

Summary of Research

Title of grant:

“Hemichordates and the origin of chordates”

Grant number:

NASA NAG2-1361

P.I.'s name:

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Institution's name and address:

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Table of contents:

1. Final summary of research
2. Final inventions/patent report
3. Final inventory/property report
4. Final SF272 cash transaction

1. Final summary of research:

The main contributors to the project have been Dr. Marc Kirschner, Dr. Chris Lowe, and myself. At the start of the period of the NASA grant three years ago, we had no information on the organization and development of the body axis of the hemichordate, *Saccoglossus kowalevskii*. Now we have substantial findings about the anteroposterior axis and dorsoventral axis, and based on this information, we have new insights about the origin of chordates from ancestral deuterostomes. We greatly appreciate the NASA support that allowed us to initiate this project, which we think is now well on its way. We will publish our results soon. The first of three articles should be submitted within a month.

On the technical side of the project, we found ways to obtain and preserve large numbers of embryos and hatched juveniles. We can now collect about 40,000 embryos in the month of September, the time of *S. kowalevskii* spawning at Woods Hole. At Dr. Kirschner's request, excellent cDNA libraries were prepared by Dr. Chris Gruber from three developmental stages. From these libraries, we directly isolated about 30 gene ortholog sequences by screening and pcr techniques, all of these sequences of interest in the inquiry about the animal's organization and development. At Dr. Kirschner's request, Dr. Eric Lander agreed to do a mid-sized EST project (60,000 randomly picked clones, many of these arrayed). About half of these have been analyzed so far by blastx and are suitable for direct use of clones. We have obtained about 50 interesting sequences from this set. The rest still await analysis. Thus, at this time we have isolated orthologs of 80 genes that are known to be expressed in chordates in conserved domains and known to have interesting roles in chordate organization and development. The orthology of the *S. kowalevskii* sequences has been verified by neighbor joining and parsimony methods, with bootstrap estimates of validity. The *S. kowalevskii* sequences cluster with other deuterostome sequences, namely, other hemichordates, echinoderms, ascidians, amphioxus, or vertebrates, depending on what sequences are available in the database for comparison. We have used these sequences to do high quality in situ hybridization on *S. kowalevskii* embryos, and the results can be divided into three sections—those concerning the anteroposterior axis of *S. kowalevskii* in comparison to the same axis of chordates, those concerning the dorsoventral axis of *S. kowalevskii* in comparison to the same axis of chordates, and those concerning the signals and transcription factors found in the endoderm of *S. kowalevskii* compared to the signals and transcription factors in the endo-mesodermal cells of Spemann's organizer of chordates.

Anteroposterior axis: We have taken 22 genes known to be expressed in the chordate central nervous system in a pattern of domains that is conserved across the chordate phylum (see for example the work of J. Rubenstein on the mouse brain). To our surprise, the *S. kowalevskii* orthologs, in 21 cases out of 22, are expressed in the same anteroposterior arrangement as chordates have. This is a surprise because chordates have a central nervous system whereas hemichordates have a diffuse (non-centralized) intraepidermal nervous system, and because chordates have a uni-coelomic body whereas hemichordates have a tri-coelomic body. The dorsoventral expanse of the domains is quite different, as might be expected from the different nervous systems. In chordates the domains are limited to the dorsal neurectoderm comprising the nervous system and are excluded from the epidermal ectoderm. In hemichordates they are expressed in rings around the dorsoventral entirety of the animal, a distribution consistent with the diffuse nervous system surrounding the animal, in which neurons and epidermal cells are finely interspersed. We will take the patterns in more detail, looking separately at anterior, middle, and posterior expressions:

1) Anterior domains: These include expression of *six3*, *bfl*, *rx*, *dlx*, *otp*, *vax*, *pitx*, and *nkx2.1*. These eight genes, which are expressed in the chordate anterior forebrain (telencephalon), are expressed in ectoderm of the hemichordate prosome, the anterior part

of the animal specialized for burrowing ("proboscis"). All these domains end before the mesosome (the collar region containing the mouth) is reached.

2) Middle-level domains: These include expression of *pax6*, *tll*, *irx*, *emx*, *lim1/5*, *otx*, *en*. These seven genes, which are expressed in the chordate diencephalon and midbrain, are expressed in ectoderm domains extending into the hemichordate mesosome and anterior metasome, including the first but not second gill slit. The metasome is the third body part, containing the branchial region anteriorly (up to 70 pairs of gill slits) and the gut posteriorly. *En*, for example, is expressed in a single ring in the metasome, just anterior to the first gill slit. In chordates it is expressed as a stripe at the midbrain-hindbrain boundary.

3) Posterior domains: These include expression of *hox1,3,4,8*, and *10*. Finally, five genes, which are expressed in the chordate hindbrain and spinal cord, are expressed in ectoderm of the *S. kowalevskii* posterior metasome, from the second gill slit, on toward the anus. With the higher number of the ortholog, the domain starts more posteriorly, similar to the colinear relationship seen in other bilaterian animals. *Hox10* deserves special mention. In chordates it (along with *hox11,12,13*) is expressed in the dorsal post-anal tail, which is a hallmark of the phylum. In hemichordates it is expressed in the ventral post-anal "extension" which is present in the juvenile (until the 7-8 gill slit stage) but not in the adult, and which has been variously called a "tail", or "holdfast", or "posterior sucker". This is new evidence for the comparability of the chordate post-anal tail and hemichordate post-anal extension.

From these data we suggest that the common ancestor of hemichordates and chordates, which was the ancestor of deuterostomes, already had a rich array of gene expression domains in the anteroposterior dimension of the body, and that this array has been conserved in both evolutionary lines. The maps of chordates and hemichordates would be homologous for these 21 domains. Approximately 16 of the domains are shared with protostomes such as *Drosophila*, and probably go back to the ancestor of all bilateral animals. The other five seem to be deuterostome innovations. We cannot yet make conclusions about morphological homologies. All we can say is that:

1. the chordate telencephalon evolved from the same parts of the domain map of the ancestor as did the hemichordate prosome nervous system,
2. the chordate diencephalon and midbrain evolved from the same parts of the domain map of the ancestor as did the hemichordate nervous system of the mesoderm and anterior metasome,
3. the chordate hindbrain and spinal cord evolved from the same parts of the domain map as did the hemichordate nervous system of the posterior metasome. And
4. the chordate tail developed from the same map domains of the ancestor as did the hemichordate post-anal extension.

We suggest that the ancestor had a diffuse nervous system, as do modern hemichordates. Centralization of the nervous system, then, must have occurred in the chordate line while preserving the domain map. We don't think centralization occurred in an ancestral deuterostome larva, as Garstang suggested, because modern deuterostome larvae don't express many domains of this map. If the ancestral larva also lacked domains and if Garstang is correct, chordates would then have independently evolved a 21-fold domain map like that of hemichordates. This is exceedingly unlikely as a convergence. The only gene of the 22 that did not show a conserved expression domain was *gbx*. In chordates this is expressed in the hindbrain up to the midbrain-hindbrain boundary, and the formation of this boundary is thought to depend on an *otx-gbx* mutual antagonism. In hemichordates *gbx* is expressed in metasome mesoderm (in the coeloms) and not in the ectoderm.

Dorsoventral axis: We have examined 20 genes known to be expressed in the chordate central nervous system in its dorsoventral dimension (see for example the work of T. Jessell on the mouse spinal cord). Several have intriguing similarities of expression

in *S. kowalevskii* whereas many others are entirely different, perhaps in ways related to the fact that the nervous system was never centralized in the evolutionary line leading to hemichordates, but was centralized in the chordate line. In chordates, *bmp2* and *4* are expressed in the dorsal roofplate of the CNS, and *Shh* and *netrin* are expressed in the ventral floorplate. The walls of the chordate neural tube are entirely neurogenic (and gliogenic). Bmps in the roofplate are thought to diffuse out in a gradient and specify dorsal neuronal fates, especially sensory interneurons, and to antagonize ventral neuronal fates such as motoneurons. *Shh* is thought to have the opposite effect: to diffuse out of the floorplate and favor ventral fates while antagonizing dorsal ones. Within the *Shh*-*Bmp* double gradient, a variety of dorsoventral domains are set up based on regulatory responsiveness to *Shh* or Bmps and to mutual antagonisms of protein products of the genes themselves. Thus, *msx*, *dbx*, *gsh*, *pax6*, *pax2*, *nkx2.2*, *en*, *nkx6.1* and others have a dorsoventral ordering. *Msx*, *gsh*, *nkx2.2*, and *netrin* orthologs have a similar ordering in *Drosophila*, and it has been suggested that the ordering is conserved from an ancestor, that of all bilateral animals. *Netrin* in chordates and *Drosophila* (and *C. elegans*) is known to diffuse from the floorplate and attract growth cones of some axons toward its source (the floorplate in chordates) and repel others away, thus contributing to dorsoventral axonal placement. However, the *shh* role in chordates, diffusing from the floorplate, is not at all comparable in *Drosophila* or *C. elegans*, which don't express it ventrally.

In *S. kowalevskii*, *Bmp2/4* is expressed in the body ectoderm in a stripe at the dorsal midline, running the length of the body. *Netrin* is expressed in body ectoderm in a ventral midline stripe. Neurogenesis occurs throughout the body wall, as indicated by the pervasive ectodermal expression of the genes *musashi/nrp*, *elav/hu*, and *sox1/2/3*, which are considered pan-neural marker genes in chordates. In these respects the hemichordate body as a whole resembles a neural tube. Namely, it has a *Bmp* stripe dorsally, a *netrin* stripe ventrally, and neurogenesis in the walls between the midlines. *Netrin* may attract axons to the hemichordate ventral midline as it does in the bodies of nematodes and arthropods where it is expressed in the ventral midline, and as it does in the ventral floorplate of the chordate neural tube. At the ventral midline of hemichordates there is a dense tract of fasciculated axons, the "ventral nerve cord", which is a through-conduction tract, not a neurogenic, informing processing cord. There is also a bundle of axons at the dorsal midline, the "dorsal nerve cord". Presumably axons arrive there by repulsion from the ventral *netrin* source, as is thought to happen in *C. elegans* to form its dorsal tract. It is suspected, though not proven, that the ventral cord contains mostly axons of motoneurons and the dorsal cord contains axons of sensory neurons. We do not know if the growth cones of axons of motoneurons and sensory neurons have different responses to *netrin*.

We think that *Bmp* diffuses from the dorsal midline the hemichordate body and serves a patterning role. Several other genes are expressed nearby as broad dorsal stripes, namely, *dlx*, *tbx2/3*, and *twg*. Vertebrate neural crest cells are dorsal derivatives arising close to, and dependent upon, the *Bmp*-producing roofplate, and they express *tbx2* and *dlx*. *Tbx2/3* is probably a *Bmp*-activated gene, as is its ortholog *omb* by *Dpp* in *Drosophila*. *Twg* is a protein that binds to and modifies *Bmp* activity. *Msx* may also be expressed dorsally in the posterior metasome, perhaps like the domain of *msx* expression in the dorsal neural tube expression of *msx* in chordates. *Msx* expression is known to depend on Bmps in chordates. In *S. kowalevskii* there is a ventral expression of *mnx*, a "motoneuron homeobox" gene (also called *H9*) known in chordates to be specific to motoneurons, which arise only in the ventral half of the nerve cord. *Bmp* is known in chordates to repress *mnx* expression in the dorsal half of the nerve cord.

However, the dorsoventral similarities of neural patterning end at this point. Hedgehog (at least the *hh* ortholog we have found) is not expressed in the ventral midline of *S. kowalevskii* but at the tip of the proboscis in a tight spot, an entirely different arrangement from that of *Shh* in chordates (in the floorplate). Thus, there can be no role

for *hh* in the dorsoventral patterning of nerves in *S. kowalevskii*. The expressions of genes such as *nkx2.2* and *nkx6.1*, which depend on *Shh* in the chordate neural tube, are completely different in hemichordates: the former is expressed only in the gut and the latter only in the telocroch ciliary band. They are not expressed in the ventral nervous system. Also we know there is no dorsoventral expression of *pax6* or *en* as there is in the chordate nerve cord (secondary domains in addition to the brain domains). As noted above these are expressed only in circumferential rings in the prosome and mesosome, comparable to the chordate brain domains.

Thus many dorsoventral aspects of the nervous system looks quite different between chordates and hemichordates. If the deuterostome ancestor of chordates had a diffuse intraepidermal system with rather limited set of dorsoventral differences, the stem members of the chordate line would have had to undergo centralization of the nervous system, which is largely a dorsoventral modification. They would have segregated dorsal neurectoderm from ventral non-neurogenic epidermis, and in the course of this centralization they probably further differentiated the dorsoventral axis of the neurectoderm itself. Thus the dissimilarities of chordates and hemichordates in this dimension may reflect their centralized versus diffuse nervous systems.

As noted above, we know that neurogenesis occurs throughout the *S. kowalevskii* ectoderm (as revealed by uniform expression of *sox2/3*, *musashi/nrp*, and *elav/huC*), consistent with presence of neurons throughout the ectoderm, detected anatomically. Hemichordates probably do not have a non-neurogenic ectoderm like that of chordates. Such non-neurogenic ectoderm was presumably a chordate innovation. If the deuterostome ancestor of chordates had a diffuse nervous system, there are various possibilities for centralization in the chordate line: the nervous system could have segregated dorsally, or it could have segregated ventrally followed by body inversion, or it could have segregated both dorsally and ventrally with some later unification of the two neural areas into one dorsal system. There has been little thinking about possible modes of centralization since it has been assumed (as is done in the inversion hypothesis) that it occurred in the ancestor of all bilateral animals, well before deuterostomes arose. The diffuse nervous system of the hemichordates (and echinoderms) may present a challenge to this assumption. An alternative possibility is that the hemichordate line lost the centralized nervous system of an ancestor—it became diffuse secondarily. We are seeking evidence for and against these alternatives. Loss of centralization, to produce the specific patterns of dorsoventral domains we see, is as difficult to explain as is centralization itself.

The organizer: We have made the first steps in an investigation of the origin of the chordate organizer. This important signaling center is thought to characterize chordates and to operate at the gastrula-neurula stage for both anteroposterior and dorsoventral patterning of the body axis and of many organs. The organizer has three parts: 1) the trunk-tail organizer (prospective notochord) which specifically expresses genes for the secreted signals *Admp*, *Noggin*, *Chordin*, *FGF4/5*, *Shh*, and *Nodals*, and for the transcription factors *Bra*, *Not*, and *HNF3beta*; 2) the head organizer (prospective head mesoderm) which expresses genes for the secreted signals *Dkk*, *Crescent*, *Noggin*, and *Chordin*, and for the transcription factors *Otx* and *Gsc*; and 3) the anterior endomesoderm which expresses genes for the secreted signal *Cerberus* and for the transcription factor *Hex*. In *S. kowalevskii*, *hex* is expressed in the dorsal midline of the gut, including the stomacord. *Otx* is expressed locally in the stomacord (as well as in the overlying ectoderm), and *dkk* is expressed in the stomacord. From these three expressions we think the stomacord is more like the prechordal plate/head organizer, rather than like the notochord/trunk-tail organizer as other have suggested over the years. The trunk-tail organizer genes *bra* and *admp* are expressed, respectively, in the posterior gut and posterior ventral gut, on the ventral midline (as well as in ventral midline ectoderm). Thus, if one is to make comparisons, the posterior and ventral endoderm is more like the

notochord in its gene expression. Other genes such as *fgf4/5* and *hh* are expressed far away, in the prosome tip ectoderm. We are considering the possibility that the hemichordate has a few genes for organizer-like signals and organizer-like transcription factors that are expressed in the dorsal and ventral midlines of the gut, not in the mesoderm. If true, early chordates may have unified these two midlines into one, as they unified the dorsal and ventral nervous systems, for example by displacement of the blastopore (and anus) along the ventral side, leaving *hex*, *otx*, *dkk*, *bra*, and *admp* expressed on the same side, the new dorsal side, relative to the blastopore/anus. We are exploring these possibility in further studies that will require experimental analysis of the mode of development of *S. kowalevskii* embryos.

Related publications of the period:

Gerhart, J. Evolution of the organizer and the chordate body plan. *Int. J. Dev. Biol.* 45: 133-153 (2001).

2. Final inventions/patent report

There was no invention or patent in this period. The appropriate forms will be submitted by the University of California Berkeley Sponsored Projects Office.

3. Final inventory/property report

The appropriate forms will be submitted by the University of California Berkeley Sponsored Projects Office.

4. Final SF272 cash transaction

The appropriate forms will be submitted by University of California Berkeley Extramural Fund Accounting.

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NASA GRANTEE NEW TECHNOLOGY SUMMARY REPORT

NASA requires each research grantee, research contractor, and research subcontractor to report new technology to the NASA Commercial Technology Office. For that purpose, the following reports and corresponding schedules are provided:

<u>Title of Report</u>	<u>Form Number</u>	<u>Timetable</u>
New Technology Disclosure	NASA Form 1679	The grantee discloses <i>each</i> discovery of new technology individually, at the time of its discovery
NASA Grantee New Technology Summary Report (checkmarked "Interim")	NASA C-3043	For multi-year grants, the grantee summarizes the previous year's disclosures on an annual basis. The first Interim New Technology Summary Report is due exactly 12 months from the effective date of the grant. Future reports are due annually, thereafter.
NASA Grantee New Technology Summary Report (checkmarked "Final")	NASA C-3043	The grantee submits a cumulative summary of all disclosed technologies. The Final New Technology Summary Report is submitted immediately following the grant's technical period of performance.

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Telephone No.: (510) 642-6382
NASA Grant No: NAG2-1361 Grant Completion Date: 8/31/02
NASA GM: _____ Report Submitted by: _____

New technology should be reported whether or not it is or may be patentable.

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Subject to approval by contractors (or subcontractors) who retain or obtain title to subject inventions or reportable items, all such reported items are evaluated for publication in NASA Tech Briefs. If an item is published in NASA Tech Briefs, the innovator receives a monetary award from NASA.

NASA GRANTEE NEW TECHNOLOGY SUMMARY REPORT

General Information

1. Type of Report: ☐ Interim ☒ Final Reporting Period: 7/15/99 to 8/31/02
2. Size of Business: ☐ Large ☐ Small ☒ College/ University ☐ Nonprofit Organization
3. Have any reportable items or subject inventions resulted from work performed under this contract during this reporting period? ☐ Yes ☒ No
4. Are New Technology Items being disclosed (NF 1679 or equivalent) with this Summary Report?
☐ Yes ☒ No

• New Technology Items

Please provide the title(s) of all new and previously disclosed new technology items conceived or first reduced to practice under this grant. Use a separate piece of paper if additional space is required.

<u>Title</u>	<u>Internal Docket Number</u>	<u>Patentable Item</u>	<u>Patent Appl. Filed</u>	<u>Patent Appl. Not Filed</u>
• _____	_____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• _____	_____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• _____	_____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
• _____	_____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

• Grant Subcontractors

Please complete the following section listing all research subcontractors participating to date. Include each subcontractor's name, address, contact person, telephone number, and the subcontract award date. Use a separate piece of paper if additional space is required.

Marc Kirschner, Ph.D.,
Harvard College-Harvard Medical School

Date of Award: 7/15/99 to 8/31/02

Date of Award:

Date of Award:

Date of Award:

• Certification

I certify that active and effective procedures ensuring prompt identification and timely disclosures of reportable new technology items have been followed. Furthermore, I certify that all new technology items required to be disclosed and conceived during the period identified on this form have been disclosed to NASA.

Name and Title of Authorized Official

Joyce B. Freedman

Signature and Date

Joyce B. Freedman
Assistant Vice Chancellor
Research Administration