A novel ontogenetic pathway in hybrid embryos between species with different modes of development

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SUMMARY

To investigate the bases for evolutionary changes in developmental mode, we fertilized eggs of a direct-developing sea urchin, *Heliocidaris erythrogramma*, with sperm from a closely related species, *H. tuberculata*, that undergoes indirect development via a feeding larva. The resulting hybrids completed development to form juvenile adult sea urchins. Hybrids exhibited restoration of feeding larval structures and paternal gene expression that have been lost in the evolution of the direct-developing maternal species. However, the developmental outcome of the hybrids was not a simple reversion to the paternal pluteus larval form. An unexpected result was that the ontogeny of the hybrids was distinct from either parental species. Early hybrid larvae exhibited a novel morphology similar to that of the dipleurula-type larva typical of other classes of echinoderms and considered to represent the ancestral echinoderm larval form. In the hybrid developmental program, therefore, both recent and ancient ancestral features were restored. That is, the hybrids exhibited features of the pluteus larval form that is present in both the paternal species and in the immediate common ancestor of the two species, but they also exhibited general developmental features of very distantly related echinoderms. Thus in the hybrids, the interaction of two genomes that normally encode two disparate developmental modes produces a novel but harmonious ontongeny.

Key words: Evolution, Direct-development, Cross-species hybrids, Sea urchin

INTRODUCTION

In order to understand the mechanistic bases for evolution, it is necessary to understand how development evolves (Raff, 1996). We have studied two congeneric species of sea urchins that diverged only about 10 million years ago (Smith et al., 1990; McMillan et al., 1993), but have very different modes of early development: *Heliocidaris tuberculata* has a typical indirect-developing feeding larva, whereas its congener *H. erythrogramma* rapidly develops directly to the juvenile adult. The radical changes in early development that these two species have undergone over a relatively short evolutionary time span both emphasizes the unexpected plasticity of embryological programs, and offers an experimental system in which we can begin to dissect the molecular and cellular mechanisms that underlie such changes.

H. tuberculata has a 90 μm diameter egg, and produces a bilaterally symmetric pluteus larva (Fig. 1A) with characteristic features including a functioning gut, arms with skeletal rods, and a complete ciliary band arrayed along the arms, surrounding the oral territory and separating oral from aboral ectoderm. The precursor tissues of the adult rudiment form on the left side of the advanced 8-armed pluteus. Metamorphosis to generate the radial, pentamerally symmetric adult does not occur until after about 6 weeks of larval growth.

At metamorphosis, most of the larval body structures are discarded.

In contrast, the 430 μm *H. erythrogramma* egg generates a highly modified non-feeding larva (Fig. 1B), in which feeding larval structures have been lost, and in which there is an accelerated development of the adult rudiment. Metamorphosis takes place in 4 days. As in indirect-developers, the rudiment of the adult urchin forms on the left side of the larva as a result of interactions between invaginating vestibular ectoderm and endomesoderm (Okazaki, 1975; S. Minsuk and R. R., unpublished data). The adult rudiment comprises most of the body of the direct-developing larva. Although *H. erythrogramma* larvae retain a few relict features of the ancestral pluteus (Bisgrove and Raff, 1989; Emlet, 1995), they have undergone extensive reorganization of morphogenesis. The direct-developing larva has not just lost or abbreviated feeding larval structures, but has gained many features specific to direct development that allow immediate development of adult structures (Raff, 1987). These include modifications both of oogenesis and of subsequent embryogenesis, including production of a large yolk-poor, but lipid-rich egg (Byrne et al., 1999); the maternal specification of the larval axes and novel maternal distribution of determinants of cell fate (Henry and Raff, 1990; Henry et al., 1990); a novel cleavage pattern (Williams and Anderson, 1975; Wray and Raff, 1989, 1990);

a novel and highly divergent pattern of cell lineages (Wray and Raff, 1989, 1990), and a novel mechanism of gastrulation (Wray and Raff, 1991). In addition to the changes in maternal and zygotic programs, there have also been changes in spermatogenesis (Raff et al., 1990). Thus remodeling of the developmental program in the evolution of direct development has involved coordinate changes in many different pathways.

There have been many studies of hybrids between different species of indirect-developing sea urchin (Brandhorst et al., 1991; Chen and Baltzer, 1975; Conlon et al., 1987; Fujisawa, 1993; Nisson et al., 1989; 1992; Tennent, 1914; Uehara et al., 1990). Such cross-species hybrids have been utilized to investigate the roles of maternal versus zygotic information in the generation of larval features, and to examine regulation of gene expression. Successful hybrids in general exhibit maternal species-specific features through gastrulation, and then start to exhibit paternal species-specific features. Although no crosses between species with differing developmental modes have been reported, the results with cross species hybrids among indirect-developers suggested that, if they could be made, hybrids between a derived direct-developing species and an indirect-developing species would have the potential to reveal the nature of genes whose expression has been lost or altered in the derived species, by restoration of their expression in the hybrids.

Here we report the generation of viable hybrid embryos obtained by fertilizing *H. erythrogramma* eggs with *H. tuberculata* sperm. We characterized the development of these hybrid embryos to test for the predicted restoration of lost regulatory functions in development. Cleavage and development in hybrids through gastrulation was similar to *H. erythrogramma*, but thereafter the hybrids underwent a complex morphogenesis distinct from both parental species (Fig. 1C,D). Thus, the *He × Ht* hybrids in effect constitute a kind of 'ontogenetic species' with a novel developmental pathway resulting from expression of the genome of the paternal indirect-developing species within the context of the maternally structured environment of the direct-developing egg.

We also examined embryos of the reciprocal cross in which *H. tuberculata* eggs were fertilized by *H. erythrogramma* sperm: this hybrid cross is lethal, resulting in arrest at gastrulation. The mode of failure of these embryos was consistent with the interpretations of the factors that interact in the viable development of the *He × Ht* hybrids. Taken together, the developmental features of the two complimentary cross-species hybrids provide insight into the kinds of genic changes that have occurred in the evolution of the direct-developing ontogeny.

MATERIALS AND METHODS

Hybrid embryos

Reciprocal cross-species hybrids were made between the two *Heliocidaris* species. Hybrids generated by fertilizing *H. erythrogramma* eggs with *H. tuberculata* sperm are designated as *He ×Ht* hybrids; hybrids generated by fertilizing *H. tuberculata* eggs with *H. erythrogramma* sperm are designated as *Ht × He* hybrids.

H. tuberculata eggs can be readily fertilized by *H. erythrogramma* sperm in the laboratory. (This suggests that the barriers to cross-

fertilization in the natural habitat, where these species occur together, might be in timing of spawning.) In contrast, *H. erythrogramma* eggs with an intact jelly coat cannot be fertilized by *H. tuberculata* sperm, although the sperm bind extensively to the *H. erythrogramma* jelly coat. Treatments of sea urchin eggs that achieve removal or modification of the jelly coat, permitting fertilization by heterologous sperm, are trypsin treatment (Conlon et al., 1987) or prolonged (several minutes) exposure to acid sea water (McMahon et al., 1985). We found that after even very brief trypsin treatment, *H. erythrogramma* eggs could not be fertilized by either conspecific or heterologous sperm, even though sperm were still able to bind to the treated eggs. However, partial dejellying of *H. erythrogramma* eggs by brief treatment with acid sea water allowed subsequent fertilization by sperm of either species. We generated *He × Ht* hybrids from *H. erythrogramma* eggs dejellied by 45 seconds exposure to sea water acidified to pH 5 with glacial acetic acid, followed by rinsing into normal sea water and fertilization with *H. tuberculata* sperm. Control *H. erythrogramma* embryos generated from dejellied eggs fertilized by conspecific sperm developed normally (Fig. 2A). The phenotype of the hybrids was reproducible and stable. Several independent sets of hybrid embryos from both reciprocal crosses were prepared from different females and males, during two spawning seasons, with consistent results as reported below.

Microscopy

For light microscopy, larvae were fixed in 2% paraformaldehyde in sea water. To analyze internal structures, whole larvae were stained for DNA with 5 μg/ml propidium iodide to display cell nuclei, imaged by confocal microscopy, and the optical stacks analyzed using the public domain program NIH Image (http://rsb.info.nih.gov/nih-image). Gut reconstructions were drawn from clay models of the optical stacks. For antibody staining, samples were embedded in paraffin wax. Six μm thick sections were stained with anti-Ecto-V (Coffman and McClay, 1990), and visualized with secondary antibody conjugated to horseradish peroxidase (HRP). For scanning electron microscopy, larvae were fixed in 2.5% glutaraldehyde in sea water.

RESULTS

H. erythrogramma × *H. tuberculata* hybrids exhibit a novel morphogenesis resembling the development of larvae of distantly related echinoderm classes

He × Ht hybrid embryos exhibited the normal *H. erythrogramma* pattern of equal cleavages, and underwent normal formation and resolution of the wrinkled blastula stage (Williams and Anderson, 1975; Henry et al., 1991). The hybrids completed early development on the same time schedule as normal *H. erythrogramma*, completing gastrulation by 24 hours. Through gastrulation, the morphology of hybrid embryos was the same as that of *H. erythrogramma* embryos, although hybrid gastrulae were somewhat more rounded than those of *H. erythrogramma*. Thereafter, however, post-gastrulation development deviated markedly from that of either parental species (Fig. 1).

Normal 2-day *H. erythrogramma* larvae are potato-shaped, and remain so until metamorphosis. The primary external morphological features are the vestibular opening (beneath which the adult rudiment is developing), and an incomplete ciliary band at the vegetal end of the *H. erythrogramma* larva (Figs 1B, 2A). By 2 days postfertilization, the *He × Ht* hybrids developed into a flattened larval form with a complex lobed body shape, and a mouth and anus separated by a complete ciliary band following the body lobes (Figs 1C,D, 2B-D). As
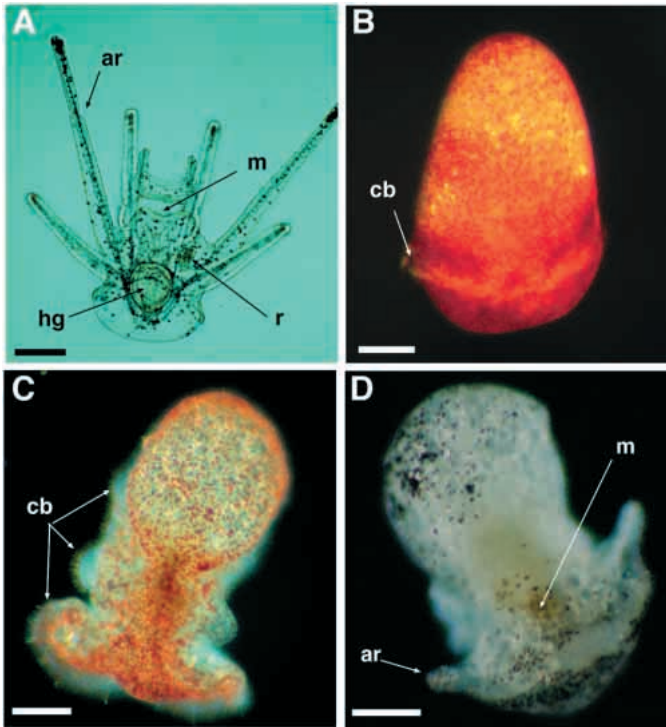
Fig. 1. Bright-field micrographs of larvae of *H. tuberculata*, *H. erythrogramma*, and *He × Ht* hybrids generated from dejellied *H. erythrogramma* eggs fertilized with *H. tuberculata* sperm. (A) Ventral view of a 17-day eight-armed *H. tuberculata* pluteus showing the skeletonized larval arms (ar), mouth (m) and tri-partite gut (hindgut, hg, visible in this view). The mass on the left side of the pluteus is the developing adult rudiment (r). Metamorphosis to generate the juvenile adult occurs at approximately 6 weeks. (B) Left lateral view of a 3-day *H. erythrogramma* larva. The direct-developing larva does not have arms or a functioning gut, and has only an incomplete ciliary band (cb) that is derived from the ciliary epaulets of the pluteus rather than from the complete ciliary band that encircles the arms (Emlet, 1995). Metamorphosis occurs at 4 days. (C) Dorsal view of a 3-day *He × Ht* hybrid larva, showing the deeply lobed body encircled by a complete ciliary band. (D) Ventral view of another 3-day hybrid, showing the oral face of the ciliary band, the stomodeal invagination that becomes the mouth, and the early development of pluteus-like larval arms. Metamorphosis of the hybrids occurred at 7 days. Scale bars, 100 μm.

shown in Fig. 2E, the striking morphological features of the early stage hybrids are similar to the dipleurula-type larva inferred to be the primitive echinoderm larval form (Nielsen, 1995), and present in other modern echinoderms, including sea cucumbers and starfish (Byrne, 1991).

Some of the ancestral features restored in the *He × Ht* hybrids are common to dipleurula-type larvae and also to the pluteus larva of the paternal species (and of the common indirect-developing ancestor of the two *Heliocidaris* species). A feature of early development that has been lost in the maternal species but is present in the hybrids and in both dipleurula-type larvae and plutei is a complete ciliary band. This ciliary band separates the mouth and anus, and, as discussed below, demarcates the boundary between the oral and aboral ectodermal territories. Like plutei of other indirect-developing sea urchins, the *H. tuberculata* pluteus has two developmentally distinct sets of

ciliated cells (diagrammed in Fig. 3): a complete ciliary band that follows the larval arms and separates the oral and aboral fields, and the ciliary epaulets that appear within the aboral region in late stage plutei. *H. erythrogramma* larvae have only an incomplete ciliary band, which appears to be homologous to the epaulets (Emlet, 1995). In the *He × Ht* hybrids, formation of a complete larval ciliary band accompanies the development of mouth and anus; an epaulet-like ciliary band does not form. The morphology of the complete ciliary band in the hybrids is less elaborate, but resembles that of dipleurula-type larvae (e.g. starfish, Fig. 2E) in that it follows the lobes on each side of the larval body, and continues over the top of the preoral lobe.

Although the 2- to 3-day *He × Ht* hybrid larvae had a unique non-echinoid appearance (Fig. 2B,C), as development continued they added a suite of feeding larval features typical of the advanced paternal pluteus stage and specific to larvae of euechinoids, the echinoid clade to which *Heliocidaris* belongs. Larval arms appeared at approximately 3 days of development (Fig. 1D), and by about 5 days postfertilization, the morphology of late stage hybrid larvae came to more closely correspond to that of a developing pluteus (Fig. 2D). As discussed below, restored pluteus-like features in late stage hybrids included a vestibule positioned left of the mouth, a pluteus-like gut, and skeletonization of the larval arms.

Control *H. erythrogramma* underwent metamorphosis to form the juvenile adult urchin at 4 days. Development of the *He × Ht* hybrids was prolonged relative to the maternal species, but many completed development and metamorphosed into juvenile adults at 7 days postfertilization (Fig. 2F). The hybrid urchins had all characteristic body parts of normal juvenile urchins, but like the example shown, most had imperfect pentameral symmetry.

Although a number of paternal pluteus-like features are restored in the *He × Ht* hybrid larvae, the hybrids do not represent a reversion to a pluteus. However, although they develop to metamorphosis very rapidly, their development is also radically different from the maternal direct mode; for example they do not develop the adult gut directly but construct a complete larval gut. We conclude that the developmental mode is truly a 'hybrid' one, in which features from both parents combine to generate a new pathway. Thus the *He × Ht* hybrid cross, although derived from parents with distinct modes of echinoid development, produced a novel synthetic morphogenetic pathway yielding a distinct but harmonious ontogeny. Moreover, in early stages of development, the hybrid larvae closely resembled those in echinoderm classes that have been separated from echinoids by over 450 million years.

Restored paternal structures in *H. erythrogramma × H. tuberculata* hybrids

The internal structure of the hybrids was radically re-organized relative to the maternal species. *H. erythrogramma* larvae produce only a blind rudimentary C-shaped gut (Fig. 4C,D), which is the precursor of the adult gut. In contrast, *He × Ht* hybrids form a complete gut with surface connections (Fig. 4A,B) and a tripartite morphology characteristic of the pluteus gut. We do not know if the gut in the hybrid larvae is functional, although in some animals we observed gut contents, demonstrating that the mouth (and possibly the anus) are open to the outside, and suggesting that at least some of the hybrids may feed. In normal development of indirect developers, the

hind- and fore-gut of the pluteus are discarded at metamorphosis, and only the midgut is retained and re-modeled during formation of the adult gut. We do not know yet if the morphogenesis of the adult gut in the *He × Ht* hybrids follows this pattern of development.

Another pluteus-like structural feature in the late stage hybrids is development of the adult rudiment within a vestibule on the left side of the mouth (Fig. 2D). In all echinoderms, the adult rudiment forms on the left side of the larva. In euechinoids, the adult rudiment develops within the vestibule, an invagination of ectodermal tissue. In both *H. erythrogramma* and *H. tuberculata*, a vestibule and rudiment form on the left side of the larva. Although, as discussed below, the timing of establishment of the left-right axis is different in the two parental species, this feature of development is retained in the *He × Ht* hybrids. The positioning of the vestibule with respect to the mouth resembles the body organization of the paternal pluteus.

Finally, the hybrids produce obvious pluteus arms (Figs 2D, 4E-G), and the ciliary band that lies between the mouth and anus extends around each of the arms (Fig. 4E), as it does in a pluteus (Fig. 3). In a normal eight-armed pluteus, two pairs of arms project from the preoral lobe, the anterolateral and preoral arms, which in *H. tuberculata* have simple spicular skeletal rods. Two additional pairs of arms project from the body of the pluteus, the posterodorsal and postoral arms. In *H. tuberculata*, these long arms have large fenestrated skeletal rods. *H. erythrogramma* larvae do not possess larval arms at any stage of development. The only larval spicular skeleton in *H. erythrogramma* larvae are four tiny fenestrated remnants of the postoral and posterodorsal rods (Emlet, 1995). There is no trace of anterolateral or preoral skeletal elements.

In plutei, the number of arms is tightly regulated, but in *He × Ht* hybrids we observed a variable number of arms, usually four or five arms (Fig. 4F), indicating imperfect spatial and meristic regulation. However, all of the larval arms of the hybrids contain long fenestrated skeletal rods (Fig. 4G), and are thus clearly homologous to the posterodorsal and postoral arms of the *H. tuberculata* pluteus both by position and morphology of skeletal rods. There are no anterolateral or preoral arms or skeletal elements in the hybrids, although a preoral lobe is present, and the ciliary band passes over its surface (as in dipleurula-type larvae).

The restored pluteus arms in the *He × Ht* hybrids thus represent those for which remnant skeletal elements are present in the maternal species, *H. erythrogramma*; the class of pluteus arms that are entirely absent in *H. erythrogramma* are not restored in the hybrids. This might mean that expression from the hybrid paternal genome is not sufficient to reactivate development of the anterolateral and preoral arms. The paternal genome may allow the hybrid to extensively develop the remnant posterodorsal and postoral arms of *H. erythrogramma*, but not to initiate arms de novo.

Restored paternal gene expression in *H. erythrogramma × H. tuberculata* hybrids

We interpret the morphology of the *He × Ht* hybrids to indicate that the paternal pattern of ectoderm differentiation has been restored. Larvae of indirect-developing sea urchins, like *H. tuberculata*, form three kinds of ectoderm, oral, aboral, and vestibular (Coffman and Davidson, 1992). The oral and aboral ectoderm territories are separated by a continuous ciliary band (Fig. 3). In contrast, *H. erythrogramma* larvae produce only two kinds of ectoderms, the vestibular ectoderm that forms the future oral side of the juvenile adult urchin, and nonvestibular ectoderm, a larval ectoderm that covers the remainder of the larva until metamorphosis (Haag and Raff, 1998). The morphological features of the *He × Ht* hybrids are consistent
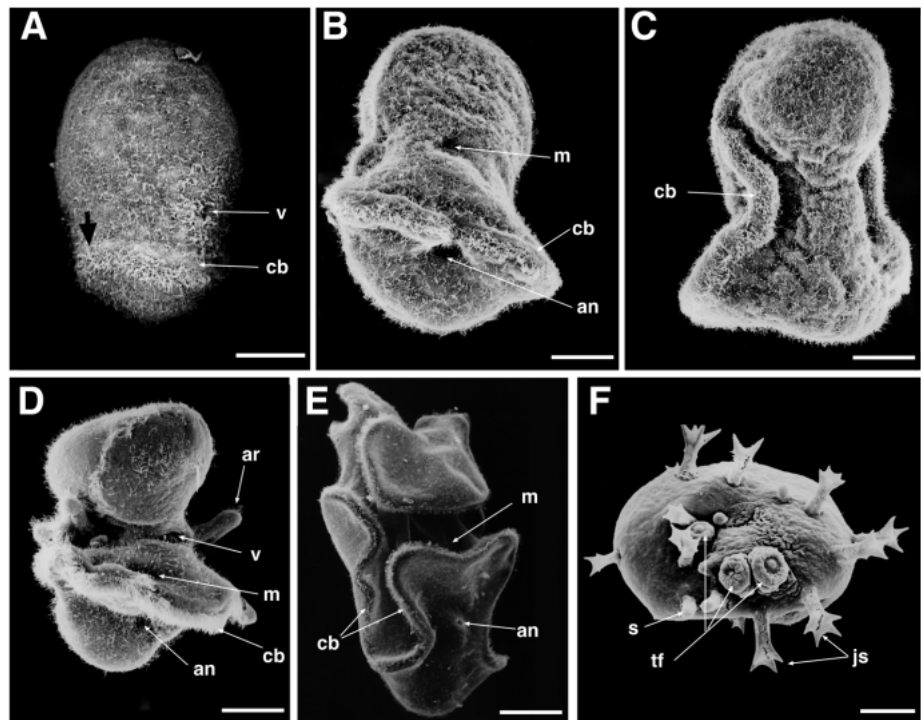

Fig. 2. Scanning electron micrographs of larvae. (A) Ventral view of a 51 hour control *H. erythrogramma* larva from a dejellied egg fertilized with *H. erythrogramma* sperm, showing the vestibular opening (v) and incomplete ciliary band (cb; arrow indicates position where the ciliary band terminates); the entire larva is also covered with short cilia. (B) Ventral view of a 54 hour *He × Ht* hybrid showing the mouth, anus (an), and complete ciliary band. (C) Dorsal view of another 54 hour *He × Ht* hybrid showing the complete ciliary band following the lobes on the lateral sides of the embryo. (D) Ventrolateral view of a 128 hour *He × Ht* hybrid showing the larval arms, ciliary band, mouth, anus, and the vestibular opening on the left side of the embryo. The ciliary band follows the body lobes and extends around each arm (see also Fig. 4E). (E) Ventral view of a 6 week bipinnaria larva (a derived dipleurula-like larval form) of the starfish *Patiriella regularis* (Byrne, 1991), showing the lobed body with complete ciliary band separating mouth and anus (provided by Maria Byrne). (F) Oral view of a 170 hour newly metamorphosed hybrid (adult mouth is not yet developed), showing three tube feet (tf), many typical four-pronged juvenile spines (js), and several adult spines (s) in the process of erupting. Pentameral symmetry is imperfect. Scale bars, 100 μm.
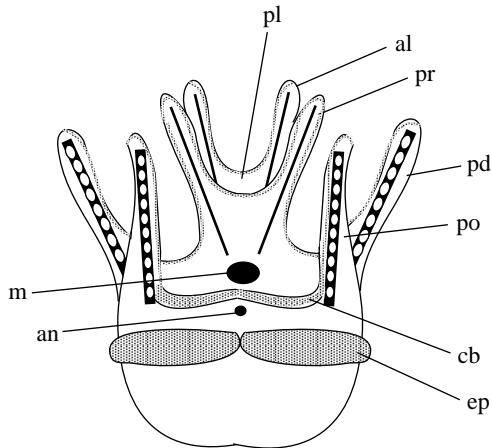
Fig. 3. Skeletal characteristics of the oral aspect of a mature *H. tuberculata* eight-armed pluteus. The four skeletal arms (al, anterolateral; pr, preoral), located on the preoral lobe (pl), have simple spicular rod skeletons. The four arms located on the body of the larva (pd, posterodorsal; po, postoral) are supported by large fenestrated rods. The complete ciliary band (cb) extends along all the arms to separate the oral territory, in which the mouth (m) is located, from the aboral territory, in which the anus (an) is located. The ciliary epaulets (ep) comprise a second ciliated band of cells, distinct from the ciliary band.

Fig. 4. Internal features of *H. erythrogramma* and *He × Ht* hybrid larvae examined by confocal imaging (A-D) or standard light microscopy (E-G). (A) Average of three optical sections from a stack of 29 sections through a propidium iodide-stained 105 hour *He × Ht* hybrid larva, viewed from a ventrolateral aspect. The gut of this hybrid resembles that of a pluteus; an esophagus (os), foregut (fg), midgut (mg), hindgut (hg) and anus (an) are discernible. The rudiment, the position of which is demarcated by a tube foot (tf), forms on the left side of the gut, as in the pluteus. (B) Reconstruction of the hybrid gut, with the position of the tube feet (four present in this larva) shown schematically. (C) A single optical section from a stack of 25 sections showing a portion of the gut of a propidium iodide-stained 71 hour *H. erythrogramma* larva, viewed from the right side of the larva. (D) Reconstruction of the *H. erythrogramma* gut. The gut is closed, with no surface connections, and is acquiring the adult gut morphology; the large intestine, or stomach (sm), lies over the hydrocoel, and the intestine (in) has begun to curve towards the ventral side of the larva. The rudiment lies below the gut, indicated schematically by the position of the tube feet. The arrowhead shows the position of the blastopore (closed by 36 hours). Completion of the gut occurs post-metamorphosis; it would take another 4-5 days before the adult stomodeal invagination begins to form and another week before the connection to the esophagus is complete (E. M. P., unpublished data). (E) Scanning electron micrograph of the larval arm of a 102 hour *He × Ht* hybrid, showing the ciliary band extending along the arm. The overall morphology of the animal is like that of the hybrid larva shown in Fig. 2D. (F) A 72 hour *He × Ht* hybrid larva under polarizing light showing the skeletal rods of the arms. (G) The skeleton of the arms of a 105 hour *He × Ht* larvae, composed of fenestrated rods much like those of the posterodorsal and postoral arms of the paternal species, *H. tuberculata*. Scale bars, 50 µm.

with the interpretation that the hybrids have regained an oral ectoderm and a complete ciliary band that demarcates the boundary between the restored oral and aboral territories. Further evidence in support of this hypothesis is provided by restoration of oral ectoderm-specific gene expression in the *He × Ht* hybrids. Ecto-V is an extracellular protein whose expression has been shown to be entirely restricted to the oral ectoderm and foregut in embryos of indirect-developing sea urchins (Coffman and McClay, 1990). Consistent with this, we found that Ecto-V is not expressed at all in *H. erythrogramma* larvae, which lack an oral ectoderm (Fig. 5A), whereas *H. tuberculata* plutei exhibit the same oral ectoderm-specific Ecto-V expression as in other indirect developers (Fig. 5B). Likewise, Ecto-V is expressed in the presumptive oral ectoderm in the *He × Ht* hybrids (Fig. 5C). We detected Ecto-V in the presumptive oral ectoderm at all stages of hybrid larvae examined. Thus the paternal pattern of oral ectoderm-specific expression of Ecto-V is restored in the hybrids. Although the antibody data do not allow us to distinguish whether the *Ecto-V* gene expressed is from the paternal or maternal genome, or both, the restoration of oral ectoderm patterning clearly results from dominant acting paternal factors in the *He × Ht* hybrids. Experiments are underway to determine the source of new genes expressed in the hybrids. Our preliminary results demonstrate that some genes are expressed from the paternal genome (K. Wilson and R. R., unpublished data).

Lethality of the reciprocal *H. tuberculata × H. erythrogramma* hybrid cross reveals the importance of the maternal contribution in *H. erythrogramma × H. tuberculata* hybrids

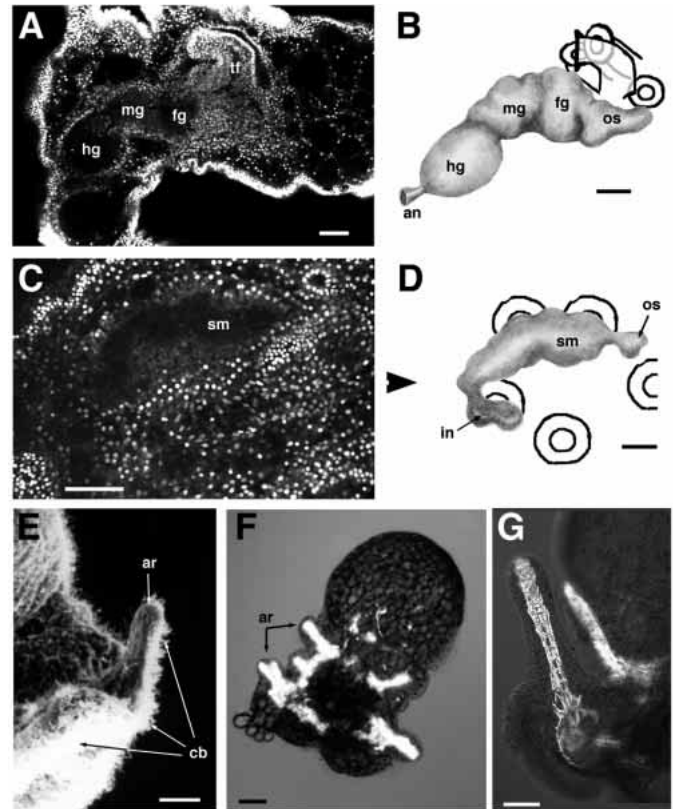In order to gain more insight into the novel developmental pathway exhibited by the *He × Ht* hybrids, we also made the reciprocal hybrid cross, by fertilizing eggs of the indirect-developer, *H. tuberculata*, with *H. erythrogramma* sperm. In contrast to the successful development through metamorphosis of the *He × Ht* hybrids, the *Ht × He* hybrids did not even

Fig. 5. Sections of paraformaldehyde-fixed larvae stained with an antiserum specific to Ecto-V, an oral ectoderm-specific protein (Coffman and McClay, 1990). Ecto-V staining was visualized with HRP-conjugated secondary antibody (brown); tissues were counter-stained with hematoxylin (blue). (A) 69 hour *H. erythrogramma* larva showing the adult rudiment, including two tube feet, and the ciliary band. Ecto-V is not present at this stage, nor did we find it expressed at any other stage in *H. erythrogramma*. (B) 86 hour *H. tuberculata* pluteus showing two larval arms adjacent to the oral hood of the pluteus, plus a portion of the gut. Ecto-V (brown staining) is present throughout the oral ectoderm (oe) but is not present in the aboral ectoderm (abe). (C) 51 hour *He × Ht* hybrid larva showing the ciliary band and an oblique section through the rudiment. Similarly to *H. tuberculata*, Ecto-V (brown staining) is present throughout the presumptive oral ectoderm but not in the presumptive aboral ectoderm; the ciliary band marks the boundary between Ecto-V-expressing and non-expressing tissue. Scale bars, 50 μm.
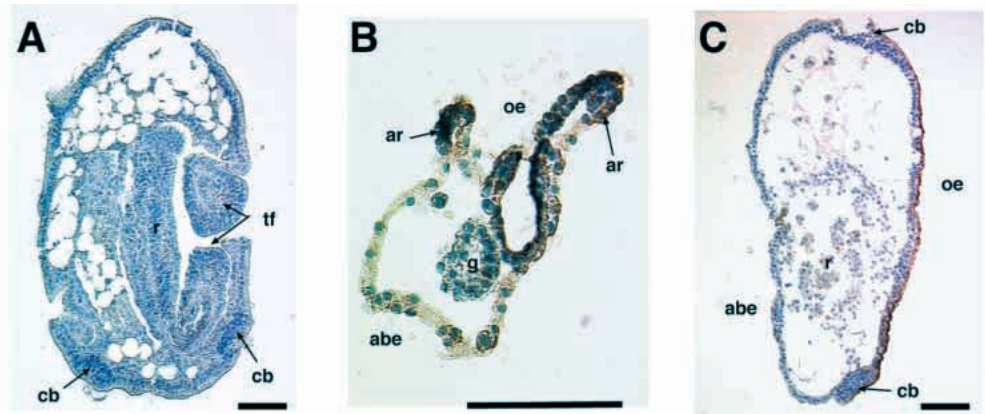
complete embryogenesis, but instead arrested at gastrulation. Almost all of the terminal stage *Ht × He* gastrulae exhibited radialization (Fig. 6), showing that they were unable to properly establish the embryonic axes necessary to generate a bilaterally symmetric larva. We interpret this early developmental failure to be a consequence of the fundamental difference in the way in which the embryonic axes of asymmetry are determined in the maternal and paternal species. In *H. erythrogramma*, some and potentially all of the embryonic axes are pre-established during oogenesis (Bisgrove and Raff, 1989; Henry and Raff, 1990; Henry et al., 1990; Emlet, 1995). In *H. tuberculata* and other indirect developers, only the animal-vegetal axis is maternally established, the other axes being established zygotically (Hörstadius, 1973; McCain and McClay, 1994). Thus the fact that the *Ht × He* cross results in radialization of the embryo indicates that in these embryos, zygotic gene expression necessary to properly establish dorsal-ventral polarity fails to occur properly.

DISCUSSION

The unique developmental mode of *He × Ht* hybrid embryos can be understood in terms of gene expression territories (Fig. 7A). Development of indirect-developing sea urchins proceeds by rapid differentiation of polyclonal groups of cells to form a small number of discrete territories, each characterized by a unique pattern of histo-specific gene expression (Davidson, 1989, 1990; Coffman and Davidson, 1992; Davidson et al., 1998). In plutei of indirect-developing sea urchins, ectodermal polarity provides organizing signals to internal tissues

(Armstrong et al., 1993; Hardin and McClay, 1991; Wikramanyake and Klein, 1996; Tan et al., 1998). Evidence for analogous signals in *H. erythrogramma* comes from *Wnt* gene expression patterns (Ferkowicz et al., 1998) and microsurgical experiments (S. Minsuk and R. R., unpublished data).

We conclude that the paternal pattern of ectodermal territories is restored in the hybrids. In the pluteus there are three kinds of ectoderm. The ciliary band separates the aboral ectoderm territory from the oral ectoderm territory, which contains both oral ectoderm and vestibular ectoderm. *H. erythrogramma* embryos have only two ectoderms, vestibular
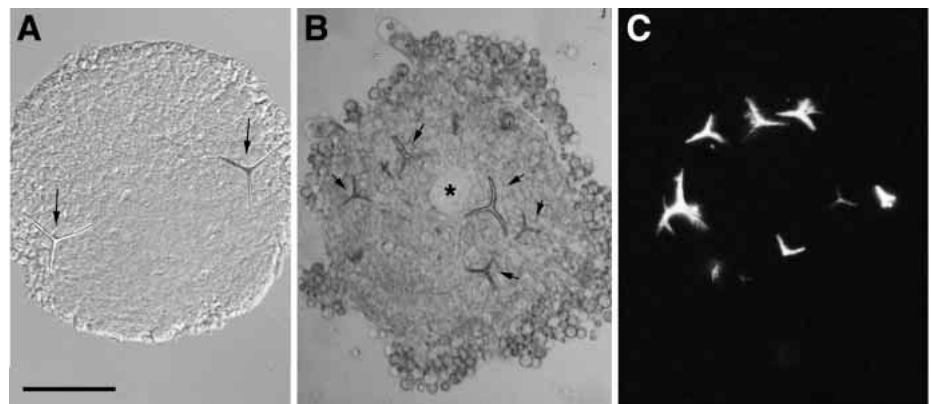
Fig. 6. Gastrula stage embryos of *H. tuberculata* and *Ht × He* hybrids generated from *H. tuberculata* eggs fertilized with *H. erythrogramma* sperm. Embryos were squashed under cover slips to show the internal skeletal elements. (A) *H. tuberculata* 25 hour embryo at early gastrula stage, viewed under bright-field optics. As shown here, skeletonization of indirect-developing sea urchin embryos begins with the formation of two tri-radiate spicules (arrows) on the lateral sides of the archenteron, which will later give rise to the complex pluteus skeleton diagrammed in Fig. 3. (B) 48 hour *Ht × He* hybrid embryo viewed under bright-field optics. This embryo is at gastrula stage, the terminal stage of development reached by this hybrid cross. The multiple skeletal spicules (arrows) arrayed around the archenteron (asterisk) demonstrate that these hybrids are radialized; i.e., have failed to form a bilaterally symmetric larva. (Normal 48 hour *H. tuberculata* embryos have reached prism stage, the early pluteus stage at which the skeletonized larval arms are forming.) (C) Another 48 hour terminal stage arrested *Ht × He* hybrid gastrula, viewed under polarizing optics to display the radially arranged multiple skeletal elements. Scale bar, 50 μm.
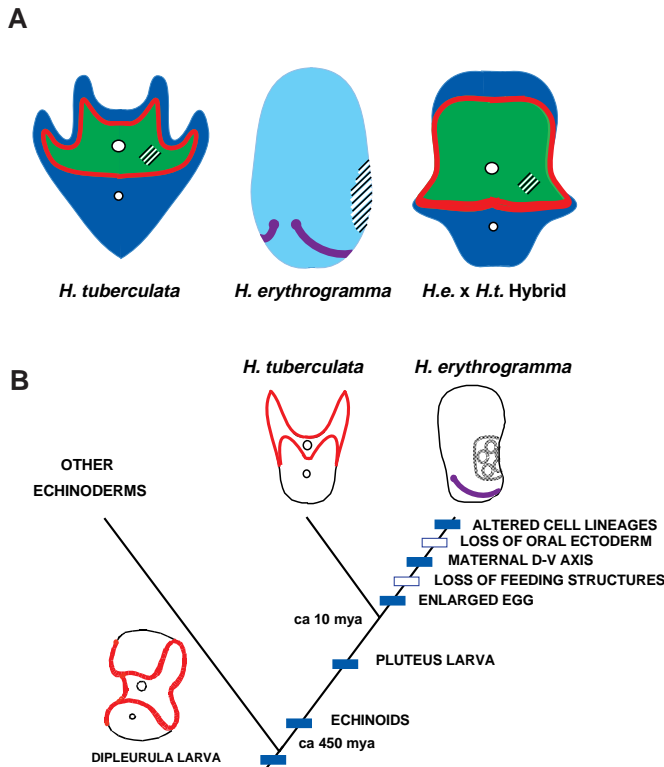
Fig. 7. (A) Ectodermal territories in sea urchin embryos and a model of the ectodermal regions in an *He* x *Ht* hybrid embryo. Left: Ectodermal territories (Coffman and Davidson, 1992) of a pluteus larva, viewed from the larval ventral (oral) aspect. The oral territory includes two kinds of ectoderm; oral ectoderm (green), and (on the larval left) the vestibular ectoderm of the juvenile rudiment (cross hatched). The larval mouth (center) forms in the oral ectoderm. The ciliary band (red) forms a boundary between the oral territory and the third larval ectoderm, the aboral territory (dark blue). The anus forms from the blastopore, in the aboral territory. Middle**:** Ectodermal territories of *H. erythrogramma* (Haag and Raff, 1998) shown from the larval ventral aspect. Only two ectoderms are present, vestibular ectoderm (cross hatched), and extra-vestibular (light blue). There is no oral ectoderm. The incomplete ciliary band (purple) lies entirely within the extra-vestibular ectoderm, and is probably homologous to the epaulettes rather than the primary ciliary band. Right: Proposed model for ectodermal territories in *He* x *Ht* hybrid larvae. We interpret the hybrids to have regained an oral ectodermal territory and a complete ciliary band that forms a boundary with aboral ectoderm, as in feeding echinoderm larvae. (B) Evolutionary changes in echinoderm larval features. The ancestral dipleurula-type larva gave rise to many larval forms. The pluteus gained stiffening calcareous rods in the cilia-bearing lobes. Evolution of the *H. erythrogramma* direct-developing larva involved both losses (open bars) and gains (solid bars) relative to the pluteus. The gain of a large egg appears to have been the requisite first step (Raff, 1996); the subsequent order of events is inferred.

and extra-vestibular ectoderm, which is likely aboral in origin (Haag and Raff, 1998). There is no oral ectoderm. The incomplete epaulet-like ciliary band of *H. erythrogramma* (Emlet, 1995) lies entirely within the extra-vestibular ectoderm. This is as expected if the extra-vestibular ectoderm is homologous to aboral ectoderm, and does not constitute a boundary between different gene expression territories. The epaulets of plutei also do not appear to constitute territory

boundaries. The morphological features of the *He* × *Ht* hybrids are consistent with the interpretation that the hybrids have regained an oral ectoderm and a complete ciliary band that demarcates the boundary between the restored oral and aboral territories. To test this hypothesis, we probed for the expression of an oral ectoderm marker, Ecto-V, found in the paternal oral territory, but not expressed in *H. erythrogramma*. Ecto-V expression in the presumptive oral ectoderm of *He* × *Ht* hybrids (Fig. 5C) provides strong support for the restoration of an oral ectodermal territory. Restoration of pluteus features also extends to internal structures, including both gut and skeletal structures, which derive from endodermal and mesodermal gene expression territories (Coffman and Davidson, 1992; Davidson et al., 1998).

The dominant restoration of pluteus features in the *He* × *Ht* hybrids is reminiscent of the restoration of the larval tail in ascidian embryos that occurs when eggs from tail-less ascidians are fertilized with sperm from typical tailed species (Jeffrey and Swalla, 1991, 1992). Restoration of the tail correlates with expression of the transcription factor encoded by the *Manx* gene, normally expressed only in the tailed species (Swalla and Jeffrey, 1996).

In the sea urchin hybrids, in addition to paternally regulated zygotic gene expression, the maternal program in the egg also plays a major role. Both *H. erythrogramma* and *H. tuberculata* have a maternally established animal-vegetal axis in the egg. In addition, however, maternal information in the *H. erythrogramma* egg specifies at least one other axis not determined until much later in indirect-developers: that is, the larval left-right axis, which corresponds to the adult dorsal-ventral axis (Bisgrove and Raff, 1989; Henry and Raff, 1990; Henry et al., 1990; Emlet, 1995; S. Minsuk and R. R., unpublished data). We propose that development in the *He* × *Ht* hybrids is possible because in *H. erythrogramma*, the polarity of the embryo is established by the maternal patterning of the egg. Restoration of the oral ectoderm by expression of paternal genes acting within the framework of the maternally determined *H. erythrogramma* embryonic axes allows the organization of differentiated larval ectodermal territories and completion of development.

The importance of maternally determined embryonic axes in *He* × *Ht* hybrids was substantiated by the results of the reciprocal hybrid cross of *H. tuberculata* eggs with *H. erythrogramma* sperm. *Ht* × *He* hybrids arrested as radialized gastrulae. Hybrids between even distantly related indirect-developing species generally develop normally to feeding pluteus larvae (Conlon et al., 1987; Fujisawa, 1993; Uehara et al., 1990); thus the failure of the *Ht* × *He* hybrids to complete development indicates a serious defect in early embryonic patterning. Radialization of indirect-developing sea urchin embryos reflects the failure to polarize the ectoderm into oral and aboral territories (Armstrong et al., 1993). Like other indirect-developing species, normal *H. tuberculata* embryos do not possess a maternally determined dorsal-ventral axis. Radialization of *Ht* × *He* embryos indicates that zygotic gene expression necessary to properly establish dorsal-ventral polarity fails to occur properly. In the *He* x *Ht* hybrids, dominant expression of the paternal *H. tuberculata* genome directs restoration of paternal features; in the reciprocal cross, it may be that in the absence of a normal paternally directed program, the necessary zygotic genes are not turned on, or that

expression from the *H. erythrogramma* genome inhibits the normal axial patterning events.

Fig. 7B places the changes in developmental mode and morphogenetic features of the hybrids in a phylogenetic perspective. We conclude that in *H. erythrogramma*, evolution of direct development from the ancestral indirect mode included gains in maternal programs that permit an accelerated developmental path to the adult rudiment, combined with losses of expression of some high level regulatory genes that determine feeding larval features. We interpret the novel developmental pathway in *He* × *Ht* hybrids to reflect the action of dominant paternal factors constrained by the pre-patterned egg cytoplasm. Further study of the reciprocal hybrid crosses will allow us to identify genes whose expression patterns have been lost, altered, or gained in the change from indirect to direct development of *H. erythrogramma*.

Based on the regain of a tail in crosses between species of ascidians with tailed and tailless larvae, (Jeffrey and Swalla, 1991, 1992; Swalla and Jeffrey, 1996), we predicted the regain of pluteus features in *He* × *Ht* hybrids. However, the reiteration of the dipleurula larva-like morphology could not have been predicted from previous work in the literature, and reveals a surprisingly long term retention of an ancient pattern of larval development. A non-expressed gene or pathway should be lost in a few million years at most, whereas genetic potential may be retained if the genes involved are utilized in other pathways (Marshall et al., 1994). The evolution of direct development in *H. erythrogramma* has involved loss of many paternal features. There are many different possible mechanisms by which loss may have occurred; our previous data and the novel developmental pathway of the *He* × *Ht* hybrids shows that alteration of developmental mode has involved multiple mechanisms. First, a gene normally expressed during development of the pluteus larva may have been actually lost; that is, a given gene might have become a pseudogene, as has happened in the case of the *H. erythrogramma* CyIII actin gene (Kissinger et al., 1997), or the gene may have been lost from the genome altogether. Other mechanisms that do not involve loss of genetic potential include a change in a pathway that normally results in activation of the gene at a specific time or place in pluteus development, or conversely, by gain of a new mechanism to repress expression of the gene. In these latter cases, the gene in question may be used in other pathways and retained in the genome, providing the potential for re-gain of expression, as we have seen in the *He* × *Ht* hybrid development.

The dipleurula larval-type morphology exhibited by the hybrids may thus represent regain of expression of a high level regulatory gene or group of genes. Alternatively, the reiteration of the dipleurula-like development may reflect the consequences of conserved epigenetic interactions expressed in new contexts. The novel morphogenesis of the *He* x *Ht* hybrids reveals an amazing and unexpected potential for integration of disparate parental pathways, providing new insight into the mechanisms by which early development can be radically reorganized while at the same time allowing a coherent ontogeny.

The question remains of the nature of the developmental constraint that has resulted in restoration of a dipleurula-like stage in the hybrids. We suggest that the key interaction may lie in the formation of the ciliary band at the boundary between oral and aboral ectoderm. Thus the ciliary band would be restored as an automatic consequence of adding an oral ectodermal domain. This of course, also happens in indirect-developing sea urchins. However, in development of a pluteus, ectodermal differentiation takes place at the same time as the growth of skeleton and arms, which transforms the shape of the larva. In the *He* x *Ht* hybrids, there is a heterochronic dissociation that results in arms appearing much later than ectodermal differentiation. The result is an early larval morphology that resembles those of other echinoderm larvae, in which there are no larval arms. There may be other constraints, because after all, the hybrids do produce a viable ontogeny. Analogous constraints may apply in cases of 'throwbacks,' such as in the occasional development of horses with three toes. It would seem that these atavisms can be absorbed in ontogeny to yield a harmonious whole. This capacity of development to absorb perturbation may play a crucial role in the evolution of development. The unique morphogenetic pathway of the hybrids suggests that interplay of new features within long term constraints might be a way in which developmental novelties can arise.

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