Characterisation of homologs of nervous system patterning genes in the staghorn coral, *Acropora millepora* (Cnidaria; Anthozoa; Scleractinia)

Thesis Submitted by

Danielle DE JONG B. Biomed Sc (Hons) JCU

in February 2005

Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy in the School of Pharmacy and Molecular Sciences at James Cook University

Statement of Access

I, the undersigned, author of this work, understand that James Cook University will make this thesis available for use within the University Library and, via the Australian Digital Theses network, for use elsewhere.

I understand that, as an unpublished work, a thesis has significant protection under the Copyright Act and I do not wish to place any further restriction on access to this work.

Signature

Date 24/02/05

Statement of Sources

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

Signature

24/02/05
Date

Abstract

The identification of a gene related to *Drosophila ind*/vertebrate *Gsx* and its expression in an axially-restricted manner implied that homologs of *vnd/Nkx2.1* and *msh/Msx* - genes known to interact with *ind*, and with it pattern the D/V axis of the nervous system - might also be present in the staghorn coral, *Acropora millepora*. Genes of the *vnd* and *Msx* types were identified, and shown to expressed in axially-restricted patterns. However, it is difficult to relate the expression patterns of these cnidarian genes with their bilaterian counterparts, as unlike the *ind/Gsx* gene *cnox-2Am*, expression was not restricted to neurons. Preliminary investigation into the conservation of function of *cnox-2Am* in *Drosophila* using the P-element system suggested that *cnox-2Am* is capable of rescuing aspects of the *ind* null phenotype, which is consistent with conservation of function.

Two further genes were isolated from *Acropora*, which had sequence identity to genes involved in nervous system development in 'higher' animals. The first of these, *cnox1Am*, is related to the *Hox11/Tlx* gene family, and also displays similarity to a predicted *Drosophila* gene, *cg13424*. *Cnox1Am* was shown to be expressed exclusively in the ectoderm of post-settlement polyps, in an axially restricted manner. Preliminary investigation into the expression pattern of *cg13424* in *Drosophila* revealed expression in the maxillary bud in early stages (stage 9), and in the lateral transverse muscles of the body wall in later stages (stage 14 – 16). The second *Acropora* gene isolated (*barhAm*), is related to the *Bar* class genes which are involved in the patterning of anterior embryonic structures, in addition to their nervous system expression. The distribution of the *barhAm* transcript was also spatially restricted, and was expressed around the oral pore in pre- and post-settlement stages, and like *cnox1Am*, was not limited to a specific cell type.

The molecular basis of eye specification is conserved across the Bilateria, with *eyeless/Pax-6* homologs specifying the early morphogenesis of eyes across the animal kingdom. Preliminary work in our laboratory implied that *Acropora PaxCam* might correspond to a *Pax-6* precursor, implying that the eyes of cnidarians and bilaterians

might be homologous. Consistent with this hypothesis, when fusion constructs of PaxC with the activation domain of EY were expressed in imaginal discs, ectopic eyes were generated. However, similar (but much weaker) phenotypes resulted from expression of PaxB/EY fusion proteins. The relationship between cnidarian and bilaterian Pax genes is therefore not simple.

Acknowledgements

The past couple of years has flown by, and it is only with the technical, practical, emotional and financial support of others that I have managed to finally finish. Firstly, a big thank-you to my supervisor Prof. David Miller for all his assistance during my project and also getting back my thesis chapter edits (surprisingly) promptly. I have derived great pleasure from clicking the 'accept all' button in response to your suggestions. I also spent a reasonable amount of time at RSBS at the Australian National Univerity during my project and I would like to thank Dr Eldon Ball and Dr David Hayward for their never-ending willingness to help and for accepting me into their lab. At both JCU and ANU, I have had the pleasure of meeting and working with some great people – I thank all past and present members of the DM lab especially Nikki, Isabelle, Niko, Hiroki, Karin, Ingo and Christina and those at ANU especially Lauretta in Eldon's Lab and members of Prof. Rob Saint's lab; Nisrine, Masha, Peter, Michael, Nelida and Rob Saint himself. You all welcomed me warmly and made me feel right at home in Canberra. I also thank Dr Rohan Baker and Melanie Morris at JCSMR, ANU. To all groups at both JCU and ANU, thank you for your generous sharing of resources, protocols and advice.

I would also like to thank those outside of the world of science who have supported me throughout, especially Mum and Dad, my brother Luke and Belinda (and my favourite nephew, Conor), my sister Kristina and Julie, Kathryn, Michelle, Jess, Emma, Kate and Andy – and to my awesome touch footy team who were always there for a chat on 'therapy hill' on a Sunday afternoon.

I also wish to acknowledge the financial support of the Australian Government in the form of an Australian Postgraduate Award.

Table of Contents

Statement of Access i
Statement of Sources ii
Abstract iii
Acknowledgements v
List of Figures x
List of Tables xiii

Chapter 1 - Introduction 1
1.1 Metazoan Phylogeny 1
1.2 Gene Duplication 4
1.2.1 Mechanisms of Gene Duplication 5
1.2.2 Fates of Duplicated Genes 6
1.3 Evolutionary Origins of Body Patterning 9
1.3.1 Hox Genes, paraHox Genes and the protoHox cluster 10
1.4 Cnidarians as Model Animals 13
1.4.1 Cnidarian Nervous Systems 14
1.4.2 Acropora millepora as a model cnidarian 15
1.4.3 Embryological development of Acropora millepora 16
1.5 Project Objectives 20

Chapter 2 - Materials and Methods 22
2.1 Coral – *Acropora millepora* 22
2.1.1 Extraction of genomic DNA from Acropora millepora sperm 22
2.1.2 Acropora millepora genomic and cDNA libraries – bacteriophage manipulation methods 23
2.1.3 Fixation and in situ hybridisation of Acropora millepora embryos 29
Rehydration of embryos and removal of lipids 30
Dehydration of embryos 30
Rehydration of embryos 31
2.2 Fruit Flies – Drosophila melanogaster 32
2.2.1 Extraction of genomic DNA from Drosophila melanogaster 32
2.2.2 Fixation and in situ hybridisation of Drosophila melanogaster embryos 33
2.2.3 P-Element Transformation 36
2.3 Bacteria 37
2.3.1 Bacterial Strains 37
2.3.2 Media Preparation 38
2.3.3 Manipulation of Bacterial Colonies 40
2.3.4 Small Scale Overnight Bacterial Cultures 40
2.3.5 Competent Cells 40
2.3.6 Transformation of Competent Cells 42
2.3.7 Glycerol Cell Stocks 43
2.4 DNA Manipulations 44
2.4.1 Plasmid Vectors 44
2.4.2 Restriction Endonucleases 44

2.4.3 Ligation Reactions 45
2.4.4 Dephosphorylation of Cut Vector 45
2.4.5 Recovery of DNA from Agarose Gels 46
2.4.6 Generation of radioactive probes 46
2.4.7 Phenol/Chloroform Extraction of DNA 47
2.4.8 Ethanol Precipitation of DNA 47
2.4.9 Isolation of plasmid DNA 48
2.4.10 Southern blotting 48
2.4.11 DNA Quantification 48
2.4.12 Agarose Gel Electrophoresis 49
2.4.13 Oligonucleotide Primer Design 49
2.4.14 DNA Sequencing 50
2.4.15 Polymerase Chain Reaction (PCR) 50
2.4.16 5' and 3' Rapid Amplification of cDNA Ends (RACE) 52
2.5 RNA Manipulations 52
2.5.1 RNA extraction 52
2.5.2 RNA Quantification 53
2.5.3 Agarose gel electrophoresis of RNA samples 53
2.5.4 Synthesis of Digoxigenin- and Fluorescein labelled Riboprobes 54
2.5.5 Dot Blot Testing of DIG- or FL- labelled riboprobes 55

Chapter 3 - Conservation of Dorsal-Ventral Patterning Genes in Cnidaria 56
3.1 Introduction 56
3.1.1 Conserved A/P and D/V patterning systems in the Metazoa 56
3.1.2 DNA-binding characteristics and mode of repression of vnd/Nk2, ind/Gsh and msh/Msx 61
3.1.3 Cnidarian genes related to vnd, ind and msh 63
3.1.4 Statement of goals 65
3.2 Results 66
3.2.1 Isolation of vnd1-Am, vnd2-Am and vnd3-Am 66
3.2.2 The Vnd1-Am, Vnd2-Am and Vnd3-Am proteins – assignment to the Vnd/NK2 family using phylogenetic analysis 76
3.2.3 The vnd1-Am and vnd2-Am genomic loci 79
3.2.4 Spatial expression patterns of vnd1-Am, vnd2-Am and vnd3-Am 79
3.2.5 Isolation of msx3-Am 81
3.2.6 The Msx3-Am protein – assignment to the Msh/Msx family using phylogenetic analysis 86
3.2.7 Isolation of a msx3-Am genomic clone 88
3.2.8 Spatial expression pattern of msx3-Am 88
3.2.9 Spatial expression pattern of cnox-2Am in post-settlement embryos 90
3.2.10 Conservation of function between cnox-2Am and Drosophila ind 90
3.3 Discussion 95
3.3.1 Structural features of the cnidarian Vnd and Msx homologs 95
3.3.2 Regulation of the vnd1/2/3-Am, msx3-Am and cnox-2Am genes 97
3.3.3 Genomic organisation of vnd/Nk-2 and msx/Msh loci 99

3.3.4 Expression patterns and possible roles of Acropora vnd1-/vnd2-/vnd3-Am, msx3-Am and cnox-2Am 103
3.4 Conclusions 105
3.5 Future Directions 106

Chapter 4 - Isolation and Preliminary Characterisation of cnox1Am and barhAm genes from *Acropora millepora* - no clear functional correlation with the Hox11-like genes or the Bar family 107
4.1 Introduction 107
4.1.1 cnox1Am and cg13424 – Related to the Hox11 and Mnx families 107
4.1.2 BarhAm – A cnidarian member of the Bar class of transcription factors 110
4.1.3 Statement of goals 111
4.2 Results 112
4.2.1 Isolation and preliminary characterisation of cnox1Am and cg13424 112
4.2.1.1 The cnox1Am cDNA 112
4.2.1.2 The Cnox1Am protein - Assignment to the Hox11/Tlx family using phylogenetic analysis 112
4.2.1.3 The cnox1Am and cg13424 genomic loci 113
4.2.1.4 Spatial expression patterns of cnox1Am and cg13424 116
4.2.2 BarhAm – a second cnidarian member of the Bar class 123
4.2.2.1 The barhAm cDNA 123
4.2.2.2 The BarhAm protein 125
4.2.2.3 Evolutionary relationships amongst the Bar class of homeodomains 125
4.2.2.4 Spatial expression pattern of barhAm 128
4.3 Discussion 128
4.3.1 Structural features of the Cnox1Am and BarhAm proteins 128
4.3.2 Expression patterns of cnox1Am, cg13424 and barhAm 130
4.3.3 Regulation of cnox1Am and cg13424 131
4.4 Conclusions 133
4.5. Future Directions 134

Chapter 5 - DNA-Binding Characteristics of Cnidarian PaxC and PaxB Proteins In Vivo and In Vitro: No Simple Relationship With the Pax6 and Pax2/5/8 Classes 135
5.1. Introduction 135
5.1.1 Common Molecular Mechanisms of Eye Development 135
5.1.2 Pax Genes 136
5.1.3 Specification of Mammalian and Drosophila Eyes 138
5.1.4 Cnidarian Pax Genes 140
5.1.5 Eyes in Cnidaria 142
5.1.6 Statement of Goals 143
5.2 Results 143
5.2.1 Expression of PaxCam in Drosophila imaginal discs results in a dominant negative-like phenotype 144
5.2.2 Chimeric PaxCam constructs encoding the EY C-terminal domain result in eye formation in Drosophila 144
5.2.3 Ectopic sine oculis expression is induced by PaxCam/EY constructs 145

5.2.4 In vitro binding properties of Pax-Cam and Pax-Bam PDs 145
5.2.5 Expression of a PaxBam/eyeless chimera in Drosophila 150
5.2.6 PaxCam binds to the sine oculis eye-specific enhancer region in a yeast one-hybrid system 150
5.3 Discussion 153

Chapter 6 - General Conclusions 158

References 161

Appendix A – Abbreviations 192

Appendix B – Creation of an *ind*-GAL4 driver line 194

Appendix C - RNAi as a tool to silence genes in Drosophila 197

Appendix D – Tandem organization of independently duplicated homeobox genes in the basal cnidarian *Acropora millepora* 200

List of Figures

Figure 1.1 Relationships between extant animal phyla 2

Figure 1.2 Schematic representation of three possible fates of duplicated genes 8

Figure 1.3 Changes in Hox and paraHox numbers associated with major metazoan transitions 12

Figure 1.4 Scanning Electron Micrographs of the embryonic development of *Acropora* .. 18

Figure 1.5 Formation of the two tissue layers in *Acropora* 19

Figure 3.1 Comparison of nerve cord development in insects and vertebrates 58

Figure 3.2 Degenerate primers for amplification of a Nk2-related gene from *Acropora* ... 67

Figure 3.3 The *vnd1-Am* cDNA 69

Figure 3.4 Virtual northern data for *vnd1-Am*, *vnd2-Am*, and *vnd3-Am* 70

Figure 3.5 The *vnd2-Am* cDNA 72

Figure 3.6 Organisation of the *vnd2-Am* cDNA clone isolated from the post-settlement cDNA library 74

Figure 3.7 The *vnd3-Am* cDNA 75

Figure 3.8 Comparison of Eh1 motifs from *Acropora* proteins with related *Drosophila* proteins 77

Figure 3.9 Comparison of the *Acropora* Vnd proteins to other Vnd/NK2-type proteins ... 78

Figure 3.10 Genomic Organisation of the *vnd1-Am* and *vnd2-Am* locus 80

Figure 3.11 Spatial expression patterns of the *vnd* genes in *Acropora* pre-settlement and post-settlement 82

Figure 3.12 Degenerate primer positions for amplification of a *msx*-related gene from *Acropora* 84

Figure 3.13 The *msx3-Am* cDNA 85

Figure 3.14 Comparison of the *Acropora* Msx proteins to other msh/Msh-type proteins .. 87

Figure 3.15 Spatial expression patterns of *msx3-Am* during development 89

Figure 3.16 Spatial expression patterns of *cnox-2Am* in post-settlement polyps 91

Figure 3.17 Even-skipped antibody staining in *arm*-GAL4 x UAS-*cnox-2Am* in an *ind* mutant background..... 94

Figure 3.18 Comparative genomic structure of *vnd* and *msh* genes 102

Figure 4.1 The *cnox1Am* cDNA 114

Figure 4.2 Comparison of the *Acropora* Cnox1Am protein to other related proteins..... 115

Figure 4.3 Transcription factor binding sites conserved between the first intron and putative promoter region of *Drosophila melanogaster* and *Drosophila pseudoobscura cg13424* genes 117

Figure 4.4 Spatial expression pattern of *cnox1Am* in post-settlement polyps..... 119

Figure 4.5 Spatial expression pattern of *cg13424* at various stages of *Drosophila* development 121

Figure 4.6 Coexpression of *cg13424* and 22c10 in the body wall of late stage *Drosophila* embryos 122

Figure 4.7 The *barhAm* cDNA 124

Figure 4.8 Comparison of the *Acropora* BarhAm protein to other Bar-related proteins.. 126

Figure 4.9 Spatial expression patterns of *barhAm* in pre-settlement and post-settlement stages..... 127

Figure 5.1 Representation of the complex regulatory network of the eye specification genes..... 139

Figure 5.2 Schematic representation of constructs expressed in *Drosophila* imaginal discs 146

Figure 5.3 Phenotypes resulting from expression of Pax-Cam in *Drosophila* wing imaginal discs..... 147

Figure 5.4 b-galactosidase expression in *sine oculis* (so)-lacZ line driven by *dpp*-GAL4 Pax-Cam constructs 148

Figure 5.5 DNA-binding assays using the Pax-Cam and Pax-Bam Paired Domains..... 149

Figure 5.6 Comparison of phenotypes resulting from expression of Pax-Bam/EY and Pax-Cam/EY constructs in leg and wing imaginal discs 151

Figure 5.7 Pax-Cam binds to the *sine oculis* eye specific enhancer *so10* in a yeast one-hybrid system..... 152

Figure 5.8 Suggested scenario of *Pax* evolution 156

Figure 7.1 Schematic representation of *ind*-GAL4 constructs 195

Figure 7.2 pWiz vector and cloning strategy to produce RNAi constructs..... 199

List of Tables

Table 2.1 Lambda vectors and their applications 23

Table 2.2 Reagents involved in phage manipulation methods 29

Table 2.3 Bacterial *E. coli* strains used and their applications 38

Table 2.4 Conditions of electroporation when using the Gene Pulser® II Electroporation System 43

Table 2.5 Plasmid vectors used and their application 44

Table 3.1 Cnidarian orthologs of *ind/Gsh* triploblastic genes 65

Chapter 1 - Introduction

1.1 Metazoan Phylogeny

Animal body forms are amazingly diverse, and for many years this was rationalised by viewing their origins as polyphyletic (Greenberg, 1959). There is now, however, an overwhelming body of data supporting the opposite view – there is no question that the animal kingdom is monophyletic, which leads to the obvious question of how such diversity of form is possible. Understanding the nature of macroevolutionary change – the relationships between the major body plans, or bauplans - has become a major focus of modern biology, spawning the field of evolutionary developmental biology, or "evo-devo". This field aims to understand how developmental processes and mechanisms have changed during evolution and how these changes have contributed to past and present biodiversity.

Ideas about relationships between the phyla put forward by 19th century zoologists were strongly influenced by the idea of recapitulation, the popular theory that embryological development essentially follows the evolutionary stages the species experienced. Therefore, great significance was attached to broad-ranging similarities in embryological stages between different species. The phyla were organised into a 'tree of life' reflecting notions of 'increasing complexity', with morphologically simple extant animals emerging at the base of the tree. Recently views of phylogenetic relationships between animals have been substantially revised, largely on the basis of molecular data (Field *et al.*, 1988). The modern 'tree of life' (See Fig 1.1) has the Parazoa (sponges) branching off at around 565 million years ago and the Cnidaria shortly after (Lundin, 1999). The Cnidaria are classically viewed as diploblasts, although an increasing body of molecular evidence substantially undermines the idea of a simple dichotomy between triploblasts and diploblasts (reviewed in Ball *et al.*, 2004). Cnidaria are essentially the sister group of 'higher' animals proper – the clade known as the Bilateria – which display many defining characteristics such as overt bilateral symmetry and the presence of true mesoderm. Other features which distinguish the bilaterians from cnidarians include the presence of a secondary body cavity (coelom), a

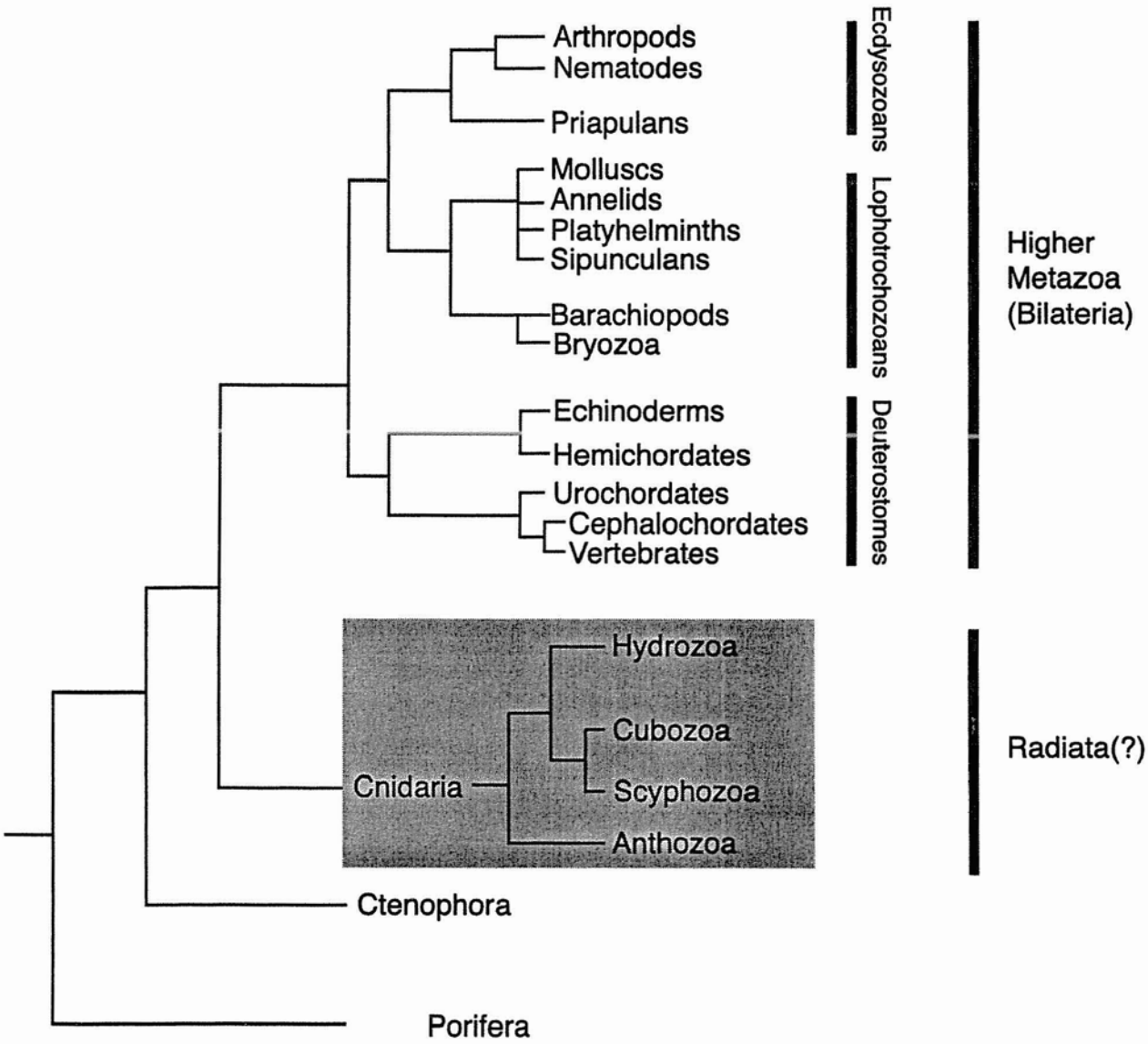

Figure 1.1: Relationships between extant animal phyla. Note that not all phyla are shown. The position of the Ctenophora is ambiguous, but in this case is based upon the analyses of Medina *et al,* 2001. Within the Cnidaria (shaded in grey), the Anthozoa are considered to be basal. The higher Metazoa/bilaterians are shown and the three major groups (ecdysozoans, lophotrochozoans and deuterostomes) are indicated. The assignment of the Cnidaria as radially symmetric is now being challenged as indicated by a question mark. (Adapted from Ball *et al,* 2004 and Lundin, 1999).

through gut with both mouth and anus, and a centralised complex nervous system. When considering the nominal evolutionary distance between the cnidarians and bilaterians, it is surprising that these characteristics apparently evolved over a relatively short period of time. The fossil record indicates that the three main clades of bilateral animals – the deuterostomes, lophotrochozoans and ecdysozoans (see Fig 1.1) – arose in a time frame of around 10 million years, the so called Cambrian "explosion" around 543±5 million years ago (Conway Morris, 2000). It is probable that the molecular basis of the bilaterian body plan substantially pre-dates the Cambrian, and also that the abundant fossil record found from the early Cambrian is an artifact caused by a rise in the fossilisation potential. If the Cambrian "explosion" did indeed occur, it is logical to assume that the ancestral genome contained all of the elements required to create the enormous diversity of animal bauplans (Ohno, 1996). The 'Cambrian Pananimalia' genome implies that the plethora of early animal morphologies simply reflected the differential usage of a nearly identical (and rather complex) set of genes (Ohno, 1996).

Comparison of the genomes of prokaryotes to eukaryotes shows eukaryotes have an increased gene number, an increase in the size and number of repetitive DNA and mobile genetic elements, and the presence of introns (Kidwell, 2002; Lynch and Conery, 2003). This has led to the assumption that greater organismal complexity directly correlates with a larger genome size. However, some closely related species with practically indistinguishable morphologies have vastly different genome sizes (Patthy, 2003), and while in general higher eukaryotes have larger genome sizes than prokaryotes, their genetic complexities may not be drastically different. For example, the size of the human genome is a 1000-fold larger than a typical bacterial genome but their gene number differs only by a factor of 10 (Patthy, 2003). Consistent with the rest of the animal kingdom, the genome size amongst cnidarians varies significantly. While there are few reliable estimates for the size of cnidarian genomes, the genome of *Nematostella vectensis* is approximately 230Mb (www.genomesizes.com) and the genome of *Acropora millepora* is approximately 100-200Mb on the basis of hybridisation with single copy probes (D. Miller, pers. comm.). However, other cnidarians (eg *Hydra spp.*) have larger genome sizes (~1800Mb).

1.2 Gene Duplication

The potential evolutionary significance of gene duplication events was realised quite recently by Ohno (Ohno *et al.*, 1968; Ohno, 1969; Ohno, 1970). Prior to the publication of Ohno's classic book, evolutionary novelty was thought to be primarily due to the accumulation of many small mutations in genes which could eventually result in phenotypic change. Ohno's ideas have gained considerable support in recent years, and the notion that single genes, chromosomal segments or entire genomes have been duplicated during evolution is now generally accepted. Such duplication processes are assumed to have facilitated corresponding increases in morphological complexity during evolution. Outside of the animal kingdom there is evidence for genome duplications having occurred during evolution; polyploidy is generally thought of as a driving force in plant evolution, and the genomic sequence of *Arabidopsis thaliana* provides clear evidence for large-scale duplications having occurred (The Arabidopsis genome initiative, 2000). Analysis of the genome of the yeast *Saccahromyces cerevisiae* suggests that a whole-genome duplication may have occurred following divergence from Kluyveromyces, although only 15% of the duplicate copies have been retained (Wolfe and Shields, 1997). In animals, the importance of duplication in early vertebrate evolution has been widely discussed, and gene duplication may have played a critical role in the transition from unicellularity to multicellularity (Lundin, 1999). It has also been suggested that duplication of some gene families (for example the Hox genes) may have enabled dramatic increases in metazoan complexity (Lundin, 1999). It seems likely that two rounds of total genome doubling occurred after the divergence from the cephalochordates (such as amphioxus) and before the radiation of fish and other bony vertebrates (the 2R hypothesis) (Ohno, 1970; Lundin, 1993; Holland *et al.*, 1994; Sidow, 1996; Meyer and Schartl, 1999). Consistent with this hypothesis, is the observation that vertebrates often possess four genes corresponding to a single invertebrate homolog, and genes from the same gene family are often arranged in linked clusters that maintain the same gene order on different chromosomes; a degree of synteny has sometimes been retained across large evolutionary distances (see (Pebusque *et al.*, 1998; Meyer and Schartl, 1999)). However, other studies have suggested that only a single round of polyploidisation has occurred (the 1R hypothesis) (Guigo *et al.*, 1996) and that multiple independent gene duplications could account for the increased gene numbers in vertebrates (Hughes *et al.*, 2001). Evidence cited in support of the 1R

hypothesis has been the earlier suggestion that the human genome contains at the most twice as many genes as those of invertebrates such as *Drosophila* and *Caenorhabditis* (Lander *et al.*, 2001; Venter, 2001), however more recent evidence has suggested that this is not the case, and the 1R hypothesis is generally considered to be redundant.

1.2.1 Mechanisms of Gene Duplication

The rate of gene duplication is not known, although it has been estimated that a gene can duplicate and become fixed in a eukaryotic population at approximately 1 gene per 100 million years (Lynch and Conery, 2000). Gene duplication can result from retrotransposition, (either through illegitimate recombination or LINE-1 (L1) element mediated recombination) (Moran *et al.*, 1999), homologous but unequal crossing-over or chromosomal (genome) duplication (Zhang, 2003). Retrotransposition can result in total or partial gene duplication (which can subsequently result in exon shuffling) and in mammals is most commonly mediated by L1 element mediated recombination (note that L1 elements have been estimated to represent 15% of the human genome) (Long, 2001). L1 is a retrotransposon that can reverse transcribe cDNA from mRNA and insert the duplicated cDNA elsewhere in the genome (Moran *et al.*, 1999). This results in loss of introns and regulatory sequences, and the presence of polyA tracts and flanking short direct repeats in the duplicated gene, although these features can be lost so the absence of these characteristics does not prove that L1 retrotransposition did not take place (Long, 2001). A duplicate gene generated in this way is unlikely to be linked to the original gene, and groups of genes cannot be duplicated together unless they were originally located together. Often a gene produced in this way will become a pseudogene as it lacks the regulatory sequences needed to function; occasionally it will be inserted in a region downstream of a promoter and thus be expressed in a new pattern, or alternately recruit nearby pre-existing coding and non-coding sequences to become part of a chimeric gene (Brosius, 1999; Long, 2001; Zhang, 2003).

Unequal crossing-over is another mechanism of gene duplication and can occur between sister chromatids of the same chromosome during mitosis, or between homologous chromosomes during meiosis. This mechanism can result in duplication of part of a gene, an entire gene or several genes depending on the position of the crossing

over event. Unequal crossing-over usually results in a tandem duplication where a duplicated gene pair are linked on a single chromosome, and will result in introns present in the original gene being present in the duplicated gene(s) (see (Zhang, 2003). Chromosomal or gene duplication most likely occurs by a lack of separation of daughter chromosomes after DNA replication, thus creating two copies of the one chromosome (see (Zhang, 2003)). Note that in the duplication of an entire genome (polyploidisation), the organism must go through a tetraploid (4N) evolutionary intermediate, before the re-establishment of the disomic (2N) state, thus leading to the apparent duplication of large portions of chromosomal regions. Therefore, this process is dependent upon the viability of a tetraploid ancestor and thus is restricted to the ancient past for most vertebrates (Eichler, 2001). Allotetraploidy results from hybridisation between closely related species, and is common in the plant kingdom (Wendel, 2000).

1.2.2 Fates of Duplicated Genes

Following gene duplication a dramatic change in the species in question will not immediately be seen, but rather the duplicated gene or genes in an individual may be fixed in the entire species and slowly diverge by either neutral genetic drift or positive selection (Lynch and Force, 2000). The classical model predicts that duplicated genes will initially have fully overlapping, redundant functions and, in this situation, one copy can shield the second copy from natural selection (as long as gene dosage is not critical) (Force *et al.*, 1999). If a duplicate gene confers no selective advantage, it has only a small probability of being fixed in the population, and will most likely become a pseudogene and ultimately lost from the genome within the first few million years after duplication (Kimura, 1983; Lynch and Conery, 2000). The long-term evolutionary fate of duplicated genes is determined by the extent and pace of their functional divergence. Duplicated genes generally face three possible fates; i) conversion into a pseudogene while the original gene remains unchanged (non-functionalisation), ii) acquisition of a novel (advantageous) function while the original gene remains unchanged (neo-functionalisation), or iii) both the duplicated and original genes may acquire complementary loss-of-function mutations and must act in concert to maintain the original function (sub-functionalisation) (see Fig 1.2) (Ohno, 1970; Force *et al.*, 1999).

Neofunctionalisation is one of the most important outcomes of gene duplication, and one that has the widest implications for evolution. Two models have been proposed to explain the process of neofunctionalisation, the first based on neutral evolution (the Dykhuizen-Hartl effect), and the second on positive selection. In the Dykhuizen-Hartl model, random mutations are fixed in the duplicate gene, which, following an alteration of the environment or genetic background, enable novel gene functions (Dykhuizen and Hartl, 1980; Kimura, 1983). The second model states that after gene duplication, a new function may confer a weak selective advantage and the fixation of these mutations is accelerated due to positive selection (Goodman *et al.*, 1975). Along with a duplicated gene evolving an entirely new function, neofunctionalisation can also mean that the duplicated gene takes on a different, but related function. An example is the ability of humans and related primates to see a wide range of colours; duplication of a parent opsin gene resulted in a duplicate red- and green-opsin pair, which allowed for a 30-nm difference in the maximum absorption wavelength of light, resulting in a wider range of colour sensitivity (Yokoyama and Yokoyama, 1989).

Subfunctionalisation has been theorised to take place via duplication, degeneration and complementation (the DDC model). In this model, either both the duplicate and original gene acquire complementary mutations so that the original function of the gene is divided between the two, or mutations restrict the expression of each of the genes, resulting in both being required to maintain a threshold level of gene expression (Force *et al.*, 1999). Examples of subfunctionalisation have been reported from most of the major animal phyla, and the phenomenon most neatly demonstrated in zebrafish. The paralogous zebrafish genes *hoxb1a* and *hoxb1b* have different functions in the nervous system, but their functions are also partially redundant. It was shown that both the original and duplicate genes were originally maintained in the genome due to the accumulation of complementary degenerative mutations in specific cis-regulatory regions (McClintock *et al.*, 2002).

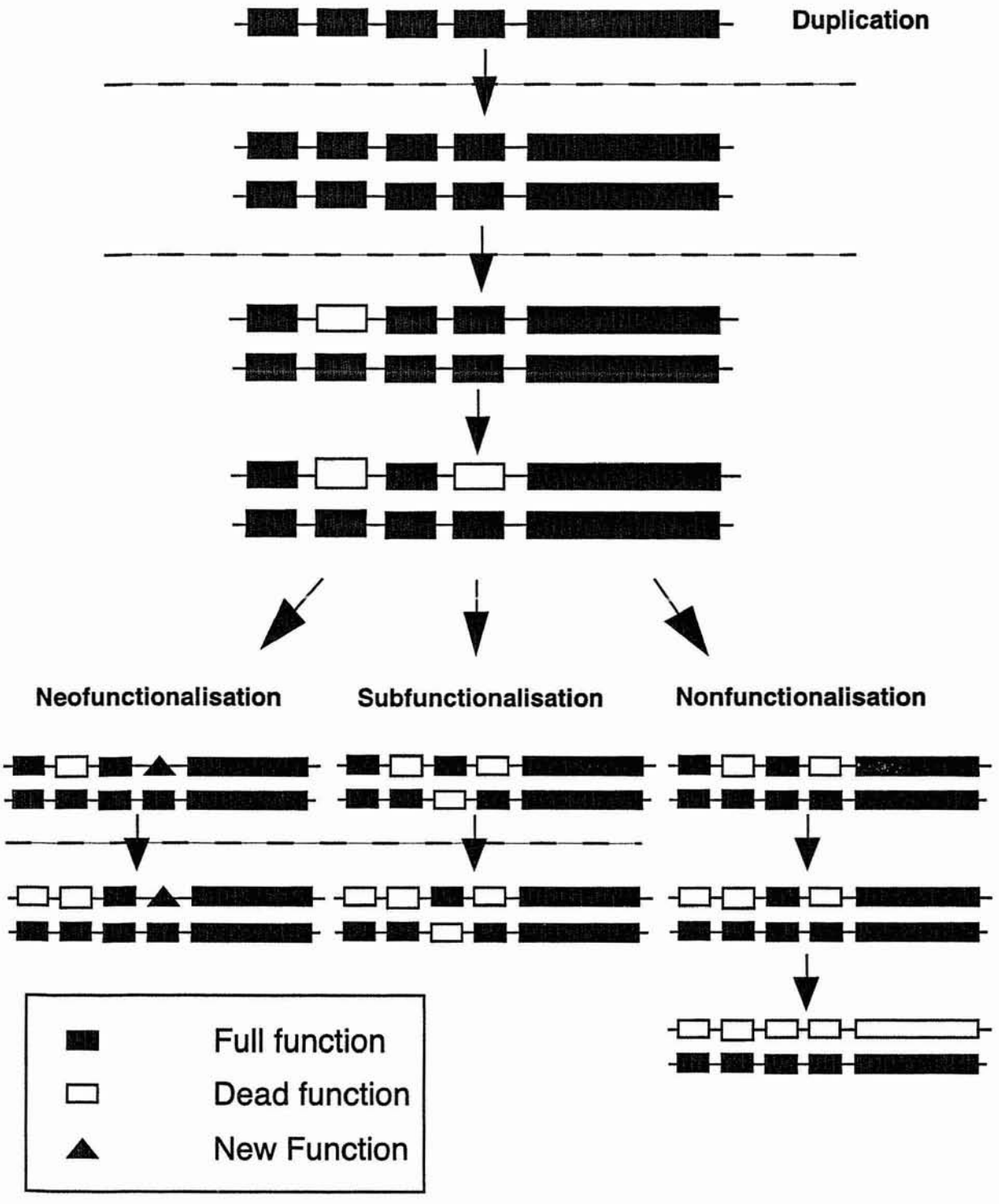

Figure 1.2: Schematic representation of three possible fates of duplicate genes. The small boxes denote regulatory elements with unique functions, while the large boxes denote transcribed regions. Black boxes represent intact regions of a gene, open boxes denote null mutations while triangles denote the evolution of a new function as shown in the figure legend. Duplication of a gene is followed by one of three alternatives. In Neofunctionalisation, the newly duplicated gene is able to acquire a new and useful function while the original remains unchanged. In Subfunctionalisation, degenerative mutations accumulate in complemenetary regulatory regions so that the original function of the gene is divided between the two. In Nonfunctionalisation, the newly duplicated gene accumulates mutations that make the newly duplicated gene a pseudogene while the original remains unchanged. (Adapted from Force *et al*, 1999).

1.3 Evolutionary Origins of Body Patterning

The origin of the second body axis has generally been seen as a major innovation which accompanied the metazoan radiation at the base of the Cambrian. Traditionally, cnidarians are considered as radially symmetric animals, possessing only one body axis (the oral/aboral (O/A) axis), but strictly radial symmetry is actually displayed by relatively few hydrozoan cnidarians. A form of bilateral symmetry has long been recognised in some cnidarians, most obviously amongst anthozoans (Stephenson, 1926), but this has often been interpreted as a case of convergent evolution. Both the revision of cnidarian phylogeny (with the Anthozoa rather than Hydrozoa as basal) and the growing amount of data on cnidarian genomes implies that the common ancestor of cnidarians and "higher" animals had a form of bilaterality, and that the radial symmetry seen in some cnidarians is a derived state (see (Finnerty, 2003; Ball *et al.*, 2004).

The A/P and D/V axes in bilateral animals are specified and patterned by conserved molecular mechanisms. For example, Hox clusters pattern the A/P axes of *Drosophila* and the mouse, and a system of this type is involved in A/P patterning in all Bilateria so far investigated (Slack *et al.*, 1993). Similarly, many aspects of both the specification and patterning of the D/V axis are achieved by molecular mechanisms that are conserved across the Bilateria. The *vnd/ind/msh* cascade controlling nerve cord fate in *Drosophila* has homologous members in vertebrates *(nkx2/gsx/msx)* and the repressive cascade appears to have been conserved despite the axis inversion between insects and vertebrates (see section 3.1.1). To investigate the origins of the axial patterning mechanisms of bilaterians, and the relationship between the axes of cnidarians and "higher" animals requires comparative molecular studies to determine if homologs of key axial patterning genes are present in cnidarians, and whether these genes are expressed in patterns that are consistent with conservation of function. Recently, genes related to the *Drosophila* gene *decapentaplegic (dpp),* which plays a central role in defining the D/V axis across the Bilateria, have been identifed in two cnidarians and shown to be expressed in patterns that are consistent with conservation of function (Hayward *et al.*, 2002; Finnerty, 2003). The fact that in basal (anthozoan) cnidarians these genes are differentially expressed in an axis that is orthogonal with respect to the

primary (O/A) body axis provides the most convincing evidence to date that the common ancestor of cnidarians and "higher" animals had a form of bilateral symmetry. However, there are no obvious morphological correlates of the cnidarian *dpp* expression patterns, complicating their interpretation. Based on the presence of key genes alone, it can be hypothesised that the Bilateria and Cnidaria are likely to share some basic axial patterning mechanisms, but that bilaterians have elaborated upon this organisational scaffold to a much greater degree than have their cnidarian sister group.

1.3.1 Hox Genes, paraHox Genes and the protoHox cluster

The zootype hypothesis proposes that the differential expression of a specific set of genes (those of the Hox cluster and genes related to *Drosophila empty spiracles* (*ems*), *orthodenticle* (*otd*) and *even-skipped* (*eve*)) along a primary body axis is a defining characteristic of the Metazoa (Slack *et al.*, 1993). It was also thought that the hierarchical application of this approach might provide a simple mechanistic key to understand the evolution of animal bauplans. Hox genes are a subset of the homeobox gene family and are characterised by three criteria; sequence similarity to genes of the *Drosophila* HOM-C complex (Antp and BX-C), serial position in a cluster, and spatial and co-linear expression along the A/P axis with respect to their position in the cluster; genes located at the 5' end of the cluster are expressed earlier and more posteriorly than those at the 3' end (Ruddle *et al.*, 1994). Hox genes have never been found in protists, fungi or plants, thus they seem to be unique to metazoans.

The isolation of three genes from Amphioxus (*AmphiGsh, AmphiXlox* and *AmphiCdx*) which were clearly Hox-like based on their sequence, but were not part of the Hox cluster (Brooke *et al.*, 1998) were postulated to form the hypothetical paraHox cluster, a paralog of the Hox cluster. The paraHox and Hox clusters were theorised to have originated from an early duplication of a hypothetical protoHox cluster (Brooke *et al.*, 1998). Duplication of the protoHox cluster to form the paraHox and Hox clusters, (and further duplication of the Hox cluster in particular) has been suggested as being the major factors in the evolution of the Metazoa (Brooke *et al.*, 1998; Akam, 2000; Kappen, 2000; Pollard and Holland, 2000; Shimeld and Holland, 2000; Garcia-Fernandez, 2004). Many theories have been put forward debating the existence of the

protoHox cluster, and much debate has focussed on the number of genes in this hypothetical cluster; some groups maintain that there were four protoHox genes (an anterior, a Group 3, a central and a posterior gene) (Finnerty, 1999; Ferrier and Holland, 2001; Yanze *et al.*, 2001; Ferrier and Holland, 2002; Martinez and Amemiya, 2002; Ferrier and Minguillon, 2003; Cook *et al.*, 2004; Seo *et al.*, 2004), while others state that the protoHox cluster consisted of just two genes, one anterior and one posterior gene (Garcia-Fernandez, 2004) (Fig 1.3). There is limited support for any of these arguments, and the evolutionary origin of the protoHox cluster is also unclear; sampling of the genomes of ctenophores, placozoans and sponges have not yet resulted in the isolation of any protoHox genes, although the isolation of a single Hox-like gene in the Ctenophores (Finnerty *et al.*, 1996) and a Hox/paraHox-like gene from a placozoan (Jakob *et al.*, 2004) have been reported, however further investigation is required in both cases. In addition, the existence of the paraHox cluster itself is an issue of contention; the clustering of the paraHox cluster described for amphioxus has not since been found conserved in other animals. For example the human paraHox genes map to four chromosomes, but only one of these contains a definitive paraHox gene cluster, while the others have only single *Cdx* or *Gsx* family genes (Pollard and Holland, 2000).

The first Hox-like genes isolated from cnidarians were from the hydrozoans, *Chlorohydra viridissima* (Schummer *et al.*, 1992) *Hydractinia symbiolongicarpus* and *Eleutheria dichotoma* (Schierwater *et al.*, 1991) and the anthozoan *Acropora formosa* (Miller and Miles, 1993). In *Acropora,* hopes of finding a conserved Hox cluster were raised when an *eve*-like gene (*eveC*) was isolated (Miles and Miller, 1992) and found to be tightly linked to an Antp-like gene (*antpC*) (Miller and Miles, 1993); this linkage also holds in the case of the sea anemone *Nematostella vectensis* (Finnerty, 1999) and is mirrored in vertebrates (Faiella *et al.*, 1991). It was thought that these genes might constitute the 5' end of a cnidarian Hox cluster, which would include the two Hox-like genes (*cnox1* and *cnox2*) from *Chlorohydra*, originally identified as *labial* (*lab)*-like and *deformed (Dfd)*-like respectively (Schummer *et al.*, 1992; Miller and Miles, 1993). However, while many more homeobox genes have since been isolated from cnidarians, definitive Hox genes have not yet been identified. In addition, those genes that were previously considered to be divergent Hox-genes now appear more like members of other, more recently identified homeobox families; for example the cnidarian *cnox2* genes were originally considered to be group 4 Hox genes, but are now regarded as

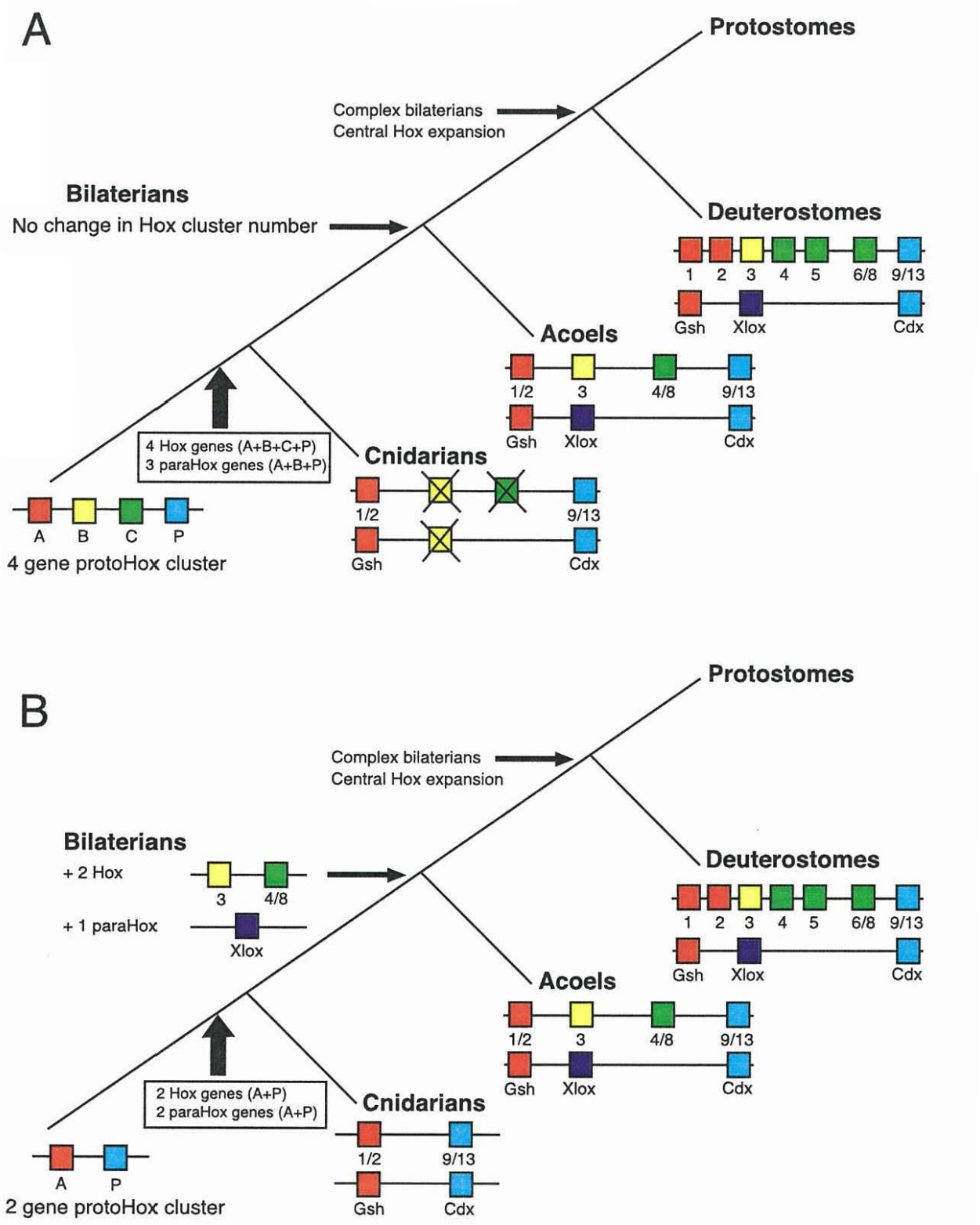

Figure 1.3: Changes in Hox and paraHox numbers associated with major metazoan transitions. (A) Under the four-gene protoHox model, the origin of bilaterians was not accompanied by changes in Hox or paraHox gene numbers. In addition, cnidarians lost at least three Hox and paraHox genes. (B) Under the two-gene protoHox model, the origin of bilaterians was coincident with the invention of two Hox (PG3 and Central groups) and one paraHox (Xlox) classes (adapted from Garcia and Fernandez, 2004).

orthologs of the *ind/Gsx* types from higher animals (Finnerty, 1999; Gauchat *et al.*, 2000; Hayward *et al.*, 2001; Finnerty *et al.*, 2003). In view of their uncertain status, it is probably most appropriate at this time to refer to the cnidarian genes that resemble true Hox genes as 'Hox-related' or 'Hox-like'.

The Hox-like genes identified thus far from cnidarians appear to fall into approximately four groups (Miller *et al.*, in preparation). Two of these resemble anterior and posterior Hox types, but their relationships with true Hox genes and other homeobox types in Bilateria are unclear. In addition, genes clearly related to *ind/Gsx* have been identified in a wide range of cnidarians (see (Gauchat *et al.*, 2000; Hill *et al.*, 2002)). The fourth type of Hox-related gene is most likely to have originated after the Cnidaria / Bilateria split. Only limited expression data are available for cnidarian Hox -like genes. In *Nematostella*, staggered expression along the O/A axis has been interpreted as reflecting conservation of Hox function, (Finnerty, 2003; Finnerty, 2004), but there is no evidence for linkage (i.e. the presence of a cluster) and the genes are actually paralogs (Miller *et al.*, in prep.).

1.4 Cnidarians as Model Animals

Cnidarians are among the earliest diverging animal phyla and are generally viewed as the 'sister-group' to the Bilateria (Medina *et al.*, 2001). As a phylum, the cnidarians have long been considered to be 'simple' animals, and as such have been used for comparative purposes to understand the origins of conserved developmental mechanisms and the nature of the last common ancestor. Characteristics of members of the phylum Cnidaria thought to represent ancestral character states include; diploblasty, a blind sac for a gut, the absence of a coelom and a decentralised nerve net (see (Willmer, 1990)). Cnidarians develop from two primary germ layers, the ectoderm and endoderm, and thus lack the true mesoderm that is characteristic of higher animals (triploblasts). Another important feature dividing animals such as cnidarians from higher animals is their body symmetry; higher animals have two axes, anterior/posterior and dorsal/ventral, whereas cnidarians have only one oral/aboral axis however as stated

before, recent molecular evidence suggests that bilateral symmetry may have evolved prior to the bilaterian/cnidarian divergence.

The cnidarians as a phylum are very successful with over 9000 species, the majority of which are marine dwellers, and includes corals, sea anemones and jellyfish. The phylum is divided into four classes; the Hydrozoa (Hydra), the Cubozoa (box jellyfish), the Scyphozoa (true jellyfish) and the Anthozoa (sea anemones and corals). On the basis of molecular data, the Class Anthozoa is now considered to be basal within the Cnidaria; the most convincing evidence for this is that, uniquely amongst cnidarians, anthozoans possess circular mitochondrial genomes like those characteristic of higher animals (Bridge *et al.*, 1992), but the basal position of the Anthozoa is also supported by ribosomal DNA phylogenies (Bridge *et al.*, 1995; Odorico and Miller, 1997; Medina *et al.*, 2001). Thus, although Hydra has a long history as the textbook cnidarian, anthozoans are arguably better positioned to reflect the traits of the last common ancestor.

1.4.1 Cnidarian Nervous Systems

The body plan of the Cnidaria consists of an external and an internal epithelium (the ectoderm and endoderm respectively in embryological stages, epidermis and gastrodermis in the adult) separated by a thin, 'acellular' intermediate layer (the mesoglea) and enclosing the central digestive cavity (Hyman, 1940). The cells of the ectoderm include sensory neurons that allow for signal transduction in either direction and are connected via synaptic junctions to a nerve plexus (Grimmelikhuijzen *et al.*, 1996). As cnidarians are the most basal animal group with a nervous system, it is thought that their nervous systems most closely reflect the ancestral state (Grimmelikhuijzen and Westfall, 1995). The cnidarian nervous system is composed of a nerve net - a loose meshwork of interconnecting neurons - which condenses to form nerve plexuses or tracts (Grimmelikhuijzen and Westfall, 1995). However, there is growing evidence that the cnidarian nervous system is more complex than was previously assumed. Cnidarian neurons are known to be multifunctional with combined sensory, motor, interneuronal and neurosecretory functions (Grimmelikhuijzen *et al.*, 1992). In Hydra, at least six neurochemically different populations of neurons exist

(Hansen *et al.*, 2000) and over 30 biologically active neuropeptides have been identified in the sea anenome, *Anthopleura elegantissima* (Grimmelikhuijzen and Westfall, 1995; Grimmelikhuijzen *et al.*, 1996). In addition to classical neurotransmitters, the sea pansy contains large amounts of the peptide Antho-RFamide which is widely distributed in its nervous system (Grimmelikhuijzen and Graff, 1986) and gonadotrophin-releasing hormone (GnRH), previously known only from molluscs and vertebrates, has been isolated from both the sea pansy (*Renilla koellikeri*) and a sea anenome (*Nematostella vectensis*) (Anctil, 2000). The recent use of immunohistochemical methods has permitted visualisation of transmitter-specific neurons; in *Acropora millepora,* neurons span the ectoderm and extend perpendicularly to the surface forming synapses with those that lie parallel and adjacent to the basement membrane (on the ectodermal side), as visualised with an antibody to the neurotransmitter RFamide (Hislop et al, in prep), and seen by *in situ* hybridisations with the homeobox genes, *Pax-Cam* (Miller, 2000) and *cnox-2Am* (Hayward *et al.*, 2001). While the development of the cnidarian nervous system was though to be largely uncoordinated, in the jellyfish *Podocoryne carnea*, the tyrosine-tubulin positive nervous system has been shown to develop in a serially repeated pattern initiating at the 'anterior' end (with respect to direction of swimming) and forming continuously along the A/P axis (Groger and Schmid, 2001). Several subsets of transmitter-specific neurons have also been reported including a ring-like set of neuron bundles around the hypostome of some hydrozoans (Grimmelikhuijzen, 1985), and electrophysiological observations link patterns of neuronal activity with specific behaviours (Mackie, 1990). For example, hydrozoan and scyphozoan medusae contain gravity sensors and photoreceptors to assist their pelagic lifestyle and when necessary propulsion is facilitated by a simultaneous, symmetrical contraction of the bell due to circular rings found at the bell margins. In addition, food can stimulate nerve rings located near the mouth and tentacles of hydroid polyps and cause a rapid action of the tentacles (see (Grimmelikhuijzen and Westfall, 1995)).

1.4.2 Acropora millepora as a model cnidarian

Classically, the freshwater species *Hydra* and *Chlorohydra* (note that most cnidarians are marine dwellers) have been the most widely studied of the cnidarians, but several features make them unsuitable as models for comparative molecular embryological

studies, and as such other cnidarians such as *Nematostella vectensis* and *Podocoyne carnea* have become more popular as the focus for molecular and embryological studies. Our research focuses on the reef-building anthozoan coral *Acropora millepora* which has several important advantages over the *Hydra* spp. Firstly, while in general the pattern of embryonic development amongst the cnidarian classes is highly variable, that of the hydrozoan class is the most derived. *Hydra* commonly reproduces by asexual budding; sexual budding is unpredictable and occurs within a thick cuticle (Martin *et al.*, 1997). In contrast acroporid corals participate in annual mass spawning events (Harrison *et al.*, 1984), which have the advantage of being predictable and result in large quantities of fairly synchronous embryonic material. Secondly, the fact that *Acropora* spp. are members of the Anthozoa implies that they may be more likely to display ancestral characteristics than the more derived hydrozoans. Thirdly, the *Acropora* genome is not strongly biased with respect to base composition (~61% (A+T) Miller pers. comm.) whereas those of *Hydra* (~71% (A+T)) (Fisher and Bode, 1989) and many other cnidarians have strong biases. Our laboratory has pioneered the use of *Acropora millepora* as a model system for understanding the evolution of many aspects of development, and our collaborator, Dr Eldon Ball has developed methods for *in situ* hybridisation and is establishing the descriptive embryology of *Acropora*. Many molecular tools are now available for *Acropora*, including a number of stage-specific cDNA libraries and genomic libraries in phage and cosmids. A recent preliminary EST analysis of *Acropora millepora* implies that the cnidarian gene set, and by implication, that of the common metazoan ancestor, are surprisingly rich and complex. Paradoxially, while anthozoan cnidarians have the simplest extant nervous systems, the *A. millepora* genome contains many of the genes known to specify and pattern the much more sophisticated nervous systems of vertebrates and the fly.

1.4.3 Embryological development of Acropora millepora

A. millepora is a simultaneous hermaprodite, and upon spawning, buoyant egg and sperm bundles are released into the water where the gametes disperse. The gametes are at least initially self-incompatible, but cross-fertilise with gametes from other colonies to form a zygote that settles to form a new polyp after passing though a number of developmental stages (reviewed in (Ball *et al.*, 2002)). Typically, the development to

the planula larva stage takes approximately 96 hours, but is strongly temperature dependent. After this point the larvae are competent to undergo settlement, but this generally requires appropriate settlement cues, which may be provided by coralline algae (Morse *et al.*, 1996), and mediated by LWamides (Iwao *et al.*, 2002). The major stages of early development in *A. millepora* are summarised in Fig 1.4 and described below.

After fertilisation, the zygote undergoes unilateral cleavage, resulting in the formation of two equal blastomeres. At approximately 7 hours post-fertilisation, the embryo has reached the 8-cell stage, and at this point cell divisions cease being regular. At 13 hours post-fertilisation, the embryo passes through the first clearly distinct morphological stage, which is colloquially known as the 'prawn-chip'. At this point, the embryo is an irregularly shaped bilayer of undifferentiated cells; note that the limited literature suggests that this morphological stage may not be typical of anthozoan development. Gastrulation typically occurs at 22 – 36 hours after fertilisation, resulting in the formation of the two germ layers, endoderm and ectoderm. Gastrulation begins with the upward folding of the two edges of the prawn-chip, as the disc both reduces in circumference and thickens (Fig 1.5A-B). As the two sides meet, cells of one of the layers become internalised and eventually lose their epithelial character and redifferentiate to form endoderm (Fig 1.5C-F). At approximately 28 hours post fertilisation the embryo becomes spherical and a blastopore becomes apparent and slowly closes, closure marking the transition from embryo to larva. After this stage, larva become pear shaped and cilia develop, enabling motility – swimming behaviour is varied and embryos are observed either spinning on their axis, swimming rapidly or slowly on one side of their body, or spiralling. The oral pore, which develops at the posterior end of the embryo with respect to the direction of swimming, is lined with cilia and appears to consist exclusively of glandular ectodermal cells, and has therefore been postulated to have a function in extracellular digestion (Ball *et al.*, 2002). At approximately 50 hours into development, other recognisable cell types first appear, including putative neurons which are positive for the neurotransmitter RFamide, as visualised by antibody staining. As development proceeds, the pear shaped embryos become spindle shaped and differentiation continues. Settlement can be delayed for months until the planula receive appropriate settlement cues and attach to the substratum by their anterior (aboral) end and sway from side to side as they flatten

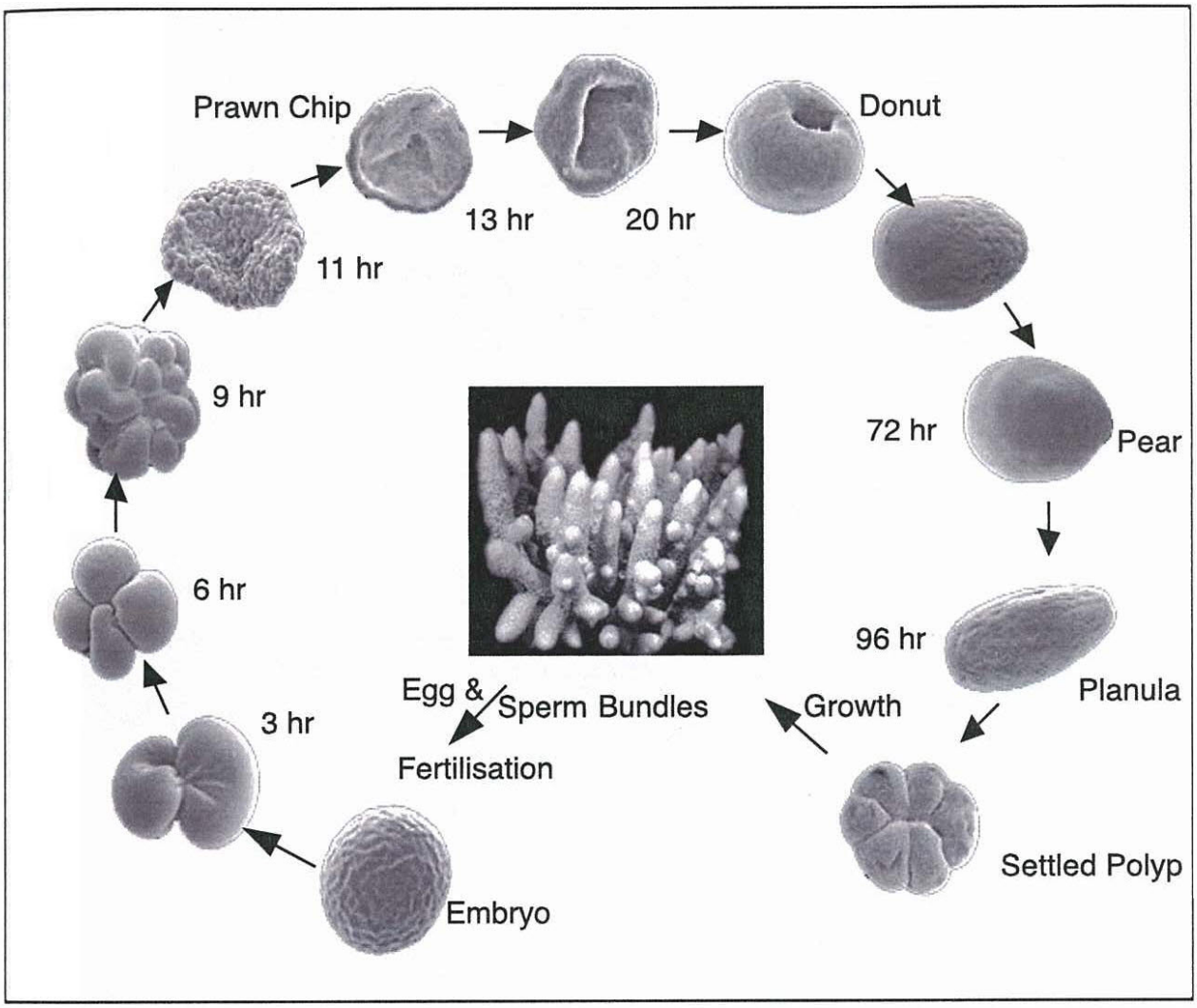

Figure 1.4: Scanning Electron Micrographs of the embryonic development of *Acropora millepora*. The stages are not to scale. Times shown are hours post-fertilisation and can vary depending upon several conditions. The morphology of late stage embryos (~96hr) is sometimes inconsistent but planula usually have an elongated spindle shape. (SEM images courtesy of Dr. Eldon Ball, RSBS, ANU).

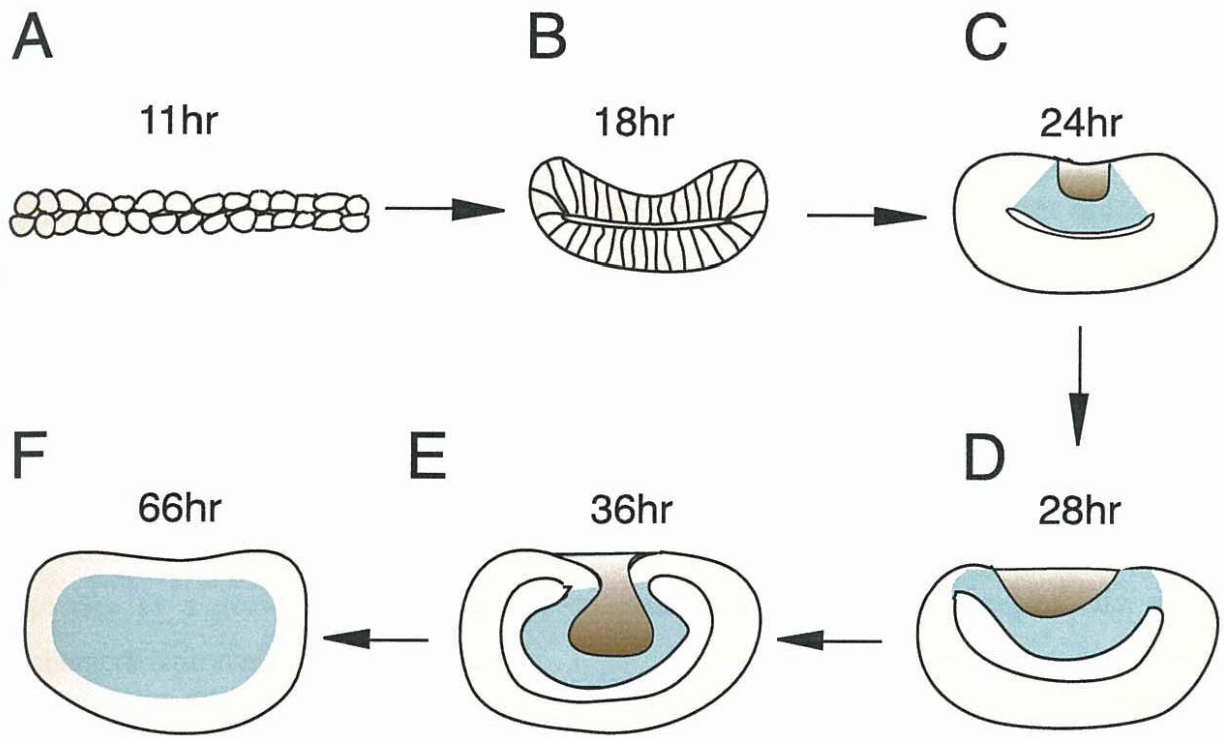

Figure 1.5: Formation of the two tissue layers in *Acropora*. Schematic representation of the process of gastrulation in *Acropora* which forms the two tissue layers. (A) The cellular bilayer of cells at approximately 11 hr post-fertilisation (prawn chip stage). (B) The cellular bilayer begins to contract and thicken and the outer edges appear to fold upwards. (C) The early donut stage of development where the edges continue to fold upwards and inwards. (C-F). From approximately 28 - 66 hr post-fertilisation the deep gastral pore is present and the outer edges gradually make contact with each other. The presumptive endoderm is pale blue and the hollow area is represented by a light brown gradient. The presumptive ectoderm is shaded pale yellow (Adapted from Ball *et al*, 2004).

along their oral-aboral axis. In the time immediately before and after the settlement process takes place, there is a dramatic reorganisation of tissues and the start of calcification, including a complete remodelling of the nervous system (Ball, Miller *et al.*, unpublished). The flattened post-settlement polyp has also been observed spinning on its O/A axis, suggesting the presence of cilia facilitating this motion (Hayward, pers. comm.). Following settlement, mesenteries appear within the flattened disc as calcification begins, a process that also appears to be coordinated with a set order of septa formation (Hayward, pers. comm.) (Hyman, 1940). Exactly where the 'mushroom' morphology (see Figs 4.4 and 4.9) fits within this scheme is unclear. A small but significant proportion of pre/post settlement larvae have the 'mushroom' morphology, but these are not seen sufficiently frequently and/or regularly enough for it to be clear whether this represents a normal stage of development. Following settlement, tentacles begin to form in the area surrounding the oral pore, which begins to rise as a growing tip; eventually, colonies of each *Acropora* species take on characteristic skeletal morphologies.

1.5 Project Objectives

While research on cnidarians has a long history, many of the techniques of molecular developmental biology have only recently been applied to cnidarians. The objectives of this study were to characterise *Acropora millepora* genes related to specific homeobox genes which play key roles in nervous system development in higher animals. As strict genetic analyses are not possible in the coral (or other cnidarians), spatial and temporal expression data were used as evidence for (or against) conservation of function. The primary focus of this work was on identifying genes related to those of the D/V patterning cascade (*vnd/ind/msh)* in order to better understand the relationship between the single overt body axis of cnidarians and the two perpendicular axes of bilateral animals. Although some of the work presented in Chapters 3 and 4 is preliminary, it provides important insights into the evolution of function in several homeobox gene families, and can form the basis of functional analyses aimed at addressing specific hypotheses. Chapter 5 provides an example of how transgenic expression can be used to test for conservation of function, extending previous ideas about the evolution of Pax

genes and their roles in nervous system patterning and eye specification. The research presented here provides further evidence of the surprisingly high genetic complexity of these morphologically simple animals, and provides insights into the ancestral functions of a subset of highly conserved genes involved in the development of the bilaterian nervous system.

Chapter 2 - Materials and Methods

2.1 Coral – *Acropora millepora*

Colonies of *Acropora millepora* were collected from Magnetic Island, north Queensland (Latitude 19°09' South; Longitude 146°49' East). At least two adult *A.millepora* collected were placed in large containers of seawater on the shore line before dusk on the evenings anticipated to precede the spawning event. If spawning did not occur, the colonies were replaced to the ocean at a common site for the next evening's collection. After spawning egg and sperm bundles were removed from the surface of the water and were mixed in containers containing fresh seawater and left overnight to fertilise and begin development. The following day, developing embryos were monitored and maintained until the appropriate developmental age was required, at which time embryos were removed and either frozen in liquid nitrogen or fixed (see section 2.1.3.1). Every 24 hours, dead embryos and resulting surface lipid were removed and the water exchanged for fresh seawater so that development could continue.

2.1.1 Extraction of genomic DNA from Acropora millepora sperm

(adapted from (McMillan *et al.*, 1988))

Approximately 3 g of *Acropora millepora* sperm was collected and washed once with 50mLs proteinase K buffer (50mM Tris, pH8.0, 1mM $CaCl_2$). The pellet was resuspended in 15mLs of Proteinase K buffer, and Proteinase K enzyme was then added to a final concentration of 100μg/mL. SDS was added to a final concentration of 0.4% (from a 20% stock), thus making the final volume 20mLs. Sperm was lysed at 55°C for 3-5 hours without shaking, after which an equal volume of phenol:chloroform was added to extract proteins. This was achieved with gentle rocking for 6-8 hours at room temperature. DNA was recovered and extracted with an equal volume of chloroform for the same period of time. The DNA was then recovered by decanting and dialysed against TE pH8.0 and stored at 4°C.

2.1.2 Acropora millepora genomic and cDNA libraries – bacteriophage manipulation methods

Lambda vectors used in the preparation of clones from *A.millepora* libraries are as follows:

Table 2.1: Lambda vectors and their applications

Vector	Application
λGEM-11	Genomic libraries
λZAP-II	cDNA libraries
ExAssist helper phage	*in vivo* excision of cDNA clone in pBluescript plasmid from λZAP-II vector

2.1.2.1 Phage Plating and Titration

Preparation of Plating Bacteria

XL-1 Blue-MRF'

50mLs of NZY media (see section 2.3.2) was inoculated with 500μL of overnight culture and grown with moderate agitation at 37°C until the OD_{600} was equal to 0.6. The cells were then recovered by centrifugation at 3000rpm for 5 minutes. The pellet was resuspended in 10mM $MgSO_4$ to a final OD_{600} of 2 (approximately 5mLs $MgSO_4$) for large (150mm diameter) Petri dishes, or OD_{600} of 1 for small (90mm diameter) Petri dishes. Alternately, host strain bacteria were prepared by inoculating 100mL of NZY media with a single colony and incubated at 37°C for 12 –16 hours with shaking. 20mL of this overnight culture was then centrifuged at 3000rpm for 5 minutes at room temperature, and the resulting pellet resuspended in 40mL of 10mM Magnesium Sulphate solution. These preparations were viable for 5-10 days when stored at 4C.

LE392

500μL of overnight culture of LE392 in LB media (see section 2.3.2) was inoculated into 50mLs of LB media, 500μL of 1M $MgSO_4$ and 500μL 20% maltose. This colony

was grown at 37°C shaking until an OD_{600} of 0.6 – 0.8 was reached. The cells were recovered by centrifugation at 3000rpm for 5 minutes and the pellet resuspended in 5mLs 10mM $MgSO_4$. Alternately, 100mL of LB media with 1mL 1M $MgSO_4$ and 1mL 20% maltose was inoculated with a single colony and grown for 12 – 16 h ours at 37°C. 20mL of this overnight culture was then centrifuged at 3000rpm for 5 minutes at room temperature and the resulting pellet resuspended in 40mL of 10mM Magnesium Sulphate solution. These preparations were viable for 5-10 days when stored at 4°C.

Titration and plating of phage

For each phage infection, 200μL aliquots of host bacteria were mixed with the appropriate volume of phage diluted in suspension medium (SM) buffer and incubated at 37°C for 20 minutes to promote infection. Generally, 10μL and 100μL of a 10^{-5} dilution of library was plated onto 90mm plates, and was sufficient to calculate the titer of the library expressed as plaques forming units per microlitre (pfu/μL). Molten top agarose (LB for LE392 cells; NZY for XL1-Blue cells) cooled to 50°C was added to the phage infection (10mL for 150mm plates; 5mL for 90mm plates) and mixed prior to pouring onto pre-warmed LB or NZY agar plates. Plates were left for at least 15 minutes to allow the top agar to set and were incubated inverted at 37°C for 12-16 hours.

2.1.2.2 Screening Phage libraries

Both the *Acropora millepora* cDNA and genomic DNA libraries were constructed by Dr David Hayward (RSBS, Australian National University). Four different cDNA libraries were available for use generated from mRNA extracted from embryos at approximately 11-13 hours of development (prawn chip stage), 96 hours post fertilisation (pre-settlement), and from the post settlement and adult stages. High molecular weight genomic DNA was extracted from frozen sperm, digested with *MboI* and size fractionated for genomic library construction in λGEM-11.

A plaque density of approximately 100 000 plaque forming units per 150mm plate was used to screen either cDNA library, for a total library screen of approximately 600 000

plaques per probe. In contrast, 10 000 plaque forming units per 150mm plate was used to screen the genomic library, for a total library screen of approximately 60 000 plaques per probe. Once the library was plated at the appropriate density from an overnight incubation at 37°C, the Petri dishes were cooled to 4°C for at least 30 minutes to minimise top agar separation from the bottom agar. Phage DNA was transferred from the agarose plates to Hybond-C+ circular nylon membranes (Amersham Biosciences) as per the manufacturers recommendations. Once on the plate, the nylon membranes were pierced asymmetrically with a needle to mark orientation before being lifted and placed in denaturation solution for 2-5 minutes, neutralisation solution for 7 minutes and 2xSSC for 1 minute before drying the membranes DNA-side up on Whatmann paper. The DNA was irreversibly bound to the membrane by being baked at 80°C for 2 hours.

Radioactively labelled probes were prepared as described in section 2.4.6. Membranes were placed in hybridisation bottles or small plastic containers containing 30 ml of hybridisation solution for at least 1 hour at 60 - 65°C prior to the addition of the radioactive probe. After addition of probe, membranes were further incubated at 60 - 65°C for 12-16 h with rotation in a hybridisation oven (Hybaid) or shaking in a heated water bath. Following hybridisation, the probe was removed and membranes were washed 2 x 15 min at 60 - 65°C in low stringency wash solution following an initial rinse in low stringency wash solution. Membranes were monitored with a Geiger counter which determined if further washes were required or a higher stringency wash was to be used. Once washes were completed, membranes were wrapped in plastic film, exposed to Phosphorimager screens (Molecular Dynamics) for 3-6 hours and then scanned in a Molecular Dynamics Storm Phosphorimager (Molecular Dynamics, Sydney). Alternatively, membranes were exposed to autoradiography film (XR; Fujifilm) with intensifying screen at –80°C overnight. Autoradiography films were manually developed by 4 minutes incubations each in Kodak developer and fixer and replenisher or were automatically processed.

Positive clones were selected by marking the phosphor image or film based on needle marks on the membranes, orientating with the plate and using a cut-off 1ml to 'plug' the plaque of interest. This plug was then placed in 1 ml of SM solution with one drop (~20 μL) of chloroform and vortexed at high speed for 1 min. Incubation at 4°C overnight or

at room temperature for 4 – 12 hours assisted phage elution from the agar before the agar plug was pelleted by centrifugation at 13,000 g for 5 min and the phage/SM solution removed. The resulting phage supernatant was then titrated and re-screened at a plaque density of 50-200 plaques per plate. This process was repeated until all plaques on a plate were shown to be positive, or until well-isolated positive plaques were obtained.

2.1.2.3 *In vivo* excision of cDNA clones

Positive plaques from cDNA library screening were excised from the phage as pBluescript phagemids, using the ExAssist helper phage and the non-supressing SOLR *E.coli* host strain, as described in protocols supplied by Stratagene. The ExAssist helper phage releases the pBluescript/cDNA particles as single stranded plasmids, recircularises them, and they are then packaged by the helper phage and secreted out of the host cells as phagemids. SOLR cells can then be transformed with these phagemids and maintained on LB/ampicillin agar plates.

Phage were eluted from single positive plaques by incubation in SM solution with chloroform overnight at 4°C as described previously. *E.coli* XL1-Blue MRF' cells were grown to an OD_{600} of 0.5 and were harvested by centrifugation at 3000xg for 10 min and resuspended in 5mLs 10mM $MgSO_4$. The excision process was carried out in 10 – 15mL tubes by addition of 200μL of XL1-Blue MRF' cells to 250μL phage stock (~10^6) and 1μL of ExAssist helper phage (10^6 pfu). The mixture was incubated at 37°C for 15mins, before the addition of 3mL of LB and incubation with shaking at 37°C overnight. The following day, the culture was heated to 70°C for 15 mins and cellular debris removed by centrifugation at 4000xg for 15 mins. The supernatant containing the excised phagemids was recovered and stored at 4°C. *E.coli* SOLR cells were grown to an OD_{600} of 1.0 throughout the day, and 200μL of SOLR cells was incubated with 100μL of the excised phagemids at 37°C for 15 mins. 50μL of the mixture was then plated on LB plates containing ampicillin and incubated at 37°C for 10 – 16 hours to allow for growth of colonies containing the excisied phagemid. Small-scale plasmid preparations from the resulting colonies were then performed to yield pBluescript

plasmid DNA containing the cDNA of interest. These cDNAs were then sequenced using T3 and T7 primers.

2.1.2.4 Extraction of bacteriophage DNA from λGEM-11 phage clones

Sequencing and characterisation of genomic clones required the preparation of large amounts of high quality λDNA. Unlike λZAP-II, λGEM-11 does not allow excision of inserts as phagemids. To prepare high quality DNA, a high titre phage suspension was generated using the liquid lysis method (Sambrook *et al.*, 1989), which involves the amplification, purification on a cesium chloride gradient (Sambrook *et al.*, 1989) and recovery of phage DNA (Lockett, 1990).

Phage Amplification

After isolating a single phage in a secondary or tertiary screen, amplification to a titre of $>10^7$ pfu/μL using the plate lysate and elution method as described in (Sambrook *et al.*, 1989) was performed. Briefly, plaques were plated to achieve complete lysis and eluted from a number of large agar plates by adding 10mL SM solution to the surface and incubated at 4°C overnight with gentle agitation. If needed, the process was repeated to allow large scale bacterophage DNA extraction.

Purification on a cesium chloride gradent

To initiate lysis, a 20mL culture of LE392 *E.coli* was grown overnight in LB containing 10mM $MgSO_4$ and 0.2% (w/v) maltose. The following day, the culture was infected with $5x10^8$pfu from a pure phage stock and incubated at 37°C for 20 mins to promote infection, before the entire phage/cell suspension inoculated into 200mL LB medium containing 10mM $MgSO_4$ in a 1.5L flask. This was incubated for 6-8 hrs at 37°C with vigorous shaking, or until lysis was evident. Sodium chloride was added to a final concentration of 1M and the lysate stored at 4°C overnight. The titre of this solution was calculated to determine the success of infection and purification of the phage DNA only continued if the titre was $>1x10^{10}$pfu/mL.

Phage particles were concentrated using the polyethylene glycol (PEG) precipitation method as described in (Sambrook *et al.*, 1989). The lysed culture was centrifuged at 10000xg for 10 mins to remove cell debris and the supernatant decanted to a new tube. PEG 8000 (Sigma) was then added and to a final concetration of 10% (w/v) and the preparation placed on ice for 2 hours to allow phage to precipitate. Phage were pelleted by centrifugation at 11000xg for 10 mins, and the pellets allowed to drain well, before being resuspended in 5mLs of SM solution. In order to remove any remaining PEG 8000 from the phage suspension, chloroform extraction was performed as follows; 1x starting volume of chloroform was added to the preparation, the solution vortexed, and the phases separated by centrifugation at 3000xg for 5 mins. The top aqueous layer containing the phage was removed to a fresh tube, and phage particles purified by caesium chloride gradient centrifugation. Solid caesium chloride was added to preparation to a final concentration of 0.75g/mL and the mixture transferred to polyallomer centrifuge tubes (Beckman). Centrifugation was performed at 35000xg for 22-24hours at 10°C in a SW41 swinging rotor bucket Beckman ultracentrifuge. After centrifugation, phage particles were present as a light blue visible band in the top third of the tube and were recovered using a 19-gauge needle and 2.5mL syringe. To remove the caesium chloride, the phage suspension was placed in dialysis tubing with a molecular weight limit of 12 000Da and dialysed against several changes of 100-fold volume excess of dialysis buffer.

Recovery of phage DNA

Following dialysis, phage were placed in microfuge tubes and the DNA eluted as follows. EDTA was added to 20mM final concentration, proteinaseK to a final concentration of 50μg/mL and SDS to 0.5% final concentration. The solution was then mixed well and incubated at 56°C for 1 hour, cooled to room temperature and the protein extracted with one phenol extraction, two phenol/chloroform extractions, and two chloroform extractions. Phage DNA was precipitated using ice-cold isopropanol, and pelleted at 8000xg for 10mins. Pellets were washed three times with 70% ethanol, and resuspended in 50 - 100μL of ddH_2O before spectroquantification and storage at -20°C.

Alternatively, smaller amounts of phage DNA were routinely prepared from 50mL liquid cultures using a Lambda Midi Kit (QIAGEN) and associated protocols.

Table 2.2: Reagents involved in phage manipulation methods

Name of solution	Composition
20x SSC	3M sodium chloride; 0.3M sodium citrate pH7.0
20x SSPE	3 M sodium chloride, 0.2M sodium dihydrogen phosphate, 20mM EDTA pH8.0
Denaturation Solution	0.5M sodium hydroxide, 1.5M sodium chloride
Neutralisation Solution	0.5M Tris.HCl, pH7.2, 1.5M sodium chloride
Suspension Medium (SM buffer)	0.1M sodium chloride, 0.01M magnesium sulphate, 0.05M Tris pH 8.0, 0.01% (w/v) gelatin
Wash Solution (Low Stringency	2xSSC; 0.1%SDS
Wash Solution (Medium Stringency)	1xSSC; 0.1%SDS
Wash Solution (High Stringency)	0.5xSSC; 0.1%SDS
Dialysis Buffer	10mM sodium chloride, 10mM magnesium chloride, 50mM Tris pH8.0

2.1.3 Fixation and in situ hybridisation of Acropora millepora embryos

The following protocols were obtained from Dr Eldon Ball and Dr David Hayward from the Australian National University, Canberra. Much of the *in situ* hybridisation work detailed in the results sections was carried out under the guidance of Dr David Hayward and Dr Eldon Ball at the Research School of Biological Sciences, ANU.

2.1.3.1 *Fixation of* Acropora millepora *embryos*

Embryos collected at spawning were kept alive in Petri dishes filled with millepore-filtered seawater (MPFSW) until fixation was ready to take place. Embryos were transferred to a small Petri dish (60 x 15mm) with 4% formaldehyde in 0.1M HEPES-buffered MPFSW and left to fix in this solution for 10 – 15 minutes. Embryos were washed in MPFSW for approximately 15 mins and were stored at 4°C for up to 7 days before being transported to the laboratory where they were transferred to a small Petri dish with 50% methanol, washed and placed in a second dish of 50% methanol. Embryos were then transferred to 2mL round-bottomed microfuge tubes for sequential dehydration steps, first with 70% methanol, which was replaced with 90% methanol 100% methanol. Embryos were stored at –20°C until required.

2.1.3.2 *Preparation of* Acropora millepora *embryos for in situ hybridisation*

Rehydration of embryos and removal of lipids

Post-fix embryos stored in absolute methanol at –20°C were transferred to a small Petri dish (60 x 15 mm) of absolute methanol at room temperature. From there, the embryos were moved to a Petri dish containing 70% MeOH followed by a dish containing 50% MeOH. Following this, they were transferred to a third Petri dish containing 1x PBS/0.1% Triton. If embryos were required to be cut, they were done so at this stage using a micro-knife in a 60 x 15mm Petri dish containing Sylgard Agar. Embryos were then transferred to a microfuge tube containing RIPA solution and were placed at 4°C with gentle shaking overnight. (RIPA Solution; 150mM NaCl, 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM EDTA, 50mM Tris pH8.0).

Dehydration of embryos

The following day, embryos were rinsed two times in PBS and then dehydrated with the following EtOH/dH2O series – 50% Ethanol, 70% Ethanol, 90% Ethanol, 100% Ethanol #1 and 100% Ethanol #2. The Ethanol was removed and replaced by a 50%

xylene: 50% absolute Ethanol mixture. This was replaced with pure xylene, and left at room temperature for 1-4hours.

Rehydration of embryos

Embryos were gradually rehydrated by passing them through the following solutions ensuring equilibration of approximately 5 minutes in each. Half of the xylene was removed and replaced with absolute ethanol. This was then replaced with pure ethanol two times to ensure all xylene was removed. Following this, 25% and then 50% of the ethanol was replaced with PBT (PBS; 0.1% (v/v) Triton X-100). After this, embryos were washed 3x5 minutes in PBT and were placed in *in situ* hybridisation solution (50% (v/v) formamide; 4xSSC; 1x Denhardt's solution; 50μg/mL heparin; 5% (w/v) dextran sulphate; 250μg/mL tRNA; 500 μg/mL denatured sonicated salmon sperm DNA; 0.1% (v/v) Tween-20) by gradual dilution as follows. Embryos were incubated for 10 minutes in 1 part PBT: 1 part hybridisation solution. This was replaced by 100% hybridisation solution and incubated for 10 minutes. The hybridisation solution was changed and embryos placed at 55°C for 1-3 hours before addition of riboprobe.

Riboprobe hybridisation and detection of transcripts

Hybridisation solution containing prepared embryos was reduced to a volume of 250μL and DIG- or Fluorescein-labelled probes added to a final concentration of 0.1 – 1.0μg/mL per hybridisation experiment. Embryos were hybridised for 48 – 72 hours at 55-58°C before addition of αDIG-AP or αFL-AP primary antibody depending upon the riboprobe used. A long wash series in hybridisation wash (50% formamide; 50% 4 x SSC; 0.1% Tween) was then begun at the hybridisation temperature beginning with a rinse of the embryos, followed by 2 x 20 minutes washes and then rotation at the slowest speed overnight. A further wash the following day was then performed followed by replacement of half the hybridisation wash in the tube with PBT and equilibration of the embryos to room temperature. The remaining hybridisation solution was gradually replaced with PBT by 3 x 15 minute washes. Alkaline phosphatase conjugated anti-DIG antibody (αDIG-AP) or alkaline phosphatase conjugated anti-Fluorescein (αFl-AP) antibody (Roche) diluted in PBS to 1:1200 – 1:1800 was added.

Incubation at room temperature for 2 hours or 4°C overnight was performed and unbound antibody was removed by 6 x 30 minute washes in PBT followed by 2 x 5 minute rinses in NTMT (100mM NaCl; 100mM Tris pH9.5; 50mM $MgCl_2$; 0.1% Tween20) prior to addition of substrate. To initiate the colour reaction, Sigma *Fast* Fast Red, BM Purple (Roche) or NTMT containing 450ng/μL NBT and 230ng/μL BCIP (Vector Laboratories) was added directly to the embryos followed by incubation at room temperature away from light. Termination of the colour reaction, which could often be seen in as little as 15 minutes or in as long as 48 hours, was achieved by placing the embryos in PBS and washing several times. Embryos were placed in 70% (v/v) glycerol to clear tissue before mounting in 90% (v/v) glycerol in preparation for photography. Embryos were viewed under a Zeiss Axioscope or Leica MZ FLIII stereomicroscope and images captured using a SPOT digital camera and processed using Adobe® Photoshop® 5.5 Software.

2.2 Fruit Flies – Drosophila melanogaster

2.2.1 Extraction of genomic DNA from Drosophila melanogaster

The method detailed below is known as Lifton Extraction as adapted from Bender *et al* (1983);

0.1g of frozen flies (approximately 100 *Drosophila*) were placed into a 1.5mL microfuge tube and 500μL of freshly made Lifton Solution (0.2M sucrose; 0.05M EDTA; 0.5% SDS; 100mM Tris pH9) was added. The flies were vigorously crushed with a small glass rod, and a further 500μL of Lifton Solution added when most of the fly matter was broken up. Vigorous agitation was continued until the tissue no longer formed aggregates. A small hole was punctured in the lid of each tube, and the tube was incubated at 65°C for 30 minutes. 200μL of 3-5M potassium acetate was added and gently mixed by inversion 4-6 times and the solution was then incubated on ice for 60 minutes. The solution was then centrifuged at 10000rpm for 10 minutes at 4C and the supernatant carefully removed and divided into two new microfuge tubes. Successive phenol, phenol/IAC and IAC extractions were performed gently with centrifugation at 5000rpm for 5 minutes to separate the phases. 1μL of RNaseI (10mg/mL) was added to each tube and incubated at 37°C for 15 minutes. An equal

volume of room temperature ethanol was then added to each tube, and gently mixed by inversion followed by incubation at room temperature for 5 minutes. The tubes were then centrifuged at room temperature for 15 minutes at 8000rpm. The supernatant was carefully removed from each tube, and the pellet washed with 80% ethanol before being dried briefly by vacuum centrifugation and resuspended in 50μL TE buffer overnight at 4°C. The preparations were recombined and spectroquantified. To ensure that the DNA obtained was of sufficiently high molecular weight, 2μg was cut with *EcoRI* and run on an agarose gel alongside 2μg of uncut DNA.

2.2.2 Fixation and in situ hybridisation of Drosophila melanogaster embryos

Drosophila melanogaster embryos of different stages were fixed in 4% paraformaldehyde fix (in 1x PBS) by Dr David Hayward at ANU according to standard protocols. Synthesis of digoxygenin labelled riboprobes was achieved according to protocols detailed in section 2.5.4. Fluorescein-labelling of riboprobes was achieved using a fluorescein-NTP mix in the place of DIG-NTP mix. Detection of fluroescein-labelled riboprobes can be achieved directly on a fluorescent microscope negating the need for addition of an enzyme-linked primary antibody and the subsequent substrate reaction.

All *in situ* hybridisation experiments conducted on *Drosophila* embyos were performed under the guidance of Dr David Hayward and Dr Eldon Ball at the Research School of Biological Sciences, ANU. Approximately 100 fixed wild type *Drosophila melanogaster* embryos per *in situ* hybridisation experiment were placed into round bottomed microfuge tubes precoated in PBS (phosphate buffered saline) to prevent embryos from sticking to the sides of the tubes. Embryos were gradually rehydrated by replacing the methanol with PBS in small increments. This process was repeated by replacing the PBS with PBS/0.1% Tween-20 solution (PT Buffer). Again this process was repeated replacing the PT buffer with *in situ* hybridisation buffer and gradually incubating the embryos at 55°C for longer periods of time. The hybridisation buffer was removed leaving only 200μL remaining and 2μL of labelled riboprobe resusended in 48μL of *in situ* hybridisation buffer was added. Embryos were left to hybridise at 55 - 60°C overnight. The following day, wash solution (1 x PT buffer; 2mg/mL BSA; 1% DMSO) was equilibrated to 60°C before removing the layer of liquid above the

hybridised embryos and filling the tube with wash solution. Embryos were washed in this way on a rotator at the hybridisation temperature 8 x 5 minutes with the final wash proceeding overnight. After washing of the unbound riboprobe was completed, embryos were placed in PT buffer by gradual dilution as before. Following this, 4 x 20 minute washes at room temperature in PBT buffer (PBS; 0.2% BSA; 0.1% Tween-20) on a rotator was performed. To a final volume of 350μL, αDIG-AP (Roche) was added to a final dilution of 1/2000 dilution; αDIG-HRP or Rabbit αFL-AP Alexa-488 to final dilutions of 1/100 or 1/300, depending upon the needs of the experiment. This was followed by incubation at room temperature with slight agitation for 1 – 3 hours. Removal of unbound primary antibody was accomplished by 2 x 1minute washes in PBT, followed by 4 x 30 minute washes in PT at room temperature. To initiate the colour reaction on those embryos hybridised to primary antibody conjugated with alkaline phosphatase, BM Purple containing 450ng/μL NBT and 230ng/μL BCIP was added directly to the embryos or Vector ™ Red (Vector Laboratories) was added according to the manufacturers instructions and incubated at room temperature away from light. To those experiments involving αDIG-HRP primary antibody, the colour reaction was initiated by addition of DAB to a final concentration of 0.5mg/mL (from a stock of 5mg/mL DAB/1 x PBS), incubation for 2 minutes at room temperature and then addition of 1/250 final dilution of 3% H_2O_2. Embryos were viewed under a Zeiss Axioscope and images captured using a SPOT digital camera and processed using Adobe® Photoshop® 5.5 Software

2.2.2.1 Double *in situ* hybridisations on *Drosophila melanogaster*

Double *in situ* hybridisation experiments utilising two riboprobes were performed at the Research School of Biological Sciences, ANU under the supervision of Dr David Hayward and Dr Eldon Ball. The following protocols involve different combinations of primary antibodies and colour substrate reactions and were performed after simultaneous hybridisation with fluorescein and digoxygenin labelled riboprobes and subsequent washing steps as detailed above. After the procedure, embryos were cleared in 90% glycerol 1 x PBS and were viewed under a Zeiss Axioscope, images captured using a SPOT digital camera and processed using Adobe® Photoshop® 5.5 Software

αFL-AP: vector red/ αDIG-AP: BCIP/NBT

Embryos were incubated for 2 hours with Rabbit αFL-AP Alexa-488 to a final diulution of 1/100 or 1/300 before being washed 8 x 15 minutes in washing solution (1 x PT buffer; 2mg/mL BSA; 1% DMSO). Equilibration for 3 x 5 minutes was then performed in 100mM Tris pH 8.2; 0.1% Tween-20 before staining with Vector™ Red according to the manufacturers instructions. The reaction was stopped by washing the embryos 2 x 1 minute in PT buffer and then heating to 65°C for 30 minutes to inactivate the alkaline phosphatase enzyme. The stain was fixed in paraformadehyde (PFA) fix for 20 minutes before embryos were blocked in PBT for 60 minutes. Embryos were then Incubated with αDIG-AP to a final dilution of1/2000 for 2 hours before antibody was washed off with 8 x 15 minute washes in AP buffer. Staining in BCIP/NBT then followed and the reaction was stopped by rinsing the embryos in PBT.

αDIG-AP:vector red/αFL-AP:direct fluorescein visualisation

Embryos were incubated for 3 hours in αDIG-AP to a final dilution of 1:2000 before being rinsed twice in PT buffer and washed 5 times over three hours in PT buffer on a rocking platform. Two washes in 100mM Tris pH 8.2 was then performed before staining with Vector™ Red according to manufacturers instructions. Staining was stopped by washing twice in PT buffer before it became too intense as to quench the fluorescein fluorescence.

αDIG-HRP:DAB/αFL-AP:BCIP/NBT

Embryos were incubated for 2 hours with αDIG-HRP antibody and the antibody washed by 8 x 15 minute washes in PT buffer (1x PBSl 0.1% Tween-20). Incubation for 2 minutes in DAB staining solution (1/10 dilution 5mg/mL DAB in 1 x PBS) was followed by addition of 1/100 – 1/200 final dilution of 3% H_2O_2. Staining was monitored and stopped by rinsing in PT buffer. The PT buffer was replaced with blocking solution (1x PBS; 0.1% Tween-20; 2mg/mL BSA; 5% Sheep Serum; 1% DMSO) and incubated at room temperature for up to 1 hour with gentle agitation. Incubation with αFL-AP for 2 hours at room temperature or overnight at 4°C with

gentle agitation was followed by 8 x 15 minute washes with PT buffer and 3 x 5 minute washes with AP buffer. Staining was achieved by adding NBT/BCIP coloured substrate as detailed above and stopped by washing with PT buffer. The stain was fixed in PFA fix in PBS overnight.

2.2.2.2 *Antibody/in situ hybridisation doubles on* Drosophila melanogaster *embryos*

Mouse α22c10 primary antibody (Roche) was added to a subset of embryos (1:5 dilution) to visualise cells of the peripheral nervous system in relation to cells which showed expression of the gene of interest. Embryos were prepared and DIG-labelled riboprobe added. Simultaneously αDIG-AP to a final dilution of 1/2000 dilution and mouse α-22c10 antibodies were added. The alkaline phosphatase catalysed reaction was initiated by addition of BCIP/NBT as previously described. This was followed by washing the embryos as usual, and addition of goat αmouse-HRP secondary antibody to a final dilution of 1:300. The coloured reaction was initiated by addition of DAB and H_2O_2 as previously described and the reaction stopped when the colour had developed to an appropriate level by washing 4 times with 1 x PBT, followed by a rinse in 1 x PBS and then clearing the embryos in 70% glycerol/PBS.

2.2.3 P-Element Transformation

P-element transformation was utilised in order to introduce different enhancer/promoter fragments of the *Drosophila ind* gene into a wild type background to generate stable fly lines. Ultimately, it was hoped that one of these fragments could drive *ind* expression in a spatially restricted manner, which could then be used to drive expression of the coral *ind* homologue (*cnox-2Am)* in an *ind* null mutant background, to rescue the null mutant phenotype. In addition, a *ind*-UAS construct was also generated in order to provide a positive control for these experiments. The appropriate construct was purified according to standard protocols (see section 2.4.9) and 6μg was co-precipitated with 6μg helper plasmid (by ethanol precipitation – see section 2.4.8) which codes for the transposase gene, enabling insertion of the desired construct into the germline of *Drosophila*. The resulting DNA was recovered by resuspending in 30μL injection buffer (0.1mM $NaPO_4$ pH7.8, 5mM HCl) and injected into the posterior pole of w^{1118}

Drosophila melangoaster embryos by Dr William Warren at JCU. Embryo injection and subsequent care were performed as described in Roberts, 1986. Embryos that survived the injection procedure were allowed to develop into flies that represented unique P-element insertion events. Individual flies were backcrossed to the injection stock to select for germline transformants resulting in flies with red eyes if the construct had been successfully inserted into the genome. Isolation of germline tranformants then allowed generation of a homozygous stock. This was achieved by crossing each red-eyed male transformant with double balancer virgins, the genotype of which is shown below;

$$\left\{ \begin{array}{ll} \underline{\text{w; CyO}}; & \underline{\text{TM6b}} \\ \text{w} \quad \text{IF} & \text{MKRS} \end{array} \right\}$$

Progeny which displayed the CyO (curly wing) and TM6B (humeral) phenotype or the CyO and MKRS (stubble bristles) phenotype were selected and homozygous stocks prepared. To determine on which chromosome the insert was inserted, one male from each insertion event with the CyO and TM6b phenotype was crossed back to a small number of wild type (W^{1118}) virgin females and the phenotype of the progeny observed, indicating which chromosome the P-element had been incorporated into. The fly stocks were maintained at 25°C on standard cornmeal-treacle-agar. Note that all fly stocks and fly-specific reagents were kindly provided by Dr William Warren (JCU) and the crosses and care of flies was performed with the assistance of Mr Niko Frank (*ind*-promoter-GAL4) and by Ms Lucija Tomljenovic (*ind*-UAS).

2.3 Bacteria

2.3.1 Bacterial Strains

A number of *E. coli* strains were used in this project as listed below in Table 2.2.

Table 2.3: Bacterial *E.coli* strains used and their application

Strain	Source	Use
NM522		General cloning and plasmid manipulation
DH5∝		General cloning and plasmid manipulation
SURE	Stratagene; D. Hayward (Australian National University)	Cloning of RNAi constructs
M15[pREP4]	QIAGEN	Kanamycin-resistant pRE4 carrier for recombinant protein expression using pQE vectors
LE392	Stratagene	Host for λGEM-11 phage for genomic library screening, amplification and DNA extraction
XL1-Blue MRF'	Stratagene	Tetracylin-resistnat host of λZAP-II phage
SOLR	Stratagene	Non-supressing strain for λZAP-II phagemid rescue of the pBluescript containing cDNA insert

Bacterial strains were stored in aliquots at -70°C and on LB agar plates at 4^0C.

2.3.2 Media Preparation

2.3.2.1 Luria-Bertani (LB) Media (1 litre)

(prepared as described in (Sambrook *et al.*, 1989))

- 10g tryptone
- 5g yeast extract
- 10g NaCl

- distilled water (dH_2O) added to 1 liter and the solution adjusted to pH 7.5 with 10M NaOH.

2.3.2.2 NZY Media (1 litre)

(prepared as described in (Sambrook *et al.*, 1989))
- 10g NZamine (casein hydrolysate enzymatic)
- 5g NaCl
- 5g yeast extract
- 2g $MgSO_4.7H_2O$
- distilled water (dH_2O) added to 1 litre and the solution adjusted to pH 7.5 with 10M NaOH.

LB or NZY media was prepared as described above and 1.5g (1.5%) agar or 0.7g (0.7%) agar was added per 100mLs medium after pH adjustment. If addition of antibiotics was required, the agar was cooled to 50°C. Sufficient 1.5% agar medium was added to each plate to a depth of approximately 5mm and any bubbles were flamed with a bunsen burner. The plates were then left to set for approximately 15-20 mins and stored in an inverted position at 4°C for up to 4 weeks.

2.3.2.3 Antibiotics

All antibiotics used were filter sterilised before use, except in the case of tetracycline, which was prepared as a stock solution in Ethanol, and therefore did not require sterilisation.
Ampicillin was stored as a stock solution at 100mg/mL at -20°C and added to media to a final concentration of 100μg/mL (Sambrook and Russell, 2001).
Kanamycin was stored as a stock solution at 25mg/mL at -20°C and added to media to a final concentration of 25μg/mL (Sambrook and Russell, 2001).
Tetracycline was stored as a stock solution at 5mg/mL at -20°C and added to media to a final concentration of 50μg/mL (Sambrook and Russell, 2001).

2.3.3 Manipulation of Bacterial Colonies

Bacteria were picked from a single colony and streaked onto LB agar plates containing an appropriate antibiotic using a sterilised platinum wire loop. Between each set of parallel streaks, the loop was flamed, and the colony was streaked again in parallel streaks which crossed the previous set. In this way, colony number was gradually reduced in order to gain single colonies. Plates were then incubated overnight at 37^{0}C.

To inoculate liquid media, a single bacterial colony was picked from a plate with a sterilised platinum wire loop and placed in the media which contained the appropriate antibiotic, ensuring that bacteria became released into the broth. Liquid bacterial cultures were spread over plates with the use of pasteur pipettes sealed at one end, and bent at 90^{0}C. Before use, the spreader was sterilised in 100% ethanol and flamed. The sample was then spread evenly over the entire plate and the plates allowed to dry before being incubated at 37°C.

The surface of frozen -70°C cell stocks were scraped with a sterilised platinum wire loop and streaked onto a LB agar plate or inoculated into broth, which was then incubated overnight at 37^{0}C.

2.3.4 Small Scale Overnight Bacterial Cultures

5mL aliquots of LB medium with appropriate antibiotics were placed in 50mL sealable tubes, and inoculated with the appropriate bacterial colony. The tubes were then placed on their side to allow sufficient aeration in a 37^{0}C incubator and shaken at 200 cycles/minute overnight.

2.3.5 Competent Cells

2.3.5.1 Preparation of Ca^{2+} Competent E.coli

The following protocol is modified from (Sambrook *et al.*, 1989);

The cell line was selected and inoculated into 3mL LB broth overnight. 1mL of overnight culture was then inoculated into 50mL broth, and grown till cells reached a

density of between A_{600} 0.4 – 0.6. The solution was placed on ice and cooled to 0°C. The mixture was then transferred to a 50mL ice-cold Falcon Tube and spun at 3500rpm for 10minutes at 4°C. The supernatant was drained well and the pellet retained. The pellet was then resuspended in 10mL ice-cold 0.1M $CaCl_2$ solution, stored on ice and gently resuspended over 1 hour. Cells were recovered by centrifugation at 4000rpm for 10minutes at 4^0C. Again the supernatant was drained and the pellet retained. The pellet was then resuspended in 2mL ice-cold 0.1M $CaCl_2$ over 1 hour. Glycerol was added to a final concentration of 20%, and the resulting solution dispensed into aliquots (eg 500μL and 100μL) and snap-frozen in Liquid Nitrogen. The competent cells were stored at -70^0C.

2.3.5.2 Preparation of electrocompetent E.coli

Preparation of electrocompetent E.coli for cell stocks

100mL of LB broth was inoculated from *E.coli* (eg DH5α) stock and grown overnight at 37^0C. 1L of LB broth was then inoculated with 10mL of the overnight culture in a 200mL or larger flask. Cells were grown at 37^0C with vigorous shaking until A_{600} = 0.5 – 0.6. The flask was chilled on ice for 15 to 30 minutes and centrifuged at 4000g for 15 minutes at 4^0C. From this point on, the cell cultures were kept on ice and the equipment pre-chilled where possible.

The supernatant was removed and tubes stood in an inverted position for 1 minute to allow the last traces of media to drain away. Pellets were resuspended in a total of 1 L of ice-cold autoclaved water, and centrifuged at 4000g for 15 minutes. The supernatant was removed and pellets resuspended in a total of 0.5 L of ice-cold water, and again centrifuged at 4000g for 15 minutes. The supernatant was removed and pellets resuspended in a total of 30mL of ice-cold 10% glycerol. They were then again centrifuged at 4000g for 15 minutes. The resulting pellets were resuspended in ice-cold 10% glycerol and combined to give a final volume of 3 mL. The cell concentration was between 1-3 x 10^{10} cells/mL. The 10% glycerol suspension was then aliquoted to sterile microfuge tubes and snap frozen on dry ice, or with liquid nitrogen and were stored at -70^0C.

Preparation of electrocompetent E.coli *for same day use*

3mLs of LB media was inoculated with DH5α cells and grown overnight. 50mLs of LB was then inoculated with 1mL of this overnight culture and grown to 2/3 log phase [OD_{660} ~ 0.6]. For every transformation 1.5mL of cells were pelleted by a 30 second pulse spin and 1mL of MilliQ H_2O (ice-cold) added. The cells were resuspended by vortexing and repelleted by a 30 second pulse spin. This step was repeated using 500μL MilliQ H_2O. Cells were kept on ice and used within one hour of being made electrocompetent.

2.3.6 Transformation of Competent Cells

2.3.6.1 Heat Shock

Transformations were carried out as described (Sambrook *et al.*, 1989) using a 10μL ligation reaction and 60μL of Ca^{2+}-competent cells. The two solutions were added together in a 10mL Falcon Tube and heat shocked at 42-45^{0}C for 2 minutes. 1mL of LB medium was then added and the transformed cells allowed to recover at 37^{0}C in an incubator, shaken at 200rpm for one hour. The transformation reaction was then transferred to a microfuge tube and cells pelleted at 13200rpm for 1 minute. 800μL of supernatant was removed and the pellet resuspended and plated on LB agar with the appropriate antibiotic added to allow for selection of transformed cells. Alternately, 10ng of circular DNA was added to 60μL competent cells and incubated on ice for 5 minutes. The mixture was then spread onto LB agar plates with the appropriate antibiotic, which had been prewarmed to 37°C for at least 30 minutes.

2.3.6.2 Electroporation

Electroporation of 1μL of a ligation reaction into 40μL of DH5α cells was performed using the Gene Pulser®II Electroporation System (Bio-Rad) under particular conditions depending upon the cuvette size as detailed below in Table 2.4;

Table 2.4: Conditions of Electroporation when using the Gene Pulser®II Electroporation System

Width of Cuvette	**0.1cm**	**0.2cm**
Resistance (ohms)	200Ω (100 Ω if does not pulse)	200Ω
Voltage	1.8kV	2.5kV
Capacitance (farads)	25μF	25μF

Before electroporation, ligation reactions were glycogen precipitated as detailed in section 2.4.3.

2.3.7 Glycerol Cell Stocks

Aseptic 80% (w/v) glycerol was added to an aliquot of a small scale overnight culture to a final concentration of 20% in a microfuge tube. The solution was mixed well and snap frozen in liquid nitrogen before being stored at -70°C. Once frozen, the samples were not allowed to thaw with all subsequent steps performed while keeping the sample on dry ice.

2.4 DNA Manipulations

2.4.1 Plasmid Vectors

The following plasmid vectors were used in this project, as listed in Table 2.4

Table 2.5: Plasmid vectors used and their application

Plasmid	Source	Special Features	Use
pBluescript SK (+/-)	Stratagene	Ampicillin resistance, blue/white selection	General cloning
pGEM-T/pGEM-Teasy	Promega	Ampicillin resistance, blue/white selection	General cloning
pP(UAST)	Dr Serge Plaza, University of Basel	Ampicillin resistance, blue/white selection, UAS-binding sites	P-element insertion and expression in *Drosophila*
pG4PN (derived from pCaSpeR4)	Dr Coral Warr, Monash University	GAL4-hsp70 activator, mini*white* gene, ampicillin resistance, 3' and 5' transposase incorporation sites	P-element insertion and expression in *Drosophila*
pBM2389	Dr Rohan Baker (ANU)	Ampicillin resistance HIS3 Reporter Plasmid	Yeast One-Hybrid system
pBM2463	Dr Rohan Baker (ANU)	Ampicillin resistance, URA3, ADE5 genes. Activator Plasmid.	Yeast One-Hybrid system
pHR307a	Dr Rohan Baker (ANU)	Ampicillin resistance. Reporter Plasmid	Yeast One-Hybrid system
pWiz	Ms Masha Smallhorn, (ANU)	Ampicillin resistance, White intron, Upstream Activator Sequence (UAS)	RNAi constructs

2.4.2 Restriction Endonucleases

All digestions were performed as described in (Sambrook *et al.*, 1989) using Promega restriction enzymes and buffers. Reactions were performed for between 2 and 8 hours at the appropriate temperature – typically 37°C. In the case of multiple digests using

enzymes requiring different buffering conditions, the digested products were precipitated and resuspended between successive digestions.

2.4.3 Ligation Reactions

Ligations were performed as described by (Sambrook *et al.*, 1989) using Promega T4 DNA Ligase and 10x Ligase Buffer, using a 1:1 molar ratio of plasmid vector:insert DNA. The reactions were carried incubated at 4^0C overnight or 25°C for 4 hours and the entire ligation reaction (10μL) used for transformation of competent cells. If the ligation was to be electroporated into competant cells, it was first glycogen precipitated in the method detailed below as modified from (Ausubel *et al.*, 1996);
1-2μL of glycogen (20μg/μL); 2.5 volumes of absolute EtOH and 0.1 volumes of 3M NaAc pH 7.0 was added to the ligation reaction. The reaction was left for 5 minutes on ice and the pellet precipitated. The pellet was then washed 1x in 70% EtOH and 1x in 100% EtOH and allowed to air dry. The pellet was resuspended for approximately 5 minutes in 20μL RNase- and DNase-free water.

2.4.4 Dephosphorylation of Cut Vector

The method for dephosphorylation of the ends of a blunt end cut vector are modified from Sambrook *et al.*, (1989), as follows;

eg. 19μL of digested vector

161μL of ddH_2O and 20μL Calf Intestinal Alkaline Phosphatase (CIAP) Buffer (supplied by Promega) was added, and 20μL was removed and retained as the non dephosphorylated vector control. 5μL (0.5U) CIAP enzyme was added to the remaining 180μL and incubated at 37^0C for 60 minutes. The reaction was then heated to 75^0C for 10 minutes with 5mM EDTA. ddH_2O was added to a volume of 800μL and the DNA phenol/chloroform extracted once, followed by one chloroform extraction.

The solution was precipitated with 0.7 volumes of ice-cold isopropanol and NaOAc (3M, pH 5.2) to a final concentration of 0.3M. The DNA was allowed to precipitate at -20^0C for 30 mins, and then spun at 14000 rpm for 20 minutes.

The supernatant was removed, and the pellet washed in 1mL of 70% ethanol.
The solution was then spun at 14000rpm for 5 minutes, the supernatant removed again, and the pellet dried in a vacuum centrifuge. The DNA was resuspended in TE buffer (10mM Tris.HCl pH8.0; 1mM EDTA) to a concentration of 0.1μg/μL and stored at 4^0C.

2.4.5 Recovery of DNA from Agarose Gels

DNA was recovered from Agarose Gels by filtration through Whatman Filter Paper. The Whatman paper was cut to a circular size of approximately 5cm in diameter and made into a cone shape by folding the paper around a 2 -20μL pipette tip which had the tapered end cut off. This was placed inside a 500μL PCR tube which had a needle hole pierced in the bottom. The filter paper/PCR tube contraption was then placed inside a 1.5mL microfuge tube and the agarose gel slice placed in the filter paper and immediately spun at 5000rpm for 5 minutes in a microcentrifuge. The eluate was then used in further experiments.

2.4.6 Generation of radioactive probes

Radioactive probes were prepared by random oligonucleotide-primed synthesis (oligolabelling) using α-^{32}P dATP (Geneworks; 10 mCi/ml, specific activity ~3000 Ci/mmol). Generally, 25 ng of linear DNA recovered from agarose gel fragments was radioactively labelled using the Megaprime (Amersham Biosciences) or Prime-a-Gene (Promega) oligolabelling kits as described in the accompanying protocols. After labelling, unincorporated α-^{32}P-dATP was separated from the labelled probe by spermine precipitation. Precipitation of the labelled probe was achieved by adding spermine to a final concentration of 7.5 μM and incubating on ice for 20 min before centrifugation at 15,000 g for 15 min. The supernatant containing unincorporated label was removed and the probe resuspended in 100 μl probe resuspension solution (10 mM EDTA; 0.5% SDS). Prior to use, the labelled probe was denatured at 100°C for 5 min and placed on ice or an appropriate volume added immediately to the hybridisation to prevent re-annealing.

2.4.7 Phenol/Chloroform Extraction of DNA

Phenol/chloroform extraction was performed to remove contaminating proteins from the DNA sample, using the premise that phenol and water are not miscible, and will form separate phases when mixed. When the aqueous phase containing the DNA is mixed with phenol, the proteins partition into the phenol phase. The aqueous phase containing the DNA is removed to a new tube, and chloroform added which acts to remove any remaining phenol and/or contaminating proteins.

The procedure, adapted from Barker, 1998 is as follows;
An equal volume of TE-saturated phenol/chloroform was added to the DNA sample taking care that the total volume did not exceed 500μL for a 1.5mL microfuge tube. The sample was vortexed vigorously for 20 seconds before being centrifuged for 5 minutes at maximum speed (13200 rpm). As much of the aqueous layer as possible was removed without disturbing the protein layer with a pipette and added to a new tube. An equal volume of chloroform was then added to the aqueous layer and the sample vortexed, centrifuged and removed as before.

2.4.8 Ethanol Precipitation of DNA

Precipitation of DNA allows for resuspension in a smaller volume and also removes residual chloroform which can inhibit many enzymatic reactions.

The procedure as adapted from Barker, 1998 is as follows;
To a maximum volume of 450μL of DNA in water/TE buffer, 1/10 volume of 3M NaAc pH 4.8 was added and inverted briefly to mix. Two volumes of 95% or 100% ethanol were added and the solution was mixed by inversion.

The DNA was precipitated by placing the sample in the cold either at;

-20^{0}C overnight;

-70^{0}C 30 minutes;

-dry ice 5 minutes.

The sample was centrifuged at high speed (>12000rpm) for 15-30 minutes at 4^{0}C and the supernatant decanted. Tubes were drained by inverting and blotting with a paper

towel. The pellet was washed with cold 70% EtOH and dried with the use of a vacuum centrifuge and allowed to resuspend in TE buffer pH 8.0 overnight at 4°C.

2.4.9 Isolation of plasmid DNA

In general, plasmid DNA was extracted using QIAGEN or BIORAD Miniprep Spin Kits which gave typical yields of approximately 100μg/mL of plasmid DNA. QIAGEN Maxiprep Kits which gave a yield of approximately 500μg/mL of DNA, were also used when greater concentrations of DNA were required.

2.4.10 Southern blotting

DNA was transferred from agarose gels to nylon membranes as described in Sambrook *et al.* (1989) and Amersham membrane protocols as modified from Southern (1975). The DNA of interest was electrophoresd on an agarose gel and transferred to Nytran-N (Schleicher & Scheull) or Hybond-N, -N+ or -NX (Amersham) using the capillary blot transfer method overnight in 20xSSC or 0.4M sodium hydroxide (Hybond-N+). DNA was covalently linked to membranes by baking at 80°C for 2h.

Following fixation of DNA, membranes were prepared for hybridisation by incubating in 100 ml of DNA hybridisation solution at 60°C for 2 - 3 hours, prior to the addition of radioactively labelled probe (see section 2.4.6). The DNA was hybridized to the membrane at 60 - 65°C for at 12 - 16 hours, after which it was washed (2 x 15 min) with 200 ml of low stringency wash solution (2xSSC; 0.1% SDS) at 60 - 65°C. Membranes were monitored with a Geiger counter and exposed to Phosphorimager screens for 3 - 5 hours. Phosphorimager screens were read by a Phosphorimager (Molecular Dynamics) and images were processed using ImageQuant software (Molecular Dynamics).

2.4.11 DNA Quantification

DNA quantification was determined spectrophotometrically using the BIORAD Smart Spec™ spectrophotometer, or estimated by comparing the ultra-violet fluorescence

emitted by ethidium bromide when intercalated with DNA against the fluorescence emitted by a series of standards.

2.4.12 Agarose Gel Electrophoresis

Agarose Gel Electrophoresis was performed as described in Sambrook *et al.* (1989) using Bio-Rad Mini-Sub Cells, with the following modifications:

- Ethidium Bromide (EtBr) was added to the molten agarose to a final concentration of 0.5mg/mL
- DNA was visualised using a 312nm ultraviolet transilluminator

Agarose gels were typically run at 5V/cm (distance between the electrodes) in 1x TAE-buffer (Tris, Acetate, EDTA) for 45 minutes.

2.4.13 Oligonucleotide Primer Design

Primers for use in routine experimental procedures such as PCR and sequencing reactions were designed using MacVector™ with parameters limiting the length to 18 – 25 nucleotides, the melting temperature to 55 - 60°C and the G/C content to 45 – 55%. Degenerate primers designed from amino acid alignments from various species were designed to contain a degeneracy of less than 1000-fold where possible and with melting temperatures of 50 - 60°C. Degeneracy was calculated by multiplying the number of possibilities at each nucleotide position with those at other positions. Melting temperature was calculated assuming each degenerate position contained the base with the higher melting temperature, that is, a guanine (G) or a cytosine (C) if these were possibilities. At nucleotide positions where all four bases could exist, an Inosine (I) was included and was assumed to have a melting temperature of 4°C. Oligonucleotide primers were supplied by ProOligo or Sigma and were stored as 100μM or 200μM stock solutions at –20°C for dilution and use in reactions.

2.4.14 DNA Sequencing

DNA sequencing was done in a thermocycler using the ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit by Perkin Elmer, or the DYEnamic™ ET Dye Terminator Sequencing Kit from Amersham Biosciences. These kits use four different fluorescent dyes to discriminate between the dideoxynucleotide A-, C-, G- and T- terminators during the reading of the sequence. The reactions were analysed on an ABI PRISM™ 310 Genetic Analyser, or a MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences) via a laser induced fluorescence capillary system. The sequences were manually checked and assembled with Sequencher Version 3.1or 4.0.

The cycle sequencing protocol for the BigDye Terminator kit is as follows;

96°C 10 seconds
50°C 5 seconds
60°C 4 minutes
} Cycles were repeated 25 times

The cycle sequencing protocol for the DYEnamic™ ET Dye kit is as follows;

95°C 20 seconds
50°C 15 seconds
60°C 3 minutes
} Cycles were repeated 35 times

2.4.15 Polymerase Chain Reaction (PCR)

2.4.15.1 General Reaction

The Polymerase Chain Reaction (PCR) was used for a variety of tasks:

- PCR-mutagenesis to insert cutting sites onto the end of DNA inserts to be cloned into a vector
- Colony PCR – a rapid method to determine if the insert of interest was present in plasmids of a bacterial colony
- To amplify a target sequence
- To radiolabel a target sequence

A typical PCR reaction mix consisted of the following

1μL DNA-template (10ng/μL)

1μL primer 1 (10μM)

1μL primer 2 (10μM)

2.5μL dNTPs (2mM each)

2.5μL 10x enzyme-buffer eg Taq buffer

2.0μL MgCl2 (25mM)

0.1 - 0.5U Enzyme eg Taq polymerase

DNase/RNase-free H_2O up to 25μL

Typical cycling-conditions involved denaturing and annealing times of 30 seconds and extension times which allowed 1 minute/kilobase of DNA, followed by a final extended extension incubation time of 2 minutes per kilobase. The number of cycles required depended upon the template DNA, but generally 20 cycles was sufficient for the amplification of plasmid DNA. Annealing temperatures used were 2 - 3°C below the melting temperature of the primers involved.

When using *Pfu* Polymerase approximately two minutes was allowed for every 1kb to be amplified and 25-35 cycles were used as *Pfu* polymerase has a lower extension rate than other enzymes.

2.4.15.2 Colony PCR

A colony was picked off a transformed plate with a sterile toothpick and resuspended in 3μL dH_2O. The solution was then incubated at 95^{0}C for 5 minutes, centrifuged and placed on ice before being used in a typical PCR reaction with primers specific to the insert of interest or primers which annealed to vector sequence surrounding the insert. After the PCR was complete, the samples were run on an agarose gel, and positive colonies identified by the presence of bands on the gel which indicated amplification of the insert had taken place. The colonies which were positive for the insert of interest were then used in further manipulations.

2.4.16 5' and 3' Rapid Amplification of cDNA Ends (RACE)

Occasionally, while an internal coding sequence of a gene was known, the 5' and/or 3' sequences surrounding this frament were not known. In these cases, the BD SMART™ RACE cDNA Amplification Kit was utilised (BD Biosciences Clontech). This method provided a mechanism for generating full-length cDNAs in reverse transcription reactions from the joint action of the BD SMART II™ A Oligonucleotide and the BD PowerScript™ Reverse Transcripase. Briefly, total RNA was extracted from the embryonic stage of interest (or pooled from a number of embryonic stages) and reverse transcribed according to the manufacturers instructions to give first-strand 5' or 3' RACE-ready cDNA. Gene specific primers were designed from the gene fragment of interest according to the primer design recommendations accompanying the kit protocols. Touchdown PCR with a gene specific primer and the universal primer provided was used for the first amplification, and if necessary nested PCR was performed on this initial PCR reaction using a second gene specific primer, and the nested universal primer supplied. For these reactions, the BD Advantage™ 2 PCR Kit was used which includes the BD Advantage™ 2 Polymerase Mix (BD Biosciences, Clontech). Products were cloned and a number of colonies sequenced to ensure the longest fragment containing the start methionine (5'RACE) or stop codon (3'RACE) were isolated.

2.5 RNA Manipulations

2.5.1 RNA extraction

Total RNA was extracted from *Acropora millepora* embryos collected at various stages of development and snap frozen in liquid nitrogen. Extraction of RNA was achieved using Ambion RNAwiz™ RNA isolation reagent and associated protocols. Briefly the sample of interest was homogenised with a metal mortar and pestle in liquid nitrogen until a fine sand-like appearance was gained. 1mL of RNAwiz™ was added per 0.1g of sample and left to sit at room temperature for at least 10 minutes to dissociate the nucleoproteins from the nucleic acids. Chloroform 0.2x the starting volume was added and the sample vigorously shaken for 20 seconds, followed by a second room temperature incubation of 10 minutes. The mixture was centrifuged at >10 000 xg for

15 minutes to separate the sample into 3 phases; the upper colourless phase containing the RNA, the semi-solid interphase containing most of the DNA and the lower organic phase. Without disturbing the interphase, the aqueous phase was carefully transferred to a clean RNase-free tube and 0.5x starting volume of RNase-free water added. RNA was precipitated at room temperature for 10 minutes after addition of 1 starting volume of isopropanol. The sample was centrifuged at >10 000 xg for 15 minutes to pellet the RNA, and the pellet washed with 1 starting volume of 75% ethanol by vortexing. Again, the sample was centrifuged at >10 000 xg for 5 minutes, and the supernatant discarded. The RNA pellet was air dried for approximately 10 minutes, with care taken not to let the pellet dry completely making it difficult to resuspend. The RNA pellet was dissolved in an appropriate amount of RNase-free water, being heated to 60°C if necessary.

2.5.2 RNA Quantification

RNA quantification was determined spectrophotometrically using the BIORAD Smart Spec™ spectrophotometer at a wavelength of 260nm where an absorbance of 1.0 = 40μg/mL RNA.

2.5.3 Agarose gel electrophoresis of RNA samples

After extraction and quantification of RNA was achieved, samples were run on a formaldehyde/agarose gel to determine integrity of sample. For a 50mL gel, 36mLs RNase-free H_2O was added to a baked flask and 0.5g agarose added. The mixture was boiled in a microwave oven and left to cool to approximately 50°C before 5mLs 10x MOPS buffer (20.6g MOPS, 40mL 1MNaOAc, 20mL 2M NaOH, H_2O to 500mLs; filter sterilize) and 9mLs formaldehyde was added. 1μL of RNA sample was mixed with 4μL H_2O, 1μL 10xMOPS, 3.5μL formaldehyde, 10μL formamide and 0.5μL EtBr, before being heated at 60°C for 15 minutes to reduce any secondary structure the RNA may have formed. 2μL loading dye was added and the entire mixture loaded onto the set gel and run at normal agarose gel conditions in RNA running buffer (15mLs 10x MOPS, 27mLs formaldehyde, H_2O to 150mLs).

2.5.4 Synthesis of Digoxigenin- and Fluorescein labelled Riboprobes

Digoxigenin (DIG)-labelled of Fluorescein (FL)-labelled RNA probes were synthesised using run-off transcription as described in Kucharski *et al* (2000). cDNA templates in pBluescript SK+ or pGEM-T were linearised with an appropriate enzyme to create a 5'overhang or blunt end at the 5' end of the cDNA. Approximately 5μg of plasmid was linearised and 500 – 1000ng of this was subjected to agarose gel electrophoresis to ensure complete digestion. DIG-labelling was modified from Stratagene protocols and performed following the reaction shown below:

1μg template DNA
4μL 5x Labelling Buffer (Promega)
(50mM Tris, pH 8.0; 8mM magnesium chloride; 2mM Spermidine; 50mM sodium chloride)
2μL 10x DIG-NTP or FL-NTP mix (Roche)
(6.5mM UTP; 3.5mM DIG/FL-UTP; 10mM ATP; 10mM CTP; 10mM GTP)
1μL (40U) RNasin (Promega)
2.4μL DTT (74mM - Promega)
1.5μL (30U) T7/T3/SP6 RNA polymerase (Promega)
Rnase-free H_2O to 20μL

The labeling reaction was incubated at 37°C for 2-3 hours before being stopped with 2μL 0.2M EDTA. 2μL was removed and 1μL of this run on an RNA gel to check efficiency of labelling while the remaining reaction was precipitated at -20°C for 2 hours with 2.2μL 3M NaOAc and 50μL 100% Ethanol after which the riboprobe was centrifuged and the pellet resuspended in 50μL RNase-free ddH_2O.

Following synthesis, riboprobes were partially hyrdolysed to facilitate penetration of the probe into treated embryo tissue. Hydrolysis was carried out in a total reaction volume of 55.5μL, containing 50μL of precipitated RNA probe and 5.5μL sodium carbonate buffer (0.4M sodium hydrogen carbonate; 0.6M disodium carbonate, pH10.2). The reaction was incubated at 60°C for the length of time denoted by the following equation (usually 30 – 40 mintues);

$$\mathbf{t = Lo - Lf / K(Lo)(Lf)}$$

Where t = time of incubation in minutes

Lo = Initial length in kilobases

Lf = desired length in kilobases (usually 200 – 300bp)

k = approximately 0.11 strand scissions/kilobase/minute

After incubation for the appropriate amount of time, the reaction was stopped by addition of 2μL 3M sodium acetate, pH 5.2 and precipitated by the addition of 2μL 10mg/mL tRNA (20μg), 150μL absolute ethanol and placing at -20°C, for 2 hours, followed by centrifugation at 13,000g for 15 minutes. Probes were resuspended in 80μL RNA probe resuspension buffer (50% formaldehyde; 50% TE Buffer; 0.1% Tween) for long term storage at -20°C and use.

2.5.5 Dot Blot Testing of DIG- or FL- labelled riboprobes

A serial dilution of riboprobe was performed in RNase-free H_2O (1, 1/10, 1/100, 1/1000, 1/10000). 1μL of each dilution was then added to 5μL 5xSSC and heated at 80°C for 5 minutes. The samples were then cooled on ice and pulse-centrifuged. 1μL of each sample in 5xSSC was then spotted onto a nitrocellulose strip (eg Hybond-C), and baked between blotting paper at 80°C for 2 hours. After fixation of RNA, the filter was placed briefly in 2x SSC, washed 2 x 5 minutes in PT buffer (PBS + 0.1% Tween-20) before being placed into a 10mL tube. A blocking step was then performed by incubation in PT buffer for 30 minutes followed by incubation with αDIG-AP or αFL-AP (Roche) primary antibody at a final dilution of 1:5000 for 60minutes. The primary antibody was removed by washing 4 x 15minutes in PBT (PBS + 0.1% TritonX-100) and then equilibration before adding substrate achieved by 2 x 5 minute washes in NTMT. The colour substrate reaction was initiated by placing the membranes in NBT/BCIP solution (Vector Laboratories). The reaction was stopped by washes in PT buffer.