To: BAC_Library_Requests@mail.nih.gov
o   ABSTRACT

     We propose that a BAC library should be constructed for the
larvacean urochordate Oikopleura dioica. This animal may be the most
similar among extant animals to the last common ancestor of all
chordates, and thus, its investigation can illuminate the mechanisms
causing the vertebrate genome expansion, the principles that guide
the evolution of functions of duplicate genes in development, and the
origin of vertebrate developmental innovations. Oikopleura dioica has
a genome of about 70 Mb, the smallest of any chordate.

o   IMPORTANCE OF THE ORGANISM TO BIOMEDICAL OR BIOLOGICAL RESEARCH.
     The Animal. Oikopleura dioica is a 3 mm long urochordate, an
abundant filter-feeder in marine plankton. Oikopleura and other
members of its phylogenetic class, the larvaceans (or
appendicularians) remain free swimming and tadpole-like into sexual
maturity. Because larvaceans are basally diverging among the
urochordates, this free-swimming life style may have been ancestral
(Wada 1998). Larvaceans have more functional organ systems than do
ascidian larvae; their embryos, tadpole larvae, and adults are
transparent, which facilitates observations on all aspects of
development (Fig. 1); embryonic development takes only 14 hours; and
the generation time is less than two weeks. Furthermore, Oikopleura
dioica is readily available from plankton tows for much of the year
off Northern hemisphere coasts. Oikopleura dioica can be among the
most abundant animals in offshore waters, even more than copepods,
amounting to several animals per liter (Dagg & Ortner 1994; Capitano
& Esnal 1998). With Oikopleura, one can perform single-pair matings,
and culture the offspring in the lab continuously in the presence of
fresh sea water (Fenaux & Gorsky, 1983; Bassham, unpubl). The
optically clear embryos are excellent for in situ hybridization to
mRNAs (Fig. 1, Bassham & Postlethwait 2000). And it is highly likely
that gene disrupters, such as morpholino antisense oligonucleotides,
will permit functional analysis in Oikopleura as they do in other
aquatic embryos (Corey & Abrams 2001; Nasevicius et al. 2000).

     In addition to the importance of its developmental anatomy,
the phylogenetic position of Oikopleura is significant because this
organism belongs to a basally diverging class within a basally
diverging sub-phylum. As Figure 2 shows, the phylum Chordata has
three sub-phyla. Urochordata diverge lowest in the tree, while
Cephalochordata and Vertebrata are sister groups diverging more
recently. Urochordates include the classes Ascidia, Thaliacea, and
Larvacea, including Oikopleura. The larvaceans diverge basally among
urochordates, and the ascidians are likely not a monophyletic group
(Wada 1998; Swallla et al. 2000). The cephalochordates include
amphioxus, and the vertebrates (or craniates) include fish, and of
course, humans.
     Significance. For many or most genes in the human genome,
there are several other genes that are very similar in sequence and
overlap in function. In some cases, mutations in such gene
duplicates, or paralogues, lead to related human diseases, for
example, craniosynostosis is caused by mutation in MSX2 and Witkop
syndrome is caused by mutation in MXS1 (Li et al 1993; Jumlongras
2001). We currently have insufficient understanding of the origin or
evolution of duplicated genes, and it seems reasonable to expect that

such knowledge might help us to better understand diseases that involve paralogous genes.

Gene phylogenies show that many groups of paralogues have arisen from genome expansion events that occurred after the origin of our phylum, the Chordates, but before the divergence of the human and teleost fish lineages, and probably before the origin of the sub-phylum Vertebrata (e.g., Holland & Garcia-Fernandez 1996; Serluca et al. 2001). The leading, but controversial, hypothesis is that vertebrate genome expansion occurred by two whole genome duplication events that took place early in vertebrate evolution (Holland & Garcia-Fernandez 1996; Hughes et al. 2001; Wolfe 2001; Serluca et al. 2001; Sankoff 2001). Because whole chromosomes were duplicated under this double-duplication hypothesis, the human genome should have four copies of each chromosome segment that was present in single copy in the genome of the pre-expansion Chordate. According to the model, after each round of genome duplication, some paralogues in each duplicated chromosome segment were lost, so that many genes have only two or three, and rarely four copies on apparently paralogous chromosome segments. Examples include portions of the four chromosomes that contain HOX clusters (Hsa2q, 7, 12, and 17), and parts of the four chromosomes that contain NOTCH paralogues (Hsa 1, 6, 9, and 19), each of which have copies of many other paralogous groups (Lundin 1993; Katsanis et al., 1996; Kasahara et al. 1997;). The question, however, is whether the genome expansion occurred by tandem duplications followed by gene dispersal, in which case the current locations of paralogous genes in the human genome arose largely by chance chromosome rearrangements or due to some functional constraint, or whether these proposed paralogous chromosome segments are in fact the remnants of the hypothesized genome duplications. Resolution of this issue requires the analysis of an extant chordate whose genome, as an outgroup to other chordate sub-phyla, can help us infer the situation in the genome of the last common ancestor of all chordates, the Eochordate.

To understand the mechanisms that led to the vertebrate genome expansion and that guide the evolution of gene duplicates, we need to study the genome organization, the molecular genetic structure, and the developmental and physiological functions of paralogous gene copies in vertebrates compared to an outgroup, an organism that diverged before the genome expansion events. We believe that Oikopleura dioica provides a key opportunity to conveniently explore these various levels of genome structure and function.

Why use Oikopleura to address these issues when cephalochordates are the sister group of the vertebrates and there is a larger literature on ascidians than on larvaceans? The urochordates and cephalochordates both generally have single copies of genes that are present in multiple copies in vertebrates (Holland & Garcia-Fernandez 1996; Simmen et al. 1998), and so animals from both sub-phyla are useful for investigating the mechanisms of origin and evolution of human paralogues. Cephalochordates, especially the amphioxus Branchiostoma floridae, have the advantage that they shared a more recent common ancestor with vertebrates than do the urochordates and have been the subject of much important developmental genetic work (e.g., Holland et al., 2000; Kozmik et al., 1999; Kusakabe et al., 1999; Pollard & Holland 2000.Sharman & Holland 1996; Wada & Saiga 1999; Corbo 1997). A BAC library should certainly be made for this organism. Ascidians are also good models for evolutionary developmental genetics, but they generally do not

have a complete chordate body plan even as embryos, and they go through a complex, but interesting, metamorphosis in which they destroy most of the nervous system, lose many features of the chordate body plan, and become sessile adults. This means that much of the ascidian developmental program (i.e., that leading to the highly modified adult) might be substantially derived relative to the ancestral chordate state.

We suggest that Oikopleura dioica has some very strong advantages for probing questions of vertebrate genome origin and function in development. 1) The genome size of amphioxus (580 Mb containing 20,000 genes) (Boeddrich et al. 1999; Schidtke et al. 1979) is about 8 times larger than that of Oikopleura dioica, estimated at about 70 MB, the smallest of any Chordate (Chourrout 2001). Other urochordates also have small genomes, including ascidians, whose small genome (162 Mb containing 15,000 genes) (Simmen, et al. 1998) is still about twice as big as that of Oikopleura. 2) Embryos necessary for mechanistic developmental studies are difficult to obtain from amphioxus because it is not possible to breed amphioxus in the laboratory, and so embryos must be obtained from fecund animals taken from nature on a few nights in late spring .

Molecular genetic analysis of Oikopleura is in its infancy, but molecular genetic analyses have begun to appear (Bassham and Postlethwait 2000; Thompson et al. 2001; Nishino et al. 2001) and the phylogenetic position of Oikopleura, coupled with its wide distribution, ease of laboratory culture, complete chordate body plan, and availability for functional genomic analysis, promise to make it a key player in attempts to understand the origin of the vertebrate genome expansion, the evolution of gene duplicates, the evolution of the chordate body plan from early deuterostomes, and the evolution of vertebrate developmental innovations.

o   USES FOR THE BAC LIBRARY.

An Oikopleura dioica BAC library would be immediately applied to three main issues: the mechanisms that gave rise to the vertebrate genome expansion; the evolution of functions of duplicate genes in development; and the origin of vertebrate developmental innovations.

The Vertebrate Genome Expansion. The double-duplication hypothesis predicts that the pre expansion animal had a set of chromosomes, each of which would be represented by four paralogous chromosome segments in the human genome. Several candidates have been proposed for such chromosome parology groups. Oikopleura BACs containing the key genes in two of these paralogy groups (the HOX clusters and the NOTCH genes) will be isolated and two or more steps in a BAC genome walk will be conducted. These BACs will be shotgun sequenced to determine if they contain the set of genes predicted from the content of the proposed human chromosome parology groups.

The Evolution of Functions of Duplicate Genes in Development. The DDC model for the evolution of gene duplicates predicts that duplicated genes will frequently be preserved because the functions of the unduplicated parental gene copy have been shared by the two duplicates (subfunctionalization)(Force et al. 1999). In other cases, both duplicates will be preserved because one or both have evolved new positively selected functions (neofunctionalization). Investigation of the expression patterns of genes present in multiple copies in vertebrates and in single copy in Oikopleura could reveal how the developmental regulation of the genes have changed after

duplication. The isolation of Oikopleura BACs carrying the single copy orthologue of vertebrate gene duplicates will allow probes to be made for the expression analysis. In addition, the BACs will provide the genomic DNA needed for functional studies by transformation of Oikopleura to identify regulatory elements, and investigation of conserved regulatory elements by introducing the Oikopleura BAC clones into a suitable vertebrate, such as zebrafish, Xenopus, or mouse.

The Origin of Vertebrate Developmental Innovations. Vertebrates possess a number of features thought to be lacking in Oikopleura and other non-vertebrate chordates, including a three-part brain, neural crest, and epidermal placodes (Northcutt & Gans 1983). What molecular genetic changes caused developmental innovations that allow vertebrate embryos to form these features? The vertebrate genes that cause those features to develop have orthologues in Oikopleura, but the genes have changed functions in some key ways that allow the vertebrate novelties to develop. How have the genes changed in structure and function? To answer this question, one needs to isolate from Oikopleura BACs containing the orthologues of vertebrate essential for the construction of vertebrate developmental innovations. The structures and functions of the Oikopleura genes must then be compared to the structure and function of the vertebrate paralogues. These experiments will involve reciprocally transforming the vertebrate and Oikopleura genes into the other species to identify regulatory pathways. In some cases, new regulatory elements may have evolved, and in other cases, new downstream targets will have been evolved.

o   THE RESEARCH COMMUNITY.
        Traditionally, biologists studying urochordates have chosen to investigate ascidians rather than larvaceans because the adults are sessile and long lived, making them easier to maintain in the laboratory. This has led to a large and important body of data about ascidian development. Recently, biologists have worried, however, that the radical metamorphosis that obliterates most chordate features from the adult, and the failure of the larva to display all chordate features, may be related to key differences in the genome or in some developmental mechanisms when compared to the rest of the chordates. Thus, developmental evolutionary biologists have called for investigations of larvaceans, especially Oikopleura (see Galt & Fenaux, 1990; Holland et al., 1994; Kozmik et al., 1999; Shimeld, 1999). The first molecular developmental investigation of Oikopleura was the isolation of the brachyury gene, a gene necessary for the development of the notochord, the eponymous feature of our phylum (Bassham & Postlethwait, 2000), and other molecular genetic investigations have followed (Thompson et al., 2001; Nishino et al., 2001). Currently there are groups working on the development of Oikopleura in Canada, Norway, France, Japan, the US, plus numerous laboratories around the world that study the ecology of larvaceans, which are among the most important grazers in the plankton.

o   STATUS OF OTHER PUBLICLY FUNDED BAC-BASED GENOMIC SEQUENCING.
        We know of no other BAC based genomic sequencing from publicly funded sources in the US. Rumor has it that Hans Lehrach (Max Planck Institute for Molecular Genetics (MPIMG), Berlin) has a BAC library for Oikopleura dioica and is sequencing it, but he has not responded to my requests for information about that project, and

no data are available at the RZPD website
(https://www.rzpd.de/cgi-bin/my_rzpd.cgi). If workers in the United
States are to be able to use Oikopleura to answer the questions
raised above, they must have a publicly funded source of BAC clones.

o   OTHER GENOMIC RESOURCES THAT WILL COMPLEMENT THIS RESOURCE.
        We have constructed a cDNA library from mixed age embryos and
a cosmid genomic library for Oikopleura dioica. These will complement
the BAC library.

o   THE STRAIN OF THE ORGANISM PROPOSED AND RATIONALE FOR ITS SELECTION.
        We will provide DNA from sperm collected from animals
captured at the Oregon Institute of Marine Biology, Charleston,
Oregon.

o   THE SIZE OF THE GENOME.
        The genome of Oikopleura dioica is about 70 Mb (Chourrout
2001). This is the smallest genome known for any chordate, about half
that of ascidians, which also have very small genomes. This is half
of the size of the Drosophila genome, less than that of C. elegans,
and about 2.5% of the human genome.

o   THE SOURCE OF DNA.
        We can supply sperm DNA. About half of the body of a sexually
mature male consists of its 3 million haploid sperm, and they release
their sperm readily in vitro for collection uncontaminated by body
parts or other species. We get about 200 ng DNA per male. Because of
the small body size and small genome size, we will have to combine
sperm from several hundred males. Gels show that the sperm DNA is in
high molecular weight form.

o   SPECIFICATIONS FOR THE LIBRARY
        A library suitable for complete genome sequencing would be
best, say 11 fold coverage, but 5 fold coverage would be acceptable
for other usages. BAC inserts of 100 to 200 kb would be acceptable in
standard sequence-ready BAC vectors.

o   THE TIME FRAME IN WHICH THE LIBRARY IS NEEDED
        The library would be put to immediate use in the shortest
possible time NIH is able to make the library.

o   OTHER SUPPORT THAT IS AVAILABLE OR HAS BEEN REQUESTED
        We have agreed to provide DNA to L. Holland and P. DeJong to
support their request to the NSF program "Genomic Resources:
Bacterial Artificial Chromosome Library
Construction (BAC) (NSF 01-145) due December 3, 2001. Of course we do
not know the outcome of that proposal at this point. We urgently need
a single BAC library funded by one or the other of the two sources.

o   THE NEED FOR AN ADDITIONAL BAC LIBRARY IF ONE OR MORE ALREADY EXISTS
        As mentioned above, Hans Lehrach apparently has a library,
but it is unavailable.

o   ANY OTHER RELEVANT INFORMATION.

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NSF Training Grant in Evolution, Development, and Genomics:
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Fig. 1 Oikopleura tadpole larva expressing brachyury

Fig. 2 A chordate phylogeny.