Reverse homeosis in homeotically reconstructed ribbonworms

Michel Tarpin*†, Walter J. Gehring‡, and Jacques Bièrne*

*Laboratoire de Biologie Cellulaire et Moléculaire, Université de Reims Champagne-Ardenne, F-51 687 Reims, France; and ‡Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Contributed by Walter J. Gehring, July 15, 1999

Homeosis is the replacement of one body part by another, which may be caused by either developmental or genetic variations. It is particularly obvious in segmented animals, like insects, in which one body segment may be transformed into another. However, homeosis also occurs in animals without overt segmentation that also have detailed positional information specifying their body plan. By grafting, we have artificially generated homeotic ribbonworms of the species *Lineus ruber* with a duplicated ocellar region replacing the postocellar region anterior to the brain. Such chimeric animals are capable of complete morphogenetic regulation of the anterior–posterior (A–P) pattern. The missing postocellar region is restored by intercalary regeneration, and the anterior duplicated ocellar region is eliminated by a process called transgeneration. Thus, homeosis is reversed, and a completely normal pattern along the A–P axis is restored. This reverse homeosis involves the elimination of the syngeneic eyes and the survival of the grafted allogeneic eye region. *LsPax-6*, the *Lineus sanguineus* ortholog of the mammalian *Pax-6* gene, which is considered to be a master control gene for eye morphogenesis, is expressed specifically in regenerating, regenerated, and intact eye regions. Our data show that ribbonworm eyes are either maintained or they regress according to their position along the A–P axis, even though there are no obvious segmental boundaries. This system allows us to test the function of LsPax-6 protein not only during eye regeneration but also during maintenance and regression of the eyes.

In 1894, William Bateson coined the term "homeosis" and gave it a very broad definition as a type of variation in which "something has been changed into the likeness of something else" (1). Later, after the rediscovery of Mendel's work, it became obvious that homeotic variations had to be subdivided into genetic variations and those that are caused by developmental abnormalities that are not heritable. Such developmental abnormalities also occur in animals without overt segmentation, like planarians, in which they have been described as polar heteromorphosis. In flatworms, heteropolar homeosis can be experimentally induced or found to occur spontaneously in nature. The most spectacular heteromorphoses are those of "Janus-heads" and "Janus-tails" produced by demecolcine (2) and those generated by regeneration of small pieces from the middle region of the body, which produce double heads or tails with opposite polarity (3). Similar heteromorphoses also occur in arthropods, mandibles growing in place of the antenna, or antennae regenerated from amputated eye stalks in crustaceans (4, 5). The discovery of the homeotic *bithorax* and *Antennapedia* mutations in *Drosophila* showed that this change of likeness often generates a duplication of one body region and the corresponding deletion of another. The generation of the four-winged fly from a wild type with two wings and two halteres has become the paradigm of homeosis. Homeotic mutations have led to the discovery of the homeobox and opened up a new approach for the study of development and evolution (6). Over the past 20 years, evidence has accumulated indicating that the body plan of most animals, including chordates, arthropods, platyhelminths, nematodes, and nemertines, is controlled by a set of master control genes that were first identified by the respective homeotic mutations and/or the presence of a homeobox (7–13).

The difficulties that prevent reconstruction of animals by piecing together body fragments from several adult specimens have been overcome by using nemertines of the genus *Lineus* (14). *Lineus*, a marine ribbonworm, is a representative genus of the invertebrate phylum Nemertini. Recent molecular studies strongly suggest that the nemertines are clearly distinct from platyhelminths (15, 16) and may be in an evolutionary transition zone between protostomes and deuterostomes—i.e., similar to the last common ancestor between invertebrates and vertebrates (13, 17).

Lineus is highly suited for reconstruction experiments and can also be used to generate homeotic constructs. Unlike natural amputations that can induce regeneration of the missing parts, reconstruction experiments allow the generation of discontinuities in the series of anterior–posterior (A–P) positional values and the testing of morphogenetic responses under altered conditions, such as: (*i*) duplications of a body region, (*ii*) internal deletions, (*iii*) introduction of several discontinuities in the body plan of the same animal, e.g., a duplication plus a deletion, and (*iv*) inversions (heteropolar insertions) of body regions.

In this study, we describe a homeotic reconstruction experiment that involves a duplication of the ocellar region plus a deletion of the postocellar region. In this experimental situation, *Lineus ruber* is capable of reverse homeosis, by intercalary regeneration of the missing postocellar region and by elimination of the duplicated ocellar region. The latter phenomenon, designated as *transgeneration*, causes the elimination of the syngeneic ocellar region and the survival of the grafted allogeneic tissue. Therefore, transgeneration is not the result of a graft rejection. This dual capacity for both intercalary regeneration and transgeneration is the most complex reprogramming of the body plan in an adult animal studied so far.

Materials and Methods

Animals. *Lineus ruber* worms were collected from the English Channel along the coast of Brittany, near Roscoff (France), kept at constant temperature (12°C) under continuous darkness, and fed calf liver once a week. The *Lineus* body plan lacks segmentation, but it is characterized by 10, nonoverlapping, anatomical regions serially placed from the rostral to the caudal end (13, 14). Transplantation experiments were performed by altering the pattern of the head region anterior to the brain. This antecerebral region is made up from two A–P components (Fig. 1), an anterior ocellar region containing a variable number of eyes and a postocellar region lacking eyes. Both regions are characterized by the complete absence of nerve cell bodies of the central nervous system, which are confined to the more posterior regions.

Abbreviation: A–P, anterior–posterior.

†To whom reprint requests should be addressed. E-mail: michel.tarpin@univ-reims.fr.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

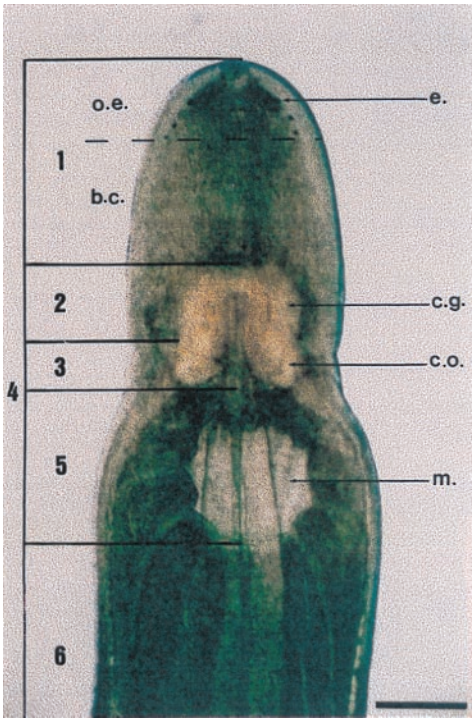

Fig. 1. Morphology of the first six body regions of a *L. ruber* specimen photographed between slide and cover glass. Regions: 1, the antecerebral end is made up of the anterior ocellar region (o.e., oculated end), which bears a few well-anchored eyes (e.) on each side, and the postocellar region (blind component, b.c.) without eyes; 2, the cerebral ganglia region (c.g.); 3, the sensory cerebral organs region (c.o.); 4, the postcerebral, preesophageal connective tissue's region (absent in this species); 5, the anterior esophagus region, characterized by the presence of the mouth (m.); and 6, the posterior esophagus region, where nephridia are located. (Scale bar: 300 μm.)

Transplantation Experiments. Transplantations were performed according to grafting procedures described previously (14). Allogeneic grafts inserted in the antecerebral end allowed us to manipulate the pattern of *Lineus* so that increasing order and/or decreasing discontinuities were present in the sequence of A–P positional values (18, 19). The precise design of the grafting experiments leading to the homeotic ribbonworms is indicated in Fig. 2 and leads to the replacement of the blind postocellar region of the recipient animal by a second ocellar region from a different donor animal (Fig. 2). Each homeotic construct was made between two specimens differing in pigmentation, one being pale, the other dark, so that host and graft tissues can be distinguished.

Whole-Mount *in Situ* Hybridization. Worms were anesthetized in 8% magnesium chloride and photographed, rinsed in PBS, and treated with 0.1 M cysteine chloride for 15 min to remove the mucus on their surface. Whole-mount *in situ* hybridization was done with digoxigenin-labeled *LsPax-6* RNA antisense probes (17).

Results

Reverse Homeosis. The design of the grafting experiment leading to the homeotic chimera is indicated in Fig. 2; the postocellar region of the recipient animal was replaced by a second ocellar region from a different donor animal, one being pale the other dark. Over a period of 14 weeks, complete morphogenetic regulation took place restoring the normal pattern (Fig. 3). The missing postocellar region was reconstituted by intercalary regeneration, and the additional anterior ocellar region was eliminated by a process called transgeneration (20). Previous studies (14) have shown that in *L. ruber* a duplication of the esophagus region generated by grafting is maintained stably over a period of more than 2 years, and neither intercalary regeneration nor transgeneration was observed. In contrast, our present data show that a rostral duplication results in both intercalary regeneration and transgeneration, leading to a restoration of the normal A–P pattern. Surprisingly, the morphogenetic regulation occurs by elimination of syngeneic eyes, whereas the allogeneic eyes from the donor are maintained, indicating that transgeneration cannot be attributed to graft rejection, but rather to an alteration in cell fate and/or removal of excess tissue.

In a preliminary biometrical study (data not shown), we have found that the syngeneic ocellar region steadily decreases in length during transgeneration, which is because of cell death and/or cell migration. During intercalary regeneration, however, there is a steady increase in length of the postocellar region, presumably because of proliferation of the regenerating cells. However, the dynamics of these processes have to be analyzed in more detail.

Expression of the *Lineus Pax-6* Gene During Regeneration. In our experiments, the eyes served as useful morphological markers to follow their regression during transgeneration and the extent of intercalary regeneration of the postocellar region. The structure of the eyes in *Lineus* is similar to that found in turbellarians and consists of a small number of inverted photoreceptor cells in a cup of pigment cells forming an inverted pigmented ocellus (21).

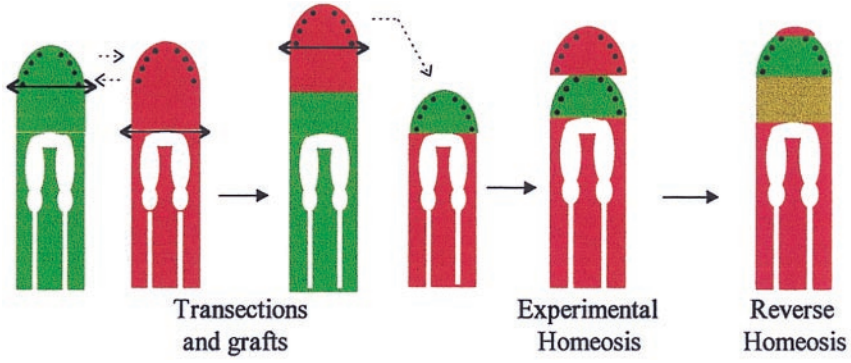

Fig. 2. Experimental design of the homeotic construction and morphogenetic regulation pattern. The homeotic construction was made in the antecerebral end by transections and grafting between two individuals of *Lineus ruber* to substitute the anterior ocellar region from the donor for the posterior postocellar region of the recipient. This resulted in an experimental homeotic chimera with a duplication of the ocellar region and a deletion of the postocellar region. Pairs of worms differing in pigmentation were chosen to distinguish host and donor tissue.

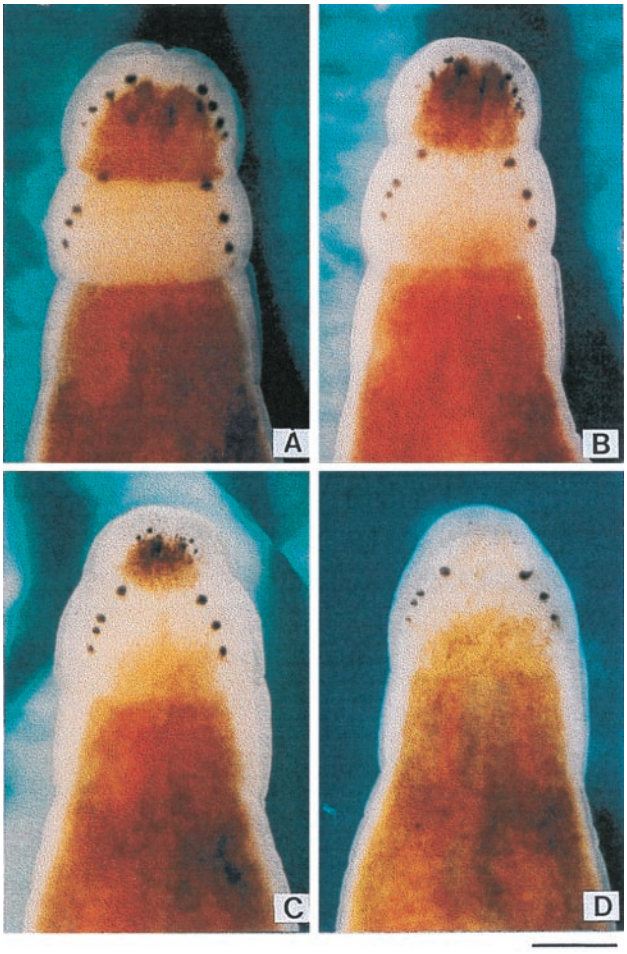

Fig. 3. Reverse homeosis in a homeotically reconstructed *L. ruber* chimera. Over a period of about 3 months, morphogenetic regulation occurred by both intercalary regeneration and compensatory transgeneration. The postocellar region was restored by intercalary regeneration behind the posterior ocellar region. The compensatory regression occurred in the anterior ocellar region leading to the degeneration of the syngeneic eyes. Pictures were taken at: 1 week (*A*), 3 weeks (*B*), 6 weeks (*C*), and 14 weeks (*D*) after the homeotic reconstruction. (Scale bar: 300 μm.)

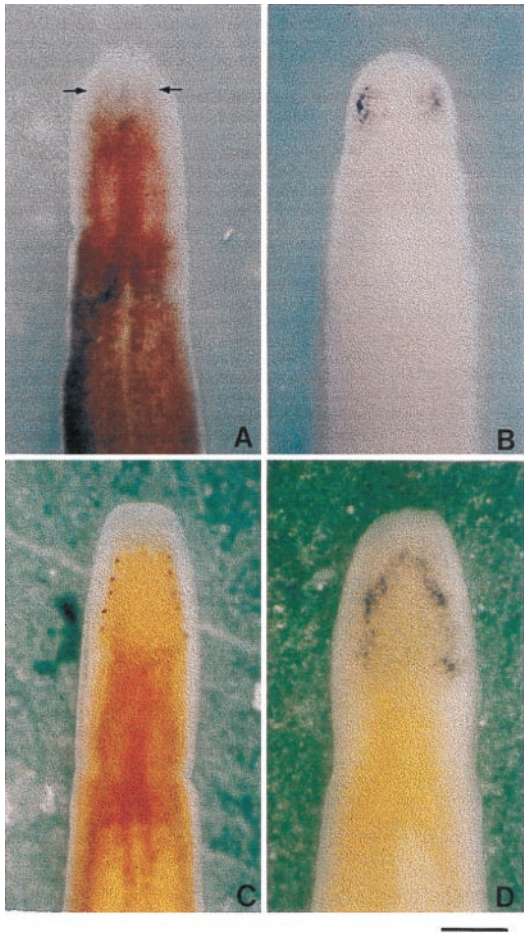

Fig. 4. Whole-mount *in situ* hybridization with digoxigenin-labeled *LsPax-6* antisense RNA probes derived from a reverse transcription-PCR clone (17). (*A*) Dorsal view of a 17-day regenerating antecerebral end of *L. sanguineus* adult. Location of the red pigment spots, which are the earliest signs of the regenerating eyes, are indicated by arrows. (*B*) *LsPax-6* expression in 17-day regenerating antecerebral end (same worm as *A*). Whole-mount *in situ* hybridization using digoxigenin-labeled *LsPax-6* anti-sense RNA probe. (*C*) Fully regenerated antecerebral end (38 days of regeneration). Five eyes are regenerated on each side. (*D*) *LsPax-6* expression after 38 days of regeneration (same worm as *C*). Whole-mount *in situ* hybridization with digoxigenin-labeled *LsPax-6* anti-sense RNA probe. The labeling is confined to the ocellar region, but extends beyond the pigment cells into the photoreceptor cells. Scale bar: 300 μm.

However, the eyes not only serve as markers but also provide an entry point for studying the genetic basis of these regeneration phenomena, because the *Pax-6* ortholog of *Lineus sanguineus*, *LsPax-6*, has recently been cloned (17), and *Pax-6* has been identified as a master control gene of eye morphogenesis. The spatial and temporal expression pattern of *LsPax-6* was analyzed during antecerebral regeneration in *L. sanguineus* by whole-mount *in situ* hybridization with digoxigenin-labeled *LsPax-6* RNA probes derived from a reverse transcription-PCR clone (17). At day 6 of regeneration, *LsPax-6* expression was first observed in the regeneration blastema of the antecerebral end as two dorsolateral spots (data not shown). At a later stage of regeneration (day 17), the two spots were clearly visible in all individuals analyzed (Fig. 4*B*). The spots are located dorsolaterally, leaving the median part of the head unstained, and the spots of *in situ* hybridization correlate well with the location of patches of red pigment, which are the earliest sign of eye regeneration (Fig. 4*A*, arrows). No staining posterior to the position of these patches was detectable; particularly in the brain, there were no signs of *LsPax-6* expression. After 5 weeks, the antecerebral end is completely regenerated and eyes have appeared also. The position of the spots of *LsPax-6* expression corresponds to those of the regenerated eyes (Fig. 4 *C* and *D*). The same staining pattern is observed in long-term regenerated heads (Fig. 4*D*) and also in intact heads of control worms (data not shown). In several heads of long-term regenerated or intact animals, additional staining was detected in a more ventral position of the antecerebral end, but the identity of the expressing cells could not be determined. The sense RNA probe, used as a negative control, did not give any staining (data not shown), indicating that the staining with the antisense probe is not because of endogenous phosphatases. These results indicate that *LsPax-6* is expressed specifically not only during eye regeneration, but also after regeneration is completed, as well as in intact ocelli. Such an expression pattern is observed in many vertebrates and suggests PAX-6 protein may have a conserved function not only in eye development but also in regeneration and maintenance of the mature eye (22–24). Previous work has shown that ectopic *Pax-6* expression in *Drosophila* can induce homeotic eyes in *Drosophila* (25–27).

Reverse homeosis in *Lineus* allows us to test the role of *LsPax-6* in developmental reprogramming during regeneration and regression as well in homeostatic maintenance of ribbonworm eyes.

Discussion

Regeneration vs. Transgeneration. In *Lineus*, terminal or intercalary deletion of sections of the body leads to terminal or intercalary regeneration restoring the positional values that were missing (20). This regeneration of the missing parts is because of epimorphosis (*de novo* formation), as in limb regeneration in amphibians (28) and insects (28, 29), and can be described by the intercalation rule (30, 31). In contrast, the morphogenetic responses to a surplus of positional values differ in arthropods, amphibians, and ribbonworms. *Lineus* is capable of downregulating a duplication of positional values, unlike cockroaches, in which intercalation of even more positional values occurs, or in axolotls or newts, in which duplication of positional values does not lead to a morphogenetic response (28). In *Lineus*, the duplication of a body region that lacks central nervous system (nerve cell bodies), like the antecerebral end, induces transgeneration and eliminates the excess of positional values (14, 20, 32). This regulatory process, accompanied by a decrease in cell number, has not, to our knowledge, been reported previously in any animal phylum. It is a unique case of morphogenetic regulation by targeted elimination of cells in an adult animal. It is a morphallactic event (developmental rearrangement) and does not convert one body region into another, as in transdifferentiation, but it reduces excess tissue positioned at the rostral end of the body.

Reverse Homeosis. The discontinuities of positional values generated in the homeotic construct (Fig. 2), a duplication plus a deletion, were rapidly reversed by both intercalary regeneration and transgeneration (local regression). Over a period of 14 weeks, complete morphogenetic regulation took place, and the normal pattern was restored. This dual capacity for both intercalary regeneration and transgeneration is the most complex reprogramming of the body plan found in any adult animal studies so far. In regenerating or regulatory systems, the behavior of boundaries is of key importance (33). In our homeotic construct, we have generated two new boundaries, discontinuities opposing cells of different positional values. Many Metazoa are capable of homeostatic maintenance of their body plan either by epimorphosis (*de novo* formation) or morphallaxis (developmental rearrangement) (28, 29). In morphallaxis, new boundaries may be formed and new positional values specified without the necessity for growth. By contrast, in epimorphosis, new positional values are generated by growth, and the boundaries play a lesser role. In *Lineus*, the two processes occur concomitantly and compensate for each other. The morphallactic events not only convert one body region into another, as usually described, but also remove excess tissue, which so far as we know has not been described in any animal, not even hydra.

The gene-regulatory mechanisms underlying reverse homeosis can now be studied, even though the genetic tools applicable to *Lineus* are still rather limited; an entry point for studying regeneration has been provided by *LsPax-6*. Whether the same Hox genes specifying the body plan in the embryo are also involved in maintenance of the adult body remains to be examined.

We thank P. Baumgartner and U. Kloter for their helpful technical advice on whole-mount *in situ* hybridization and A. Carlier and D. Brossard for technical assistance. This work was supported in part by an European Molecular Biology Organization short-term fellowship.

1. Bateson, W. (1894) *Materials for the Study of Variation* (Macmillan, New York).
2. Kanatani, H. (1958) *J. Fac. Sci. Univ. Tokyo*, Sect. 4.8 Part 2, 254–272.
3. Morgan, T. H. (1904) *Arch. Entw. Or.* **18,** 261–264.
4. Morgan, T. H. (1901) in *Regeneration* (Macmillan, New York).
5. Needham, A. E. (1965) in *Regeneration in Animals and Related Problems*, eds. Kiortsis, V. & Trampusch, H. A. L. (North–Holland, Amsterdam), pp. 283–323.
6. Gehring, W. J. (1987) *Science* **236,** 1245–1252.
7. McGinnis, W. & Krumlauf, R. (1992) *Cell* **68,** 283–302.
8. Krumlauf, R. (1994) *Cell* **78,** 191–201.
9. Garcia-Fernandez, J. & Holland, P. W. H. (1994) *Nature (London)* **370,** 563–566.
10. Carroll, S. B. (1995) *Nature (London)* **376,** 479–485.
11. Bayascas, J. R., Castillo, E. & Salo, E. (1998) *Dev. Genes Evol.* **208,** 467–473.
12. Salser, S. J. & Kenyon, C. (1994) *Trends Genet.* **10,** 159–164.
13. Kmita-Cunisse, M., Loosli, F., Bièrne, J. & Gehring, W. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3030–3035.
14. Bièrne, J. (1985) *Am. Zool.* **125,** 135–144.
15. Turbeville, J. M., Pfeifer, D. M. & Raff, R. R. (1991) *Mol. Biol. Evol.* **8,** 669–686.
16. Carranza, S., Baguna, J. & Riutort, M. (1997) *Mol. Biol. Evol.* **14,** 485–497.
17. Loosli, F., Kmita-Cunisse, M.-T. & Gehring, W. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2658–2663.
18. Wolpert, L. (1969) *J. Theor. Biol.* **25,** 1–47.
19. Wolpert, L. (1996) *Trends Genet.* **12,** 359–364.
20. Bièrne, J. (1988) in *Control of Cell Proliferation and Differentiation During Regeneration*, ed. Anton, H. J. (Karger, Basel), pp. 230–234.
21. Gibson, R. (1972) *Nemerteans*, ed. Cain, A. J. (Hutchinson University Library, London).
22. Del Rio-Tsonis, K., Washabaugh, C. H. & Tsonis, P. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5092–5096.
23. Hitchcock, P. F., Macdonald, R. E., VanDeRyt, J. T. & Wilson, S. W. (1996) *J. Neurobiol.* **29,** 399–413.
24. Macdonald, R. E. & Wilson, S. W. (1997) *Dev. Genes Evol.* **206,** 363–369.
25. Halder, G., Callaerts, P. & Gehring, W. J. (1995) *Science* **267,** 1788–1792.
26. Tomarev, S. I., Callaerts, P., Koss, L., Zinovieva, R., Halder, G., Gehring, W. J. & Piatirgorsky, J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2421–2426.
27. Glardon, S., Callaerts, P., Halder, G. & Gehring, W. J. (1997) *Development (Cambridge, U.K.)* **124,** 817–825.
28. Bryant, P. J., Bryant, S. V. & French, V. (1977) *Sci. Am.* **237** (1), 66–81.
29. Bart, A. (1988) *Development (Cambridge, U.K.)* **102,** 71–84.
30. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. (1983) *Molecular Biology of the Cell* (Garland, New York), pp. 856–870.
31. Bryant, S. V. & Muneoka, K. (1986) *Trends Genet.* **2,** 153–158.
32. Bièrne, J. (1990) *Int. J. Dev. Biol.* **34,** 245–253.
33. Wolpert, L. (1971) *Curr. Top. Dev. Biol.* **6,** 183–224.