Development 112, 669-678 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

Gradient fields and homeobox genes

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Summary

We review here old experiments that defined the existence of morphogenetic gradient fields in vertebrate embryos. The rather abstract idea of cell fields of organ-forming potential has become less popular among modern developmental and molecular biologists. Results obtained with antibodies directed against homeodomain proteins suggest that gradient fields may indeed be visualized at the level of individual regulatory molecules in vertebrate embryos.

Key words: homeobox, gradients, embryonic fields, *Xenopus laevis*, zebrafish embryos, limb development, feather development.

Introduction

The purpose of this essay is to increase awareness among modern developmental biologists of the old concepts of morphogenetic gradient fields. Fig. 1 shows a 1934 view of the amphibian neurula. Experimental embryology revealed the existence of fields of organ-forming potential at these very early stages, which precede any signs of overt differentiation. By transplanting newt embryo fragments into heterotopic positions, fields of cells that were able to give rise, at later stages, to various organs such as forelimb, hindlimb, tail, balancers and gills were identified. Cells in these 'morphogenetic fields' have the interesting property that they can 'regulate', i.e. produce a normal structure, after a number of surgical manipulations. For example, if part of the field is excised, or if a fragment of uncommitted tissue is introduced within it, a normal structure is still formed. If the field is divided into multiple fragments, multiple copies of the whole structure ensue. Entire books relate these observations (Huxley and de Beer, 1934; Weiss, 1939; Child, 1941). In particular, 'The Elements of Experimental Embryology' by Huxley and de Beer contains a lode of information and is highly recommended.

In the 1940s embryonic fields gradually lost center stage. Perhaps it was because they were considered to be the result of abstract, almost metaphysical, morphogenetic forces that could only be revealed after transplantation. The same set of properties can also be explained by graded positional information models, which have attracted wide attention (Wolpert, 1969, 1989). Another important advance in the analysis of cell fields undergoing differentiation was the proposal of the polar coordinate model (French *et al.* 1976) in which a circular and a radial set of positional information can explain, without invoking gradients, the regulation events observed after manipulation of a field.

The advent of new molecular markers has now made it possible to follow visually fields of embryonic cells that give rise to organs in later development. It may be useful to review some of the properties of embryonic fields in this light.

Fields in the embryo

The concept of morphogenetic fields originated from Ross Harrison's (1918) studies on newt forelimb development. He showed that at the early neurula stage a disc of cells in the mesodermal mantle (also called lateral plate or somatopleure) had acquired the potential to form a forelimb bud when transplanted into a different region of the embryo. Although the lateral plate mesoderm at this stage consists of an entirely uniform layer, the region giving rise to the forelimb occupies a very precise location, ventral to the third and fifth somites (Harrison, 1918; Stocum and Fallon, 1982). Fig. 2 shows the position of the circular forelimb field in an *Ambystoma maculatum* neurula, which is the same material employed by Harrison, except that in his day this American newt was called *Amblystoma punctatum*. When mesodermal cells from this region are implanted through a slit in the skin ectoderm into a more posterior site (Fig. 2), an additional forelimb, indicated by an arrowhead in Fig. 3A, grows from the flank at the swimming larva stage.

The forelimb field has very interesting properties. After complete removal of the central disc of cells that would normally give rise to the limb, the surrounding mesodermal cells retain the potency to regenerate the lost information, producing a complete forelimb. In the words of Harrison (1918): 'around the limb-forming

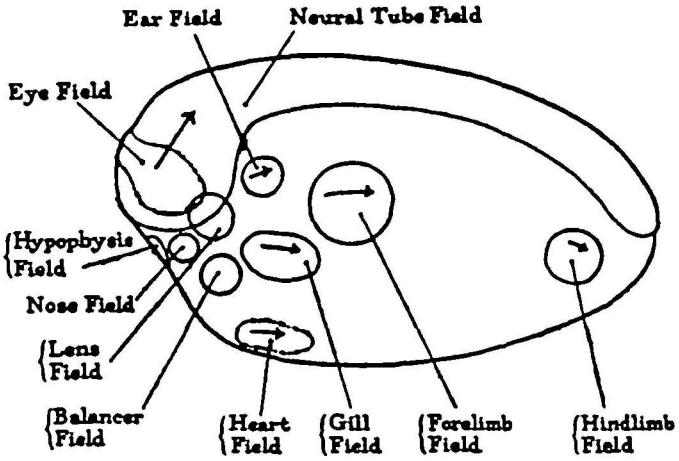

Fig. 1. Diagram of an amphibian neurula showing the localization of the main morphogenetic fields discovered by experimental analysis. Reproduced from Huxley and de Beer, 1934, with permission of Cambridge University Press.

cells there is thus a zone of tissue which has the power, in gradually diminishing intensity towards the periphery, to form a limb vicariously'. If a limb field is cut into two halves and transplanted elsewhere, two perfect limbs are obtained. Conversely, a single limb can be obtained from two half-fields grafted in the correct orientation. Harrison called this a 'self-differentiating equipotential system', in which each part can give rise to any part. The term 'field' was coined later to account for these properties, first by Spemann (1921) to describe the region of organizer activity present on the dorsal side of the amphibian gastrula, and then by Paul Weiss

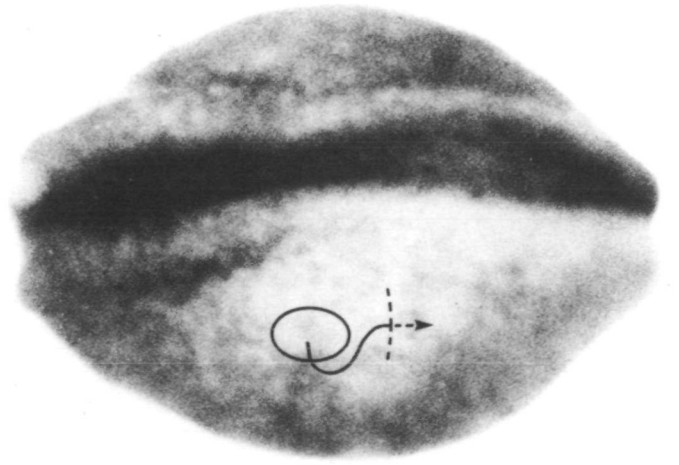

Fig. 2. Harrison's limb field transplantation experiment. A photograph of an *Ambystoma maculatum* neurula is shown. At this stage the potency to form forelimb is located in a circular region of the lateral plate mesoderm just ventral and posterior to the pronephros. If this mesoderm is surgically removed and inserted through a slit in the ectoderm into a more posterior region (arrow), an extra forelimb will grow at the site of the transplant (shown in Fig. 3A). Photo courtesy of Christopher Wright.

Fig. 3. Forelimb and pectoral fin fields in newt and fish embryos. (A) *A. maculatum* tadpole resulting from a limb field transplantation at the neurula stage (Fig. 2); note the supernumerary forelimb bud indicated by the arrowhead. The balancer (bal.) and external gills are indicated. (B–D) Zebrafish embryos stained with anti-XlHbox 1 antibodies (photos courtesy of Anders Molven). (B) Low magnification view of a 19 h embryo, the arrow indicates a circular area of the lateral plate mesodermal mantle in which nuclei contain XlHbox 1 antigen. (C) High power magnification of B, showing that the cells that will give rise to the future pectoral fin express XlHbox 1 antigen. (D) Section of a pectoral fin bud of a 48 h zebrafish embryo, XlHbox 1 protein is expressed in the anterior and proximal mesoderm as well as in ectodermal skin; this staining pattern is very similar to that found in the tetrapod forelimb.

to explain observations concerning regeneration and the formation of organ rudiments (reviewed by Weiss, 1939). Detailed transplantation studies showed that the maximal limb-forming potency is located in the anterodorsal region of the forelimb field and gradually decreases away from this point (Swett, 1923). This led to the view that each field consisted of a gradient of organ-forming potential, i.e. a 'gradient-field' (Huxley and de Beer, 1934).

A recent example of the power of regulation of the limb field was provided by tadpoles from a particular pond in Northern California which had supernumerary legs (Sessions and Ruth, 1990). Both tree frog (Hyla) and salamander tadpoles were afflicted. It was shown that the malformations were due to parasitic flatworms (trematodes) which burrowed into the developing limb buds, subdividing them into multiple regions. In some cases a single limb bud gave rise to five well-formed legs (Sessions and Ruth, 1990).

Gradient fields in the adult

The existence of gradient fields can also be revealed in adult organisms which are capable of regeneration. We shall consider here only two cases: the section of planarians in half and the consequence of nerve deflections in adult newts.

The experiments with planarians are discussed here because they provide evidence for positional information of a *graded* nature along the main body axis. If planarians are cut transversely, as is well known, the front end will regenerate a tail and the hind piece a head. This is also true if two different animals are sectioned along the closely located planes indicated as *a* and *b* in Fig. 4. The cells located between *a* and *b* will proliferate and in one case will form a head and in the other a tail. Because the cells that proliferate in both cases are essentially the same (Fig. 4), the formation of head or tail does not depend on the type of cell present in the wound, but rather on their relationship to the anteroposterior (A–P) axis of the rest of the embryo. This differs somewhat from the limb field transplantation experiments described earlier, in which the cells


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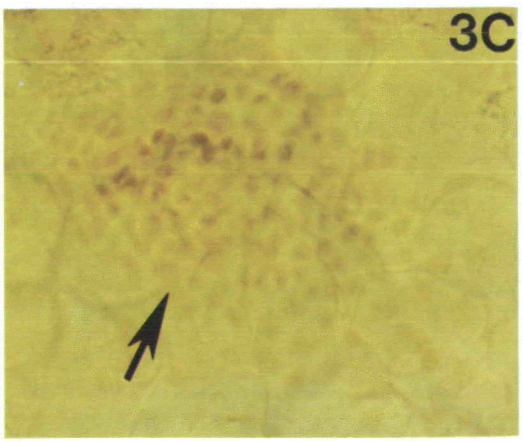
bal
gills


3B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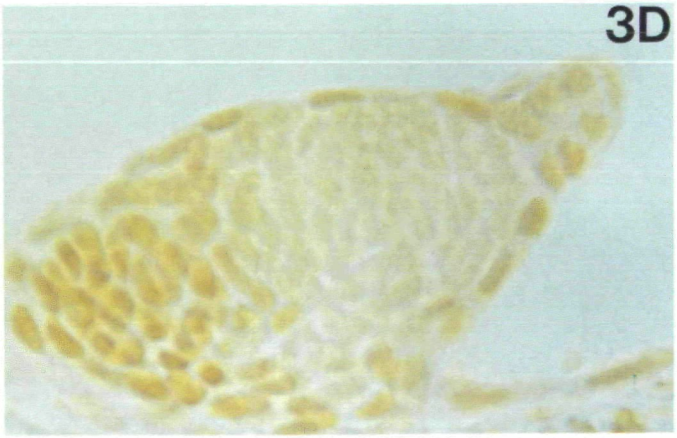
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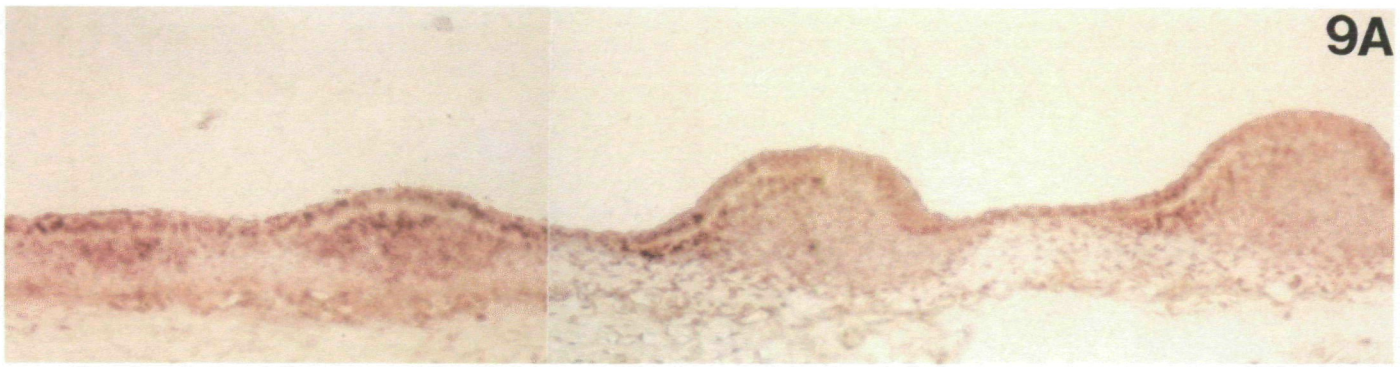
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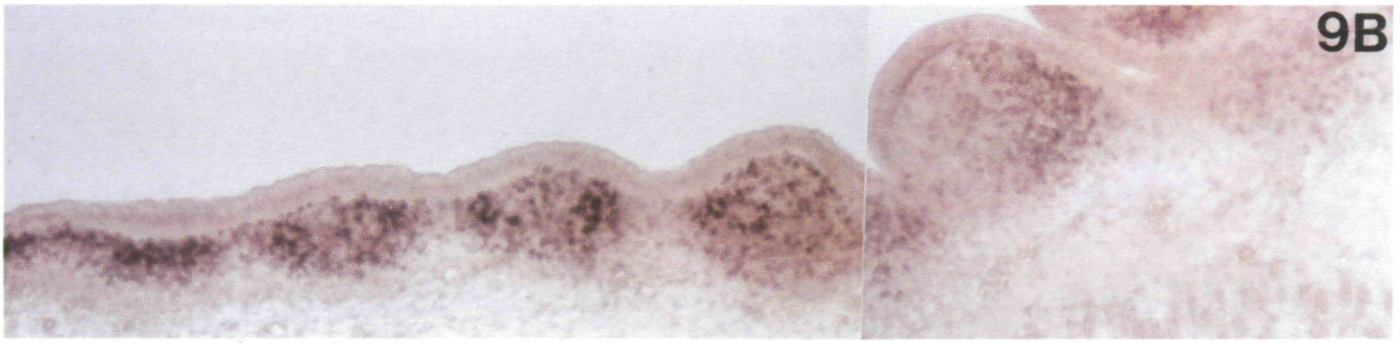
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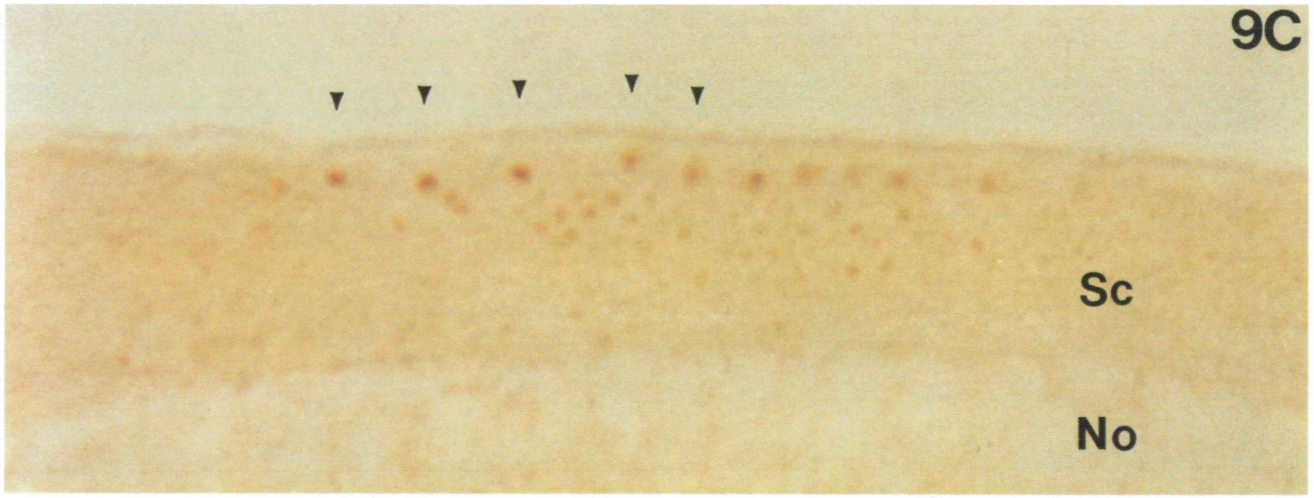
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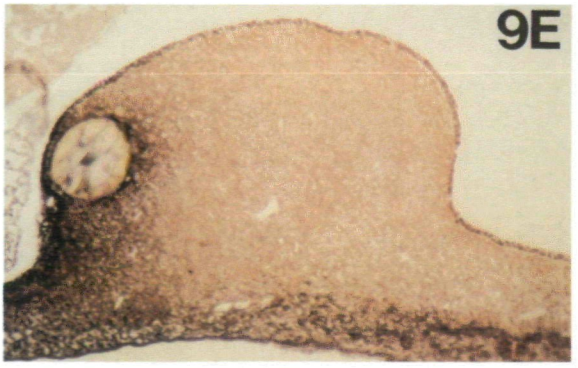
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Fig. 9. Gradients of homeodomain protein expression. Anterior is always to the left and posterior to the right. (A) Formation of a gradient of XlHbox 1 protein during feather development in a day 8 chicken embryo. Development proceeds from left to right. Initially a patch of mesodermal cells starts expressing the homeodomain protein before any morphological changes are detectable. As the feather bud begins to grow, XlHbox 1 antigen loses its homogeneous staining of the feather field and forms a gradient of nuclear staining in mesoderm, with a maximum in the anterior and proximal region of the bud. The ectoderm is stained uniformly. (B) Expression of *Hox 4.4* protein in developing feather buds. Development proceeds from left to right. Hox 4.4 initially is present in all mesodermic nuclei, but at later stages becomes localized preferentially in the posterior and distal regions of the developing feather bud. The ectoderm is negative. (C) Sagittal section of a 25 h zebrafish embryo showing a row of Rohon-Beard sensory neurons in the dorsal spinal cord (large nuclei indicated by arrowheads). The cells are separated and arranged in a row. Note that XlHbox 1 staining starts abruptly and gradually decreases in the posterior direction until it fades entirely. Sc, spinal cord; No, notochord. (D) Developing chicken wing bud immunostained with XlHbox 1 antibodies. Mesodermal expression is maximal in the anterior and proximal region. (E) Contralateral wing bud of the same embryo shown in the previous panel, into which a Dowex bead containing retinoic acid was implanted 18 h previously. Note that the intensity and area of XlHbox 1 expression in mesoderm is greatly increased by local treatment with retinoic acid. This plate shows color photographs of observations reported in full elsewhere: Chuong *et al.* 1990 (A and B), Molven *et al.* 1990 (C), and Tickle, 1990 (D and E).

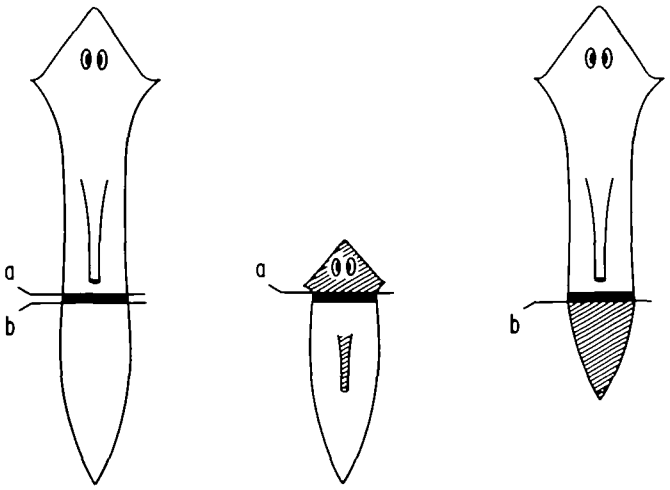

Fig. 4. Regeneration in Planaria. The same set of cells (located between section planes *a* and *b*, indicated in black) can regenerate either a head or a tail (shaded area) after cutting the animal in half. The experiment shows that cells are not predetermined to form head or tail, but rather can sense their relationship to the A–P body axis field. In the animal in the center a new pharynx is regenerated at a distance under the influence of the head regenerate. Drawing based on a paradox discussed by Huxley and de Beer, 1934.

still formed limb after being placed in an ectopic position. The planarian body, however, shares with other fields the capacity to regulate after surgical manipulation.

The rate and completeness of regeneration in Planaria depends on the A–P level of the cut, suggesting the existence of an axial gradient of regeneration potential (Child, 1915, 1941). The percentage of animals able to regenerate a head decreases in a graded way as the transection is carried out in progressively more posterior regions (reviewed by Slack, 1987). Furthermore, this axial gradient behaves like a field, because, if for example deep cuts are introduced into the head region, as shown in Fig. 5, multiple heads are formed. Unfortunately one of the main methods used to study potential gradients in Planaria was that of 'differential susceptibility' (Child, 1941). This would involve, for example, exposing intact or regenerating planarians to various amounts of KCN, strychnine or ethanol, in order to determine which region stopped regenerating or died first (usually the head was more sensitive). These findings were interpreted in terms of 'physiological gradients of metabolic activity' (Child, 1941) and may have contributed to the loss of interest in gradient fields by modern biologists.

Embryonic fields persist in adult urodeles. Their existence can be shown experimentally in *Triturus cristatus* by deflecting nerves from their normal course so that they end in the dermis instead. If a sciatic nerve is deflected to a region close to the base of the leg, a supernumerary hindlimb is induced (Guyenot and Schotte, 1926). (In the newt, hindlimbs can be recognized because they have five digits while forelimbs have only four). However, if the nerve is introduced into the tail region, an extra tail is induced. If the sciatic nerve is deviated into the dorsal crest, a supplementary dorsal crest is induced. Similarly, if the brachial nerve is deflected close to the arm or shoulder, a supernumerary forelimb (with four digits) is induced, as shown in Fig. 6. If the brachial nerve terminus is placed close to the dorsal midline an additional dorsal crest is induced instead, and if placed at a distance from the forelimb

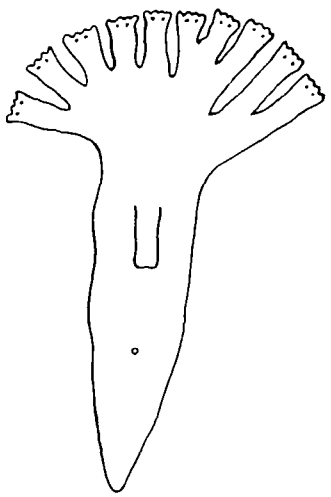

Fig. 5. Regulation in the head field of a Planarian. The anterior end received a number of deep cuts; the animal produced ten heads. Reproduced from Huxley and de Beer, 1934, with permission of Cambridge University Press.

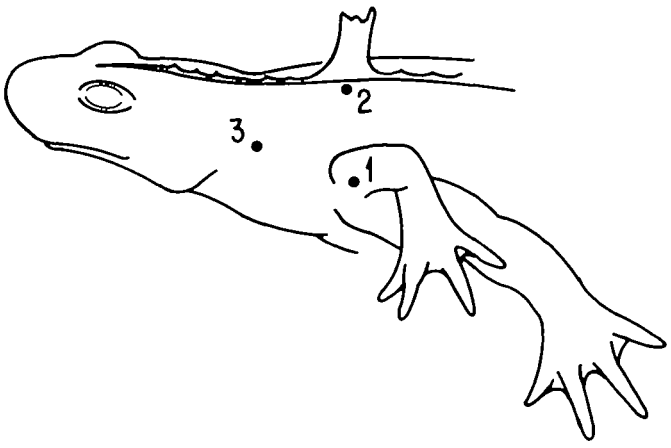

Fig. 6. Nerve deflections in the adult newt can stimulate the growth of new structures. If the brachial nerve of *Triturus cristatus* is cut and deflected so that it now ends close to the base of the forelimb (1), a supernumerary forelimb is induced. If the nerve is placed in the dermis of the dorsal crest (2), an additional dorsal crest is formed. If the nerve is deflected at a distance of the limb (3), it exits the forelimb field and is unable to induce growth of extra structures. After experiments performed by Guyenot *et al.* 1948.

and dorsal crest – outside of their respective fields – it does not induce any growth (see Fig. 6, Guyenot, 1927; Guyenot *et al.* 1948).

In these experiments, the nerve itself is thought to have a non-specific trophic action, its effect depending on the type of mesoderm that it stimulates. The latent potentialities of the mesoderm can only be revealed by experimental manipulation. The conclusion from these studies is that newts contain, even as adults, a forelimb field, a hindlimb field, a dorsal crest field, and a tail field (Guyenot, 1927).

Homeobox genes in vertebrates

Our interest in gradient fields started with the observation that a homeodomain protein, XlHbox 1, was expressed as an A–P gradient in the forelimb of several tetrapods (Oliver *et al.* 1988*a*). XlHbox 1 was the first gene isolated from vertebrate DNA by virtue of its homology to the *Drosophila* homeobox (Carrasco *et al.* 1984). In time we obtained antibody probes that detected the protein products of this *Xenopus laevis* gene (Oliver *et al.* 1988*b*). Importantly, the antibodies also reacted with the homologous protein in a number of other species such as mouse, chick and zebrafish, affording a molecular glimpse into the comparative embryology of vertebrates.

There are about 40 homeobox genes of the *Antennapedia*-type in the genomes of the mouse and most other vertebrates (reviewed by Wright *et al.* 1989*a*; De Robertis *et al.* 1990; Kessel and Gruss, 1990). They encode transcription factors which are expressed in specific A–P regions of the embryo. The genes are located in four clusters of about 10 genes each, with genes located at the 5′ end of the complexes expressed in posterior regions of the embryo and those in more 3′ positions expressed in progressively more anterior regions (Gaunt *et al.* 1988; Graham *et al.* 1989; Wilkinson *et al.* 1989). This genomic organization is strikingly similar to that of *Drosophila* homeotic gene clusters (Lewis, 1978; Gehring, 1987), suggesting that this gene arrangement arose in a common ancestor. Because mammals did not evolve from insects, these common ancestors must go back at least to organisms such as flatworms. It would be interesting to know whether primitive metazoans such as rotifers, which have well-defined A–P polarity, possess homeobox gene complexes.

The function of at least some vertebrate homeobox genes is to specify cell identity along the A–P axis. Both loss-of-function (obtained by microinjection of antibodies into *Xenopus* embryos, Wright *et al.* 1989*b*) and gain-of-function (obtained by overexpression in transgenic mice, Kessel *et al.* 1990) phenotypes suggest that the vertebrate genes have similar functions to their *Drosophila* homeotic counterparts. Furthermore, overexpression of mouse and human homeobox genes in transgenic fruit flies leads to homeotic transformations of cell fate (Malicki *et al.* 1990; McGinnis *et al.* 1990).

A gradient field of homeodomain protein in vertebrate limbs and fins

The XlHbox 1 protein is expressed in a narrow band (or belt) of cell nuclei in the anterior trunk of the *Xenopus* embryo at the tailbud stage. The band comprises mesoderm, anterior spinal cord and neural crest, with the A–P borders of expression quite well aligned between germ layers (De Robertis *et al.* 1989). The forelimb field is located entirely within the region of XlHbox 1 expression in the lateral plate mesoderm (Oliver *et al.* 1988*a*). When forelimbs grow out of this region some three weeks later, we unexpectedly found that the XlHbox 1 protein was distributed as an A–P gradient in the nuclei of an otherwise apparently uniform expanse of forelimb bud mesoderm. The gradient is maximal at the anterior bud and becomes increasingly proximal as the limb grows outward (Oliver *et al.* 1988*a*).

Fig. 7 shows mouse limb buds stained with an antibody prepared against the putative human homolog of XlHbox 1 (called *Hox 3.3*); identical results are obtained with anti-*Xenopus* antibodies. The protein is strongly expressed in mesodermic nuclei in sections of the anterior forelimb bud (panels A and B) but more weakly in posterior ones (panels C and D). Mesodermal expression is not detectable in the hindlimb (panels E to H); therefore XlHbox 1 is a rare example of a gene expressed in the arm but not in the leg mesoderm (Oliver *et al.* 1988*a*). The homologous gene, NvHbox 1, has been isolated in the newt and shown to be strongly induced during forelimb regeneration (Savard *et al.* 1988).

The fact that XlHbox 1 protein is expressed in the lateral plate mesoderm long before the forelimb bud is formed, together with the finding of a protein gradient later in development, led us to suggest that there might be a relationship between homeobox genes and the

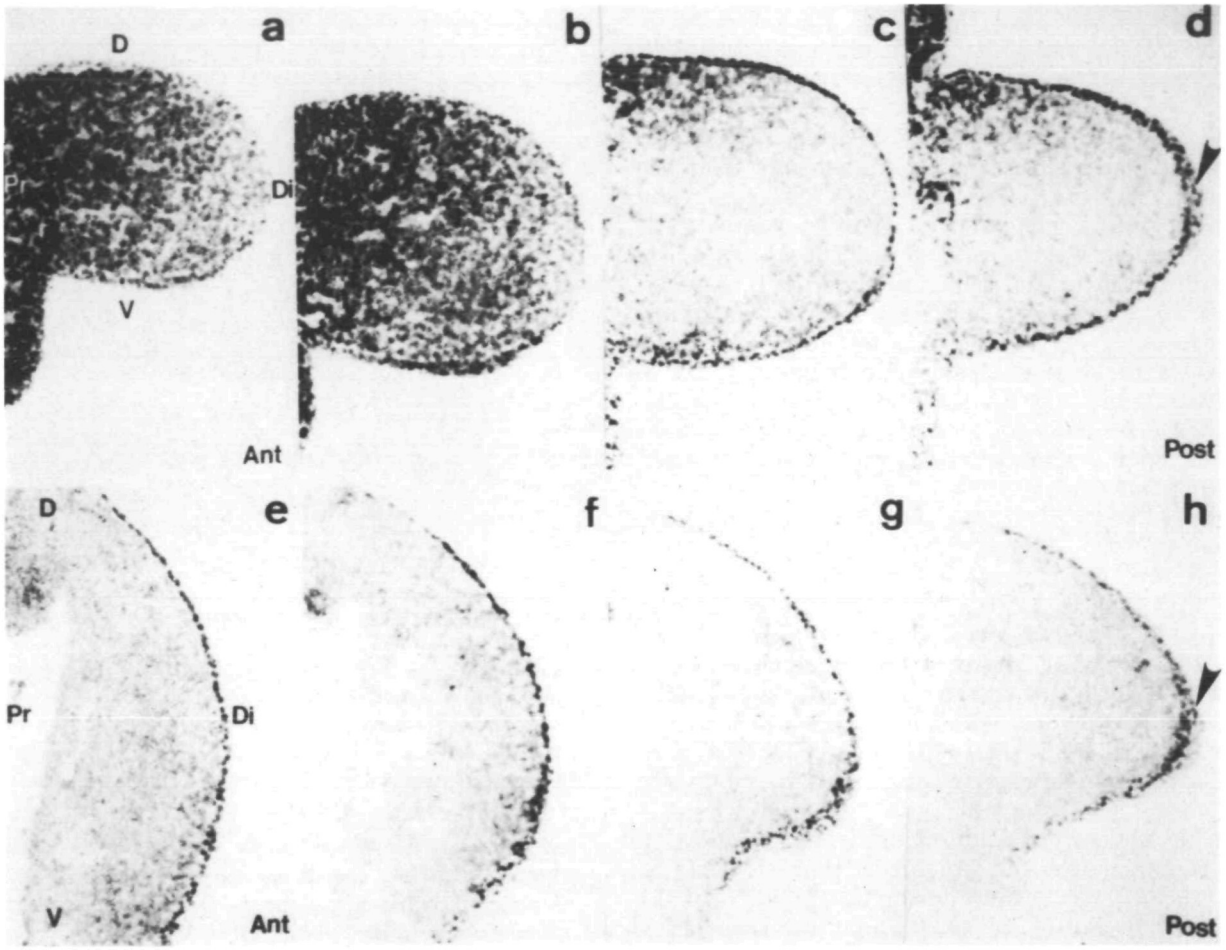

Fig. 7. A gradient of XlHbox 1 antigen is present in nuclei of the anterior mesoderm of developing mouse forelimbs (panels *a* through *d*) but is absent in hindlimbs (panels *e* through *h*). The ectoderm is stained in both fore- and hindlimbs. Transverse sections of a day 10 mouse embryo are shown. D, dorsal; V, ventral; Di, distal; Pr, proximal; Ant., anterior; Post., posterior. Reproduced, with permission, from Oliver *et al. Cell* **55**, 1017–1024 (1988).

gradient fields described by experimental embryologists (Oliver *et al.* 1988*a*). The best indication that this might be the case came from studies on the development of the fish pectoral fin bud (Molven *et al.* 1990). The fish pectoral fin, which is the evolutionary precursor of the tetrapod forelimb, develops by proliferation of a group of cells of the lateral plate mesoderm, as demonstrated initially in the salmon embryo (Harrison, 1895).

The zebrafish embryo is better material than *Xenopus* for these studies because the pectoral fin bud forms early on in embryogenesis, rather than three weeks later as part of the metamorphosis process in the case of the tadpole forelimb. In addition, in the early fish embryo, the lateral plate mesoderm extends as a thin homogenous cell layer that surrounds the yolk, greatly facilitating its study. Figs 3B and 3C show that in the 19 h embryo a circular region of XlHbox 1-positive nuclei can be distinguished in the lateral plate mesoderm (indicated by an arrow). This is about 10 h before the pectoral fin bud itself is morphologically recognizable. This circular patch of cells can be followed throughout development and corresponds to the pectoral fin region (Molven *et al.* 1990). In 25 h embryos staining becomes stronger in the anterior region and cells start to proliferate; by 48 h a well-developed finbud is present. At the latter stage, as shown in Fig. 3D, expression is maximal in the anterior and proximal fin bud. This pattern of expression is very similar to what one would find in, say, frog, chicken or mouse forelimb buds. In addition to pointing to a conservation of basic developmental mechanisms during vertebrate evolution, the zebrafish study suggests that expression of a homeobox gene can demarcate a morphogenetic field (Molven *et al.* 1990).

Many other homeobox genes are also expressed during limb development (Eichele, 1989). An antibody against the *Hox 4.4* gene of human origin detects a protein gradient that has the opposite polarity to that of XlHbox 1 in the forelimb (Oliver *et al.* 1989). The Hox 4.4 gradient is maximal in the distal and posterior region of *Xenopus*, mouse, and chick fore- and hindlimb buds. Although direct proof is still lacking, the opposing gradients of *Hox 4.4* and XlHbox 1 proteins could be involved in specifying positional values in developing limbs.

Local application of retinoic acid to the anterior

region of chick wing buds leads to changes in morphogenesis, and it has been proposed that A–P positional information in the limb bud is provided by a diffusible gradient of retinoic acid (reviewed by Eichele, 1989). Recent experiments suggest that retinoic acid applied to the anterior limb bud does not establish a new retinoic acid gradient throughout the bud, but rather changes the fate of nearby cells (Waneck *et al.* 1991; Noji *et al.* 1991). Thus the existence of a diffusible morphogen gradient has been challenged. The gradients of homeodomain proteins, although not yet proven to affect morphogenesis, strongly argue that graded positional information of some sort must exist in limb buds. Thus it may be worthwhile to study the positional signalling systems that set up the gradients of homeoproteins in limb development.

Duboule and colleagues have shown that following expression of *Hox 4.4*, the three genes located 5′ to it in the chromosome (recently renamed *Hox 4.5*, *4.6* and *4.7*, Duboule *et al.* 1990) are sequentially activated at the tip of the limb as it grows longer (Dolle *et al.* 1989). Thus, the timing of expression of these genes follows the order that they occupy in the gene complex. Hox 4 genes are also expressed along the main body A–P axis, where they are deployed in the same order (*4.4* more anterior than *4.5*, *4.5* more anterior than *4.6*, and so on).

An unexpected conclusion from the studies on homeodomain protein gradients in limbs is that the same set of genes utilized during development of the main body A–P axis are also brought into play during limb growth.

Limb fields in the fruit fly

The early *Drosophila* embryo, which has such tremendous advantages for genetic studies on development, does not lend itself easily to transplantation studies of the type that are possible, say, in Amphibia. It is therefore not surprising that embryonic field conterparts have not been found. However, experiments on regeneration of cockroach legs strongly argue that, at least at later stages in life, insect legs do have the regulatory properties of cell fields (Bohn, 1974; French *et al.* 1976). Recent studies on the gene *Distalless* suggest that fly embryos indeed have fields of cells involved in the formation of appendages.

Mutations in *Distalless* result in the loss of distal leg segments. Collections of alleles have been obtained that result in a graded series of defect severity (Cohen and Jurgens, 1989). The *Distalless* gene encodes a homeodomain protein (Cohen *et al.* 1989). Its homeobox is significantly divergent from the archetypal *Antennapedia* one. In the early embryo, a circular patch of *Distalless*-expressing cells clearly demarcates the position of the future leg, maxillar, labial and antennal appendages, as shown in Fig. 8. The location of these circular fields on the surface of the embryo is precisely controlled. For example, a row of *wingless*-expressing

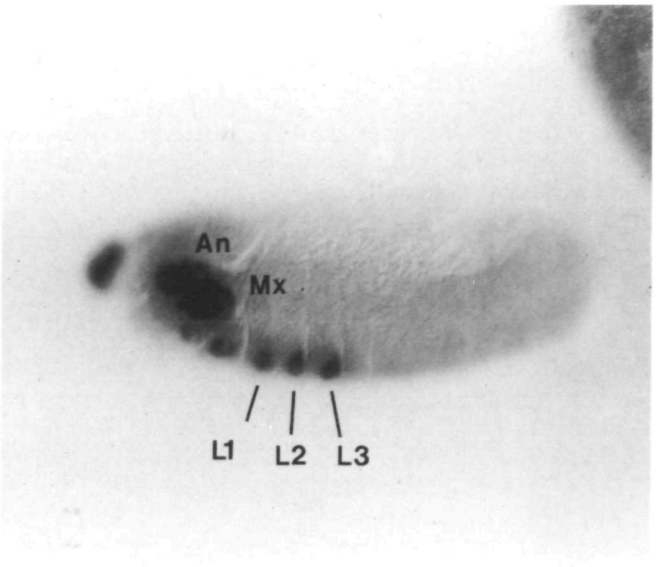

Fig. 8. Expression of *Distalless* mRNA in a *Drosophila* embryo. The whole-mount *in situ* hybridization preparation shows expression of this homeobox gene in a number of segments that give rise to appendages. Note circular patches of expression in the anlagen for legs 1, 2, and 3 (L1, L2, L3), antenna (An), and maxillary process (Mx). Photograph courtesy of Stephen Cohen, HHMI Houston.

cells passes exactly through the center of the leg anlagen. *wingless* expression is necessary for correct *Distalless* activation (Cohen, 1990). Thus, a gene coding for an extracellular, presumably signalling, protein is involved in the correct expression of a field of nuclear transcription factor. The mammalian homolog of *wingless* is the oncogene *int-1* (Rijsewijk *et al.* 1987; McMahon and Moon, 1989). Other genes, such as those of the dorsoventral positional system and those of the Bithorax complex are also involved in the control of *Distalless* expression in leg primordia (Cohen, 1990).

Gradient fields in developing feather buds

In both plants and animals, new organs such as leaves, roots, tentacles and limbs, as well as in some cases entirely new individuals, may develop from local thickenings called 'buds'. When this happens new axial patterns are laid down (Child, 1941), affording an opportunity to study how positional information is specified.

Avian feathers develop from a flat sheet of embryonic skin. Initially a 'field' of mesodermic cells acquires inductive properties, interacting with the epithelial layer, which thickens and becomes the placode epithelium (Sawyer and Fallon, 1983). The mesodermic cells increase in number, producing a feather bud that protrudes on the skin surface. Although feather bud mesoderm is entirely homogeneous by histological analysis, molecular heterogeneities had been noted. Fibronectin is enriched in the posterior (Mauger *et al.* 1982), while N-CAM is concentrated in the anterior mesoderm (Chuong and Edelman, 1985). The similarities between the polarized distribution of N-CAM in

feather buds and the gradient of XlHbox 1 in forelimb buds provided the initial impulse for analyzing the expression of homeodomain proteins in developing feathers.

Fig. 9A shows the expression of XlHbox 1 antigen in feather buds at various stages of development. The first sign of feather formation is the appearance of patches of XlHbox 1-positive cells in the dermis or feather field. Nuclei over the entire field express XlHbox 1 protein, even as a visible bud begins to form. As the buds grow staining is lost in the posterior and XlHbox 1 adopts a graded distribution, with maximal expression in the anterior and proximal region (Chuong *et al.* 1990). Detailed analysis showed that N-CAM expression occupies a smaller region of the anterior feather bud than that of the XlHbox 1 gradient. Fig. 9B shows that *Hox 4.4* is expressed uniformly and very strongly in the initial field and early feather buds. As development proceeds, *Hox 4.4* protein becomes polarized to the posterior and distal mesoderm (Chuong *et al.* 1990). This complementary distribution of the two homeodomain proteins is very reminiscent of the gradients they adopt during the development of an entirely different structure, the forelimb bud (Oliver *et al.* 1989).

Gradients of homeobox expression along the main body axis

Most *in situ* hybridization studies on the expression of mouse homeobox genes in mid-gestation embryos show a graded distribution of mRNA in the spinal cord. The maximum corresponds to the anterior border of expression, with levels usually diminishing posteriorly (e.g. Breier *et al.* 1988). While this is true for total mRNA levels, it has been difficult to extend this to the single cell level. In the zebrafish, there is a population of large sensory neurons called Rohon-Beard neurons. Their function is to process the sensory input from swimming movements and can be easily recognized because of their large size and dorsal position in the spinal cord. Rohon-Beard neurons express XlHbox 1 antigen, and display an interesting pattern (Molven *et al.* 1990). Very anterior ones are devoid of the antigen but, as shown in Fig. 9C, they abruptly start expressing XlHbox 1 within the anterior spinal cord and exhibit a graded decrease in intensity in the posterior direction until expression fades entirely. Thus, although all Rohon-Beard neurons serve the same function, they differentially express XlHbox 1 according to their position along the A–P axis. It is as if the XlHbox 1 gene in these cells were able to sense a gradient of positional information present along the body axis which affects its level of expression.

A gradient of expression in the opposite direction of the body axis exists for another homeobox gene, Xhox3, during early *Xenopus* development (Ruiz i Altaba and Melton, 1989*a*). This gene, which has a homeobox related to *Drosophila evenskipped*, is expressed very early in development, with maximal mRNA levels in the posterior end of mid-neurula embryos. Antibody staining has shown that this gradient is established in embryonic mesoderm and that it fades in the anterior direction (Ruiz i Altaba *et al.* 1991). Both loss- and gain-of-function experiments support the view that this gene plays an important role in A–P axis formation (Ruiz i Altaba and Melton, 1989*b*; Ruiz i Altaba *et al.* 1991).

Thus the main body axis of the vertebrate seems to have A–P gradients of positional information during the course of embryogenesis, which are able to activate homeobox genes.

Spemann's organizer field

A field of organization potential is present on the dorsal side of the early amphibian gastrula (Spemann, 1921; Holtfreter and Hamburger, 1955; Wakahara, 1989; Stewart and Gerhart, 1990). It determines the extent to which cells will invaginate through the blastopore lip and consequently the extent of the future A–P body axis. This dorsal organizer region exhibits many of the properties of a morphogenetic field: if one organizer is divided into several fragments each will lead to the formation of a new body axis after transplantation, part of the organizer field can be removed and a well-proportioned axial system can still be formed, uncommitted embryonic cells grafted into the dorsal lip can become part of the organizer, two organizer fields can be fused to form a single axial system (Spemann, 1938; Holtfreter and Hamburger, 1955).

The generation of the organizer field has been the subject of intense investigation. It can be traced back to fertilization, which elicits a rotation movement of the egg cortex (reviewed by Gerhart *et al.* 1989). The future dorsal side forms usually on the opposite side of the sperm entry point, where the cortical rotation brings large yolk platelets and animal pole cytoplasm into close contact. By the 32-cell stage, the two most dorsal and vegetal blastomeres acquire the potential to induce other cells. This 'Nieuwkoop center' (Gerhart *et al.* 1989) is thought to release growth factors of the TGF-β family, inducing 'Spemann's organizer' (or, in other words, anterodorsal mesoderm) activity in overlying cells (Smith *et al.* 1989; Thomsen *et al.* 1990). If the size of the organizer is decreased by surgical removal at the late blastula stage, tadpoles with a graded series of anterior axial defects are obtained (Stewart and Gerhart, 1990).

The organizer is the morphogenetic field most amenable to molecular analysis, at least in amphibians. While interesting patterns of antibody stainings suggest a correlation between homeodomain proteins and gradient fields, there is no direct evidence as yet that this has a causal effect in morphogenesis. In the case of the organizer it is known that growth factors are involved in its generation. It is also known that growth factors can activate the expression of certain homeobox genes in *Xenopus* (Rosa, 1989; Ruiz i Altaba and Melton, 1989*c*; Cho and De Robertis, 1990). The recent

findings that overexpression of homeodomain proteins can confer axis-inducing properties to *Xenopus* embryonic cells (Cho *et al.* 1991) and that organizer-specific homeobox genes are expressed in *Xenopus* gastrulae (Blumberg *et al.* 1991) encourage us to think that progress in this area will be forthcoming.

Conclusions and prospects

One of the main challenges for the future will be understanding what sets up a circular field or a gradient of *nuclear* protein in an otherwise homogeneous population of mesenchymal cells. Unravelling the cell-to-cell signalling mechanisms that achieve this should shed light on the nature of positional information.

One obvious candidate is retinoic acid, which has profound effects on limb development (Eichele, 1989) and on the expression of homeobox gene complexes (Simeone *et al.* 1990). Implantation of a bead containing retinoic acid into developing chick wing buds greatly expands the gradient of XlHbox 1 expression, as shown in Figs 9D and 9E. This is accompanied by malformations in which excess anterior shoulder structures (which are the normal fate of XlHbox 1-expressing cells) are formed at the expense of the rest of the limb bud (Oliver *et al.* 1990). In the case of Hox 4 complex, implantation of a retinoic acid bead in the anterior of the chick wing bud induces the sequential activation of homeobox genes. This leads to a mirror image duplication of Hox 4 expression which correlates very well with the digit duplications caused by retinoic acid (Izpisua-Belmonte *et al.* 1991; Nohno *et al.* 1991).

Many other molecules could be involved as well. N-CAM, peptide growth factors, and the extracellular protein *wingless* (*int-1* in vertebrates) have been mentioned already. Many orphan nuclear receptors, for which the ligands are as yet unknown, have been isolated (Evans, 1988). Cell surface molecules involved in the activation of nuclear regulatory proteins in *Drosophila* (such as *notch*, *sevenless* and *bride-of-sevenless*, see Banerjee and Zipursky, 1990) either have (Coffman *et al.* 1990) or can be presumed to have vertebrate homologs. About 50 mutations are known to affect limb development in the mouse; perhaps some of them affect this intercellular signalling system. Transgenic mice expressing gradients of reporter genes fused to homeobox gene promoters would facilitate this analysis.

In this essay, we have dealt mostly with the XlHbox 1 gene. There are about 40 different homeobox genes of the *Antennapedia*-type in vertebrates, and there is no reason to think that their expression patterns will be less rich or informative. What made XlHbox 1 special was the early availability of antibodies that cross-reacted with the homologous genes in a wide spectrum of vertebrates.

During embryogenesis XlHbox 1 is expressed in several regions of very different developmental potential. First, it subdivides the body axis into a band of homeodomain protein expression in the anterior trunk region which spans the mesoderm, CNS and neural crest. In some cases (Fig. 9C), it can be seen to gradually decrease towards the posterior end. Second, it is expressed in the region of the lateral plate mesoderm that will give rise to the forelimb and, as a limb bud develops, it forms an A–P gradient of expression in mesodermal nuclei. Finally, in the case of feather development, circular patches of expression in the dermis are followed by bud growth and a new A–P gradient in each feather bud. Thus, the morphogenetic gradient fields defined by experimental embryology (Harrison, 1918; Huxley and de Beer, 1934) seem to have a molecular substratum that can be followed visually with antibody markers. It seems that the problem of setting up pattern in the vertebrate is resolved within fields of cells. Gradients of homeodomain proteins are utilized again and again during embryogenesis, perhaps to provide A–P polarity, in these cell fields undergoing pattern formation.

We have discussed correlations between gradients of expression of homeodomain proteins and the behavior of morphogenetic fields defined by transplantation experiments. We have emphasized that there is no direct evidence linking the two in a causal way at present. The purpose of this essay was to stimulate thought and research in gradient fields, an area of development ripe for molecular studies.

We are indebted to several colleagues that made important contributions to the XlHbox 1 localization studies reviewed here: G. Oliver (now at the University of Uruguay), C.V.E. Wright (now at Vanderbilt University), A. Molven (University of Bergen, Norway), C.B. Kimmel (University of Oregon), C.M. Chuong (University of Southern California), and C. Tickle (University College, London). We are grateful to Stephen Cohen (HHMI, Houston) for Fig. 8, and to Christof Niehrs and Dennis Bittner for suggestions. Particular thanks to Phil De Vellis, a high school student, who recovered this essay from the depths of a floppy disk after the entire text was accidentally erased. Work in our laboratory is supported by the N.I.H. (HD 21502-06). K.W.Y.C. was supported by the UCLA Human Genetics Postdoctoral Training Program (GM 08243) and E.A.M. by Training Program GM 07104.

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(*Accepted 28 March 1991*)