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Transformations in null mutants of Hox genes: do they represent intercalary regenerates?

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Transformations in null mutants of *Hox* genes: do they represent intercalary regenerates?

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Summary

In the minds of many, *Hox* gene null mutant phenotypes have confirmed the direct role that these genes play in specifying the pattern of vertebrate embryos. The genes are envisaged as defining discrete spatial domains and, subsequently, conferring specific segmental identities on cells undergoing differentiation along the antero-posterior axis. However, several aspects of the observed mutant phenotypes are inconsistent with this view. These include: the appearance of other, unexpected transformations along the dorsal axis; the occurrence of mirror-image duplications; and the development of anomalies outside the established domains of normal *Hox* gene expression. In this paper, *Hox* gene disruptions are shown to elicit regeneration-like responses in tissues confronted with discontinuities in axial identity. The polarities and orientations of transformed segments which emerge as a consequence of this response obey the rules of distal transformation and intercalary regeneration. In addition, the incidence of periodic anomalies suggests that the initial steps of *Hox*-mediated patterning occurs in Hensen's node. As gastrulation proceeds, mesoderm cell cycle kinetics impose constraints upon subsequent cellular differentiation. This results in the delayed manifestation of transformations along the antero-posterior axis. Finally, a paradigm is sketched in which temporal, rather than spatial axial determinants direct differentiation. Specific, testable predictions are made about the role of *Hox* genes in the establishment of segmental identity.

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Introduction

Following the formation of an antero-posterior polarity, vertebrate embryos undergo a series of subdivisions which lead to morphological and functional segmentation. The mechanisms that underlie these events are still not clear; however both somitogenesis and the subdivision of the central nervous system coincide with the expression of homologs of the *Drosophila* segment selector genes. These genes, called *Hox*genes in mammals, are arrayed in four complexes. Each complex consists of a series of homeobox-containing genes which are arranged as paralogues in the same order as the genes in the *Drosophila HOM* cluster.

Generally, *Hox* genes are expressed in the order of their appearance within the clusters. Their domains of expression overlap to form a nested series, with the earlier expressing 3' genes

reaching up into anterior regions, and the later-expressing 5' genes being restricted to the posterior regions. It has been suggested that anterior borders of *Hox* gene expression arise as the genes undergo sequential activation in Hensen's node as it regresses during gastrulation (1,2). Just how these borders arise remains a matter for conjecture. However, recent evidence may provide a hint: some of the early expressing genes activate in two phase (3-5). In the first phase, they are expressed in the posterior primitive streak (presumptive extra-embryonic mesoderm). This expression domain expands anteriorly until it meets Hensen's node, whereupon the second phase of expression is initiated. Expression in the primitive streak subsides, and a new domain comes into prominence in the tissues left behind as the node regresses posteriorly. In other words, the anterior border of expression for these particular *Hox* genes seems to be a function of when and where the first expression wave intersects the regressing node. Much as Drosophila selector genes act to specify the identity of body segments, *Hox* genes seem to specify the identity of murine body segments, the somites and, ultimately, the vertebrae (6). A '*Hox* code' is commonly invoked to explain how cells in vertebrate embryos are regulated as they differentiate along the neural axis (7). There are two variations of this paradigm but, in both, *Hox* genes define discrete spatial domains which serve to establish an initial map, or plan, which leads to specification of distinct morphological characteristics of the vertebrae.

When these genes are mutated or are expressed ectopically, vertebrae form which exhibit characteristics typical of more anterior or posterior segments. Within a given domain, the most posterior expressing *Hox* gene (the most 5', and the most recently activated gene) tends to set the agenda for axial development there. This phenomenon of 'posterior prevalence' has confirmed for many the idea that sequentially activated *Hox* genes specify unique domains and consequently direct the formation of unique structures. Each gene within a cluster is envisaged as defining progressively more posterior structures (8). Although paralogous genes from different clusters often share similar expression patterns, they nevertheless appear to perform distinct functions during development (9-10). Indeed, another feature that argues in favour of each gene playing a unique patterning role lies in the high conservation which extends to the regulatory elements in organisms as diverse as mouse and *Drosophila* (11).

Unfortunately, this model is not without its deficits since the transformations which arise in mutant mice do not always occur in the predicted direction. An alternative explanation de-emphasizes the tendency to posterior prevalence and instead focuses upon the idea that specific combinations and levels of *Hox* gene expression specify segments in a mosaic fashion (12). However, there are several other features of *Hox* gene function that are not predicted by either model. These inconsistencies indicate that discontinuities in *Hox* gene expression patterns can elicit an intercalary regenerative response. Rectification of expression pattern discontinuities is constrained by mesoderm cell cycle kinetics in the node, where the *Hox* genes exert their first

influence and provide the temporal cues for morphogenesis.

Problems

The current models fall short in explaining several features of the mutant phenotypes. Firstly, many vertebral transformations observed in *Hox* mutant mice either do not correlate with the anterior boundary, or even, in some instances, with the domain of wild-type gene expression. Typically, transformations tend to occur near major body transition zones (where the cephalic bones give way to the cervical vertebrae, the cervical vertebrae give way to the thoracic vertebrae, and so on). Secondly, where the abrogated expression and function of one gene might be expected to cause anteriorized development in a region, some mutations appear to cause posteriorization or even transformations in both directions. Thirdly, in some instances antipodal transformations arise which result in mirror image duplications: in these cases, not only are some vertebral and bone elements transformed to anterior or posterior identities within the same embryo, but also the orientations of these transformed elements are inverted. Antipodal effects are not limited to knock-out mutant mice lines, but also occur in ectopically expressing transgenic lines (see note at the end of ref. 13). Finally, *Hox* gene mutations do not always lead to 'transformation', but may on occasion result in inhibited development of structures normally within their domain of expression (8, 14).

The appearance of compound transformations which are periodic in nature may be particularly significant, but has received scant attention. For example, mutagenesis of murine *Hox*a-5 results in the anterior transformation of cervical vertebra six (C6) to C3, or 4, and the posterior transformation of C7 to thoracic vertebra one (T1) (l5). This posterior transformation of identity results in the generation of an extra pair of anterior ribs. Furthermore, lumbar vertebra one (LI) is anteriorized to T13.

Other *Hox* mutants exhibit similar such compound transformations (Fig. 1). The homozygous *Hox*c-8 null mutation causes T8 to T7 and L1 to T13 transformations (l6), and the mutation of *Hox*d-4 causes C2 to C1 and C7 to T1 transformations (17); *Hoxd-3* mutation causes C1 and C2 to transform one vertebra anteriorly and TI to T7 to develop ribs which meet abnormally at the sternum: and *Hox*a-7 1 nulls transform TI3 to L1 while also inducing generation of a supernumerary L7 (12). It is a curious feature of compound transformations that anomalies often arise with a periodicity of six to seven vertebrae, or cover regions seven vertebrae long. *Hox*d-11 disruption can create either a supernumerary L6 or an S1 (18). Each of these mutants exhibits effects well outside the domain of developing tissue in which their respective transcript presumably exerts a unique influence. For instance, *Hox*a-11 disruption causes abnormal attachment of the first thoracic ribs, a region well outside the gene's putative expression domain. Additionally, some null mutants appear to produce phenotypes that are consistent with neither a strict anteriorization nor posteriorization of axial identity. *Hox*a-2 homozygous nulls yield mirror-image duplications of the bones making up the ear (l4, l9) though the latter study also revealed a posterior transformation of the hyoid bone.

Our problem is threefold: how can we explain the generation of both posterior and anterior transformations by mutation of a single gene; why are these transformations sometimes antipodal or even mirror-image in orientation; and why do mutations occasionally result in the appearance of perturbations, which arise in periodic fashion along the antero-posterior axis

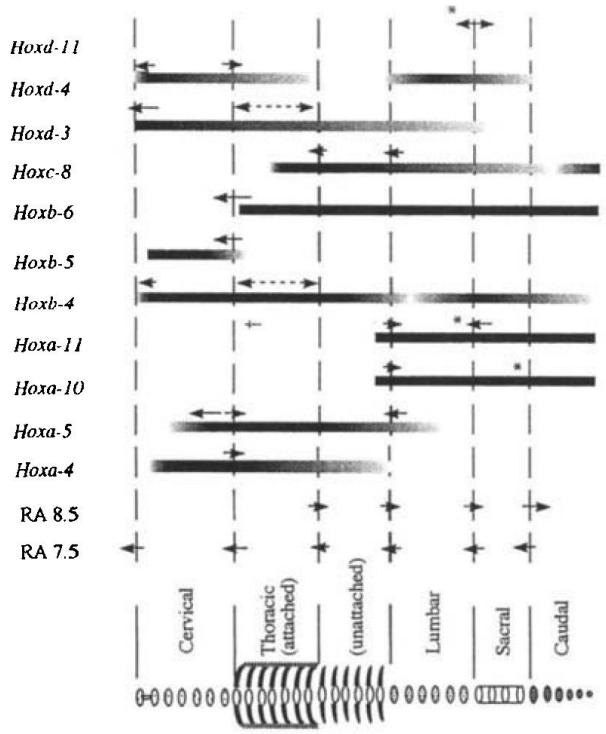

Fig. 1. Transformations of the vertebral column. In this representation of the vertebral column, morphological transition zones between major body domains are demarcated. The regions are divided according to vertebral morphology, including the cervical, thoracic (ribs attached to sternum), thoracic (ribs unattached), lumbar, sacral and caudal zones. The apparent direction of transformation following retinoic acid treatment (RA) on day 7.5 or 8.5 p.c. or disruption of *Hox* gene activity is indicated with arrows. Dashed lines indicate regions where perturbations were seen throughout a domain and asterisks denote the presence of a supernumerary element. The † denotes abnormal rib/sternum attachment. Shaded bars approximate wildtype expression patterns in paraxial mesoderm at day 12.5 of development (except *Hox*a-11, which is shown at day 9.5). Note: transformations tend to commence at borders of morphological transition; compound transformations occur in vertebrae separated by multiples of roughly seven. References for these transformations and expression boundaries are listed in the text, with the addition of refs 41 and 45-48.

axis? The answer to the first two questions may be surprisingly simple: the results are consistent with a model devised to explain regeneration of missing positional information by intercalary regeneration. The answer to the third, the question of periodic reiteration of anomalies, may come from a consideration of specific epigenetic features of somite development.

Distal transformation and intercalation by the shortest route

The rules of distal and intercalary transformation were devised to explain properties of positional identity evident in limb and tail regenerates following amputation, or amputation in conjunction with limb segment recombination. Cells at a plane of amputation exhibit properties, in some amphibians, which permit them to dedifferentiate, proliferate and re-differentiate missing structures. Limb stumps will always regenerate missing elements in a proximal-to-distal manner. This is called the rule of distal transformation (20,21).

There are, however, a few exceptions. Vitamin A and some of its derivatives appear to be capable of proximalizing the perceived starting point, with the result that proximodistal duplications occur. There are other unusual cases where the distal transformation rule is violated and these demanded the formation of a second rule: that of intercalation by the shortest route. Intercalation was useful in explaining why positionally uncontiguous insect and amphibian leg grafts intercalated intervening or extra limb elements of reverse polarity or handedness, respectively (22,23). Briefly, when a ‘positional’

discontinuity exists between two abutted amputation planes, in some instances the discontinuity is smoothed. This means that a system will regenerate the missing values, but it will occasionally necessitate the generation of values and orientations not normally present.

More recently these rules have been discussed with regard to the apparent homeotic transformation of tadpole tail stumps by retinoic acid (24). In this example (see Fig. 2), an amputated tail stump, which might normally regenerate missing posterior elements, is treated with retinoic acid. The cells which accumulate in the regeneration blastema behave as if they were of a more anterior identity. This creates an axial discontinuity, as the differentiated stump cells immediately underneath the blastema are still relatively posterior in phenotype. Blastema cells, consequently, do two things. First, they regenerate perceived missing parts in an anterior-to-posterior manner, but commencing from a more anterior identity due to the influence of retinoic acid. In effect, they recapitulate the formation of structures which already exist more anteriorly. Second, and as a consequence of this resetting of their 'axial address', anteriorized blastemal cells abutting the posteriorly differentiated stump tissues must respond to another discontinuity. Their axial identity is no longer contiguous with the underlying stump cells: they must regenerate, through intercalation, positional values missing between their respective and disparate identities. The tissues which form from these latter interactions are in reverse orientation to the rest of the animal. However, all of this occurs within a very short axial distance, with the result that the reverse orientation limbs formed by intercalation and the normally oriented limbs recapitulated in a distal manner appear in close proximity. These regeneration phenomena might be useful in explaining the puzzling murine knockout phenotypes. In short, a similar set of interactions may operate when segment selector genes are mutated, and cells which normally sit within one positional context are forced to behave in a chronologically aberrant manner, as if they were more anteriorly specified.

Generally, each cluster of murine *Hox* genes expresses in a pattern which delimits unique domains. For example, the 3' *Hox*b complex defines regions in the head which are about two presumptive rhombomeres in length. The genes expressed most rostrally tend to be activated at the earliest phase of gastrulation. One might imagine that mutational inactivation of a locus responsible for specifying one of these domains would cause cells within that domain to respond to developmental cues as if they were more anterior. However, just as two discontinuities were created (and resolved) in the amphibian tail, targeted disruption of a *Hox* gene might be expected to cause discontinuities in axial identity to occur in two places as well: namely at the anterior and posterior boundary of unique expression. For the sake of argument, let us assume that, as with the 3' *Hox*b genes, a certain *Hox* gene normally delimits a unique domain of expression two somites in length. Its anterior expression limit defines one boundary, and the place where its 5' neighbour in the cluster commences expression defines the next. Although we might disrupt this gene, the tissues anterior to its normal expression domain are

presumably specified in normal fashion by the previous *Hox* gene in the cluster. Segmentation and regression of Hensen's node continue, however, and paraxial mesoderm becomes

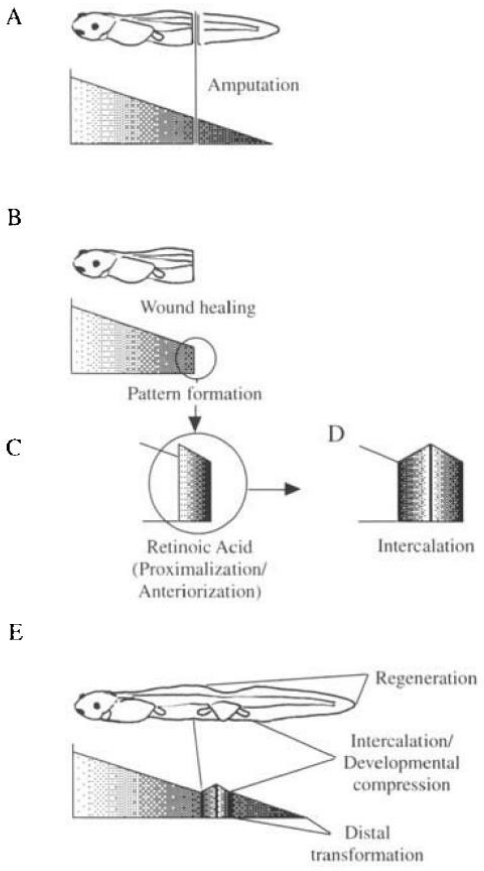

Fig. 2. Intercalation and distal transformation following amputation and subsequent treatment of amphibian tails with retinoic acid. Modified from ref. 24, this diagram illustrates how discontinuities in positional information along the dorsal axis can be created by treatment of a regeneration blastema following treatment with retinoic acid. Amputation of a tail causes loss of posterior positional information represented by a hypothetical concentration curve (A and B). Treatment with retinoic acid (C) proximalizes (anteriorizes) the positional identity of cells at the amputation plane so that a discontinuity is created. When this discrepancy is smoothed, positional information is intercalated by the shortest possible route, resulting in the production of a gradient of reverse orientation (D). The biological effect of this is to cause a regenerating tail blastema to recapitulate structures in an antipodal fashion (E). In tadpoles, the mirror image duplicated region is compressed developmentally, yielding structures which contain anteriorly and posteriorly oriented supernumerary limbs in close proximity.

entrained to form somites, but now in the absence of the mutated *Hox* gene cue. We might expect that the next two somites which form will remain, therefore, under the influence of the gene previously expressed. In essence, they begin to recapitulate the characteristics which defined the previous two somites. As this region undergoes the initial phase of differentiation, it becomes apparent that a discontinuity exists where the anterior zone of the recapitulated axial mesoderm abuts the posterior margin of the previously specified somites. This necessitates the first instance of regeneration of positional information by intercalation. Intercalation of the values missing at this discontinuity would induce these cells to differentiate into more anterior phenotypes. Then, when the next *Hox* gene in the cluster is activated, these forming somites are confronted with a second urgent cue to differentiate, but this time into tissues very much more posterior to that which they are competent to achieve in short order (Fig. 3). Cells at the posterior end of the respecified region would be far too anterior relative to their more normally specified posterior neighbours. Again by intercalation, cells must transform, but this time to more posterior lineages.

So a mechanism may exist whereby a single *Hox* cluster expression discontinuity emerging within an improper context might give rise to antipodal transformations. If there is sufficient time for cells in the disrupted region to regenerate missing positional attributes, no mutant phenotype need necessarily be obvious. Conflicts in specification of axial coordinates will be rectified before morphological differentiation commences. If there is insufficient time, however,

cells will be caught midway through the regeneration process, and positional values or attributes achieved up to that point will become fixed. This may explain results seen in the *Hox*d-4 mutants. Here, although the neural arches of C1 appear to undergo mirror-image duplication, other aspects of vertebral orientation appear normal. In the instance of other mutated *Hox* genes, the timing of wild-type gene expression and the entrainment of axial mesoderm to form somites would combine to determine whether regenerated positional information might lend vertebrae the appearance of having been only partially transformed to an anterior identity. In addition, just as tadpole tail coordinates appear to be intercalated within a very short axial distance, intercalary somitic specification and differentiation might also be compressed into a short region. However, there are at least two instances where the 'transformation' is unequivocally a mirror image duplication: disruption of *Hox*d-4 causes the formation of two neural arches arising in a splayed array from C1 (17); and mutational inactivation of *Hox*a-2 leads to symmetrical duplications in the structures comprising the middle ear (3).

Unfortunately, intercalation alone does not explain why some antipodal or reiterative transformations occur six or seven somites apart. To understand this, it may be helpful to review the temporal features of somite formation.

Periodic reiteration of developmental anomalies

The appearance of periodic vertebral (segment) anomalies has engendered curiously little discussion in the literature. The phenotype is unexpected and not immediately transparent to a simple analysis. There are several other instances, however, of developmental defects that arise in the axial skeleton and which have a period of 6 to 7 vertebrae.

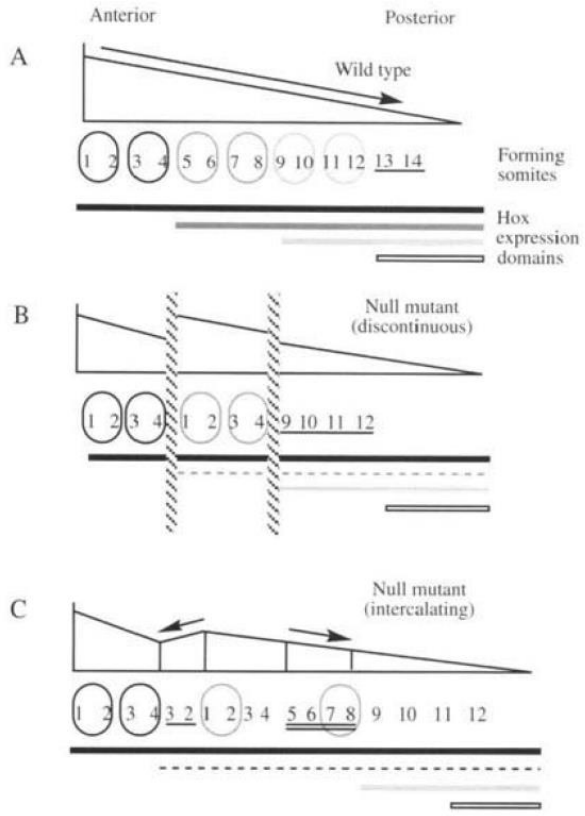

Fig. 3. *Hox* gene perturbation might result in positional identity discontinuities, which must be resolved by intercalation and distal transformation. (A) Different *Hox* genes are transcribed in overlapping domains to specify discrete zones of expression (shaded bars). Somites are first specified and then differentiated in an anterior-to-posterior manner. One of each somite pair is represented here with numbers inside denoting positional coordinates (1 is anterior, 12 is more posterior, etc.). Underlined numbers denote regions undergoing positional specification as distinct from morphological differentiation. A gradient of positional information established by *Hox* genes is represented above the somites in arbitrary units. What comprises this gradient remains unknown. (B) The mutational inactivation of one of the *Hox* genes (dashed line) initially results in a reiteration of positional information since, though axial specification is aberrant, segmentation presumably continues. Specification, however, repeats, using information established by the more anteriorly expressing, intact *Hox* gene. The anterior edge of one 'respecified' somite abuts the posterior edge of the previously formed somite, which is of a more posterior axial value. A discontinuity (represented by cross-hatching) is formed which must be smoothed by intercalation. Similarly, a more posterior discontinuity is also created and must be

smoothed. (C) When positional information is intercalated (underscored numbers), two new zones arise, one of which is of reverse orientation to the normal axial progression of coordinates (arrows). The anterior discontinuity is resolved through creation of a mirror image duplication of variable phenotypic penetrance. The posterior discontinuity may be resolved within the same cell cycle (doubly underscored numbers), resulting in an posterior differentiation pattern normal to external appearances. However, if this perturbation is severe, or the next *Hox* gene to impinge upon the pacesetting cells arrives late in the cell cycle, a discontinuity might not be resolved within that pre-somite and cells will have to wait for one cell cycle (or seven somites) to complete the intercalation. This would yield reiterated vertebral perturbations. Cells fated to contribute to the next somite presumably will have more time to smooth positional discontinuities and will ultimately give rise to more contextually appropriate axial morphologies. Although the diagram represents the rectification of discontinuities over a chronological and spatial distance on the order of somites, pattern respecification might be directed by only a few 'pioneer cells' at the anteriormost boundary of the aberrant specification domain. Consequently, intercalation in both directions might be accomplished in very short order, as evidently occurs in tadpole tail regenerates.

Some examples include: vertebral dismorphogenesis in heat shocked chick embryos (25), somite anomalies in notch-1 mutant mice (R. Conlon, personal communication) and the truncation of structures in some *brachyury* mutants (26). Moreover, in mammals, major body zones comprise six or seven vertebrae: there are seven cervical vertebrae, seven thoracic vertebrae that have ribs attached to the sternum, six that have unattached ribs, six lumbar vertebrae and finally four sacral vertebrae. Clearly the period length changes in the sacral region, possibly because caudal development is under the influence of a different kind of organizing activity. What is it about somitogenesis which constrains development to a 6-7-somite period in all of these instances?

Cell cycles and delayed manifestations of positional identity: a model

Segmentation in both Drosophila and vertebrates occurs independently of *Hox/HOM* gene activity (27). In vertebrate embryos, paraxial mesoderm emerging from the node is rapidly entrained to form epithelialized somites. Only a narrow developmental window will be open during which segmenting mesoderm can be specified, as witness the tendency of explants of presegmental chick cervical mesoderm to differentiate cervical vertebrae when transplanted to a thoracic domain (28). Rectification of positional discontinuities is liable to consume precious time. This is going to be particularly problematic if cells undergoing (re)specification are required to progress through a stereotypical series of steps before arriving at the appropriate end point. In vitro, even the *Hox* clusters themselves appear to pass through a sequential series of gene activations before achieving a state appropriate to specific axial levels, developmental times, or retinoid concentrations (2, 29, 30). Cells situated near axial discontinuities are not necessarily going to have sufficient time to regenerate positional information or to attain competence to respond to cues perceived to be contextually aberrant. An axial address might be partially or completely regenerated, but subsequent differentiative events might have to be postponed for one cell cycle. A respecification event might, for example, posteriorize a sub-population of cells, but they might not achieve the competence to differentiate immediately. The result? The differentiative step is delayed until the appropriate context arises for expression of a more posterior characteristic. A simple hypothesis is that completion of axial specification or differentiation might be postponed until the next cell cycle. Lineage analysis of cells emerging from Hensen’s node in chick demonstrates that clonal clusters are deposited along the axis with a cell cycle period equivalent to

6 or 7 somites (25). If a similar periodicity is present during mouse embryogenesis, this could explain the 6 or 7 somite/vertebrate periodicity of *Hox* mutant transformations. There are other examples of clonal periodicity in development which strongly support a role for this phenomenon

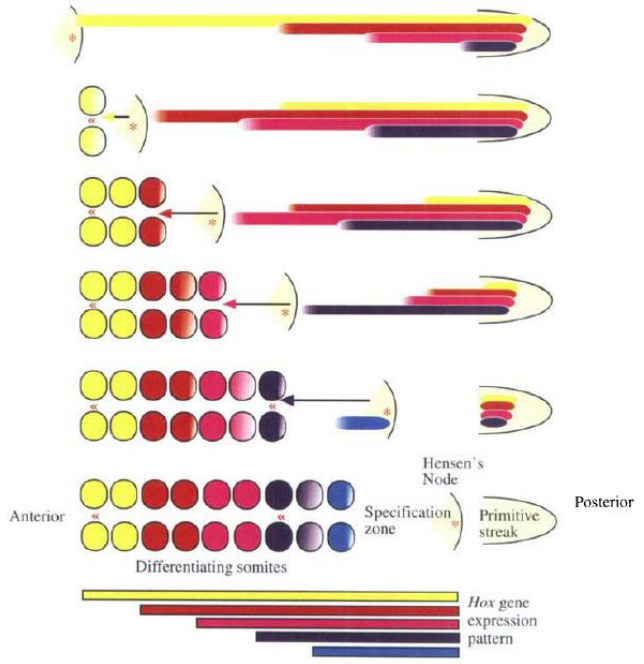

Fig. 4. Sequentially expressed *Hox* gene domains are represented in different colours as they arise from the posterior primitive streak. When their domains of expression intersect with the node two things occur: (1) expression in the posterior domain diminishes; and (2) new expression commences in the cells which have just passed through the node. Presumably under the influence of paralogs and orthologs, *Hox* genes might commence expression directly in the node (light blue domain). Cells, cued by a given *Hox* gene, are deposited by the node, and are entrained to form somites (coloured arrows). Whenever a more 5' gene is expressed, it dominates the developmental agenda and directs morphogenesis in the somites which are forming. As a consequence, the dorsal axis acts as if it has been subdivided into regions uniquely specified by different *Hox* genes (coloured somites). When cells in the early stages of specification are perturbed (red asterisk), their attempts to rectify anomalies are constrained by the rapid rate of cell division and somite epithelialization. Sometimes, partial pattern respecifications or transformations are 'fixed' (anterior double red arrowheads) and cannot be completed until one cell cycle later. In this case, a reiteration of the anomaly occurs a developmental distance of seven somites later (posterior double red arrowheads).

in cellular morphogenesis (31-33) Given the division of the mouse trunk into regions approximately 6-7 somites/vertebrae long, the hypothesis begins to enter the realm of possibility. Additionally, if this resetting of axial address is effected by changes to a cell sub-population, and these changes persist, then the segment identity perturbation might be reiterated for more than one cell cycle. Consequently, segmental anomalies would be expected to recur with a periodicity determined by cell cycle length: once every six or seven somites as Hensen's node regresses along the dorsal axis. Posterior Another noteworthy point arises in the cases where *Hox* gene inactivation results in aberrant morphologies 6 or 7 vertebrae posterior to the first (expected) anomaly: the transformations which occur are contextually appropriate. In other words, when *Hox*c-8 disruption produces a T8 to T7 transformation, the second anomaly six vertebrae later at L1 does not also exhibit characteristics of an L1 to T7 transformation, but is transformed into a morphology appropriate to one position more anterior, namely into that of T13. We infer from these sorts of transformations that the action of specific *Hox*genes is to specify not absolute vertebral identity, but relative axial position. Furthermore, segment respecification in this manner would entail limits that are imposed by the duration of periods of cellular competence and cell cycle times.

An important attribute of *Hox* gene activity must lie in the precise timing of their expression in Hensen's node. An interesting corollary to this hypothesis is that the *Hox* genes play the relatively prosaic role of time-keeper, and define not what specific type of segment can form, but when a generic type of segment posteriorization can occur.

Possibly, *Hox* genes act to trigger a change in morphology, but do not specify the identity of that segment perse. The fascinating results from the studies by Kessel and Gruss (34,35) also might be re-interpreted in this light. In these studies, administration of retinoic acid to pregnant mice resulted in progeny exhibiting periodic vertebral transformations. Retinoic acid administered early during dorsal axis formation transformed vertebrae anteriorly, while administration late during axis formation transformed elements posteriorly. These investigators interpreted their results in the context of a spatial respecification of patterns of *Hox* gene expression. An alternative explanation is that retinoic acid induces a temporal respecification: earlier-expressing 3 *Hox* genes are more easily induced by retinoic acid than the later expressing 5' When retinoids retard the initial stages of gastrulation, they simultaneously alter the timing of sequential *Hox* gene activation. In effect, two timed processes are thrown out of conjunction. Treatment with retinoic acid early during gastrulation will speed up the rate of 3' *Hox* gene activations relative to segmentation, resulting in anterior transformations. Treatment later in development will slow gastrulation relative to the activation of retinoid-resistant 5' *Hox* genes. Segments will consequently be specified in an aberrantly posterior manner. An apparent alteration in somite/ganglia *Hox* expression domain registry in retinoic-acid-treated embryos (34) tends to support the notion of heterochronic effects. Furthermore, retinoids may also directly affect cell cycle rates. For example, retinoids affect the activity of an intrinsic cell-cycle-associated clock during rat oligodendrocyte differentiation (36). Cell cycle perturbations may also be indicated by the generation of periodic segmental anomalies in chick embryos exposed to heat shock during gastrulation (25).

Implications of the model

Parts of the model outlined here have been touched upon by several different investigators (3,4,24,37). This, however, is the first time that all of the elements have been brought together and used to explain morphological anomalies following targeted disruption of *Hox* genes within the context of an intercalation It is important to bear in mind that, in whatever manner it occurs, segment specification is accomplished in a progressive manner as Hensen's node progresses posteriorly along the presumptive dorsal axis. Critical to this aspect of the model is an assumption that routes of differentiation open to cells are emergent properties of the system. In other words, cells and tissues will only reach their 'end state' of differentiation after passage through a stereotypical progression of steps. This occurs as a consequence of other features which arise during gastrulation and which impinge upon the node, for instance the tendency of presumptive notochordal and somitic cells to deposit clonal clusters with a periodicity equivalent to 1.5-2 and 6-7 somites respectively (38,25). Another might involve the waves of *Hox* gene activity which are sequentially propagated from the posterior primitive streak to reach the node at intervals as it makes its way posteriorly. As any given somite (or for that matter notochordal) cell is liable to have relatives spaced with regularity along the dorsal axis, morphological elements are likely to be defined by two temporal

considerations: namely, which clonal cluster was the first to be influenced by *Hox* gene activation (and thereby became the first to set the pace for subsequent patterns of differentiation); and when a new *Hox* gene is activated, which pre-somitic cells are in a state to be receptive to this cue, and for how long (see Fig. 4).

The morphologies which arise in the *Hox* mutant mice are consistent with a model in which axis specification arises as *Hox* genes are sequentially activated in cells in Hensen's node. In some cases the timing of this expression might be a function of when posterior expression domains expand anteriorly to meet the node. The cells in the node continue to express the genes as the node regresses caudally. The node cells which first express a gene set the agenda for subsequent local differentiation. In effect, they act to specify neighbouring cells within that cohort as they are entrained to epithelialize during somitogenesis. This specification is initially generic in nature, in the sense that *Hox* genes induce differentiation of structures which are one increment more posterior than exists already.

Several general observations and predictions arise from this model that have particular bearing upon how *Hox* gene disruptions are liable to affect subsequent patterns of morphogenesis. Firstly, if regarded as disruptions that must be surmounted by regeneration of identity by intercalation, then zones lacking in normal *Hox* gene expression patterns are liable to have to contend with discontinuities at two faces: the plane where the 'disrupted' zone abuts the normally specified anterior zone, and the plane where it must jump to meet patterns of differentiation set in motion by the next *Hox*gene activated. Presumably, the morphogenetic machinery set in motion by different *Hox* genes might lead to effects of greater or lesser expressivity and persistence, depending upon the degree of functional redundancy that can be accommodated by remaining paralogues. The time permitted for intercalation will also have important bearing upon which potential morphology gets 'fixed' at a disruption border.

Secondly, if anomalies are reiterated, or cover a range of vertebrae, then maximally expressive phenotypes will exhibit a period of 6 to 7 somites/vertebrae. The rectification of anterior domain discontinuities by intercalation might persist over several cell cycles, with the result that segment identity problems are reiterated with a periodicity of 6-7 somites. Similarly, a discontinuity at the posterior boundary demands a degree of competence which might be unattainable by cells in this region if they have not had time to pass through the requisite steps. Differentiative events are postponed one cycle although the developmental 'clock' has been set one increment forward.

Finally, the model invokes a degree of communication between different cell types. Cells expressing *Hox* genes, and cells of the presumptive somitic and notochordal mesoderm and the neural tube, may both play a role in timing and demarcating the progression of developmental decisions. Since it seems likely that a degree of functional overlap occurs between these Compartments, then combinations of null mutants affecting both *Hox* genes and the communication between those compartments should prove

catastrophic to the embryo. As the model also implies a degree of overlapping responsibility between cell cycle rates and patterns of *Hox* gene expression, then impaired function in either compartment should still permit rudimentary morphologies to develop. The phenotype of *HNF3ß* mutants may attest to this: in some instances, discernible head and trunk regions are elaborated in the absence of a node or notochord (39). From these results one might predict that embryos homozygous null for an entire *Hox* cluster would nevertheless achieve some semblance of axial differentiation.

Several specific predictions devolve from the observation that *Hox* mutant mice respond to pattern discontinuities in a regenerative manner. Currently, intercalation is regarded as a response to positional confrontations: confrontations have been proposed to play a role in maintaining cellular proliferation rates in normally developing embryos, as well as in regenerating systems (40). If this is true, then inactivation of orthologous, or chronologically offset paralogous *Hox* genes should have the effect of inhibiting the development of structures within their normal domains of expression. Certainly, this has recently appeared to be the case, as double-mutant mice appear to lose structures in a gene dose-dependent manner (41). However, if double mutants are derived in which the loci disrupted are normally close in chronological order of expression, then repetition of ‘anteriorized’ morphologies might ensue - cells would have an incrementally longer period of adjustment, and so intermediate morphologies would be prevalent over longer axial distances. Discontinuities at the posterior boundary of the domain of unique expression would, as before, have to wait one cell cycle to be corrected, and presumably will be more severe (less phenotypically ambiguous and more stereotypically posteriorized). Moreover they might be expected to be more prone to undergo periodic reiteration, since the discontinuity to be bridged is a large one to remedy in one step.

Recent experiments by Gaunt and Strachan (5) would be worth following further, particularly with regard to temporal aspects of *Hox* gene expression. Specifically, if the node receives cues in sequence from anteriorly expanding domains of *Hox* expression originating from the streak, then a glass microbarrier interposed between the primitive streak and the node could be useful in discriminating between two possibilities. First, the experiment would inform us whether or not waves of *Hox* gene activation are due to intercellular communication or to genetic cascades set in motion early in development. Second, it would disclose whether or not the node is dependent upon these cues for subsequent expression patterns and development. As it is, our present understanding suggests merely that pursuant to expansion of the streak expression domain, the node has the ability to sequester cells along the dorsal axis that can autonomously regulate gene expression.

Perhaps the most radical prediction arises from the observation that *Hox*gene mutations appear to cause reiterated anomalies that are contextually appropriate. If *Hox* genes specify relative rather than specific axial co-ordinates, then their activity on somitic cells must be generic in nature. The *when and where* is more important that *what* gene is activated. Within an identical

regulatory context, genes of similar evolutionary derivation should perform in much the same fashion. A way to test this would be to make transgenic mice utilizing constructs which express on null mutant backgrounds. For example, one might expect that ectopically expressed *Hox*a-4 or c-4 transgenes could 'rescue' *Hox*d-4 mutants if the constructs utilized complete *Hox*d-4 regulatory regions. Indeed, perhaps any 3' *Hox* cluster gene could substitute given an appropriate regulatory context. (The presence of a hexapeptide domain in the only the 3' region genes suggests that 5' genes might lack the ability to interact with other proteins such as Pbx which may be involved with 3' genes in pattern formation (43). Nevertheless, the same predictions would hold true for substituted function and rescue using 5' cluster genes on a 5' null mutant background.)

The notion of *Hox* genes as regulators of developmental heterochronies is not a new one (37). However, the present model outlines how these genes might play a role in providing generic temporal cues for the relative axial specification of segments. The model also demonstrates how temporal discontinuities might combine to cause anomalies of an antipodal or repetitive nature. The molecular nature of these cues remains obscure. However, Duboule's speculations that *Hox* genes control patterns of cellular proliferation are consistent with a temporal model (44). Indeed, Bryant and Gardiner's conception of pattern formation following regeneration by intercalation explicitly links discontinuities, in their words 'positional confrontations', with growth control. It is amusing to entertain the possibility that, like the progesterone receptor (45). Hox proteins modulate chromatin structure independently of the role they play as transcriptional activators. We can imagine a scenario in which Hox proteins render domains of chromatin accessible to transcription factors, in a sense opening genetic regulatory modules which are critical to growth and development. The manner in which *Hox* genes themselves are arrayed, activate and, possibly, interact, supports this possibility.

The *Hox* genes do not perform their respective functions in isolation from other factors, genetic or epigenetic. Documented cell cycle characteristics of pre-somitic mesoderm may be involved in the 6-7-somite periodicity seen in *Hox* gene, and *brachyury* mutations. The combined activity of these genes, and the synchronous division of presomitic mesoderm cell sub-populations, might both be necessary to invoke the conditions required to specify and differentiate vertebral identity.

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