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Original Article

cdx4/lacZ and cdx2/lacZ protein gradients formed by decay during gastrulation in the mouse

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ABSTRACT Expression of the mouse caudal genes *cdx4* and *cdx2* is examined by use of *lacZ* reporter constructs expressed in transgenic mouse embryos. During early gastrulation, up to at least 8.5 days of development, reporter mRNA distributions are apparently similar to those of endogenous *cdx* mRNAs. By 8.25 to 8.8 days, cdx/lacZ protein activities have become distributed as posterior-to-anterior gradients along the neural and mesoderm tissues. The gradients form by decay of activity as cells become distanced from the regressing tailbud. *In situ* hybridization studies indicate that the decay is primarily in cdx/lacZ protein activities rather than mRNAs. As gastrulation proceeds, the locations of the gradients regress progressively posteriorly along the growing axis. Our results indicate how cdx4 and cdx2 protein gradients might be generated by decay during normal development. The smoothness of the gradients that we detect shows that there cannot be extensive mixing of cells once they leave the tailbud to contribute to the growing axis. An enhancer element located in the first intron of the *cdx4* gene is essential for correct transgene expression.

KEY WORDS: *cdx4, cdx2, lacZ, transgenic mice, gradient*

Introduction

Instructional (morphogen) gradients may form part of the mechanism whereby transcripts of developmental genes, such as Hox genes, become positioned along the developing vertebrate axis. The characterization of Hox gene regulators that are expressed in gradients, and the analysis of how these gradients arise, may therefore be important steps in the elucidation of vertebrate pattern formation. The *cdx* genes are upstream regulators of the Hox genes (e.g., Subramanian *et al*., 1995; Epstein *et al*., 1997; Charité *et al*., 1998; Isaacs *et al*., 1998; van den Akker *et al*., 2002) and the cdx proteins become distributed in posterior-to-anterior (P-A) gradients along the axis of gastrulating embryos (Gamer and Wright, 1993; Meyer and Gruss, 1993; Beck *et al*., 1995). There is evidence that Hox expression boundary positions respond to cdx proteins in a dose-dependent way and cdx gradients might therefore serve as instructional (morphogen) gradients for setting of Hox expression patterns (Charité *et al*., 1998; Gaunt *et al*., 2004).

For *cdx1*, the expression gradients have been studied by our earlier use of *cdx1/lacZ* transgenes expressed in transgenic mouse embryos (Gaunt *et al*., 2003; Gaunt, 2004). We presented evidence that the *cdx1/lacZ* gene is expressed mainly in the proliferative, posterior parts of the embryo and that gradients form by decay of gene product within neurectoderm and mesoderm cells as these become left behind by the regressing tailbud. We suggested that a similar mechanism could generate the gradients of endogenous cdx1 protein and that formation of gradients by decay might thereby be an important mechanism in patterning along the embryonic axis. To test whether this mechanism could also apply to the other caudal genes, we now examine expression of *cdx4/lacZ* and *cdx2/lacZ* constructs in transgenic mouse embryos.

Results

cdx4/lacZ expression gradients formed by decay

Dot matrix comparison of mouse and human *cdx4* genomic sequences shows that in addition to the coding regions there is extensive sequence conservation within the immediately upstream sequence and also throughout the large first intron (Fig. 1A). We considered these conserved non-coding regions likely to contain the *cdx4* regulatory elements. A construct was therefore made which incorporated these regions from mouse DNA and which had the *lacZ* gene spliced in frame to the *cdx4* exon 2 sequence (construct 1; Fig. 1B). This construct, which encodes 71% of the cdx4 protein coding region coupled to lacZ, was expressed in transgenic mouse embryos as shown in Fig. 2A-K. While only one *cdx4/lacZ* transgenic line is illustrated in this paper, identical

Abbreviations used in this paper: cdx, caudal; P-A, posterior-to-anterior; Fgf, fibroblast growth factor.

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distributions were obtained with four independently-derived lines (not shown). There were, however, differences between lines in the intensity of lacZ activity and, in particular, some lines showed more speckled expression of construct 1 (not shown, but like Fig. 2L).

Expression commences at 7.5 days at the base of the allantois, in the adjacent embryonic region and in occasional cells scattered along the length of the primitive streak (Fig. 2A and not shown). LacZ activity at 8 to 8.25 days is more extensive, including neurectoderm, mesoderm and endoderm germ layers and with the staining in neurectoderm and endoderm extending anterior to the limits in mesoderm (Fig. 2B-D). Similar patterns of distribution have been described for the endogenous cdx4 protein, as detected using an antibody (Gamer and Wright, 1993). The anterior limit of lacZ staining at 7.75 days is apparently the same as that of endogenous cdx4 mRNA, as detected by *in situ* hybridization (Fig. 3A,B).

The lacZ activity at these early stages appears to be rather uniform in intensity along the P-A axis (Fig. 2C,D), but with a slight reduction in the anteriormost neural staining, at the level of somite 5 (Fig. 2C). The position of this boundary was found by removal of an adjacent somite, followed by embryo sectioning to locate the address of the extracted somite (somite 5; Fig. 2C,D).

By 8.8 days (ca. 14 somite stage), clear P-A gradients in staining are seen in neurectoderm, paraxial and lateral plate mesoderms (Fig. 2E-K). The anterior limits are at the levels of somite 5 (neurectoderm), somite 9 (paraxial mesoderm) and somite 7 (lateral plate mesoderm). The gradients have, at least in part, formed by decay since anterior expression in both neurectoderm (e.g., at the level of somite 7) and mesoderm is now clearly reduced in intensity (Fig. 2E,F,G,H) relative to that seen at earlier times (Fig. 2D). Further evidence for decay is given by the observation that at later times the staining at these positions has disappeared. Thus, by 9.25 days the anterior boundaries have regressed in neurectoderm to about the level of somite 12 and in paraxial mesoderm to about the level of somite 17 (Fig. 5I).

cdx2/lacZ expression gradients formed by decay

Comparison of mouse and human cdx2 genomic sequences (Fig. 1C) revealed regions of homology within the first intron, the immediate upstream sequence and also more distant upstream sequence. Construct 5 includes the first two of these regions and construct 6 includes all three (Fig. 1D). In both constructs, lacZ is spliced in frame to the cdx2 exon 2 sequence such that they encode the first 69% of the cdx2 protein linked to lacZ protein. This paper illustrates only one transgenic line which expresses construct 5, but identical patterns of expression, in embryos of 7.5 to 9 days, were obtained for two additional lines expressing construct 5 and one line expressing construct 6 (not shown).

At each of the stages 7.5 days to 8.25 days, cdx2/lacZ expression (Fig. 4A-D) extends further forwards than the corresponding stages expressing cdx4/lacZ (Fig. 2A-C). The anterior limit of staining seen at 7.75 days is the same as that of endogenous cdx2 gene expression, as detected by *in situ* hybridization (Fig 3C,D). This boundary, at the posterior limit of the headfold, is located just posterior to that for cdx1 (Fig 3E,F and Gaunt *et al.*, 2003).

The anterior boundary of cdx2/lacZ staining in 8.25 day neurectoderm, at the level of somite 2 (Fig. 4C-E), is anterior to that of cdx4/lacZ (Fig 2C,D). The position of this boundary was found

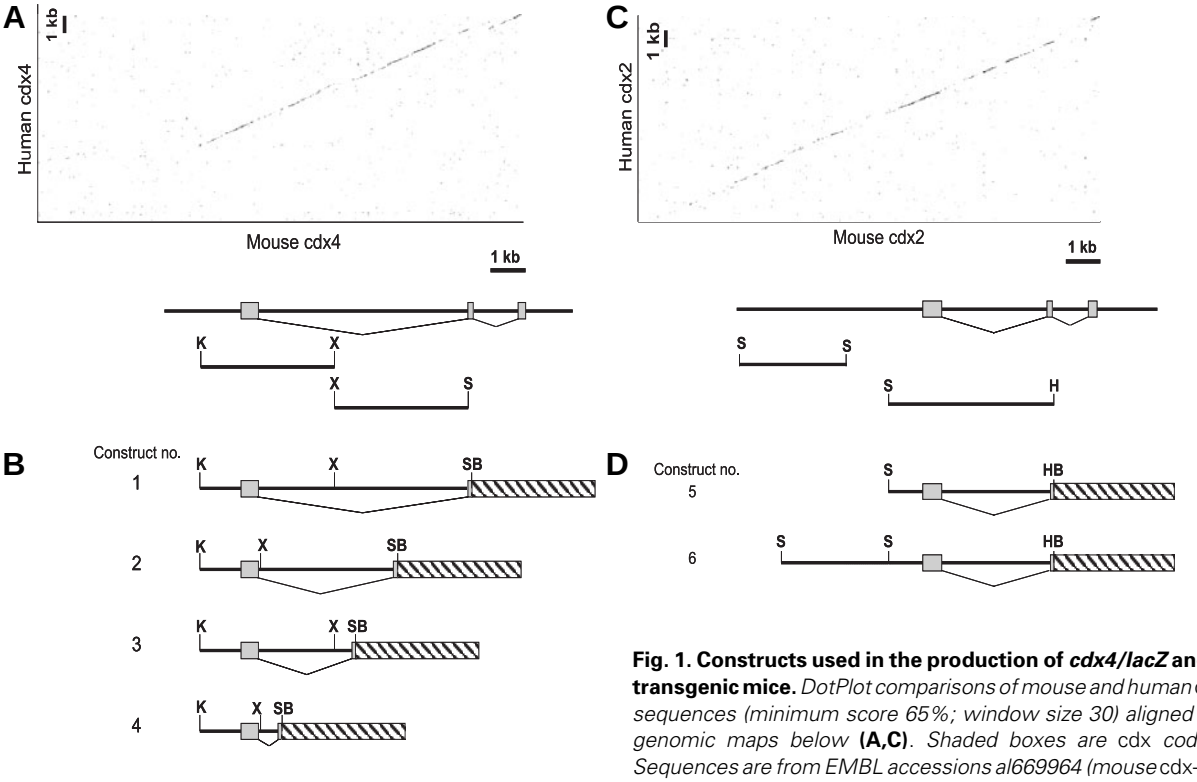

Fig. 1. Constructs used in the production of *cdx4/lacZ* and *cdx2/lacZ* transgenic mice. *DotPlot comparisons of mouse and human* cdx *genomic sequences (minimum score 65%; window size 30) aligned with mouse genomic maps below* **(A,C)**. *Shaded boxes are* cdx *coding regions. Sequences are from EMBL accessions al669964 (mouse* cdx-4*), al450108 (human* cdx-4*), ac127549 (mouse* cdx-2*) and al591024 (human* cdx-2*). K-X, X-S, S-S and S-H are DNA fragments prepared by PCR.* cdx4 *constructs* **(B)** *and* cdx2 *constructs* **(D)** *used for preparation of transgenic mice. Striped boxes are* lacZ/SV40polyA *DNA. K, Kpn1; X, Xho1; S, Sal1; B, BamH1; H, Hind3.*

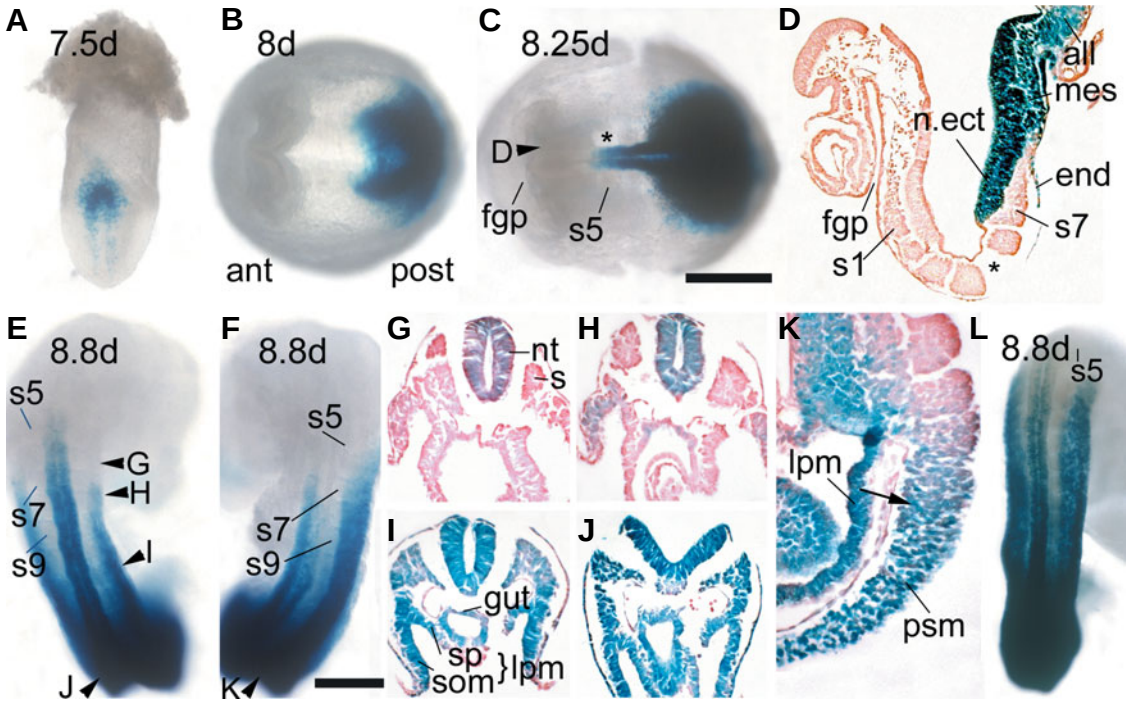

Fig. 2. Establishment of graded cdx4/lacZ protein activity over the period of gastrulation. *Embryo at 7.5 day* **(A)** *is viewed posteriorly. Embryos at 8 and 8.25 day are viewed ventrally within the yolk sac after removal of Reichert's membrane* **(B,C)**. *Arrowheads shown in* **(C,E,F)** *indicate the planes of sections* **(D,G-K)**. *Asterisk in (C) indicates where a somite was removed to allow location of its address after sectioning (D). Arrow in* **(K)** *show latest-formed somitic furrow. Embryos shown in (A to K) are from the same transgenic line, expressing construct 1 and were all stained for 1 hour 10 min.* ***(L)*** *is a transient transgenic embryo expressing construct 2. ant, anterior; post, posterior; s, somite; fgp, foregut pocket; all, allantois; mes, mesoderm; n.ect, neurectoderm; end, endoderm; nt, neural tube; sp, splanchnic mesoderm; som, somatic mesoderm; lpm, lateral plate mesoderm; gut, hindgut; psm, presomitic mesoderm. Bars, 0.2 mm*

by removal of an adjacent somite, followed by embryo sectioning to locate the address of the extracted somite (somite 4; Fig. 4D,E). Graded expression in somites is already evident by 8.25 days, with an anterior boundary at the level of somite 4 (Fig. 4C,E). Early lateral plate mesoderm expression is prominent for cdx2/lacZ (relative to cdx4/ and cdx1/lacZ) and extends forwards to the level of somite 3 (Fig. 4C,D).

Posterior-to-anterior gradients of cdx2/lacZ activity are apparent within neurectoderm and paraxial mesoderm over the period 8.25 to 9 days (Fig. 4C-L). Evidence that these form by decay is given, for example, in the observations that anterior limits of labelling are seen to move progressively posteriorly along the embryo (cf. Fig. 4C,G,H). Thus, the changing limits in neural tube are shown at the levels of somite 2 (8.25 days), somite 4 (8.5 days) and somite 12 (9 days). For paraxial mesoderm, limits are shown at somite 4 (8.25 days), somite 7 (8.5 days) and somite 15 (9 days).

cdx4 and cdx4/lacZ mRNA distributions compared by in situ *hybridization*

Having seen that gradients form by decay of cdx4 and cdx2 reporter gene product as cells become distanced from the regressing primitive streak, the question remains as to the nature of the molecule that decays. In theory this could be cdx/lacZ protein, cdx/lacZ mRNA, or there might be decay in either the protein or mRNA of an upstream activator of cdx genes, such as Wnts (e.g., Lickert *et al.*, 2000) or Fgf (e.g., Pownall *et al.*, 1996; Bel-Vialar *et al.*, 2002). In attempt to distinguish between these possibilities, we proceeded to compare the distributions of cdx mRNA, cdx/lacZ mRNA and cdx/lacZ protein.

At 8.5 days (ca. 10 somites), the mRNA expression from both the endogenous *cdx4* gene and transgene are similar in distribution and are almost entirely confined to the region of the tailbud (Fig. 5A,B). Thus, both genes show expression in paraxial and lateral plate mesoderms that is posterior to the level of the latest-

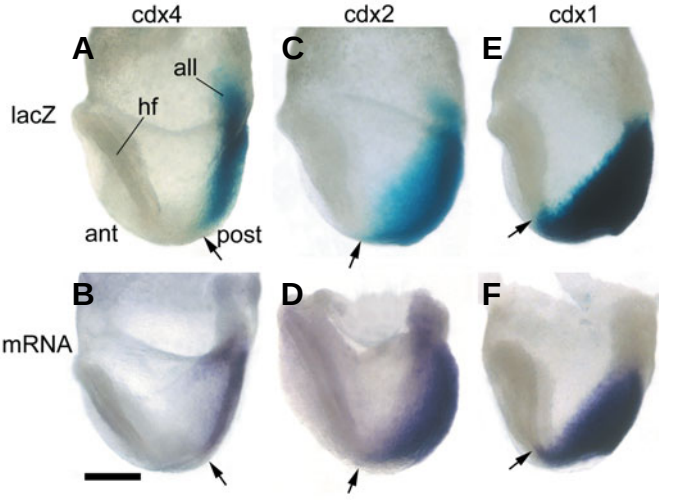

Fig. 3. Comparison of cdx/lacZ protein and cdx mRNA distributions at 7.75 days. *Embryos, viewed laterally, are either* cdx4/lacZ *transgenic* **(A)**, cdx2/lacZ *transgenic* **(C)**, cdx1/lacZ *transgenic (line described in Gaunt* et al., *2003)* **(E)** *or wild-type* **(B,D,F)**. *Embryos in (A,C,E) were stained for lacZ protein activity. Embryos in (B,D,F) were stained for mRNA by* in situ *hybridization, using probes to the endogenous* cdx *genes. Arrows show the anterior limits of stainings. hf, head fold; other labelling as for Fig. 2. Bar, 0.2 mm.*

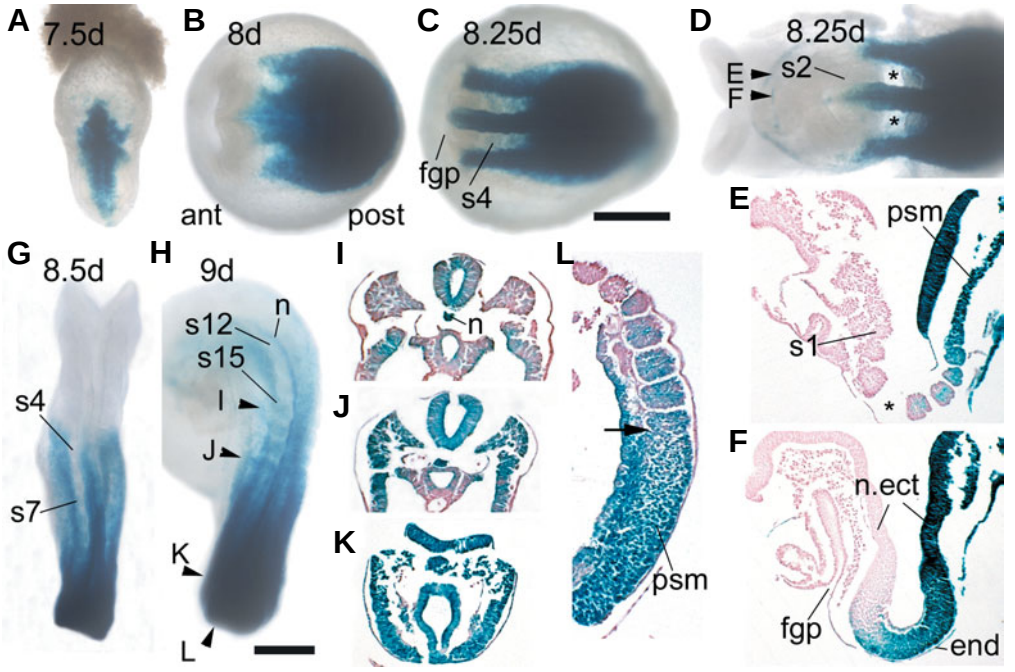

Fig. 4. Establishment of graded cdx2/lacZ protein activity over the period of gastrulation. *Embryos at 7.5 to 8.25 days* **(A-C)** *viewed as in Fig. 2. Embryo* **(D)** *is dissected from the yolk sac and has somites removed (asterisks) for location of address after sectioning* **(E,F)**. *Arrowheads shown in* **(H)** *indicate the planes of sections* **(I-L)**. *All embryos are from a single line, expressing construct 5 and stained for 2 hours 30 min. n, notochord; other labelling as for Fig. 2. Bars, 0.2 mm.*

formed somitic furrow and a boundary in expression within neurectoderm that is located posterior to the position of complete neural tube folding. However, by this stage, unlike earlier (Fig. 3A,B), the *cdx4* (and *cdx4/lacZ)* mRNA distributions now correspond with only the posteriormost part of the lacZ protein domains (Fig. 5C). In the 9 day embryo (ca. 18 somites) similar results are obtained (Fig. 5D-F). In transverse sections of the posterior embryo, the same tissue distributions of expression in neurectoderm, hindgut, presomitic and lateral plate mesoderms are seen for *cdx4* mRNA (Fig 5J), *cdx4/lacZ* mRNA (Fig. 5L) and lacZ protein activity (Fig. 2I,J). These patterns of labelling were the same in all embryos. Variably between embryos, small patches of weaker labelling were sometimes detected, using both *cdx4* and */acZ* mRNA probes, in the midline of the neural tube anterior to the strong posterior domain (Fig. 5D,E). Sectioning shows that these are confined to dorsal-most parts of the neural tube (Fig. 5K,M). Similar patches of weaker labelling, located anterior to the strong posterior domains, are not seen in somites or lateral plate mesoderm.

At 9.25 days (ca. 24 somites) *cdx4/lacZ* and *cdx4* mRNAs again show similar distributions in the tailbud, with expression in neurectoderm, paraxial and lateral plate mesoderms remaining posterior to the level of somite separation (Fig. 5G,H). Apparently similar distributions of *cdx4* mRNA at this stage were found by Abu-Abed *et al.* (2003). In contrast, the lacZ protein staining at 9.25 days continues to extend forward as gradients in neurectoderm, paraxial and lateral plate mesoderms considerably anterior to the tailbud (Fig. 5I).

Why is cdx4/lacZ protein and *cdx4* mRNA similarly distributed in the early embryo (7.75 days, Fig. 3A,B), yet graded cdx4/lacZ protein extends so far forward of *cdx4* (and *cdx4/lacZ*) mRNA distribution at 8.5 to 9.25 days? The observation is readily explained if both mRNA and protein are synthesised only within the posterior part (vicinity of the primitive streak), but due to a longer half-life of decay only the protein persists to form a distinct gradient within the cells that leave, and become progressively distanced from, the regressing tailbud. In an alternative explanation, mRNA synthesised only in the posterior part does not have a short half-life and itself forms a gradient by decay, but reduced sensitivity in the *in situ* hybridization technique may allow detection of only the most abundant, posterior transcripts. These explanations differ in whether the decay is primarily in protein or mRNA.

To investigate this further, we conducted short-term lacZ labelling (20 min.) of some embryos. At even low levels of staining, graded lacZ activity was seen to proceed smoothly forward along the neural tube (Fig. 5C,F) with no sharp anterior boundary at the position of the mRNA boundaries (Fig. 5A,B,D,E). Overall, therefore, the experiments suggest that protein decay is important in the generation of *cdx4/lacZ* activity gradients. It is not easy, however, to rule out a smaller contribution caused by mRNA decay.

cdx2 *and* cdx1 *transgenes examined by* in situ *hybridization*

At 8.5 days, *cdx2* and *cdx2/lacZ* mRNAs are apparently identical in their distribution (Fig. 5N,O), with strong labelling confined only to the tailbud, posterior to the level of neural tube closure. Similar distributions of *cdx2* mRNA at this stage were found by Prinos *et al.* (2001). In contrast, short-term staining for lacZ shows a protein distribution which does not share this distinct boundary, but instead extends forward to the level of somite 4 in neural tube, somite 3 in lateral plate mesoderm and somite 7 in paraxial mesoderm (Fig. 5P).

For *cdx1* at 8.5 days, strong *cdx1* mRNA and *cdx1/lacZ* mRNA labellings are seen in the tailbud posterior to the point of neural tube closure. For both probes, much lower levels of signal are also seen as speckled labelling along the neural tube up to the position of somite 1, but labelling is not seen in somites (Fig. 5Q,R). Short-term staining for cdx1/lacZ protein does not show a similarly marked change in the intensity of labelling within the anterior tailbud. Instead, graded labelling extends continuously forward to the level of somite 1 or 2 in the neural tube and somite 6 in the paraxial mesoderm (Fig. 5S).

We conclude that results for *cdx1* and *cdx2* support our findings for *cdx4* that posterior-to-anterior gradients of lacZ staining form primarily by decay of cdx/lacZ protein, although we do not

rule out the possibility of a lesser contribution made by decay of *cdx/lacZ* mRNA.

cdx4/lacZ reporters require an intron element for cdx-like expression in transgenic embryos

We prepared further *cdx4/lacZ* constructs that resemble construct 1 except that they are deleted in various parts of the intron. Deletion of 2 kb from the 5' end of the intron (construct 2; Fig. 1) does not apparently change the pattern of expression as seen in two independently derived transient transgenic embryos at 8.8 days (Fig. 2L and not shown). These two embryos showed speckled expression, as seen in some of the transgenic lines expressing construct 1 (not shown). However, deletion of 3.3 kb from the 3' region of the intron (constructs 3 and 4; Fig. 1) prevents the described pattern of *cdx4/lacZ* expression. Instead, a consistent pattern of ectopic expression was seen in the visceral arches of two independently derived embryos (one each with constructs 3 and 4; not shown). The significance of this is unclear since no expression at this position was detected for either constructs 1 or 2, nor after localization of endogenous *cdx4* mRNA by *in situ* hybridization (Fig. 5D,G).

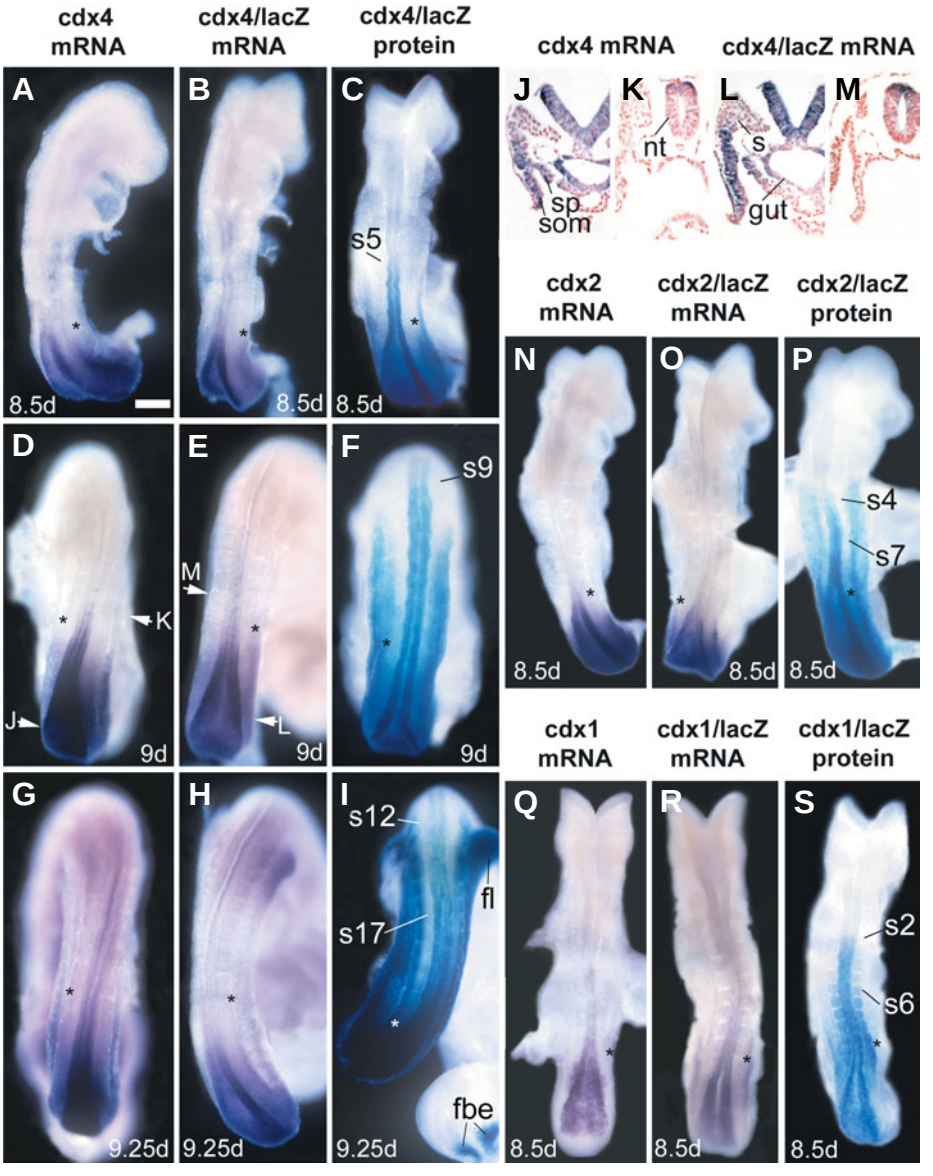

Fig. 5. Comparison of *cdx* mRNA, *cdx/lacZ* mRNA and cdx/lacZ protein distributions during gastrulation. *Embryos are either wild type* **(A,D,G,J,K,N,Q)**, cdx4/lacZ *transgenic* **(B,C,E,F,H,I,L,M)**, cdx2/lacZ *transgenic* **(O,P)** *or* cdx1/lacZ *transgenic (line described in Gaunt* et al.*, 2003)* **(R,S)**. *lacZ staining was for either 20 min (C,F) or 40 min (I,P,S). Asterisks indicate positions of the latest-formed somitic furrow in each embryo. Expression of* cdx4/lacZ *in forebrain and forelimbs (I; and seen, after 9 days, in all four* cdx4/lacZ *transgenic lines examined) is apparently ectopic since it is not found using the* cdx4 *mRNA probe (G). Arrows in (D,E) indicate the positions of the transverse sections shown in (J-M). fl, forelimb bud; fbe, forebrain expression domains; other labels as for Fig. 2. Bar, 0.1 mm.*

Discussion

Cdx/lacZ gradients formed by decay

The results presented here for cdx4/ and cdx2/lacZ reporters support our earlier proposals for cdx1/lacZ regarding the way in which cdx/lacZ protein gradients arise in mouse embryos. This proposal is 1) that cdx products are made only, or mainly, within the posterior, proliferative zones of the embryo and 2) that cells moving out of this region undergo a time-dependent decay of cdx/lacZ protein as they are distanced from the regressing tailbud (Gaunt *et al.*, 2003; Gaunt, 2004). Our findings do not exclude the possibility that decay of cdx/lacZ mRNA also makes a lesser contribution to gradient formation. mRNA decay was found to be the primary cause of the Fgf gradient in vertebrate tailbuds (Dubrulle and Pourquié, 2004).

By use of an antibody, Gamer and Wright (1993) showed that the mouse cdx4 protein is distributed as P-A gradients along the embryonic axis. They report that the graded nature of the signal made it difficult to identify the precise anterior limits, but they show a boundary in 8.5 day neurectoderm that is located anterior to the position of complete neural tube folding (their Fig. 5D,6A) and report that cdx4 protein in mesoderm appeared to extend, after prolonged staining (legend to their Fig. 5), into the posteriormost somites. Similarly, an antibody to mouse cdx2 protein (Beck *et al.*, 1995) produced staining that extended forward in the neural tube anterior to the point of complete tube closure. All of these boundaries are clearly located anterior to the cdx4 and cdx2 mRNA boundaries detected in our *in situ* hybridization studies (our Fig. 5A,5N). In contrast, the published wholemounts of cdx4 protein staining at 8.5 days appear similar to our cdx4/lacZ staining pattern (cf. Fig

5D,E of Gamer and Wright, 1993, with our Fig 5C). Similar conclusions can be drawn for the endogenous cdx1 protein. This extends abundantly forwards, most notably within somites, to positions more anterior than the cdx1 mRNA domains detected in our study, similar to our cdx1/lacZ staining pattern (cf. Fig. 4G,H of Meyer and Gruss, 1993, with our Fig 5S). Thus, although cdx and cdx/lacZ proteins may not be the same in their half-lives, we consider it a likely possibility that both generate their graded expression domains, at least in part, as a result of protein decay, and lacZ staining may provide a sensitive method to determine the initial anterior limits of the endogenous cdx protein gradients.

In the chick, as in mouse, cdx mRNAs apparently remain largely confined to the tailbud (Morales *et al.*, 1996; Marom *et al.*, 1997). In *Xenopus*, however, cdx mRNAs extend forward as gradients along the neural tube (e.g. Pillemer *et al.*, 1998). In this species, therefore, mRNA decay might make a greater contribution than in mouse or chick to the development of cdx protein gradients.

The decay mechanism can only generate a smooth gradient if there is not extensive cell mixing within the columns of cells left behind by the regressing tailbud. The fact that smooth gradients may be observed (e.g., Fig. 5F,P,S) provides evidence that extensive cell mixing cannot be occurring. Cell marker studies have shown progressive reduction in A-P mixing of cells in the developing neurectoderm and coherent growth after 8 days in more anterior parts of the spinal cord (Mathis and Nicholas, 2000). The speckling in intensity of lacZ staining that we have noted (e.g., Fig. 2L) could reflect low levels of cell mixing. This explanation of the 'salt and pepper' pattern was earlier made by Dubrulle and Pourquié (2004) with respect to irregularities in the gradient of FGF mRNA. This explanation may not be complete, however, since some of our lines and transient transgenics clearly showed much less evidence of speckling than others, both in neurectoderm and mesoderm (c.f. Fig. 2E,F and 2L).

The cdx4 intron enhancer

We found that correct expression of the mouse *cdx4/lacZ* reporters requires an enhancer element located within the first intron. Normal expression of *Xcad3,* the *Xenopus* structural homologue of mouse *cdx4* (e.g. Marom *et al.*, 1997), depends upon both upstream (Reece-Hoyes *et al.*, 2005) and intron 1 (Haremaki *et al.*, 2003) elements. The critical elements in *Xcad3* intron 1 are Fgf response elements that are widely scattered (Haremaki *et al.*, 2003). Our findings may therefore indicate that the most important of these elements for posterior expression of the mouse *cdx4* gene lie within a 3.3 kb region of the intron that is located towards its 3' end. We question, however, whether *Xcad3* is truly a functional homologue of mouse *cdx4* since its expression pattern as the most anterior of the *Xcad* genes (e.g. Pillemer *et al.*, 1997) makes it appear more like *cdx1*.

cdx gradients and Hox expression boundaries in the developing embryo

Hox expression boundaries respond to cdx proteins in a dose-dependent way and cdx gradients might serve as instructional (morphogen) gradients for setting of Hox expression patterns (Charité *et al.*, 1998; Gaunt *et al.*, 2004). For *cdx1* and *cdx2*, there is also evidence from knockout studies that both genes contribute to patterning all along the vertebral column (van den Akker *et al.*, 2002). Fig. 6 shows one way in which this might occur, in a mechanism that combines both cdx instructional gradients moving progressively posteriorly as the axis develops and also 'temporal colinearity' in the timing of initial expression of Hox genes (Izpisúa-Belmonte *et al.*, 1991). The scheme can explain two earlier observations upon the initial expression of a Hox gene. Thus, a Hox gene's expression may at first be graded (Gaunt, 2001) because the gene is responding to a gradient of cdx activator; and Hox expression may subsequently regress posteriorly (Bel-Vialar *et al.*, 2002; Gaunt *et al.*, 2004) because of posterior regression in the cdx protein gradients.

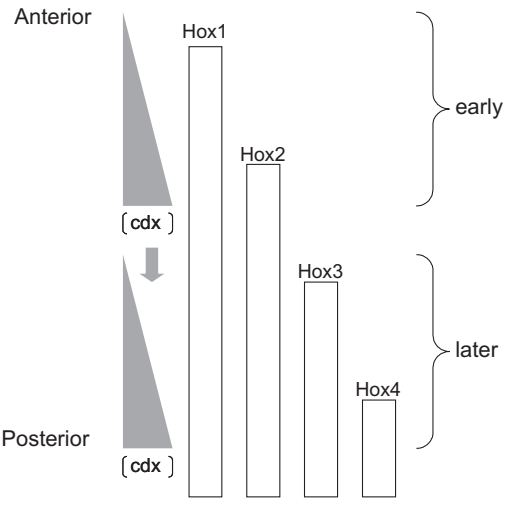

Fig. 6. Possible scheme for the establishment of Hox expression boundaries along instructional cdx gradients. *During gastrulation, the cdx2 and cdx4 gradients regress posteriorly along the developing axis (indicated here by a vertical arrow). At early times only 3'-located Hox genes are expressed and their boundaries may become differentially sorted by the cdx protein gradients as they then exist over prospective anterior parts of the embryo. This may occur, for example, because each Hox gene is activated by the products of more 3' Hox genes (Hooiveld* et al.*, 1999; McNulty* et al.*, 2005), but only above a threshold cdx protein concentration. At later times, more 5'-located Hox genes become expressed and their boundaries become sorted over the more posterior structures which are then displaying the cdx gradients. The protein gradients from all three cdx genes likely overlap (this paper and Gaunt* et al.*, 2003). A null mutation in a single cdx gene will therefore cause only a limited posterior shift in the total cdx protein concentration gradient and hence may produce only limited posterior shifts in Hox expression boundaries. These shifts may, however, be enhanced when multiple cdx genes are mutated, as has been reported (van den Akker* et al.*, 2002).*

Members of the Hoxc cluster are expressed in different spatial domains along the length of the neural tube and the positions of these are sensitive to Fgf concentration (Liu *et al.*, 2001). Since abundant levels of Fgf are found only in the tail region (Dubrulle and Pourquié, 2004) it is possible that the role of Fgf in patterning is normally mediated by cdx decay gradients. Both *cdx1* and *cdx4* are responsive to Fgf (Bel-Vialar *et al.*, 2002).

In short and intermediate germ band insects, as in vertebrates, the caudal gene similarly remains expressed in the posterior growth zone while segmentation proceeds (Dearden and Akam, 2001; Shinmyo *et al.*, 2004). It remains unclear, however, whether gradients formed by decay of invertebrate tail proteins could also potentially provide instructional gradients for the es-

tablishment of developmental gene expression boundaries.

Materials and Methods

Preparation of cdx4/lacZ reporter constructs

Mouse *cdx4* gene was obtained as Bac clone RP23-11P22 (EMBL accession al669964), purchased from BACPAC Resources Centre, Children's Hospital Oakland, Ca., USA. Two PCR fragments were prepared (Fig. 1A). Fragment 1, of 3838 bp, commences 1176 bp upstream of the coding region, extends 2166 bp into the large first intron and introduces Kpn1 and Xho1 restriction sites at the upstream and downstream ends, respectively. Primers (excluding added restriction sites) for fragment 1 were: ATTTGAACTCAGGACCTTCGGAAGAG (upstream) and GTGAGGAGAAAGCTTAGGAAGCTCTG (downstream). PCR fragment 2, of 3894 bp, contains the remaining intron 1 sequence, extends 107 bp into the second exon and introduces Xho1 and Sal1 sites at the upstream and downstream ends, respectively. Primers for fragment 2 were: CCATTGCACATGGACATAGCGTTTAG (upstream) and CTTCCTCCTGATGGTGATGTATC (downstream).

Constructs were assembled in Bluescript KS$^-$. For preparation of construct 1 (Fig. 1B), the two PCR fragments were assembled as in the normal *cdx4* gene, but with *lacZ/SV40* DNA (Gaunt *et al.*, 1999) spliced at the 3' end. Construct 2 (Fig. 1B) resembles construct 1 except that PCR fragment 1 is deleted of intron 1 sequence in all but its first 41 bp. This was prepared using, as 3' primer: GGTAGAGAAGAGAAGAAACAG. Construct 3 (Fig. 1B) resembles construct 1 except that PCR fragment 2 is deleted of intron 1 sequence in all but its last 503 bp. This was prepared using, as 5' primer: CTATGGTGGTGCACACTTTTAG. Construct 4 (Fig. 1B) resembles construct 1 except that intron 1 is reduced to its first 41 and its last 503, bp. For all constructs, sequencing reactions confirmed that there are no mutations within the coding regions and that exon 2 is in the same reading frame as *lacZ.* Constructs were cut from vector using Kpn1 plus Not1.

Preparation of cdx2/lacZ reporter constructs

Mouse *cdx2* gene was obtained as Bac clone RP24-510G5 (EMBL accession ac127549) from BACPAC, as above. Two PCR fragments were prepared (Fig. 1C). Fragment 1, of 3063 bp, commences 5301 bp upstream of the coding sequence and introduces Sal1 sites at both ends. Primers (excluding added Sal1 sites) were CCCCTAGAAGGTGTTTACTATG (upstream) and GCTAATT ACACGACGTATTCGGTTTG (downstream). Fragment 2, of 4862 bp, commences 1050 bp upstream of the coding sequence and extends 107 bp into exon 2. This introduces Sal1 and Hind3 sites at the upstream and downstream ends, respectively. Primers were: CCAGCCATCCACTAATTACTGCCTTC (upstream) and TTCCTCCTGATGGTGATGTATCGAC (downstream). Two separate *cdx2/lacZ* constructs (constructs 5 and 6) were assembled in Bluescript KS$^-$ as shown in Fig. 1D. Constructs were cut from vector using Xho1 plus Spe1.

Transgenic embryo production, staining and sectioning

These were carried out as described earlier (Gaunt *et al.,* 2003). Embryos were taken to be at 0.5 day of development at midday on the day of the copulation plug. Transient transgenics, each expressing one of the constructs 2 to 4, were examined at 8.8 days only.

Positions of cdx/lacZ expression boundaries were located relative to somite address. Due to flexure of early embryos (8.25 days in this study), somite 1 could not be positively identified in wholemount alone. As earlier described (Gaunt *et al.*, 2004), we therefore removed a somite adjacent to the lacZ expression boundary and then subsequently identified the address of the extracted somite as seen in parasagittal sections (Fig. 2D,3E). After about 8.7 days, localization of the first somite was facilitated by reference to the position of the newly formed otic vesicle (Theiler, 1989).

In situ *hybridization*

Wholemount *in situ* hybridizations were carried out as described by Henrique *et al.* (1995). *cdx4* probe, of 850 bp, comprised the entire coding region. *cdx2* probe, of 516 bp, was of non-coding 3' sequence (IMAGE Consortium cDNA clone no. 4217425; Lennon *et al.*, 1996). *cdx1* probe, of 250 bp, was as described by Gaunt *et al.* (2003). A 450bp *lacZ/SV40polyA* probe (Gaunt, 2001) was used to detect all cdx/lacZ mRNAs and gave no background labelling on non-transgenic embryos.

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