Original Article

Additional enhancer copies, with intact cdx binding sites, anteriorize *Hoxa-7/lacZ* expression in mouse embryos: evidence in keeping with an instructional cdx gradient

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ABSTRACT   **Expression of a *Hoxa-7/lacZ* reporter construct in transgenic mouse embryos is shifted anteriorly when the upstream enhancer is multimerized. The shift occurs in spinal ganglia, neurectoderm and in both paraxial and lateral plate mesoderms. Much of the multimer effect is inhibited by destruction of a single caudal (cdx) binding motif in the additional copies of the enhancer. These observations are in agreement with earlier enhancer multimerization analyses made for *Hoxb-8* (Charité *et al.,* 1998). Our findings therefore provide further evidence that the anterior limit of a Hox gene's expression domain is normally dependent upon and is determined by, the dosage of transcription factor(s) which bind to its enhancer element(s) and that these factors may be, or must include, the cdx proteins. We consider these findings in terms of both instructional (morphogen-like) gradient and timing models for the establishment of Hox gene expression domains. Enhancer multimerization results in an earlier onset of Hoxa-7/lacZ activity in the embryo. In neurectoderm at 8.7 days and in mesoderm at 10.5 days, the anterior boundaries of expression are located posterior to those seen at some earlier stages of development. We discuss how these findings are in keeping with a model where Hox expression boundaries become set along instructional cdx gradients, formed by cdx decay in cells moving away from the primitive streak region.**

KEY WORDS: *Hoxa-7/lacZ, transgene, enhancer multimer, cdx*

Introduction

It remains uncertain as to how Hox expression boundaries become established during vertebrate embryogenesis (e.g. Gaunt, 2000). In 'instructional (morphogen-like) gradient' models, there is a posterior-to-anterior gradient of Hox-inducer along the embryo, with each Hox gene expression boundary becoming set at its own unique concentration threshold. Graded proteins that could potentially function as such Hox gene inducers include caudal (cdx) (Charité *et al.,* 1998; Gaunt *et al.,* 2003), Wnt3A (Kiecker and Niehrs, 2001; Aulehla *et al.,* 2003) and FgF8 (Dubrulle *et al.,* 2001; Liu *et al.,* 2001). In the alternative 'timing model', boundary positions become established according to the time at which a Hox gene is first expressed in the posterior founder tissues of the embryo: the earlier the expression, the more anterior the eventual boundary position (Dollé *et al.,* 1989). It is quite possible that both models have a role to play and that the relative importance of these roles may differ between neurectoderm and mesoderm. We use the term instructional gradient here because of a trend to confine the definition of morphogen to organizer substances that form their gradients by spreading between cells (e.g., Vincent and Briscoe, 2001). Gradients of cdx proteins are not formed in this way (Gaunt *et al.,* 2003; Gaunt, 2004).

Charité *et al.* (1995, 1998) found that multiple copies of a *Hoxb-8* enhancer cause a forward shift in the expression boundaries of a *Hoxb-8/lacZ* transgene. The shift was seen in both neurectoderm and mesoderm. This enhancer dosage-effect must be interpreted differently in terms of the two models mentioned above. The timing model predicts that multiple enhancer copies result in earlier expression of the transgene, thereby generating a more anterior boundary. The instructional (morphogen) gradient model predicts that increased enhancer copies make the transgene more sensitive to the Hox inducing morphogen gradient, thereby setting expression at a more anterior position along the axis. Charité *et al.* (1998) argued in terms of the second of these two mechanisms and

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suggested that the transducer of positional information to the Hox genes (the morphogen) may be cdx proteins. In support of this, they produced evidence that cdx protein binding activity is graded in concentration along the embryo, that ectopic expression of cdx-4 is able to activate ectopic transcription of *Hoxb-8*, and that the *Hoxb-8* enhancer dosage-effect depends critically upon an intact cdx protein binding motif.

Other studies have also shown that cdx proteins are upstream regulators of Hox genes and that they have a dose-dependent effect upon the position of Hox expression boundaries (Deschamps *et al.,* 1999; Lohnes, 2003, for reviews). Thus, cdx knockouts may give posterior shifts in Hox gene expression boundaries (Subramanian *et al.,* 1995; Chawengsaksophak *et al.,* 1997; van den Akker *et al.,* 2002; Houle *et al.,* 2003), while cdx over-expressions and mis-expressions may cause anterior shifts (Pownall *et al.,* 1996; Epstein *et al.,* 1997; Charité *et al.,* 1998; Ehrman and Yutzey, 2001; Bel-Vialar *et al.,* 2002). Posterior-to-anterior gradients of cdx gene products have been shown in both protein immunohistochemistry (Gamer and Wright, 1993; Meyer and Gruss, 1993) and *cdx/lacZ* reporter studies (Gaunt *et al.,* 2003). These gradients apparently arise by decay of cdx activity (or by decay of an upstream regulator of cdx activity) as, during gastrulation, cells move anterior to the vicinity of the primitive streak to populate the growing neurectoderm and mesoderm germ layers (Gaunt *et al.,* 2003).

In the present paper, we examine the effect of enhancer dosage on the expression boundaries of a *Hoxa7/lacZ* transgene. The upstream enhancer element of the *Hoxa-7* gene has been identified in previous studies (Püschel *et al.,* 1991; Knittel *et al.,* 1995; Gaunt *et al.,* 1999; Kim *et al.,* 2002). We now show that adding three extra copies of the enhancer to a *Hox/lacZ* reporter causes a forward shift in its expression within spinal ganglia, neurectoderm and both paraxial and lateral plate mesoderms. Much of this effect depends upon the integrity of a single cdx protein binding motif. Enhancer multimerization also results in an earlier onset of Hox/lacZ activity. However, this change in timing may not be responsible for the forward shift in Hox/lacZ expression.

Results

Multimerization of the Hoxa-7 upstream enhancer element causes forward shift in Hoxa-7/lacZ expression

We earlier reported a chick *Hoxa-7/lacZ* reporter construct (construct 1 in Fig. 1) that is expressed in embryos of a transgenic mouse line with anterior boundaries at the levels of spinal ganglion 5 (sg5) in neural tissue, and prevertebra 13 (pv13) in paraxial mesoderm (Gaunt 2001; Fig. 2 A,H,I). In Fig. 2, boundary positions in paraxial mesoderms are also given (in brackets) as somite (s) addresses. Pv13 corresponds to s17/18 (Burke *et al.,* 1995).
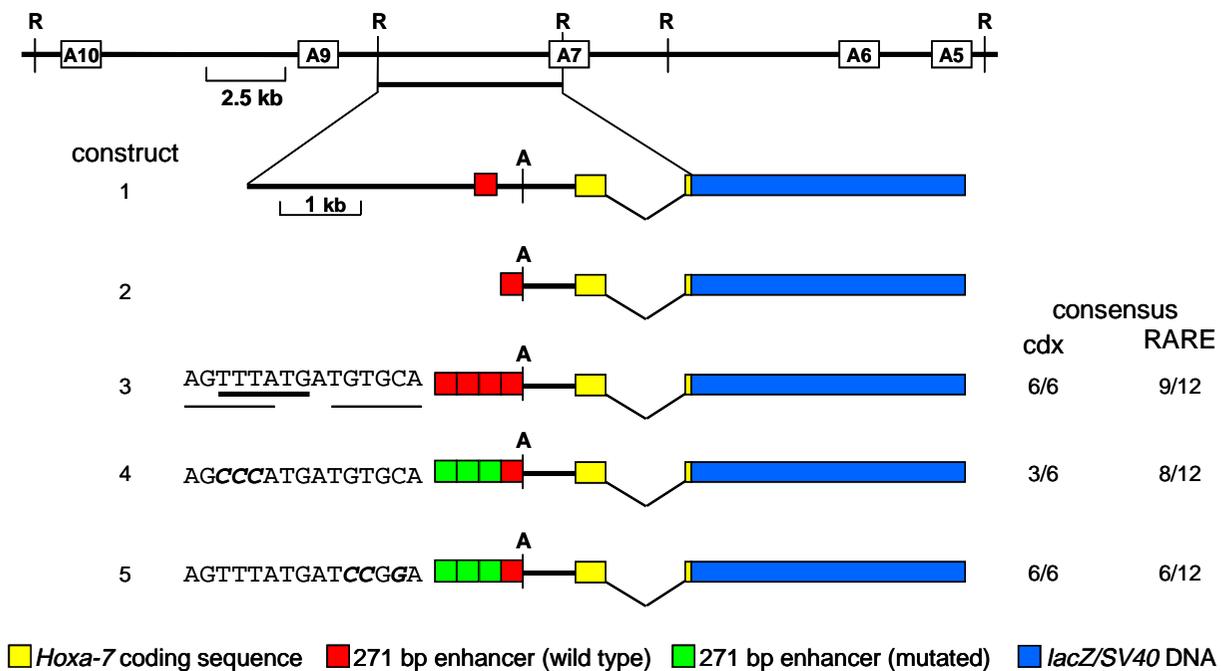

Fig. 1. Constructs used in the production of Hoxa-7/lacZ transgenic embryos. *The chick Hoxa map and construct 1 are as shown by Gaunt* et al. *(1999). Construct 2 has one normal copy of the 271 bp enhancer region. Construct 3, with four normal copies of the enhancer region, shows the wild-type sequence of the cdx binding motif (thick underline; Margalit* et al., *1993) and the repeats of the putative DR3-type RARE (thin underline; Kim* et al., *2002). The lower constructs resemble construct 3 except that they are mutated at the bases shown in bold italics, within either the cdx motif (construct 4) or the RARE (construct 5) of the three additional copies of the enhancer fragment. 'Consensus' shows the fraction of the bases within either the cdx binding motif or the RARE repeats that are in accordance with the consensus sequences, as defined in Margalit* et al. *(1993, for cdx) and Chambon (1994, for RARE). The consensus sequence for a RARE consists of direct repeats of PuGG/TTCA, usually spaced by either two or five nucleotides. R, EcoR1, A, Apa1.*
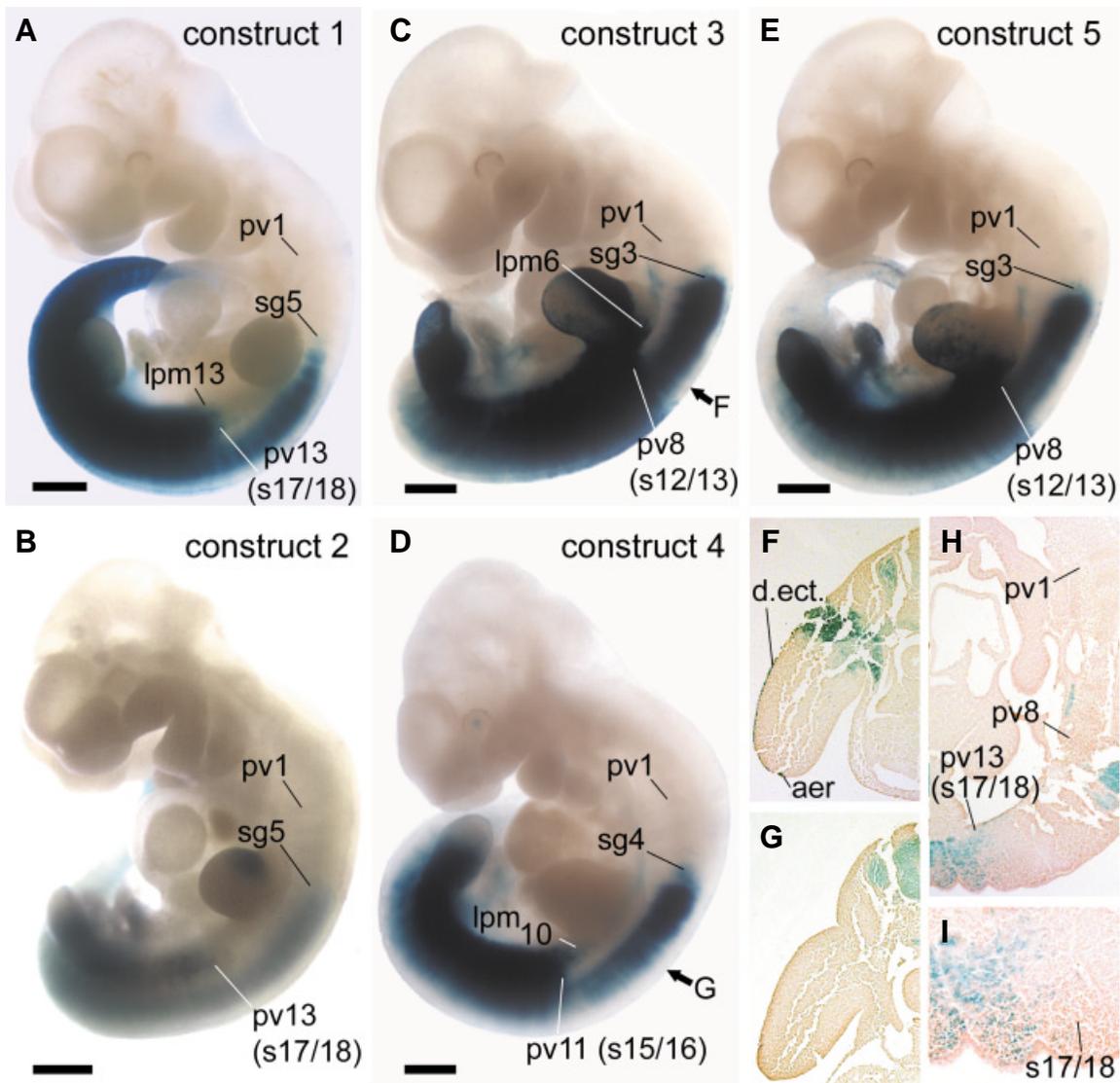
Fig. 2. Constructs 1 to 5 expressed in the 10.5 day mouse embryo. *Four copies of the enhancer unit (construct 3)* **(C)** *anteriorizes the expression boundaries in spinal ganglia and mesoderm relative to a single copy (constructs 1 and 2* **(A,B)** *respectively). Much of this anteriorization is lost after mutation of the cdx binding motif within the additional copies of the enhancer unit (construct 4)* **(D)**, *but not after mutation within a putative RARE (construct 5)* **(E)**. *Boundaries in paraxial mesoderm are shown both as prevertebral (pv) and somite (s) addresses, as in Burke* et al. *(1995). Boundaries in lateral plate mesoderm are labelled relative to prevertebral position. Abbreviations: aer, apical ectodermal ridges; d.ect., dorsal ectoderm; sg, spinal ganglion; lpm, anterior boundaries in lateral plate mesoderm. Arrows in (C,D) mark the positions of the transverse sections shown in* **(F,G)** *respectively. A parasagittal section through the embryo shown in (A) is presented in low* **(H)** *and high* **(I)** *power views. Bars, 0.5 mm.*

Three transient transgenic embryos showed similar neural expression boundaries and boundaries in paraxial mesoderm that varied from pv12 to pv13 (Gaunt *et al.,* 1999).

The upstream enhancer of *Hoxa-7* has been characterized both structurally and functionally (Knittel *et al.,* 1995). It is seen as a region of sequence homology when chick and mouse (Gaunt, 2001) or chick and human (Fig. 3) DNAs are compared. To make construct 2 (Fig. 1) much of the upstream sequence of construct 1 is replaced with the 271 bp conserved fragment shown in brackets in Fig. 3. In transgenic mouse embryos, construct 2 is expressed with the same anterior boundaries as construct 1 in both spinal ganglia and mesoderm tissues (Fig. 2B). This same

observation was made in a second, independently-derived transient transgenic embryo (not shown). The embryo shown also has lacZ activity in the anterior part of the forelimbs, but we attach no significance to this finding since the full-length construct (construct 1) also produces lacZ expression in the anterior forelimb in a proportion of independently produced transgenic embryos (Gaunt *et al.,* 1999).

Construct 3 (Fig. 1) differs from construct 2 in that it contains four identical copies of the 271 bp fragment, arranged in tandem. When analysed in embryos from a transgenic line, this multimerization of the enhancer element results in clear anterior shifts in expression (Fig. 2C). In three independently-derived

transgenic embryos (two of which are not shown), the anterior boundary in spinal ganglia varied from sg2 to sg3 and the boundary in paraxial mesoderm was at the level of pv8 (s12/13). The boundary in paraxial mesoderm was therefore located two vertebra positions anterior to the posterior edge of the forelimb. In contrast, the corresponding boundary for constructs 1 and 2 is located three vertebra positions posterior to the forelimb (Fig. 2 A,B). Construct 3 expression, unlike that of constructs 1 and 2, extends into the flank over the posterior part of the forelimb. This appears as an extension of the staining in lateral plate mesoderm derivatives, up to the level of pv 6 (s10/11). This is anterior to the corresponding boundary of construct 1, located at the level of pv13 (s17/18). Within the forelimb, construct 3 is expressed in the posterior AER (Fig. 2C) and the dorsal ectoderm (Fig. 2F).

Much of the multimer effect depends upon an intact cdx protein binding motif

Construct 4 differs from construct 3 in that the three additional copies of the 271 bp fragment are each mutated in the cdx binding motif as shown in Fig 1. In a transgenic line (Fig. 2D) this resulted in a posterior shift from the expression boundaries reached by
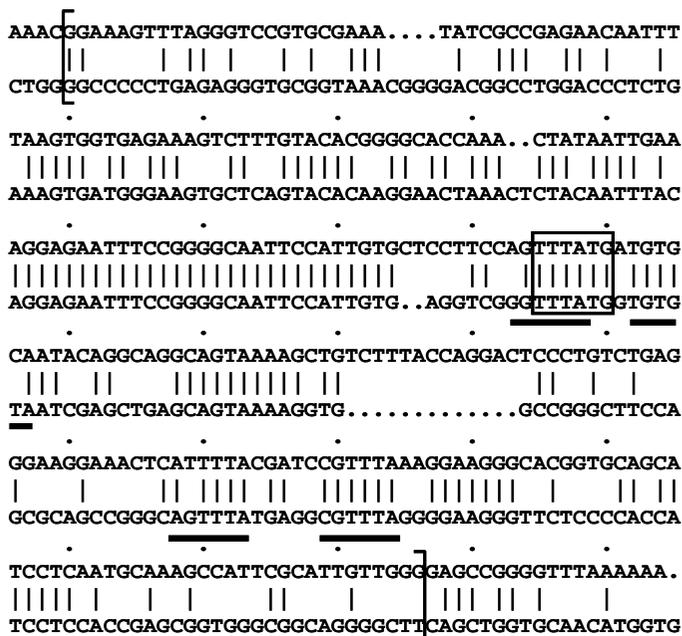

Fig. 3. The upstream enhancer element of *Hoxa-7* compared in chick (upper sequence) and human (lower sequence). *The region in brackets shows the 271 bp fragment of chick DNA used in the production of constructs 2 to 5 (Fig. 1). The cdx protein binding motif (Margalit* et al., *1993), first identified for* Hoxa-7 *by Subramanian* et al. *(1993), is shown boxed. Repeats of the putative DR3 and DR5 retinoic acid response elements (Kim* et al., *2002) are underlined. Deletion of the DR5 element does not change the position of the neural lacZ expression boundary generated in transgenic mice (Knittel* et al., *1995; Kim* et al., *2002). The sequences, from EMBL accessions AJ291729 (chick) and AC004080 (human), were compared using Gap from the GCG analysis package (gap weight, 5.0; length weight, 3.0; average match, 1.0; average mismatch, 0.0). The chick sequence shown commences 1227 bp upstream of the coding region.*

TABLE 1

SUMMARY OF ANTERIOR BOUNDARY POSITIONS FOR HOXa-7/ LACZ EXPRESSION

	Somite address of anterior boundary position		
	Construct 1	**Construct 3**	**Construct 4**
Neurectoderm			
8.25 days	5/6 (Fig. 4B)	5 (Fig. 4E)	5 (Fig. 4H)
8.7 days	7 (Fig. 5 A,A')	5 (Fig. 5B)	6 (Fig. 5C)
Paraxial mesoderm			
8.7 days	14 (Fig. 5 A',D)	11 (Fig. 5B')	12 (Fig. 5C')
10.5 days	17/18 (Fig. 2 A,H,I)	12/13 (Fig. 2C)	15/16 (Fig. 2D)
Lateral plate mesoderm			
8.7 days	13 (Fig. 5A')	10 (Fig. 5B')	10 (Fig. 5C')
10.5 days	17/18 (Fig. 2A)	10/11 (Fig. 2C)	14/15 (Fig. 2D)

construct 3 (Fig. 2C). This posterior shift is greater within paraxial mesoderm (where it is three segments) and lateral plate mesoderm (four segments) than in spinal ganglia (one segment). Six independent transient transgenic embryos (not shown) had similar boundaries in spinal ganglia and had mesodermal boundaries that varied from pv10 to pv11 (paraxial mesoderm) and pv9 to pv10 (lateral plate mesoderm). Construct 4, unlike construct 3, is not expressed in any part of the forelimb (Fig. 2G). The expression boundaries for construct 4 remain, however, slightly anterior to those of constructs 1 and 2.

These results indicate that much, although not all, of the forward shift due to enhancer multimerization may be nullified by destruction of a single cdx binding motif within each of the three extra copies of the enhancer. A potential problem with this conclusion, however, is that this cdx binding motif forms part of a putative retinoic acid response element (RARE) (Fig. 3). This is the first and least RA-responsive, of the two RAREs described by Kim *et al.* (2002). The posterior shift of construct 4 expression relative to construct 3 might therefore be due to destruction of a RARE rather than a cdx binding motif. To distinguish between these possibilities, a new construct was made in which a mutation was introduced to disrupt the RARE but not the cdx motif. Construct 5, containing this mutation in the three additional copies of the 271bp fragment (Fig. 1), shows identical anterior boundaries of lacZ activity in spinal ganglia and mesoderm (Fig. 2E) as those generated by construct 3 (Fig. 2C). This same result was obtained in three additional transient transgenic embryos (not shown). Overall, therefore, these results indicate no evidence for a functional RARE at this position and show instead that the forward shift in lacZ expression caused by multimerization of the 271bp element requires the intact cdx protein binding motif.

Effect of multiple cdx binding sites on the timing of initial expression

Transgenic lines expressing either constructs 1, 3 or 4 were compared in their times of initial expression. Construct 3 (Fig. 4D) commenced expression at the early headfold stage, while constructs 1 (Fig. 4A) and 4 (Fig. 4G) commenced expression later, at the late headfold stage. In all cases, this initial expression was mainly confined centrally to the primitive streak. It therefore appears that multiple copies of the enhancer increase the sensi-
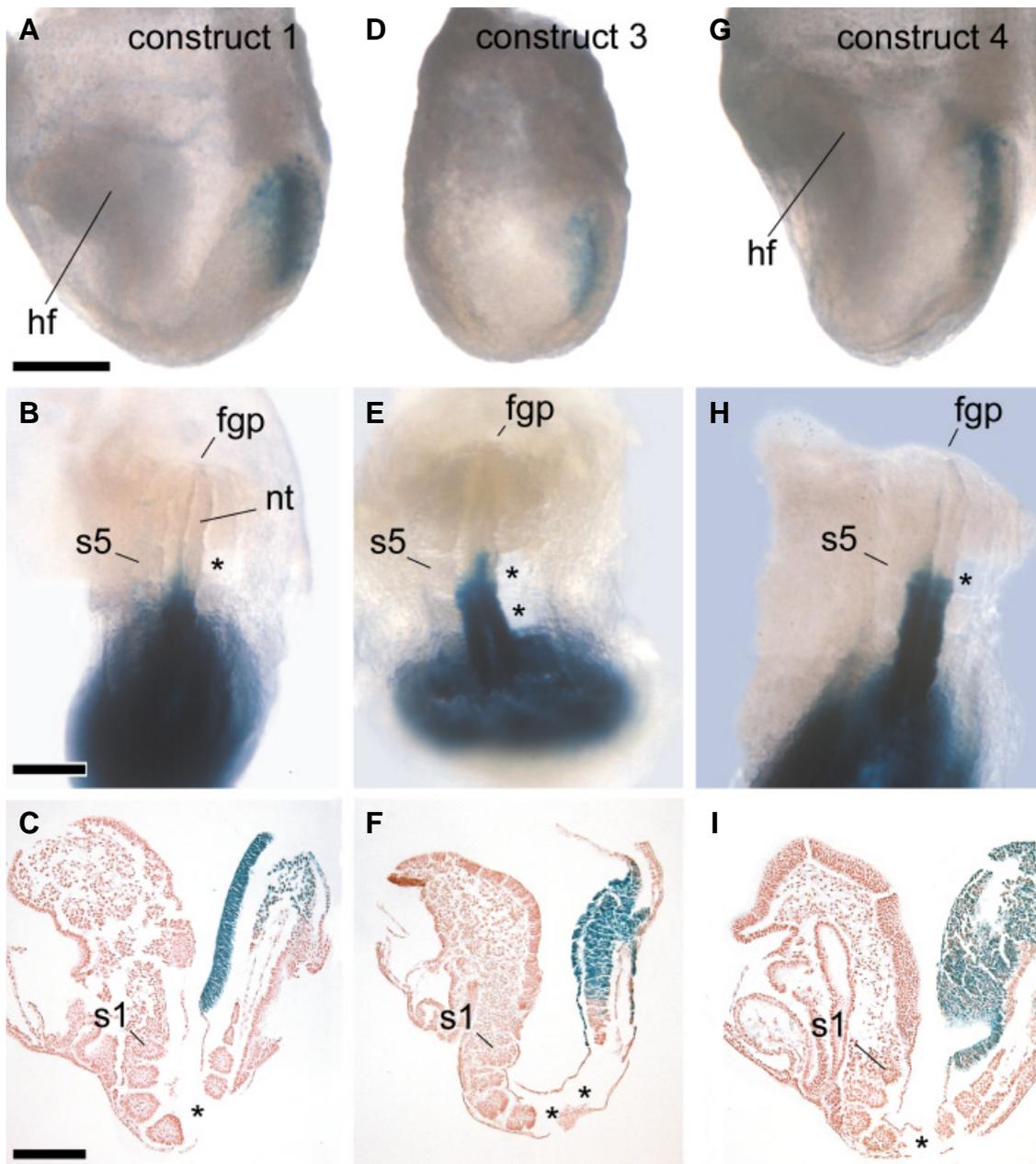
Fig. 4. Early expression of constructs 1, 3 and 4. *For each construct, expression is shown at the stage of its earliest detection* **(A,D,G)** *and also at 8.25 days* **(B,C,E,F,H,I).** *The lines of mice examined are the same as those shown in Fig. 2 A,C,D. The positions of the expression boundaries in neurectoderm at 8.25 days (B,E,H) were assessed by removal of adjacent somites (asterisks), followed by location of the gaps in parasagittal sections (C,F,I, respectively). Abbreviations: fgp, foregut pocket; hf, headfold; nt, neural tube; s, somite. Bars, 0.25 mm.*

tivity of the transgene to transcription factors, causing earlier expression, but that this effect is not seen if the extra copies are debilitated by destruction of the cdx binding site. We considered the possibility that constructs 1 and 4 commence expression at the early headfold stage, but at much lower levels than construct 3. While this possibility is difficult to rule out, we were unable to detect even low levels of lacZ staining before late headfold stage (not shown). Constructs 1 and 4 did not appear to be expressed any less intensely than construct 3 at later stages.

Early expression of Hoxa-7/lacZ extends anterior to its definitive boundaries

At the 9 somite stage (8.25 days), construct 1 expression has an anterior boundary in neurectoderm at the level of the junction between somites 5 and 6 (Fig. 4B). This somite address was identified by removing an adjacent somite (Fig. 4B) and then locating the initial position of this extracted somite in parasagittal sections (Fig. 4C; see Materials and Methods). At this same stage, constructs 3 (Fig. 4 E,F) and 4 (Fig. 4 H,I) showed
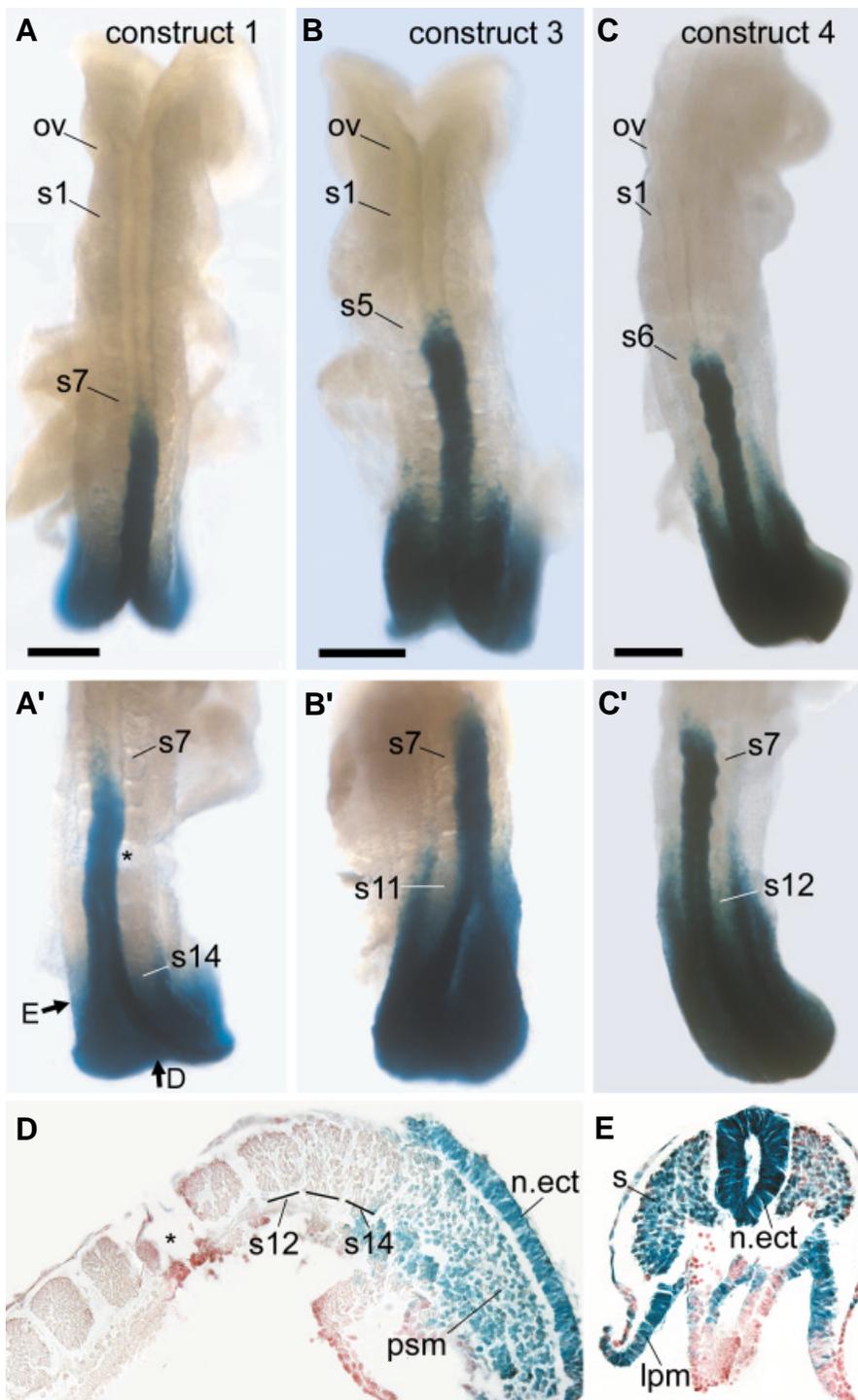
Fig. 5. Constructs 1, 3 and 4 expressed in 8.7 day mouse embryos. *The lines of mice examined are the same as those shown in Figs. 2 A,C,D and 4. The embryos shown in* **(A, B, C)** *are positioned below* **(A', B', C')**, *respectively, to better illustrate boundaries in paraxial and lateral plate mesoderms. In A' and its accompanying section* **(D)**, *somite 10 has been removed (asterisk) and arrows show the planes of section illustrated in (D), which is a parasagittal section of embryo A', and* **(E)** *which is an obliquely transverse section of a similar embryo, also expressing construct 1. The more weakly staining (right) side of (E) indicates a more anterior position, since lacZ is expressed in a posterior to anterior gradient along the developing paraxial mesoderm (Fig. 5D; Gaunt, 2001). Abbreviations: lpm, lateral plate mesoderm; n.ect, neurectoderm; ov, otic vesicle; psm, presomitic mesoderm; s, somite. Bars, 0.5 mm.*

apparently similar anterior boundaries of neural expression, at levels corresponding to about the middle of somite 5. These boundaries are located about half a somite level anterior to the neural boundary of construct 1.

In Fig. 5, expression boundaries in neurectoderm and mesoderm are located at 8.7 days (ca. 15 somite stage). The appearance of the otic vesicle at this stage facilitates localization of somite 1 (Theiler, 1989). At 8.7 days, the anterior boundary of construct 1 expression in neurectoderm has clearly shifted posteriorly, to the level of somite 7 (Fig. 5A, A'; c.f. Fig. 4B at 8.25 days). This anterior-to-posterior regression in neural tissue was less evident for constructs 3 and 4. Hence, the neural boundary for construct 3 remains at about the level of mid somite 5 (Fig. 5 B,B'), while the boundary for construct 4 appears to move posteriorly only slightly, to the level of mid somite 6 (Fig. 5 C,C').

For constructs 1, 3 and 4, anterior boundaries in paraxial mesoderm at 8.7 days are detected in somites 14 (Fig. 5 A',D), 11 (Fig. 5B') and 12 (Fig. 5C'). The corresponding boundaries in lateral plate mesoderm are at the levels of somites 13 (Fig. 5A'), 10 (Fig. 5B') and 10 (Fig. 5C'). We conclude that these boundary positions must later regress posteriorly in order to reach the levels seen at 10.5 days (Fig. 2 A,C,D).

Table 1 summarizes the positions of these anterior expression boundaries for constructs 1, 3 and 4 in neurectoderm, paraxial and lateral plate mesoderms. The data indicate that there is a time in the early activity of the transgenes when expression over-reaches its eventual boundary position. Subsequent A-P regression of the expression boundary is greatest, in both neural and mesoderm tissues, for the most posteriorly expressed construct (construct 1) and is least for the most anteriorly expressed construct (construct 3).

Discussion

Extra copies of an upstream enhancer anteriorize Hoxa7/lacZ expression

We show that expression of a *Hoxa-7/lacZ* reporter construct is shifted anteriorly when an upstream enhancer element is multimerized and that this occurs in spinal ganglia, neurectoderm, paraxial mesoderm and lateral plate mesoderm. Similar results were earlier obtained for *Hoxb-8* by Charité *et al.* (1995, 1998). These findings are readily

explained if the anterior limits of a Hox gene's expression domain are normally dependent upon and determined by, the dosage of transcription factor(s) that binds to its enhancer element(s). Thus, the binding may be increased (and hence the boundary shifted anteriorly) either by an increased concentration of transcription factor, such as cdx protein (e.g. Epstein *et al.,* 1997), or else by an increase in the number of copies of the enhancer monomer unit.

The role of caudal (cdx) proteins in the regulation of Hox expression boundaries

Much of the multimer effect is inhibited by destruction of the cdx binding site within the additional copies of the *Hoxa-7* enhancer element. Charité *et al.* (1998) obtained a similar result for *Hoxb-8*. One possibility is that cdx proteins themselves are the dose-dependent regulatory factors which determine Hox expression boundaries. The results do not rule out an alternative possibility that it is some other enhancer-binding factor that is limiting on the setting of Hox boundaries, with cdx being an essential co-factor. However, since increased cdx protein alone is able to anteriorize Hox gene expression (Pownall *et al.,* 1996; Epstein *et al.,* 1997; Charité *et al.,* 1998; Ehrman and Yutzey, 2001; Bel-Vialar *et al.,* 2002) it does seem that cdx proteins themselves are likely to be important regulators of Hox gene expression boundary positions.

Anterior-to-posterior regression of Hox/lacZ activity in neurectoderm and mesoderm

Table 1 summarizes the positions of anterior expression boundaries observed within both neurectoderm and mesoderm at various times of development. For each tissue, it is seen that the anterior boundary tends to be more anterior at the earlier stages. The subsequent anterior-to-posterior (A-P) regression is more evident for constructs that eventually adopt a more posterior expression boundary (constructs 1 and 4). The regressions that we describe may have a similar mechanism to the regressions noted earlier, by *in situ* hybridization, for the anterior boundaries of *Hoxa-4* in the chick paraxial mesoderm (Gaunt, 2000) and *Hoxb-9* in the chick neurectoderm (Bel-Vialar *et al.,* 2002). A related mechanism might account for regression in the expression of a *Hoxb-4/lacZ* transgene (Brend *et al.,* 2003). A prediction of these observations is that a cell located at the anterior boundary of a Hox gene's expression at an early developmental stage will, at least in the case of some Hox genes, be situated some distance anterior to the boundary as it will exist at a later stage. This prediction is consistent with the mesodermal fate mapping studies of Forlani *et al.* (2003).

A-P regression of Hox expression boundaries is apparently opposite to the forward spreading that has been described in several earlier papers (Deschamps and Widgerde, 1993; Gaunt and Strachan, 1994, 1996). Forward spreading occurs in certain situations. For example: 1) expression of anterior Hox genes in the neural tube, where forward extension of expression is induced as a secondary event by induction from underlying somites (Itasaki *et al.,* 1996; Grapin-Botton *et al.,* 1997) in a retinoid-dependent mechanism (Liu *et al.,* 2001; Oosterveen *et al.,* 2003). 2) During the early phase of a Hox gene's expression along the primitive streak (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994, 1996; Forlani *et al.,* 2003). 3) As a forward spread in the abundance of transcripts within an initially graded Hox

expression domain (Gaunt, 2001). A possible explanation for A-P regression of Hox gene expression boundaries is suggested by the expression pattern of their upstream activators, the cdx proteins. Thus, cdx gene expressions also show A-P regression (Pillemer *et al.,* 1998). We have suggested that this is a feature of the mechanism whereby cdx gene expression gradients are set up along the axis (Gaunt *et al.,* 2003). Our proposal was that cdx proteins are synthesised mainly within the vicinity of the primitive streak and that gradients form by decay of cdx proteins (or their upstream activators, such as Wnt in the case of cdx1; Lickert *et al.,* 2000) within cells once they move anteriorward of the primitive streak region. In support of this, we showed that the cdx-1/lacZ activity gradient in transgenic mice forms in this way, by decay of activity as cells move forward of the primitive streak region in order to contribute to the growing body axis (Gaunt *et al.,* 2003). The time over which we now observe anterior-to-posterior regression of Hox/lacZ activity (8.25 to 8.7 days) shows good correspondence with that over which there is regression and gradient formation in the lacZ activity of *cdx-1/lacZ* transgenic embryos (Gaunt *et al.,* 2003).

The forward shift of construct 3 expression into the posterior AER and dorsal ectoderm of the forelimb (Fig. 2F) provides additional evidence for the role of cdx as activator of the *Hoxa-7* enhancer. Thus, these parts of the forelimb are both sites of cdx-1 activity, as indicated in *cdx-1/lacZ* reporter embryos (Gaunt *et al.,* 2003).

A timing or an instructional gradient mechanism to set Hox expression boundaries?

A novel observation in this paper is that manipulations made upon the regulatory elements of a transgene can change not only its anterior limits of expression, but also the time of its initial expression in the embryo. Thus, expression of a transgene with four copies of the enhancer is first detected earlier in time (early headfold stage) than is either a transgene with only one copy, or else is a transgene with one normal copy plus three mutated within a cdx binding motif (late headfold stage). This particular observation means that it is not possible to say with certainty whether the findings reported are best in-keeping with the 'instructional gradient' or 'timing' models (Introduction; see also Gaunt, 2000). Thus, the forward shift in Hox gene expression caused by four enhancer units might be due either to activation earlier in time (timing model) or else to activation at a lower concentration along a posterior-to-anterior instructional gradient along the embryo (instructional gradient model).

Certain observations do, however, seem to be more in keeping with the instructional gradient model. For example: 1) Construct 3 shows earlier expression than construct 4, yet both apparently display rather similar anterior boundaries of expression in neurectoderm at 8.25 days (Fig. 4 E,H) and in paraxial mesoderm at 8.7 days (Fig. 5 B',C'). 2) Constructs 1 and 3 show neural expression boundaries differing by a distance of only half to one somite at 8.25 days (Fig. 4 B,E), but of two to three somites at 8.7 days (Fig. 5 A,B). 3) Constructs 3 and 4 show paraxial mesoderm expression boundaries differing by only about one somite at 8.7 days (Fig. 5 B',C'), but by the equivalent of three somites at 10.5 days (Fig. 2 C,D).

The findings are consistent with a model (e.g., Fig. 6) in which anterior boundaries of Hox gene expression become positioned

along a developing cdx protein instructional gradient. A cdx gradient forming by decay (Gaunt *et al.,* 2003) could account for the observed A-P regression of both cdx and Hox expression. As shown in Fig. 6, more anteriorly expressed Hox genes activated at lower concentrations of cdx (such as construct 3, with its multiple cdx binding sites) might be expected to show less A-P regression than more posterior Hox genes activated at higher cdx concentrations (e.g., constructs 1 and 4). Although we discuss a cdx gradient here, it is also possible that other Hox-activating transcription factors (e.g., FGF, Pownall *et al.,* 1996; Liu *et al.,* 2001) form gradients as a result of a decay mechanism. A Wnt3A gradient may form by decay within the presomitic mesoderm and it is apparently a fall to a critical threshold level of Wnt3A that provides the signal for somite separation (Aulehla *et al.,* 2003). The formation of gradients by decay may therefore provide a mechanistic link between the parallel processes of segmentation and Hox boundary formation. However, while decay models predict that segmentation occurs at a fixed distance from the primitive streak, they would also predict that the boundaries of

different Hox genes may form at dissimilar distances from the primitive streak. It is uncertain in this scenario how Hox expression boundaries would eventually become stabilized.

In summary, at least three observations, discussed above, are in keeping with an instructional cdx gradient model for the setting of Hox gene expression boundaries. 1) Hox/lacZ expression boundary positions are apparently determined by the dosage of cdx protein that binds to the Hox enhancer(s) (this paper and Charité *et al.,* 1998). 2) Concentrations of cdx gene products form posterior-to-anterior gradients along the embryo (Gamer and Wright, 1993; Meyer and Gruss, 1993; Charité *et al.,* 1998; Gaunt *et al.,* 2003). 3) There is a good temporal correlation between the time period over which Hoxa-7/lacZ expression boundaries regress along the neural tube (8.25 to 8.7 days - this paper) and that over which the cdx-1/lacZ activity gradient becomes established (Gaunt *et al.,* 2003).

An instructional gradient model can, therefore, accommodate changes in the initial positions of Hox expression boundaries as these evolve during embryonic development. The timing model, on the other hand, predicts that once Hox expression patterns are established in the posterior embryo they become fixed in a lineage-dependent way and it seems less able to accommodate the findings, now described, of changes in the anterior limits of developing Hox gene expression boundaries. Our observations suggest a need for similar studies on other Hox genes, comparing the position of their expression boundaries at early and later stages in gastrulation. The findings can provide support for either the timing or gradient model.

Materials and Methods

Preparation of chick Hoxa-7/lacZ reporter constructs

Construct 1 (Fig. 1) has been described earlier (Gaunt *et al.,* 1999). The upstream enhancer element of chick *Hoxa-7* (Fig. 1,3) was cloned by PCR as a 271 bp fragment using the primers GTCACGAGATCTGGAAAGTTTAGGGTCCGT (5') and TATACAGGGCCCAGTATCATCGGATCCCCAACAATGCGAATGG (3'). This incorporated a BglII site at the 5' end and both ApaI and BamHI sites at the 3' end. After cutting with BglII and ApaI, the fragment was subcloned into the BamHI/ApaI sites in the polylinker of pCDNA3 plasmid (Invitrogen). To make construct 2 (Fig. 1), the insert was cut from pCDNA3 using KpnI and ApaI, then used to replace the EcoRI/ApaI fragment of construct 1.

Multimers of the *Hoxa-7* enhancer were prepared as described by Charité *et al.* (1998), making use of the fact that BglII/BamHI ligation sites are not re-cut by BamHI. Thus, the monomer in pCDNA3 was cut with BamHI plus ApaI and a second copy of BglII/ApaI cut enhancer fragment was inserted to make a dimer. This process was repeated, producing trimer, then tetramer. The monomer units used at each step were, as required, either normal or contained a defined mutation generated by site-directed mutagenesis (QuikChange, Stratagene). To make construct 3 (Fig. 1), the tetramer consisted of four normal enhancer monomers. It was cut from pCDNA3 using KpnI plus ApaI, then used to replace the KpnI/ApaI fragment of construct 1. To make construct 4 (Fig. 1), the 3'-most monomer was normal, but other units in the tetramer contained a mutation in the cdx binding motif that is known to abolish binding (Charité *et al.,* 1998). To make construct 5 (Fig. 1), the 3'-most monomer was normal, but others contained a mutation in the second repeat of the putative RARE. This mutation incorporated a diagnostic AccIII site.

Transgenic embryo production and staining

This was carried out as described earlier (Gaunt *et al.,* 2003), except that all staining reactions were allowed to proceed to apparent completion (18
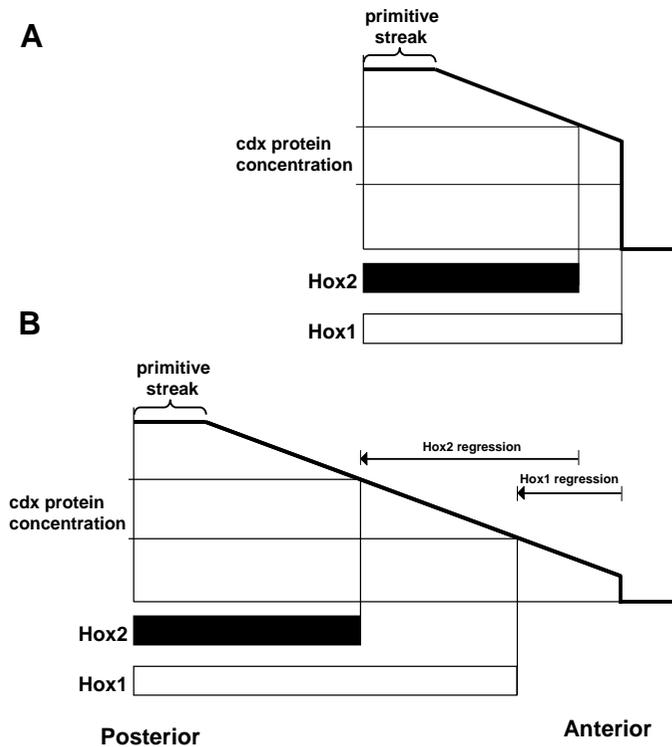

A

B

Posterior   **Anterior**

Fig. 6. A scheme for the establishment of Hox expression boundaries along an instructional cdx gradient, formed by decay. *With regression of the primitive streak* **(A to B)**, *the cdx protein gradient forms by a process of decay (Gaunt* et al., *2003). Expression of the* Hox2 *gene (* Hoxa-7/lacZ *reporter with single cdx binding site, or a normal 5'-located* Hox *gene) is activated at a high threshold concentration of cdx protein. Expression of the* Hox1 *gene (* Hoxa-7/lacZ *reporter with multiple cdx binding sites, or a normal 3'-located* Hox *gene) is activated at a lower threshold concentration of cdx protein. After time (A to B), the anterior boundaries of* Hox *expression show anterior-to-posterior regression and this may be greater for more posteriorly expressed genes. The exact kinetics of cdx protein decay is unknown.*

to 30 hours). The embryos shown in Figs. 2 A,C,D, 4 and 5 of this paper were from three lines of transgenic mice that express either constructs 1, 3 or 4. Embryos were taken to be at 0.5 days of development at midday on the day of the copulation plug. Transient transgenics, each expressing one of the constructs 2 to 5, were examined at 10.5 days only.

At stages up to 8.7 days, neural boundaries of Hox/lacZ expression 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. A special procedure was adopted in order to positively identify somite 1. We removed one or two somites adjacent to the lacZ expression boundary and then subsequently identified the address of the extracted somites as seen in parasagittal sections (Fig. 4). At 8.7 days, localization of the first somite was facilitated by reference to the position of the newly formed otic vesicle (Theiler, 1989).

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