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Increased Cdx protein dose effects upon axial patterning in transgenic lines of mice

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To investigate the link between Cdx protein concentration and axial patterning in embryos, we made lines of mice OE1, OE2 and OE4 that overexpress each of the Cdx genes *Cdx1*, *Cdx2* and *Cdx4*, respectively. The lines carry Cdx transgenes under the transcriptional control of their own promoter/enhancer elements. Transgenic embryos show Cdx transcription at 8.5 to 8.7 days within normal spatial domains for Cdx expression (primitive streak/tailbud), yet, overall, they contain elevated levels of Cdx proteins. Increased doses of Cdx proteins result in homeotic shifts in vertebral types along most of the vertebral column, with transformations being most obvious within the cervical region. Most of the shifts are anterior-to-posterior transformations and the anterior limits of these are commonly skull/vertebra 1 (v1) for OE1, v1/v2 for OE2 and v7 for OE4. OE embryos display anterior shifts in the expression of a *Hoxa7/lacZ* reporter within neural, paraxial and lateral plate mesoderm tissues. *Hoxa7/lacZ* expression commences at the normal time in OE1 and OE4 embryos. OE2 embryos display a forward shift in the gradient of Cdx2 protein along the axis, suggesting that a Cdx morphogen gradient model could account, at least in part, for the homeotic shifts in vertebral types. OE mice display additional defects: forelimb deficiencies in OE1, multiple tail axes, vertebral mis-alignments and axial truncations in OE2.

KEY WORDS: Cdx, Overexpression, Homeotic transformation, Hox, Mouse

INTRODUCTION

Organization of the developing head-tail axis of the mouse embryo is mediated, at least in part, by the expression patterns of the 39 Hox genes. Each Hox gene exerts its main effect in the anterior part of its expression domain and hence the anterior boundaries of Hox expression must be positioned with precision (Kmita and Duboule, 2003). The establishment of vertebrate Hox gene expression patterns along the developing AP axis is poorly understood. Two plausible models are the instructional (morphogen) gradient model and the timing model (Gaunt, 2000). In the gradient model, Hox expression boundaries establish at threshold concentrations of a Hox gene activator. Suggested activators are retinoic acid, Fgf, Wnt and Cdx proteins (Deschamps and van Nes, 2005). The timing model, by contrast, proposes that patterns are generated by the sequential activation of Hox genes within the growing primitive streak/tailbud region (Dollé et al., 1989). Consistent with this model, Hox genes have been shown to be activated sequentially, 3′ to 5′, along their clusters (temporal co-linearity) (Izpisúa-Belmonte et al., 1991).

The Cdx proteins are regulators of Hox genes within both neurectoderm and mesoderm (Bel-Vialar et al., 2002; Charité et al., 1998; Ehrman and Yutzey, 2001; Epstein et al., 1997; Isaacs et al., 1998). Knockout of Cdx genes may result in posterior shifts of both Hox gene expression and vertebral morphologies (Chawengsaksophak et al., 1997; Subramanian et al., 1995; van den Akker et al., 2002; van Nes et al., 2006). Posterior shifts of vertebral types are commonly described as posterior-to-anterior homeotic transformations (van den Akker et al., 2002). The Cdx proteins are distributed as posterior-to-anterior concentration gradients along the developing embryo (Beck et al., 1995; Gamer and Wright, 1993; Meyer and Gruss, 1993), and evidence from Cdx/*lacZ* reporter mice has shown that these gradients may form by decay of Cdx protein in cells once they leave the region of intense Cdx gene expression within the primitive streak/tailbud (Gaunt et al., 2003; Gaunt et al., 2005).

An important issue is whether the Cdx protein gradients function as morphogen gradients for the specification of Hox gene expression boundaries. A proposal here is that the expression boundary of a Hox gene may move forward in the gradient until its enhancer elements bind a minimal threshold level of Cdx protein. In this scenario, it is the dose of Cdx protein in a cell that can determine whether or not it will express a given Hox gene. Struhl et al. (Struhl et al., 1989) identified criteria to test for a morphogen gradient in *Drosophila*, and these can also be applied to the mouse. Thus, two experimental predictions are that a mouse Hox expression boundary might be shifted forwards: (1) by increase in the number of Cdx-binding elements within the Hox gene enhancer, so making the gene more sensitive to the endogenous Cdx protein gradient; and (2) by increase in the dose of Cdx product within the normal tailbud domain, so shifting the Cdx protein decay gradient forward along the embryo. The first of these two predictions has already been examined (Charité et al., 1998; Gaunt et al., 2004). Multimerization of the *Hoxb8* or *Hoxa7* enhancer elements results in forward shift in the expression boundaries of Hox/*lacZ* reporters in transgenic mice. This effect depends upon intact Cdx-binding motifs within the additional enhancer elements. These studies do not, however, provide clear-cut evidence for the Cdx morphogen gradient model as enhancer multimerization also causes earlier activation of the transgene (Gaunt et al., 2004), making the main findings also explicable in terms of the timing model.

In the present paper we adopt the second approach described above. We describe lines of mice (OE1, OE2 and OE4) overexpressing *Cdx1*, *Cdx2* and *Cdx4* transgenes under the control of their own promoter/enhancer elements (Gaunt et al., 2003; Gaunt et al., 2005). These OE transgenes are transcribed in early embryos within normal Cdx expression domains where they generate elevated levels of Cdx proteins. For *Cdx2*, we show that raised levels of Cdx protein in tailbuds result in a forward extension of the protein

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gradient along the embryonic axis. When crossed with a *Hoxa7/lacZ* reporter line, OE1, OE2 and OE4 lines show anterior shifts in Hox/*lacZ* expression boundaries without, at least for OE1 and OE4, any accompanying change in the initial timing of *Hoxa7/lacZ* activation. This is consistent with a role for Cdx protein morphogen gradients in the positioning of Hox expression boundaries. The new transgenic lines display homeotic, mainly anterior-to-posterior, transformations in vertebral types, and also forelimb (OE1) and tail (OE2) abnormalities.

MATERIALS AND METHODS

Preparation of overexpresser (OE) transgenic mice

Overexpresser (OE) constructs designed to express full-length Cdx-coding regions are shown in Fig. 1A-C. For each Cdx gene, the second and third exons, together with the second intron and the 3′ end of the first intron was prepared as a PCR fragment using as template Bac clone RP24-211A8 (*Cdx1*), RP24-510G5 (*Cdx2*) or RP23-11P22 (*Cdx4*) (BACPAC Resources Centre, Children's Hospital, Oakland, CA, USA). These PCR fragments extended downstream of the Cdx termination codons by three bases (*Cdx1*), six bases (*Cdx2*) or four bases (*Cdx4*), and upstream of the second exon (*Cdx1*) by 134 bases. As shown in Fig. 1, these fragments, prepared with *Hin*dIII/*Eco*RI (*Cdx1*) or *Bgl*II/*Eco*RI (*Cdx2* and *Cdx4*) ends were used to replace regions of DNA with similar ends in our previously characterized Cdx/*lacZ* reporter constructs (Gaunt et al., 2003; Gaunt et al., 2005). The 450 bp fragment of *lacZ/SV40* DNA remaining in the 3′ untranslated regions (UTRs) of the OE constructs provides a polyA signal and permits detection by PCR and in situ hybridization. Constructs were cut from Bluescript vectors using *Kpn*I plus *Spe*I (*Cdx1*), *Xho*I plus *Spe*I (*Cdx2*) or *Kpn*I plus *Not*I (*Cdx4*).

Transgenic embryo production was as described (Gaunt et al., 2003), injecting DNA into embryos of F1 (CBAxC57Bl6) mice. Transgenic lines were maintained as heterozygotes by crossing with normal F1 mice. Embryos were taken to be at 0.5 days of development at midday on the day of the copulation plug. Genotyping of embryos by PCR was carried out on DNA prepared from extra-embryonic membranes (for embryos over 8.5 days) or from the embryos themselves after *lacZ* staining and photography (for embryos up to 8.5 days).

Cdx protein detection in embryos

For western blotting, tissue lysates were prepared from pooled stage-matched embryo samples. These were run on 10% SDS/PAGE gels and then blotted on to Immobilon-P (Millipore) membranes. Membrane blocking and antibody incubations were as described in Abcam protocols. Primary antibodies were anti-Cdx1 (ab24000, Abcam), anti-Cdx2 (CDX2-88, BioGenex), anti-Cdx4 (ARP32765, Aviva Systems) and anti-GAPDH (ab9484, Abcam). Secondary antibodies were HRP-conjugated anti-mouse or anti-rabbit IgG (Abcam), and detection was by luminescence using Millipore Immobilon HRP substrate. The fold-levels of Cdx overexpression for each OE line were estimated by densitometry scans using the Scion Image programme.

For immunohistochemistry, embryos were fixed for 2 hours in 80% methanol and 20% DMSO (v/v), and then washed/stored in methanol prior to embedding in paraffin wax. Sections (10 μm) were de-waxed in xylene and rehydrated prior to staining with anti-Cdx2 monoclonal antibody and a mouse-on-mouse HRP detection kit (Vector labs). Sections were counterstained with 0.5% Neutral Red.

Skeleton preparations, *lacZ* staining and in situ hybridization

Most skeleton preparations were made upon pups within the first 2 days of life (van den Akker et al., 2001). The *Hoxa7/lacZ* reporter mouse line and the staining method to detect *lacZ* activity were as described (Gaunt et al., 2004). This mouse line carries a chick *Hoxa7/lacZ* transgene containing at least one functional and evolutionarily-conserved Cdx-binding motif. In situ hybridization was carried out as reported previously (Gaunt et al., 1999). Transgene expression was detected using a 450 bp *lacZ/SV40* probe (Gaunt et al., 2003). The *Mox1* probe was prepared from IMAGE clone 3984366 (Lennon et al., 1996). *Hoxb4* and *Hoxa10* probes were as described by Gaunt et al. (Gaunt et al., 1989; Gaunt et al., 1999).

RESULTS

Production of mouse lines OE1, OE2 and OE4

Fig. 1A-C shows the constructs used for overexpression of *Cdx1*, *Cdx2* and *Cdx4* in the transgenic mouse lines OE1, OE2 and OE4, respectively. Two independent OE1 founder mice showed forelimb abnormalities: a slight in-turning of one forelimb seen in one, and a severe in-turning of both forelimbs in a second (Fig. 2A). The least-affected OE1 founder was used to sire transgenic offspring (OE1 line 1). These showed forelimb defects at penetrance varying from no obvious abnormality to severely affected rudimentary limbs (Fig. 2B). Six other founders of normal appearance were screened for defects in the vertebral patterning of their newborns. One such founder was identified (OE1 line 2) whose transgenic pups also showed the same range of forelimb defects described above. Two independent lines of OE1 mice could therefore be compared in these studies. OE1 limb defects are first detected as forelimb buds of reduced size in 10.5 day embryos. Thus, normal (Fig. 2E) forelimb buds form at the level of prevertebrae 4 to 9, whereas OE1 forelimb buds are variable in size with posterior boundaries that are usually shifted forwards (Fig. 2F).

Three independent OE2 founder mice showed kinked tails. Only two of these allowed production of independent lines for analysis in this study. Offspring of both show kinked tails with terminal scabs in some but not all transgenic pups (Fig. 2C). The most severely affected newborns also have shortened tails with multiple protuberances, haemorrhages and scabs at the tip (Fig. 2D). The third OE2 founder mouse sires only non-transgenic live pups. All his transgenic offspring die at 8.5 to 11 days gestation displaying large clubbed tailbuds (Fig. 1G). We find a similar pattern of embryonic death and club-tailing in a smaller proportion of the transgenic embryos produced by the OE2 transgenic lines.

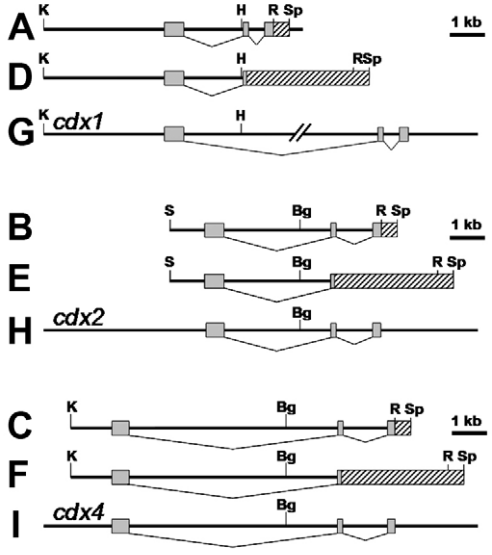

Fig. 1. Mouse Cdx gene overexpresser (OE) constructs for preparation of transgenic mice. The OE constructs (**A-C**) were derived as modifications of Cdx/*lacZ* reporter constructs (**D-F**) previously found to mimic expression of endogenous Cdx genes in early embryos (Gaunt et al., 2003; Gaunt et al., 2005). (**G-I**) *Cdx1*, *Cdx2* and *Cdx4* genomic maps. Grey boxes indicate Cdx exons; hatched boxes indicate *lacZ/SV40*polyA DNA; K, *Kpn*I; H, *Hin*dIII; R, *Eco*RI; Sp, *Spe*I; Bg, *Bgl*II; S, *Sal*I.

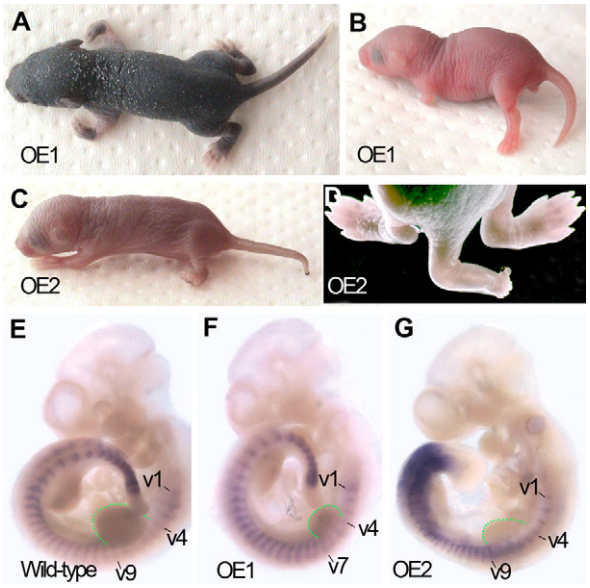

Fig. 2. OE1 and OE2 mice and embryos display externally visible abnormalities with penetrance varying from severe to non-detected. (**A**,**B**) OE1 transgenic mice commonly display defective forelimbs. (**C**,**D**) OE2 mice commonly display kinked tails with terminal scabs in newborns. (**E-G**) Smaller forelimb buds (with dotted green outline) are commonly seen at 10.5 days in OE1 embryos (F) relative to wild type (E) and OE2 (G). Embryos are stained for *Mox1* mRNA to facilitate assignment of prevertebral (v) addresses. The OE2 embryo possesses a club-tail.

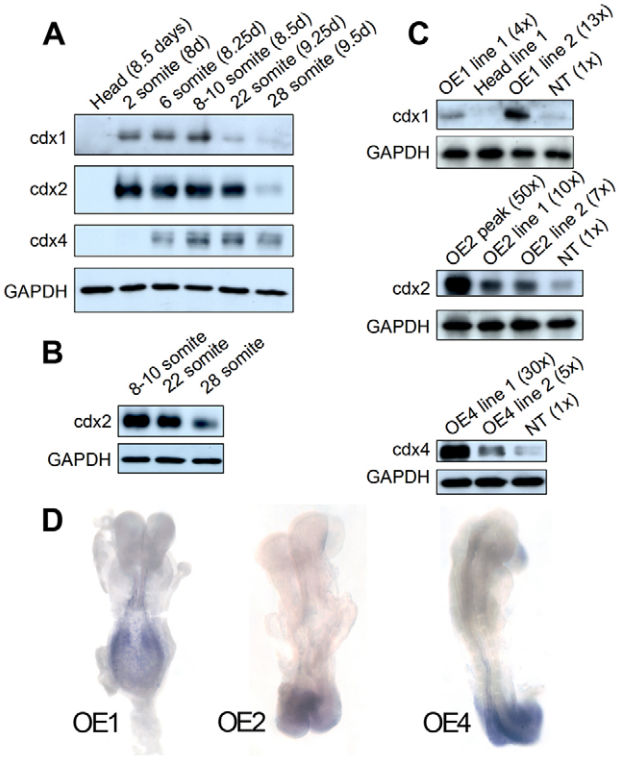

Fig. 3. OE embryos display elevated Cdx protein concentrations and normal location of mRNA expression. (**A**) Western blots showing time-course of Cdx1, Cdx2 and Cdx4 protein levels in the primitive streak/tailbud region of normal embryos. Replicate tissue samples are examined for each protein. Cdx1 bands lie just below a 37 kDa marker protein (not shown); Cdx2 and Cdx4 bands lie just above. (**B**) Western blot showing time-course of Cdx2 protein in OE2 line 1 embryos. (**C**) Western blots comparing Cdx protein concentrations in tailbuds of stage-matched transgenic versus non-transgenic (NT) littermates sired by Cdx overexpresser (OE) mice. Results are shown for lines 1 and 2 of each OE type, and the Cdx2 blot also includes embryos sired by the peak-expresser stud. The fold-levels of overexpression for each OE line, assessed by densitometry, are indicated. (**D**) Expression of OE transgenes (line 1 for each) detected by in situ hybridization of the *lacZ/SV40* probe to 8.5 to 8.7 day embryos.

Independently derived OE4 founder mice were screened for defects in the vertebral patterning of their offspring. Two lines, producing similar defects, were examined in this study.

OE1, OE2 and OE4 transgenes upregulate Cdx expression within the normal domains of embryonic expression

Western blotting was used to compare the levels of Cdx proteins at different stages of normal mouse development (Fig. 3A). Tissue lysates were prepared from embryonic material posterior to, and including, the last two-formed somite pairs. The allantois was not included. Equality of protein loading is assessed by staining for GAPDH. Protein levels for Cdx4 appear to rise slightly later than for Cdx1 and Cdx2, but this is probably due to the smaller, more posterior domain of *Cdx4* expression at the earliest time-point (Gaunt et al., 2005). Protein levels for Cdx1 decline earlier than for Cdx2 and Cdx4. All three proteins form doublets on SDS gels, suggestive of post-translational modification (Gross et al., 2005).

Fig. 3B shows a time-course study with the OE2 line 1. Transgenic embryos show clear reduction in total Cdx2 protein by 28-somite stage, which is similar to that seen with normal embryos (Fig. 3A). The earlier fall in Cdx1 protein and later fall in Cdx4 (Fig. 3A) are also likely to be mimicked in, respectively, the OE1 and OE4 transgenic embryos as similar time-courses have already been described for mRNA expression from the endogenous promoter sequences used in our constructs (Gaunt et al., 2003; Gaunt et al., 2005).

The above studies showed the importance of using embryos at the same developmental stage when comparing transgenic and non-transgenic embryos sired by OE studs. We used 15- to 16-somite stages (OE2 and OE4) and 10- to 12-somite stages (OE1). For each mouse line, the levels of the corresponding Cdx proteins are seen to be higher in the transgenic embryos (Fig. 3C). For Cdx2, the study included embryos sired by the OE2 founder whose transgenic progeny always die at 8.5 to 11 days gestation. These embryos show much higher levels of Cdx2 protein than those produced by the two OE2 lines. We therefore distinguish between OE2 peak-expressers and OE2 lines in this and subsequent experiments.

The OE transgenes include a 450 bp non-translated fragment that contains *SV40* polyA and terminal *lacZ* sequences. Riboprobes to this fragment allow detection of transgene expression by in situ hybridization (Fig. 3D). OE1, OE2 and OE4 transgenes are mainly confined in their expression to the primitive streak/tailbud region of 8.5 to 8.7 day embryos, with mRNA boundaries in paraxial mesoderm located some distance posterior to the presomitic/somitic boundary (not shown, but as previously found for endogenous *Cdx1*, *Cdx2* and *Cdx4* genes) (Gaunt et al., 2005).

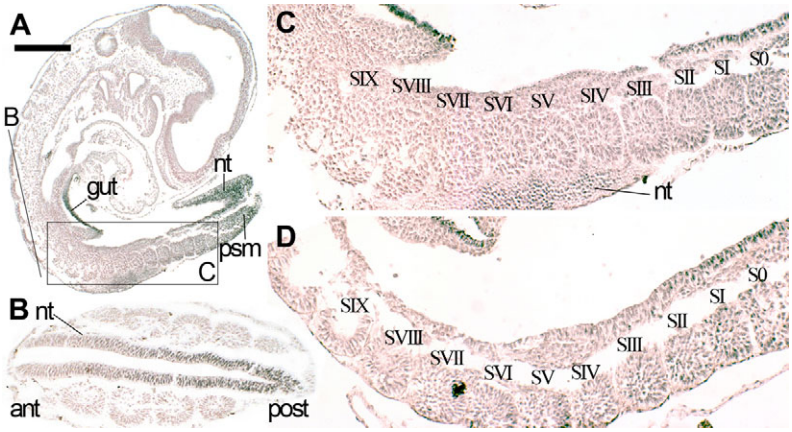

Fig. 4. Cdx2 protein gradients detected by immunohistochemistry in 8.7 day embryos. (**A-C**) Wild-type embryos. (**D**) OE2 transgenic embryo. C is an enlargement of the field boxed in A. B is a section from a similar embryo cut along the plane shown in A. nt, neural tube; psm, presomitic mesoderm; ant, anterior; post, posterior; S, somite. Scale bar: 0.25 mm.

Forward shift in the Cdx2 protein gradient along the axis of OE2 embryos

Of the three anti-Cdx antibodies examined in this study, the anti-Cdx2 antibody provided the highest signal-to-background ratio when tested in immunohistochemistry. This antibody allowed clear visualization of the Cdx2 protein gradient within sections of normal 8.7 day embryos (Fig. 4A-C). The distribution is similar to that described earlier for Cdx2/β-galactosidase protein expression in reporter mice (Gaunt et al., 2005). Posterior neural and mesoderm tissues are intensely labelled and the level of labelling declines on proceeding forward along the embryo. Clear posterior-to-anterior gradients are seen within the newly formed somites (Fig. 4C) and developing neural tube (Fig. 4B).

For comparison, OE2 line 1 transgenic embryos at 8.7 days were also examined. These show a similar pattern except that Cdx2-positive nuclei extend more anteriorly in the embryo. In paraxial mesoderm, heavily labelled nuclei extend forwards to SVII [seven somites anterior to the presomitic/somitic boundary; nomenclature of Pourquié and Tam (Pourquié and Tam, 2001)] in OE2 (Fig. 4D) but only to SIV in wild type (Fig. 4C).

Axial skeletal defects in OE1, OE2 and OE4 newborn mice

Table 1 summarizes the defects observed. Most of the defects may be classified as anterior-to-posterior transformations, symbolized P←A in Table 1 and van den Akker et al. (van den Akker et al., 2002). However, some of the more caudal defects are posterior-to-anterior (P→A) transformations. For each OE type we examined skeletal defects in newborns of the two independent transgenic lines. As the range of defects observed was apparently the same for lines 1 and 2, the data are pooled in Table 1.

In the cervical region, OE1 mice usually show the most anteriorly located defects, with fusions of v1 to the basioccipital (Fig. 5A,D) and splits in v1 (Fig. 5B). These defects are also seen, but much less commonly, in OE2 (Fig. 5F). OE2 defects usually extend forward only to the level of v1/v2 (Fig. 5E). Fusions of v1 and v2, and splits in v2 are seen in both OE1 and OE2, and more posterior cervical vertebrae may be fused in OE1 (Fig. 5D). The cervical vertebra bearing anterior tuberculi, normally v6 (Fig. 5H), may be shifted anteriorly in both OE1 and OE2 mice (Fig. 5C,F). The first vertebra bearing sternal ribs, normally v8, may be shifted anteriorly to the level of v5 for OE1 (Fig. 5A), v6 for OE2 (Fig. 5F) and v7 for OE4 (Fig. 5G). OE4 defects are therefore the most posterior in location, extending forward only as far as v7. All of these cervical defects are anterior-to-posterior transformations.

In the thoracic region, the nuchal ligament normally extends from v9 (Fig. 5H) to the skull. The thoracic attachment may be shifted forward in OE1, OE2 and OE4 mice (Fig. 5A,C,E,F,G). The caudal-most vertebra with rib attached to the sternum is normally v14 (Fig. 6F). This vertebral phenotype is shifted forward in some OE1 (Fig. 6C), OE2 and OE4 mice. The caudal-most vertebra bearing ribs is normally v20 (Fig. 6B,F). This vertebral phenotype may be shifted anteriorly in OE1 (Fig. 6A,C), OE2 (Fig. 7A,B) and OE4 mice. All of these thoracic vertebral defects are anterior-to-posterior transformations. However,

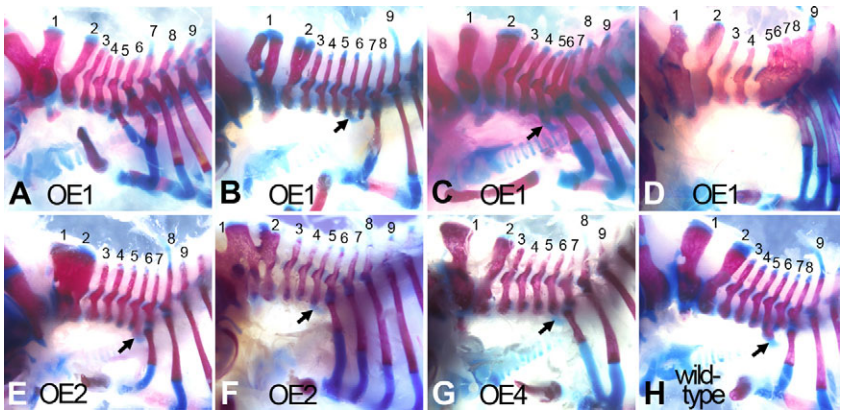

Fig. 5. Vertebral homeotic transformations in the neck and anterior thoracic region of OE mice compared with wild type. (**A-H**) Lateral views of skeletons. There is wide variability within OE1 (A-D), OE2 (E,F) and OE4 mouse lines in the extent of their defects. Arrows indicate anterior tuberculi, which are normally on vertebra 6.

some OE1 and OE4 mice show posterior-to-anterior transformations in the thoracic region. Thus, some OE1 mice show posterior shift in the thoracic attachment of the nuchal ligament (not shown), and some OE1 and OE4 mice show posterior shifts in both the caudal-most vertebra with rib attached to sternum, and in the caudal-most vertebra bearing ribs (Fig. 6D). Rib fusions and mis-alignments of ribs on the sternum are common in all OE mice (Fig. 6E).

In the lumbar region there is normally some homeotic variation. Thus, wild-type mice may show either five or six lumbar vertebrae, so that the first vertebra contributing to the sacrum may be either v26 or v27 (Fig. 6B). Vertebra 27 of normal animals may sometimes show a lumbar phenotype on one side and sacral on the other. In some OE1 mice the first vertebra with a sacral phenotype is shifted forward, in an anterior-to-posterior transformation, up to the level of v24 (Fig. 6A). However, some OE4 mice show a posterior shift

Table 1. Vertebral phenotypes of Cdx gene overexpresser (OE) mice compared with wild type

	Type of transformation (posterior-anterior)	Wild type % (*n*=22)	OE1 % (*n*=16)	OE2 % (*n*=19)	OE4 % (*n*=25)
Abnormalities of vertebra 1					
Fusion to basioccipital (bo)	C1←bo	0	69	5	0
Split	C2←C1	0	6	5	0
Abnormalities of vertebra 2					
Fusion to V1	C2←C1	0	6	26	0
Split	C3←C2	0	31	26	0
Anterior tuberculi on					
V4+V5 unilaterally	C6←C4,C5	0	7	0	0
V5 bilaterally	C6←C5	0	7	16	0
V5+V6 unilaterally	C6←C5	0	53	21	0
V6 bilaterally	None	100	33	63	100
First vertebra with rib attached to sternum					
V5 bilaterally	T1←C5	0	6	0	0
V5+V6 unilaterally	T1←C5,C6	0	6	0	0
V6 bilaterally	T1←C6	0	6	11	0
V6+V7 unilaterally	T1←C6,C7	0	13	5	0
V7 bilaterally	T1←C7	0	13	58	12
V7+V8 unilaterally	T1←C7	0	25	11	28
V8 bilaterally	None	100	31	15	60
Nuchal ligament on					
V7	T2←C7	0	7	0	0
V8	T2←T1	0	13	53	16
V9	None	100	40	47	84
V10	T3→T2	0	40	0	0
Most caudal vertebra with rib attached to sternum					
V12 bilaterally	T7←T5	0	13	0	0
V12+V13 unilaterally	T7←T5,T6	0	6	0	0
V13 bilaterally	T7←T6	0	6	16	8
V13+V14 unilaterally	T7←T6	0	0	26	0
V14 bilaterally	None	100	38	58	80
V14+V15 unilaterally	T8→T7	0	6	0	0
V15 bilaterally	T8→T7	0	31	0	12
Most caudal vertebra bearing ribs					
V18 bilaterally	T13←T11	0	6	0	0
V18+V19 unilaterally	T13←T11,T12	0	0	0	0
V19 bilaterally	T13←T12	0	13	26	8
V19+V20 unilaterally	T13←T12	0	6	0	0
V20 bilaterally	None	100	31	74	80
V20+V21 unilaterally	T14→T13	0	0	0	0
V21 bilaterally	T14→T13	0	44	0	12
First vertebra contributing to sacrum					
V24	S1←L4/5	0	6	0	0
V25	S1←L5/6	0	6	0	0
V26	None	73	31	37	72
V27	None	27	57	63	16
V28	S1→L6	0	0	0	12

Percentages are given to the nearest whole number. V, vertebrae. For each OE type, data are pooled from lines 1 and 2 as no differences were found between them in the extents of their defects. C, cervical; T, thoracic; L, lumbar; S, sacral.

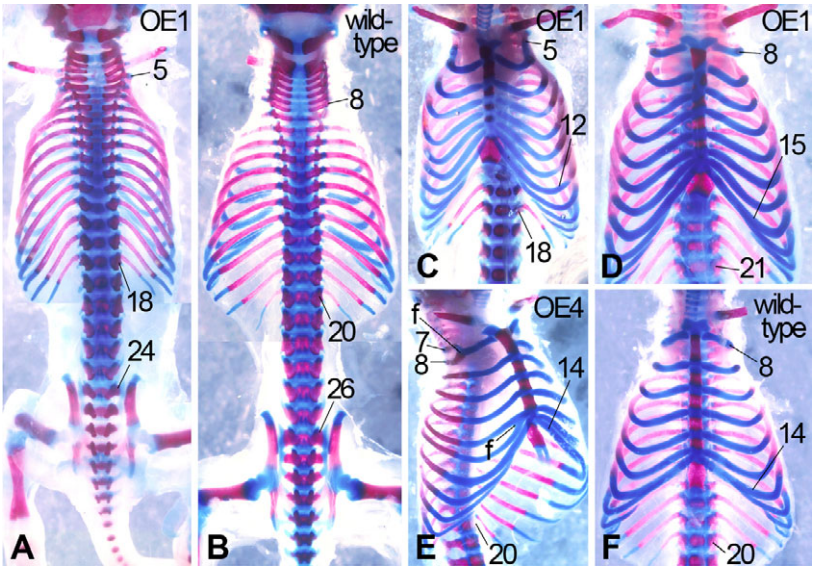

Fig. 6. Vertebral homeotic transformations in the posterior thoracic and lumbosacral region of OE mice compared with wild type. (**A**,**B**) Dorsal views; numbers indicate the most anterior vertebrae bearing ribs, most posterior vertebrae bearing ribs and the first sacral vertebrae. (**C-F**) Ventral views; numbers indicate the most anterior vertebrae bearing ribs, most posterior vertebrae with ribs attached to sternum, and most posterior vertebrae bearing ribs. f, fused ribs. A, C and Fig. 5A show different views of the same skeleton.

in the first sacral vertebra (posterior-to-anterior transformation; not shown). Like wild-type, OE1 and OE4 mice show five or six lumbar vertebrae. OE2 mice may show seven lumbar vertebrae (Fig. 7A,B) and mis-alignments of lumbar vertebrae (Fig. 7C).

Defects in the tail region are seen in OE2 mice. These may simply be mis-alignment of caudal vertebrae resulting in tail kinks (Fig. 7D), but in more severe cases there is splitting of the tail tip to produce either two rows of ossification (Fig. 7F) or multiple separate axes of caudal vertebrae (Fig. 7G,H). There may also be premature termination of the OE2 axis with reduced numbers of caudal vertebrae (Fig. 7B). Overall, 68% (n=19) of OE2 transgenic offspring showed skeletal defects in the tail.

OE1, OE2 and OE4 effects upon expression of a Hoxa7/*lacZ* reporter

Heterozygous OE1 line 1, OE2 peak-expresser or OE4 line 1 stud males were crossed with homozygous *Hoxa7/lacZ* females, and the resultant embryos examined by β-galactosidase protein staining (Fig. 8). Each embryo was genotyped by PCR, allowing OE1-, OE2- or OE4-positive embryos to be compared directly for β-galactosidase distribution with their OE1-, OE2- or OE4-negative littermates.

At 10.5 days, the anterior limits of β-galactosidase protein activity are readily assessed relative to the boundaries of the forelimb, which normally extends from prevertebra (v) 4 to 9. In OE non-transgenic embryos (Fig. 8A) (Gaunt et al., 2004) the boundaries are located four somites posterior to the forelimb in paraxial and lateral plate mesoderms (at the level of v13) and at a level just behind the anterior border of the forelimb in spinal ganglia (sg) (at the level of sg5). β-galactosidase activity boundaries in OE1-, OE2- and OE4-positive embryos are shifted anteriorly relative to OE-negative littermates, and these forward shifts occur in spinal ganglia, prevertebrae and lateral plate mesoderm (Fig. 8B-D). The shifts are greatest in OE1 embryos: up to the level of sg2, and up to the levels of v2 in lateral plate mesoderm and v11 in paraxial mesoderm (Fig. 8B).

The *Hoxa7/lacZ* transgene commences expression in wild-type embryos at headfold stage (Fig. 8E) (Gaunt et al., 2004). To test for an effect of OE1, OE2 and OE4 upon the initial timing of *Hoxa7/lacZ* activation, OE transgenic embryos sired by the same studs as used in Fig. 8B-D were examined at pre-, early- and later-headfold stages (8 to 8.25 days of gestation) (Fig. 8E). OE1 and OE4 transgenic embryos show no difference from wild-type in the time of initial *Hox/lacZ* activation: all commence expression at the later headfold stage. By contrast, OE2-positive embryos commence expression earlier, at the pre-headfold stage.

OE1 and OE4 effects upon endogenous Hox gene expressions

We examined expressions of *Hoxb4* and *Hoxa10* by in situ hybridization upon sections of 12.5 day OE1 and OE4 embryos (see Fig. S1 in the supplementary material). Expressions of these Hox genes mark, respectively, the second cervical and first lumbar vertebrae (Gaunt et al., 1989; Gaunt et al., 1999). OE1, but not OE4, embryos showed forward shift of *Hoxb4* to the level of the first cervical vertebra. *Hoxa10* expression is apparently normal in both OE1 (not shown) and OE4 embryos.

Forelimb and tailbud defects in OE mice

The forelimb defects in OE1 (lines 1 and 2) mice most commonly appear as in-turning of the feet with reduction in the number of digits, from one to four (Fig. 2A). Seventy-nine percent (n=16) of OE1 transgenic offspring showed forelimb defects after staining of skeletons (see Fig. S2 in supplementary material). OE1 hindlimbs are normal. There is no consistent defect in forelimb skeletons. Loss of the radius is common, apparently causing in-turning of the foot. The humerus or humerus plus radius and ulna may be absent. It is likely that absence of skeletal elements in the forelimbs of OE1 mice can be explained by the small size of the forelimb buds seen at 10.5 days gestation and caused by their restriction posteriorly (Fig. 2F). The posterior and anterior limits of the limbud are normally specified by the position of Hox expression boundaries within the flank mesoderm (Cohn et al., 1997). For OE1, Hoxa7/β-galactosidase protein expression is dramatically shifted forward in flank mesoderm (Fig. 8B). It therefore seems likely that forward shifts in endogenous Hox expression are responsible for shifting anteriorly the posterior limits of OE1 limbuds. Occasional forelimbs in OE1, OE2 and OE4 mice show increased numbers of digits, with otherwise normal limb skeletons (see Fig. S2D in supplementary material). We considered that these might result from larger limbuds,

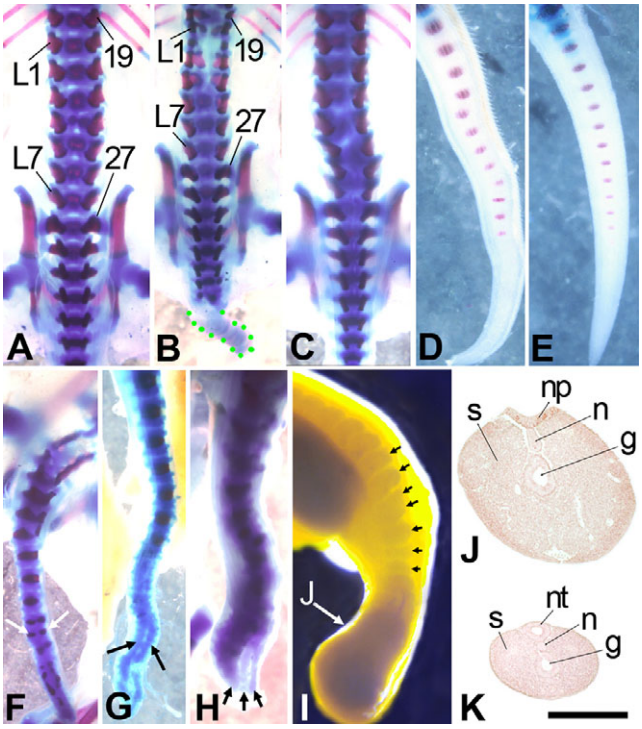

Fig. 7. Abnormalities in the lumbar, sacral and tail regions of OE2 mice. (**A**,**B**) Numbers indicate the most posterior vertebrae bearing ribs and the first sacral vertebrae. Green dots in B show the outline of the tail. Mis-alignments of lumbar (**C**) and caudal (**D**) vertebrae. (**E**) Wild-type tail. (**F-H**) Arrows show split centres of ossification (F) and multiple tail axes. (**I**) Club-tail of Bouin's fixed 10.5 day OE2 peak-expresser sired embryo showing irregularly sized and spaced somites (small arrows) and, in transverse section (**J**), failure of neural tube closure and increased mass of mesodermal component relative to wild type (**K**). Large arrow in I shows the level of section J. L, lumbar vertebra; np, neural plate; nt, neural tube; s, somitic mesoderm; n, notochord; g, hindgut. Scale bar: 0.2 mm in J,K.

caused by forward shift in Hox boundaries that specify their anterior limits. However, we did not detect larger limbuds in either OE1, OE2 or OE4 embryos.

The club-tails of OE2 embryos, seen especially in the offspring of the peak-expresser stud, display irregularities in somite size and spacing (Fig. 7I). The neural tube remains open in the tailbuds of these OE2 embryos (Fig. 7J), while it is normally closed at 10.5 days (Fig. 7K). OE2 club-tails are much larger in cross section than normal owing mainly to increase in mesodermal tissue (Fig. 7J,K).

DISCUSSION

Elevation of Cdx gene activity within the normal domain of expression

We describe lines of mice (OE1, OE2 and OE4) in which *Cdx1*, *Cdx2* and *Cdx4* transgenes are expressed under the control of their own promoter and enhancer elements. Transcription of the transgenes in 8.5 to 8.7 day embryos occurs within normal Cdx expression domains (primitive streak/tailbud), and this results in an overall increase in Cdx protein levels. Previous studies have shown that ectopic (Bel-Vialar et al., 2002; Charité et al., 1998; Ehrman and Yutzey, 2001) or ubiquitous (Epstein et al., 1997; Isaacs et al., 1998) activation of Cdx product anterior to the normal domains of Cdx expression will induce more anterior expression of Hox genes. These findings include some of the earliest evidence that Cdx genes are upstream regulators of Hox genes. An important difference in our work, however, is that we now show that elevation of Cdx gene transcription within the normal spatial domain is also able to anterioriorize Hox expression, with accompanying homeotic transformations.

Our findings are complementary to Cdx gene knockout analyses (Chawengsaksophak et al., 1997; Subramanian et al., 1995; van den Akker et al., 2002; van Nes et al., 2006) in which the effects of reduced Cdx expression are analysed. The knockout studies have not, however, examined for shifts in Cdx protein gradients along the embryo, or provided any evidence for changes in the timing of Hox gene activation. The Cdx knockout studies presented so far cannot, therefore, shed light on the relative merits of gradient- or temporal co-linearity-based mechanisms for the positioning of Hox expression boundaries.

OE1, OE2 and OE4 homeotic defects extend to different anterior limits

Increased doses of Cdx proteins result in homeotic transformations in vertebral types. These transformations are usually most anterior for OE1 (skull/v1 level), more posterior for OE2 (v1/v2 level) and most posterior for OE4 (v7 level). It is unlikely that these differences simply reflect different levels of overexpression within individual lines of transgenic mice, as (1) for overexpressers of each Cdx gene, the same results were obtained for two independently derived lines; and (2) OE1 line 1 (4-fold normal Cdx1 levels) shows more anterior defects than does OE4 line 1 (30-fold normal Cdx4). We consider it more likely that the different positions of defects in OE mice reflect the different anterior limits of expression normally shown by the various Cdx genes (Gaunt et al., 2005).

Nature of OE1, OE2 and OE4 homeotic transformations

Apart from a spatial difference, the nature of the homeotic transformations are apparently similar for all three OE lines, supporting earlier conclusions that there is normally overlap in function between the three Cdx proteins (van den Akker et al., 2002; van Nes et al., 2006). The effects of increased Cdx protein dose on homeotic shifts are largely opposite to those of reduced Cdx gene product, as examined in Cdx knockouts (Subramanian et al., 1995; van den Akker et al., 2002). However, in contrast to the clear homeotic transformations found in OE4 embryos, *Cdx4* mutants display transformations only when combined with mutations in *Cdx1* or *Cdx2* (van Nes et al., 2006).

Many of the obvious homeotic effects in OE mice are located in the neck and anterior thoracic vertebrae and may be classified as anterior-to-posterior transformations. For example, all three transgenic lines show forward shift of thoracic-type vertebrae, with attached ribs, into the region that is normally cervical. The reasons for cervical vertebral fusions and splits are less apparent and we suggest the following. Fusion between v1 and the skull is common in OE1 mice. v1 forms normally from the posterior half of somite 5 (s5) and the anterior half of s6, and the occipital condyles of the skull form from anterior s5 (Couly et al., 1993). If the level of Cdx protein is elevated in anterior s5 then we suggest that this tissue acquires v1 characteristics and therefore fails to split from v1, so producing a skull/v1 fusion. Splits within v1 are also seen in OE1 mice. If the level of Cdx protein is elevated in anterior s6 then we suggest that this tissue acquires v2 characteristics and fails to fuse properly to s5, so producing a split. Both of these fusions and splits can therefore

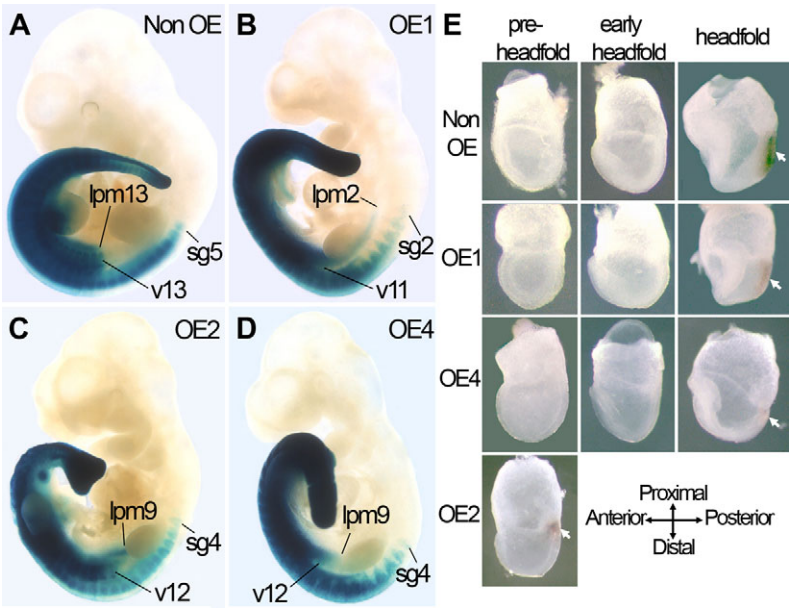

Fig. 8. Cdx OE embryos show forward shifts in *Hoxa7/lacZ* expression without, necessarily, earlier activation. (**A-D**) At 10.5 days, the anterior boundaries in prevertebrae (v), lateral plate mesoderm (lpm) and spinal ganglia (sg) of wild-type (A) and OE (B-D) embryos are shown. The OE2 embryo possesses a club-tail. (**E**) At 8 to 8.25 days, OE1 and OE4 embryos commence *Hox/lacZ* activity in the primitive streak region (white arrows) at apparently the same developmental stage (headfold) as wild-type (non OE) embryos.

be explained as anterior-to-posterior transformations, and the mechanisms we describe may also explain vertebral (and rib) fusions and splits observed at other levels of the vertebral column. Anterior-to-posterior transformations are also seen in posterior thoracic and lumbosacral regions. For example, the most caudal thoracic vertebrae with ribs attached to sternum and the most caudal vertebrae bearing ribs are shifted forward in some OE1, OE2 and OE4 mice, and the first sacral vertebra is shifted forward in some OE1 mice.

Some OE mice also show posterior-to-anterior transformations. For example, the nuchal ligament attachment normally on v9 is shifted posteriorly in some OE1 mice. In addition, the most caudal vertebrae with rib attached to the sternum, and the most caudal vertebrae bearing ribs may be shifted posteriorly in some OE1 and OE4 individuals. The first vertebra contributing to the sacrum may be shifted posteriorly in OE4 mice.

The extent of the transformations is highly variable between individual OE1, OE2 or OE4 mice. Similarly there is much variability between the vertebral patterning of individual Cdx knockout mice (Subramanian et al., 1995; van den Akker et al., 2002) and of mice with defects in *Cdx1* gene regulation (Pilon et al., 2007). In striking contrast, however, the vertebral formula and patterning of normal mice is highly conserved. We suggest that overlapping and interacting patterns of developmental gene expression in embryos may, over evolutionary time, have reached a state of mutual compatibility and equilibrium. When embryos are tipped out of this equilibrium by, in our case, overexpression of Cdx genes, the end result is wide variability between individuals in the exact nature and extent of the defects.

Retinoic acid administered to pregnant mice generates phencopies in their offspring of the vertebral transformations that we now find in OE mice. Thus, retinoic acid given to mothers at 7.3 days pregnancy results in anterior-to-posterior transformations, while administration at 8.5 days also produces some posterior-to-anterior transformations in the posterior thoracic and lumbosacral regions (Kessel and Gruss, 1991). It is known that *Cdx1* is upregulated by retinoic acid (Houle et al., 2000; Houle et al., 2003). Our results therefore indicate that effects of retinoic acid on vertebral patterning could, at least in part, be mediated by its effect on *Cdx1*. Transpositions in vertebral morphologies have also been reported in *Wnt3a* and *Fgfr1* mutant mice (Ikeya and Takada, 2001; Partanen et al., 1998). As *Wnt3a* and *Fgf* are expressed in the primitive streak/tailbud (Deschamps and van Nes, 2005), and both Wnt3a (Pilon et al., 2006) and Fgf (data from chick) (Bel-Vialar et al., 2002) proteins are known activators of *Cdx1* and *Cdx4*, it is likely that at least some of their effects on vertebral patterning could also be mediated by Cdx protein levels (Lohnes, 2003). Pilon et al. (Pilon et al., 2007) have recently shown that normal *Cdx1* expression in embryos depends upon both Wnt and retinoic acid stimulation.

The defects in the tail vertebrae of OE2 mice seem unlikely to be homeotic transformations. Vertebral mis-alignments and tail splits in OE2 mice might instead be due to our observed effects of *Cdx2* overexpression on abnormal growth and segmentation in the tailbud mesoderm. *Cdx2* knockout in mice (van den Akker et al., 2002) and *Cad* knock-down in insects (Shinmyo et al., 2005) both result in premature axial termination. Our results suggest that this effect in mice may, as in insects, be due to impaired growth in the tailbud.

Cdx proteins as regulators of Hox expression

When crossed with *Hoxa7/lacZ* reporter mice all three OE lines produce forward shifts in *Hoxa7/lacZ* expression in 10.5 day embryos. We also find a forward shift in the expression of endogenous *Hoxb4* in OE1 embryos, but not OE4. These shifts in Hox expression boundaries are opposite in direction to those reported in Cdx knockout mice (Subramanian et al., 1995; van den Akker et al., 2002) and in mice defective in *Cdx1* regulation (Houle et al., 2003; Pilon et al., 2007). We propose that dose of Cdx protein normally plays a key role in the positioning of Hox gene expressions. Differential dose of Cdx protein along the axis is provided by the additive effects of the three Cdx proteins, as each has a different anterior boundary of expression. In addition, differential dose in gradients is provided by the decay of Cdx proteins in cells left behind by the regressing tailbud (Gaunt et al., 2003).

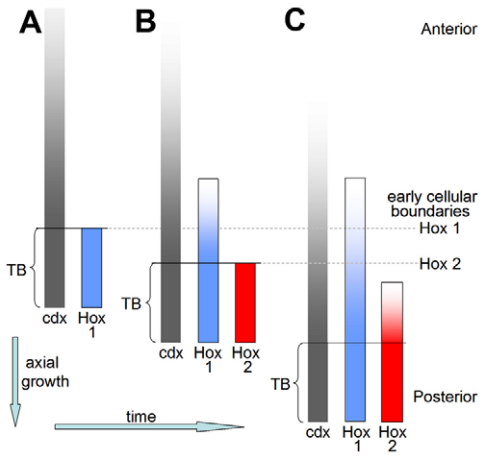

Fig. 9. Possible role of Cdx protein gradients in the refinement of Hox expression boundaries. It is proposed that the rate and extent of co-linear activation of Hox genes (temporal co-linearity) varies with the concentration of Cdx protein (grey). Early expression of each Hox gene (**A**,**B**) occurs in the primitive streak/tailbud (TB), assumed here to extend forward to the level of the node. Anterior to this, within neurectoderm, presomitic, somitic and lateral plate mesoderms, each Cdx protein forms a posterior-to-anterior gradient by time-dependent decay. A Hox gene with high sensitivity to Cdx dose may become progressively activated along the Cdx gradient in a spreading wave that moves forward ahead of cell position (such as Hox1, blue in B). An example would be *Hoxb8* expression in the mouse neural tube (Forlani et al., 2003). The spreading wave stops when Cdx protein concentration becomes limiting, and the time at which this occurs is influenced by the continuous regression of the Cdx gradient. A Hox gene less sensitive to Cdx dose may not spread forward, and may indeed show some posterior regression relative to cell position owing to Cdx gradient regression (Hox2, red in **C**). Examples here might be *Hoxb8* expression in mouse paraxial mesoderm (Forlani et al., 2003) and *Hoxb9* expression in chick neurectoderm (Bel-Vialar et al., 2002). The Cdx morphogen gradient may thus operate by adjusting Hox boundaries forwards or backwards according to Hox gene sensitivities. We assume here that similar mechanisms operate in neural and mesoderm tissues, and the more anterior Hox boundaries in neural tissue may then be explained by the more anterior boundaries of Cdx proteins in neural versus mesoderm tissues (Fig. 4). At a specific point in the maturation of neural and mesoderm tissues, it is envisaged that the boundaries of Hox gene expression become fixed by mechanisms such as auto- and crossregulation between Hox genes and their products (Gould et al., 1997; Zappavigna et al., 1991), and/or by establishment of the Polycomb silencing mechanism (Akasaka et al., 2001).

We next consider whether Cdx effects are mediated in embryos by morphogen gradient or timing mechanisms, reviewed as models 1 and 2 by Gaunt (Gaunt, 2000). A Cdx morphogen gradient model predicts (1) that Hox boundary positions are responsive to dose of Cdx proteins, (2) that Cdx proteins are expressed in gradients along the head-tail axis, and (3) that upregulation of Cdx protein should shift forward the position of the gradient, thereby generating a forward shift in Hox expression boundaries. These predictions are consistent with the observations described in this paper. In terms of the timing model, it is less obvious why increased dose of Cdx protein should shift Hox expression boundaries forwards. Such a shift would presumably be achieved by an earlier initial activation of Hox expression. We did detect earlier onset of *Hoxa7/lacZ* expression in OE2 embryos sired by the peak-expresser line, but not in either OE1 or OE4 embryos. We earlier reported precocious expression of *Hoxa7/lacZ* constructs that contain multiple Cdx-binding sites (Gaunt et al., 2004). Together, these findings lead us to conclude that dose of Cdx protein binding to a Hox enhancer may indeed influence its time of initial expression. A similar conclusion has been reached in *Xenopus* (Isaacs et al., 1998). However, OE1 line 1 embryos show no precocious expression of *Hoxa7/lacZ* yet display greater forward shifts in their later expression than OE2 embryos sired by the peak-expresser stud. This lack of clear correlation between the time of *Hoxa7/lacZ* first expression and the final position of its expression boundary leads us to conclude that Hox expression boundaries cannot simply be set by the time of their initial expression (temporal co-linearity model).

This conclusion is supported by studies demonstrating forward or backward shifts in the position of Hox boundaries that occur independently of cell lineage (Bel-Vialar et al., 2002; Forlani et al., 2003). The model of Fig. 9 can accommodate these observations. It assumes that temporal co-linearity and Cdx gradients operate together in the establishment of Hox boundaries. The proposal is that Cdx protein concentration can regulate the rate and extent of sequential Hox gene activation (temporal co-linearity). A forward shift in the Cdx protein concentration gradient of OE mice would result, in this model, in a forward shift in Hox gene expression boundaries. For both OE1 and OE4, embryos from lines showing modest (four- to fivefold) increase in Cdx protein levels show the same magnitude of homeotic vertebral shifts as do embryos of higher expresser lines. This appears inconsistent with a simple morphogen gradient model but may be explained if temporally co-linear Hox gene activation becomes the rate-limiting step. In the proposal of Fig. 9 the co-linear activation of Hox genes in the tailbud can alone generate approximately correct patterns of Hox gene expression but then these later become refined or focused along Cdx protein gradients. Refinement of Hox boundaries initially generated by temporal co-linearity in the tailbud affords Cdx gradients an important yet possibly subtle contribution, and could explain why some authors have concluded that the effects of Cdx gene knockout on anterior boundaries of Hox expression are modest (Deschamps and van Nes, 2005) or even not detected (Tabariès et al., 2005).

Cdx overexpresser mice display homeotic shifts most clearly over anterior parts of the vertebral column. Other morphogens might regulate Hox boundaries more posteriorly. *Gdf11* encodes one possible candidate (McPherron et al., 1999) as knockout mice show posterior-to-anterior transformations in the anterior lumbar region. As for Cdx, Gdf11 product apparently exerts a dose-dependent effect upon vertebral shifts.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/15/2511/DC1

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