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A1–THE BIOLOGY OF DEUTEROSTOMIAN INVERTEBRATES: A POST-GENOMIC PERSPECTIVE

Organised by M.R. Elphick and M.C. Thorndyke for the Animal Biology Section

A1.1 cDNA and genome resources of *Ciona intestinalis* and their application in biological studies

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Ascidians, or sea squirts, are sessile marine invertebrate chordates found throughout the world seas. Although the adults are simple filter feeders, the fertilized eggs develop quickly into so-called tadpole larvae, which represents the most simplified chordate body plan. Recently the *Ciona intestinalis* draft genome has been sequenced (1). Analysis of the *Ciona* genome indicates that the ~159 Mbp genome contains 15 852 protein-coding genes (1). Among these is a fundamental set of conserved chordate proteins involved in cell signaling and development. A thorough examination of *Ciona* gene expression (the transcriptome) is ongoing, including large scale EST analyses (~500 000 ESTs), cDNA sequencing, and in situ hybridization screens (2). The 17 834 independent cDNA clones were re-arrayed in 36 384-well plates for release as “*Ciona intestinalis* Gene Collection”. To facilitate research of basic gene networks involved in various processes of biology, “*Ciona* oligonucleotide chip” covering 17 834 cDNAs has been made. In addition, function of genes can be determined by suppression of their function with specific morpholino oligonucleotides. These research circumstances make ascidians a good experimental system for investigating the molecular mechanisms that underlie various processes of biology (2).

(1) Dehal, P., Satou, Y., et al. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298: 2157–2167.

(2) Satoh, N. et al. (2003) *Ciona intestinalis*: an emerging model for whole-genome analyses. *Trends Genet.*, 19: 376–381.

A1.2 Eutely and the mitotic history of cells forming the neural tube in *Ciona intestinalis*

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The tadpole larva of *Ciona intestinalis* has about 2,600 cells. Of these, exactly 40 are notochord and 36 are muscle cells in all larvae, a constancy referred to as eutely. The larva's central nervous system (CNS) has about 350 cells, distributed amongst three structures, the caudal nerve cord, rostral sensory vesicle and, between these, visceral ganglion. The entire neural tube comprises some of the progeny of three blastomeres: vegetal blastomere A4.1—most of the nerve cord, visceral ganglion, and posterior sensory vesicle; animal blastomere b4.2—the dorsal parts of the nerve cord; and blastomere a4.2—the populous anterior sensory vesicle. All arise after only 9–13 divisions, which have been followed using confocal microscopy of wholemount embryos at stages between neurulation and hatching. Lineage is invariant in neural cells derived from the A- and b-line blastomeres. The fates of some cells are known: five pairs of presumed motoneurons within the visceral ganglion arise from left and right A10.57 (one pair), and A9.30 (four pairs). Other fates are more tentative: neck cells between sensory vesicle and visceral ganglion are likely progeny of A9.16; 18 coronet cells in the sensory vesicle are probable left lateral cell progeny of a9.33 and a9.37; and 18 photoreceptor cells are probable dorsal progeny of right lateral cells a9.33 and a9.37. This mitotic record documents the lineage of 226 of the larva's CNS cells, predominantly those from A-line blastomeres. [Supported by NSERC grant OGP0000065.]

A1.3 Formation of the neural tissue in ascidians: when embryology meets imaging, bioinformatics and functional genomics

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The aim of our group is to generate a precise description of the ontogeny of the larval central nervous system of the ascidian *Ciona intestinalis*. Because this CNS contains less than 100 neurones, it constitutes the only chordate system in which a complete description of the formation of the CNS and its connectivity can be achieved with a single-cell resolution level. We focus our attention on the formation of the anterior central nervous system, which forms from the a-line. I will first report on the projects that we have achieved in the understanding of the mechanisms that restrict the anterior neural programme to the a-line blastomeres, in the identification of the neural inducer and its transcriptional logic, and in the *in silico* search for novel transcriptional targets of the inducer. I will then present our attempts at modelling the ascidian embryo by a combination of imaging and bioinformatics approaches.

References: Bertrand et al., 2003, *Cell* 115, 615–627; Hudson et al., 2003, *Development* 130, 147–159; Hudson and Lemaire, 2001, *MOD* 100, 189–203.
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A1.4 The diet of worms: *Xenoturbella* is a deuterostome that eats molluscs

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Xenoturbella bocki is a small marine worm up to 3cm in length, typically yellow-occasionally greenish or brown. *Xenoturbella* is extremely simple morphologically; externally it has two lateral furrows with a dense underlying nerve plexus and, immediately behind the mouth, a pronounced circumferential groove. Internally it has three loosely organised tissue layers, a single opening into the gastral cavity, no coelomic cavities and no defined organs apart from an anterior statocyst. *Xenoturbella* has no condensed brain or nerve chords the nervous system being composed of a basal, intra-epidermal nerve net. *Xenoturbella* was first discovered off Sweden in mud at 60 metres depth by Sixten Bock in 1915 and was first described in print by Westblad in 1949 who considered it a primitive flatworm. Subsequent authors considered it variously as related to the

hemichordates and echinoderms, the acoelomorph flatworms or as amongst the most primitive of Bilateria. In 1997 two papers seemed finally to have solved this riddle, claiming, on the basis of molecular and embryological comparisons, that *Xenoturbella* is a highly derived bivalve mollusc, so derived that the adult *Xenoturbella* retains no molluscan characteristics. Using data from three genes, we suggest that the samples in these studies were contaminated by the food source of *Xenoturbella*: the embryos and adults of a bivalve mollusc. We show that *Xenoturbella* is a member of the deuterostomes related to the hemichordates and echinoderms.

A1.5 Current controversies in deuterostome phylogeny

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In this review, I report comparisons of the results of phylogenetic analyses of four independent data sets: 18S sequences, mitochondrial genomes, sequences of HOX genes, and morphological characters. Sequence data are analyzed for their contents of phylogenetic signal and their noise-signal ratios. In the 18S sequences the noise-signal ratio is high and supported phylogenetic hypotheses depend on alignment parameters, taxa composition, and method of analysis. The traditionally recognized taxa Chordata and Craniata are not recovered as monophyletic in the analyses of 18S sequences, excluding Cephalochordata and Hyperotreta respectively. Support for a sister group relationship between Hemichordata and Echinodermata is weak. Analyses of mitochondrial genomes reveal a high variability of gene arrangements in Tunicata and Brachiopoda, show some support for a sister group relationship between enteropneusts and chordates, and strong support for a sister group relationship between Cephalochordata and Craniata. HOX gene sequences are too short to yield high phylogenetic resolution, but analysis of combined sequences of several HOX genes supports monophyly of Chordata and a derived position of Appendicularia within Tunicata. Phylogenetic analysis of morphological characters supports a sister group relationship between Enteropneusta and Chordata. The organization and development of pharyngeal arches and mesodermal complexes including excretory systems are discussed. It is concluded that these are complex anatomical structures that support the hypothesis that Enteropneusta is closely related to Chordata. [Supported by the Royal Swedish Academy of Sciences and the Smithsonian Institution.]

A1.6 Properties of the neural network that controls locomotion in the larvae of the ascidian (*C. intestinalis*); combining physiological and genomic approaches

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Larval ascidians represent the most primitive chordate nervous system available for study. With a recently sequenced genome and powerful new tools available for genetic manipulation, the ascidian *Ciona intestinalis* represents an exciting experimental model to understand basic nervous system development and physiology. We have begun an analysis of the 'input-output' properties of the neural net controlling swimming behaviour in *Ciona* in order to understand how the larva controls swimming behaviour and settlement. We find that 'random' or 'spontaneous' swimming is mediated by a glutamate sensitive pathway (p1) that has a firing rate of around 20 Hz. A second pathway (p2), is activated by step-down light responses and becomes apparent 20–24 hours post fertilization (pf). p2 is insensitive to ionotropic glutamate receptor blockers and has an output period that is extended by GABA receptor antagonists and a firing rate of around 40 Hz. Activation of p2 apparently cross inhibits p1 while no evidence was found for cross inhibitory action of p1 over p2. A draft model of the network will be presented that is based on pharmacological, mutation and gene knock down experiments.

A1.7 Cannabinoid signalling: a deuterostomian invention?

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The human genome encodes ~ 800 G-protein coupled receptors (GPCRs) that belong to five distinct families (Fredriksson et al. 2003; Mol. Pharmacol. 63, 1256–1272). Orthologues of some human GPCRs are also present in protostomian invertebrates (e.g. *Drosophila*), indicating that these receptors originated early in animal evolution before the divergence of protostomes and deuterostomes. Other human GPCRs do not have protostomian orthologues, indicating that they may have originated more recently. An example of the latter are cannabinoid receptors, which are molecular targets for endogenous cannabinoids and psychoactive constituents of the drug cannabis. Humans and other vertebrates have two cannabinoid receptors, known as CB₁ and CB₂, but *Drosophila* and *C. elegans* do not have orthologues of

these receptors. Recently, we identified an orthologue of vertebrate cannabinoid receptors in the urochordate *Ciona intestinalis*, which we refer to as CiCBR (Elphick et al., 2003; Gene 302, 95–101). On-going studies are investigating the expression of CiCBR in *Ciona* using immunocytochemistry and the pharmacological properties of CiCBR using assays for G-protein activity and second messenger formation. CiCBR is the first putative cannabinoid receptor gene to be identified in an invertebrate and the discovery of CiCBR indicates that the ancestry of cannabinoid receptors can be traced back at least as far as the common ancestor of the chordates. However, there is also pharmacological evidence of cannabinoid receptors in sea urchin sperm and therefore analysis of the emerging sea urchin genome sequence may reveal if the ancestry of cannabinoid receptors extends back further to the common ancestor of deuterostomes.

A1.8 Genome wide gene expression analysis of vegetalised, radialised and animalised sea urchin embryos

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Using an array representing about 20 000 genes of the sea urchin *Strongylocentrotus purpuratus* we have analysed the molecular mechanisms underlying radialisation (excess of oral ectoderm), animalisation (excess of ectoderm) and vegetalisation (excess of endomesoderm). Gene expression profiles were generated from nickel, lithium and zinc treated embryos. We found 5770 clones in lithium, 4036 in nickel and 1666 clones significantly differentially expressed in zinc treated embryos, respectively. Significance of differential expression is calculated from statistical tests using 4608 *Arabidopsis thaliana* clones on the same array where a false positive error rate of 5% is not exceeded. Clones with a reproducibility value of e^{-5} or better, which we consider as highly significantly regulated, were analysed by whole mount *in situ* hybridisation. We selected 646 clones from the lithium and 874 clones from the zinc experiment. In total 53% and 49% of the clones selected from the lithium and zinc experiments respectively show a restricted expression pattern during embryogenesis. For example 87% of these clones upregulated in lithium treated embryos localise to the endomesoderm domain, while 67% of the clones upregulated in zinc treated embryos localise to an ectodermal domain. Only about half of the selected regulated clones display BLAST matches to known genes. Besides the identification of a high number of novel tissue specific genes the data indicate that the ectoderm in lithium treated embryos is effectively oralised. The effect of zinc treatment on the ectoderm is not as clear and might indicate a general inhibitory mode of action of zinc treatment.

A1.9 Features of the sea urchin genome: a preliminary view

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That the sea urchin is an appropriate candidate for a whole genome sequencing project almost goes without saying. Features like the availability of gametes and the ease of gene transfer are among many that lead to the recognized suitability of the purple sea urchin, *Strongylocentrotus purpuratus* for this project. Phylogenetically, sea urchins diverge from near the base of the deuterostomes and offer an excellent outgroup to the chordates. But it is our understanding of the endomesoderm specification gene regulatory network that is the most compelling reason. As of January 2004, more than 40 genes have been linked together in this network which is now a sufficient number to describe all of the experimentally verified developmental states up to about 24 hours post fertilization. This is the most completely described such network among animals. Complete genome sequences and advancing computational methods offer a way to speed up these descriptions even more. The effort to sequence the sea urchin genome is at a midpoint. There are over 6 million traces deposited in public databases and the genome coverage exceeds 5-fold. A preliminary assembly of the first 3.8 traces (2.9X) has resulted in over 90 000 contigs greater than 1Kb and a calculated genome coverage of 0.3X (<http://www.hgsc.bcm.tmc.edu/projects/seaurchin/>). From these sequences emerge a preliminary view of the sea urchin genome in terms of gene variety, gene number and haplotype differences.

A1.10 How often do gene duplicates adopt a novel role? Views from a comparative whole mount *in situ* hybridisation screen of single copy amphioxus genes and their duplicated zebrafish orthologs

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Gene duplication is a major force driving the evolution of genomes. Gene duplicates account for 8–20% of the genes in eukaryotic genomes while the rates of gene duplication are estimated at 0.2–2% per gene per My. We have grouped the duplicated human genes that have an invertebrate ortholog in 3044 groups. In the same

orthologous groups we have assigned the zebrafish and amphioxus orthologs. Teleost fish have undergone additional recent gene duplications relative to the other vertebrates thus being an ideal system to study the functional diversification that can be reached after duplication. Amphioxus is the closest living invertebrate chordate to vertebrates whose genome has not undergone the extensive gene duplications, which are hypothesised at the origin of vertebrates thus serving as a reference point for deducing ancestral gene function. We are carrying out a whole mount *in situ* hybridisation screen of the genes in 293 orthologous groups that include a single amphioxus and multiple zebrafish orthologs. It is hypothesised that functional diversification via either amino-acid substitutions in coding regions or changes in the number and type of shared regulatory motifs of duplicates can secure their retention in genomes. Through the above screen we intend to answer: How often do duplicated genes adopt a non-overlapping expression domain? How often is this expression domain novel? Do novel expression patterns correlate with the rate that genes evolve? Provided that candidate regulatory regions can be recognised via the comparison of *Fugu* and zebrafish genomic non-coding sequence, how many regulatory elements are shared between duplicates?

A1.11 Role of the MAPK signalling pathway in mesenchyme formation and differentiation in the sea urchin embryo

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The epithelial-mesenchyme transition (EMT) — i.e. the formation of mesenchymal cells from a primitive epithelium — is an essential process that occurs during critical phases of embryonic development in most metazoans. We have chosen to study EMT during sea urchin embryo development, as the formation of primary (PMCs) and secondary mesenchyme cells (SMCs) in the sea urchin embryo represents a relatively simple model for studying transcriptional regulation during EMT. PMCs, the first cells to enter the blastocoel in the sea urchin embryo, are destined to give rise exclusively to the larval skeleton, whereas SMCs, which delaminate from the tip of the archenteron during gastrulation, produce a variety of differentiated mesodermal cell types: pigment, blastocoelar, circumesophageal muscle and coelomic pouch cells. We have identified the MAP kinase ERK as a key component of the regulatory machinery that controls the formation of all mesenchymal cell types during sea urchin embryogenesis. ERK is activated in a spatial-temporal manner which coincides with the EMT of the prospective PMCs and SMCs. Loss and gain of function experiments demonstrate that ERK

signalling is not required for the early specification of either PMCs or SMCs, but controls the maintenance and/or the enhancement of expression levels of regulatory genes which participate in the process of specification of these cell types. In addition, ERK-mediated signalling is essential for the transcription of terminal differentiation genes encoding proteins that define the final structures generated by PMCs and SMCs. Our findings suggest that ERK has a central pan-mesodermal role in coupling EMT and terminal differentiation of all mesenchymal cell types in the sea urchin embryo.

A1.12 Hemichordate axial patterning and early deuterostome nervous system organization

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The early evolutionary history of deuterostomes has been the subject of an unresolved debate for over 150 years. Despite major advances in our understanding of deuterostome relationships, the adult morphologies of the four remaining deuterostome phyla; chordates, echinoderms, hemichordates and xenoturbellids, are highly divergent and reconstruction of plausible ancestral character states on morphological criteria alone remains very difficult. Developmental genetic data shows great promise to help advance our understanding of this problematic group. We have begun a comprehensive developmental genetic study of a direct-developing enteropneust hemichordate *Saccoglossus kowalevskii*. By directed cloning and an extensive EST project, we have cloned and examined expression by *in situ* hybridization of a large number hemichordate orthologues of developmentally important patterning genes, that have conserved roles in patterning the central nervous system of arthropods and chordates. The expression of these orthologues during the development of the diffuse, intra-epidermal nerve net of hemichordates exhibits strikingly similar relative domains in hemichordates in the A/P axis to those in arthropods and chordates, despite fundamental differences in their nervous system organization. However expression is not restricted dorsally or ventrally, but rather circumferentially uniform. Our results suggest that early deuterostomes may have been characterized by a diffuse, but highly patterned nervous system, inconsistent with the prevailing view of dorsoventral axis inversion.

A1.13 Regeneration and development in adult ascidians and echinoderms

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Echinoderms and ascidians have a long history as models in developmental biology. This has taken advantage of their ease of manipulation as embryos and larvae as well as their facility for laboratory culture. Adult members of these two groups show remarkable powers of regeneration that essentially comprises an adult developmental phenomenon. We have been using a multidisciplinary approach to study this process. Our chosen models include *Ciona intestinalis*, a range of colonial budding ascidians as well as crinoid, ophiuroid and asteroid echinoderms. Detailed morphological analysis combined with immunocytochemistry indicates the existence specific sites for stem cell proliferation that appear to include the possibility of both re-programming of cell lineage and/or transdifferentiation. One common feature in both groups appears to be the ubiquity of blood cells or coelomocytes as potential stem cell sources. Parallel studies using molecular genetic approaches indicate that at least some genes employed in normal embryonic development are re-expressed during adult development. We are also exploring the expression of a range of patterning genes in regeneration and budding and as a first step we are cloning homologues from our chosen models. Currently, our data includes a range of Hox genes, BMP/TGFbeta homologues and genes in the Wnt pathway. [Supported by BBSRC, VR, STINT and KVA]

A1.14 The origin of the neurotrophic system within chordates

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Neurotrophins (NT) and their Trk receptors are known to play a crucial role in the development and maintenance of the vertebrate nervous system, regulating not only neuronal survival but also axonal growth and guidance, synaptic plasticity, and long-term potentiation events. Evidence for an authentic NT-Trk signaling system has not been identified so far in invertebrates. Furthermore, the lack of NTs and Trks in the urochordate genome strengthened the idea that the NT-Trk signaling system was an invention of vertebrates, linked to nervous system complexity. Between urochordates and vertebrates there is still a living invertebrate clade, the cephalochordates (amphioxus), with a simple vertebrate-like body plan and a genome that is regarded as the prototypical and pre-duplicative vertebrate-like genome. Early chordates were likely to be, as amphioxus still

remains, gentle, filter-feeding marine animals. Their vertebrate descendants include intelligent and resourceful predators and their prey, with complex brains and paired sense organs. We will show that this evolutionary transition is coincident with the duplication and diversification of the neurotrophic signalling system, plus the recruitment of particular signal transduction pathways by Trk receptors. One is tempted to speculate then that duplication of a *ProtoTrk* and its ligand has permitted the amplification and co-evolution of differentially expressed neurotrophins and Trk receptors, thereby allowing the formation of specific and distinct functions in selective neuronal populations during the evolutionary elaboration of the vertebrate nervous system. The appearance of functional gene families involved in regulating CNS complexity in the invertebrate ancestors of vertebrates may thus have provided the genetic basis for a key aspect of vertebrate evolution, namely the development of higher neural functions and cognitive ability.

A1.15 A structure-activity analysis of starfish SALMFamide neuropeptides using NMR spectroscopy and *in vitro* pharmacology

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The SALMFamide neuropeptides S1 (GFNSALMFamide) and S2 (SGPYSFNSGLTFamide) occur in the star-

fish *Asterias rubens*. Both S1 and S2 cause relaxation of the starfish cardiac stomach in a dose-dependent manner, but S2 is about ten-times more potent than S1. We thought that this difference in potency might be due to the presence of a N-terminal tetrapeptide amino-acid sequence (SGPY) in S2 that is not present in S1. To test this hypothesis, a S2 analogue (SS2) without the N-terminal tetrapeptide and a S1 analogue (LS1) with a N-terminal extension were tested on starfish cardiac stomach. The results indicate that N-terminal extension of S1 does not increase its potency, nor does removal of the N-terminal tetrapeptide of S2 affect its potency. Therefore, the N-terminal SGPY sequence in S2 is probably not responsible for its increased potency with respect to S1 and the difference in the potency of S1 and S2 may be due to sequence differences in the C-terminal region of these peptides. To examine the structure-activity relationships of SALMFamide neuropeptides, we are using NMR spectroscopy to determine three-dimensional solution structures. The structure of LS1 has been obtained, revealing a fold at the C-terminus between Phe₆ and Phe₁₂ that favours clustering of the side chains of the hydrophobic amino-acids Leu₁₀, and Met₁₁ together with the aromatic side chain of Phe₆. To observe such secondary structure without disulphide linkages is unusual for small peptides. On-going studies will investigate the importance of this structure for the biological activity of SALMFamide neuropeptides.