2
Materials and Methods

2.1. ANIMALS

2.1.1. Animal collection

Colonies of *Acropora millepora* were collected from inshore reefs at Magnetic Island (Latitude 19°09' South; Longitude 146°49' East) and mid-shelf reefs at Orpheus Island (Latitude 18°28' South; Longitude 146°25' East), both located off the coast near Townsville, Australia. At least two colonies were placed in large containers of seawater before dusk on the anticipated night of spawning. To facilitate fertilisation, gametes from at least two separate colonies were mixed in buckets containing fresh seawater and were left overnight to begin development. The following day, developing embryos were transferred to larger containers of seawater where they were maintained until the appropriate developmental age. Every 24 hours, dead embryos and resulting surface lipid were removed and the healthy embryos were transferred to fresh seawater.

2.1.2. Embryo fixation

Embryo fixation was carried out in the field, during annual coral spawning events. *A. millepora* embryos were collected at each developmental stage of interest and fixed for 10 min in Millepore-filtered seawater (MPFSW) containing 3-4% formaldehyde. Immediately following fixation, the embryos were washed three times in 10 volumes of MPFSW for 10 min prior to storage in HEPES-buffered seawater (pH 8.0) at 4°C (for 3 to 6 days) awaiting transportation to the laboratory. For long-term storage, embryos were dehydrated through a methanol series: 50%; 70%; 90%; 100% and stored at –20°C.

2.2. BACTERIA

2.2.1. Bacterial strains

Bacterial strains used for various applications in this project are given in Table 2.1.

Table 2.1. Bacterial strains and associated applications.

NM522	General plasmid manipulation
LE392	λGEM-12 phage screening, amplification and DNA extraction
DH5α	General plasmid manipulation and electroporation
XLI-Blue MRF	Tetracycline-resistant host of λZAP-II phage
M15 (pREP4)	Kanamycin-resistant pREP4-carrier for expression of recombinant fusion proteins using pQE vectors
SOLR	Non-suppressing strain for λZAP-II phagemid rescue of the pBluescript containing cDNA insert

2.2.2. Media and Solutions

All media and solutions were prepared as described in Sambrook et al. (1989). Protocols for preparation of media and solution are given in Table format at the end of each section.

Cultures were incubated in sterile containers at least twice the culture volume to enable sufficient aeration. Cultures were made selective by the addition of suitable antibiotics from 1000x stock solutions to final 1x concentration. Plates were inoculated using a single bacterial colony or -70°C glycerol stock using a platinum wire loop or from a bacterial suspension using a glass spreader as described in Ausubel et al. (1996). Plates were allowed to dry and were incubated inverted at 37°C for 12-16 h to allow growth.

2.2.3. Competent cells

Calcium chloride competent cells were prepared using the Sambrook et al. (1989) modifications to the methods of Cohen et al. (1972). 50 ml of LB media was inoculated with 500 µl of a 3 ml overnight culture of the selected bacterial strain and grown at 37°C in a shaking incubator until an OD_{600} of 0.4-0.6 was attained. The cells were then chilled on ice and pelleted by centrifugation for 5 min at 3000 g at 4°C. The pellet was then gently resuspended in 10 ml ice-cold 0.1M calcium chloride over 1 h on ice. Following centrifugation, the cells were gently resuspended in 2 ml of ice-cold 0.1M calcium chloride containing 20% glycerol over 1 h. Aliquots of competent cells were snap frozen in liquid nitrogen and stored at -80°C. Transformation efficiencies of 10^5 colonies per 1 µg of DNA were regularly observed.

2.2.4. Bacterial transformations

Aliquots of competent cells were transformed as described in Sambrook et al. (1989). After thawing on ice, 50 µl aliquots of cells were added to ligation mixtures or DNA controls (in a maximum volume of 10 µl) and incubated on ice for a further 30 min. The transformation mixture was then subjected to a heat shock at 42°C for 2 min prior to the addition of 900 µl of LB medium and incubation with shaking at 37°C for 1 h. After this time, cells were pelleted at 3000 g for 30 s and all but 100 µl of supernatant removed. The pellet was subsequently resuspended and used to inoculate an appropriate selective agar plate. Once dry, the plates were incubated inverted at 37°C for 12-16 h.

2.2.5. Bacterial glycerol stocks

Bacterial cell stocks were prepared by adding 300 µl of 80% (v/v) sterile glycerol (20% final concentration) to 900 µl of a freshly grown 3 ml overnight culture. The mixture was then snap frozen in liquid nitrogen and stored at -80°C.

Table 2.2. Media and solutions for bacterial manipulation methods.

Ampicillin (1000x stock)	50 mg/ml in ddH_20
Kanamycin (1000x stock)	10 mg/ml in ddH_20
Chloramphenicol (1000x stock)	34 mg/ml in ethanol
Luria-Bertani (LB) media	(per litre) 10 g tryptone; 5 g yeast extract; 10 g sodium chloride; pH 7.5
NZY media	(per litre) 10 g casein hydrolysate; 5 g yeast extract; 5 g sodium chloride; 2 g magnesium sulphate heptahydrate; pH 7.5
LB plates	LB medium; 1.5% (w/v) select agar
LB ampicillin plates	LB plates with 50 μg/ml ampicillin
LB amp/X-gal/IPTG	LB plates with 50 μg/ml ampicillin; 20 μg/ml X-gal; 200 μg/ml IPTG
LB kanamycin plates	LB plates with 50 μg/ml kanamycin

2.3. BACTERIOPHAGE MANIPULATION METHODS

Lambda vectors used in the preparation of *A. millepora* libraries are in Table 2.3.

Table 2.3. Lambda vectors and their applications

λGEM-12	Genomic libraries
λZAP-II	cDNA libraries
ExAssist helper phage	*in vivo* excision of cDNA clone in plasmid from λ vector

2.3.1. Phage plating and titration

Plating of phage was performed as described in Ausubel et al. (1996). Host bacteria were prepared by inoculating 50 ml of LB media containing 0.2%(w/v) maltose and 10 mM magnesium sulphate (which are required for optimal lambda phage receptor expression on the surface of the host bacteria) with 500 µl of a 3ml overnight bacterial culture and grown on a shaker at 37°C to an OD_{600} of 0.6-0.8. Bacterial cells were pelleted by centrifugation at 3000 g for 10 min at 4°C and resuspended in 5 ml of 10mM magnesium sulphate solution.

Alternatively, host bacteria were prepared by inoculating 100 ml of LB media with a single colony and incubating with shaking at 37°C overnight. 25 ml of this overnight culture was then centrifuged at 3000 g for 5 min at room temperature and the pellet resuspended in 40 ml of 10mM magnesium sulphate solution. When stored at 4°C, these preparations of bacteria were viable for up to five days. For each phage infection, 100 µl aliquots of host bacteria were mixed with the appropriate volume of phage diluted in SM and incubated at 37°C for 20 min to promote infection. Molten top agarose (cooled to 50°C; 10 ml per 15 cm plate; 5 ml per 8 cm plate) was added to the phage-infected bacteria and mixed well prior to pouring onto pre-warmed agar plates. Plates were left for 15 min to allow top agar to set, and then incubated inverted at 37°C for 12-16 h.

The titre of a phage stock or library was determined by plating a series of 10-fold serial dilutions. Generally 10 µl aliquots of 10^{-4}, 10^{-5} and 10^{-6} phage dilutions were incubated

with 100 µl of host bacteria and then plated on 8 cm plates. After an overnight incubation at 37°C, plaques appearing on each plate could be counted to determine the titre of the original phage solution, expressed as plaque forming units per microlitre (pfu/µl).

2.3.2. cDNA libraries

Three *A. millepora* cDNA libraries were constructed by Dr. David Hayward at the Research School of Biological Sciences at the Australian National University using tissue from three different stages of coral development. These were generated using mRNA extracted from embryos at approximately 11-13 h of development (colloquially known at the 'prawn chip' stage), approximately 96 h post fertilisation ('spindle' or 'pre-settlement' stage) and early adult polyps ('post-settlement' tissue).

For the construction of cDNA libraries, total RNA was prepared from frozen tissue ground under liquid nitrogen using the acid guanidinium thiocyanate / phenol-chloroform extraction method of Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated from total RNA using the PolyATract mRNA Isolation System IV (Promega) as described in the accompanying protocol for small-scale mRNA isolation. mRNA extracted in this way was used to generate directionally cloned cDNA inserts in Uni-ZAP XR vector, using the λZAP-cDNA Synthesis Kit (Stratagene). A feature of the Uni-ZAP XR vector is an internal pBluescript SK(-) phagemid that, together with the cDNA, can be excised in vivo using the ExAssist interference-resistant helper phage with the non-suppressing SOLR *E. coli* host strain. The ExAssist helper phage contains an amber mutation, which prevents replication of its phage genome in the SOLR cells and only allows replication of the excised phagemid.

2.3.3. Genomic library

High molecular weight genomic DNA was extracted from frozen sperm as described by McMillan et al. (1988). Genomic DNA was digested with *Mbo*I and size fractionated for library construction in λGEM-12 (Promega) by Dr. David Hayward (RSBS, ANU) following the manufacturer's recommended protocols.

2.3.4. Screening phage libraries

For screening of the genomic library, LE392 cells and LB media/agar were used, while XL1-Blue cells and NZY media/agar were used for screening cDNA libraries. NZY agar plates were aged at least three days at room temperature before use to ensure they were sufficiently dried to allow adhesion of the top agarose layer. For primary screening of cDNA libraries, plates were prepared with a plaque density of approximately 100,000 plaques per 130 mm plate for initial library screens of approximately 600,000 plaques per probe. Approximately 50,000 plaques of the genomic library were examined in each genomic screen.

Phage DNA was transferred from the agarose plates to Hybond-N or -NX neutral nylon membranes (Amersham) as described in accompanying protocols for plaque blotting. Hybond-N membranes are recommended for use in radioactive applications such as plaque blots while Hybond-NX membranes are specifically designed for low hybridisation buffer volumes and multiple re-probing applications. Phage DNA was transferred to membranes using the alkali fixation method (as modified from Benton and Davis, 1977). Following overnight incubation at 37°C, plates were first cooled to 4°C to minimise top agar separation, after which nylon membranes were placed on the agar for one min and needle punctures made to enable orientation of the membrane with the plate. Once lifted, the membranes were placed in denaturation solution for 5 min, neutralisation solution for 5 min and 2xSSPE for 2 min before drying (DNA side up) on Whatman 3MM paper. DNA was then covalently linked to the membrane by exposure to UV light at 312nm for 3 min on a Spectroline TC-312A transilluminator (Medos Company, Brisbane). Alternatively, membranes were baked at 80°C for 2 h in a vacuum oven.

Radioactively labelled probes were prepared as described in section 2.4.8. Membranes were placed in hybridisation bottles containing 30 ml of hybridisation solution for at least 1 h at 65°C prior to the addition of the radioactive probe. After addition of probe, membranes were further incubated at 65°C for 12-16 h with rotation in a hybridisation oven (Hybaid). Following hybridisation, the probe was removed and membranes were washed 3 x 20 min at 65°C in low stringency wash solution, and monitored using a

Geiger counter. Membranes were wrapped in plastic film, exposed to Phosphorimager screens (Molecular Dynamics) for 3-12 h 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 –70°C overnight.

Positive clones were selected by orientating the phosphor image or film with the plate and using a cut-off 1ml tip to 'plug' the plaque of interest. This plug was then placed in 1 ml of SM solution with 20 µl of chloroform and vortexed at high speed for 1 min. Incubation at 4°C overnight 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 observed.

2.3.5. Identification of overlapping genomic clones

In some cases, genomic clones isolated did not contain the entire region of interest. In these cases, overlapping genomic clones were isolated using probes generated using fragments derived from the end of the initial genomic clone. Approximately 50,000 pfu were screened and primary positives then screened with a second probe corresponding to the opposite end of the clone. Plaques hybridising to both fragments were discarded. Plaques hybridising with only the first probe were screened to 100% purity and DNA prepared from these clones in bulk.

2.3.6. Phage amplification

After isolating to homogeneity, phage were amplified to a titre of > 10^7 pfu/µl using the plate lysate and elution method as described in Sambrook et al. (1989). Briefly, plaques were first eluted from a number of large agar plates in 10 ml SM solution at 4°C overnight. The process was repeated until sufficient phage had been accumulated to allow large-scale bacteriophage DNA extraction (see section 2.3.5).

2.3.7. In vivo excision of cDNA clones

The cDNA clones isolated from the λZAP-II library were excised from the phage as pBluescript phagemids, using the ExAssist helper phage and the non-suppressing SOLR *E. coli* host strain, as described in protocols supplied by Stratagene. The ExAssist helper phage releases the pBluescript sequences and circularises them as single stranded plasmids, which 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 plated out onto LB-ampicillin plates to select for transformants.

Phage were eluted from single plaques by incubation in SM solution with chloroform overnight at 4°C. Cultures of XL1-Blue MRF and SOLR *E. coli* were grown to OD_{600} of 0.5 and 1.0 respectively. XL1-Blue MRF cells were harvested by centrifugation at 3000 g for 10 min and resuspended in 10mM MgSO4 at an OD_{600} of 1.0. *In vivo* excision was carried out in 15 ml Falcon tubes by the addition of 200 µl of XL1-Blue MRF cells to 250 µl of phage stock (~10^6 pfu) and 1 µl of ExAssist helper phage (10^6 pfu). The mixture was incubated at 37°C for 15 min to promote infection, before the addition of 3 ml of LB and incubation with shaking at 37°C for 10-16 h. The culture was then heated to 70°C for 15 min and debris removed by centrifugation at 4000 g for 15 min. The supernatant, (approximately 400 µl) containing the excised phagemid was recovered and stored at 4°C in microcentrifuge tubes. Aliquots (100 µl) of the excised phagemids were then incubated with 200 µl of freshly grown SOLR cells at 37°C for 15 min. 50 µl of the mixture was then plated on LB plates containing ampicillin and incubated at 37°C for 10-16 h to allow for growth of colonies containing the excised phagemid. Small-scale plasmid preparations from the resulting colonies were then prepared to yield pBluescript plasmid DNA containing the cDNA of interest.

2.3.8. Extraction of bacteriophage DNA from λGEM-12 phage clones

Sequencing and characterisation of genomic clones required the preparation of large amounts of high quality λDNA. Unlike λZAP-II, λGEM-12 does not allow phagemid excision of inserts. Instead, DNA was prepared from high titre phage suspensions generated using the liquid lysate method (Sambrook et al., 1989). This method involves the concentration of phage DNA (Lockett, 1990) and their purification on caesium

chloride gradients (Sambrook et al., 1989) prior to lysis and recovery of phage DNA (Lockett, 1990).

High-titre phage suspensions of λGEM-12 clones were generated using the liquid lysate method. A 20 ml culture of LE392 *E. coli* was grown overnight in LB containing 10mM $MgSO_4$ and 0.2% (w/v) maltose, and was then infected with $5x10^8$ pfu. This mixture was incubated at 37°C for 20 min to promote infection before the entire phage/cell suspension was used to inoculate 200 ml of LB medium containing 10mM $MgSO_4$ in a 1.5L flask and incubated at 37°C for 6-8 h with vigorous shaking (300 g) until lysis was evident. Sodium chloride was added to a final concentration of 1M and the lysate stored at 4°C overnight. The phage titre of this solution was calculated to determine the success of the amplification step before purification was continued. Purification was 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). Cell debris was pelleted by centrifugation at 10,000 g for 10 min and the supernatant decanted to a new tube. After this step, PEG 8000 (Sigma) was added and dissolved to a final concentration of 10% (w/v) and the preparation placed on ice for 2 h. PEG-precipitated phage were pelleted by centrifugation at 11,000 g for 10 min, the supernatant decanted and the tubes inverted for 5 min to allow the pellets to drain well. Phage pellets were resuspended in 5 ml aliquots of SM solution. PEG was removed from the phage suspension by chloroform extraction; an equal volume of chloroform was added and the preparation vortexed, the phases were well separated by centrifugation at 3000 g for 5 min and the top aqueous layer removed. Phage particles were purified by caesium chloride gradient centrifugation to ensure high template quality for sequencing. Solid caesium chloride was added to the preparation to give a final concentration of 0.75 g/ml and the mixture transferred to polyallomer centrifuge tubes (Beckman). Centrifugation was performed at 35,000 g for 22-24 h at 10°C in an SW41 swinging rotor bucket Beckman ultracentrifuge. After centrifugation, the visible band of phage particles in the top third of the tube was recovered using a 19-gauge needle to pierce the tube and draw out the band into a 2.5 ml syringe. Caesium chloride was removed by dialysis; the phage suspension was placed in dialysis tubing and dialysed against several changes of 100-

fold volume excess of dialysis buffer. For this purpose, dialysis tubing with a molecular weight limit of 12,000 Da was prepared as described in Sambrook et al. (1989).

Phage suspensions recovered after dialysis were placed in microfuge tubes and DNA eluted as follows; EDTA was added to 20mM final concentration, Proteinase K to a final concentration of 50 μg/ml, and SDS to 0.5% final concentration. The solution was then mixed well and incubated at 56°C for 1 h. After cooling to room temperature, the protein was extracted once with an equal volume of phenol, twice with an equal volume of phenol/chloroform, and twice with an equal volume of chloroform. Phage DNA was then precipitated using ice-cold isopropanol, and pelleted at 8,000 g for 10 min. Pellets were washed three times with 70% ethanol, resuspended in 50-100 μl of ddH20 and quantitated spectrophotometrically.

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

Table 2.4. Media and solutions for phage manipulation methods

20xSSC	3M sodium chloride; 0.3M sodium citrate; pH 7.0
20xSSPE	3M sodium chloride; 0.2M sodium dihydrogen phosphate; 20mMEDTA pH 8.0
Denaturation Solution	0.5M sodium hydroxide; 1.5M sodium chloride
Dialysis Buffer	10mM sodium chloride; 10mM magnesium chloride; 50mM Tris pH 8.0
Neutralisation Solution	0.5M Tris.HCl, pH 7.2; 1.5M sodium chloride; 10mMEDTA pH 8.0
SM solution (Suspension Medium)	0.1M sodium chloride; 0.01M magnesium sulphate; 0.05M Tris pH 8.0; 0.01% (w/v) gelatin
Top agarose	Appropriate medium with 0.7% (w/v) agarose
Wash solution (low stringency)	2X SSC; 0.1% SDS

Wash solution (medium stringency)	1X SSC; 0.1% SDS
Wash solution (high stringency)	0.5X SSC; 0.1% SDS

2.4. DNA MANIPULATION METHODS

2.4.1. Plasmid vectors

Vectors used in various applications during this project are given in Table 2.4.

Table 2.5. Plasmid vectors and their applications.

pUC18/19 (New England Biolabs)	Ampicillin resistance, blue/white colony selection, DNA manipulation
pBluescript SK/KS (+/-) (Stratagene)	Ampicillin resistance, blue/white colony selection, DNA manipulation
pGEM-T (Promega)	Ampicillin resistance, T-overhangs to facilitate direct cloning of Taq Polymerase amplified PCR products
pQE vectors (Qiagen)	Ampicillin resistance, expression of recombinant protein with poly His-tag
pBM2389, pBM2463	Yeast-One hybrid system: reporter and activator vectors
PGEX-6P-2 (Pharmacia)	Ampicillin resistance, expression of recombinant protein with GST moiety

2.4.2. Restriction endonucleases

DNA digestions were performed as described in Sambrook et al. (1989), using restriction endonucleases and buffers from Promega or Boehringer. Generally, restriction digests contained 0.5-5 U of enzyme; appropriate buffer (1x); DNA (0.1-10

□g) and ddH_2O to a final volume of 10-50 □l. Reactions were incubated at the temperature optimal for enzyme activity (generally 37°C) for 1-4 h, and DNA fragments were visualised after agarose gel electrophoresis. If digestion were carried out in large volumes, digests were precipitated and resuspended in smaller volumes prior to electrophoresis.

2.4.3. Agarose gel electrophoresis of DNA

Gels were prepared by dissolving molecular biology grade agarose in 1x TAE buffer to a final concentration of 0.8 – 1.2% (w/v) by boiling for 1 min. After cooling to 50°C, ethidium bromide was added to a final concentration of 1 □g/ml, and the gel cast in the appropriate tray and allowed to set. Tracking dye was added to DNA samples prior to loading in the wells, and agarose gels run submerged in the electrophoresis tank under 1x TAE buffer. Electrophoresis was performed at 40-100 V for 30 min to 12 h, and ethidium bromide fluorescence visualised using a 340nm UV transilluminator and recorded either photographically or using the Biorad Fluor-S gel documentation system.

2.4.4. Purification of DNA fragments from agarose gels

DNA fragments of interest were excised from gels using sterile scalpel blades and stored in sterile 1.5 ml microfuge tube. Small (3 cm in diameter) circles of filter paper (Whatmann 3MM), were shaped into cups around a cut-off yellow tip and pushed into a 0.6 ml microfuge tube, in to which a hole had been made in the base with a needle. The tube containing the paper cup was then placed in a clean 1.5 ml microfuge tube, and the agarose gel slice placed into the paper cup and the complete assembly spun at 5000 g for 5 min. DNA was recovered in solution in the 1.5 ml tube with recoveries generally >70%. Recovered DNA was used successfully in ligations, restriction digestions and in the generation of radioactive probes without subsequent purification.

2.4.5. DNA quantification

DNA concentrations were determined by spectrophotometric analysis as described in Sambrook et al. (1989) using OD (260nm; 280nm). A_{260} = 1 = 50 □g/ml of double stranded DNA. Ratios of OD_{260}:OD_{280} were used as an indicator of the purity of DNA samples.

2.4.6. Phenol:chloroform extraction

Organic solvents were used to extract protein and lipid from DNA preparations to improve the purity of the sample. Phenol was distilled from a crystalline product and equilibrated with 1M Tris pH 8.0, or purchased in pre-equilibrated form (Astral). Phenol:chloroform (1:1) solution was prepared by mixing equal volumes of equilibrated phenol and AR grade chloroform (BDL).

Prior to extraction, the volume of DNA samples was adjusted to 800 µl with ddH20, the solution transferred to a sterile 1.5 ml microfuge tube and then 300 µl of organic solvent added and followed by vortexing. The phases were separated by centrifugation at 13,000 g for 5 min and the aqueous phase removed to a new tube. For volumes larger than 1.5 ml, extractions were performed in SS-34 tubes with equal volumes of organic solvents and centrifugations carried out at 20,000 g for 10 min in a Sorvall centrifuge. Generally, phenol extractions were followed by two separate subsequent phenol:chloroform extractions, and a final chloroform extraction before the DNA sample was precipitated and described in section 2.4.7.

2.4.7. DNA precipitation

DNA precipitation was performed by adding 0.1 volumes of 3M sodium acetate (pH 5.2) and either 0.7 volumes of ice-cold isopropanol or 2 volumes of absolute ethanol, the solution was then mixed well and incubated at -20°C for 30 min. The DNA sample was centrifuged at 13,000 g for 30 min and the supernatant removed. DNA pellets were washed with 70% ethanol and allowed to air dry at room temperature, after which time they were resuspended in the appropriate volume of TE buffer or ddH20. Alternatively, DNA was precipitated from large volumes by centrifugation in SS-34 tubes at 20,000 g for 30 min.

2.4.8. Generation of radioactive probes

Radioactive probes were prepared by random oligonucleotide-primed synthesis (oligolabelling) using α-32P dATP (Geneworks; 10 mCi/ml, specific activity ~3000 Ci/mmol). Probes were radioactively labelled using the Megaprime (Amersham) or Prime-a-Gene (Promega) oligolabelling kits as described in the accompanying protocols, as modified from Feinberg & Vogelstein (1983). Generally, 25 ng of linear DNA recovered from agarose gel fragments was used as template for bulk synthesis.

After labelling, unincorporated dATP was separated from the labelled probe by 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 10 mM EDTA, 0.5% SDS. Prior to use, the labelled probe was denatured at 100°C for 5 min and placed on ice immediately for 3 min to prevent re-annealing. An appropriate volume of the probe was then used in the hybridisation experiment.

2.4.9. Determination of specific activity

The determination of specific activity of labelled probes was carried out using the TCA precipitation technique as described in Ausubel (1996). Two glass fibre filters (Whatman) were spotted with 12 µl of reaction mix; one was placed in a vacuum manifold and washed three times with 10 ml 10% trichloroacetic acid to precipitate labelled DNA and remove any unincorporated nucleotides; the second was left untreated. Both filters were then dried using a spot lamp and placed in scintillation vials containing 10ml of biodegradable Starscint scintillation fluid (Packard). Specific activity was calculated as described in Sambrook et al. (1989) after scintillation counting. When using 25 ng of DNA and 50 µCi of radiolabelled dATP, typical specific activities were ~1.5 x 10^9 cpm/µg.

2.4.10. Cloning and ligation reactions

When necessary, plasmids with identical ends were dephosphorylated to prevent re-ligation. Using Calf Intestinal Alkaline Phosphatase (CIP) (Promega) as described in Sambrook et al. (1989), 50 µl reactions containing 1-5 µg of digested vector, 10 µl 5x CIP buffer and 0.1 U CIP were incubated at 37°C for 1 h. The enzyme was then inactivated by heating at 65°C for 10 min in the presence of EDTA. Ligation reactions were performed using T4 DNA ligase (Promega) as described in Sambrook et al. (1989). Generally reactions were carried out in 10 µl total volumes with a 3:1 insert:vector ratio using 1 µl 10X T4 Ligase buffer (containing 10 mM ATP), T4 Ligase (0.3 U) and ddH_2O to 10 µl. Reactions were incubated 4-16°C for 16 h.

2.4.11. Plasmid DNA preparation

Plasmid DNA was prepared using the SDS-alkaline lysis method as modified by Birnboim & Dolby (1979). Plasmid DNA was isolated from small-scale (1-10 ml) and large-scale (>100 ml) cultures using Qiagen Spin Miniprep Kit and Maxiprep Kit columns respectively, as described in Sambrook et al. (1989) or accompanying protocols.

Plasmid DNA prepared as described in Sambrook et al. (1989) required the bacterial cells to be pelleted by centrifugation at 3000 g for 10 min. The bacterial pellet was then resuspended in Solution I (300 µl small scale; 10 ml large scale) and incubated at room temperature for 15 min. Cells were lysed by the addition of Solution II (300 µl small scale; 10 ml large scale). Ice-cold Solution III was added (300 µl small scale; 10 ml large scale) to denature bacterial proteins and chromosomal DNA. The sample was mixed well and incubated on ice for 15 min. Precipitated material was pelleted by centrifugation at 13,000 g for 15 min and the supernatant extracted with phenol:chloroform and chloroform (section 2.4.6) before plasmid DNA was subjected to ethanol precipitation (section 2.4.7). The resulting DNA pellet was resuspended in an appropriate volume of ddH_2O or TE buffer and the concentration determined spectrophotometrically.

2.4.12. DNA sequencing

Nucleotide sequencing of plasmid or phage DNA was performed using the dye terminator cycle sequencing method. This process utilises the dideoxynucleotide-mediated chain termination reaction (Sanger et al., 1977) to alternatively incorporate one of four nucleotides, differentially labelled with a fluorescent tag that can be detected and identified as they pass by a laser within the DNA sequencing apparatus.

Sequencing reactions were generally 20 µl and contained template DNA (200 ng plasmid DNA or 1 µg phage DNA); primer (3.2 pmol for plasmid sequencing or 20 pmol for phage sequencing), 4 µl (1/2 reaction) Big Dye Terminator reaction mix (Applied Biosystems) and ddH_2O to 20 µl. Thermal cycling conditions consisted of 30 cycles of 94°C for 30 s, 50°C for 15 s and 60°C for 4 min. Reactions were ethanol precipitated (section 2.4.7) and loaded onto an Applied Biosystems 310 DNA sequencer

according to the suppliers' instructions. Resulting chromatograms were edited using EditView software (Applied Biosystems), and corrected sequences aligned and analysed using Sequencher software.

2.4.13. 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). DNA of interest was subjected to agarose gel electrophoresis and transferred to Nytran-N (Schleicher & Scheull) or Hybond-N, -N+ or NX (Amersham) using the capillary blot transfer method overnight in Southern transfer solution or 0.4 M sodium hydroxide (Hybond-N+). DNA was covalently linked to membranes by baking at 80°C for 2 h, or by exposure to UV light (312 nm) on a Spectroline TC-312A UV-transilluminator (Medos Company, Brisbane) for 3 min.

Membranes were prepared for hybridisation by incubating in 100 ml of DNA hybridisation solution at 60°C for at least 1 h, prior to the addition of radioactively labelled probe (see section 2.4.8). The final volume of hybridisation solution was no greater than 200 µl per cm^2 membrane and the denatured probe was added at a final concentration of no less than 5 x 10^6 cpm/ml. The hybridising membrane was incubated at 65°C for at least 16 h, after which it was washed (3 x 30 min) with 200 ml of wash solutions at 65°C. Hybridised membranes were exposed to Phosphorimager screens for 3-12 h. Phosphorimager screens were read by a Phosphorimager (Molecular Dynamics) at 100-micron resolution. Images were processed using ImageQuant software (Molecular Dynamics).

2.4.14. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was carried out as described in Ausubel et al. (1996), as modified from Saiki et al. (1988). Generally, reaction volumes were 25 µl and contained template DNA (50 ng of plasmid or fragment; 500 ng of phage DNA); dNTPs (0.2 mM each); 2 mM magnesium chloride; 0.5 U Taq polymerase (Promega or Fisher Biotech); 20 pmol of each primer and ddH_2O. Reactions were performed in either 0.6 ml or 0.2 ml microfuge tubes using a MJ Research Inc. PTC-100 Thermal Cycler or Hybaid Thermal Cycler. Programs varied depending on reactions but

generally the thermal cycler was programmed to perform one denaturing cycle of 94°C for 2 min, then 30-35 cycles of 94°C for 30 s, annealing (45-65°C) for 30 s, and extension at 72°C for 1 min/kb of DNA to be amplified. All programs contained a final hold cycle at 4°C. Amplified DNA was analysed using agarose gel electrophoresis (0.6-1.2%) and fragments of interest were excised and purified from gel for further experiments.

2.4.15. Oligonucleotides

Primers used for PCR and DNA sequencing were designed using MacVector software and custom designed by various companies (Gibco BRL, Sigma). Primers were resuspended from dried pellets in ddH_2O to a final stock solution concentration of 200 μM and stored at -20°C. Working solutions were diluted from this stock for use in reactions.

Table 2.6. Solutions associated with DNA manipulation methods.

50x TAE Buffer	(per litre) 242 g Tris base; 57.1 ml glacial acetic acid; 100ml 0.5M EDTA pH 8.0
DNA hybridisation Solution	0.5% (w/v) BLOTTO; 2x SSC; 0.5% (w/v) SDS
Ethidium bromide	Stock 10mg/ml
Solution I	50 mM glucose; 10 mM EDTA; 25 mM Tris pH 8.0; RNase 10 μg/ml; lysozyme 10mg/ml
Solution II	0.2 M sodium hydroxide; 1%(w/v) SDS
Solution III	(per litre) 600 ml 5 M potassium acetate; 115 ml glacial acetic acid
Southern Transfer Solution	1 M ammonium acetate, 20 mM sodium hydroxide
TE Buffer	10 mM Tris.HCl pH 8.0; 1 mM EDTA
Tracking dye	(6x) 40% (w/v) sucrose; 0.25% (w/v) bromophenol blue
Wash solution	2x SSC; 0.1% (w/v) SDS

2.5. RNA MANIPULATION METHODS

2.5.1. Removing ribonucleases from equipment and work area

Standard procedures were followed when working with RNA to ensure samples were not contaminated with RNases. Clean gloves were worn at all times, and changed frequently. All containers were either sterile disposable plastic-ware, or glassware that had been baked at 200°C for at least 12 h. Filter pipette tips were routinely used. Solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) for 12 h and autoclaved. Solutions that could not be treated with DEPC were prepared using DEPC-treated H_2O and sterilised by passing through 0.2 μm filters. RNases were removed from plasticware using RNA-zap (Ambion) followed by rinsing in DEPC-treated dd$H_2$0.

2.5.2. Extraction and purification of RNA from coral tissue

Total RNA was extracted from frozen embryonic and adult tissue using the acid guanidinium thiocyanate/phenol:chloroform extraction method as described by Chomczynski and Sacchi (1987). Coral tissue or embryos were ground to a fine powder under liquid nitrogen and mixed with RNAwiz (Ambion) to prepare total RNA as described in accompanying protocols. Poly(A)+ RNA was extracted from total RNA using the PolyATract mRNA Isolation System IV (Promega) as described in the accompanying protocols for small-scale mRNA isolation.

2.5.3. RNA quantification

Samples of RNA were quantitated spectrophotometrically as described in Sambrook et al. (1989). OD (260nm) 1 = 40 μg/ml RNA.

2.5.4. Quantitative PCR

Quantitative PCR was routinely used to compare mRNA levels throughout development. For this purpose, first-strand cDNA was prepared from 5 μg aliquots of total RNA using the Amersham First-Strand cDNA Synthesis Kit. The resulting single-stranded cDNAs were used as templates in quantitative PCR reactions. PCR reactions were carried out using primers designed with Primer Express™ 1.5 software (ABI

Applied Biosystems), and SYBR® Green PCR Master Mix (ABI Applied Biosystems). Reactions were run on an ABI 7700 Quantitative PCR System, using SDS Sequence Detection System analysis software (ABI Applied Biosystems). Non-specific amplification products were minimised by optimising the primer pair concentrations prior to experimentation. Optimal primer concentrations were determined to be 50 nm. Product specificity was also checked after each experimental assay using a dissociation curve to determine the melting temperature of the specific product.

First-strand cDNA was prepared from thirteen different stages of coral development, from egg to post-settlement, collected during a single spawning event of November 2001 at Geoffrey Bay, Magnetic Island. As temporal development of coral embryos and planulae is strongly dependent of a number of factors, most importantly water temperature, embryos and planulae from different years were not be compared.

The level of expression of each gene of interest (the target) was expressed relative to that of *integrin-β* subunit (the endogenous reference), a uniformly expressed coral gene, in order to control for variations in the quantity and quality of target mRNA in different samples. Relative levels of gene expression were calculated as outlined in User Bulletin #2 of the ABI Prism 7700 Sequence Detection System compendium. Relative quantitation of both the experimental target and the endogenous reference was determined using the relative standard curve method. Stock DNA of each gene of interest was accurately diluted ten-fold to give five stock DNA samples with relative dilution values of 1, 0.1, 0.01, 0.001 and 0.0001, of which the resulting values can be used to form a standard curve. For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard curve. The target amount was then divided by the endogenous reference amount to obtain a normalised target value, without units, expressed as mRNA copy number. The standard deviation of the quotient is calculated using the standard deviations of the target and endogenous reference values using the following formula:

$$cv = \frac{s}{\overline{X}} = \frac{std.dev}{mean.value} \qquad \text{where:} \qquad cv = \sqrt{cv_1^{\,2} + cv_2^{\,2}}$$

Real-time quantitative PCR assays were carried out in 96-well plate with optical caps (ABgene), suitable for use with the ABI SDS 7700. Each assay was performed in triplicate while pipetting errors were reduced by increasing pipetting volumes and working from master mixes for each gene to be assayed.

Each PCR reaction consisted of 50 nm of forward primer, 50 nm reverse primer, 15.75 µl SYBR Green PCR Master Mix (Applied Biosystems), 31.25 µl Milli-Q H2O and 1 µl of template first-strand cDNA. Thermal cycler conditions were as follows: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C (15 sec), 60°C (1 min). Completed reactions were then held at 15°C. The resulting data was analysed using the SDS 7700 software and exported to Microsoft Excel for normalisation to the endogenous control.

With each new first-strand cDNA preparation, the endogenous reference control must also be repeated with the target genes before data can be normalised.

2.1.5. Agarose gel electrophoresis of RNA

For electrophoresis of RNA, agarose gels were prepared by dissolving 0.5 g agarose in 36 ml of boiling DEPC-treated water and adding 5 ml 10x MOPS; 9 ml formaldehyde and DEPC-treated water to a final volume of 50 ml. After cooling to 50°C, the mixture was poured into gel casting apparatus and allowed to set. The set gel was placed in an electrophoresis tank and submerged in 1x RNA agarose gel running buffer. Prior to loading, RNA samples and markers (5 µl) were prepared for electrophoresis by the addition of 1µl 10X MOPS; 3.5 µl formaldehyde; 10 µl formamide; 0.5 µl ethidium bromide (10 mg/ml) and incubating at 65°C for 15 min before chilling on ice for 5 min. 2 µl of tracking dye was then added to each sample before being loaded onto the gel. Electrophoresis was carried out at 100 V for 60-90 min. The RNA/fluorescence was documented using a 312 nm UV transilluminator and a photographic gel documentation system.

2.1.6. Northern blotting

After electrophoresis, RNA was transferred to Nytran-N nylon membranes (Schleicher & Scheull) by capillary blotting. RNA was irreversibly linked to the nylon membrane by

cross-linking using a 2 min exposure to UV light (312 nm) and baking at 80°C under vacuum for 2 h. Membranes were prepared for hybridisation by soaking in 25 ml of RNA hybridisation solution at 48°C for at least 1 h. Radioactively labelled probes were prepared as described in section 2.4.8 using the Prime-a-Gene oligolabelling kit (Promega). Hybridisation was carried out in 15 ml of RNA hybridisation solution at 48°C for 16-24 h. Membranes were washed (3x30 min) at 65°C with 200 ml of wash solution before exposure to a Phosphorimager screen for 4-16 h. Phosphorimager screens were processed at 100-micron resolution and the images manipulated using the ImageQuant software.

2.1.7. Coral embryo in situ hybridisation

Spatial and temporal gene expression patterns were examined by in situ hybridisation of coral embryos, at various stages of development using RNA probes (riboprobes) containing digoxigenin (DIG). After hybridisation, the distribution of riboprobes was determined using enzyme-coupled antibodies directed against the incorporated DIG.

Prior to in situ hybridisation, coral embryos were treated with xylene and detergents to partially damage cell membranes and remove lipid bodies that provide buoyancy to embryos but interfere with the probing process. Riboprobes were generally hydrolysed prior to use, to allow greater penetration into coral tissue.

2.1.7.1. Embryo preparation

Prior to use, embryos were allowed to come to room temperature in the 100% methanol in which they had been stored at -20°C, after which they were rehydrated through an ethanol series to 50%. Prior to a 12 h treatment at 4°C in RIPA (although this step was sometimes emitted), embryos were dissected if necessary with "micro-knives" fashioned using the thinnest available razorblades, broken into to widths of around 5 mm, and attached to the end of wooden skewers. All sectioning was carried by hand, orientating embryos and planulae in a groove in an agar dish and sectioning through the plane of interest. Embryos were never mounted in paraffin or resin, nor were they frozen for sectioning purposes. In general, embryos and planulae were sectioned prior to staining, but in some instances sectioning of stained samples was carried out for the purposes of microscopy and photography.

After the RIPA treatment, embryos were then dehydrated through a methanol series and transferred to 100% ethanol prior to treatment with xylene for 2 h. Residual xylene was removed by washing (3 x 30 min) with 100% ethanol, after which the embryos were rehydrated through an ethanol series and washed with PBT. The PBT solution was then gradually replaced with hybridisation solution, in which the embryos were incubated at 56°C for 3 h.

2.1.7.2. Riboprobe synthesis

Digoxigenin (DIG)-labelled RNA probes were synthesised using run-off transcription as described in Kucharski et al. (2000). The vector containing the cDNA corresponding to the probe template was linearised such that digestion produced a 5'- overhang at the 5'- end of the cDNA insert. Template DNA (1 µg) was then added to 50 mM Tris, pH 8.0 containing 8 mM magnesium chloride, 2 mM spermidine, 50 mM sodium chloride, 9 mM DTT, 40 U RNAsin (Promega), 2 µl 10x DIG RNA labelling mixture (Boehringer Mannheim) (6.5 mM UTP; 3.5 mM DIG-UTP; 10 mM ATP; 10 mM CTP; 10 mM GTP) and 30 U of either T3 or T7 RNA Polymerase (Promega) in a total reaction volume of 20 µl. The reaction was then incubated at 37°C for 2 h and stopped by the addition of 2µl of 0.2 M EDTA. 2 µl of the reaction was then analysed by RNA agarose gel analysis, 2 µl was used for spectrophotometric quantitation and the remaining sample was salt/ethanol precipitated (see section 2.4.6) and resuspended in 100 µl RNase-free ddH_2O for use in hybridisation.

After synthesis, riboprobes were partially hydrolysed to facilitate penetration of the probe into treated tissue. Hydrolysis was carried out in a reaction volume of 55.5 µl containing 50 µl of precipitated RNA probe and 5.5 µl sodium carbonate buffer. The reaction was incubated at 60°C for 30-40 min and stopped by neutralisation with 4 µl 3 M sodium acetate, pH 5.2. After this, the RNA probe was precipitated by the addition of 40 µg tRNA and 300 µl absolute ethanol followed by centrifugation at 13,000 g for 15 min. Probes were resuspended in RNA probe resuspension buffer at a final concentration of 50 µg/ml for storage at -20°C and use.

To determine the extent of DIG incorporation, serial dilutions of the hydrolysed probe were prepared using RNase-free ddH_2O and dot blotted onto nitrocellulose membrane (Schleicher & Scheull) alongside a standard. 0.5 µl aliquots of dilutions were spotted onto the nitrocellulose and then permanently immobilised by baking at 80°C for 2 h. Baked membranes were placed in blocking buffer for 30 min at 4°C, and then transferred to a solution of alkaline phosphatase conjugated anti-DIG antibody (Boehringer-Mannheim), diluted 1:1500 in PBS, for 60 min at room temperature. Afterwards, the membrane was washed several times in PBS to remove residual antibody.

2.1.7.3. Riboprobe hybridisation and detection

Hydrolysed probes, suspended in hybridisation solution, were added to the embryos to a final concentration of 0.1-1.0 µg/ml and incubated at 56°C for 48-72 h. This was followed by extensive washing in hybridisation wash solution at 56°C (2 x 5 min; 4 x 30 min; 1 x 12 h; 3 x 30 min) to remove residual unbound probe before the hybridisation solution was gradually replaced with PBT (3 x 10 min) and cooled to room temperature. In order to reduce non-specific binding by the anti-DIG antibody, embryos were first blocked for 30 min in 5% (v/v) normal goat serum in PBT, before transfer to alkaline phosphatase conjugated anti-DIG antibody (Boehringer-Mannheim) diluted in PBS to 1:1500. After incubation at room temperature for 2 h, unbound secondary antibody was removed by washing (5 x 30 min) with PBT and embryos then rinsed (2 x 5 min) in NTMT prior to the addition of the substrate. The colour reaction was initiated by transferring the embryos into either BM purple solution (Boehringer Mannheim) or NTMT containing 450 ng/µl NBT and 230 ng/µl BCIP followed by incubation in the dark for up to 48 h. Placing the embryos in PBS and washing several times terminated the colour reaction. 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 microscope and images were captured using a SPOT digital camera and processed using Adobe Photoshop.

Table 2.7. Solutions associated with RNA manipulation methods.

Denhardt's Solution	(50X Stock): 1% (w/v) bovine serum albumin; 1% (w/v) Ficoll; 1% (w/v) PVP
Embryo hybridisation solution	50% (v/v) formamide; 4x SSC; 1x Denhardt's solution; 5 µg/ml heparin; 5% (w/v) dextran sulphate; 250 µg/ml tRNA; 500 µg/ml denatured sonicated salmon sperm DNA; 0.1% (v/v) Tween 20
Embryo hybridisation wash solution	4x SSC; 50% (v/v) formamide; 0.1% (v/v) Tween 20
Ethidium bromide stock	10 mg/ml ethidium bromide in ddH_20
MOPS (10x Stock)	0.2 M MOPS; 0.1 M sodium acetate; 0.1 M sodium hydroxide, pH 7.0
NTMT	100 mM sodium chloride; 5 mM magnesium chloride; 0.1% (v/v) Tween 20; 100 mM Tris, pH 9.5
PBS	137 mM sodium chloride; 27 mM potassium chloride; 10 mM disodium hydrogen phosphate; 2 mM potassium dihydrogen phosphate; pH 7.2
PBT	PBS; 0.1% (v/v) Tween 20
RNA agarose gel	1% (w/v) agarose; 1x MOPS; 18% formaldehyde
RNA gel running buffer	1x MOPS; 20% (w/v) formaldehyde
RNA hybridisation solution	0.25 M sodium phosphate; 7% SDS, 50% formamide; 1 mM EDTA, pH 8.0
RNA tracking dye	50% (v/v) glycerol; 1 mM EDTA; 0.25% (w/v) bromophenol blue; 0.25% cyanol orange
RIPA	150 mM sodium chloride; 1% (v/v) Nonidet P-40; 0.5% (v/v) sodium deoxycholate; 0.1% (w/v) SDS; 1 mM EDTA; 50 mM Tris, pH 8.0
Sodium carbonate buffer	400 mM sodium hydrogen carbonate; 600 mM disodium carbonate; pH 10.2

TE buffer	10 mM Tris; 1 mM EDTA; pH 7.5
Wash solution (low stringency)	2x SSC; 0.1% SDS

2.6. PROTEIN MANIPULATION METHODS

2.6.1. Generation of recombinant fusion proteins

Fusion proteins were generally generated by cloning appropriate PCR products into Qiagen His-tag expression vectors (pQE-30, -31 or -32) or pGEX vectors (Pharmacia), and expressed either in M15 or BL21 *E. coli*.

2.6.1.1. Protein expression using pQE vectors

Expression of recombinant fusion proteins from pQE vectors was performed as described in The QIAexpressionist handbook (Qiagen), modified from Janknecht et al. (1991). The solubility of proteins expressed in this way was determined by fractionation of the cell lysate under non-denaturing conditions. This allowed the selection of appropriate conditions for small-scale expression, which in turn allowed optimisation of conditions for large-scale expression. Large-scale cultures were then grown and protein expression induced. In order to achieve large-scale cultures, a single bacterial colony was used to inoculate 10 ml of LB containing amp/kan, which was then placed in a 37°C shaking incubator overnight. A 3 ml aliquot of this culture was then used to inoculate 100 ml of LB containing amp/kan in a 250 ml flask, which was incubated at 37°C with shaking until OD_{600} reached 0.6. At this point, protein expression was induced by the addition of IPTG to a final concentration of 1 mM, and the culture incubated for a further 4-5 h. Cells were then harvested by centrifugation at 4000 g for 20 min, and the pellet stored at -20°C overnight.

For protein extraction, pellets were thawed and then resuspended in 500 µl Buffer B then freeze/thawed twice to lyse the bacteria. Insoluble material was then removed by centrifugation at 10,000 g for 10 min and the supernatant removed to a fresh microfuge tube. Recombinant proteins were purified from the supernatant by the addition of 50 µl

of a 50% slurry of Ni-NTA agarose beads (Qiagen) to 500 µl of supernatant and mixed at room temperature for 30 min to allow binding of the His-tagged protein. The agarose beads were pelleted briefly (30 s) at 4,000 g and washed three times with 1 ml of Buffer C. The bound protein was then eluted off the beads in 50 µl Buffer E at room temperature for 2 min, pelleting the beads as before and removing the supernatant to a new tube. Affinity-purified proteins, together with induced and uninduced lysates, were then analysed using SDS-PAGE.

2.6.1.2. Protein expression using pGEX vectors

Expression of recombinant protein from pGEX vectors (Pharmacia) was performed as described in Ausubel et al. (1996) as modified from Smith and Johnson (1988). 50 ml aliquots of LB containing ampicillin were inoculated directly with a single colony from a bacterial streak and the culture incubated at 37°C overnight with shaking. The entire 50 ml culture was then used to inoculate 1 litre of ampicillin-containing LB and the culture incubated at 37°C with shaking until it reached an OD_{600} of 0.7-0.9 (approx 3 h). Protein expression was then induced by the addition of IPTG to a final concentration of 1 mM, and incubation continued for a further 5-8 h at 37°C with shaking, after which the cells were pelleted at 4,000 g for 10 min at 4°C and resuspended in 10 ml ice-cold PBS. Cells were lysed by sonication (6 x 10 s using a 5 mm diameter probe; Thomas Optical & Scientific Co.), after the addition of Triton X-100 to 1%. Insoluble components were pelleted at 10,000 g for 10 min and the supernatant removed to a new tube.

GST-fusion proteins were purified by the addition of 1/10 volume of 50% slurry of glutathionine-agarose beads and incubated at room temperature for 1 h to allow the GST-fusion protein to bind to the beads. The agarose beads were then pelleted at 500 g for 5 min and washed three times with 50 ml PBS. The bound protein was then eluted by incubation at room temperature for 2 min in 1 ml GST elution buffer, and the beads removed by centrifugation. Elution was repeated twice, and affinity purified proteins analysed by SDS-PAGE.

2.6.2. SDS-Polyacrylamide gel electrophoresis

Protein analysis was performed using electrophoresis on SDS-polyacrylamide gels with Mini-PROTEAN II dual slab gel apparatus (BioRad) following accompanying protocols adapted from Laemmli (1970). Discontinuous 0.1% (w/v) SDS gels with a resolving layer (8-12% (w/v) polyacrylamide 29:1; 375 mM Tris pH 8.8) and stacking layer (5% (w/v) polyacrylamide 29:1; 125mM Tris pH 6.8) were prepared in a gel casting apparatus, using APS and TEMED to initiate the polymerisation reaction. Protein samples and molecular weight standards were prepared for electrophoresis by the addition of an equal volume of 2x Laemmli buffer and heating at 95°C for 10 min.

After polymerisation, SDS-PAGE gels were transferred to an electrophoresis tank containing SDS running buffer covering the gel and electrodes. Loading wells were rinsed to remove residual gel prior to loading samples. Electrophoresis was performed at 200V for approximately 45 min, using bromophenol blue to track the progress of electrophoresis. After electrophoresis was complete, gels were removed from the apparatus and soaked in staining solution for 1 h followed by destaining for 2 h to overnight. Gel images were captured using a fluorescent light box and photographic gel documentation, or vacuum-dried at 80°C for 2 h and stored dried.

2.6.3. Protein quantification

Protein concentrations were determined using the BioRad Protein Assay (BioRad), as modified from the Bradford method (Bradford, 1976). Reactions were performed in a 96-well microtitre plate. Samples and standards were prepared in a 160 µl final volume in separate wells and 40 µl of diluted BioRad Protein Assay reagent added followed by mixing. The colour reaction was incubated at room temperature for 15 min and the absorbance of each well measure on a microtitre plate reader at 595 nm. Protein concentrations were then determined using bovine serum albumin (BSA) or IgG to create a standard curve using Microsoft Excel software.

2.6.4. Electrophoretic Mobility Shift Assays (EMSA)

Binding of oligonucleotides by various DNA-binding domains was assessed by EMSA using fusion proteins generated as described in section 2.6.1-3.

2.6.4.1. Radioactive labelling of oligonucleotide probes

Double stranded DNA sequences corresponding to potential binding sites were synthesised as two complementary oligonucleotides (Sigma), one of which had a 5'-GATC- overhang to facilitate radioactive-labelling using Klenow enzyme to end-fill the overhang with α-^{32}P-dATP. The complimentary oligonucleotides were annealed by heating 2 pmol of each primer to 95°C for 5 min in a thermal cycler, followed by slow cooling. Double-stranded oligonucleotides were then labelled using Klenow and unincorporated radionucleotides removed using the QIAQuick nucleotide removal kit (Qiagen) prior to storage at -20°C and use.

2.6.4.2. Oligonucleotide binding

The protein and labelled oligonucleotide were added to a binding reaction (see Table 2.8) and incubated on ice for 30 min prior to loading on the gel. Labelled oligonucleotides were used at a final concentration of 2 nM in the binding reaction. Unlabelled competitor probes were used in a dilution series at a final concentration of 800, 400 and 200 nM. When unlabelled competitor probes were included in the binding reaction, they were added 15 min prior to the addition of the labelled probe and incubated on ice in some cases. Protein concentrations were varied in a dilution series in order to determine the affinity of the binding reaction. Binding reaction conditions were optimised for each reaction; those for the emx-Am homeodomain are listed in Table 2.8 (EMX).

2.6.4.3. Non-denaturing electrophoresis of protein/DNA complexes

Continuous, non-denaturing 8% polyacrylamide gels (2.5% v/v glycerol; 0.5x TBE) were prepared in a 15 cm gel-casting apparatus using 10% ammonium persulphate and TEMED to initiate gel polymerisation. After polymerisation, the gel was transferred to the electrophoresis apparatus with 0.5x TBE running buffer such that the gel and electrodes were submerged. Prior to electrophoresis, the wells of the gel were first flushed with running buffer (using a needle and syringe) and the gel pre-run at 200 V for at least 30 min at 4°C prior to loading of samples. After the pre-run, wells were again flushed with running buffer to remove any residual acrylamide that may interfere with the running of the sample.

Binding reactions were loaded to the gel without the addition of tracking dye); loading dye (10 µl) was loaded to a separate well to enable the progress of electrophoresis to be followed. Gels were subjected to electrophoresis at 200 V for approximately 2 h at 7°C before drying onto Whatman paper (80°C for 2 h) and exposure to a phosphorimager screen for 10-16 h. Images were captured using a Molecular Dynamics Storm Phosphorimager and ImageQuant software.

Table 2.8. Solutions associated with protein manipulation methods.

2X Laemmli Buffer	2% (w/v) SDS; 10% glycerol; 20 mM DTT; 0.1% (w/v) bromophenol blue; 250 mM Tris pH 6.8
10x TBE	0.89 M Tris; 0.89 M boric acid; 20 mM EDTA
Binding Buffer (EMX)	25 mM HEPES (pH 7.6); 10% glycerol; 50 mM potassium chloride; 5 mM magnesium chloride; 1 mM DTT; 0.1 mM EDTA
Binding Reaction	16 µl binding buffer; 2 µl protein sample; 1 µl radioactively-labelled oligonucleotide; 1 µl poly dI-dC (1 µg/µl)
Buffer B	8 M urea, 100 mM sodium dihydrogen phosphate, 10 mM Tris, pH 8.0
Buffer C	8 M urea, 100 mM sodium dihydrogen phosphate, 10 mM Tris, pH 6.3
Buffer E	8 M urea, 100 mM sodium dihydrogen phosphate, 10 mM Tris, pH 4.5
Destaining Solution	50% (v/v) methanol; 10% (v/v) glacial acetic acid
Elution buffer	50 mM Tris (pH 8.0); 15 mM reduced glutathione
LB ampicillin	LB media, 50 µg/ml ampicillin
LB amp/kan	LB media, 50 µg/ml ampicillin, 50 µg/ml kanamycin
PBS	137 mM sodium chloride; 27 mM potassium chloride; 10 mM disodium hydrogen phosphate; 2 mM dihydrogen phosphate, pH 7.2

SDS Running Buffer	192 mM glycine; 0.1% (w/v) SDS; 25 mM Tris pH 8.3
Staining Solution	50% (v/v) methanol; 10% (v/v) glacial acetic acid; 0.25% (w/v) Coomassie Brilliant Blue R-250
Tracking Dye	80% (v/v) glycerol; 0.5% (w/v) bromophenol blue

2.7. ANTIBODY METHODS

2.7.1. Generation of antibodies

Polyclonal antibodies were generated in rabbits at the Institute of Medical and Veterinary Services, Adelaide, South Australia. Recombinant proteins were expressed, purified (section 2.6.1) and quantified (section 2.6.3) then lyophilised as 300 µg aliquots before sending these to IMVS for antibody production. Immunisation was carried out at 3-4 week intervals. The primary dose was administered as a 1:1 emulsion with Freund's Complete Adjuvant, while the remaining three doses were administered as 1:1 emulsions with Freund's Incomplete Adjuvant. Animals were sacrificed three weeks after the final dose, serum extracted and stored as aliquots at -70°C.

2.7.2. Indirect ELISA

Indirect ELISA assays were carried out in 96-well Immunosorb microtitre plates (Nunc). 3 µg of protein was diluted in 2 ml of coating buffer and 200 µl of this antigen/coating buffer added to each well in column 1. 2-fold serial dilutions were then prepared across the plate in columns 2-11 using a multichannel pipette. Column 12 was treated as a negative control and received no protein solution. After loading, the plate was covered in plastic wrap and incubated at room temperature overnight. Plates were washed three times in distilled water. Wells were then blocked in PBSTB solution overnight at room temperature and washed as above.

Primary antibody was diluted 1:500 in PBSTB (3 µl primary antibody in 1.5 ml PBSTB) and 100 µl added to each well in row A. Two-fold serial dilutions were then prepared across the plate in rows A-F. Row G was treated as a negative control without primary antibody, and row H was treated as a negative control without primary or secondary antibody. After the addition of the primary antibody, the plate was wrapped

in plastic, incubated at room temperature for 1-5 h and washed as described above. The HRP-conjugated secondary antibody (5 µl) was diluted in PBSTB (5 ml) (1:1000 dilution) and 50 µl added to each well, except row H, prior to incubation at room temperature for 1 h and washing as described above. The antigen-antibody interaction was then determined by assaying peroxidase activity. Immediately before use, developing solution was prepared and 100 µl aliquots of this added to each well. Reactions were incubated for 1 h, and the colour change measured spectrophotometrically at 405 nm.

2.7.3. Western blotting

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membranes for use in immunodetection assays. Transfers were performed using the Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) as described in accompanying protocols, as modified from Towbin et al. (1979). After SDS-PAGE, both the gel and nylon membrane were equilibrated in transfer buffer for 30 min prior to blotting. The gel was then placed in contact with the nylon membrane in the transfer cassette, between wetted filter paper and transfer pads. The assembled transfer cassette was then placed into the Trans-Blot apparatus such that the electrodes were submerged in transfer buffer. Electrophoresis was performed at 90 V for 2 h at 4°C. Once the apparatus was disassembled, the nitrocellulose membrane was rinsed in PBS and dried. Protein transfer to the nitrocellulose membrane was confirmed by staining with Ponceau-S and destaining in several changes of ddH_2O. Ponceau-S was completely removed in several washes with PBS.

Nitrocellulose membranes were blocked in PBSTB at room temperature for 1 h, and then rinsed in PBS solutions (3 x 5 min), prior to incubation for 1 h at room temperature in primary antibody diluted in PBSTB. Unbound primary antibody was removed by washing with PBS (3 x 15 min) prior to the addition of the peroxidase conjugated secondary antibody in PBSTB. All incubations were carried out at room temperature for at least 1 hour with gentle agitation. Unbound secondary antibody was then removed by washing with PBS (3 x 15 min). The membrane was then submerged in DAB reagent solution, the peroxidase reaction initiated by the addition of hydrogen

peroxide and incubated at room temperature until a colour reaction was observed. This colour reaction was terminated by transfer of the membrane into PBS and rinsing well.

2.7.4. Affinity purification of antibodies

This technique uses immobilised antigen as an affinity matrix to purify antibodies specific for that antigen. Antigen was transferred to nylon membrane (section 2.7.3), visualised by staining with Ponceau-S, and the section of membrane excised with a scalpel and destained in PBS. The resulting membrane strip was dried and stored at room temperature until required.

In order to minimise non-specific binding of antibodies, membrane strip were incubated in blocking solution for 50 min with gentle agitation in a plastic Petri dish, followed by washing with PBS (3 x 5 min) prior to incubation in 3 ml of antiserum at room temperature for 3 h with gentle agitation in a 10 ml tube. The solution removed at this stage (the 'depleted fraction') was retained as a control for the efficiency of the affinity purification. The strip was then washed (3 x 10 min) in PBS before the purified antibodies were eluted by incubating in 1ml low-pH buffer at room temperature for 10 min with gentle agitation. The eluted fraction was then neutralisation by addition of 150 □l 1M Tris.HCl pH 8.0 and stored at 4°C. The strip was washed in PBS (3 x 5 min) and dried, then stored at 4°C for reuse. The specificity of untreated serum, depleted fraction and the purified fraction were then tested by probing strips of a blot of antigen and lysates from coral embryos and larvae.

2.7.5. Embryo immunohistochemistry

Fixed embryos were rehydrated from 100% methanol to 70% methanol for dissection and then fully rehydrated through a methanol series to PBS. Embryos were washed in PBS (3 x 2 min), then PBT (3 x 2 min; 3 x 10 min) and then incubated at 4°C for 36 h with the primary antibody diluted to the appropriate concentration (1:100 – 1:1000) in PBT with gentle agitation. Unbound primary antibody was removed by washing in PBT (5 x 20 min), then embryos were incubated for 2 h at room temperature with the secondary antibody at the appropriate concentration (Goat-anti-rabbit-Cy-5 at 1:100; DAPI stain at 1:100; Goat-anti-rabbit-biotin at 1:200).

Unbound secondary antibody was removed by washing in PBT (5 x 20 min). In some cases a tertiary antibody (Streptavidin-Cy-5 at 1:200) was also used and was treated in the same way as the secondary antibody. Embryos were then rinsed in PBS before being placed in nickel intensified DAB reagent solution and incubated at room temperature to allow colour development (5-30 min). The colour reaction was terminated by transferring the embryos to PBS, and the embryos cleared for photography in a glycerol series. For photography, embryos were mounted on slides in 70% (v/v) glycerol. Embryos stained with DAB were examined using a Zeiss Axioscope microscope and digital images captured using a SPOT digital camera and processed using Adobe Photoshop software. Where immunofluorescent secondary or tertiary antibodies were used, embryo staining did not require a chemical reaction and was instead visualised on a DeltaVision deconvolution microscope. Between five and fifty Z-series optical sections (of 0.1 – 0.5 □M resolution) were captured for each sample using a digital camera. With softWoRx 2.5 imaging software, each image was then construct by deconvolution of a suitable selection of Z-series optical section to create the final image. Using this method, the protein of interest was detected using a secondary or tertiary antibody with a Cy-5 label while DNA within each cell was localised using a DAPI stain. Other fluorescent labels (including Alexa-568) could not be used on coral embryos as confocal wavelength scans of the embryos indicated a high level of autofluorescence emission between 400 and 600 nm when excited with a number of wavelengths between UV and 633nm. For solution recipes see Table 2.9.

Table 2.9. Solutions associated with antibody manipulation methods.

Blocking solution	0.5% non-fat milk powder in PBS
Coating buffer	0.1 M sodium carbonate (Na2CO3); 0.1 M sodium bicarbonate (NaHCO3); 3 mM sodium azide
DAB reagent solution	100 mM Tris pH 7.5; 0.8mg/ml DAB; 0.4 mg/ml nickel chloride; 0.01% (v/v) hydrogen peroxide
Developing solution	Sequentially add 9.6 ml ELISA buffer; 200 □L of 30 mg/ml ABTS (2'2'azino-di-[3-athul-benzolin-sulfonat(6)] ; 200 □l 0.9% hydrogen peroxide solution and use immediately
ELISA buffer	29.4 ml of 0.1M citric acid; 20.6 ml of 0.2M disodium orthophosphate (Na2HPO4); 50 ml ddH20

Low-pH Buffer	0.2 M glycine; 1 mM EGTA; pH 2.3-2.7
PBS	137 mM sodium chloride; 27 mM potassium chloride; 10 mM Na2HPO4; 2 mM KH2PO4
PBT	PBS with 0.1% Tween 20
PBSTB	PBS with 0.05% (v/v) Tween 20; 2% (w/v) BSA
Ponceau-S solution	0.2% (w/v) Ponceau-S dye; 0.3% trichloroacetic acid
Transfer solution	192 mM glycine; 25 mM Tris; 1.3 mM SDS; 20% methanol

2.8. YEAST METHODS

Yeast-One Hybrid System

The yeast-one hybrid system is an *in vivo* yeast genetic assay to identify target sequences for DNA binding proteins. The method used was based on Liu et al. (1993).

2.8.1. Yeast strains

YM4271 was used in screens to select DNA binding sites. The genotype of this strain is *MAT*a *ura3-52 his3-Δ200 ade2-101 lys2-801 trp1-901 gal4-Δ512 gal80-Δ538 ade5::hisG.*

2.8.2. Yeast media

All media used in the yeast-one hybrid system were prepared as described in Burke et al. (2000), and are given in Table 2.10.

2.8.3. Preparing competent yeast cells

5 ml aliquots of YPD liquid media were inoculated with the appropriate yeast strain and the culture incubated at 30°C with shaking overnight. The OD_{600} of overnight cultures was determined and yeast diluted into 100ml liquid YPD to a final OD_{600} = 0.1, prior to incubation at 30°C with shaking until an OD_{600} of 0.8-1.0 was achieved. Yeast cells were then pelleted by a centrifugation for 5 min at 2,000 g at room temperature, and briefly resuspended in 10 ml TEL buffer before being re-centrifuged at 2,000 g for 5 min. The pellet was then resuspended in 1 ml of TEL Buffer and incubated at 30°C for 20 min with gentle agitation. These cells were either used immediately, or else glycerol

was added to 15% final concentration and material stored at -80°C. Freshly prepared cells, however, give the highest transformation efficiency.

2.8.4. High efficiency transformation of yeast

Yeast cells were transformed with activator and reporter plasmids as described in Burke et al. (2000) adapted from Gietz and Schiestl (1995). 100 µl aliquots of competent cells (section 2.8.3) were mixed with 5 µl plasmid DNA and 5 µl sheared salmon sperm DNA (10mg/ml) followed by incubation at 30°C for 20 min. 700 µl of 40% PEG4000 in TEL buffer was then added to the mixture and incubated at 30°C and continued for a further 20 min. Transformation was achieved through a heat shock at 42°C for 5 min before the cells were pelleted (5 s spin) and resuspended in 150 µl H20. Transformed yeast were then plated on selective media and incubated at 30°C until colonies appeared.

2.8.5. Yeast-one hybrid plasmid construction

Activator plasmids were constructed by inserting DNA sequences, encoding DNA-binding domains, between the *Not*I and *Xho*I sites of pBM2463 such that the activator plasmid produced an in-frame lexA-X-Gal4 fusion protein (where X is the protein of interest). This insert fragment was generated by PCR and restriction sites were introduced at either end to facilitate cloning. Prior to expression, the sequence of the DNA insert was verified.

An *Acropora millepora* reporter plasmid library was constructed in pBM2389. High molecular weight genomic DNA (5 µg) was partially digested with *Sau*3AI (50 U) at 37°C for 1 h to generate fragments in the size range 0.1 to 5 kb. The digested DNA was size fractionated by agarose gel electrophoresis and the 0.1-1 kb fragments purified by spinning through Whatman paper (section 2.4.4). These fragments were ligated into pBM2389 that had been digested with *Bam*HI and treated with alkaline phosphatase, and transformed into DH5α cells by electroporation. In six separate pools, 50,000 transformants were generated, representing approximately 5 x 10^7 bp of genomic sequence. Estimates of the size of the *Acropora* genome are 1-4 x 10^8 bp (Miller, unpublished). Each pool was maintained as a glycerol stock and large-scale plasmid preparations were prepared for each pool using Qiagen Maxiprep Kits.

2.8.6. Verification of activator-dependent interactions

Identification of DNA-binding sites of the Emx-Am homeodomain was carried out as described in Liu et al. (1993). The yeast-one hybrid system utilises two plasmids: an activator plasmid expressing the DNA-binding domain of interest fused to the yeast Gal4 protein and carrying a *URA3* selectable marker gene; and a reporter plasmid containing an inactive *HIS3* gene, lacking its UAS element, and a *TRP1* selectable marker. If the DNA fragments inserted upstream of this reporter gene contain binding sites for the DNA-binding protein, this provides UAS function and activates *HIS3* expression. Therefore, a DNA fragment that interacts with the DNA-binding domain can be selected from a library of genomic fragments cloned upstream of the *HIS3* gene.

Yeast cells (strain YM4271), transformed with the activator plasmid were selected on media lacking uracil, and these were then transformed with a reporter library. 10% of each transformation mixture was plated on media lacking uracil and tryptophan to determine the number of reporter plasmids screened, while the remaining 90% was screened for His+ transformants containing plasmids carrying DNA binding sites for the protein of interest. Only a small proportion of His+ colonies contain plasmids with binding sites for the protein encoded by the activator plasmid; the majority of transformants generally contain plasmids with binding sites for endogenous yeast transcription factors. A second screening step is then also required to identify rare transformants with activator-dependent *HIS3* expression.

If *HIS3* expression is activator-dependent, colonies will become His- if the activator plasmid is lost. Two methods were employed to detect the loss of the activator plasmid and, therefore, if *HIS3* expression is activator-independent. The first of these employs the *ADE5* gene on the activator plasmid, which causes *ade2 ade5* colonies to be red on media lacking histidine, uracil and tryptophan. Plasmid loss was identified if white sectoring was observed on red colonies (colonies required the activator plasmid for *HIS3* expression remain red). The second method is based on the toxicity of 5'-fluoroorotic acid (5-FOA) to cells carrying the URA3 gene, which is present in the activator plasmid. Yeast, which grow on media lacking histidine, uracil and tryptophan, are replica plated onto the same media, which also contains 5-FOA. Activator-dependent colonies are those that cannot grow on –His media in the presence of 5-FOA.

2.8.7. Isolation of plasmid DNA from yeast cells

Plasmid DNA was isolated from yeast cellular lysate as described in Hoffman and Winston (1987). Small-scale overnight cultures of yeast cells grown in appropriate media were harvested by centrifugation and resuspended in a small volume of culture media. 200 µl of lysis buffer was then added, followed by 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 g of acid-washed glass beads (0.45-0.52 mm). Before use, the glass beads were soaked in nitric acid for 1 h, rinsed well and baked dry. The mixture was vortexed for 2 min and spun for 5 min at 14,000 g. The aqueous layer was then used to transform competent *E. coli* cells (no more than 5 µl of solution per transformation).

Table 2.10. Media and solutions associated with yeast manipulation methods.

100X amino acid stock solution	0.2 g arginine	0.1 g methionine
	0.6 g isoleucine	0.5 g threonine
	0.4 g lysine	0.6 g phenylalanine
	Dissolve in 100 ml ddH_2O, filter sterilised using 0.2 µM filter	
500X Uracil Stock	1 g uracil in 100 ml 0.1 M sodium hydroxide, filter sterilise	
500X Adenine Stock	1 g adenine in 100 ml 0.1 M sodium hydroxide, filter sterilise	
500X Histidine Stock	1 g L-histidine in 100 ml ddH_2O, filter sterilise	
500X Tryptophan Stock	1 g L-tryptophan in 100 ml ddH_2O, filter sterilise	
400X Leucine Stock	1.2 g L-leucine in 100 ml ddH_2O, filter sterilise	
Lysis Buffer	2% Triton-X; 1% SDS; 100 mM NaCl; 10 mM Tris pH 8.0; 1 mM EDTA	
YPD liquid media (for routine growth)	(per litre) 10 g yeast extract; 20 g peptone; 20 g dextrose (glucose); ddH_2O to 1000 ml	
YPD Agar	YPD liquid media; 1.5-2.0% agar	
SD liquid media (A synthetic minimal media for selective	(per litre) 1.7 g yeast nitrogen bases without amino acids or ammonium sulphate; 5.0 g ammonium sulphate; 20 g dextrose (glucose); ddH_2O to 1000 ml. It must also be supplemented	

media for selective growth)	with amino acids not produced by yeast strains or provided by transforming plasmids.
SD Agar	SD minimal liquid media; 2.0% agar
TEL Buffer	10 mM Tris, pH 8.0; 1 mM EDTA; 100 mM Lithium Acetate
40% PEG/TEL Solution	10 mM Tris, pH 7.5 or 8.0; 100 mM Lithium Acetate; 40% (w/v) polyethylene glycol 4000