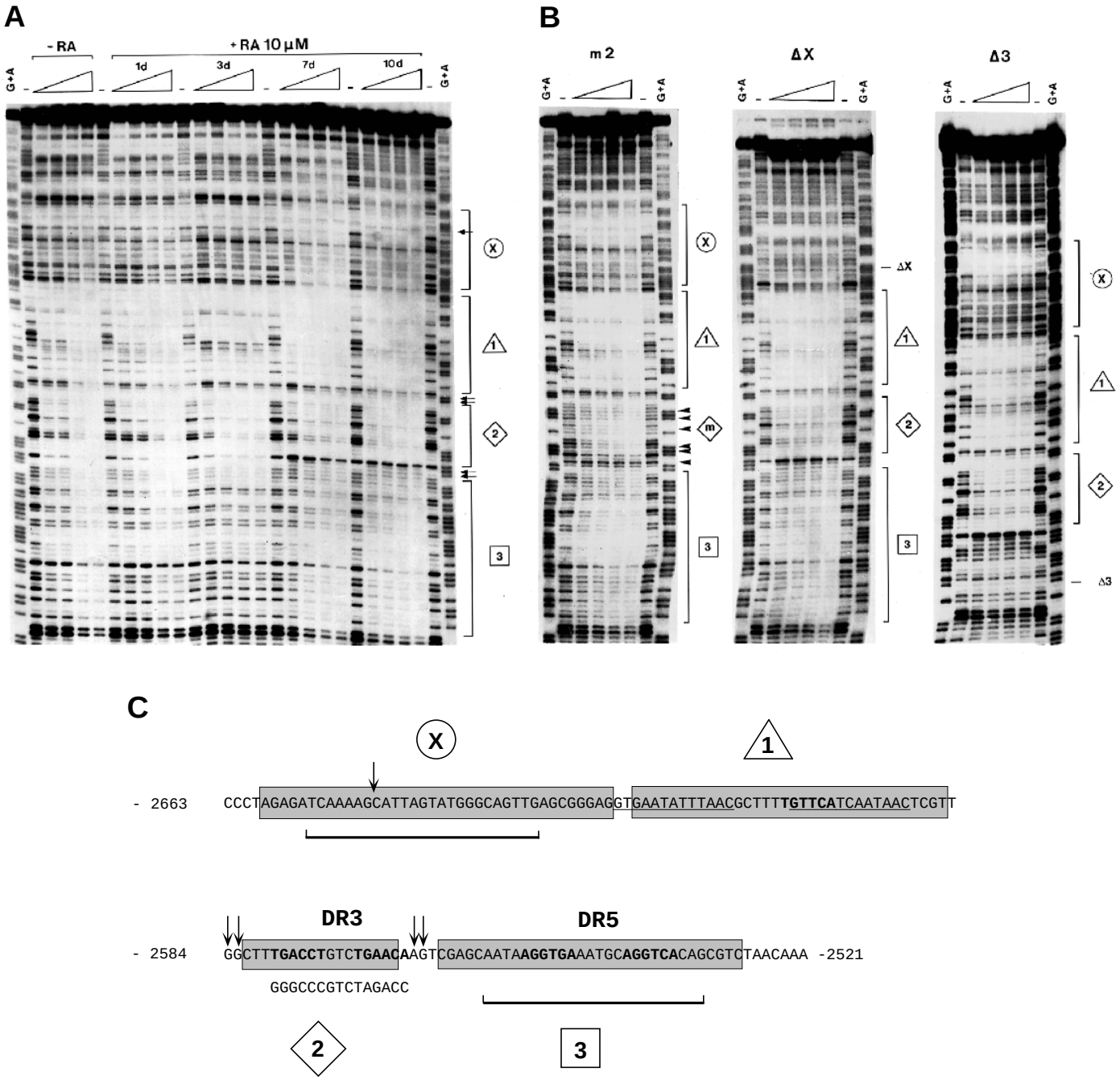

Fig. 6. (A) DNase I footprinting analysis of the *HOXD4* upstream genomic region spanning nucleotides –2720 to –2449 from the proximal (P2) transcription start site. The 3′ end-labeled fragment was incubated with 12, 24, 36 or 54 µg of nuclear extracts from NT2/D1 cells, uninduced (–RA) or induced with 10 µM retinoic acid (+ RA 10 µM) for 1, 3, 7 or 10 days, before digestion with DNase I. Unprotected DNA is shown by –. A G+A Maxam and Gilbert sequence reaction is shown in the first and last lane. The regions protected by nuclear extracts of RA-induced NT2/D1 cells are indicated as X, 1, 2 and 3. Arrows indicate DNase I hypersensitive sites. (B) DNase I footprinting analysis of the same *HOXD4* region, mutated in element 2, or deleted of element X or 3. Nuclear extracts were obtained from NT2/D1 cells induced by RA for 10 days, and used in the same amounts as in A. The point mutations in region 2 are indicated by arrowheads. The position of each deletion is indicated on the right side of the respective panels (ΔX or Δ3). A G+A sequence reaction is shown in the first and last lane in each panel. (C) Nucleotide sequence of the *HOXD4* upstream region from position –2663 to position –2521 with respect to the proximal (P2) transcription start site (Cianetti et al., 1990). The regions footprinted in A and B are shaded, and indicated with X, 1, 2, and 3, and a symbolic notation (circle, triangle, diamond and square, respectively). The arrows indicate DNase I hypersensitive sites. The DR5 RARE in element 3, the DR3 site in element 2 and the hemisite in the element 1, are shown in bold face. Two putative HNF-1 binding sites are underlined in element 1. The sequence mutated in the p*HOXD4*-2892m2 constructs (see Fig. 1, construct no. 6) is indicated below the wild-type sequence. The regions deleted in the p*HOXD4*-2892ΔX and Δ3 mutants are indicated by brackets.

with construct no. 5 were also treated with RA in utero at 9.5 d.p.c.. None showed expression in the neural tube at 10.5 d.p.c., although ectopic expression was observed in one case (data not shown). This shows that the X element is essential for the enhancer activity in vivo. Thus while deletion of the X element leaves some residual enhancer activity in the transfection assay, it appears that this is insufficient to direct gene expression in the neural tube, even in the presence of exogenous RA.

Of 8 expressing embryos obtained with construct no. 6, in which the DR3 sequence of element 2 was mutated, all showed *lacZ* expression in the neural tube (Fig. 7C), in a pattern indistinguishable from that seen with constructs no. 1, 3 or 4. Founder embryos carrying construct no. 6 were also treated at 9.5 d.p.c. with RA, and analysed at 10.5 d.p.c. Fig. 6C-H shows, in whole-mount embryos and coronal sections, that treatment with RA induces a rostral shift in the anterior boundary of expression up to approximately the r6/7 junction, as with the wild-type enhancer. These data show that the DR3 in element 2 has no influence on the enhancer activity in vivo, in generating either the neural tube-specific expression or the response to exogenous RA.

Finally, we tested the role of the DR5-containing element 3 by generating transgenic mice with construct no. 7. Of a total of eight transgenic founder embryos containing this construct, none showed expression in the neural tube. One embryo did however show ectopic expression indicating the reporter was capable of functioning (Fig. 7B). Administration of exogenous RA in utero at 9.5 d.p.c. to these embryos had no effect on *lacZ* expression. Because we obtained no detectable expression in transgenics when either element 3 or X were mutated, unlike the basal level seen in EC cells when X is mutated (construct no. 5, Fig. 1), it is not possible to determine if elements X and 3 are both involved in the RA response. These experiments show that element 3 containing the DR5 RARE is also a critical component of the *HOXD4* enhancer, and suggests that interactions between factors binding to element X and the RARE are important in directing neural-specific expression and RA response.

DISCUSSION

In this paper we show that a conserved 5′ flanking region of the *HOXD4* gene active in EC cells is capable of directing spatially restricted neural expression in vivo. Expression of a *lacZ* transgene under the control of the 2.9-kb *HOXD4* upstream region is activated in the neural tube with a graded distribution. The highest levels of staining appear in the anterior domain which has a border caudal to the otic vesicle, and the posterior boundary maps in the mid-thoracic region. Expression increases with time, and progressively spreads forward until it reaches an anterior boundary in the region of the hindbrain-spinal cord junction, which is just posterior to the anterior limit of expression of the endogenous *Hoxd-4* gene at the r6/r7 junction (Gaunt et al., 1989; Hunt et al., 1991). Therefore, this pattern represents a major subset of the neural domain of endogenous *Hoxd-4* expression (Fig. 2E,F), and suggests that this enhancer region normally regulates a component of *Hoxd-4* neural expression.

In support of this idea, transgenic analysis of the paralogous gene, *Hoxb-4*, has shown that the domains of neural expression are not generated by a single enhancer element. Rather, the expression is the result of overlapping patterns produced by the combinatorial action of at least two different enhancers, one of which (Region A) specifies the correct anterior boundary of expression at the r6/r7 junction and the other (Region C) which produces the more posterior domain (Region C) (Aparicio et al., 1995; Whiting et al., 1991). The pattern of *HOXD4* transgene expression closely resembles that generated by the *Hoxb-4* region C enhancer, and we predict by analogy that the elements which regulate anterior expression at the r6/r7 boundary must reside elsewhere in the locus. Furthermore, analysis of the *Hoxa-4* gene has also shown that multiple 5′ elements regulate its neural expression (Behringer et al., 1993), so the requirement for different enhancers in generating the full neural pattern may be a common property of the paralogous group 4.

We performed a detailed analysis of the ability of the *HOXD4* regulatory region to respond to exogenous RA in utero, because of its RA sensitivity in EC cells (Moroni et al., 1993; Pöpperl and Featherstone, 1993). Our experiments clearly demonstrate that this region is able to mediate a rapid and stable anterior shift in neural-restricted *HOXD4* transgene expression, up to the r6/r7 boundary (Figs 3 and 4). This response occurs when embryos are treated with RA at 9.5 d.p.c., but not when they are treated at 7.5 d.p.c.. This is interesting because other *Hox* genes, such as *Hoxb-1* and *Hoxb-2*, have been shown to respond to RA treatments at 7.5 d.p.c., but not later stages (Conlon and Rossant, 1992; Marshall et al., 1992). This implies that there are temporal differences in the competence of specific *Hox* genes to respond to RA, and that these can be linked to particular enhancers.

Identical *cis*-acting elements are required for the *HOXD4* enhancer activity in vivo and in EC cells

In a direct comparative analysis, we mapped sequences within the 2.9-kb *HOXD4* upstream region which are necessary for its activity both in RA-induced EC cells and in transgenic mice. The region contains two previously identified domains which might be involved in the generation of the expression pattern we observed in vivo. These are a potential autoregulatory element at the 3′ end of the region (Pöpperl and Featherstone, 1992), and a conserved 185-bp transcriptional enhancer associated with RA response in EC cells (Moroni et al., 1993; Pöpperl and Featherstone, 1993). A possible reason why the entire *HOXD4* upstream region functioned effectively in transgenic mice could have been that the autoregulatory element served as a readout for part of the endogenous *Hoxd-4* expression. However, deletion of the conserved 185-bp block, leaving the autoregulatory element and the remaining sequences in the 2.9 kb region unaltered (construct no. 2 in Fig. 1), specifically abolished the spatially restricted expression even in the presence of exogenous RA in vivo. Hence, the autoregulatory motif is not sufficient for the expression we observed in transgenic mice, although it is able to direct region-specific expression in transgenic flies (McGinnis et al., 1990). Furthermore, we found that the conserved 185-bp block alone could function as an enhancer in an orientation-independent manner linked to a heterologous promoter, and mediates all the regulatory activities observed in vivo and in cell culture with the full 2.9-kb fragment. Combined, these results indicate that

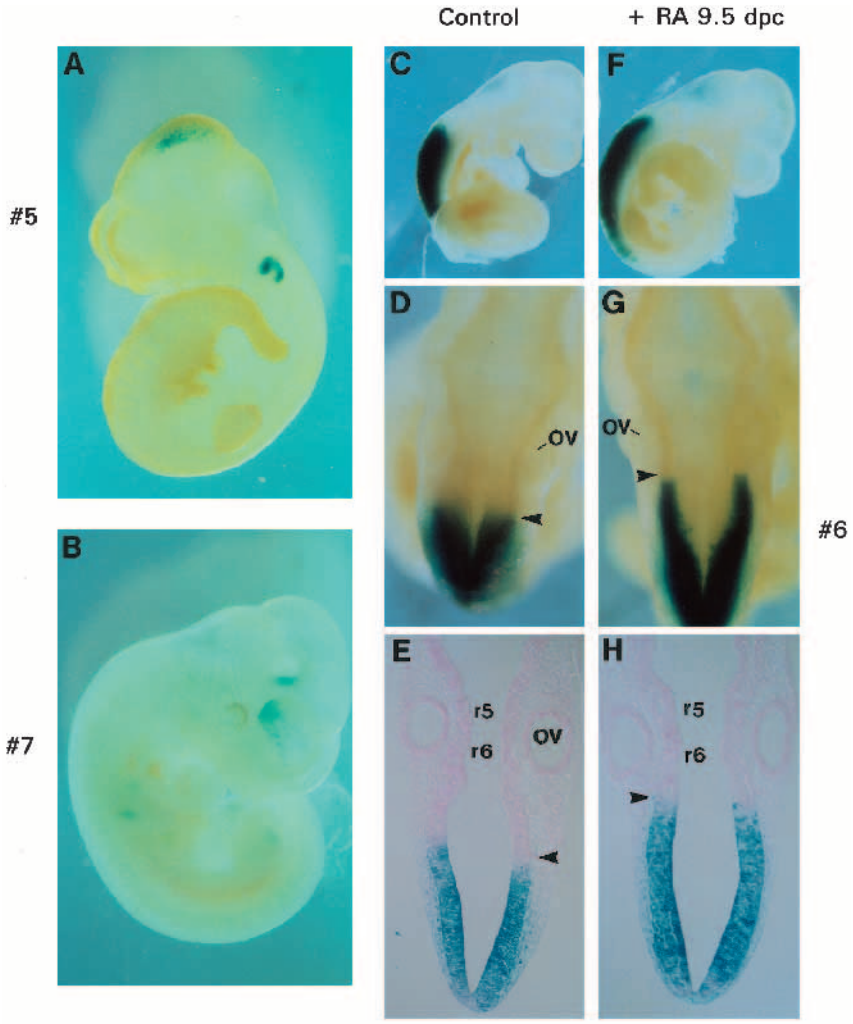

Fig. 7. The footprinted elements X and 3 are essential for *lacZ* expression in transgenic mice. (A) One of the 13 transgenic embryos carrying construct no. 5, none of which showed expression in the neural tube. The 10.5 d.p.c. embryo shown here has ectopic expression in the superior colliculi and around the otic vesicle, indicating that the reporter gene was capable of activity. (B) One of eight 10.5 d.p.c. embryos carrying construct no. 7. Again, no expression is observed in the neural tube. (C) A 10.5 d.p.c. embryo carrying construct no. 6 showing expression in the neural tube in a pattern identical to that seen with construct no. 1, suggesting that element 2 is not required for enhancer activity in transgenic mice.(D,E) Dorsal view and coronal section respectively of the embryo shown in A, showing that the anterior boundary of *lacZ* expression (arrowhead) relative to the otic vesicle (OV) and the r5 and 6 bulges. (F) The effect of RA treatment at 9.5 d.p.c. in utero on the expression pattern at 10.5 d.p.c. of a founder transgenic embryo carrying construct no. 6. (G,H) A dorsal view and coronal section respectively of the embryo shown in F. The RA-treated embryo containing construct no. 6 shows a rostral shift in the anterior *lacZ* expression boundary (arrowhead) towards the otic vesicle up to the r6/7 junction. This is the same response to RA seen with construct no. 1.

the minimal 185-bp block is both necessary and sufficient for generating the spatially restricted expression pattern in the neural tube. It is possible that the autoregulatory element could be involved in controlling other aspects of *HOXD4* expression, in conjunction with regions outside the 2.9-kb upstream fragment, but our analysis clearly shows that it has limited activity by itself and does not appear to work co-operatively with the 185-bp enhancer.

Previous footprinting analysis (Moroni et al., 1993), and work carried out in this study, show that nuclear extracts obtained from RA-induced EC cells protect four specific elements within the 185-bp enhancer. One of these (element 2) contains a DR3 consensus binding site of the type recognised by vitamin D or thyroid hormone receptors, but mutations abolishing the DNase I footprint in this element (Fig. 6B) have no effect on the enhancer activity or RA response in either transgenic mice or EC cells (construct no. 6; Figs 1 and 7C-H). Similarly, we have no evidence for a functional role of element 1, containing a consensus hormone receptor binding half-site, and this site appears not to be required in the homologous element of the mouse *Hoxd-4* enhancer (Pöpperl and Featherstone, 1993). Conversely, mutation or deletion of either of the two remaining elements (X and 3) specifically abolished expression and RA response in both EC cells and transgenic mice (constructs no. 5 and no. 7 in Figs 1 and 7A,B). Therefore, an identical subset of elements within the conserved 185-bp enhancer, acting independently or co-operatively, are responsible for regulating the *HOXD4* expression and RA response in both systems.

Requirement of a DR5 RARE for *HOXD4* regulation

The response of the *HOXD4* enhancer to exogenous RA in embryos and cell culture raised the question of whether the ectopic induction was mediated by the same or different elements from those required for spatially restricted expression in vivo. Our results demonstrated that footprint element 3, which contains a DR5 RARE that interacts with RARs (Moroni et al., 1993; Pöpperl and Featherstone, 1993), is absolutely required for the neural-restricted expression and RA response directed by the enhancer in transgenic mice and EC cells. There is no expression from constructs which specifically mutate this element (construct no. 7, Figs 1 and 7), implying that the DR5 RARE is likely to be essential in the normal regulation of part of the *HOXD4* neural expression pattern.

In agreement with these findings in *HOXD4*, different RAREs have also been identified close to the most 3′ genes in two mouse complexes, *Hoxa-1* (Frasch et al., 1995; Langston and Gudas, 1992) and *Hoxb-1* (Marshall et al., 1994; Studer et al., 1994). The RAREs in *Hoxb-1* are highly conserved in the human, mouse, chick and puffer fish homologous genes,

implying that they are functionally important (Marshall et al., 1994; Ogura and Evans, 1995a,b; Studer et al., 1994). Indeed, the conserved DR2 RARE at the 3′ end of *Hoxb-1* is required for establishing early neural expression in vivo and the early RA response (Marshall et al., 1994), which is similar to the role of the DR5 in *HOXD4*. Together these results of transgene expression show that retinoid-responsive elements of the DR2 and DR5 type are involved in the generation of neural-specific expression of multiple *Hox* genes. The fact that the sequences required for spatial aspects of *Hox* gene regulation are the same as those required for ectopic response to exogenous retinoids in both cultured cells and embryos, further supports the idea that endogenous retinoids are instrumental in establishment and spatial restriction of *Hox* gene expression.

The DR5 RARE requires additional factors for *HOXD4* enhancer activity in vivo

The patterns of transgene expression we observed with the *HOXD4* enhancer are not simply a readout of the DR5 ability alone to respond to endogenous retinoids. Multimerized RARE elements conferring an immediate-early response to ligand-activated RARs, such as the DR5 RARE from the RARβ gene, are capable of directing broad expression of a reporter gene in transgenic mice (Balkan et al., 1992; Colbert et al., 1993; Mendelsohn et al., 1991; Reynolds et al., 1991; Rossant et al., 1991). Furthermore, transgene expression directed by the RARβ retinoid-responsive element induced *lacZ* activity in the entire embryo upon exposure to excess RA in utero (Rossant et al., 1991). These types of observations previously reported for minimal RAREs contrast with those we obtained using the *HOXD4* enhancer. In transgenic mice, deletion of the X element from the enhancer (construct no. 5 Fig. 7) completely abolishes spatially restricted expression, even though the DR5 RARE is still present. In addition, treatment with excess RA had no effect on the inability of construct no. 5 to direct expression in vivo. Hence, the *HOXD4* DR5 RARE is not sufficient to mediate spatial expression in vivo or response to RA. In agreement with these findings, mutational analysis of the RAREs in *Hoxb-1* has also shown that they are necessary but not sufficient for the function of their respective enhancers in transgenic mice (Marshall et al., 1994; Studer et al., 1994).

The essential role of element X in these experiments points to a critical requirement for additional factors in potentiation of the DR5-dependent activity in vivo. In cell culture, deletion of the X element significantly decreases, but does not abolish, the RA inducibility of the enhancer, suggesting that the X element may work by increasing the basal promoter activity upon which the DR5 element can impose an RA-dependent response. It is difficult to ascertain if the same is true in transgenic embryos, because the basal activity is undetectable in all (13) of the independent integrations with the same construct. The in vivo analysis suggests that the X element may, rather, be involved in co-operative interactions with the DR5 RARE, by affecting the binding specificity/affinity of RARs themselves or by recruiting other positive or negative acting proteins to the RARE sites. In fact, RARα, β and γ bind the isolated *HOXD4* DR5 in vitro much less efficiently than the RARβ RARE (Moroni et al., 1993). In agreement with the requirement for additional factors, the ability of the human *HOXB1* RAREs to confer an RA response in EC cells is also influenced by the presence of adjacent binding sites for unknown factor(s) (Ogura and Evans, 1995a,b). The X element has no homology with the accessory sequences surrounding the *Hoxb-1* RAREs, nor does it contain binding sites for characterised transcription factors, so it will be important to identify these accessory factor(s) to understand the mechanism by which they modulate RARE activity.

Retinoid-responsive elements and activation of the *Hox* complexes

Hox genes are sequentially activated in development, display ordered or colinear domains of expression along the embryonic axis, and show a colinear response to retinoids in cell culture (reviewed by Krumlauf, 1994; McGinnis and Krumlauf, 1992). Understanding the basis of the sequential RA response in vitro could therefore be important in understanding the mechanisms which generate colinearity, a concept reinforced by the finding that the same RAREs required for RA response are also essential for spatial regulation of gene expression in vivo. However, multiple mechanisms are likely to be responsible for the retinoid-dependent activation of the *Hox* complexes (reviewed by Langston and Gudas, 1994). This could include indirect RA responses (Pöpperl et al., 1995), putative master 3′ RAREs involved in opening the complexes (Langston and Gudas, 1992), multiple RAREs with different potential (Frasch et al., 1995; Marshall et al., 1994; Studer et al., 1994), and a cascade of cross and autoregulatory interactions between the *Hox* genes (Faiella et al., 1994; Pöpperl et al., 1995; Pöpperl and Featherstone, 1992).

The results presented here show that in addition to the RAREs flanking the 3′-most genes, RARE-dependent enhancers capable of mediating restricted expression can also be present within a cluster. Since the 3′ genes are flanked by RARE-containing elements and show a most rapid and protein synthesis-independent response to RA treatment (La Rosa and Gudas, 1988; Simeone et al., 1991), it is logical to consider them as direct targets for the retinoid receptors. The late response in genes in the middle or at the 5′ end of the clusters could require indirect factors or progressive ‘opening’ of the cluster triggered by 3′ gene activation. We found that the endogenous *Hoxd-4* gene, and the *lacZ* transgene under the control of the *HOXD4* enhancer, do respond to RA treatment in utero, but under different conditions with respect to *Hoxb-1*. Ectopic expression of *Hoxb-1* is activated by exposure to RA at 7.5 d.p.c., whereas *Hoxd-4* and the *Hoxd4-lacZ* transgene respond only if embryos are treated two days later, at 9.0-9.5 d.p.c.. The fact that RAREs, and additional sequence elements, are required for both the early and late response, suggests that there is a restriction on the ability of the *HOXD4* DR5 element to respond to ligand-activated RARs present during the earlier period. Similarly, additional factors in the *Hoxb-1* enhancer may facilitate its early response and block activity later. Our in vitro binding studies show that the complex footprint observed on the *HOXD4* enhancer in RA-induced EC cells is not generated immediately, as soon as the ligand is given to the cells. Rather there is a time lag (approx. 3 days) necessary for the factors binding to the four elements, including the DR5 RARE, to be assembled in the characteristic ‘induced’ configuration. This might entail de novo synthesis and/or post-translational modification of nuclear factors in addition to the RARs, required for binding and enhancer activity. Therefore, we favour the idea that specific *trans*-

acting factors synergising with RARs could temporally regulate the activity of RAREs, and binding such factors could be one of the roles the X element plays in the *HOXD4* enhancer.

In conclusion, this work has implicated a DR5 RARE and its co-operating components in the normal regulation of neural expression of the *HOXD4* gene from a 5′ enhancer. It serves to provide further support for the role of endogenous retinoids in regulating normal domains and boundaries of *Hox* expression, particularly in the nervous system. Studying the induction of *Hox* genes by RA in EC cell culture appears to be a valuable means for investigating normal regulation of these genes, as we found that EC cells and mouse embryos require the same *cis*-elements for appropriate regulation. This is an important advantage as it provides a valid culture system for identifying factors, such as those interacting with region X, in the face of limited amounts of embryonic tissue.

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