Quantitative assessment of *Hox* complex expression in the indirect development of the polychaete annelid *Chaetopterus* sp.

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A prediction from the set-aside theory of bilaterian origins is that pattern formation processes such as those controlled by the *Hox* cluster genes are required specifically for adult body plan formation. This prediction can be tested in animals that use maximal indirect development, in which the embryonic formation of the larva and the postembryonic formation of the adult body plan are temporally and spatially distinct. To this end, we quantitatively measured the amount of transcripts for five *Hox* genes in embryos of a lophotrochozoan, the polychaete annelid *Chaetopterus* sp. The polychaete *Hox* complex is shown not to be expressed during embryogenesis, but transcripts of all measured *Hox* complex genes are detected at significant levels during the initial stages of adult body plan formation. Temporal colinearity in the sequence of their activation is observed, so that activation follows the 3′–5′ arrangement of the genes. Moreover, *Hox* gene expression is spatially localized to the region of teloblastic set-aside cells of the later-stage embryos. This study shows that an indirectly developing lophotrochozoan shares with an indirectly developing deuterostome, the sea urchin, a common mode of *Hox* complex utilization: construction of the larva, whether a trochophore or dipleurula, does not involve *Hox* cluster expression, but in both forms the complex is expressed in the set-aside cells from which the adult body plan derives.

Much speculation on animal body plans has focused on the *Hox* gene cluster, because of its remarkably conserved genomic structure and the powerful effects of *Hox* genes on development (e.g., ref. 1). Until recently, however, *Hox* gene utilization had been studied exclusively in direct developing systems, mainly *Drosophila* and vertebrates. These studies have shown that one of the major roles of *Hox* complex genes is assignment of positional values along the anterior-posterior axis of the developing body plan (e.g., refs. 2 and 3). The similar utilization of the *Hox* gene cluster in *Drosophila* and vertebrates has led to the notion that the *Hox* gene cluster is the defining feature of animals, absolutely required for both their development and evolution (4).

In all bilaterians so far studied, expression of the *Hox* complex genes is associated with regional specification processes underlying construction of the adult body plan (5, 6). In general, the function of these genes is to control patterning processes by affecting the expression of other genes that execute spatial regulation in development (e.g., ref. 7). In the "set-aside cell" theory of bilaterian origins (6), it was proposed that early stem-group bilaterians were a small microfauna of relatively simple construction, which developed solely by Type 1 embryogenesis (8), and that the development of this microfauna would not have required the kinds of pattern-formation processes in which *Hox* genes participate. Evolution of the adult body plans of modern organisms of bilaterian grade must have involved the appearance of a much more complex regulatory apparatus. Such an apparatus would have included the complete *Hox* gene set, and co-evolving with more advanced patterning mechanisms would have been populations of cells "set aside" from embryonic specification events, to which these mechanisms could be productively applied (ref. 6; see ref. 9, which is in this issue of PNAS). A modern surrogate for the hypothetical stem-group bilaterian microfauna is at hand in the larvae of indirectly developing forms. The prediction followed that expression of the *Hox* gene complex would not be required for formation of the free-living larvae of indirectly developing bilaterians, but only for formation of the adult body plan to which the larval set-aside cells give rise in postembryonic development (6). This idea was tested and indeed confirmed in an indirectly developing sea urchin, *Strongylocentrotus purpuratus*. Development of the pluteus larva of *S. purpuratus* cannot require the utilization of the *Hox* gene cluster because most *Hox* genes are not significantly expressed at all during its development (10). Activation of the *Hox* cluster genes in *S. purpuratus* occurs during the initial stages of adult body plan formation in the feeding larva, in which their transcripts are localized primarily in the progeny of endomesodermal set-aside cells (C. Arenas-Mena, R.A.C. and E.H.D., unpublished data). However, this could be a peculiarity of indirectly developing echinoderms, and the generality of this mode of *Hox* cluster utilization is important: it should be a general property of indirectly developing larvae if the ancestors of all bilaterians developed in a similar fashion. To broaden the phylogenetic scope of the argument, we have now examined *Hox* cluster usage in the embryogenesis of an indirectly developing protostome. The protostomes are monophyletic (11), and, because all of the ecdysozoan protostomes are direct developing (12, 13), this study was carried out on a lophotrochozoan, specifically a polychaete annelid.

The phylum Annelida consists of two major groups: namely, the marine polychaetes and the marine, freshwater, and terrestrial clitellates (i.e., the oligochaetes including earthworms, and leeches; see refs. 14 and 15 for reviews of annelid anatomy and development). Although all clitellates are direct-developing, many of the marine polychaetes still undergo maximal indirect development by means of a feeding trochophore larva (16). One such polychaete is *Chaetopterus* sp. (ref. 17; in this work, we used the same species of *Chaetopterus* as studied by these workers). In *Chaetopterus* a simple "prototrochophore" is produced at the end of a spiralian developmental program that, although somewhat modified, includes definitive features such as generation of a 4d cell (at about 4.5 h), and subsequent formation of endomesodermal germ bands (17–19). By 9 h the embryo is completely ciliated, and by 12 h the animals are swimming. By 18 h the "L1" larval stage is reached, and larval-specific structures such as the apical tuft have appeared. It is at this stage that development of the adult body plan begins. By 36 h the "L2"

Abbreviation: WMISH, whole-mount *in situ* hybridization.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF223402).

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(metatrochophore) stage is attained, and a tripartite gut is formed. Feeding commences by 72 h (the "L3" larval stage). The larvae have now produced a trochal band of cilia, which aids in feeding, and adult body plan formation continues for the next two months. Irvine and colleagues had cloned much of the *Hox* gene complex in this species (20) and have recently demonstrated that the initial locus of expression of the five 3′ *Hox* genes is in the region of the teloblastic endomesodermal cells (21). Our objective here was to measure quantitatively the amount of *Hox* gene transcripts throughout the first 48 h of development, including the period before the initiation of adult body plan formation.

Materials and Methods

Obtaining Gametes and Collection of Embryonic Total RNA. Adult *Chaetopterus* sp. were obtained from Cape Fear Biological Supply Company (Southport, NC). The adult worms were removed from their tubes, were sexed according to the morphology of their gonads, and were placed individually in finger bowls at 15°C for at least 24 h with at least two changes for fresh sea water. Collection of eggs and sperm followed the protocol of Eckberg and Anderson (22). In brief, oocytes were obtained by snipping the parapodia near the proximal end and manually pipetting the oocytes and parapodial material up and down to release the oocytes from their surrounding tissue. The oocytes were then filtered from the surrounding tissue, first by using a 150-μm nylon mesh, then through two layers of cheesecloth, and finally were washed three times in sea water. Germinal vesicle breakdown was manually checked on a Zeiss Axioskop microscope equipped with DIC optics and was usually apparent after about 30 min. Ripe sperm were obtained by snipping a male parapodium. About 100 μl of dry sperm were diluted into 100 ml of sea water. The sperm were allowed about 10 min for activation, which was also checked on the same Zeiss microscope. Approximately 1,000–1,500 μl of the sperm dilution were added to the egg suspension (usually around 1×10^7 oocytes in 300 ml). The mixture was checked and adjusted so that a 10:1 ratio of sperm to egg was achieved. After about 15 min, the eggs were gravity-washed twice, were transferred to 1-liter glass jars, and were stirred vigorously at 23–25°C.

Total RNA was prepared from various embryonic and early larval stages by using RNAzol B (Leedo Medical Laboratories, Houston, TX). Approximately 1 ml of embryonic or larval material was obtained from the culture and was homogenized in 40 ml of RNAzol B. Total RNA was isolated by using the manufacture's protocol.

Preparation of Probes and Probe Excess Titrations. Probes for *Hox1-Hox5* and β-tubulin are described in the works by Irvine and Martindale (21) and Irvine *et al.* (17). A 353-bp fragment of the *Brachyury* cDNA (*CH-Bra*) was obtained by using "touchdown" reverse transcription–PCR (23) in which 6-h *Chaetopterus* embryo cDNA served as template, in the presence of 2.0 mM $MgSO_4$. After an initial denaturation at 96°C, the reactions were "hot started" at 80°C by using 2.5 units of AmpliTac DNA polymerase (Perkin–Elmer). Annealing began at 52°C for two cycles (94°C for 1 min, 72°C for 1 min), then 51°C for another two cycles, and continued with a drop of one degree every two cycles until 40°C was reached. The primers were designed against highly conserved portions of the T-box: forward, WKYVNGEW; reverse, NPFAKAF. Secondary PCR consisted of 30 cycles of 94°C for 1 min, 52°C for 2 min, and 72°C for 1 min using 5 μl of the primary PCR as the template and 2.5 mM $MgSO_4$. The PCR product was gel-excised and cloned into a pGEM-T vector (Promega) following the manufacturer's instructions. Sequencing was done by Applied Biosystems PRISM dye terminator cycle sequencing, using an Applied Biosystems 373 automatic sequencer. *Brachyury* orthology was determined by phylogenetic analysis using maximum parsimony [PAUP 3.0s (24)]. The GenBank accession number for the partial coding sequence for *CH-Bra* is AF223402.

Single-stranded riboprobes were made by using Riboprobe *in vitro* Transcription Systems (Promega) using [^{32}P]UTP at 400 Ci/mmol (Amersham) following the manufacturer's instructions. Linearization of the template and probe excess RNA titrations were performed as described (25) except for the following details. Increasing amounts of total RNA from each developmental stage were analyzed (0, 6, 12, 24, and 48 h of development) and sufficient yeast tRNA to a total of 100 μg were coprecipitated overnight at −20°C in 2.5 V ethanol with 0.05–1.00 ng of purified riboprobe in the presence of 0.2 M ammonium acetate. The precipitated RNA was pelleted and was dried for 30 min in a 37°C water bath. The hybridization was performed in 20 μl of 50% formamide, 0.4 M NaCl, 1 mM EDTA, and 8 mM Pipes (pH 6.8) at 55°C for at least 18 h in a hybridization oven. Unhybridized RNA was removed by digestion with 300 μl of 500 unit/ml RNase T1, 216.7 μg/ml RNase A, and 2.5× SET [1× SET = 0.15 M NaCl, 30 mM Tris (pH 8.0), and 2 mM EDTA] at 37°C for 1 h. The precipitated RNA was collected, and the amount of radioactive RNA was determined according to Arenas-Mena *et al.* (10).

Number of Cells per Embryo, and Amount of Total RNA per Embryo. For quantification of cells/embryo and total RNA/embryo at different stages, we used RNA STAT-60 (Leedo Medical Laboratories), which allows for the isolation of both total RNA and genomic DNA in the same preparation. Ten thousand embryos at each embryonic stage were counted out by means of a dilution series and were homogenized in RNA STAT-60. One-hundred thousand cpm of radioactive labeled riboprobe were then added to the homogenate as a recovery and contamination standard. Total RNA was isolated according to the manufacture's protocol. A sample of the dissolved total RNA preparation was counted with a scintillation counter to determine loss, and the amount of total RNA was determined by UV spectrophotometry. Genomic DNA was then extracted from the same preparation; the amount of DNA from each stage was determined by UV spectrophotometry, and the amount of RNA contamination was assessed by scintillation counting. The procedure was repeated with another 10^4 embryos homogenized in RNA STAT-60, but this time with a known amount of radioactive labeled DNA. A 1.7-kb fragment of DNA was random labeled with [^{32}P]dCTP (Pharmacia), and 10^5 cpm of the labeled DNA was added to the homogenate. The procedure was repeated, but this time allowing for the determination of genomic DNA loss, and the amount of DNA contamination in the RNA preparation. These controls showed that the amount of RNA and DNA cross contamination was minimal and that the amount of RNA and DNA loss was approximately 20%. We determined that there is about 6.5 ng of total RNA per embryo with no significant addition or loss of total RNA during the first 48 h of development (i.e., before feeding commences at 72 h). The number of cells/embryo at each stage was calculated, given the diploid genome size of 2×10^9 bp (26).

Whole Mount *in Situ* Hybridizations. The whole mount *in situ* hybridizations (WMISH) shown in this paper were generated in the course of the comprehensive study of *Hox* gene expression in *Chaetopterus* by Irvine and Martindale (21). For this work, some of the stained embryos they had prepared for WMISH were sectioned, and then photographed, following Peterson *et al.* (27).

Results

Irvine and Martindale (21) previously showed by WMISH that the first five 3′ *Hox* genes (*CH-Hox1*, *-2*, *-3*, *-4*, and *-5*) are all

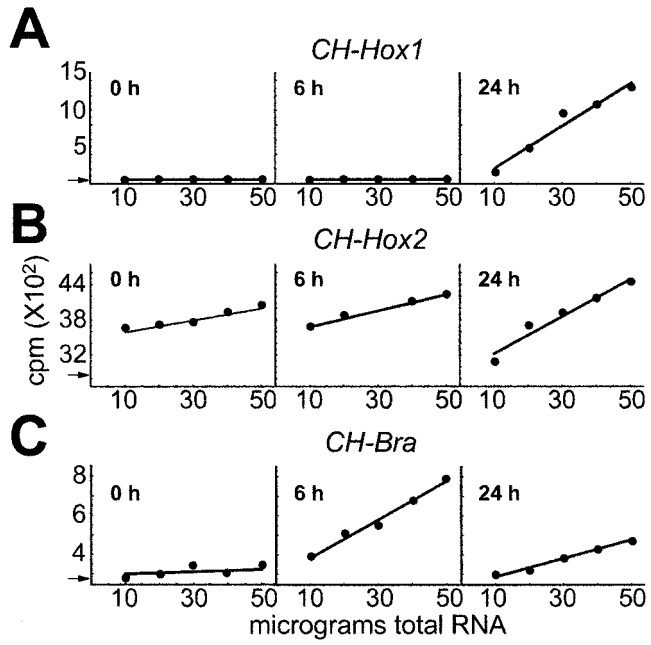

Fig. 1. Single-strand probe excess titrations for transcripts of two *Hox* genes and the *Brachyury* gene in the polychaete worm *Chaetopterus*: *CH-Hox1* (*A*), *CH-Hox2* (*B*), and *CH-Bra* (*C*). These examples are representative of the data sets that provided the measurements reported in this paper. Total RNA was extracted from unfertilized eggs and embryos at the indicated stages and in increasing amounts reacted with ^{32}P-labeled antisense RNA probes (see ref. 21 and *Material and Methods* for probes and procedures). Mean background levels (averages of two measurements on samples containing yeast RNA alone) for each probe are indicated with an arrow: for *CH-Hox1*, 38 and 41 cpm; for *CH-Hox2*, 2,792 and 2,889 cpm; for *CH-Bra*, 236 and 239 cpm.

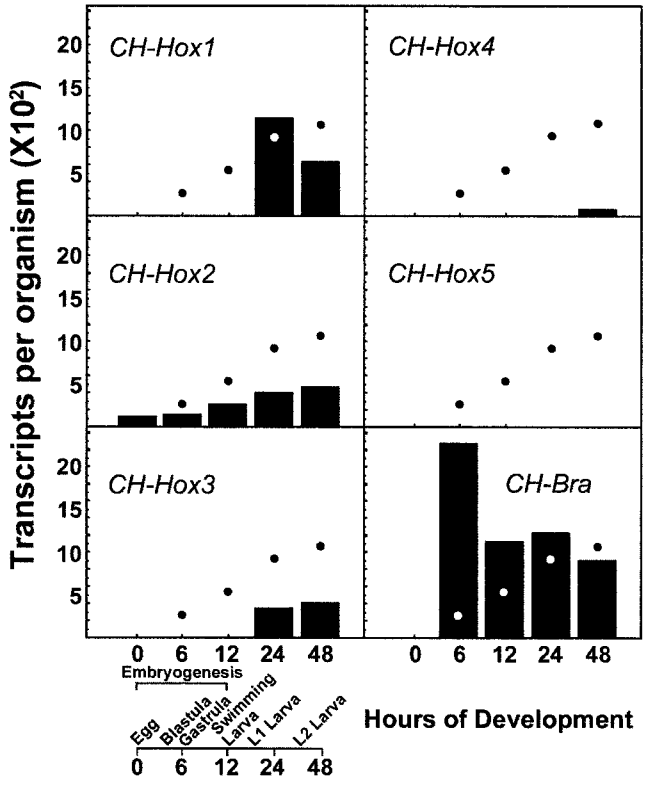

Fig. 2. Transcripts of *Hox* cluster genes and the *Brachyury* gene, per embryo or larva. Histograms indicate transcript numbers for the indicated stages of embryogenesis: 0 h, unfertilized eggs; 6 h, early gastrula; 12 h, swimming prototrochophore; 24 h, L1 larva; and 48 h, L2 larva. The dots indicate the number of cells per organism (same ordinates). Each panel represents a single data set. Transcript numbers were calculated from the slopes as described (ref. 25; see Fig. 1), taking into account the amount of total RNA per embryo (6.5 ng; see *Materials and Methods*) and the lengths and specific activities of the probes. *Hox* probes are from Irvine and Martindale (21); the *Brachyury* probe is described in *Materials and Methods*.

expressed in the region of the teloblastic progeny by 3 days of larval development (initiation of feeding). However, embryonic stages were not analyzed. To determine *Hox* gene usage in embryogenesis, we measured the number of transcripts of each of the five *Hox* genes in the RNA of embryos and early larvae by using single-stranded probe excess titration (25). This is the method of choice for accurate determination of the number of transcripts present, because it can reliably detect <0.05 mRNA molecules per average embryonic cell, and because hybridization is kinetically independent of the level of expression, depending only on the probe concentration. Furthermore, the method is impervious to any but the most severe RNA degradation (10, 25). Transcript numbers were calculated from probe excess titrations by using antisense RNA probes specific for each *Hox* gene (21). For controls, we used the β-tubulin gene (17), which is expressed copiously throughout development, and the *Brachyury* gene. The *Brachyury* gene expression profile has been determined in other maximal indirect developers: namely, in echinoid and asteroid echinoderms (refs. 27 and 28; K.J.P., unpublished data) and in an enteropneust hemichordate (29). The same *Chaetopterus* embryo RNAs were used for *Hox* gene, *Brachyury*, and tubulin transcript determinations.

Representative titration data are shown for *CH-Hox1*, *CH-Hox2*, and *CH-Bra* in Fig. 1. Transcript numbers per unit mass RNA and per embryo are directly proportional to the absolute slopes in each data set. As illustrated in Fig. 1*A*, *CH-Hox1* is not expressed significantly at any embryonic stage; the slopes remain close to 0 until 24 h of development (L1 stage larva). In contrast, transcripts for *CH-Hox2* were detected in all RNA preparations, the amount of total transcripts increasing as development progressed (Fig. 1*B*). Finally, although *CH-Bra* was not detected in the maternal RNA preparation, *CH-Bra* was found to be copiously expressed by 6 h of development (Fig. 1*C*). Expression then gradually decreases as development continues. Correlation coefficients for these titration curves were $>90\%$ (most were $>95\%$) for all of the measurements reported in this paper, except where the slopes of the titration measurements were close to zero.

Results for all five *Hox* genes and for the *Brachyury* gene are summarized in Fig. 2. The ordinates show the number of transcripts per embryo for each gene at the indicated stage of development. The dots in Fig. 2 indicate the total number of cells at each stage measured, read off the same ordinates, and thus the number of transcripts required for a single molecule per average cell. As in all free-living embryos before the onset of feeding (30), the mass of total RNA, and the amount of mRNA, remain essentially constant throughout the period to which these measurements pertain. The measurements shown in each panel in Fig. 2 were carried out simultaneously on the whole set of RNA preparations with the same probe preparations. Similar results were obtained with several different RNA preparations. Except for *CH-Bra*, the same probe preparations were used for the WMISH results reported by Irvine and Martindale (21) and Irvine *et al.* (17).

Expression of the *Chaetopterus Hox* Gene Complex in Embryos and Larvae. The main result illustrated in Fig. 2 is that *CH-Hox1*, *CH-Hox3*, *CH-Hox4*, and *CH-Hox5* are not expressed signifi-

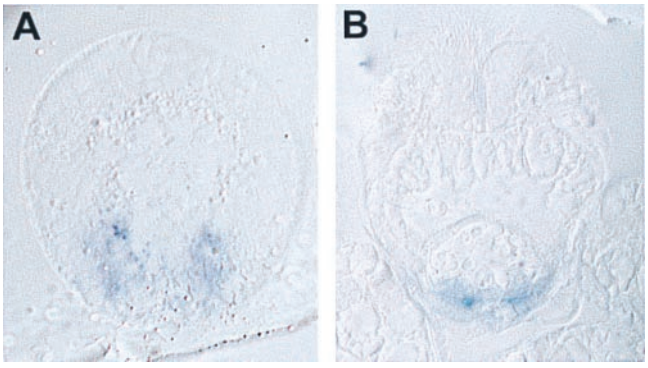

Fig. 3. *Hox* gene expression in larvae of *Chaetopterus*: *CH-Hox2* (*A*) and *CH-Hox4* (*B*). Plastic thin sections were made from WMISH prepared by Irvine and Martindale (21). (*A*) *CH-Hox2* expression pattern in an L1-stage larva sectioned horizontally with the anterior end upward. The cells expressing the gene are in the posterior region occupied by the teloblasts, which generate much of the adult endomesodermal derivatives that constitute the body wall of the segmented trunk. (*B*) *CH-Hox4* expression in an L2-stage larva. The plane of section is slightly oblique to the longitudinal axis, and the gut appears almost in cross section. As for *CH-Hox2*, the cells expressing the *CH-Hox4* gene product appear to be the progeny of the endomesodermal teloblastic set-aside cells.

cantly at any stage of embryogenesis (here defining embryogenesis as the period before the L1 larval stage). The only *Hox* gene that is expressed during embryogenesis is *CH-Hox2*. Its expression pattern is also unusual in that *CH-Hox2* transcripts are present maternally as well. The expression of the other *Hox* complex genes does not begin until around 24 h of development (L1 larval stage), when the progeny of the teloblastic set-aside cells begin proliferating. This can be regarded as the initial step in formation of the adult body plan itself, and it occurs at the same time as the activation of the *Hox* gene complex. At 24 h, *CH-Hox1* is abundantly expressed at around 1,100 transcripts per embryo; *CH-Hox2* at 400 transcripts per embryo; and *CH-Hox3* at 350 transcripts per embryo. However, *CH-Hox4* and *CH-Hox5* transcripts are not detected at 24 h of development. By 48 h of development (L2 larval stage) *CH-Hox1* expression has dropped to 640 transcripts per embryo. The level of *CH-Hox2* and *CH-Hox3* transcripts increased slightly, to 480 and 400 transcripts per embryo, respectively. *CH-Hox4* expression is now barely detected at 80 transcripts per embryo, but *CH-Hox5* transcripts are still not detected.

Expression of *Brachyury*. In contrast to the *Hox* genes, *CH-Bra* shows a very different pattern of transcriptional activity (Fig. 2). As in early sea urchin development (K.J.P., unpublished data) the maximum level of expression in *Chaetopterus* is around the time of gastrulation. There are 2,290 *Brachyury* transcripts per embryo at 6 h of development, significantly more than for any of the *Hox* genes studied. This number gradually decreases until there are only about 900 transcripts detected at 48 h of development.

Spatial Localization of *Hox* Transcripts. Irvine and Martindale (21) have previously discussed in detail the spatial expression profiles of each *Hox* gene in *Chaetopterus* larvae. By examining sectioned material, we have confirmed their suggestion that expression of the *Hox* complex in larvae is limited to the region of teloblastic set-aside cells, as illustrated in Fig. 3. In Fig. 3*A* is shown a section of an L1 larva hybridized with the *CH-Hox2* probe, and in Fig. 3*B* is an L2 larva hybridized with the *CH-Hox4* probe. It is clear that the labeling is restricted to the posterior cell mass on either side of the nascent gut. Although lineage marking studies are clearly required to confirm this supposition, the bilaterial posterior position of the labeled regions suggests that the cells initially expressing the *Hox* complex are those of the endomesodermal teloblasts, the derivatives of the 4d blastomere (also see ref. 21).

Discussion

The reliability of the results presented in this paper depends on the quality of both the probes and the total RNA used. However, our measurements are qualitatively consistent with the pattern of expression measured by WMISH (21). Both studies show that *CH-Hox1* and *CH-Hox2* are expressed at higher levels earlier in ontogeny than are *CH-Hox3*, *-4*, or *-5*. They further agree in demonstrating that, by around 48 h of development, *CH-Hox4* is expressed at a lower level than is *CH-Hox3*, but higher than *CH-Hox5*, the expression of which cannot be detected via WMISH for at least another 12 h (21). In fact, each of the qualitative observations of Irvine and Martindale (21) is supported by the values reported in this paper (Fig. 2). Thus, it can be safely concluded that, where no *Hox* gene expression is observed, there are no, or almost no, transcripts present. The two-fold higher expression level of *CH-Hox1* with respect to *CH-Hox2* at 24 h probably reflects the fact that there are two spatial domains of *CH-Hox1* expression: namely, the teloblastic set-aside cells plus the boundary between the foregut and midgut (21). A minor exception to the general convergence between the two data sets is the decrease of *CH-Hox1* expression between 24 and 48 h of development, which was not predicted by the available WMISH data.

The same RNA preparations yield qualitatively different expression profiles for different kinds of genes. Thus, the peak of *CH-Bra* expression is at 6 h development, and expression gradually decreases throughout the development stages analyzed. As in the sea urchin (K.J.P., unpublished data) *CH-Bra* is not maternally expressed. It is important to note that the low levels of *CH-Hox2* transcripts detected at 0 h cannot be attributable to problems with the maternal RNA preparation. Thus, we detect around 8.9×10^4 transcripts of β-tubulin in the 0 h preparation; there are 4.1×10^4 transcripts at 6 h, and 4.1×10^5 transcripts by 12 h of development (results not shown). As expected, there are differences of one to two orders of magnitude in the amounts of transcripts representing this cytoskeletal gene, compared with the amounts of transcripts encoding the two different classes of transcription factors: namely, the *Brachyury* and *Hox* regulators.

***Hox* Gene Expression and Temporal Colinearity.** When the data reported in this paper are combined with the expression data reported by Irvine and Martindale (21), it appears that, except for the expression of *CH-Hox2* during embryogenesis, the five 3′ *Hox* genes obey a temporal colinearity rule. Temporal colinearity is the general phenomenon associated with the sequential activation of *Hox* genes from 3′ to 5′ (or anterior to posterior) (see ref. 31 for review). In the polychaete *CH-Hox1* transcripts are detected at around 18 h of development with WMISH, but *CH-Hox3*, *-4*, and *-5* transcripts are not detected. By 24 h, *CH-Hox3* transcripts are detected by probe excess titration. By 48 h of development *CH-Hox4* transcripts are detected, but *CH-Hox5* expression is still not observed. By WMISH expression of *CH-Hox5* begins soon after, but remains relatively weak (21). In sum, the sequence of expression profiles in the 24- to 48-h period of development parallels the assumed chromosomal arrangement of the genes, and so also does the amplitude of expression measured in terms of transcripts per embryo. Thus, *CH-Hox1* > *-3* > *-4* > *-5* (see Fig. 2).

The expression of *CH-Hox2* is anomalous. *CH-Hox2* apparently has an embryonic role, as its transcripts are detected in increasing amounts throughout embryogenesis (we are so far

unable to localize these transcripts by WMISH). Curiously, a similar anomalous expression of particular *Hox* genes was encountered in the embryo of the sea urchin *S. purpuratus*. Here, two *Hox* genes of more posterior classes—namely, *SpHox7* and *SpHox11/13b*—are activated by blastulation in non-overlapping spatial domains: *SpHox7* transcripts are found in the aboral ectoderm territory (32) whereas the initial activation of *SpHox11/13b* is ubiquitous, with transcripts eventually localized to several different and unrelated parts of the embryo (ref. 33; also see ref. 34). Activation of neither gene occurs in the context of any known embryonic specification function (10). It could be relevant that in *Caenorhabditis elegans* embryogenesis the role of the *ceh-13* gene, a *Hox1* orthologue, is regulation of cell affinities rather than cell type specification (35, 36). The embryonic expression patterns of *SpHox7* and *SpHox11/13b* in *S. purpuratus* are transient and are unrelated to the deployment of these genes during adult body plan formation. Thus, by 2 weeks of development *SpHox7* and *SpHox11/13b* are expressed in nested spatial domains in the expanding progeny of the larval set-aside cells, specifically in the mesoderm of the somatocoels (C. Arenas-Mena, R.A.C. and E.H.D., unpublished data). Something similar is probably true for *CH-Hox2* in the polychaete: although we do not know its embryonic spatial pattern, because its transcripts are maternal they are unlikely to be confined to the endomesodermal set-aside cells, as they are by 24 h of development. Unlike *CH-Hox2*, neither *SpHox7* nor *SpHox11/13b* in the sea urchin are expressed maternally (10); neither is *ceh-13* in *C. elegans* (36). In both the indirectly developing sea urchin and the polychaete, it would appear that individual *Hox* genes have been co-opted for special embryonic roles during evolution of these clades and that these roles are independent of the function that these same *Hox* genes execute during the postembryonic development of adult body plan structures. The same phenomenon, i.e., separate and unrelated embryonic and postembryonic phases of expression, was described for the *Brachyury* gene in *S. purpuratus* and in an indirectly developing enteropneust hemichordate, and also for the gene *Not* in *S. purpuratus* (27, 29).

***Hox* Genes and Primary vs. Secondary Larvae.** Some confusion exists in the literature with respect to the usage of the word "larva." The primary marine larvae of modern bilaterians are qualitatively different from the secondary larvae found in several taxa, such as arthropods, ascidians, and vertebrates (6, 16). We erected the term "maximal indirect development" to distinguish the process of indirect development as found in many marine invertebrates that use primary larvae, from the metamorphosis of the secondary larvae of insects, ascidians, or amphibians. The morphological differences between primary and secondary larvae are usually obvious: for example, primary larvae, such as the trochophore larva of *Chaetopterus* or the dipleurula larva of *S. purpuratus*, never display multilayered mesodermal structures or a central nervous system, which are often found in secondary larvae. Secondary larvae, as recognized by Jägersten (37), are modified adult (or juvenile) forms comparable to the adults of other bilaterians. Essentially, they represent extended phases of direct development. An excellent example of a secondary larva is the ascidian tadpole (37). As we would predict for a phase of adult body plan development, in the secondary ascidian larva the *Hox* complex is deployed in nested spatial domains along the A/P axis of the central nervous system, much as in its vertebrate and cephalochordate cousins (38–41). Examination of the utilization of the *Hox* complex would appear to provide a genetic criterion for determining whether a larva is of primary or secondary type. Taken together with the earlier work on sea urchin embryos and larvae (10), our present results support the generalization that primary larvae do not use the *Hox* complex for their development. We would further predict that in all primary larvae activation of the *Hox* complex will coincide with adult body plan formation, and that *Hox* complex transcripts will be found in progeny of the localized set-aside cell populations from which the adult body plan will form. In secondary larvae, however, the *Hox* complex seems always to be expressed during development of the larva, along its A/P axis, in neuronal and/or mesodermal structures. The distinction could be useful, for example, in taxa such as inarticulate brachiopods, where the status of the larva is more equivocal (see ref. 42). Were the *Hox* gene cluster found to be used during the formation of these larvae (which seems likely given that they are equipped with the lophophore, an adult feeding structure), then the larvae of inarticulate brachiopods should be considered secondary larvae.

Evolutionary Implications. We have now shown that the expression of the *Hox* complex in the set-aside cells of indirectly developing animals is not a peculiarity of sea urchins (as some have argued; e.g., ref. 43), but rather is likely to be a primitive feature, because it is widely shared. The *Hox* complex is not used to build the embryo/larva itself, irrespective of whether that larva is a trochophore or dipleurula, but is used during the construction of the adult body plan. This is impressive because trochophore and dipleurula larvae are very different in their morphology and their cell lineage, as well as in the adult forms to which they give rise. The observation implies an underlying similarity in mechanisms shared between distantly related forms that undergo maximal indirect development. Furthermore, it confirms the argument that simple free-living bilaterian forms can indeed be constructed without the use of patterning mechanisms mediated by the *Hox* gene cluster; Type 1 specification processes suffice. We imagine that the earliest stem-group bilaterians would have been constructed mechanistically like the primary bilaterian larvae of today. The subsequent co-evolution of set-aside cells and of the *Hox* gene cluster, together with the remainder of the bilaterian repertoire of regional specification mechanisms, would have potentiated the appearance of macroscopic bilaterian body plans by the latest Precambrian, as discussed elsewhere in this issue (9).

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