Shaping up with hedgehogs – How a fruit fly gene and its vertebrate homologues function in generating patterns during animal development

Bhagwati P. Gupta* and K. VijayRaghavan* †

*Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay 400 005, India
†National Centre for Biological Sciences, TIFR Centre, IISc Campus, Bangalore 560 012, India

Recent experiments in animals as diverse as fish, flies, chicken and mice have examined the role of the *hedgehog* gene in specifying the shape of tissues. These studies show, once again, how many of the elements of inter-cellular signalling are conserved between species. In addition, they show how intrinsic properties of cells and the effects of their environment combine to specify pattern during animal development. This brief review summarizes recent studies on *hedgehog* and its vertebrate homologues.

You boil it with sawdust: you salt it in glue:
You condense it with locusts and tape:
Still keeping one principal object in view–
To preserve its symmetrical shape

Lewis Carroll, *The Hunting of the Snark*

THERE has been much excitement and expectation from recent studies that have emerged from diverse systems: zebrafish, chicken, mouse and fruit fly[1–4]. These studies demonstrate the crucial role played by a fruit fly gene called *hedgehog* (*hh*) and genes similar to it, loosely called 'homologues', in other systems. They make important advances in our understanding of the mechanisms underlying the patterning of organs during development. We will try to summarize the main features of these studies and their implications.

How are patterns formed in biological systems? What are the mechanisms that determine the shape of an insect wing, the pattern of digits on a limb and the position of a cat's whiskers? These questions have dominated the thoughts of developmental biologists for over a century. The traditional tools for analysis have been experiments that ablate or excise regions of the developing egg or transplant parts of it to other regions. The effect of removal of a region of the developing embryo can have two effects. First, there is the obvious effect; tissues and structures that are directly derived from the removed chunk of cells may not form. But there is, sometimes, a more profound result; the death or removal of one group of cells affects the differentiation of neighbouring cells and the patterns they form. This suggests that a group of cells have an *inductive* effect on their neighbours' fate. This induction can be further demonstrated by transplantation experiments where the grafted cells influence their new neighbours to take on fates and shapes they normally would not take. In other kinds of transplantation experiments, if cells take on the same fate irrespective of their position, then the implication is that they are not subject to inductive influences but their properties are determined by mechanisms intrinsic to them.

Orthodox developmental biologists used to fall into two camps. Those that gave principal importance to the role of inductive interactions and other forms of cell–cell communication and those that felt that the major mechanism that operated were the ones whereby cells passed on information about their fate to their daughters. That is, in one case the position of a cell decided its fate and in the other, its lineage. Results from worms, flies and vertebrates have provided a remarkably similar picture about the ways in which cells eventually take on specific fates and these results have shaken and mixed the two kinds of biologists together vigorously. This shaking-up has resulted in the spewing out of a large number of publications, but has also demonstrated how position- and lineage-based mechanisms both operate to specify pattern in many organisms.

The recent enthusiasm about the *hh* gene stems from its apparently similar mode of action to pattern diverse tissues in many different animal species. The *hh* gene was originally identified as one of the genes that function to pattern the embryo of the fruit fly *Drosophila melanogaster*. We will first summarize results from work on the *Drosophila* embryo and on the precursors of the *Drosophila* adult appendages. We will then look at the reports on the function of 'homologues' of the *hh* gene in chicken and zebrafish.

First, let us see how the fly's egg develops[5]. Maternally contributed mRNA molecules play a crucial role in the specification of anterior-posterior axis of the *Drosophila* embryo. The proteins derived from these maternally synthesized mRNAs activate a set of genes in the genome of the developing egg. This set of genes in turn activates others eventually patterning the egg into stripes of cells. Next, these stripes themselves acquire

polarity: their anterior is distinguished from their posterior and finally each cell in the stripe acquires a specific fate (Figure 1).

The gene *hh* plays a role in two events during early *Drosophila* development: in conjunction with the genes *engrailed* (*en*) and *wingless* (*wg*), *hedgehog* acts to polarize each stripe or parasegment[6]. Cells that express *en* also express *hh*. This expression of *hh* at this time is required to stabilize *wg* expression in other cells. Similarly, *wg* expression is required to stabilize the expression of *en* and *hh* in the cells which express them (Figure 2 *a*, *b*). The important conclusion from these experiments is the suggestion that the early function of *hh* depends on cell–cell contact. Thus, while mis-expression of *hh* leads to the *mis*-expression of *wg*, during *normal* development the expression of *wg* is limited to cells adjacent to *hh* expressing cells. This suggests that the early *hh* signal is local in nature. The second role of *hh* during embryogenesis very likely depends on the concentration gradient of the *hh* product (Figure 2 *c*). At this stage, *mis*-expression of *hh* can change the fate of cells in a manner that depends on the level of *hh* gene expression[7]

The *hh* gene is used again in the development of the adult fruit fly, in a manner remarkably similar to its role in vertebrates. The adult fly's epidermis, or cuticle, is derived from a group of cells which divide, but do not differentiate, during larval life. After the onset of metamorphosis these cells, in response to hormones, differentiate into specific parts of the adult cuticle. The *patterning* of the groups of cells that will give rise to the adult epidermis takes place much before they differentiate. In the larva, the progenitors of the adult thoracic

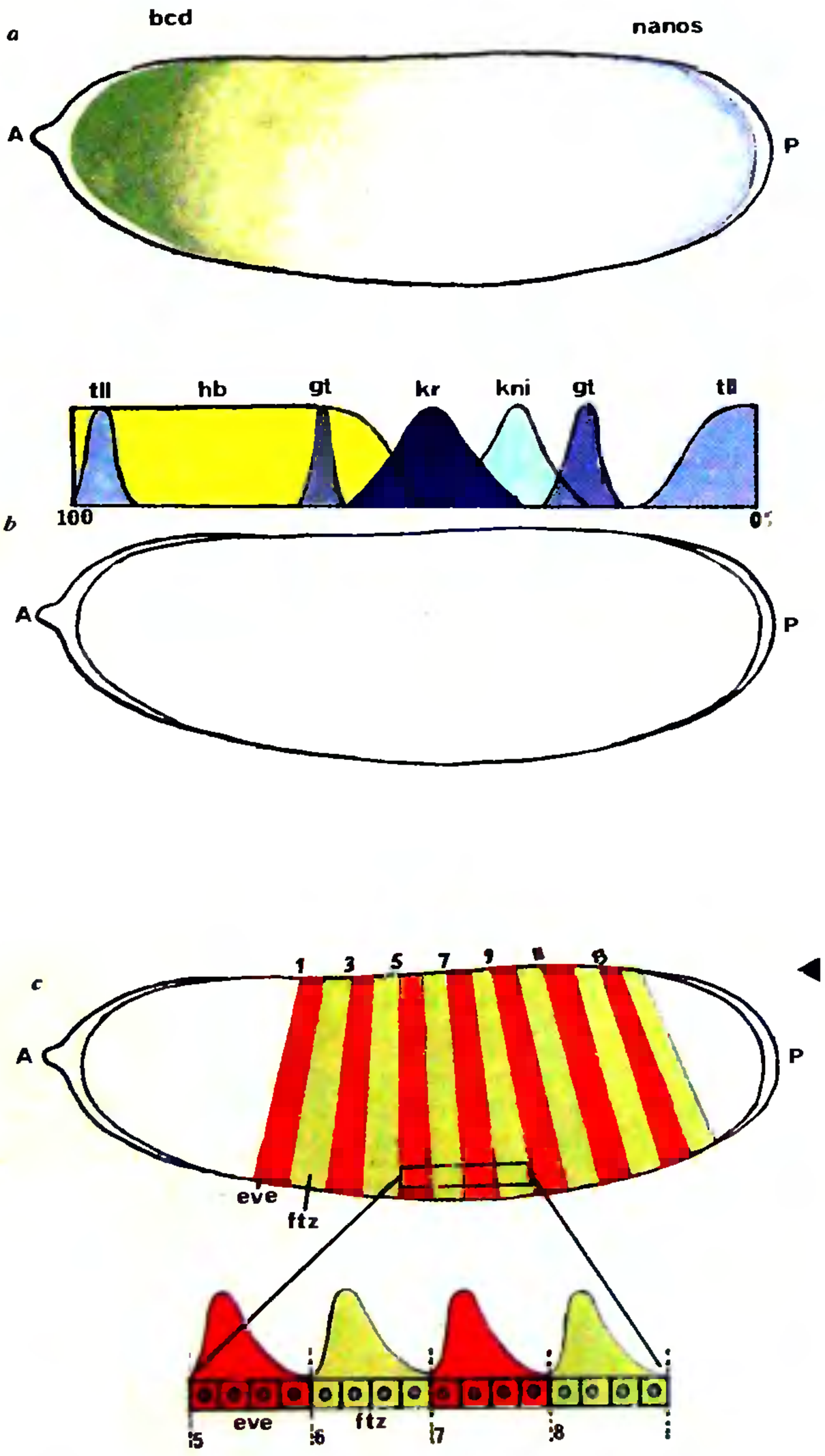

Figure 1. *Drosophila* development. *Early events in patterning the embryo of the fruit fly Drosophila melanogaster*. If you would like to read a simplified view of early *Drosophila* development, go through Figure 1. Otherwise move on to Figure 2 without loss of continuity or face. *a*, *Maternal contributions to patterning the fly embryo*. mRNA from the *bicoid* (*bcd*) gene is synthesized in the mother and deposited in the anterior of the unfertilized egg. The translation of this anterior localized *bcd* mRNA results in deposition of BCD protein in the anterior of the egg. This protein diffuses towards the posterior and forms a gradient of BCD concentration. This is shown as a gradient of green colour. In the posterior of the egg maternally derived mRNA from the *nanos* gene is present and translated to form NANOS protein. This is shown in blue colour in the figure. In both ends of the embryo, the termini, a maternally contributed signal transduction cascade (not shown) acts to define these ends. The anterior of the egg is marked with A and the posterior, P. *b*, *Maternal contributed information results in the activation of the egg's genes in a specific pattern along the anterior-posterior axis of the egg*. In response to the localized activity of genes such as *bcd* and *nanos*, a group of genes called the gap genes are activated along the egg's length (defined as 0% at the posterior end and 100% egg length at the anterior and shown in the figure as 0 and 100). In the anterior, the best analysed genes are *hunchback* (*hb*) and *Kruppel* (*kr*). These two genes and the gene *giant* (*gt*) respond to maternal cues to set the initial differences along the anterior of the egg. Similarly the genes, *knirps* (*kni*) and *gt* set up differences in gene expression along the posterior of the egg. The gene *tailless* (*tll*) is the zygotic gene which is activated at the termini of the egg. The expression of each of these gap genes is shown in a different colour to mark the domain of their expression. In addition, their levels of expression vary over the length of the egg where they are expressed (modified from ref. 5, p. 58); *c*, *The level and overlapping pattern of expression of gap genes results in the activation of stripes of pair rule gene expression*. The gap genes appear to function in a combinatorial manner to activate and repress their targets, the pair rule genes along the axis of the egg. This results in the division of the egg into metameric units called parasegments. The pattern of expression of two pair rule genes *even-skipped* (*eve*) and *fushi-tarazu* (*ftz*) are shown in orange and green respectively. The rectangle marked in the top of *c* is expanded below and shows that, as for the gap genes, pair rule expression shown here in parasegments 5–8 (each four cells wide), is also regulated along the region of its expression (Figure 1 *c* is modified from ref. 11, p. 575, Figure 19 *b*).

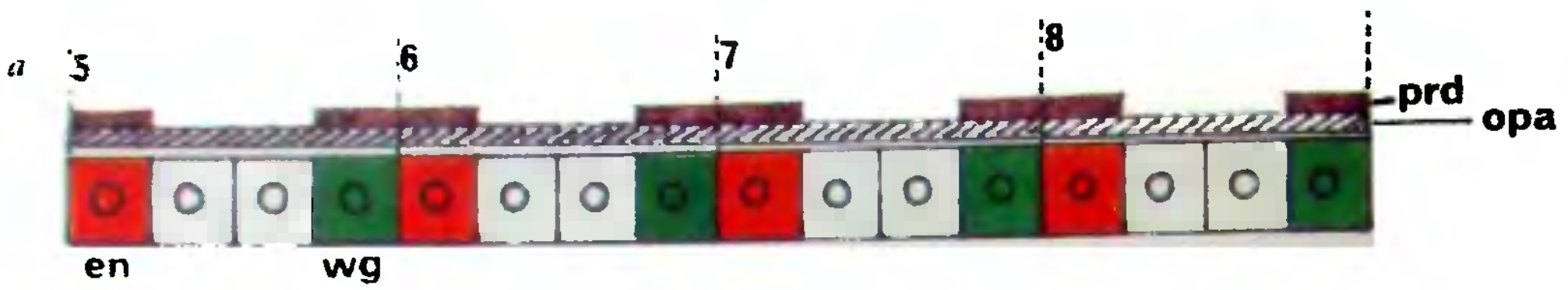

Figure 2. In the *Drosophila* embryo the *hedgehog* gene product is likely to play two roles: a local one and another in which it can act over a distance. ***a****. The early function of hedgehog in the Drosophila embryo is a contact-dependent process that stabilizes the expression of wingless and engrailed.* The expression of the pair-rule genes *paired* (*prd*) and *odd-paired* (*opa*) determine the pattern of expression of the segment-polarity genes *engrailed* (*en*) and *wingless* (*wg*). The domains of expression of *prd* is shown in purple and that of *opa* is shown below. The *opa* expression is uniform. *en* and *wg* are not expressed in the region where only *opa* is expressed. In the region where both *opa* and *prd* are expressed, *en* is expressed in the anterior of each parasegment and *wg* is expressed in the posterior of each parasegment. The stippled lines show the parasegment boundaries. *hedgehog* is expressed in *engrailed* expressing cells. Signals from cells that express *hh* and *en*, and from cells that express *wg*, act locally to reciprocally stabilize the early expression of these genes. (Figure modified from ref. 11, p. 575, Figure 19*b*). ***b*** **&** ***c****. The late function of hh and wg is in cell fate specification where the gradient of concentration of their products appears to allow these genes to act over a distance.* A single parasegment is shown between the dotted vertical lines in *b*. The cells, in red, that express *en* also express *hh*. The cells that express *wg*, shown in green, and those that express *hh* act to specify the fate of cells in their neighbourhood. A suggested mode of action of *hh* in this pathway is illustrated in *c*. More than one parasegment is shown. The cells that express *wg* are shown in green and the cells that express *hh* are marked blue. A gradient of *hh* expression is shown in the blue triangle. The posterior-most *hh* expressing cells adopt the 1° fate. As shown in *b*, these cells also express *en*. A gradient of *hh* product specifies the fate of more posterior cells to 2° and 3° fates. Cells that adopt the 4° fate can also respond to levels of *hh* product. The blocking sign indicates that *wg* expression may prevent *hh* from acting in the direction of *wg* expressing cells. (Modified from ref. 7).

cells and those of the head are grouped into bags called imaginal discs. The wing, or the dorsal-mesothoracic, imaginal disc, gives rise to the structures of the dorsal thorax, the notum and the wing blade shown in Figure 3 *a*. The leg imaginal disc and the structures that are derived from it are shown in Figure 3 *b*. The dissection of the role of *hh* reveals its importance in patterning the wing and leg discs. Perhaps the earliest

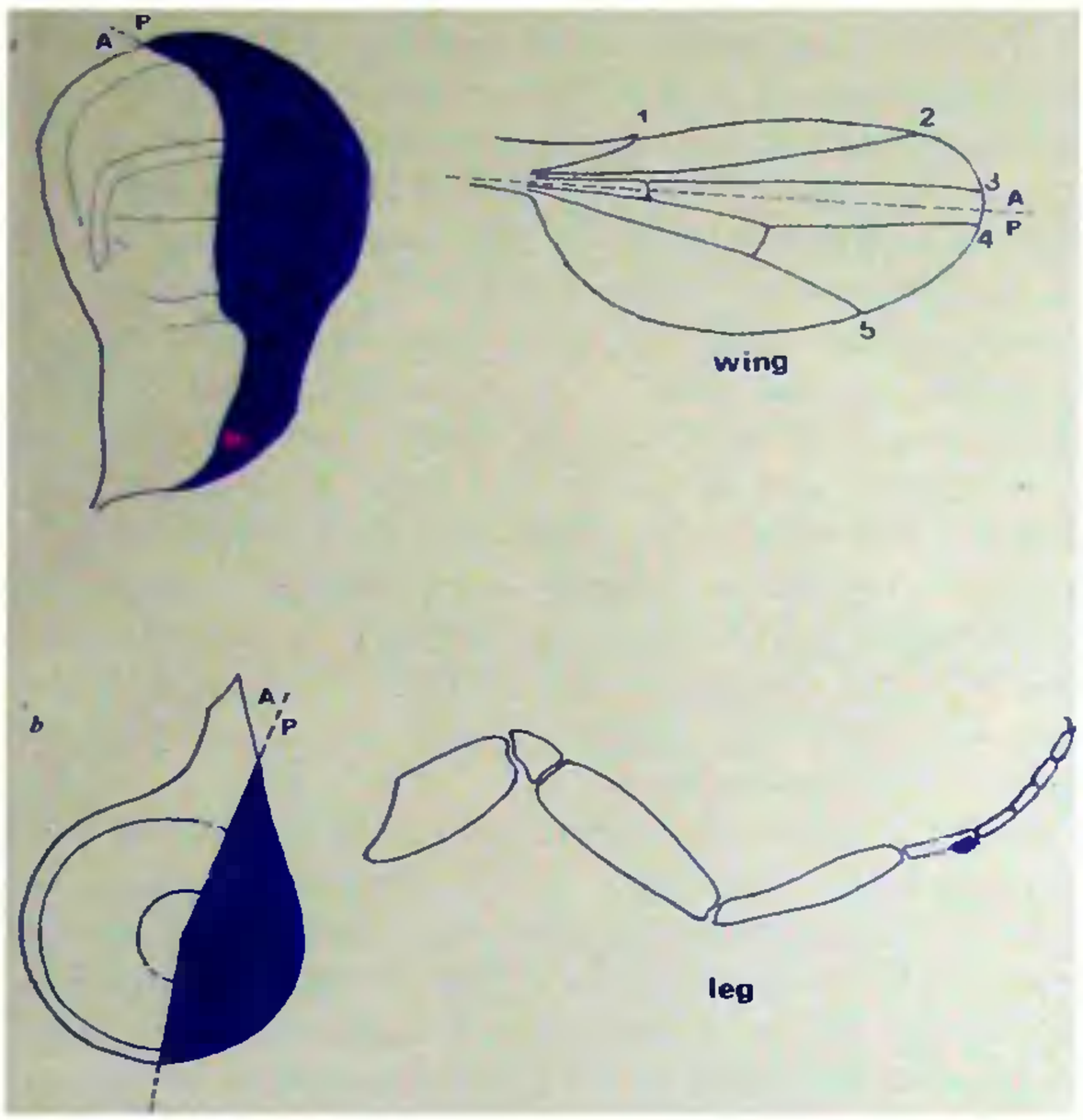

Figure 3. The *hh* gene is expressed in the posterior compartment of imaginal discs. *a*, A representation of the wing imaginal disc is shown at left and that of the wing blade at right. The posterior compartment is marked blue; *b*, A representation of the leg imaginal disc is shown at left and that of the leg at right. The posterior compartment is marked blue; The orientation of the discs and their derivatives do not correspond in this figure. The Roman numerals in the wing disc are marked to show the polarity of the wing blade. This is evident from the landmarks on the blade. Other landmarks, bristle patterns. on the leg allow the identification of its anterior and posterior structures.

patterning events in the imaginal discs are the demarcation of anterior and posterior domains (see ref. 5, for a recent review). The *en* gene is expressed in posterior cells and is required for the 'posterior' label. In the absence of *en* function, progeny of cells in the posterior can cross to the anterior of the anterior/posterior (A/P) border. In a manner similar to that seen in the embryo, *en*-expressing cells in the imaginal discs also express *hh*. Studies on *mis*-expressing the *hh* gene and also experiments that look at the effect of removing its function in the imaginal discs point to the role of the *hh* protein as a signalling molecule that functions in a pathway in which the products of the *wg* and *decapentaplegic* (*dpp*) genes also function[4]. The *mis*-expression of *hh* in the imaginal discs was done in two ways. In the first method, transgenic animals were used in which the *hh* gene is expressed under the control of the regulatory sequences of the α1-Tubulin gene. α1-Tubulin is expressed ubiquitously but the time of onset of this expression in the *hh* expression studies was

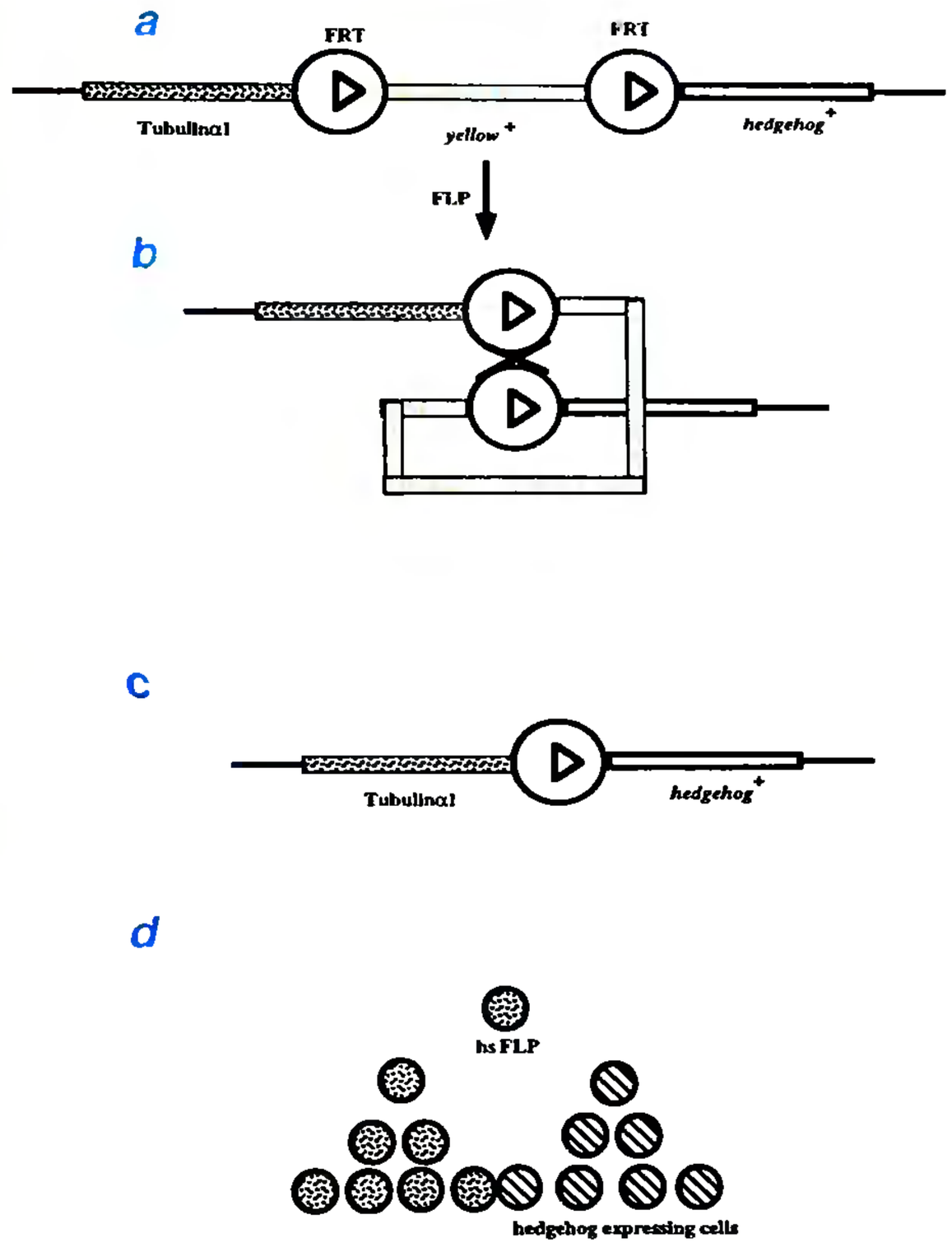

Figure 4. The FLP out technique and its use to generate clones of cells that express *hh* ectopically. *a*, Flies that carry the gene construct shown in this panel express the *yellow* gene under the control of the α1-Tubulin regulatory sequences. The yellow gene is flanked by FRT sites (shown as circles with arrowheads inside). The FRT (FLP recombinase target) sites are DNA targets for the FLP recombinase protein. The gene for the FLP recombinase, under the control of the heat-shock promoter is also genetically introduced into the animals which have the α1-Tubulin>*yellow*>*hh* construct. Upon heat-shocking the animals, the FLP recombinase is expressed and recombines out one FRT site (*b*), and in doing so removes the *yellow* gene. The presence of a transcription termination sequence after the *yellow* gene [in the construct shown in (*a*)] prevented the expression of the *hh* gene. The removal of the *yellow* gene, and of this transcription terminator, juxtaposes the *hh* gene downstream of the α1-Tubulin regulatory sequences. Thus, after FLP induced recombination, *hh* is expressed continuously in the progeny of the cells where recombination has taken place (*c*); *d*, A schematic representation of a clone of cells expressing *hh* is shown adjacent to cells that continue to express *yellow* but not *hh*.

controlled by using the 'FLP-out' method[4]. This method is outlined schematically in Figure 4. Using this method it was thus possible to 'switch-on' *hh* expression at different times and places during development. Once this expression was activated in a cell, this cell and its progeny continued to express the *hh* gene during development. The other method involved the expression of *hh* under the control of a heat-shock promoter

(Ingham, unpublished, referred to in ref. 8). The effect of *hh* mis-expression by these methods, on posterior cells (the blue region in Figure 3) of wing and leg imaginal discs was not remarkable. This is not surprising since the native *hh* gene is expressed in these cells and additional expression does not seem to matter. However, when *hh* expression was induced in anterior cells, in particular when these cells were far from the anterior–posterior compartment boundary, the effect was dramatic. Cells which thus expressed *hh* acted as an organizing centre and induced the formation of additional anterior tissue in neighbouring cells[4]. This is illustrated in Figure 5 *a* for the wing and in Figure 5 *b* for the leg. What is the mechanism of *hh* action to pattern appendages of the *Drosophila* adult? As of now we know only of some of the elements of what will probably be a complex story. In the wing disc *hh* acts by inducing the expression of *dpp* in anterior cells. During normal development, *dpp* is expressed, in the wing disc, in a stripe of anterior cells abutting *hh*-expressing cells. In the ectopic expression experiments the anterior expression of *hh* results in the activation of expression of *dpp*, as seen by the expression of a *dpp-lacZ* transgene, in anterior cells. All anterior cells are able to respond to *hh* signals and express *dpp*[4]. This is reminiscent of the experiments in the embryo where *hh* expression in posterior, *en* expressing cells is required for the maintenance of *wg* expression in cells which are in contact with it, but ectopic expression of *hh* results in the ectopic activation of *wg*. Indeed, in the leg disc, the expression of *hh* in anterior cells causes the expression of β-galactosidase from a *wg-lacZ* reporter gene in anterior-ventral cells and from a *dpp-lacZ* reporter gene in anterior-dorsal cells[4]. Thus, the conclusion is that anterior cells in wing and leg discs respond differently to *hh* signals: *dpp* is activated in the wing disc and *dpp* or *wg* is activated in leg discs. Just as *hh* expression in posterior cells acts through *dpp* and *wg* to organize anterior cell proliferation and pattern, it seems likely that *dpp* and *wg* expression in the anterior acts on *en* and *hh* expressing posterior cells to organize their proliferation and pattern.

Usually, major discoveries regarding mechanisms of developmental regulation in animals are postulated in flies and then tested in animals with a spinal cord. In the case of *hh*, though the gene itself was first identified in flies (hence its catchy name that is uninformative to non-*Drosophila* workers), its most dramatic effects were first

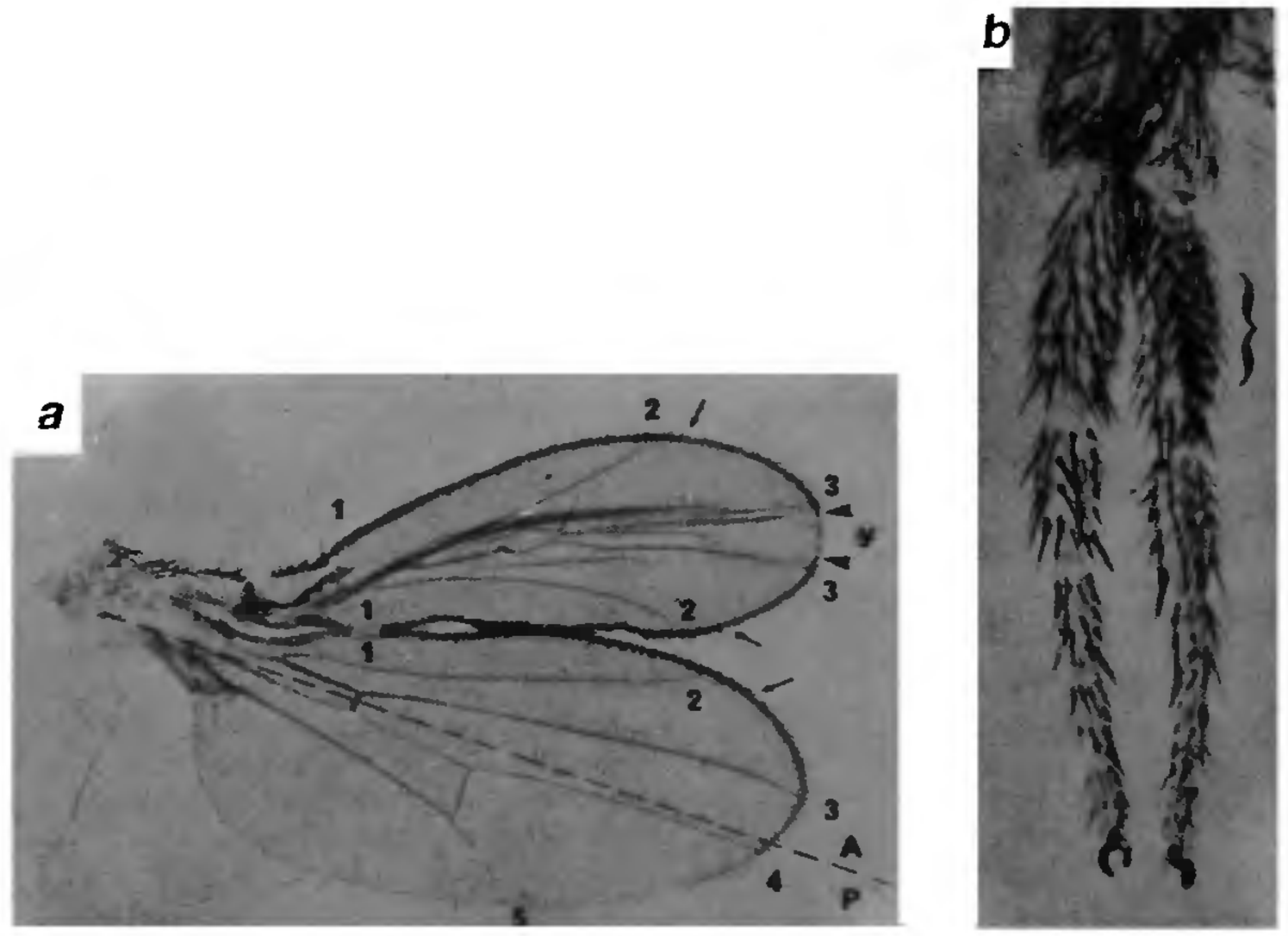

Figure 5. *hh* Expression in anterior cells of wing and leg imaginal discs results in the formation of duplicated anterior structures[4]. *a*, An alteration in the anterior compartment pattern in response to ectopic expression of *hh* (The pattern of the wild-type wing is shown in Figure 3 for comparison.) The arrowheads mark the boundaries of the tissue that expresses *hh* under the Tubulin α1 promoter. The wing blade below is the 'normal' one, has the posterior region and the anterior/posterior compartment boundary is marked A/P. The numbers on the wing blade denote, in increasing order (1–5) the polarity of the wing disc. See Figure 3 for the wild-type order. The altered order in *hh* induced anterior wing is seen as 123321 with the *hh* clone at the centre. *b*, The anterior leg pattern is also re-organized by the ectopic expression of *hh* in the developing anterior leg. The normal (right) and the supernumerary (left) leg are shown. The bracketed structure is of posterior provenance and is not present in the supernumerary leg. The supernumerary structure has double anterior structures and no posterior structures showing that, as in the wing, ectopic expression of *hh* results in duplicated anterior structures but has no effect on posterior structures.

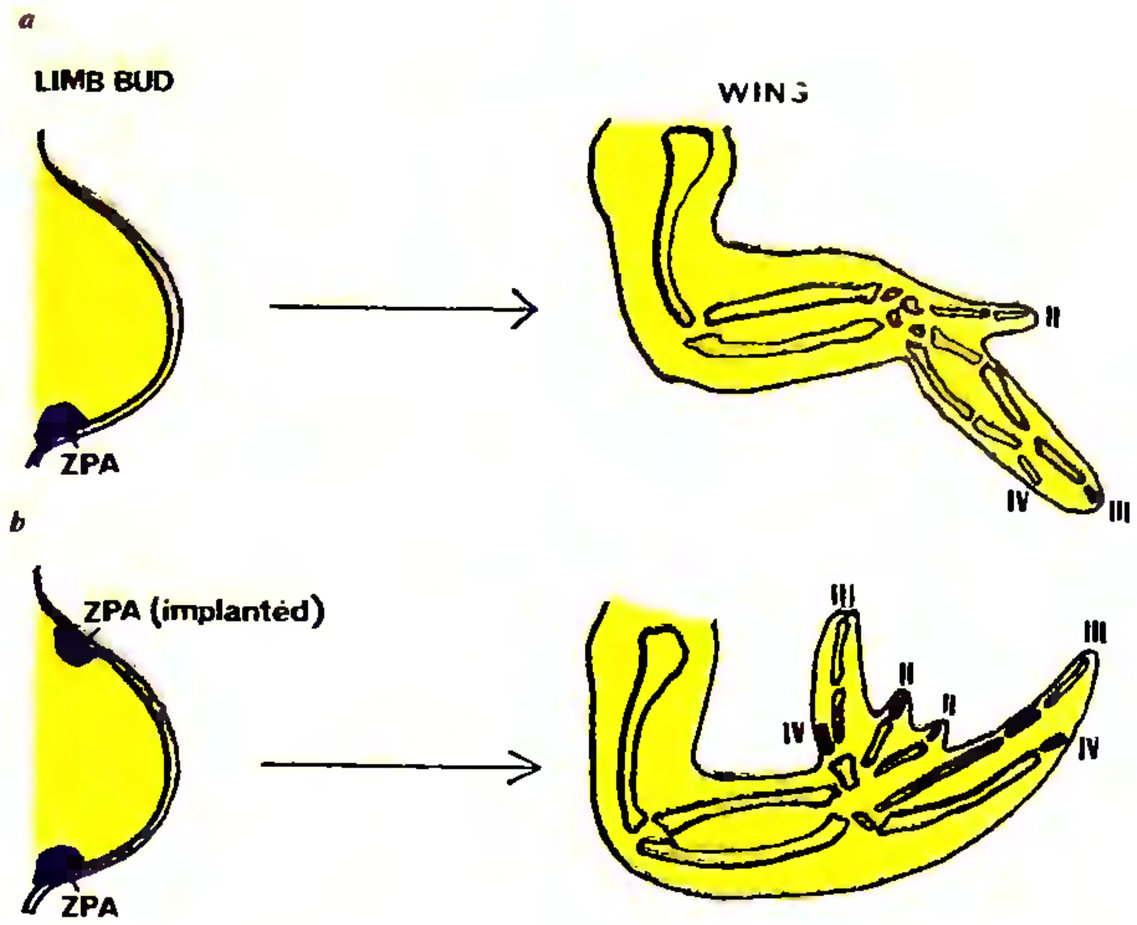

Figure 6. The chicken homologue of *hh*, sonic hedgehog functions to pattern limbs: ectopic expression results in ectopic limbs. (Figure modified from ref. 3); *a*, A schematic limb bud is shown at left and the mature wing is shown in the right of the panel. The ZPA is marked blue. The Roman numerals II-III-IV mark the polarity seen in the mature wing; *b*, When ZPA, from another limb bud, is transplanted to the top (anterior) of the wing bud, a duplication in pattern of the mature wing is observed: IV-III-II-II-III-IV. *shh* expresses in the ZPA and the transplantation of *shh* expressing cells has an effect very similar to that seen in ZPA transplants.

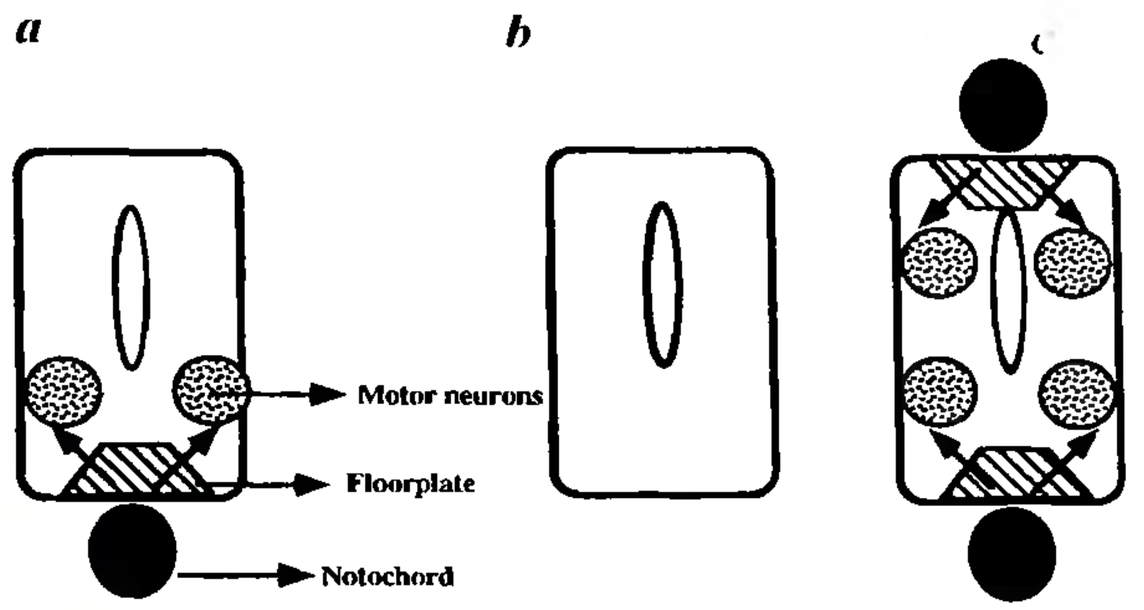

Figure 7. The induction of floorplate by the notochord and the induction of floorplate motor neurons by the floorplate: events in which *shh* may play an important role (Modified from ref. 8) *a*. The notochord, shown in black, are cells of mesodermal origin. The spinal cord is the large rectangular structure in which the floorplate and the floorplate motor neurons are marked. The floorplate is in contact with the notochord while the floorplate motor neurons are at some distance from the floorplate: *b*. The removal of the notochord results in the absence of the floorplate and floorplate neurons; *c*. When the notochord is transplanted dorsal, an ectopic floorplate is induced, which in turn induces the formation of floor plate motor neurons.

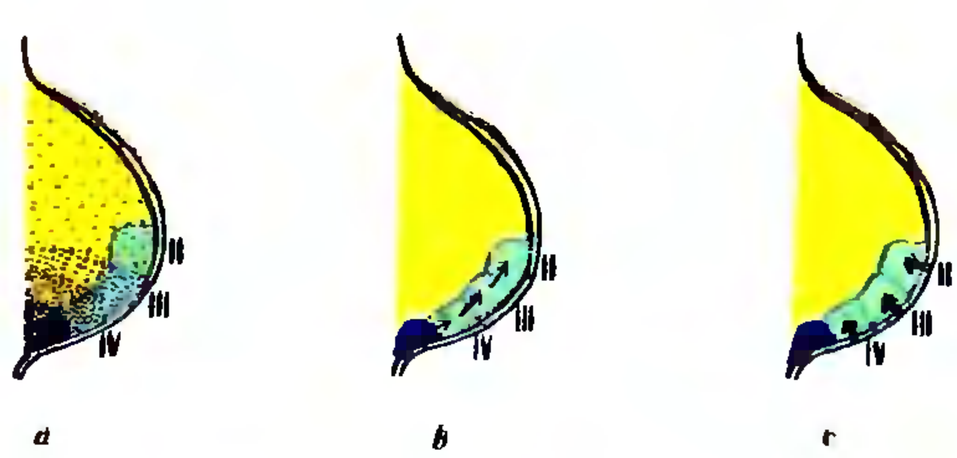

Figure 8. Some mechanisms by which *shh* may act to pattern limbs (Figure modified from Riddle *et al.*, 1993). *a*, *shh* may function as a morphogen whose concentration at a site determines its effect. The source of *shh* in these schematics is the ZPA shown in blue. The highest concentration of *shh* in the developing wing bud is in region IV, lower in III and lowest in II. This is denoted by the intensity of stippling. Compare this with the model for *hh* action in the *Drosophila* embryo (Figure 2); *b* and *c*. Another way in which *shh* may act is by a series of cell–cell interactions. These interactions could take place by directly affecting the limb mesenchyme as shown in *b* or could affect the limb by intermediates in the AER as shown in *c*. The arrows suggest a signalling cascade. The ZPA where *shh* is expressed is shown in blue. Compare these with the early role of *hh* in maintaining *wg* expression in the *Drosophila* embryo (Figure 2).

demonstrated in vertebrates. The chicken homologue of the *hh* gene was isolated and named *sonic hedgehog*[2]. In the developing chick limb bud *sonic hedgehog* (*shh*) expression is seen in the posterior, in a region known as the zone of polarizing activity (ZPA, Figure 6). The ZPA is an example, often used by developmental biologists, to demonstrate the patterning capabilities of cells. Grafting the ZPA to the anterior will result in the alteration of the pattern of digits in a manner very similar to that seen from *mis*-expression of *hh* in flies (Figure 5). It turns out that *shh* expression is seen in the ZPA and *shh mis*-expression in the anterior results in the alteration of limb patterning exactly as seen for ZPA grafts. It seems that we have at hand the beginnings of a molecular explanation of a classical developmental phenomenon.

The floorplate–notochord interaction in the vertebrate spinal chord is another example of patterning that has been well studied by grafting and ablation experiments (see ref. 8 for a summary). Along the ventral midline of vertebrates is a structure of mesodermal origin, called the notochord. This structure plays an important role in organizing the neural tube and it structures along the dorsal-ventral axis (Figure 7). The notochord induces the formation of the floorplate. This seems to be a contact-dependent process. Removal of the notochord results in the absence of floor plate formation (Figure 7 *b*). Transplantation of the notochord results in ectopic floorplate formation (Figure 7 *c*). This is a contact-dependent process. The floorplate, in turn, induces the formation of floorplate motor neurons (Figures 7 *a*, *c*) and this induction does not seem to require contact. These events thus have similarities to the two proposed modes of action of *hh* suggested in the

Drosophila embryo, the early contact-dependent role of *wg* maintenance and the later role in specifying cell fate. In zebrafish, *shh* is expressed in the notochord and the floor plate and here too, ecotopic expression results in the ectopic expression of floorplate and motor neuron markers[1]. In addition, functional conservation of *hh* is demonstrated by studies that introduce the zebrafish *hh* gene in fruit flies: *mis*-expression of *shh* in fruit flies results in the same phenotype as the *mis*-expression of *hh*[1].

How does *hh* act in the process of patterning diverse elements in many species? Most of the clues towards where the answer lies come from studies on *Drosophila*. As seen above the *wg* and *dpp* gene products are crucial players. The *wg* gene product is a member of a family of growth factors, the Wnt family and could function in very similar ways in flies and mice[9]. The *dpp* gene product belongs to the TGFβ family of growth factors and has the greatest similarity to the bone morphogenetic protein 2 (BMP2); this protein has recently been reported to be expressed in the ZPA[10]. We also know, from studies on the *Drosophila* embryo that there could be more than one way in which *hh* acts. Thus, secreted hedgehog protein may, in a contact-dependent process, signal the maintenance of *wg* expression in neighbouring cells. Later on, a gradient of protein concentration could specify cell-fate. In the limb bud and notochord too, these mechanisms may operate. Some of the possible ways in which *shh* acts are shown schematically in Figure 8 (ref. 3). One possibility is that a gradient of concentration of the gene product determines limb pattern in a concentration-dependent manner; another is that a series of cell–cell interactions involved and finally, *shh* could specify polarity in the region adjacent to the developing limb, the apical ectodermal ridge (AER) which in turn could act to specify cell-fate. We do not know if any one or a combination of these mechanisms are actually involved, nor do we know many of the elements of these modes of action. We know that 'fields' or group of cells in a layer are patterned and we do know that these elements of pattern are translated into the 'Hox code', or the patterned expression of homeotic genes. Homeotic genes are thought to be the final regulatory step before the expression of the structural genes that encode the components of limbs, wings, etc. Retinoic acid (RA) was once thought to be the morphogen present in the ZPA. But a number of experiments have questioned this conclusion and the issue is contentious. It appears likely that *endogenous* RA function, if present, could be upstream of *shh*. This has been shown by transplantation experiments where RA beads cause the expression of *shh*[3]. Working away from both ends of the regulatory hierarchy, developmental biologists have provided us with important clues about patterning at the molecular level.

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