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Evolutionary shifts of vertebrate structures and *Hox* expression up and down the axial series of segments: a consideration of possible mechanisms

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ABSTRACT The term 'transposition' describes how, during vertebrate evolution, anatomical structures have shifted up or down the axial series of segments. For example, the neck/thorax junction and the position of the forelimb in the chicken have shifted posteriorly, relative to mouse, by a distance of seven somites or vertebrae. By examining the expression boundaries of some chick *Hox* genes not previously described, we provide new evidence that axial shifts in anatomical structures correspond with shifts in *Hox* expression domains. These shifts occur both in mesodermal components (somites, vertebrae, and lateral plate mesoderm) and neural components (spinal ganglia). We discuss morphogen gradient, timing, spreading, and growth models for the setting of *Hox* expression boundaries, and consider how evolutionary shifts in boundary positions might have been effected in terms of these models.

KEY WORDS: *Hox expression, transposition, chick, mouse*

Transposition of structures along the segmented body axis of vertebrates

The segmented parts of the vertebrate body include the hindbrain, the somites and their derivatives (vertebrae, segmental muscles and dermal bands), and the spinal nerves. The spinal cord itself has lost the segmental arrangement found in primitive forms (cephalocordates, agnathans and gnathostome fishes), and the segmental positioning of the spinal nerves –both motor neurons and sensory spinal ganglia– is imposed secondarily upon the developing spinal cord by the adjacent somites (Lim *et al.,* 1991; Stern *et al.*, 1991). The spinal nerves simply develop at positions corresponding to the anterior half of each somite (Keynes and Stern, 1984).

Somites and spinal nerves develop according to their position along the axial series. For example, some somites give rise to neck vertebrae, some to thoraco-lumbar, and some to sacral. Similarly, some spinal nerves remain separate, while others –at the levels of both the fore and hindlimb– group together to form plexuses. The relative number of segments contributing to these various parts, and their position along the axial series, are notoriously variable between different species. There is also variability between species in the total number of segments. This evolutionary flexibility accounts for much of the variability between species in the axial proportions of different vertebrates. As an extreme example, the very long neck of *Elasmosaurus* (a sea reptile from the Cretaceous period) contained 76 neck vertebrae.

This whole subject was carefully studied by earlier generations of comparative anatomists. Goodrich (1913, 1958) noted that the fins of different fish species often lie in quite different relative positions along the body. However, whatever the position of a fin, he found that it was almost always supplied by nerves and muscles from its own axial level. He concluded that, during evolution, fins are able to move up or down the segmental series. Two main theories were proposed to account for this. According to the 'intercalation theory', a fin is actually formed by the *same segments* in all species of fish, but the position of these segments along the axial series may change by the addition of new segments (growth), or loss of segments, at any location along the axis. Goodrich argued that this model could not explain why the dorsal and ventral fins of different fish species can shift quite independently of each other. He favoured instead a 'transposition theory' in which a fin moves in its location because it is derived from *different segments*. This theory was originally developed by Rosenberg (cited in Goodrich, 1958) to account for the varying extension of different regions of the vertebral column.

Transposition of *Hox* expression domains along the body axis

Goodrich (1958) said: *"When structures appear to move up or down a segmental series, the shifting is due to a change in the incidence of formative stimuli which determine the development of*

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and differentiate the equipotential segments."

Nowadays, we know that these 'formative stimuli' are the selector genes which operate early in embryogenesis. The *Hox* genes are the best known group of selector genes. Tetrapod vertebrates possess 39 *Hox* genes arranged in 4 separate, but homologous, clusters. During embryogenesis, *Hox* genes are expressed in domains which overlap posteriorly but which extend to different anterior limits along the A-P axis. There is a strict correspondence between the order of these expression domains (anterior to posterior) and that of the genes (3' to 5') along their chromosomal clusters (Gaunt *et al*., 1988; Gaunt, 1991). This phenomenon is known as structural colinearity. Commonly, each *Hox* gene exerts its major effect as a 'formative stimulus' in the anterior part of its overall expression domain.

Hox transpositions within segmented mesoderm

Gaunt (1994) and Burke *et al*. (1995) compared *Hox* expression boundaries in two vertebrates, chick and mouse, which have different vertebral formulae (that is, different numbers of vertebrae of each morphological type). They found that *Hox* expression boundaries correspond with vertebral identity, rather than simply with the segment number along the axial series. For example, in both species, the *Hoxb-3* expression boundary marks the anterior cervical region, and the *Hoxc-6* boundary marks the anterior thoracic region. This is remarkable because the thorax in chick is, relative to mouse, shifted posteriorly by 7 segments along the axial series (the thoracic region commences at vertebrae 15 in chick and 8 in mouse). In complete accordance with this, however, the *Hoxc-6* expression boundary in chick is also shifted posteriorly by 7 segments. Moreover, Burke *et al*. (1995) found that the *Hoxc-6* boundary marks the neck/thorax junction in three other species with different vertebral formulae (goose, *Xenopus* and zebra fish). These findings suggest that *Hoxc-6* has a role as a 'formative stimulus' in the specification of thoracic structures. Consistent with this, Jegalian and De Robertis (1992) showed that *Hoxc-6* overexpression in lumbar vertebrae results in their development of ribs.

More recently, we have examined some additional chick *Hox* genes *(Hoxa-6 and b-6)* whose homologues in mouse are also known to have expression boundaries at around the neck/thorax junction (Gaunt *et al*., 1988; Graham *et al*., 1989; Gaunt, 1991). The data are shown in Figure 1, together with results for *Hoxa-4* and *a-5.* Figure 2 summarises the anterior limits of expression for these *Hox* genes, together with those for *Hoxa-7* (Gaunt *et al*., 1999). It is seen that *Hoxa-6* and *a-7* fit the pattern published earlier for *Hoxc-6:* their expression boundaries are shifted posteriorly by 4 and 6 vertebrae respectively, a finding in general accordance with the 7-vertebra shift in the position of the chicken thorax. In contrast, however, the chick *Hoxb-6* expression boundary – at the level of vertebra 6 (Fig. 1A) – is apparently not shifted posteriorly relative to mouse (Graham *et al*., 1989). Indeed, weak expression of *Hoxb-6* seen in vertebra 5 of the chick may even indicate that this expression boundary is more anteriorly located than in mouse. The significance of this particular observation for the chicken is unclear, but it may help in distinguishing between different models for the mechanism of the shift (see below).

For *Hoxa-7* expression, clear posterior boundaries can also be seen (e.g. Fig. 1 in Gaunt *et al.,* 1999). As summarised in Figure 2, the block of mesodermal segments that express *Hoxa-7* corresponds with those that contribute to the flank (region between the fore- and hindlimb). This is true both for mouse and chick, even though the chick flank is shorter and is shifted posteriorly along the body (Gaunt *et al*., 1999).

In addition to differences in the axial positions of *Hox* gene transcripts, we also note differences between chick and mouse in the abundance of transcripts. It is not yet clear, however, whether these differences are real, or due to a non-equivalence in the developmental stages examined in chick and mouse (5 1/2 and 12 1/2 days of development, respectively). In chick, *Hoxa-4* transcripts (Fig. 1E) are seen to be more abundant, and they do not display the steep anterior-to-posterior decline found in the mouse (Gaunt *et al*., 1988). In contrast, chick *Hoxa-6* (Fig. 1B) and *c-6* (Gaunt, 1994) transcripts are less abundant than have been found in mouse (Gaunt *et al.,* 1988). These observations are discussed further below.

Hox transpositions within segmental spinal nerves

Figure 2 also summarises our recent finding (Gaunt *et al*., 1999) that the anterior boundary of *Hoxa-7* expression is shifted posteriorly not only in mesoderm, but also in spinal ganglia (spinal ganglia are neural crest derivatives). This is the first indication that evolutionary transposition in *Hox* boundaries occurs within neural components. As summarised in Figure 2 we find, for both mouse and chick, that spinal ganglia expressing *Hoxa-7* most strongly are apparently those that contribute to the brachial plexus for innervation of the forelimb (Gaunt *et al*., 1999).

Spinal nerves (at least motorneurones) bear a memory of their A-P address that dictates their target tissue (Lance-Jones and Landmesser, 1980; Stirling and Summerbell, 1985). Since this A-P address is likely to be specified by *Hox* genes, it makes good sense that where the limb and brachial plexus are shifted posteriorly (as in chick relative to mouse; Fig. 2) there is a corresponding posterior shift in *Hox* expression boundaries within the spinal ganglia.

We therefore consider that the axial address of spinal nerves –and hence their structure and function– is specified by their patterns of *Hox* gene expression. This view was also favoured by Rijli *et al*. (1995) who noted that *Hoxa-10* mice display homeotic mutations in spinal nerves within the lumbar region.

Hox transpositions within unsegmented lateral plate mesoderm

The location of *Hox* expression boundaries in lateral plate mesoderm probably specifies the axial levels of limb bud initiation (Charité *et al*., 1994; Rancourt *et al*., 1995; Cohn *et al*., 1997). During evolution, limb buds can be transposed relative to somite level (e.g. Fig. 2). We must therefore assume that *Hox* expression boundaries in lateral plate mesoderm, being markers of the limb buds (Cohn *et al*., 1997), can also shift.

It is clearly important that the expression boundaries of *Hox* genes must be spatially coordinated between lateral plate mesoderm, somitic mesoderm and spinal ganglia. Thus, for example, the limb must form at the level of the junction of neck and thoracic vertebrae, and it must form adjacent to the brachial plexus (Fig. 2). The mechanism of this coordination, which is not understood, is considered further below.

The mechanism by which *Hox* boundaries are established

An evolutionary transposition of *Hox* expression domains seems likely to be effected by perturbations within the normal molecular

mechanisms that are responsible for the establishment and maintenance of *Hox* expression boundaries. However, these normal mechanisms still remain unclear. This continues to be one of the central mysteries in vertebrate embryology. That is, how do cells within the various germ layers sense their position within the embryo in order to activate the appropriate developmental selector genes? We do not attempt here to list all the possible ways in which *Hox* patterning may occur. Instead, we discuss four different possibilities, and some of these are illustrated in Figure 3. A feature in the appearance of *Hox* expression patterns, at least for 3'-located *Hox* genes, is that they develop by spreading from the posterior end of the embryo (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994,1996; Gaunt *et al.,* 1999). Figure 4, for example, presents a time course for the development of the *Hoxb-4* expression pattern, showing forward spreading along primitive streak, neural tube and lateral plate mesoderm. Our recent evidence for forward spreading within paraxial mesoderm will be presented elsewhere (Gaunt *et al.,* 1999).

A gradient of a diffusible morphogen along the length of the developing embryo: model 1

Hox boundaries could be set according to a gradient of morphogen which is established by diffusion along the length of the embryo. Each *Hox* gene might, for example, be activated above a critical threshold concentration of the morphogen (Fig. 3A). In theory, the gradient could extend along the total length of the developing embryo during the entire period that *Hox* expression patterns become established. Alternatively, an early morphogen gradient (existing before expression of *Hox* genes) could establish a series of coordinates as 'stripes' of regulatory gene expression, and these could then persist to set the *Hox* expression patterns at a later time. The source of the gradient would presumably lie at the posterior end of the embryo since this is the point around which *Hox* expression patterns radiate. Forward diffusion of the morphogen could, in theory, account for the observation that *Hox* expression domains become established by forward spreading from posterior parts of the embryo (Fig. 4).

A morphogen gradient setting coordinates along the early embryo might require that the germ layers at this time are present as a miniature of the final form. Fate maps of the early (definitive streak) stage embryo indicate that the neural plate may indeed first exist, at least to some extent, as a miniature of its final form (Spratt, 1952; Nicolet, 1971; Tam, 1989; Schoenwolf and Sheard, 1990). However, there is no evidence at this stage for such a preformation of the A-P axis within presumptive paraxial mesoderm (e.g. Selleck and Stern, 1991; Schoenwolf *et al.*, 1992; Nicolas *et al.,* 1996).

Evidence against diffusion of a morphogen along the full length of later-stage embryos is provided by the fact that physical barriers inserted across embryonic tissues, although presumably impermeable to diffusing morphogens, do not prevent the forward spread of *Hox* expression domains. We showed this first for the neural tube (Fig. 5A,B; Gaunt and Strachan, 1994), and in Figure 5C-F we report a similar finding for the lateral plate mesoderm. Thus, between the 5-somite and the 15-somite stages, *Hoxb-4*

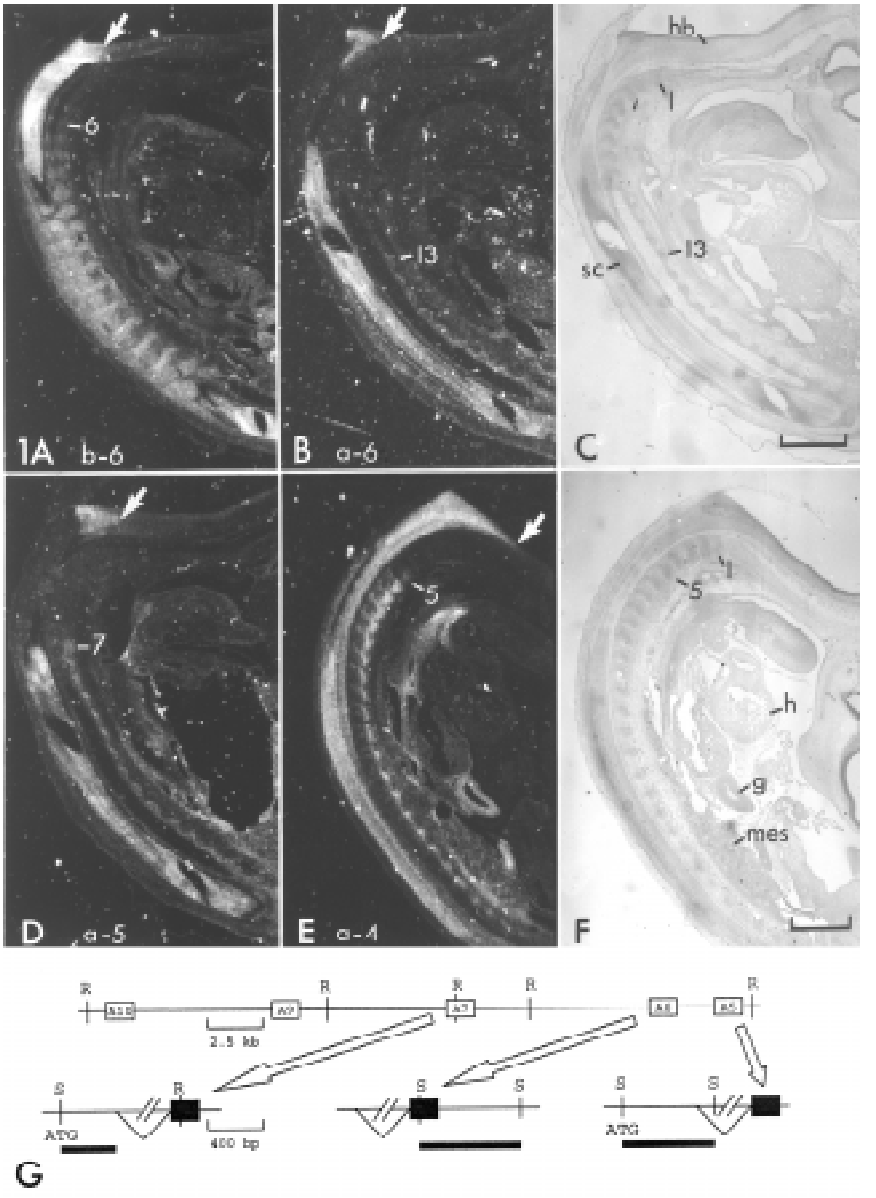

Fig. 1. *Hoxb-6* (A), *a-6* (B), *a-5* (D) and *a-4* (E) expression detected by *in situ* hybridisation on sagittal sections of 5 1/2 day chick embryos. *The autoradiograms are viewed under darkfield illumination.* **(A,B** *and* **D)** *are cut from the same embryo;* **(E)** *is from a different embryo. Brightfield views* **(C,F)** *are shown of both embryos. Numbers denote vertebrae at the anterior boundaries of the Hox expression domains. Arrows indicate anterior boundaries in the central nervous system. hb, hindbrain; sc, spinal cord; mes, mesonephros; g, gut; h, heart. Bar, 1.0 mm. Hybridisations were carried out using* ^{35}*S-riboprobes, as by Gaunt et al. (1988). For Hoxb-6 and a-4, probes were as used by Gaunt (1994).* **(G)** *Hoxa-5, a-6 and a-7 probes used for in situ hybridisations are represented by thick bars. These were subcloned from a genomic DNA clone that had been isolated from a chick cosmid library (Stratagene). R, EcoR1; S, Sac1.*

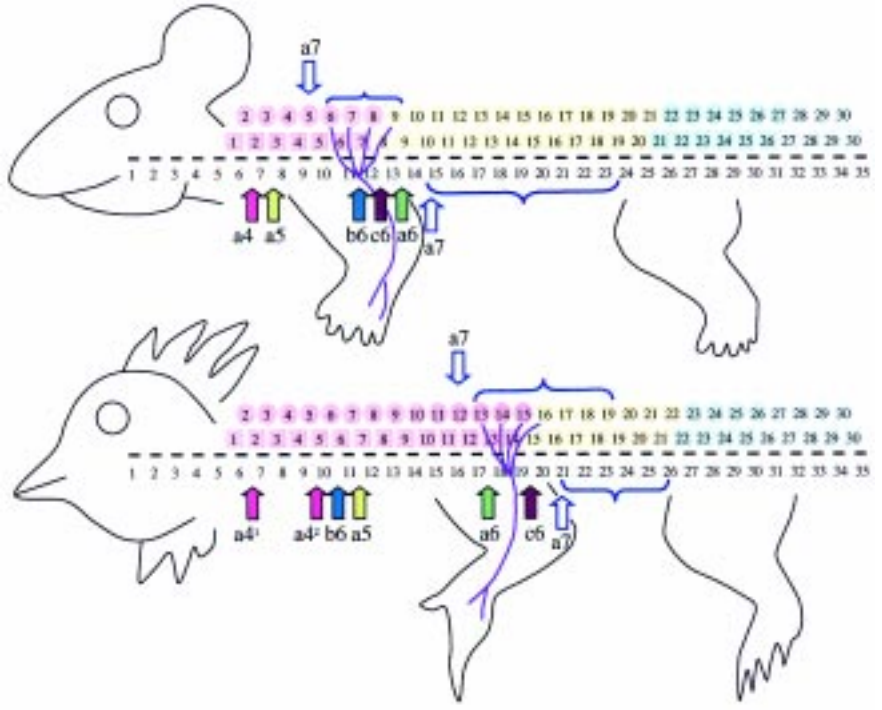

Fig. 2. The relationship between somites, vertebrae, spinal ganglia, and the anterior boundaries of expression for *Hoxc-6, a-4, a-5, a-6, a-7* and *b-6*. *Somites, vertebrae and spinal ganglia are shown as lines, squares and ovals respectively. Cervical, thoracic, lumbar and sacral structures are shown pink, yellow, green and blue respectively. Arrows show the anterior boundaries of Hox gene expression, and the brackets show those spinal ganglia and vertebrae that most strongly express Hoxa-7 (Gaunt et al., 1999). Positions of the limbs relative to contributing somites are derived from Burke et al. (1995) and Cohn et al. (1997). Positions of brachial plexuses relative to spinal ganglia are derived from Burke et al. (1995) and Gaunt et al. (1999). Results shown are derived from Figure 1 (for chick Hoxa-4, a-5, a-6 and b-6), from Gaunt (1994, for chick Hoxc-6 and a-4), and from Gaunt et al. (1988). The anterior boundary of chick Hoxa-4 expression is apparently slightly different when observed at 1 1/2 days (a-4^1;Gaunt, 1994) and 5 1/2 day (a-4^2; Fig. 1E) of development.*

expression normally spreads forwards in lateral plate mesoderm to occupy a domain which includes the area adjacent to the first few somites (see Fig. 4C-F). Insertion of an impermeable barrier at the 1-somite stage (Fig. 5C), followed by culture for 16 h (Fig. 5D), does not prevent expression appearing anterior to the boundary (Fig. 5E,F). The diffusible morphogen model shown in Figure 3A predicts that the posterior region of the embryo, at least in the early stages, should be the source of this morphogen. Yet we have found that posterior primitive streak tissue grafted to a more anterior site adjacent to presomitic mesoderm does not disturb the subsequent development of normal *Hox* expression domains in host somitic mesoderm (Fig. 6A-C). Similarly, grafts of anterior primitive streak and Hensen's node (although generating their own somitic mesoderm and *Hox* patterns) do not prevent *Hox* boundaries forming at normal positions in adjacent host somitic mesoderm (Fig. 6D-F).

A timing (temporal colinearity) model: model 2

Consider the 'opening zone' in the model shown in Figure 3B. Suppose that this is a growth centre, and also the region where *Hox* genes become activated. Since *Hox* genes are activated sequentially along their clusters ('temporal colinearity'; e.g. Duboule, 1994), only the 3'-most gene *(Hox 1)* is initially active. After a time interval a group of cells has moved out of the opening zone, ceasing to activate further *Hox* genes but maintaining expression of *Hox 1*. Meanwhile, cells remaining in the zone have now activated *Hox 2*. With successive intervals of time a partially overlapping set of *Hox* expression domains is established. This is the model originally proposed by Dollé *et al.* (1989), who also suggested that within the opening zone the *Hox* clusters might commence in a closed chromatin state, and that the progressive activation of *Hox* genes may be due to a wave of opening moving in the direction 3' to 5'. Duboule (1994, 1995) has suggested that opening of the *Hox* clusters depends upon rapid cell growth, a characteristic of cells in the opening zone.

Two recent observations support the timing model. First, movement of a *Hox* gene to a more 5' location within its cluster, by homologous recombination, does result in a delayed expression of the gene (van der Hoeven *et al.*, 1996). Second, studies upon *polycomb/M33*-deficient mice have suggested that the *Hox* clusters may indeed commence in a closed state (Coré *et al.*, 1997).

Other factors are not so easily accommodated within this model, at least not in its simplest form (Fig. 3B). The model supposes that the embryo develops by the continuous generation of new (and progressively more posterior) parts at a posterior growth zone. While this may be true, at least in part, for the paraxial mesoderm (e.g. Nicolas *et al.*, 1996), it may not be so for the neural tube, which apparently develops mainly by a re-assortment of parts that are rather widely distributed at the gastrula stage (e.g. Tam, 1989). As one possibility, therefore, the timing mechanism may be used to pattern only paraxial mesoderm, and the neural tube (and possibly also the lateral plate mesoderm) may then be patterned secondarily by vertical signals from the adjacent somites ('heterogenetic induction'; Gaunt and Strachan, 1994). Such a mechanism could certainly explain how the axial limits of *Hox* expression boundaries become coordinated in abutting paraxial mesoderm, lateral plate mesoderm and neural tube. Recent work has given credibility to this view: somites transplanted to more anterior locations in the embryo will induce new patterns of *Hox* expression in the adjacent neural tube (Itasaki *et al.*, 1996; Grapin-Botton *et al.*, 1997; see also Ensini *et al.*, 1998). There does, however, remain an obstacle in this line of reasoning. Work on *Xenopus* has shown that *Hox* expression patterns in neurectoderm can develop, at least to some extent, in the absence of any underlying mesoderm (e.g. Doniach *et al.*, 1992; Ruiz i Altaba, 1994).

The somite transplantation experiments mentioned above present a possible objection to the timing model. If *Hox* expression boundaries in neurectoderm form in response to diffusible intercel-

lular signals (Itasaki *et al*., 1996; Grapin-Botton *et al*., 1997) then it might seem surprising if boundaries in mesoderm were established by an unrelated mechanism, entirely dependent upon timing. A further problem, particularly in our own work, is that the timing model does not suggest an obvious explanation for the finding that *Hox* expression patterns can be seen to spread forward in the embryo, in a process that occurs independently of cell movement. It was an attempt to overcome this difficulty that led Gaunt and Strachan (1996) to suggest a timing and spreading model.

A timing and spreading model: model 3

This model (e.g. Fig. 3C; Gaunt and Strachan, 1996), which is based upon the timing model (Fig. 3B), assumes that functional opening (activation) of the Hox clusters is not limited to the posterior part of the embryo, but that gene activation over the clusters, in direction 3' to 5' (temporal colinearity), occurs along most or all of the embryo, and in all germ layers. The model further assumes that any given embryonic cell is advanced in this activation process when compared with any more anterior cell, and that any given state of functional opening therefore proceeds as a spreading wave (posterior to anterior) along the embryonic cell sheets (Fig. 7 of Gaunt and Strachan, 1996; Fig. 6B of Gaunt *et al*., 1999). It is unclear how such a wave would be set up. As one possibility, the wave might be initiated in the early embryo by an intercellular inducer of Hox cluster activation that spreads forward along the axis, reaching posterior cells before anterior. Once a cell is reached by inducer, an *intra*cellular cascade of *Hox* gene activation would then proceed by progressive, 3' to 5', activation of genes along their clusters (see Fig. 7 of Gaunt and Strachan, 1996). This mechanism probably requires that the cells of the early embryo are located in a pre-pattern of their final arrangement. While this may be true for neurectoderm (e.g. Tam, 1989), it is not apparently so for paraxial mesoderm (e.g. Nicolas *et al*., 1996).

As a second possibility, more easily applicable to paraxial mesoderm, a forward spreading wave of *Hox* expression would be generated if Hox clusters commence functional opening as in model 2, but then temporal colinearity in expression continues as cells move out of the posterior (opening) zone, with the 3' to 5' serial activation of genes proceeding at an ever-decreasing rate as cells become more distant from the posterior zone. Such a wave of activation would cause *Hox* expression domains to spread forward (3' genes before 5' genes) along the length of the developing embryo, without the need for corresponding movement of cells.

Some additional factor must be incorporated into timing and spreading models in order to explain why each *Hox* expression domain stops its forward spreading upon reaching the definitive anterior boundary. This is the least clear aspect but, again, there are several possibilities. One possibility, most applicable to paraxial mesoderm, is a 'spreading to chromatin pre-pattern' scenario. This proposes that the final extent of Hox cluster expression in any cell is determined by a chromatin pre-pattern which is established while it resides in the posterior opening zone. As proposed by Dollé *et al*. (1989), this pre-pattern could be a time-dependent structural opening of the Hox chromatin that proceeds so long as cells remain within the opening zone. Unlike the proposal of these authors, however, functional opening of the Hox clusters (temporal colinearity in expression of their Hox genes) would not be directly linked to this earlier structural opening, and there would be a lag period between structural (chromatin) and functional opening along the Hox clusters. Functional opening would continue in cells that have left the opening zone, and generate forward-spreading waves of *Hox* gene activation as described in the previous paragraph.

In other scenarios, the extents of forward spreading in *Hox* expression may not be pre-determined at such an early developmental stage. In Figure 3C, a 'spreading to refractoriness' version, it is suggested that each *Hox* expression domain arrests in its posterior-to-anterior spreading upon reaching an anterior-to-posterior spreading wave of refractoriness. Cells might become refractory to further opening of their Hox clusters when, for example, they reach a certain stage of maturation, such as when mesoderm becomes segmented as somites, when neural plate undergoes folding, or when cell cycle times become lengthened (Duboule, 1994, 1995). In an alternative 'spreading to equilibrium' version there is no wave of refractoriness, and it is envisaged instead that there is an intrinsic stability in the final pattern of *Hox* expression due, perhaps, to cross- and autoregulatory interactions between *Hox* genes and the products of their activation. That

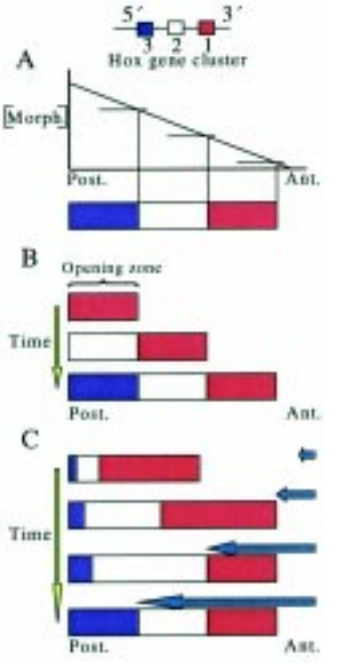

Fig. 3. Three different models for the establishment of *Hox* expression patterns. *See text for details. In each model, the final Hox expression pattern generated is the same, and is drawn to resemble Wolpert's French Flag (e.g., Wolpert, 1996). This gives a good representation of how vertebrate Hox expression domains extend to different anterior limits along the body axis, although it does not show how they usually overlap posteriorly. In* **(A)**, f*orward diffusion of a posterior morphogen establishes a concentration gradient along the embryo. Each Hox gene is activated above a critical threshold concentration. In* **(B)**, *Hox genes are activated sequentially in time along their cluster, but only in cells that lie within a posterior 'opening zone'. Cells move forward from here to form the embryonic tissue layers, maintaining the Hox expression pattern that they acquired in the opening zone. In* **(C)**, *Hox boundaries are established when anteriorly spreading waves of Hox gene activation confront a posteriorly spreading wave of refractoriness (green arrows). In (B), once cells have left the opening zone their Hox expression patterns are linked to cell lineage. In (A and C), Hox expression patterns are established independently of prior cell lineage.*

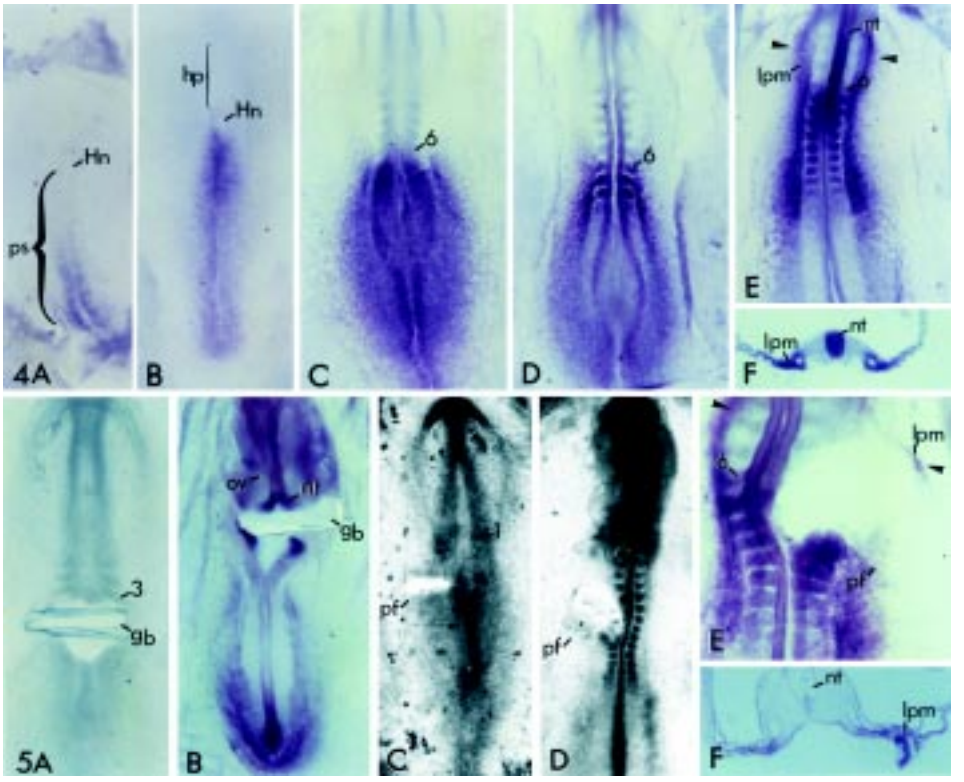

Fig. 4. Forward spreading in the establishment of a vertebrate *Hox* expression domain. **(A-E)** *A time-course for chick Hoxb-4 expression, detected by whole-mount in situ hybridisation.* **(A)** *Definitive streak stage (18 h of incubation);* **(B)** *Head process stage (21 h);* **(C)**, *5-somite stage (29 h);* **(D)**, *9-somite stage (33 h);* **(E)**, *15-somite stage (48 h).* **(F)** *Section through embryo at the level of the arrows shown in E. (A) and (B) show how expression first spreads forward along the primitive streak. (C-E) show how expression spreads forward along both neural tube and lateral plate mesoderm adjacent to the anteriormost somites. Expression of other Hox genes also spreads forward, and in an overall temporal sequence that is colinear with gene position along the Hox clusters (Gaunt and Strachan, 1996). Hn, Hensen's node; ps, primitive streak; hp, head process; nt, neural tube; lpm, lateral plate mesoderm. Numbers denote somite addresses.*

Fig. 5. Forward spreading in *Hox* expression is not blocked by embryo transection or impermeable barriers. **(A)** *A 3-somite stage chick embryo was cut through the neural tube and adjacent mesoderm at a position anterior to the spreading wave of Hoxd-4 expression. The forward progression of Hoxd-4 expression (shown in Gaunt and Strachan, 1994) is rather similar to that shown in Figure 4 for Hoxb-4. After transection, a glass block was inserted to keep the tissues apart.* **(B)** *The same embryo shown after 16 h of culture and then whole-mount in situ hybridisation to detect Hoxd-4 expression. Expression is seen to have moved across the barrier with the same kinetics as if it had not been there (Gaunt and Strachan, 1994).* **(C)** *A 1-somite stage embryo was cut unilaterally through the lateral plate mesoderm, paraxial mesoderm and half of the neural plate at a position posterior to presumptive somite 4. After transection, a wedge of plastic film (cling film) was inserted to keep the tissues apart. The embryo was cultured for 16 h* **(D)**, *followed by whole-mount in situ hybridisation to detect Hoxb-4 expression* **(E)**. **(F)** *Section through the embryo at the level of the arrows shown in E. In (E) and (F), Hoxb-4 expression in lateral plate mesoderm (seen at the right arrow in E) is seen to have spread across the barrier as if it had not been there (c.f., Fig. 4E,F). (A,C,D) ventral views; (B,E) dorsal views. ov, otic vesicle; gb, glass block; pf, plastic film; other labels as for Figure 4.*

is, there is an intrinsic stability in the Hox code within any particular geographical zone of the embryo. This might require interaction between different *Hox* expression domains, and could involve both intra- and intercellular signalling. So far, however, there is little direct evidence in favour of an equilibrium model, and mice knocked out for (e.g. Horan *et al.*, 1995; Rancourt *et al.*, 1995; Rijli *et al.*, 1995) or overexpressing (e.g. Jegalian and De Robertis, 1992) *Hox* genes have shown no apparent changes in the expression boundaries of their neighbour genes.

Timing and spreading models have some advantages over the simple timing model (Fig. 3B). First, they can readily explain how *Hox* expression domains can move forward without corresponding movement of cells. Second, they can explain how a given *Hox* expression domain can move across a surgically implanted barrier if we assume that, at the time of surgery, cells anterior to the boundary are already activated to commence opening of their Hox clusters. An important feature of timing and spreading models is that each geographical zone of the body establishes its pattern of expressing *Hox* genes while any more posterior *Hox* genes are effectively locked away in a functionally closed condition (Fig. 7B of Gaunt and Strachan, 1996; Fig. 6B of Gaunt *et al.*,1999). In these models, temporal colinearity in *Hox* gene expression is not primarily a mechanism for setting the position of *Hox* expression boundaries (as in model 2), but instead it may provide a safety mechanism to prevent posterior (dominant; Duboule and Morata, 1994) genes from becoming ectopically expressed anterior to their normal expression domains.

A morphogen timing and spreading model: model 4

This is really a timing and spreading model, but it combines aspects of all three models above. It is illustrated in Figure 6 of Gaunt *et al.* (1999). A morphogen gradient is generated along the length of the paraxial mesoderm (and also, perhaps, along lateral plate mesoderm) as it emerges from the primitive streak, with early-emerging (anterior) cells containing a lower concentration of the morphogen than later cells. In this model (unlike model 1) the proposed morphogen is not necessarily a diffusible substance. It could, instead, be a trranscription factor (currently, the caudal proteins are potential candidates here: Charité *et al.*, 1998). Establishment of the morphogen gradient might depend, for example, upon time spent by cells within the primitive streak, or upon a shorter-range diffusible gradient marking position along the primitive streak. Subsequently, Hox clusters become functionally activated, 3' to 5', within the emergent mesodermal cells at a rate, and to a final extent, that is proportional to their endogenous concentration of morphogen. Such a mechanism would cause *Hox* expression patterns to become established by forward spread-

ing (Fig. 4), with each *Hox* gene being advanced in its spreading relative to any more 5'-located genes (as in Figs. 6 of Gaunt and Strachan, 1996, and Gaunt *et al.*, 1999). In this model, neural tube would (by the same arguments used above for model 2) presumably become patterned by heterogenetic induction from the adjacent somitic mesoderm. Similarly, the same mechanism might induce, or at least modify, *Hox* expression patterns in the adjacent lateral plate mesoderm. The model incorporates the same two starting assumptions as model 3 and, like model 3, can therefore readily explain forward movement of *Hox* expression across surgically implanted barriers.

The mechanism of evolutionary shifts in *Hox* boundaries

In terms of the models given above, we consider some possible ways by which *Hox* expression boundaries might have become shifted during evolutionary time.

Change in a morphogen gradient along the anteroposterior axis

From Figure 3A it is clear that either change in the slope of the morphogen gradient or change in the response of individual genes to morphogen (models 1 and 4, above) could result in shifts in *Hox* expression domains. Whilst the results of our embryo transection and grafting experiments argue against long-range diffusion of a morphogen (model 1), the works of Itasaki *et al.* (1996) and Grapin-Botton *et al.* (1997) suggest that a gradient of *Hox* gene inducer may indeed exist within paraxial mesoderm and neural tube. However, it is not clear whether this inducer of *Hox* expression acts upon any tissues other than neural tube, nor whether the inducer has an instructional role (functioning as a morphogen) or a permissive role (for example, by regulating the state of refractoriness in the model shown in Figure 3C). The possibility is discussed elsewhere (Gaunt *et al.*, 1999) that transposition of a *Hox* gene's expression boundary might be caused by a change in the response of its *cis* regulatory elements to morphogen.

Change in the timing of Hox expression

In terms of the timing model (Fig. 3B; Dollé *et al.*, 1989), or the 'spreading to refractoriness' scenario (Fig. 3C), a delay in the onset of any given *Hox* gene's expression will result in an eventual elongation of the region of cells which express the 3'-neighbour gene. In theory, this therefore provides a simple mechanism whereby a particular region of the body could become extended during evolution (Dollé *et al.*, 1993; Duboule, 1994; Gaunt, 1994). Duboule and his colleague have recently provided good evidence for a direct relationship between the time that a *Hox* gene is activated and the position of its anterior boundary of expression. They used homologous recombination experiments to delete or change enhancer elements that regulate *Hoxd-11*. This resulted in either the delayed (Zákány *et al.*, 1997) or the premature (Gérard *et al.*, 1997) expression of this gene. These perturbations resulted, respectively, in posterior and anterior transpositions of the lumbosacral junction (with lumbar vertebrae varying in number from 5 to 7) since it is the anterior boundary of *Hoxd-11* expression that specifies, in part, the position of the first sacral vertebra.

These findings may also be accommodated within the spreading to equilibrium scenario (model 3) and within model 4. Here, change in the time for activation of a *Hox* gene results in change in the rate of forward spreading of its transcripts. The eventual expression boundary will not, in these models, be transposed, but a delay in *Hox* expression at a critical stage in a tissue's development could itself result in a homeotic change (Zákány *et al.*, 1997). These predictions of models 3 and 4 seem, at least in part, to be supported by the findings of van der Hoeven *et al.* (1996) and Zákány *et al.* (1997). Here, experimental perturbations used to postpone onset of a *Hox* gene's expression had the effect of delaying –but not of eventually preventing– the establishment of a normal anterior boundary of expression.

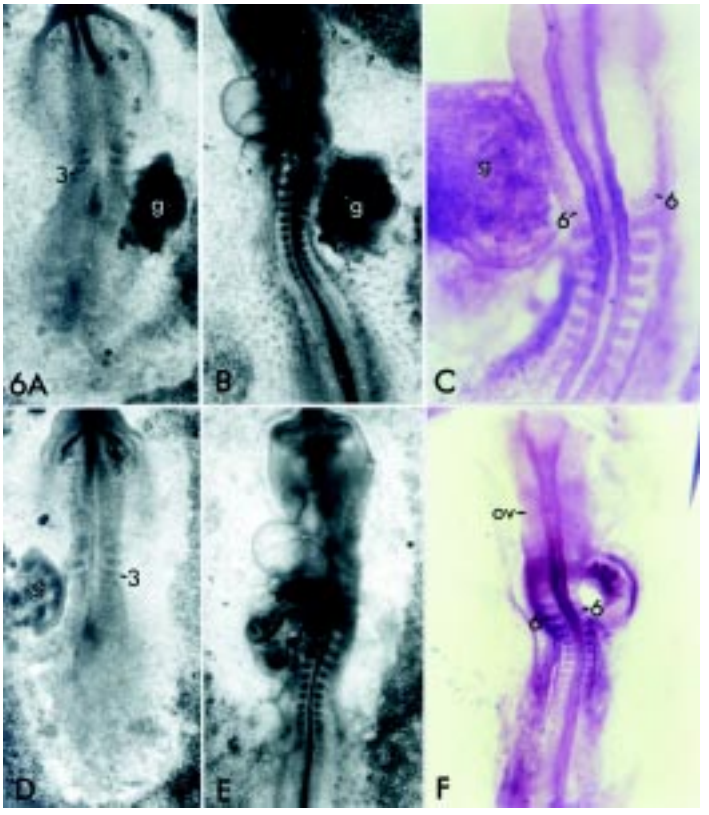

Fig. 6. Effect of primitive streak tissue grafts upon the *Hoxb-4* expression boundary in somitic mesoderm. (A) *Posterior primitive streak tissue from a head process stage chick embryo was inserted as a graft into an incision, made through both ectoderm and mesoderm layers, adjacent to the presomitic mesoderm of a 3-somite stage embryo. The embryo was cultured for 16 h* **(B)**, *followed by whole-mount in situ hybridisation to detect Hoxb-4 expression* **(C)**. *The expression boundary in somitic mesoderm, at the level of somite 6, is unaffected by the graft (c.f., Fig. 4E).* **(D-F)**, *as in A-C, but using anterior primitive streak and Hensen's node as the graft tissue. The graft generates its own somitic mesoderm and Hox expression, but it does not disrupt development of a normal Hoxb-4 expression boundary, at the level of somite 6, in the adjacent host somitic mesoderm. (A,B,D,E) ventral views; (C,F) dorsal views. Numbers denote somite addresses; g, graft; ov, otic vesicle.*

Change in a chromatin pre-pattern

In terms of the 'spreading to chromatin pre-pattern' scenario (model 3), change in the rate of Hox chromatin opening within the posterior opening zone will cause transpositions in the final boundaries of *Hox* gene expression. It will not necessarily change the subsequent timings at which *Hox* genes become expressed.

Change in the equilibrium position of the Hox code

In terms of the 'spreading to equilibrium' scenario, discussed above, a change in the equilibrium position of the *Hox* code could cause transposition in the final location of a *Hox* expression boundary without necessarily any change in the timing of *Hox* gene activation. The apparently different levels in the abundance of *Hox* gene transcripts now noted between mouse and chick might possibly be an indication of different equilibrium states for their respective *Hox* codes.

A change in tissue growth

For the 3'-located *Hox* genes studied so far (e.g. *Hoxd-4*, Gaunt and Strachan, 1994; *Hoxa-4*, Gaunt *et al.*, 1999), anterior boundaries of *Hox* expression are apparently first formed in paraxial mesoderm prior to the process of somitogenesis. These boundaries then maintain their axial positions as somites develop into vertebrae.

Suppose that a particular Hox protein, whilst still confined to presomitic mesoderm, were to stimulate mesodermal cell growth, then this gene's domain of expression would extend over a greater number of somites after mesoderm had completed segmentation. This would therefore be one way in which a neck-specifying *Hox* gene could regulate development of a longer neck, resulting in increased number of neck vertebrae. A pleasing aspect of this model is that it offers some possible unity in the mechanism adopted for evolutionary change in neck length within both mammals and birds. In almost all mammals the number of neck vertebrae remains constant at seven, and longer necks here might result from a neck-specifying *Hox* gene stimulating growth in *post*-somitic mesoderm, resulting in larger neck vertebrae.

While proposals 1 to 4 in this section, correspond with the transposition model discussed by early anatomists (e.g. Goodrich, 1958), proposal 5 corresponds with their intercalation model. Goodrich's objection to this model (because it cannot account for the apparently independent axial movement of dorsal and ventral fins) may also be applied to the new data on *Hox* expression boundaries. Thus, while *Hoxc-6*, *a-6* and *a-7* are shifted posteriorly by rather similar distances in chick relative to mouse, *Hoxb-6* is not shifted correspondingly (Figs. 1,2). For validity of the growth model, this indicates the unlikely conclusion that all extra growth in the chick must occur within that region of mesoderm that corresponds to the interval between vertebrae 7 and 8 of the mouse.

Concluding remarks

Comparisons made upon chick and mouse show how the three primary embryonic tissues, paraxial mesoderm, lateral plate mesoderm and neural plate, display evolutionary transpositions in both their anatomical derivatives and their boundaries of *Hox* gene expression. The mechanisms by which *Hox* boundaries are normally established, and the mechanism of their evolutionary transposition, remain uncertain. Duboule and his colleagues have suggested, and have presented supporting evidence, that transpositions are primarily due to changes in the timing of *Hox* gene activation. However, as we have discussed, it does not seem entirely clear that such a mechanism can operate in the same way in each of the three primary embryonic tissues mentioned above, and some important questions remain unanswered. Thus, do *Hox* boundaries arise by the same or different mechanisms in these three embryonic tissues? How are *Hox* expression boundaries spatially coordinated in the three tissues? And how can we explain the finding that *Hox* boundaries in neural tube respond to vertical signalling from somites, yet they also apparently develop rather normally in the absence of underlying mesoderm?

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References

BURKE, A.C., NELSON, C.E., MORGAN, B.A. and TABIN, C. (1995). Hox genes and the evolution of vertebrate axial morphology. *Development 121*: 333-346.

CHARITÉ, J., DE GRAAFF, W., CONSTEN, D., REIJNEN, M.J., KORVING, J. and DESCHAMPS, J. (1998). Transducing positional information to the Hox genes: critical interaction of cdx products with position-sensitive regulatory elements. *Development 125:* 4349-4358.

CHARITÉ, J., DE GRAAFF, W., SHEN, S. and DESCHAMPS, J. (1994). Ectopic expression of *Hoxb-8* causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures. *Cell 78*: 589-601.

COHN, M.J., PATEL, K., KRUMLAUF, R., WILKINSON, D.G., CLARKE, J.D.W. and TICKLE, C. (1997). Hox 9 genes and vertebrate limb specification. *Nature 387*: 97-101.

CORÉ, N., BEL, S., GAUNT, S.J., AURRAND-LIONS, M., PEARCE, J., FISHER, A. and DJABALI, M. (1997). Altered cellular proliferation and mesoderm patterning in *Polycomb-M33* -deficient mice. *Development 124*: 721-729.

DESCHAMPS, J. and WIJGERDE, M. (1993). Two phases in the establishment of Hox expression domains. *Dev. Biol. 156*: 473-480.

DOLLÉ, P., DIERICH, A., LEMEUR, M., SCHIMMANG, T., SCHUHBAUR, B., CHAMBON, P. and DUBOULE, D. (1993). Disruption of the *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. *Cell 75*: 431-441.

DOLLÉ, P., IZPISUA-BELMONTE, J.-C., FALKENSTEIN, H., RENUCCI, A. and DUBOULE, D. (1989). Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature 342*: 767-772.

DONIACH, T., PHILLIPS, C.R. and GERHART, J.C. (1992). Planar induction of anteroposterior pattern in the developing central nervous system of *Xenopus laevis. Science 257*: 542-545.

DUBOULE, D. (1994). Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Development (Suppl.):* 135-142.

DUBOULE, D. (1995). Vertebrate Hox genes and proliferation: an alternative to homeosis? *Curr. Opin. Genet. Dev. 5*: 525-528.

DUBOULE, D. and MORATA, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet. 10*: 358-364.

ENSINI, M., TSUCHIDA, T.N., BELTING, H. and JESSELL, T.M. (1998). The control of rostrocaudal pattern in the developing spinal cord: specification of motor neuron subtype identity is initiated by signals from paraxial mesoderm. *Development 125:* 969-982.

GAUNT, S.J. (1991). Expression patterns of mouse Hox genes: clues to an understanding of developmental and evolutionary strategies. *BioEssays 13*: 505-513.

GAUNT, S.J. (1994). Conservation in the Hox code during morphological evolution. *Int. J. Dev. Biol. 38*: 549-552.

GAUNT, S.J. and STRACHAN, L. (1994). Forward spreading in the establishment of a vertebrate Hox expression boundary: the expression domain separates into anterior and posterior zones, and the spread occurs across implanted glass barriers. *Dev. Dyn.199*: 229-240.

GAUNT, S.J. and STRACHAN, L. (1996). Temporal colinearity in expression of anterior Hox genes in developing chick embryos. *Dev Dyn. 207*: 270-280.

GAUNT, S.J., SHARPE, P.T. and DUBOULE, D. (1988). Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. *Development 104 (Suppl.):* 169-179.

GAUNT, S.J., DEAN, W., SANG, H. and BURTON, R.D. (1999). Evidence that Hoxa expression domains are evolutionarily transposed in neurectoderm, and that they

are established by forward spreading in paraxial mesoderm. *Mech. Dev. 82:* 109-118.

GÉRARD, M., ZÁKÁNY, J. and DUBOULE, D. (1997). Interspecies exchange of a Hoxd enhancer *in vivo* induces premature transcription and anterior shift of the sacrum. *Dev. Biol. 190*: 32-40.

GOODRICH, E.S. (1913). Metameric segmentation and homology. *Q. J. Microsc. Sci. 59*: 227-248.

GOODRICH, E.S. (1958). *Studies on the structure and development of vertebrates.* Vol 1. Dover Publications, Inc. New York.

GRAHAM, A., PAPALOPULU, N. and KRUMLAUF, R. (1989). The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. *Cell 57*: 367-378.

GRAPIN-BOTTON, A., BONNIN, M-A. and LE DOUARIN, N.M. (1997). Hox gene induction in the neural tube depends on three parameters: competence, signal supply and paralogue group. *Development 124*: 849-859.

HORAN, G.S.B., RAMIREZ-SOLIS, R., FEATHERSTONE, M.S., WOLGEMUTH, D.J., BRADLEY, A. and BEHRINGER, R.R. (1995). Compound mutants for the paralogous *hoxa-4*, *hoxb-4*, and *hoxd-4* show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. *Genes Dev. 9*: 1667-1677.

ITASAKI, N., SHARPE, J., MORRISON, A. and KRUMLAUF, R. (1996). Reprogramming Hox expression in the vertebrate hindbrain: influence of paraxial mesoderm and rhombomere transposition. *Neuron 16*: 487-500.

JEGALIAN, B.G. and DE ROBERTIS, E.M. (1992). Homeotic transformations in the mouse induced by overexpression of a human *Hox-3.3*transgene. *Cell 71*: 901-910.

KEYNES, R.J. and STERN, C.D. (1984). Segmentation in the vertebrate nervous system. *Nature 310*: 786-789.

LANCE-JONES, C. and LANDMESSER, L. (1980). Motorneurone projection patterns in the chick hindlimb following early partial reversals of the spinal cord. *J. Physiol. 302*: 581-602.

LIM, T.M., JAQUES, K.F., STERN, C.D. and KEYNES, R.J. (1991). An evaluation of myelomeres and segmentation of the chick embryo spinal cord. *Development 113*: 227-238.

NICOLAS, J.F., MATHIS, L. and BONNEROT, C. (1996). Evidence in the mouse for self-renewing stem cells in the formation of a segmented longitudinal structure, the myotome. *Development 122*: 2933-2946.

NICOLET, G. (1971). Avian gastrulation. *Adv. Morphogen. 9:* 231-262.

RANCOURT, D.E., TSUZUKI, T. and CAPECCHI, M.R. (1995). Genetic interaction between *hoxb-5* and *hoxb-6* is revealed by nonallelic complementation. *Genes Dev. 9*: 108-122.

RIJLI, F.M., MATYAS, R., PELLEGRINI, M., DIERICH, A., GRUSS, P., DOLLÉ, P. and CHAMBON, P. (1995). Cryptorchidism and homeotic transformations of spinal nerves and vertebrae in *Hoxa-10* mutant mice. *Proc. Natl. Acad. Sci. USA 92*: 8185-8189.

RUIZ I ALTABA, A. (1994). Pattern formation in the vertebrate neural plate. *Trends Neurosci.17*: 233-243.

SCHOENWOLF, G.C. and SHEARD, P. (1990). Fate mapping the avian epiblast with focal injections of a fluorescent-histochemical marker: ectodermal derivatives. *J. Exp. Zool. 255*: 323-339.

SCHOENWOLF, G.C., GARCIA-MARTINEZ, V. and DIAS, M.S. (1992). Mesoderm movement and fate during avian gastrulation and neurulation. *Dev Dyn. 193*: 235-248.

SELLECK, M.A.J. and STERN, C.D. (1991). Fate mapping and cell lineage analysis of Hensen¢s node in the chick embryo. *Development 112*: 615-626.

SPRATT, N.T. (1952). Localization of the prospective neural plate in the early chick blastoderm. *J. Exp. Zool. 120*: 109-130.

STERN, C.D., JAQUES, K.F., LIM, T.M., FRASER, S.E. and KEYNES, R.J. (1991). Segmental lineage restrictions in the chick embryo spinal cord depend on the adjacent somites. *Development 113*: 239-244.

STIRLING, R.V. and SUMMERBELL, D. (1985). The behaviour of growing axons invading developing chick wing buds with dorsoventral or anteroposterior axis reversed. *J. Embryol. Exp. Morphol. 85*: 251-269.

TAM, P.P.L. (1989). Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. *Development 107*: 55-67.

VAN DER HOEVEN, F., ZÁKÁNY, J. and DUBOULE, D. (1996). Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. *Cell 85*: 1025-1035.

WOLPERT, L. (1996). One hundred years of positional information. *Trends Genet. 12*: 359-364.

ZÁKÁNY, J., GÉRARD, M., FAVIER, B. and DUBOULE, D. (1997). Deletion of a *HoxD* enhancer induces transcriptional heterochrony leading to transposition of the sacrum. *EMBO J. 16*: 4393-4402.