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Analysis of the Life Cycle, Genome, and Homeo Box Genes of the Leech *Helobdella triserialis*

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Leeches, like all annelids, have a segmented body plan. The developmental basis of this segmental plan has been analyzed by cell lineage analysis of glossiphoniid leeches, beginning with C. O. Whitman's descriptions (1878, 1887, 1892) of cleavage and embryogenesis in *Clepsine* (now *Glossiphonia complanata*). More recently, Weisblat et al. (1978, 1980) showed that benign molecular markers such as horseradish peroxidase or fluorescently labeled peptides can be microinjected into one of the large precursor cells (teloblasts) of the *Helobdella* embryo to mark the progeny of that cell during subsequent development. More easily injected and detected tracers, based on dextran as the carrier molecule, have also been devised (Gimlich and Braun, 1985; Ho and Weisblat, 1987a). Lineage tracers have been used alone and in conjunction with other cell marking techniques to refine and extend Whitman's lineage studies (Weisblat et al., 1984; Zackson, 1984; Weisblat and Shankland, 1985; Kramer and Weisblat, 1985; Stuart et al., 1987; Shankland, 1987a,b,c; Braun and Stent, 1989a,b; Bissen and Weisblat, 1989). Moreover, the development of techniques that selectively perturb normal development, especially by ablating (Blair, 1983; Shankland, 1984; Shankland and Weisblat, 1984) and duplicating (Ho and Weisblat, 1987b) selected cells or cell lines in the leech embryo permit us to examine details of how *individual* cells in the *Helobdella* embryo develop, from early stages to adulthood.

The embryonic development of *Helobdella* has been divided into 11 stages based on visible morphological changes such as cell divisions and migrations, and tissue differentiation (Fig. 9.1). On fertilization (which occurs internally), a clear *vitelline membrane* lifts from the surface of the zygote. Development is arrested until the egg is laid, after which time the zygote gives off two polar bodies and then undergoes a series of stereotyped, holoblastic cleavages. The eight-cell embryo contains four micromeres and four macromeres (stage 4a).

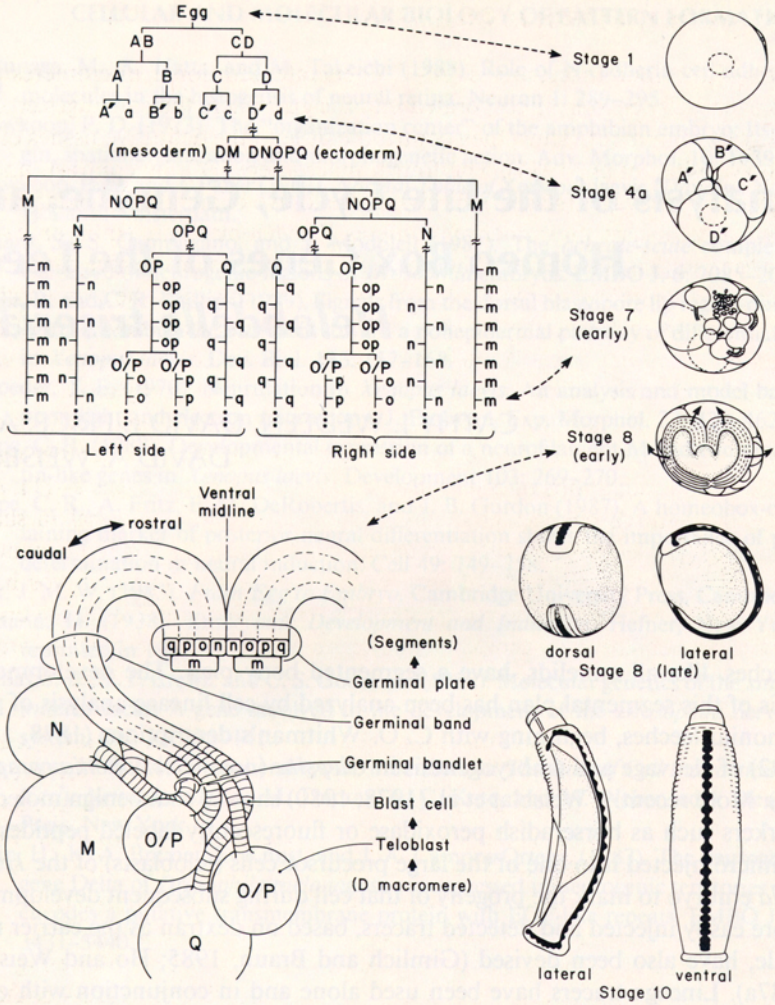

Figure 9.1. Schematic summary of development. Several stages of embryogenesis are illustrated in the right-hand margin of the figure, beginning with the uncleaved egg (stage 1). The egg contains a domain of yolk-free cytoplasm called teloplasm (indicated by the dashed circle), which is passed on selectively to the D′ macromere of the eight-cell embryo (stage 4a) and then to its teloblast progeny, M, N, O/P, O/P, and Q (medium-sized circles in the stage 7 embryo). The many small, closed contours in the upper mid-portion of the stage 7 embryo indicate the micromere cap. During stage 8, the heart-shaped germinal bands move across the surface of the embryo in the directions indicated by the arrows. An enlargement of a hemilateral portion of an embryo at this stage is shown in the lower left of the figure, indicating the relative positions of the teloblasts and their blast cell progeny. The blast cells are arranged into bandlets that join to form the germinal bands. Contralateral germinal bands merge at the future ventral midline, forming the germinal plate. In the late stage 8 embryo (right-hand margin of figure), the larval integument is shown as the stippled area lying between the germinal bands. The germinal plate is shown along the ventral midline, with the nascent ventral nerve cord and its ganglia and ganglionic primordia indicated by the filled area. The stippled larval integument covers the entire embryo, from one

Three of the four macromeres (A′–C′) generate additional micromeres and then eventually become incorporated into the gut, where they are digested during late development. Thus, they contribute little to the definitive tissues of the leech (Weisblat et al., 1984). The fourth macromere (D′), by contrast, undergoes further cleavages (stages 5–6) giving rise to five bilateral pairs of large cells known as teloblasts, plus other micromeres. The teloblasts are embryonic stem cells; each undergoes a series of highly asymmetric divisions to give rise to long columns (or *bandlets*) of *blast cells* (stages 7–8), from which the segmental tissues arise. On each side the set of five bandlets forms a curving ridge of cells called the *germinal band.* As new blast cells are formed, the germinal bands lengthen and move across the surface of the embryo, eventually coalescing rostrocaudally along the future ventral midline into a multilayered sheet of cells called the *germinal plate* (stage 8). The segments arise by further sterotyped divisions and migrations of cells within the germinal plate (stages 9–11). As a result, the embryo changes gradually from spherical to cylindrical in shape. Closure of the leech body tube occurs as the lateral edges of the expanding germinal plate fuse along the dorsal midline (stage 10). The embryos hatch from the vitelline membrane during stage 9 and attach themselves to the underside of their parent via a temporary adhesive spot near their ventral anterior end. The exhaustion of the yolk supply and maturation of the gut mark the final stage of embryogenesis, stage 11. By this time, the anterior and posterior suckers are developed and functional and the juvenile leeches use them to attach to the parent, where they remain to be carried to their first few meals.

We are interested in how genetic and environmental factors interact during development to effect the phenotypic changes summarized above. *Helobdella* is a good experimental animal for such studies for the following reasons: (1) its embryos contain accessible, identifiable cells whose fates can be precisely determined using microinjected lineage tracers, and (2) the opportunity exists to use lineage tracers in conjunction with manipulations aimed at interfering with gene expression and altering cell fate. For example, lineage tracers could be coinjected with mRNA, antisense RNA, or antibodies against a specific gene product. However, the genetics of *Helobdella* has received very little attention. In this chapter, we lay the groundwork for molecular biological studies of *Helobdella* development, by describing our studies of its life cycle, its karyotype, and its genome size. In addition, we describe our initial efforts to identify one set of genes that may be involved in regulating aspects of *Helobdella* development.

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edge of the germinal plate to the other. In the stage 10 embryo shown, body closure is nearly complete. Here, the stippled areas signify the yolky remnant of the macromeres and teloblasts, now enclosed in the gut of the embryo. The chain of ganglia linked via connectives (filled area) already closely resembles the adult nerve cord. In the upper left portion of the figure is a cell pedigree denoting the stereotyped cleavage pattern by which the teloblasts arise from the fertilized egg and how each teloblast, in turn, gives rise to the bandlets. Dashed double headed arrows connect equivalent points in each part of the figure.

LIFE CYCLE

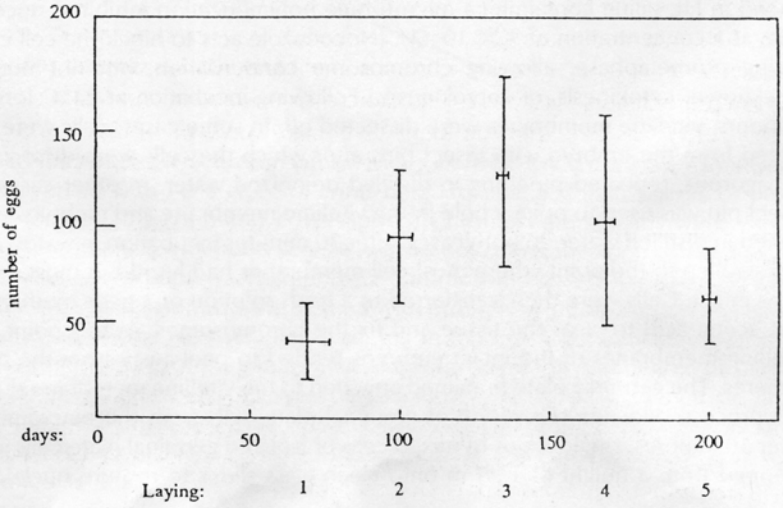
Data on the life cycle of laboratory-reared *Helobdella triserialis* were collected over the course of 1.5 years. Our colony of several thousand individuals is derived from that initiated in Gunther Stent's laboratory by Roy T. Sawyer in 1975 starting with individuals obtained from Stowe Lake in Golden Gate Park, San Francisco. The leeches were fed on small physid snails obtained from a commercial supplier (Western Scientific). Since the shipments of snails occasionally contained wild *Helobdella,* we cannot be certain that the gene pool of the colony is solely derived from the Stowe Lake population. All leeches were bred and maintained in the laboratory colony at room temperature (18–25°C) or in a 23°C incubator, in natural (Black Mountain) or artificial (1% Instant Ocean) spring water. Embryos obtained from the first laying of newly matured, presumably cross-fertilized leeches from the colony were raised in isolation to adulthood and these individuals generated progeny by self-fertilization. Three or four embryos were collected from the first laying of these self-fertilized adult leeches and were themselves raised in isolation. The embryos were maintained in HL saline (Blair and Weisblat, 1984) from the time they were isolated up through stage 9–10, at which point they were transferred to spring water. Normally, juvenile *Helobdella* remain attached to the venter of their parent or other animals until they have fed several times. Once the young leeches had reached late stage 11 (eye spots formed, both posterior and anterior suckers functional, individuals motile, yolk exhausted from the gut), they were assisted to feed several times over the course of about a week until they became proficient at feeding independently. These young leeches were followed during their maturation and as they produced successive clutches of eggs, until they died. Individual embryos were selected from these clutches and followed in the manner described above. To ensure that all animals were self-fertilized, embryos were isolated between embryonic stages 1 and 10 (well before sexual maturation). Leeches were followed for 7 successive generations, over the period of 1.5 years. The intervals from egg to first clutch and between subsequent clutches, the number of eggs per laying, and the life span of the leeches were all measured and are presented in Figure 9.2. The most layings made by any leech followed in this study was five, and the longest life span recorded was 319 days (the average life span was 241 days). The mean intervals between successive layings

Figure 9.2. Life cycle analysis. (a) Flow chart indicating the stages of the life cycle from hatching to death (central column). The mean interval between egg layings is shown in the column to the left and the mean number of eggs laid at each stage is shown in the column to the right. The number of individuals contributing to each mean is designated by *n*. The range of clutch sizes is also designated for each laying. (b) Graph of the life cycle. The data shown in (a) is summarized in graphic form. The average number of eggs produced per individual is plotted against mean times of laying (in days). Crossbars designate the standard deviations around the mean for each data point.

a

n	mean interval ±s.d. (days)	stage of life cycle	number of eggs: mean ± s.d.	range	n
		egg			
16	69±9.0				
		1st laying	43±16	(17-65)	15
15	30±5				
		2nd laying	99±36	(49-166)	13
12	32±4				
		3rd laying	133±58	(44-228)	12
8	34±5				
		4th laying	101±54	(15-198)	9
4	35±3				
		5th laying	67±26	(24-101)	6
		death			

b



were not significantly different, and combining all measurements of interlaying intervals gave a mean of 32 (± 5 SD) days. On average, the largest clutches (mean = 133 eggs) were produced in the third laying. Because most leeches in this study lived for long periods after the fifth laying without producing any more eggs, and since those individuals that laid fewer than five clutches of eggs were those that died in the interval before their next anticipated laying, we conclude that intrinsic factors normally regulate the number of clutches produced to five. The individuals examined in this life cycle study, reared in isolation and fed *ad libitum*, grew to approximately twice the maximum size of adults obtained from our standard breeding colony, and the largest clutch size (233 eggs) obtained from these experimental animals is approximately four times larger than any obtained from individuals in the colony.

KARYOTYPE

To determine the chromosome number of *Helobdella*, chromosome spreads from 48 different nuclei were counted. These 48 spreads fell into three categories. Twenty spreads (42% of the total) made up the first category of nuclei, containing exactly 36 chromosomes. An example of one such spread where 36 chromosomes were counted is shown in Figure 9.3. Of the remaining 28 spreads, 18 (38% of the total) contained fewer than 36 chromosomes, in a range from 22 to 35; 10 spreads (21% of the total) were counted as containing more than 36 chromosomes, in a range from 40 to 63. Assuming that *Helobdella* is normally diploid (but see below), we interpret these results to mean that the

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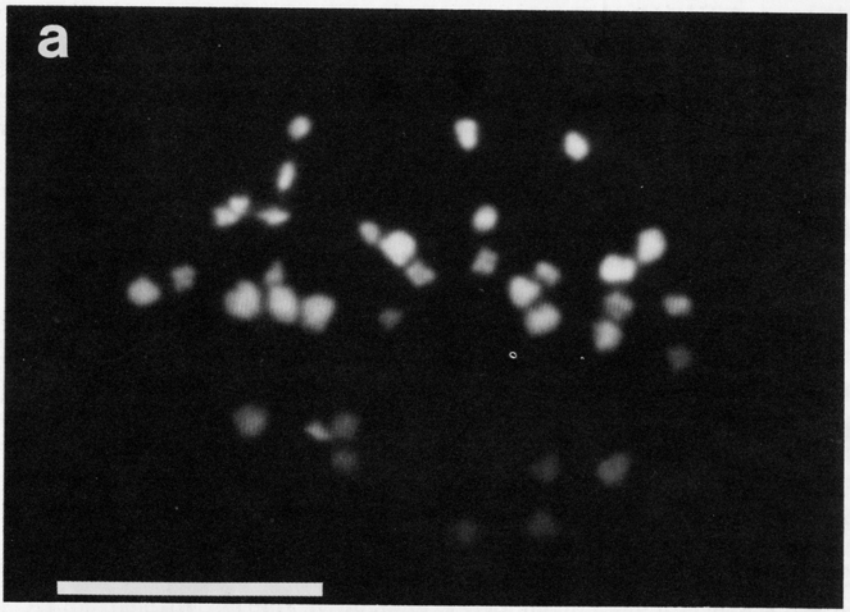
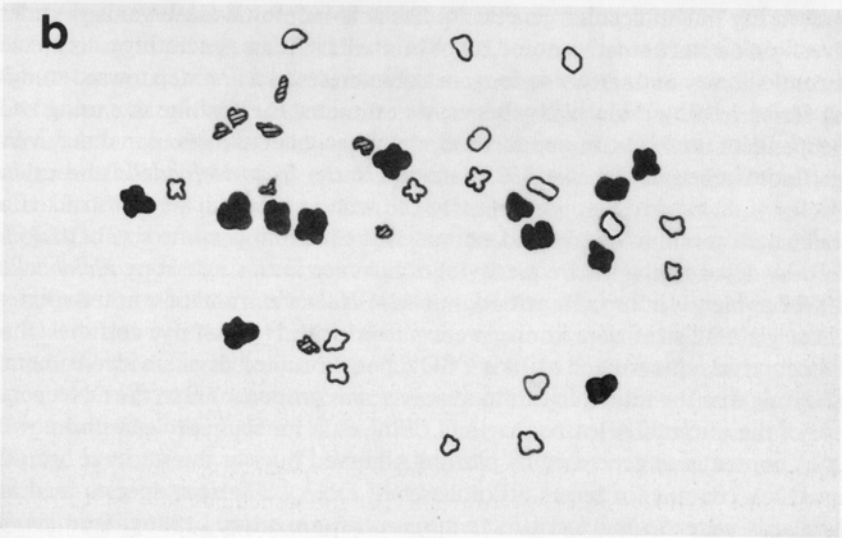
Figure 9.3. Chromosome analysis. To determine the chromosome number of *Helobdella*, we used a modification of standard procedures for karyotyping chromosomes (see Macgregor and Varley, 1983). Stage 9 embryos were used as a source of actively dividing cells. Embryos were dissected from their cocoons and placed in HL saline containing a microtubule polymerization inhibitor, nocodazole, at a concentration of $3 \times 10^{-7}M$. (Nocodazole acts to block the cell cycle during prometaphase, allowing chromosome condensation, but not nuclear breakdown, cytokinesis, or karyokinesis.) Following incubation at 24°C for 15–40 hours, vitelline membranes were dissected off. In some cases, cells were dissected from the embryo with insect pins after which the cells were dissociated by vigorous, repeated pipetting in distilled deionized water. In other cases, an insect pin was used to poke a hole in the vitelline membrane and embryos were placed in distilled water. In both cases, after 30 minutes incubation in water, cells had swollen to the point where most cell membranes had lysed but most nuclei were intact. Cells were then transferred to a fresh solution of 3 parts methanol:1 part acetic acid to clear the tissue and fix the chromosomes. At this point, the vitelline membranes of the intact embryos tended to peel away from the macromeres. The germinal plate remained attached to the vitelline membrane during this process, allowing separation of germinal plate cells from the macromeres. After 30 minutes, single drops of mixed cells or isolated germinal plate cells were dropped from a height of 1.25 m onto clean glass slides to rupture nuclei and





spread the condensed chromosomes. Slides were air-dried (for about 5 minutes), then overlaid with 70% glycerol containing Hoechst 33258 (1 μg/ml) or propidium iodine (10 μg/ml) and coverslipped. Chromosome spreads were viewed and photographed with fluorescence microscopy, using the appropriate filter set. (a) Fluorescence photomicrograph of a chromosome spread revealing 36 chromosomes. Chromosomes were stained with propidium iodide and photographed using a rhodamine filter set. The scale bar in the lower left indicates 10 μm. (b) Pen and ink tracing of the photomicrograph shown in (a). The degree of shading designates different size classes of chromosomes, loosely defined as large, medium, and small. Six pairs of chromosomes (filled) were classified as large; eight pairs (open) were classified as medium-sized; four pairs (hatched) were classified as small.

normal diploid chromosome number (2*N*) is 36, and therefore, the haploid number (1*N*) is 18. Cases were fewer chromosomes were counted could be attributed either to (1) poor spreading, in which case overlapping chromosomes might be counted as one, or (2) overspreading, in which case several chromosomes from one nuclear complement may have been separated a large distance from the major group when the nucleus burst. Cases where greater than 36 chromosomes were counted could be attributed to either of the above mentioned possibilities in combination with either of the following: (1) the chromosomes from two nuclei may have been very close or overlapping or (2) polyploid cells may have arisen in the embryo either normally or as a result of nocodazole treatment. Although we have not yet directly tested this possibility, candidates for such polyploid cells might be the macromeres or their micromere derivatives, since the macromeres are known to generate multinucleate syncytia by stage 9 of development.

GENOME SIZE

In carrying out molecular genetic studies it is helpful if the organism under investigation has a small genome, since this will facilitate generating a complete genomic library and screening for genes of interest. As a first step toward studying *Helobdella* by molecular genetics, we estimated the genome size using two independent methods. In one method, we quantitated and compared the average fluorescence of Hoechst 33258-stained nuclei from *Helobdella* and other species with known genome sizes. By the second method, we constructed a *Helobdella* genomic library and estimated the haploid genome size of *Helobdella* by determining the frequency of occurrence for a single copy *Helobdella* DNA fragment. In the first method, nuclei of *Helobdella* and of selected species whose genome sizes were known were stained with Hoechst dye and their fluorescence was quantitated using a CCD (charge coupled device) video camera, assuming that the total nuclear fluorescence was proportional to the DNA content of the nucleus. A least-squares fit of the data for four species with known DNA content was generated by plotting observed nuclear fluorescence against the DNA content (in terms of kilobases of DNA). The four species used as standards were *Strongylocentrotus purpuratus* (Davidson, 1986), *Drosophila melanogaster* (Rudkin, 1964, 1972; Rasch et al., 1971), *Caenorhabditis elegans* (Sulston and Brenner, 1974), and *Saccharomyces cerevisiae* (Lauer et al., 1977) (Fig. 9.4), and corrections to the estimates of their precise nuclear DNA values were made to take into account the exact nature of the specimens used. For example, the source of *Drosophila* nuclei for this experiment was embryos in the earliest stages of gastrulation (i.e., following cephalic furrow and ventral furrow formation but prior to germ band extension). Nuclei prior to this stage undergo rapid rounds of DNA synthesis and division without any measurable G_1 phase (Foe and Alberts, 1983). By gastrulation the length of the cell cycle has increased dramatically, but most of the increase in cell cycle duration comes from an increase in the length of G_2 and most of the cells of the early

gastrula can be found in this phase of the cell cycle (Edgar et al., 1986; B. Edgar, personal communication). Therefore, the average ploidy of these nuclei should be close to $4N$ and the average DNA content of these nuclei should be approximately $4C$ (where C is the normal haploid DNA content). In plotting the nuclear fluorescence data for *Drosophila,* we assumed that 80% of the nuclei had a DNA content of $4C$ and 20% contained $2C$. Thus, we plotted the average fluorescence per *Drosophila* nucleus against $3.6C$ [i.e., $0.80(4C + 0.20(2C)$]. For *Drosophila,* this is equivalent to 6.1×10^5 kb. Similarly, when plotting the average nuclear fluorescence of cells from gastrula stage *Strongylocentrotus purpuratus,* we assumed that there would be a mixed population of dividing cells at different phases of the cell cycle during the gastrula stage, with an average DNA content per nucleus of $3C$. The *C. elegans* data were obtained from late stage larvae and adults. Thus, most cells were not dividing and we assumed that their average nuclear DNA content was $2C$. Because stationary phase *S. cerevisiae* were used, and such cells are known to be arrested in G_1, and because a tetraploid strain was used, we assumed the average DNA content of these yeast nuclei to be $4C$.

As for the early stages of *Drosophila* development, there is evidence that in late stage 7 *Helobdella* embryos the G_1 phase represnts $\leq$20% of the average total cell cycle (Bissen and Weisblat, 1989). Thus, we assumed that the average fluorescence of the *Helobdella* nuclei also corresponded to an average nuclear DNA content of $3.6C$. Plotting the nuclear fluorescence values obtained for *Helobdella* on the least-squares regression line generated to fit the data obtained for the other four species yields an estimated average DNA content of $1.17–1.73 \times 10^9$ bp per nucleus. Thus, assuming that *Helobdella* is entirely diploid, this corresponds to a haploid genome size of $3.25–4.8 \times 10^8$ bp. However, because *Helobdella* may contain polyploid nuclei at this stage (see previous section), the fluorescence of which would have been averaged in with the nuclei of lower ploidy, this number may be an overestimate. On the other hand, the possible inclusion of polyploid nuclei in the data for certain other species would tend to decrease our estimate of the genome size for *Helobdella.* For example, yolk nuclei in *Drosophila* embryos cease dividing after cell cycle 10 and become polyploid (Foe and Alberts, 1983) and nuclei in the gut of *C. elegans* are polyploid (Hedgecock and White, 1985) and some such nuclei may have been included in the samples measured after the tissues were dissociated.

The method of estimating the DNA content of the *Helobdella* nucleus by fluorescence quantitation has several additional uncertainties associated with it. First, the efficiency of intercalation of the Hoechst dye into a DNA molecule, and, hence, the relative fluorescence of that DNA molecule, is dependent on the percentage G/C content in the DNA, which may vary from species to species. Second, the background fluorescence due to nonspecific binding of Hoechst dye and to autofluorescence at the Hoechst wavelengths may also vary from species to species, and for each species the fluorescence efficiency may vary because of changes in chromatin condensation during the cell cycle. Finally, as discussed above, the average DNA content per nucleus can be approximated only on the basis of available cell cycle data and previous deter-

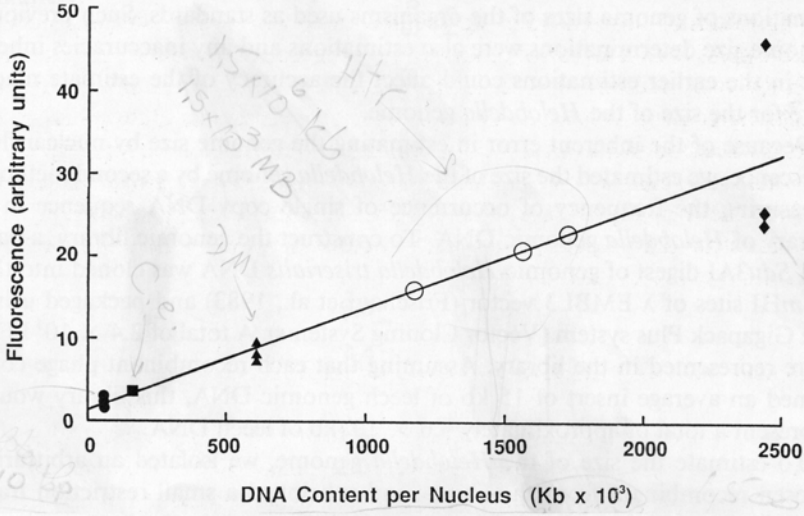


Figure 9.4. Estimation of genome size. The relative amount of DNA per nucleus was estimated by quantitating the intensity of fluorescence after staining with Hoechst 33258. Nuclei obtained from late stage 7 *Helobdella* were compared to those of early gastrulating (4–4.5 hour) *Drosophila* (Fruit Fly) embryos, gastrulating embryos of *Strongylocentrotis purpuratis* (sea urchin), mixed posthatching stages of *Caenorhabditis elegans* (nematode), and stationary phase cells from a tetraploid strain of *Saccharomyces cerevisiae* (yeast). The sea urchins and nematodes were each suspended in water for several minutes, transferred to clean glass slides, and crushed under coverslips. The coverslips were then flooded with fix (3 parts methanol:1 part acetic acid). Coverslips were removed and the nuclei were air-dried on the slides. The cells of *Helobdella* and *Drosophila* embryos were dissociated in distilled, deionized water using insect pins and vigorous pipetting. *Drosophila* embryos were first dechorionated on double stick tape (Scotch #665). They were then transferred to 3 parts methanol:1 part acetic acid and, after several minutes, placed on glass slides such that each species was repesented on each slide. After air drying, the slides were covered with 200 μg/ml RNase A in 100 m*M* Tris (pH 7.5) and incubated at 37°C in a moist chamber for 90 minutes. The slides were then rinsed in TE (10 m*M* Tris, 1 m*M* EDTA, pH 7.5) and again air-dried. Yeast cells were placed in water, then fixed in methanol–acetic acid and placed on the glass slides following the RNase treatment (since their cell membranes had not been lysed). Specimens were overlaid with 70% glycerol containing 1 μg/ml Hoechst, coverslipped, and coverslips were sealed. Slides were stored in the dark overnight before viewing under ultraviolet fluorescence using Nikon filter set UV-2A and Leitz 50× water immersion lens. Images were obtained using a CCD video camera, Pulnix model, TM-840. Images were quantitated by summing brightness over a small area containing individual nuclei and then summing the background measured from an adjacent area of equal size that did not contain a nucleus. These manipulations were achieved using a computer program, Image-1/AT (Universal Imaging Systems), to operate on the video image. The mean local background brightness was subtracted from the mean of

minations of genome sizes of the organisms used as standards. Such previous genome size determinations were also estimations and any inaccuracies inherent in the earlier estimations could affect the accuracy of the estimate made here for the size of the *Helobdella* genome.

Because of the inherent error in estimating the genome size by nuclear fluorescence, we estimated the size of the *Helobdella* genome by a second method, measuring the frequency of occurrence of single copy DNA sequence in a library of *Helobdella* genomic DNA. To construct the genomic library, a partial *Sau*3AI digest of genomic *Helobdella triserialis* DNA was cloned into the *Bam*HI sites of λ EMBL3 vector (Frischauf et al., 1983) and packaged using the Gigapack Plus system (Vector Cloning Systems). A total of 2.4×10^5 PFU were represented in the library. Assuming that each recombinant phage contained an average insert of 15 kb of leech genomic DNA, this library would represent a total of approximately 3.6×10^6 kb of leech DNA.

To estimate the size of the *Helobdella* genome, we isolated an arbitrarily chosen recombinant from the library and subcloned a small restriction fragment (a 3.9 kb *Sal*I fragment) from this phage clone into a plasmid vector (Bluescript-KS; Stratagene Cloning Systems). The resulting subclone was called pHtr-1. When a fragment of this construct was used to probe a genomic Southern blot at high stringency, it labeled a single band, indicating that the fragment represented a sequence present as a single copy in the genome (Fig. 9.5). The same fragment was then used to probe the unamplified genomic library using high stringency conditions. Out of 68,000 PFU (or, at about 15 kb of *Helobdella* genomic DNA per recombinant phage clone, approximately 10^6 kb) screened, 10 positive plaques were identified (data not shown), indicating that the size of the haploid *Helobdella* genome is around 10^5 kb. A similar estimate has been obtained by screening an amplified version of the *Helobdella* library with a different single copy sequence (I. Matsumura, personal communication).

As for the method of estimating genome size by relative nuclear fluorescence,

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the measured brightness per nucleus for each species and the difference was plotted against the estimated DNA content of the nuclei in question. The nuclear fluorescence data from the four species whose genome sizes are known (*y* axis) are plotted against the estimated average DNA content per nucleus (*x* axis). In order of ascending genome size, data points for each species are designated as follows: tetrapoloid *S. cerevisiae,* closed circle; *C. elegans,* square; *D. melanogaster,* triangle, *S. purpuratus,* diamond. Three data points for each species have been plotted, each representing the mean from at least 15 different nuclei on a single slide. The straight line represents the least-squares regression plot for the data from these four species, and three average nuclear fluorescence values for *Helobdella* were plotted as open circles along this line. The average nuclear DNA content was then estimated from the corresponding *x* axis value at these points as $1.17–1.74 \times 10^6$ kb. Assuming that the average DNA content of these nuclei is 3.6*C* (see text), we estimate the haploid (1*C*) genome for *Helobdella* by this method as $3.25–4.8 \times 10^5$ kb.

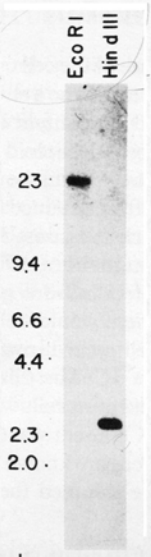

Figure 9.5. Identification of a single copy genomic sequence. Genomic DNA used in this and subsequent Southern blot analyses was prepared from adult leeches that had been starved and placed in 1% Instant Ocean containing 100 units/ml penicillin-streptomycin for 3–4 days. The DNA preparation used is a variation of a standard protocol for isolation of *Drosophila* genomic DNA (Potter and Thomas, 1977). Leeches were rinsed in 1% instant ocean, blotted dry, and frozen at −70°C. Frozen leeches were crushed to a fine powder in a porcelain mortar and pestle cooled in a bed of dry ice. The resultant powder was suspended in solution A (0.1 *M* NaCl, 30 m*M* Tris, pH 8.0, 10 m*M* EDTA, 10 m*M* 2-mercaptoethanol, and 0.5% Triton X-100), centrifuged to pellet nuclei, and the pellet was resuspended in solution B (0.1 *M* NaCl, 0.1 *M* Tris, pH 8.0, and 20 m*M* EDTA). After further centrifugation, the pellet was resuspended in a volume of solution B equivalent to the pellet volume. Sarcosyl (20%) was added to a final concentration of 1% and proteinase K (100 mg/ml) to a final concentration of 150 μg/ml. The suspension was incubated at 50°C for 4 hours. The DNA was extracted with phenol/chloroform, chloroform, and ether, and then purified on a cesium chloride gradient. This procedure yielded more than 500 μg of DNA per gram of adult leeches. Whole genomic DNA (10 μg) from *Helobdella* was digested to completion with *Eco* RI (lane 1) and with *Hin*dIII (lane 2), electrophoresed on a 1% agarose gel, and transferred to nitrocellulose. The blot was probed at high stringency [6 × SSC, 0.5% SDS, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrollidine, 10 m*M* EDTA (pH 8.0), 100 μg/ml denatured herring sperm DNA at 65°C for 16–20 hours and washed twice in 0.2 × SSC, 0.1% SDS for 15 minutes each at 68°C] with a 2.3 kb *Hin*dIII fragment isolated from the genomic subclone, pHtr-1. A single band of hybridization is detected in each lane.

there is a certain degree of uncertainty associated with the library screening method. For example, unknown inherent variations in the efficiency of cloning a particular restriction fragment, packaging a particular construct, and/or growth of a particular recombinant phage can all contribute error to this calculation.

The values obtained by the two methods used to estimate the genome size differ by less than a factor of 5. Given the numerous sources of error possible with the two methods, it is reasonable that they yield values that are not in precise agreement. It is also worth noting that, whereas the quantitative fluorescence method measures the DNA content of the nucleus, the library screening method measures the hapoid content of the genome. Thus, we cannot exclude the formal possibility that *Helobdella* somatic cells are normally tetraploid rather than diploid, and that the values obtained by these two methods are actually in close agreement.

HOMEO BOX GENES AND REVERSE GENETICS

One disadvantage in using the leech for molecular biological studies of development is that no mutant strains have been identified. But the study of mutants in *Caenorhabditis elegans* and *Drosophila melanogaster* has led to the identification of genes that play essential roles in development (reviewed in Wilkins, 1986) and a dramatic discovery that arose from the study of genes affecting the spatial organization of tissues in *Drosophila* has made it possible to identify and study putative developmental regulatory genes in the leech by a different, "reverse genetic" approach. This approach is based in part on the observation that many of these developmental regulatory genes from *Drosophila* contain highly conserved regions of about 180 nucleotides, called *homeoboxes,* which encode DNA-binding protein domains (homeo domains) of about 60 amino acids (McGinnis et al., 1984; Scott and Weiner, 1984). More than 20 different homeo box-containing genes have been identified in *Drosophila* (reviewed by Gehring, 1987; Scott et al., 1989), many of which have been shown to play a role in segment differentiation, and others of which have been shown to affect other aspects of *Drosophila* development. Although the bulk of the coding region for any of these genes bears no particular resemblance to that of any other of these genes, the homeo boxes themselves are highly conserved, i.e., with nucleotide sequences that are from 46 to 82% identical with one another. Further evidence for the fact that homeo boxes have been highly conserved in evolution is that they have been found in a very wide variety of organisms, including nematodes and mammals. The sequence identity between homeo boxes in different higher eukaryotes is also often 60% or more. As might be expected, homeo boxes have also been found in two species of leech that have been examined, *Erpobdella octoculata* and *Hirudo medicinalis* (McGinnis, 1985; Baumgarten and Macagno, 1986). Thus, the presence of homeo boxes in the leech may serve to identify a set of genes coding for developmentally important transcription factors, especially since the segments of annelids and arthropods are presumed to have arisen during evolution from those of a common ancestor. Below, we describe the use of Southern analysis to estimate the number and diversity of homeo box-containing genes in *Helobdella.*

Genomic Southern blot analysis was carried out on restricted *Helobdella* and *Drosophila* DNAs in parallel, using numerous *Drosophila* homeo box fragments as probes (see Fig. 9.6 and Table 9.1). These probes represented a wide

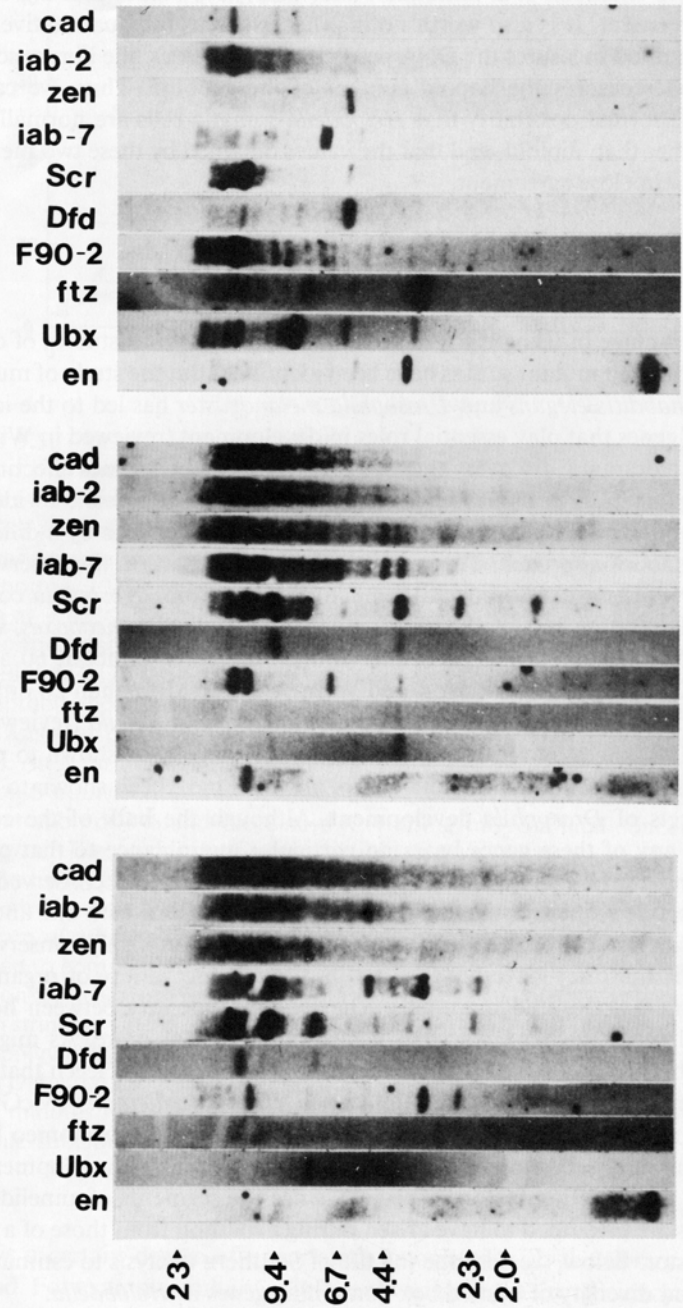
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Figure 9.6. Estimation of the number of homeo box genes. Whole genomic DNA from *Helobdella* (10 μg/lane) and *Drosophila* (1 μg/lane) was digested to completion with restriction enzymes, *Eco*RI and *Hin*dIII, and duplicate Southern blots were made according to standard methods (Southern, 1975). Nitrocellulose blots were probed with *Drosophila* homeo box probes (see Table 9.1) using identical low stringency conditions of hybridization (McGinnis et al., 1984), except in the case of the *zl* probe hybridization to *Helobdella* DNA where extra-low stringency conditions were used as follows; blots were incubated 48 hours at 37°C in 5 × SSC, 250 mg/ml denatured herring sperm DNA, 50 m*M* sodium phosphate (pH 7.0), 0.1% SDS, 11% deionized formamide, and 0.25 × BLOTTO (Johnson et al., 1984) and washed in 2 × SSC, 0.1% SDS twice for 15 minutes each at 39°C. All probes were labeled with [α-^{32}P]dCTP by nick translation (Rigby et al., 1977) to a specific activity of 5×10^7–1.5×10^8 cpm/μg and included in the hybridization buffer at a concentration of 2.0×10^6 cpm/ml. Southern blots were hybridized multiple times. Between hybridizations, blots were regenerated by washing for 5 minutes in 0.05% SDS, 0.05 × SSC at 90–95°C. (a) *Helobdella* genomic DNA digested with *Eco*RI, (b) *Helobdella* genomic DNA digested with *Hin*dIII, and (c) of *Drosophila* genomic DNA digested with *Eco*RI. The molecular weight distributions are identical in all cases since two blots made from the same gel were reused repeatedly. The molecular weight scale for (a), (b), and (c) is shown in the left margin of (a). Each lane in (a), (b), and (c) was probed with a different *Drosophila* homeo box DNA fragment, as indicated above the lane. Estimates of the homeo box gene number were based on comparisons of the original autoradiograms. In some cases, some of the faint bands visible on the autoradiograms appear less intense in the figure due to the increased contrast of the photographic reproductions.

Table 9.1. *Drosophila* Homeo Box Gene Fragments[a]

Drosophila Gene	Homeo Box Fragment	Clone and Reference
caudal (*cad* or *S67*)	450 bp *Eco*RI–*Pvu*II	pS67 (cDNA); Hoey et al. (1986)
fushi tarazu (ftz)	250 bp *Rsa*I	p523B (genomic); McGinnis et al. (1984); Laughon and Scott (1984)
even-skipped (eve)	240 bp *Sac*II–*Acc*I	p-S72 (cDNA); Frasch et al. (1987); MacDonald et al. (1986)
engrailed (en)	250 bp *Pvu*I–*Sa*II	c-2.4 (cDNA); Poole et al. (1985)
zerknüllt (*zen* or *z1*)	280 bp *Hin*dIII–*Bam*HI	zl (cDNA); Rushlow et al. (1987)
labial (*lab* or *F90-2*)	530 bp *Pst*I	pBam-2.7 (genomic); Hoey et al. (1986); Mlodzik et al. (1988)
Deformed (Dfd)	254 bp *Hpa*II	p99 (genomic); Regulski et al. (1985)
Sex combs reduced (Scr)	1.4 kb *Hpa*I–*Xho*I	pA40 (genomic); Harding et al. (1985)
Ultrabithorax (Ubx)	480 bp *Bg*II–*Cla*I	p96 (genomic); McGinnis et al. (1984)
infraabdominal-2 or *abdominal-A* (*iab-2* or *abd-A*)	1 kb *Acc*I	p93 (genomic); Regulski et al. (1985)
infraabdominal-7 or *Abdominal-B* (*iab-7* or *abd-B*)	2.5 kb *Eco*RI	pCW5 (cDNA); Wedeen (1986); Regulski et al. (1985)

[a]Fragments for hybridization probes corresponded to the homeo box genes listed in the left-hand column. The homeo box-containing fragments used as probes in this study and the clones from which they were purified are listed in the center and right-hand columns, respectively.

range of DNA sequence similarity; when compared with each other, the various *Drosophila* probes ranged from 56 to 82% similarity within the homeo box sequences. Each probe identified a set of cross-hybridizing bands in both *Drosophila* and *Helobdella,* and each probe hybridized with greater efficiency to some bands than to others. As expected, in the *Drosophila* lanes, each probe hybridized with greatest efficiency to a molecular weight band known to contain that particular homeo box and also, because of the low stringency conditions employed, with lower efficiency to other bands that presumably contain homeo box fragments of other genes (McGinnis, 1985). Two probes that hybridized well to the control *Drosophila* lanes gave no detectable cross-hybridization to the *Helobdella* lanes. These were the *even-skipped (eve)* and *zerknüllt (zl)* probes. However, when the stringency of hybridization was reduced even further, allowing 60% mismatch, the *zl* probe detected distinct bands in the *Helobdella* lanes.

A number of bands in the *Helobdella* lanes cross-hybridized with more than one *Drosophila* homeo box probe. In such cases, it would be likely that the

band contained a leech homeo box. Using this approach, we counted 16 putative homeo box genes from *Hin*dIII digests of *Helobdella* DNA and 18 such genes from *Eco*RI digests. The same analysis, carried out in parallel on *Drosophila* DNA digests that had been loaded in lanes adjacent to the *Helobdella* DNA, yielded an estimate of 21 *Drosophila* homeo box genes. This value is in agreement with current estimates for the minimum number of homeo box genes in *Drosophila* (reviewed by Gehring, 1987; Scott et al., 1989). We conclude, that there are at least 16–18 homeo box genes in *Helobdella,* and therefore that the complexity of this gene family in leeches may be about the same as in insects, or only slightly less.

There are several possible sources of error associated with the method used here to estimate the number of homeo box-containing genes in *Helobdella.* First, even though we selected *Drosophila* homeo box genes that are widely divergent in structure, there may be sequences in *Helobdella* that could be classified as homeo boxes but are different enough from the nucleotide sequence of any *Drosophila* homeo box probes that no cross-hybridization would occur under the conditions used in this study. Differences in codon preferences between *Helobdella* and *Drosophila* would be one source of homeo box sequence divergence. Second, although it seems reasonable to assume that homeo box genes in *Helobdella* will cross-hybridize at low stringency with more than one *Drosophila* homeo box, this need not always be true. For example, because of the wide diversity of homeo box sequences, even under the low stringency conditions of hybridization used in this study, each identified *Drosophila* homeo box will not hybridize to every other *Drosophila* homeo box. (Because of this sequence diversity, homeo boxes have been divided into at least 10 classes; Scott et al., 1989.) Thus, a legitimate but highly divergent leech homeo box sequence might hybridize well with a single, homologous, but equally divergent *Drosophila* homeo box, but not at all to other *Drosophila* homeo boxes. Third, if one band appears to cross-hybridize with more than one *Drosophila* probe it would be counted as one homeo box, but it could contain more than one *different Helobdella* homeo box (this is especially true for bands nearer the origin of the gel where the DNA molecules are larger and less well resolved). Finally, two comigrating bands near the origin of the gel that hybridize with non-homeo box portions of the *Drosophila* probes would be counted as one homeo box.

FUTURE PROSPECTS FOR MOLECULAR BIOLOGICAL ANALYSES OF *HELOBDELLA* DEVELOPMENT

To be optimally suited for genetic studies of development, a species should have a relatively short life cycle, be easy to culture, and have a small genome. Such properties can facilitate the generation and maintenance of mutant strains that are necessary for classical genetic studies. Two organisms, *Drosophila melanogaster* and *Caenorhabditis elegans,* have proved useful for such classical genetic analyses, in part because they possess these desired properties.

For example, the full development of *Drosophila melanogaster* takes around 14 days at 20°C and adults can lay at rates of up to 100 eggs/day for about 50 days (Ashburner and Thompson, 1978). The period from egg to egg-laying adult in *Caenorhabditis elegans* is about 65 hours at 20°C and laying rates are up to nine eggs/hour over about 60 hours (Byerly et al., 1976). The genome sizes of both species are small; that of *Drosophila* is 1.68×10^8 bp (Rudkin, 1964, 1972; Rasch et al., 1971) and that of *C. elegans* is 8×10^7 bp (Sulston and Brenner, 1974). Both species also have a small chromosome number: the haploid number in *Drosophila* is 4 (Morgan et al., 1925) and the haploid number in *C. elegans* is 6 (Brenner, 1974). Furthermore, *Drosophila* has the added advantage of very large polytene chromosomes that allow specific genes to be mapped to particular points on a chromosome.

Here we have presented a life cycle analysis of *Helobdella triserialis,* indicating approximately a 10-week period from egg to egg-laying adult. This period is about 5-fold longer than that of *Drosophila* and about 25-fold longer than that of *C. elegans.* In part because of its relatively long life cycle, the average number of eggs generated by a mature *Helobdella* individual per unit time falls far short of that for *Drosophila* or *C. elegans;* once the leech matures, even under ideal conditions it will produce only about 130 eggs per month, for about 4 months. When compared to organisms preferred for genetic studies, the egg generating capability of *Helobdella* is about 23-fold lower than that of *Drosophila* and about 50-fold lower than that of *C. elegans.* The relatively high chromosome number in *Helobdella* constitutes a further disadvantage for classical genetic studies; establishment of linkage groups and the generation of balancer stocks, for example, would be quite tedious. The combination of these properties makes *Helobdella* appear a less desirable organism for classical genetic studies. One point in favor of leeches as organisms for genetic studies is that they are hermaphroditic and capable of both self- and cross-fertilization. The capacity for self-fertilization could aid in establishing homozygous stocks, should mutants be generated. Moreover, despite the length of the life cycle and the large chromosome number of *Helobdella,* which make it suboptimal for classical genetic analyses, certain other properties make this leech a quite desirable subject for molecular genetic studies of development.

The major advantage of using *Helobdella* for studies of development has been that its embryo comprises large, identifiable, and physiologically accessible cells whose fates are highly predictable in normal development. These cell lineages have been examined in increasingly fine detail (Weisblat et al., 1984; Zackson, 1984; Kramer and Weisblat, 1985; Weisblat and Shankland, 1985; Torrence and Stuart, 1986; Shankland, 1987a,b,c; Bissen and Weisblat, 1987, 1989; Braun and Stent, 1989a,b) and appear to exhibit the degree of stereotypy and also the same degree of plasticity as those of the nematode. Hence, the development of *Helobdella* is relatively well understood and is highly approachable at the cellular level. The fact that tracers and other substances can be readily injected into individual cells in the early *Helobdella* embryo makes a molecular genetic study of development in this organism an attractive

proposition. As we report here, *Helobdella* has additional advantages as a subject for molecular genetic studies as well. First, its genome size is quite small, in fact quite close in size to that of *Drosophila.* And second, although its high chromosome number may be a disadvantage for classical genetics, when combined with the small genome size it means that the chromosomes themselves are quite small. Assuming that *Helobdella* is diploid, with a haploid genome size on the order of $1–4.8 \times 10^5$ kb, and a haploid chromosome number of 18, an average chromosome would contain approximately 5,600–26,700 kb, in the size range of chromosomes that might be separated by pulsed-field electrophoresis. For example, this technique has already been used to separate the chromosomes of *Schizosaccharomyces pombe,* the largest of which is on the order of 9000 kb (Smith et al., 1987; Turmel and Lalande, 1988). Given the small size of the *Helobdella* chromosomes, pulsed-field electrophoresis might ultimately be utilized for mapping genes or gene complexes to their particular chromosome, or for separating chromosomes to construct single chromosome libraries. In addition, this method might help to resolve the question of the ploidy of *Helobdella,* since homologous chromosomes should comigrate in a single band.

Many of the problems of initiating a molecular genetic study of development in *Helobdella* can be circumvented by taking advantage of molecular genetic advances in other species. A large number of genes have been identified that affect early embryonic development in *Drosophila.* A number of these genes have been shown to regulate aspects of segmentation along the anterior–posterior and dorsal–ventral body axes and a certain subset of these genes contains homeo boxes. In *Helobdella,* homeo boxes may also serve as landmarks for developmentally important genes. We have estimated that there are 16–18 such genes in *Helobdella* and we are now cloning these potentially important leech homeo box genes.

In the future, as these homeo box genes become available as molecular probes, the same properties that have made *Helobdella* desirable for studying developmental biology could be utilized to study the possible functions of homeo box genes in *Helobdella* development. The very properties that render *Drosophila* and *Caenorhabditis* well-suited for genetic analyses are accompanied by small cell size and rapid embryonic development, which complicate certain types of cellular manipulations that are useful for carrying out developmental analyses. For molecular genetic studies, *Helobdella* has the advantage of a relatively small genome. Moreover, the moderately slow pace of its embryogenesis means that developmental processes can be examined with a high degree of temporal resolution. Although it has neither large polytene chromosomes nor, at this point, any identified mutant strains, *Helobdella* offers advantages for combining cellular and molecular studies of development, since the embryo contains large cells that can be injected individually with lineage tracers or other substances. The presence of numerous homeo box genes in *Helobdella* should allow us to identify genes of developmental importance, and so examine the genetic control of development in this organism.

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