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Investigations on the nucleic acids coding for the sea anemone toxins, anthopleurins A and B

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INVESTIGATIONS ON THE NUCLEIC ACIDS CODING FOR THE SEA ANEMONE TOXINS, ANTHOPLEURINS A AND B

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ABSTRACT

Anthopleurin B (AP-B) is a 49 amino acid cardiotonic polypeptide produced by the giant green sea anemone, *Anthopleura xanthogrammica.* Along with AP-B, another polypeptide, anthopleurin A (AP-A), of the same length, and differing only in 7 amino acids, is produced by the animal in higher quantities. The object of this study was to investigate the the two peptides at the nucleic acid level.

Genomic and cDNA libraries of the animal were constructed in λ replacement vectors. The genomic library was constructed in the *Bam* HI site of EMBL3, while the cDNA library was constructed in the unique *Eco* RI site of λ gt10.

Synthetic nucleotides spanning the first 10 amino acids of AP-B and the complement of the codons for the last 10 amino acids of the AP-A peptides were constructed from back translation of the peptides' sequences. The primers were used to initiate a polymerase chain reaction, using cDNA as the template. A 144-147 base pair PCR product was obtained.

The PCR product was purified from the primers and used to probe the genomic DNA and to screen both the genomic and cDNA libraries. Positive clones were isolated from both libraries.

A 1.8 kb *Bam* HI genomic fragment common to 5 of the 14 genomic clones examined, and a 2.8 kb *Eco*RI genomic fragment common to 3 clones, that hybridized both to the PCR product and the primers were subcloned into M13mp18.

The PCR product was cloned into the phagemid vector, pBluescript KSII+. The sequence of a random clone showed some sequence homology with the ambiguous sequence predicted for AP-A and AP-B.

The cDNA clones were cut with two enzymes flanking the *Eco* RI site of the cloning vector and a 480 base pair fragment was subcloned into M13mp18.

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LIST OF ABBREVIATIONS

AMV	Avian myeloblastosis virus
cDNA	complementary or copy DNA
dATP	deoxyadenosine 5'-triphospahate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanidine 5'-triphosphate
DNA	deoxyribonucleic acid
dT	deoxyribothymidilic acid
dTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
EDTA	ethylene-diamine tetraacetic acid
g	grams
IPTG	Isopropyl-β-D-galactopyranoside
l	liter
µg	micrograms
µl	microliters
ml	milliliters
mV	millivolts
PCR	Polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
SDS	Sodium dodecyl sulfate
SM	Storage medium
SSC	Saline sodium citrate
TAE	Tris Acetate EDTA

TBE	Tris Borate EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-β–D-thiogalactopyranoside
℃	degree Centigrade

I. INTRODUCTION

Several species of marine organisms produce a variety of biologically active peptides. In general, these peptides are toxic; the most potent include brevetoxins, maitotoxin, paragracine or the palytoxin. Aside from the fact that they pose a serious threat to public health, these toxins and the other novel toxins have proven to be invaluable tools in studying the molecular details of ion channels and receptors of excitable cells because of their high binding affinities to ion channels. However, a more exciting possibility in the study of these toxins is their potential to be developed into pharmacologically useful drugs. The latter possibility entails thorough testing before it becomes a reality and a basic understanding of the mode of actions of these toxins needs to be achieved. The techniques of recombinant DNA technology offers the possibility of looking at these toxins at the molecular level. This review will cover briefly marine organisms, with particular emphasis on the novel polypeptides anthopleurins A (AP-A) and B (AP-B). These toxins are produced by the sea anemone, *Anthopleura xanthogrammica*. This study is aimed at investigating the molecular genetics of the sea anemone toxins A and B

The sea anemone, *Anthopleura xanthogrammica* (Brandt) produces two polypeptides, Anthopleurins A (AP-A) and B (AP-B), which are neurotoxic at high levels but exhibit a positively inotropic effect at lower levels. Of the two, AP-A has been well characterized. Anthopleurin A has been shown to have a positive

inotropic effect on the hearts of dogs (Blair *et al.*, 1978), guinea pigs (Hashimoto *et al.*, 1980) and mice (Shibata *et al.*, 1976). A potent cardiac stimulant, AP-A can potentially be used in the treatment of heart disease. In the study of cardiac muscle physiology it is useful because it is more potent than either ouabain or glucagon (Low *et al.*, 1979), and its inotropic action is not accompanied by chronotropic or hypertensive effects and it is relatively hypoxia resistant. The inotropic effect of AP-A is not inhibited by propranolol pretreatment (Shibata *et al.*, 1976). In addition to its inotropic activity, AP-A, binds to the voltage sensitive sodium channel present in the membranes of nerve, skeletal and heart cells. Detailed investigations of the excitable membranes of giant crayfish axons revealed that AP-A binds to an external site of the sodium channel and prolongs the inactivation process. The site of binding of AP-A is distinct from the tetrodotoxin binding site, where activation of the sodium channel is effected. Hence, there are two main reasons to explore these polypeptides: as a tool to investigate the mechanism of the sodium channel inactivation, and as a potential substitute for the inotropic agents currently being used in treating heart disorders. Anthopleurin B differs from AP-A in only seven of 49 amino acid residues. These substitutions confer on AP-B ten times higher inotropic activity than AP-A. Because of their potential to be developed as pharmacologically useful drugs, and because they can be used to probe the sodium channel, it was deemed important to study them at the molecular level.

II. REVIEW OF LITERATURE

A. Toxins from marine organisms

Tetrodotoxin is a heterocyclic guanidine originally isolated from the ovaries and liver of puffer fish (Brown and Mosher, 1963), but has also been found in newt (Crone *et al.*, 1976), octopus (Sheumack *et al.*, 1978), frog (Kim *et al.*, 1975), and goby (Noguchi and Hashimoto, 1973).

Saxitoxin, like tetrodotoxin, is a heterocyclic guanidine, produced by the dinoflagellates of the genus *Gonyaulax*. It is found in large concentrations in clams, mussels and other shellfish that feed on the dinoflagellates (Caterrall, 1986).

The unarmored dinoflagellate *Ptychodiscus brevis*, produces eight toxins collectively known as brevetoxins (Shimizu, 1982). Another dinoflagellate *Gambierdiscus toxicus* produces three types of toxins: ciguatoxin, scaritoxin and maitotoxin, which have been established as the cause of ciguaterra poisoning (Legrand and Bagnis, 1984). Although the three toxins are produced by the same organism, much more maitotoxin is secreted than the other two. Other marine organisms producing toxins are coelenterates: *Parazoanthus gracillis* (paragracine), *Palythoa* (palytoxins); marine snails of the genus *Conus* (conotoxins); and sea anemones (toxins I, II, III, and IV, anthopleurins A, B and C).

1. Mechanism of action of peptide toxins from marine organisms

It has been shown that tetrodotoxin at very low concentrations (10^{-7} M) selectively blocks the increase in sodium permeability caused by depolarization, but does not affect the ion permeability of the unstimulated axon, nor the delayed increase of potassium permeability caused by depolarization (Narahashi *et al.*, 1964).

Saxitoxin acts similarly to tetrodotoxin, in that it blocks action potentials without depolarization (Kao and Nishiyama, 1965). Kao and Nishiyama (1965) first proposed that the guanidinium moieties of these two toxins might enter the sodium channels like guanidine, but due to their bulkiness, they may bind tightly to the ion channel and in effect block the passage of the Na^+ ions. Henderson *et al.* (1974) extended the hypothesis of the previous authors by proposing that the receptor site for the toxins was a specific coordination site in the sodium channel. Similarly, Hille (1975) proposed that the receptor site was the ion selectivity filter, a coordination site that determines the ion selectivity of the channel.

Studies utilizing radiolabeled tetrodotoxin and saxitoxin have formed most of the current findings on the nature of the sodium channel. The finding that each of the two toxins inhibit the binding of the other provided strong evidence that they bind the same receptor (Colquohoun *et al.*, 1972; Henderson *et al.*, 1973; Barnola *et al.*, 1972).

Brevetoxins depolarize nerve and muscle membranes in a dose dependent manner (Huang *et al.*, 1984; Wu *et al.*, 1985). The extent of maximum depolarization is about 40 mV, and on crayfish giant axons the EC_{50} was reported to be 1.7 nM (Wu *et al.*, 1985). Voltage clamp experiments showed that the target of action of brevetoxins is the sodium channel. The modified sodium channel exhibits the following three characteristics: (1) the channel modified is activated at membrane potentials ranging from -160 to -180 mV, levels at which the sodium channel is normally closed, (2) the channel is activated very slowly (375-127 msec in the potential range of -80 to +10 mV) compared to that of the normal sodium channel, and (3) the channel is essentially devoid of fast inactivation (Huang and Wu, 1985). Hence, the toxins depolarize the membrane by removing the inactivation process after opening the sodium channels at potential where it is normally closed.

Ciguatoxin, at very low concentrations (0.2 -1.0 nM), induces a membrane depolarization and spontaneous action potentials in neuroblastoma cells and in frogs' nodes of Ranvier (Legrand *et al.*, 1985). Ciguatoxin modifies the sodium channel in a manner similar to that of the brevetoxins. Maitotoxin, on the other hand, causes a calcium dependent contraction of skeletal and smooth muscles. In cardiac muscles, it has a positive inotropic effect at low concentrations (0.1 to 4 ng/ml), which is completely eliminated by Co^{2+} or verapamil (Kobayashi *et al.*, 1985; Kobayashi *et al.*, 1986). Maitotoxin induces (1) an increased release of norepinephrine and dopamine from rat pheochromocytoma cells

(Takahashi *et al.*, 1982), and (2) a high uptake of Ca^{2+} by some cultured cell lines. Both effects can be inhibited by blockers of the sodium channel (Kobayashi *et al.*, 1983). These results suggest that the toxin induces an increase in the permeability of the cell membrane to calcium, which in turn triggers the release of transmitters and muscle contraction.

Paragracine is one of the troponoid substances secreted by the coelenterate species *Parazoanthus*, which are collectively known as zooxanthins. This toxin blocks the sodium channel without affecting the potassium channel, based on the observation that blockers of the sodium channel had no effect on its action (Seyama *et al.*, 1980). Paragracine blocks the sodium channels only on the axoplasmic side, and does so only when the sodium ions are flowing unidirectionally in the outward direction. This blockage of the sodium channel by brevetoxin can be alleviated if an inward sodium current is generated (Seyama *et al.*, 1980).

Palytoxin, produced by the coelenterate genus *Palythoa*, is the most lethal marine toxin known to date. When administered intraperitoneally, the LD_{50} for mice is 50-100 ng toxin/kg weight (Wiles *et al.*, 1974). Palytoxin depolarizes excitable tissues including cardiac muscle (Alsen *et al.*, 1982; Ito *et al.*, 1979; Wiedman, 1977), skeletal muscle (Ito *et al.*, 1979; Ohizumi and Shibata, 1980) and both myelinated and unmyelinated nerve fibers (Muramatsu *et al.*, 1984; Pichon, 1982). Based on the observations that the action of palytoxin is potentiated by ATP, and that dog erythrocytes which lack Na, K-ATPase are resistant

to the action of palytoxin, Habermann (1983), Habermann *et al.*, (1982), and Chattwal *et al.* (1983) have proposed that palytoxin interacts with the Na, K-ATPase and converts the pump into an ion channel.

To date, there have been twelve toxins isolated from marine snails of the genus *Conus*. In general, the conotoxins are neurotoxins. Kobayashi *et al.* (1982) surveyed 29 species of toxins and found that the conotoxins exhibit a variety of actions such as : (1) blocking acetylcholine receptors, (2) blocking muscle Na channels, (3) blocking calcium channels, (4) opening Na channels, (5) increasing Na permeability, (6) increasing calcium influx, or (7) contracting rabbit aorta. The above actions are specific for each toxin. Only toxins isolated from *Conus geographus* (conotoxin GI, GII, GIIIA and geographutoxin), *Conus magus* (conotoxin MI) and *Conus striatus* (striatoxin) pose serious threats to humans.

2. Biological importance of the peptide toxins

As mentioned earlier, the most potent toxins are serious threats to public health. For example, the catastrophic event of the red tide in the gulf of Mexico in 1946-1947 which resulted in tons of dead fish littering the coast of Florida was caused by brevetoxins. Fish poisoning in the Carribean, the South Pacifc and even in the United States , has been traced to the ciguatoxins produced by the dinoflagellates that enter the food chain via the fishes that feed on them.

Aside from the above major reasons, some of the toxins so far identified have been used as molecular tools in studying the neu-

romuscular system, because of their high binding affinity to excitable membranes. For example, alpha bungarotoxin has been useful in studying the acetylcholine receptors. Similarly, tetrodotoxin and scorpion toxins have been useful in studying the sodium channel. In the case of the novel peptides, omega conotoxin has been utilized to study the presynaptic terminal of excitable membranes. Hence, the toxins from marine organisms could provide a wealth of information on the nature and physiology of excitable membranes (Shibata *et al.*, 1976).

B. Polypeptide toxins from sea anemones

Sea anemones synthesize polypeptides which display a wide variety of biological activities, such as cardiotoxicity, cardiostimulation, cytolysis, and proteinase inhibition (Alsen, 1983). These polypeptides are classified according to their molecular weights. They are: (1) class I, <3,000 ; (2) class II, 4,000-6,000; (3) class III, 6,000-7,000; and (4) class IV, > 10,000. Classes I and II act on excitable membranes, mainly on the fast sodium channel, class III inhibits proteinase activity, and class IV exhibits mainly cytolytic activity. At present, there have been 23 such toxins isolated. Of these, those belonging to class II have elicited much interest because they exhibit actions such as cardiostimulation, cardiotoxicity, and neurotoxicity in mammals (Norton *et al.*, 1978; Beress, 1978;1982; Schweitz *et al.*, 1981). Included in this class are anthopleurins A (AP-A), and B (AP-B) from *Anthopleura xanthogrammica*, anthopleurin C (AP-C) from *Anthopleura elegantis-*

sima. and toxins I and II from *Anemonia sulcata*. The pharmacological properties of these peptides such as LD 50 on mice and crab, their binding to synaptosomes and stimulation of Na^+ uptake by neuroblastoma cells have been determined by Schweitz *et al.* (1981).

These peptides function as heart stimulants and are very potent positive inotropic agents. They increase the force of heart contraction without affecting the heart rate or blood pressure, and they are neurotoxins at somewhat higher concentrations (Norton *et al.*, 1978; Shibata *et al.*, 1976). Their mode of action has been attributed to binding to the gate of the fast sodium channel, thus causing a delay in inactivation of this channel and a prolongation of the action potential (Romey *et al.*, 1976; Kodama *et al.*, 1981). The inotropic activity has also been attributed to the peptides' binding to and affecting the slow (calcium/potassium) channel (Ohizumi and Shibata, 1981; De Barry *et al.*, 1977).

1. Primary structure of the polypeptide toxins

The amino acid sequence of AP-A (Tanaka *et al.*, 1977) and AP-B (Reimer *et al.*, 1985) have been determined. AP-C (Beress, 1978), toxin I (Wunderer and Eulitz, 1978) and toxin II (Wunderer *et al.*, 1976) primary sequences have also been reported. All of these toxins are short peptides between 46 and 49 amino acids, with considerable sequence homology. A variety of spectral techniques including NMR have been used to study the secondary structure of AP-A (Ishizaki *et al.*, 1979; Norton and Norton, 1979), toxin I (Norton *et al.*, 1980), and toxin II (Norton

et al., 1976). AP-A has been crystallized and preliminary diffraction data has been reported (Smith *et al.*, 1984). Conformational studies point to a random coil secondary structure for toxin II, while a more globular structure for that of AP-A.

Anthopleurin B, the most potent peptide heart stimulant from the sea anemone *Anthopleura xanthogrammica*, behaves anomalously on Sephadex gel filtration columns and elutes at a position indicating a molecular weight of about 2,400 (Reimer *et al.*, 1985). However, sequence determination showed that AP-B consists of a single polypeptide chain of 49 amino acids with a molecular weight of 5,257. The sequence of the peptide was shown to be: Gly-Val-Pro-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Arg-Pro-Arg-Gly-Asn-Thr-Leu-Ser-Gly-Ileu-Leu-Trp-Phe-Tyr-Pro-Ser-Gly-Cys-Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-Ala-His-Gly-Pro-Asn-Ile-Gly-Trp-Cys-Cys-Lys-Lys. Although six carboxymethyl cysteine residues were formed by reduction and alkylation of the polypeptide, no cysteine residues were detected in the native peptide indicating that there are three cystine bonds in AP-B (Reimer *et al.*, 1985). The positions of the disulfide bonds were chemically determined for toxin II (Barhanin *et al.*, 1981), but until the present time, those cystine bonds have not been localized for either AP-A or AP-B.

2. Mechanism of action of the anthopleurin polypeptide toxins

The cardiac stimulatory action of coelenterate toxins on rat atria was first reported by Shibata *et al.*, 1974, while studying the antitumor activity of the extracts from the sea animals. Most

notable was the finding that extracts from the sea anemone *Anthopleura xanthogrammica* gave the highest percent increase in inotropic activity of rat hearts, at concentrations as low as 119 parts per million (ppm). Norton *et al.* (1976) isolated the active peptide from the *Anthopleura xanthogrammica* and named it anthopleurin. The presence of two kinds of peptides from the ethanol extract from these marine invertebrates was apparent on the elution profile of the cation exchange column. The fraction that eluted at Ve/Vo (ratio of eluted volume to void volume) of 2.99-3.39 they termed AP-A, and the fraction that eluted at Ve/Vo of 6.3 to 7.01, they called AP-B.

Shibata *et al.* (1976) investigated the mechanism of the positive inotropic action of AP-A on the hearts of mongrel dogs, rabbits, rats, cats and guinea pigs. They found that the cardiotonic potency of AP-A was greater than that of glucagon and ouabain and equal to that of isoproterenol, but without an accompanying chronotropic effect on the isolated cardiac muscles of the animals examined. In addition, the cardiac contractility of the heart muscles became more resistant to the effects of hypoxia and temperature stress after pretreatment with AP-A. This latter effect was better in AP-A treated than in ouabain treated cardiac muscles.

Since cardiac glycosides, such as digitalis and ouabain increase the contractility of the heart muscle by inhibiting the Na,K-ATPase pump, Shibata *et al.* (1976) determined whether AP-A also induces positive inotropy by the same mechanism. The results from studies utilizing assays for Na,K -ATPase activity and

cAMP hydrolysis showed that neither the ATPase activity nor phosphodiesterase activity was affected by AP-A. An interesting observation was that the peptide prolonged the action potential in guinea pig atria and ventricles, without affecting all other electrical properties of membranes of the cardiac muscle (Shibata *et al.*, 1976). Blair *et al.* (1978) studied the effect of AP-A on cardiac dynamics in conscious dogs and found that AP-A does not alter mean arterial pressure, mean left atrial pressure and left ventricular end systolic diameter. They concluded that AP-A has a direct inotropic effect on the myocardium with little or no effect at all on the peripheral circulation. Hence AP-A is potentially a promising substitute for the current cardiac glycosides being used to treat heart disorders.

The other property of AP-A is its ability to prolong the action potential in excitable membranes which contain voltage gated sodium channels. Voltage clamp experiments showed that AP-A prolongs the action potential on crayfish giant axons Low *et al.*, (1979). Treatment of the axons with as little as 10^{-8} M AP-A resulted in membrane polarization and depolarization, and in repetitive firing. The prominent plateau generated on the action potential lasted up to 300 milliseconds (msec), an extension of about seven times that of the untreated controls.

Like the skeletal muscle membranes and axons from giant crayfish, cardiac muscle cell membranes contain voltage gated sodium channels. This voltage gated sodium channel is responsible for the increased permeability to sodium ions

following membrane depolarization (Ganong, 1985). The cardiac muscle cells differ from the other two, in that its membrane also contains a voltage gated calcium channel, which is responsible for prolonged repolarization. Shimizu *et al.* (1979) reported that, upon exposure to AP-A (3 x 10^{-8} g/ml) guinea pig ventricular muscle exhibited the following characteristics: 1. prolonged action potential, 2. increased tension and 3. no significant change in the maximum voltage change per unit time (dV/dT). In order to relate the mechanism of the prolongation of the action potential to the positive inotropic effect of AP-A, Hashimoto *et al.* (1980) investigated the ionic mechanism of the prolongation of action potential in isolated guinea pig ventricular muscle. They found that the prolongation of action potential by AP-A was accompanied by a decreased rate of outward current. The decreased rate of outward current could have been due to blockage of the slow inward Ca current or to a delayed outward potassium current, or to a delay in the closure of the sodium channel. Hashimoto *et al.* (1980) found that delayed increase in potassium permeability was not affected, since treatment of the ventricular muscles with blockers of the K channel had no effect on the prolonged action potential. AP-A did not alter the slow Ca channel since treatment of rabbit ventricular muscle with Verapamil, a blocker of the slow Ca channel, did not inhibit the effect of AP-A (Kodama *et al.*, 1981). In the presence of ryanodine, an agent which is known to interfere with Ca release from the sarcoplasmic reticulum, AP-A prolonged the action potential,

but failed to cause a positive inotropic effect. Hence, the increase in contractile force is probably an outcome of the alteration of the Ca kinetics by the prolonged action potential.

The other polypeptide which can be isolated from *Anthopleura xanthogrammica* is Anthopleurin B (AP-B), which can be separated from AP-A by ion exchange chromatography. AP-B is the more potent toxic polypeptide of the two.

On isolated, intraarterially perfused, spinal cord of the bullfrog, *Rana catesbiana*, AP-B caused a marked augmentation of the ventral and dorsal root potentials produced by the stimulation of the dorsal root (Kudo and Shibata, 1980). It also prolonged the excitatory post synaptic potential (EPSP), and elevated the membrane resistance, without affecting the motoneuronal action potentials. The excitatory action of AP-B was greater in low Ca^{2+} (0.9 mM) than in high Ca^{2+} media (3.6 mM). Based on these observations, Kudo and Shibata (1980) proposed that AP-B acts on subsynaptic membranes to make it more sensitive to neurotransmitters.

Ohizumi and Shibata (1981) tested AP-B on guinea pig's isolated ileum and isolated taenia caeci, in order to determine the effect of AP-B on the autonomic nervous system and the smooth muscle. In both taenia caeci and ileum, AP-B induced contraction followed by relaxation in concentrations as low as $3x10^{-9}$ M. The other sea anemone toxins effected similar actions but at much higher concentrations (10^{-7} M or higher). The AP-B induced responses were inhibited by tetrodotoxin or low Na^{+} in the medium,

suggesting that AP-B causes membrane depolarization by increasing the Na^+ permeability across cell membranes.

Contractions induced by AP-B on both the ileum and the taenia caeci were inhibited by muscarinic blockers, but not by nicotinic blockers, blockers of histamine and tryptamine release, nor by blockers of prostaglandin synthesis. This suggests that contractions on both tissues induced by AP-B were mainly mediated through endogenous acetylcholine released from cholinergic nerve terminals (Ohizumi and Shibata, 1981). In contrast, relaxation was different between the two tissues examined. In taenia caeci, relaxation was inhibited by guanethidine and phentolamine. These same agents had no effect on the relaxation induced by AP-B on the ileum. This observation suggests that in the spinal cord, the rhythmic relaxation maybe due to the release of noradrenaline, via the excitation of adrenergic nerves. On the other hand, in the smooth muscles of the mammalian gastrointestines, relaxation could be due to the excitation of adrenergic or non-adrenergic inhibitory nerves. It would appear that in this case, AP-B induced relaxation of the ileum was due to excitation of the latter.

In order to further examine the mode of action of AP-B on smooth muscles, Norton *et al.* (1981) tested its excitatory effect on guinea pig vas deferens. This study was prompted by the hypotheses concerning the nature of motor transmission in vas deferens: the presence of both adrenergic and non-adrenergic components. Norton *et al.* (1981) found that at concentrations as low as

$3x10^{-9}$ M, AP-B caused rhythmic contractions on guinea pig vas deferens. Except for toxin II, a peptide with considerable sequence homology with AP-B, the other sea anemone toxins also caused contractions but only in higher concentrations ($>5x10^{-8}$ M). A more important finding, however, was that at concentrations between 10^{-8} and 10^{-5} M, AP-B caused a dose dependent release of noradrenaline in the surrounding medium. At the higher limit, (10^{-5} M) AP-B caused increased release of noradrenaline 310 x more than that of the untreated vas deferens. Both contraction and adrenaline release induced by AP-B were inhibited by the absence of Ca^{2+}. These results suggest that the AP-B induced contraction is mediated by noradrenaline release from the adrenergic nerve endings, and that the release of noradrenaline induced by AP-B may require extracellular Ca^{2+}.

The heart stimulant activity of AP-B is over ten times higher than that of AP-A (Norton *et al.*, 1978). Therefore it is of interest to compare the sequences of these peptide heart stimulants in order to understand the chemical basis of their activity. There are only 7 amino acid differences between AP-B and AP-A, 5 of which are likely candidates for activity differences. The net charge increase at neutral pH from +3 to +5 between AP-A and AP-B may be significant as has been pointed out for the activity of the other sea anemone toxins on mammals (Schweitz *et al.*, 1981). Anthopleurin C from *Anthopleura elegantissima* and toxin II from *Anemonia sulcata* differ from AP-A and AP-B in that they consist of 47 amino acid residues. However, this shortening of the

polypeptide chain does not appear to significantly affect the heart stimulant activity since AP-C has about the same heart stimulant activity as AP-A. The following observations summarize the amino acid sequence information between AP-B and related toxins.

The amino acid sequence differs in 7 places between AP-A and AP-B. These are residues 3 (P for S), 12 (R for S), 13 (P for V), 21 (I for T), 24 (F for L) 42 (N for T), and 49 (K for Q). These differences are important since AP-B is a better heart stimulant than AP-A, even when they are produced by the same organism. Similarly, AP-B is more potent than AP-C from *Anthopleura elegantissima*, or toxin II from *Anemonia sulcata*. Since Pro (residue 3) and Ile (residue 21) are also present in AP-C, the presence of these changes in AP-B may not confer the increased potency of AP-B. This observation leads to consideration of the remaining amino acid changes from AP-A, in residues 12, 13, 24, 42 and 49.

The stability of a particular conformation of an anthopleurin toxin through primary structure factors is related to its binding and the effect of binding on the membrane receptor. Barhanin *et al.* (1981) has proposed a two step model for the activity of toxins from sea anemone. The first step in the model is the binding of the polypeptide to its receptor. The second step is proposed to be a conformational transition required to bring about the activity responsible for the cellular changes observed at the neuromuscular junctions, namely, the delay in closure of the sodium channel by stabilizing the open conformation. The

possible effects of the differences in amino acid sequence between AP-A and AP-B can be evaluated from the perspective of possible effects on the two steps. If we know more about the polypeptides at the nucleic acid level, for AP-B these differences could be exaggerated in designing changes in the amino acid sequence, with the ultimate objective of increasing the inotropic activity of the peptide, as well as reducing its antigenicity.

In studies of toxin II, the only ionizable group shown to be essential for both binding and activity was the single Arg residue at position 14 (Barhanin *et al.*, 1981). AP-B is the only toxin from sea anemone sequenced to date, which has two Arg residues in this portion of the polypeptide. Furthermore, this portion of the polypeptide seems to be the site of antigenic determinant, since there are 4 hydrophilic charged amino acid residues within an eight amino acid stretch (residues 7-14). Hopp and Woods (1981) used a computer program to predict the most antigenic portion of a polypeptide and concluded that the point of highest local average hydrophilicity is invariably located in or adjacent to the antigenic determinant site. Since aspartic acid, glutamic acid and arginine are given the highest hydrophilicity values, it is very likely that in AP-B, the antigenic determinant site is within residues 7-14.

In AP-A, both binding and activity seems to depend on Arg-14. Assuming that this is also true for AP-B, Arg-12 is a good candidate for site directed mutagenesis. Asp-7 and Asp-9 are also good candidates in decreasing the antigenicity of the peptide.

However, as will be discussed shortly, the carboxyl groups on the side chains of these residues may be important in carrying out AP-B's inotropic action.

It is interesting to note that four of the seven substitutions from AP-A to AP-B involve replacement of hydroxyl groups with residues that can not donate a hydrogen bond through their side chains (3, Ser to Pro ; 12, Ser to Arg ; 21, Thr to Ile; and 42, Thr to Asn). It seems likely that these changes , if significant, would affect the second step (conformational change) possibly by destabilizing the inactive conformation through the loss of intramolecular hydrogen bonding potential.

The C-terminal substitution of positive lysine for glutamine could be significant in binding to the receptor or possibly may affect the pK for Asp-7 or Asp-9 beta carboxyl groups , one of which was found to be unusually low in toxin I, toxin II, and AP-A (Wunderer and Eulitz, 1978; Ishizaki *et al.*, 1979; Norton and Norton, 1979). Two of the aspartyl carboxyl groups of AP-A has been shown to be essential for its cardiotonic activity in rat atria. If the pK value of one the carboxyl groups is lower because of its proximity to Lys-48 in AP-A (Lys-46 in toxin II), then the additional epsilon amino group from Lys-49 in AP-B could possibly lower the pK even more. The pK of that carboxyl group may be important for its activity because of its involvement in the conformational transition of the molecule, which occurs concomitant with the protonation of that carboxyl group (Wunderer and Eulitz,

1978; Ishizaki *et al.*, 1979; Norton and Norton, 1979), at about pH 2.

There is a slight increase in the hydrophobicity of AP-B over AP-A (34.7 % hydrophobic residues for AP-B versus 30.6 % for AP-A). A change of Phe for Leu at position 24 would not be expected to change the hydrophobic character of that region substantially. However, it has been shown that for toxin II, the two Trp residues (23 and 31) interact through pi bonding (Ishizaki *et al.*, 1979; Norton and Norton, 1979). This interaction is apparently broken during the low pH induced conformational change. These residues are also present in AP-B (23 and 33). The third Trp residue however (residue 45 in both AP-A and AP-B) becomes less random upon the changes in conformation. Such constraint maybe strengthened by the presence of an additional aromatic residue in AP-B (Phe-24).]

C. Importance of isolation and characterization of the gene for AP-B

Matsueda and Norton (1982) have shown that the synthesis of AP-A is possible through the Merrifield synthesis from t-boc amino acids. They also found that disulfides will form in the synthetic peptide. However, relative to native AP-A, the synthetic peptide has only 30 % inotropic activity. Hence it is possible to evaluate the relationship of binding and changes in amino acid structure via chemical means. This approach to the problem, however, requires long term commitment, in addition to large

expenses commonly encountered every time a natural product is synthesized chemically. The other alternative is the use of molecular techniques to isolate and translate the gene, confer changes in the amino acid structure via site directed mutagenesis, and then compare the activity of the native peptide and the synthetic peptides. This may lead to the development of peptides with more desirable properties.

The small size of these peptides along with the availability of the amino acid and activity data make them particularly attractive models for protein engineering. However, the first step to be accomplished is the isolation and characterization of the gene. The availability of the gene encoding AP-B will pave the way to single and multiple amino acid changes in the peptide by *in vitro* mutagenesis techniques. This technique involves the chemical synthesis of small sections of DNA (oligonucleotides) which are complementary to sections of the gene but, with alterations , which will result in the synthesis of a peptide whose amino acid sequence is different from that of the native peptide. These altered peptides when compared to the native peptide will reveal how certain changes in the amino acid composition will affect properties such as inotropic activity, toxicity and antigenicity. It will also allow an increased understanding not only of these molecules, but also of the sodium channel in its various electrically sensitive conformations. This will certainly provide a revolutionary approach to the neurobiology of the sodium channel. Furthermore, generation of altered peptides with

more desirable characteristics will bring them closer towards their use as therapeutic heart stimulants.

III. OBJECTIVE OF THE STUDY

Until the present time, there has been no nucleic acid studies on the anthopleurins. If these peptides are to be utilized for their pharmacological properties, as well as one of the tools to probe the sodium channel, sufficient quantities of the protein should be readily obtainable. The first step towards this goal is to have a cloned gene that can be expressed in the laboratory.

This study was conducted to investigate the sea anemone toxins, anthopleurins a and b at the nucleic acid level.

IV. MATERIALS AND METHODS

A. Biochemicals

Acrylamide, agarose, bisacrylamide, ethidium bromide, heparin, IPTG, phenol, spermidine, TEMED, Tris, and Xgal were purchased from Boehringer Mannheim, Indianapolis, Indiana.

Ammonium chloride, ammonium persulfate, ampicillin, EDTA, magnesium chloride, ficoll, glucose, magnesium sulfate, maltose, polyvinylpyrrolidone, sodium chloride, sodium pyrophosphate, and vitamin B1 were purchased from Sigma Chemical Corporation, St. Louis, Mo.

Acetic acid, n-butanol, calcium chloride, ethyl alcohol, hexane, hydrochloric acid, sarcosyl, Scintiverse cocktail mix and sodium dodecyl sulfate were purchased from Fisher Scientific.

Bacto agar, bacto tryptone, and bacto yeast extract, which are all products of Difco laboratories were purchased from Fisher Chemicals.

The oligo dT column was purchased from Collaborative Research Inc, Bedford, Ma.

Nytran and Elutip D were purchased from Sleicher and Schuell, Keene, New Hampshire.

The Eco RI adaptors and the spin columns were purchased from Promega Corporation, Madison Wisconsin.

[α-^{32}P]-dCTP and [α-^{35}S]-dATP were purchased from New England Nuclear.

B. Bacterial Strains

DH5α and JM109 were gifts from Dr. Charles Romeo.

DH5α is an *E. coli strain* that has the genotype *supE44, hsd R17, recA1, endA1, gyrA 96, thi-1, relA1,* is a recombination deficient suppressing strain and was used to propagate plasmids.

JM109 is a *recA1, supE44, end A1, hsd R17, gyrA96, relA1, thi, Δ (lac pro AB), F'[traD36 proA$^+$B$^+$ lacI^q lacZ Δ M15]* is a strain that will support growth of vectors carrying amber mutations, and will modify but will not restrict transfected DNA. This strain is recombination deficient and was used to propagate M13

C600 and C600 hfl A were purchased from Promega Corporation and were used to select λgt10 recombinants.

C600 has the genotype *supE44, hsdR, thi-1, thr-1, leuB6, lacY 1, tonA21.* C600 hfl A has the genotype *supE44, hsd R, thi-1, thr-1, leuB6, lacY 1, tonA21, hflA 150 [chr::Tn 10 (tet^r)].* The latter strain distinguishes between cI$^+$ (parent λ gt10) and cI$^-$ (recombinant). Insertion of a DNA fragment into the single Eco RI phage repressor gene generates a cI$^-$ phage which forms plaques with clear center when plated on C600. On the other hand, nonrecombinants form a turbid plaque on this strain. When plated on C600 hflA, the cI$^+$ phage is repressed so efficiently that plaque formation is suppressed, while recombinants form a regular plaque.

LE392 is a Sup E44, sup F58, hsd R514, gal K2, gal T22, met B 1, trp R55, lac Y1 and was used to propagate the genomic library.

C. Vectors

Lambda EMBL3 and λgt10 were purchased from Promega Corporation, Madison, Wisconsin.

pBluescript KS II + was a gift from Kathy Hautchins.

M13mp18 and M13mp19 were purchased from Boehringer Mannhein Corporation, Indianapolis, Indiana

D. Media

Luria Bertani (LB) medium is composed of 10 g bacto-tryptone, 5 g bacto yeast extract, and 10 g NaCl. To make plates, 15 g bacto agar was added to the above solution. This medium was used to grow and maintain *E. coli* strains C600, C600 hfl, and DH5α.

TB medium has 10 g bacto-tryptone and 5 g NaCl. This medium was used to grow and maintain *E. coli* strain LE392.

YT medium is composed of 8 g tryptone, 5 g yeast extract and 2.5 g NaCl per liter of solution. This medium was used to propagate JM109.

M9 minimal medium is composed of 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, O.5 ml of 1 M $MgSO_4$ solution, 0.05 ml of 1 M $CaCl_2$ solution, 2.5 ml of a 1 mg/ml vitamin B1 solution and 5 ml of a 20 % (w/v) glucose solution. This medium was used to maintain JM109.

E. Solutions

For the isolation of DNA from the sea anemones, the homogenization buffer contained 10 mM Tris-Cl pH 7.4, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermidine and 0.5 % (v/v) Triton-X 100.

The storage buffer for DNA isolation was TE and it was composed of 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA.

For routine agarose gel electrophoreresis, the running buffer was TAE diluted 50 times. The concentrated solution of TAE has 242 g Tris base, 57.6 ml glacial acetic acid and 100 ml of 0.5 M EDTA.

For ligation of the EMBL3 arms to genomic DNA, the ligase buffer contained: 50 mM Tris HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, and 50 µg/ml BSA.

For the isolation of total RNA, the following buffers were used: extraction buffer containing 3 M LiCl, 6 M urea, 10 mM sodium acetate, pH 5.2, 0.2 mg/ml Heparin and 0.1 % SDS, dissolving buffer made of 0.1 M sodium acetate, pH 5.2, and 0.1 % SDS and wash buffer containing 4 M LiCl and 8 M Urea.

For the isolation of polyadenylated mRNA, the equilibration buffer contained 0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5 % SDS, and 1 mM EDTA, while the elution buffer contained 0.01 M Tris-Cl, pH 7.5, 0.05 % SDS and 1 mM EDTA.

For the kinase catalysed reaction following the addition of Eco RI adaptors to cDNA, the kinase buffer contained 0.7 M Tris-Cl, pH 7.6, 0.1 M $MgCl_2$, and 50 mM dTT.

For separating the labeled primers and PCR products from the unincorporated nucleotides using Elutip-D, the buffers used were a high salt buffer containing 1.0 M NaCl, 20 mM Tris-Cl, pH 7-3-7.5, and1.0 mM EDTA and a low salt buffer containing 0.2 M NaCl, 20 mM Tris-Cl, pH 7.3-7.5, and 1.0 mM EDTA.

To store phage particles, the buffer used was SM composed of 5.8 g NaCl, 2 g $MgSO_4$. $7H_20$, 50 ml 1 M Tris-Cl, pH 7.5, and 5 ml 2% gelatin per liter of solution.

For packaging λ, the dilution buffer contained 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM $MgSO_4$.

For the isolation of plasmids and M13, the lysis buffer contained 50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl (pH8.0), and 4 mg/ml lysozyme chloride. The potassium acetate solution which is 3M with respect to K^+, 5M with respect to aceteate, pH 4.8 was prepared by adding 60 ml of 5 M potassium acetate 11.5 ml of glacial acetic acid and 28.5 ml of water.

For Southern blots, the prehybridization buffer contained 42 % formamide, Denhardt's solution at 5 times its original concentration, 200 µg/ml sheared, heated salmon sperm DNA, SSC at 5 times its original concentration, 0.15 % sodium pyrophosphate and 0.1% SDS. Denhardt's solution contained 5 g of Ficoll, 5 g of polyvinylpyrrolidone and 5 g of BSA per 500 ml water. SSC contained 150 mM NaCl, and 15 mM sodium citrate, pH 7.2.

For the sequencing reactions using sequenase, the concentrated buffer containing 200 mM Tris-Cl, pH 7.5; 100 mM $MgCl_2$; and 250 mM NaCl was used. The labeling mix concentrate contained 7.5 μM each of dGTP, dCTP, and dTTP, and the dideoxy mixes contained the following : for ddG, 8 μM each dGTP, dATP, dCTP, dTTP, 50 mM NaCl and 8 μM ddGTP; for ddA : 80 μM each of the dGTP, dATP, dCTP, dTTP, 50 mM NaCl and 8 μM ddATP, for ddC: 80 μM each of dATP, dCTP, dGTP, dTTP, 50 mM NaCl, 8 μM ddCTP, for ddT 80 μM each of dATP, dCTP, dGTP, dTTP, 50 μM NaCl and 8 μM ddTTP.

The running buffer for sequencing gels was a solution of TBE diluted 5 times. The concentrated solution has 54 g Tris base, 27.5 g boric acid and 20 ml of 0.05 M EDTA per liter of water)

For the PCR reactions, the concentrated PCR buffer contained 670 mM Tris, pH 8.0, 30 mM $MgCl_2$ and 160 mM ammonium sulfate.

F. Enzymes

All restriction enzymes, as well as exonuclease, S1 nuclease, RNAse A, DNAse I, proteinase K, calf intestinal phosphatase and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals, Indianapolis,Indiana. Taq Polymerase and Sequenase were purchased from Perkin Elmer Cetus Corporation.

G. Isolation of genomic DNA from *Anthopleura xanthogrammica*

To isolate genomic DNA from *A. x*, two methods were used.

The first method used in isolating high molecular weight DNA was that of Kaiser and Murray (1985), with minor modifications. Live sea anemones each weighing 100-120 g (fresh weight) were washed in tap water to remove sea debris and cut up into one inch cubes. The cubes were placed into a large Waring blender containing liquid nitrogen and ground to a fine powder. The powder was placed immediately in 250 ml Nalgene centrifuge bottles and frozen at -70 °C. Ten g of the powder was transferred to a 15 ml Wheaton homogenizer in an ice bath and homogenized gently with 10 ml of homogenization buffer. The homogenate was filtered through two layers of sterile cheese cloth. The filtrate was transferred to a 30 ml Corex tube and centrifuged for 7 minutes at 5,921 x g at 4 °C. The supernatant was discarded and the nuclear pellet rehomogenized in 20 ml of the same buffer. The homogenate was centrifuged as previously. The supernatant was discarded and the nuclear pellet was resuspended in 16.5 ml homogenization buffer. This homogenate was transferred to a preweighed 50 ml Falcon tube. Sarkosyl was added to a final concentration of 2 %, and was mixed with the solution by gentle inversion. The tube was incubated at 50 °C for 1 hour. Because the animal can harbor two different kinds of intracellular symbionts (Zoochlorellae), the lysate was examined under the microscope to be certain that none of the algal cell walls were disrupted. After incubation, the tube was weighed and the weight

weight of the lysate determined by difference. Cesium chloride was added to the tube (1.25 g/g lysate) and the tube was inverted gently to dissolve the CsCl crystals. When the CsCl was completely dissolved, the solution was divided into two 13.5 ml Beckman tubes. Four hundred µl of Ethidium bromide (10 mg/ml) was added to each tube before sealing. The tubes were centrifuged for 40 hours at 144,794 x g, at 18 °C in an L8-70 Beckman Ultracentrifuge.

The genomic DNA was visualized in the tubes by using a hand held uv lamp and was aspirated through a 16 gauge needle. To remove the ethidium bromide from the DNA, the sample was extracted exhaustively with n-butanol. The DNA was allowed to precipitate overnight at -20 °C with an equal volume of deionized water and 2 volumes of ethanol. The pellet obtained was dissolved in TE, extracted twice with phenol-chloroform, twice with chloroform, and precipitated in ethanol by adding 5M ammonium sulfate until the final concentration of 0.25 M was reached.

The method reported by Maniatis *et al.* (1982) was also used to isolate sea anemone DNA except the homogenization buffer was that used by Kaiser and Murray (1985), and minor modifications were also added. Forty four g of a powdered preparation of the homogenized animal was suspended in 80 ml of a solution containing 0.5 M EDTA and 0.5 % Sarcosyl. Twenty mg of proteinase K was added and the suspension was incubated at 50 °C for 3.5 hrs. After incubation, the proteinase K digest was filtered through two layers of sterile cheesecloth. The tissue remaining in

the cheesecloth was removed and homogenized and the homogenate was filtered. The combined filtrate was centrifuged for 20 minutes at 12,085 x g and at 4 °C. The pellet obtained was rehomogenized in homogenization buffer. The final volume was determined by pouring in a graduated cylinder. Cesium chloride was added at a concentration of 1 g/ml homogenate. The final density of the solution was 1.6547. The solution was subdivided into 13.5 ml Beckman tubes, and to each was added 400 µl of ethidium bromide (10 mg/ml). The tubes were centrifuged in the Beckman L8-70 ultracentrifuge for 65 hrs, at 183,254 x g and at 20°C in a 50 Ti rotor. The DNA band was visualized with a hand held uv lamp and the DNA band removed from the tube with the use of a 16 gauge needle. Ethidium bromide was removed from the DNA by extensive butanol extraction. The DNA in solution was precipitated with ethanol. The pellet obtained was redissolved in TE and dialyzed extensively in TE at 4 °C, with four changes of buffer. Routinely, DNA was visualized in agarose gels electrophoresed with TAE buffer diluted 50 times.

H. Genomic Library Construction

The genomic library of *Anthopleura xanthogrammica* was constructed according to the method of Maniatis *et al.* (1982). High molecular weight DNA isolated from *Anthopleura xanthogrammica* was partially digested with Sau 3A to generate 17-20 kb fragments. In order to optimize the number of 17-20 kb fragment for insertion into the vector the digest was centrifuged

in a sucrose gradient (5-20 %) for 18 h at 58,423 x g at 15 °C using the swinging bucket rotor (SW28) in the Beckman L8-70 ultracentrifuge. At the end of the centrifugation, the tubes were removed gently from the rotors and a 50 µl capillary pipette attached to a plastic tubing was inserted gently into the 30 ml tube, until the tip was about 3 mm from the bottom. A 5 ml syringe was used to suction the fluid until a steady flow was obtained. 1.0 ml aliquots were collected until all the gradient was removed from the tube. Eight µl of these aliquots were added with 2 µl of stop dye and sized on a 0.7 % agarose gel. The tubes containing the 17-20 kb fragments were pooled and the DNA from these samples precipitated with ethanol.

The fraction consisting mainly of 17-20 kb was inserted into a λ vector EMBL3 which had been cut with Bam H1 and treated with calf intestinal phosphatase. The ligation reaction mixture consisted of the following : 3.6 µg *Anthopleura xanthogrammica* DNA, 3 µg EMBL3 vector, 3 µl ligase buffer and 5 units of T4 DNA ligase, and sterile double distilled water in a final volume of 30 µl. The ligation mixture was incubated at room temperature for 4 hours. As a control, an aliquot (10 µl) of the ligation mixture was taken out before the enzyme was added. Two µl of the ligation mixture containing 300 ng DNA were packaged by adding 2 µl sonicated extract and 12.5 µl freeze thaw lysate and incubating at room temperature for 2 hours. After the ligation reaction, 150 µl λ dilution buffer was added to the packaging mixture. The mixture was extracted with 25 µl $CHCl_3$, and

centrifuged for 3 minutes at 2000 x g at room temperature. The supernatant was transferred to a fresh tube, and stored in the dark at 4 °C. To test for background, unligated controls were treated similarly. The library was titered in *E. coli* strain LE392. The titer of the library was determined as the difference in plaque counts between samples that received the enzyme and those that did not.

I. Isolation of RNA from *Anthopleura xanthogrammica*

For RNA isolations, all glasswares were baked at 450 °F for 6 hours before use.

To construct the cDNA library, total RNA was first isolated as in the method of [Haymer *et al.*, 1990] and poly-adenylated mRNA was isolated on an oligo dT column as in the method of Maniatis *et al.* (1982).

A 2 g sample of finely ground *Anthopleura xanthogrammica* stored at -70°C was homogenized in 10 ml of extraction buffer. Homogenization was done manually at 0°C, in order to prevent shearing and degradation of RNA. The pestle and the homogenizer were rinsed with another 10 ml of extraction buffer. The homogenates and washes were pooled into a 30 ml Corex tube and kept at -20 °C overnight. After precipitation, the homogenate was centrifuged at 10,000 x g for 15 minutes at 4 °C. The supernatant was drawn off carefully, to prevent disturbance of the pellet. Ten ml of wash buffer was added to the pellet. The pellet was broken gently with the tip of a 5 ml pipette. The suspension was

vortexed briefly, and then centrifuged for 15 minutes at 10,000 x g at 4 °C. The supernatant was decanted and the pellet was dissolved in 3 ml dissolving buffer. The solution was vortexed briefly and then shaken gently at room temperature, until the pellet was completely dissolved. To remove the remaining proteins from the sample, the solution was extracted with 5 ml of phenol saturated with TE, and 5 ml of a solution of chloroform isoamyl alcohol (24:1 v/v). Phenol-chloroform extraction was done by allowing the mixtures to shake gently in a capped tube, at room temperature. The mixture was centrifuged at 10,000 x g for 5 minutes. The aqueous phase and the interface were both retained and extracted with the chloroform isoamyl alcohol solution. From the chloroform extract, the aqueous phase was transferred to a fresh Corex tube and precipitated by addition of 1/20 th volume of a 5 M sodium acetate solution pH 5.2, and 2.5 volumes of 95 % ethanol. Precipitation was allowed to proceed at -20 °C overnight. The precipitate was recovered by centrifugation at 10,000 x g for 20 minutes at 4 °C. This pellet contained total RNA. The pellet was washed twice with ice cold 70 % ethanol, and allowed to air dry.

Total RNA was resuspended in 1-2 ml equilibration buffer The solution was applied to an oligo-dT column. The column was washed with 10 bed volumes (20 ml) of the equilibration buffer. The eluates from the column were collected in 1.5 ml fractions and the absorbance determined at 260 nm in a Beckman Model 25 Spectrophotometer. Poly-adenylated mRNA was eluted from the

column with 6 ml of elution buffer. The sample collected was ethanol precipitated as above. The poly-adenylated mRNA pellet was dissolved in sterile double distilled water, and stored at -70 °C until use.

J. Construction of the cDNA library

The poly-adenylated mRNA was retrotranscribed using AMV reverse transcriptase as in the method of Gubler and Hofman (1983), and the second strand was synthesized using the Klenow fragment of DNA polymerase 1. For first strand synthesis, the reaction mixture contained : 2.4 µg poly-adenylated mRNA, 1 µl RNAse inhibitor, 10 µM each of dATP, dCTP, dGTP and dTTP, 600 ng oligo $(dT)_{15}$ primer and reverse transcriptase buffer. The mixture was incubated at 42 °C for 60 minutes. In order to determine the efficiency of reverse transcription, a reaction with α^{32}P-dCTP was also done. The percent dCTP incorporated was calculated by taking measurements of radioactive counts at 0 and 1 hour incubation times after precipitation with 5% TCA and 20 mM sodium pyrophosphate. To determine the efficiency of second strand synthesis, radioactive dCTP was included in an unlabeled sample of first strand cDNA. The reaction mixture for the synthesis of the second strand contained all of the first strand synthesis components, and also RNAse H, to degrade the RNA template in the DNA/RNA hybrid. Second strand synthesis was accomplished using *E. coli* DNA polymerase 1, and incubation at 12 °C for 60 minutes. In order to fill up recessed ends, T4 DNA

polymerase was added after the second strand synthesis, and the reaction mixture was incubated for 10 minutes at 37 °C. The reaction was terminated by addition of 10 µl of 0.2 M EDTA solution, pH 7.2, and 2 µl of a 10 % (w/v) sarkosyl solution. The reaction mixture was extracted once with phenol/chloroform, once with chloroform and then precipitated by addition of ethanol and sodium acetate, and storing at -70 °C for 30 minutes.

The resulting double stranded, blunt-ended cDNA was ligated to Eco R1 adaptors (Promega Corporation). The adaptor is a duplex DNA molecule with one blunt end which ligates to the cDNA and one sticky end which is an Eco R1 site. The ligation reaction contained: 1 µg of cDNA, 30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM dithithreitol, 0.05 mM ATP, 1 µg/ml BSA, 10 picomoles of Eco-R1 adaptors and 7.5 units of T-4 DNA ligase in a total volume of 30 µl. Ligation of adaptors to cDNA was allowed to proceed at 15 °C for 18 hours. The ligase was inactivated by heating the mixture for 10 minutes at 70 °C. Immediately after ligation, the cDNA ligated to adaptors was subjected to a kinase catalyzed reaction in order to phosphorylate it for attachment into Eco R1 restricted and dephosphorylated λ gt10 arms. Four µl of kinase buffer was added to the 30 µl reaction mixture. The excess adaptors were removed from the reaction mixture by the Spin column procedure (Maniatis *et al.*, 1982). The eluted cDNA containing adaptors was inserted into λ gt10, using a molar ratio of 1:0.5 λ arm to insert.

Since λgt10 is a vector that can accommodate from 0-7.6 kb cDNA, the packaged phages were titered on two bacterial strains that distinguish between recombinant and non-recombinant phages. The bacterial strains used were *E. coli* C600 and *E coli* C600 hflA.

K. Primer Construction and Labeling

Synthetic oligomers were constructed using Applied Biosystems 380 B DNA synthesizer, and were either gifts from Dr. Earl Davie of the University of Washington, Seattle Washington, or synthesized by Dr. Neil Reimer of the University of Hawaii.

Three sets of primers were used in the study. The first set of primers consisted of two 30-mer overlapping nucleotide oligomers chosen from the backtranslation of the segment of the peptide that is least degenerate. This consists of amino acids 33 to 49 for APA:

33

CCAGGCTGGTAGCCAACCAC(A,G)AC(A,G)TT(T,C)GT(T,C)

H_2N-Trp-His-Asn-Cys-Lys-Ala-His-Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln-COOH

TGGCA(T,C)AA(T,C)TG(T,C)AA(A,G)GCTCACGGTCCGACC

49

The second set of synthetic nucleotides was constructed from the backtranslation of the first 10 amino acids of the peptides using the codons of highest frequency in the sea urchin.

5' GGA-GTC-CCA-TGC-CTC-TGC-GAC-AGC-GAC-GGA-3'

Gly-Val-Pro-Cys-Leu-Cys-Asp-Ser-Asp-Gly-

3' GT-ACG-GAG-ACG-CTG-TCG-CTG-CCT-5'

The third set of nucleotides were constructed to be used as the primers for the polymerase chain reaction, and thus consisted of all the possible codons for the first and last 10 amino acids of the peptides.

The first primer (KY-1) consisted of all the possible codons for the first 10 amino acids of AP-B when peptide was backtranslated.

(KY-1) 5'GGN-GTN-CCN-TG(C,T)-CTN-TG(C,T)-GA(C,T)-AGN-CA(C,T)-GG-3'

Gly-Val-Pro-Cys-Leu-Cys-Asp-Ser-Asp-Gly-

The second primer (KY-2) was constructed from backtranslating the last 10 amino acids of the AP-A peptide and then obtaining its complement .

Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln

GGN-CCN-ACN-AT(T,C)-GGN-TGG-TG(T,C)-TG(T,C)-AA(A,G)-CA(A,G)

(KY-2) 5'(C,T)TG-(C,T)TT-(A,G)CA-CCA-NCC-(A,T,G(AT-NGT-NGG-NCC-3'

The nucleotides were labeled using the random priming kit from Boehringer Mannheim, according to the standard random primed DNA labeling reaction as described by Feinberg and Vogelstein (1983). Two hundred fifty to 500 ng template DNA was denatured by heating for 2 minutes at 95 °C. The denatured DNA dissolved in sterile double distilled water was incorporated into a 20 µl reaction volume containing:Tris-Cl pH 7.2, (50 mmol/l), dithiothreitol (0.1 mmol/l), $MgCl_2$ (10 mmol/l), bovine

serum albumin (200 µg/ml), random hexanucleotides (3.1 mg/ml), dATP (25 µmol/l), dGTP (25 µmol/l), dTTP (25 µmol/l), α ^{32}P-dCTP (50 µCi, or 5 µl of 3000 Ci/mmol labeled dCTP) and Klenow enzyme (2 units). The reaction was allowed to proceed for at least 30 minutes and then stopped by addition of 2 µl of 0.2 mol/l EDTA.

In order to separate the labeled DNA fragments from the unincorporated nucleotides, a column of Elutip-D was used. After stopping the reaction 480 µl sterile water was added to the reaction mixture. The solution was mixed thoroughly by repeated aspiration and discharge via a 16 gauge needle attached to a 5 ml disposable syringe. Two µl were aliquoted into 10 ml of Scintiverse scintillation cocktail mix (Fisher Chemicals). The solution was then passed in Elutip previously hydrated with a high salt and. After applying the sample to the column, the column was washed with 5 ml of a low salt buffer, and the DNA was eluted from with 1 ml high salt buffer. Two µl of the eluate was aliquoted into 10 ml Scintiverse cocktail mix, and the samples were counted in a Beckman LS8100 scintillation counter.

The percent incorporation was determined as the ratio of the the counts before and after passing the samples through the column. Typically $4x10^7$-$2x10^8$ cpm/ µg of DNA was obtained.

L. Polymerase Chain Reaction

Since initial results using oligonucleotide primers alone yielded equivocal results in the screening of both genomic and cDNA clones, two highly degenerate oligonucleotides were constructed for use as primers in the polymerase chain reaction. The oligonucleotide sequences were constructed by back translation of the first and the last 10 amino acids of AP-B peptide. Ten to 20 ng of either genomic DNA or cDNA was used as template. The reaction vessel contained 500 ng of the two primers (approximately equivalent to 50 picomoles each), 0.2 mM each of dATP, dCTP,dGTP and dTTP, and 10 µl PCR buffer and 0.125 units of Taq polymerase, in a total volume of 100 µl. To prevent evaporation, a drop of mineral oil was placed on top of the solution. The tubes were incubated in a Perkin Elmer Cetus Thermal cycler set to 50 cycles of 1 minute at 94 °C, 2 minutes at 50 °C and 2 minutes at 72 °C, for denaturation of template, annealing of template and primers, and primer extension, respectively.

M. Isolation of Phage DNA

Several different phage preparation procedures were used. The method described by Chrisholm (1989) gave the highest yields. The method as used by Chrisholm (1989) was for large scale preparation of phage DNA; in the present work the procedure has been scaled down for 5 ml cultures. A multiplicity of infection (MOI) of 10 phages per 1000 bacteria was used in all

isolations. A 5 ml aliquot of LB medium was inoculated with the appropriate host, such that the bacteria has been diluted 1:100. Phage stocks maintained in SM and chloroform were then added. The tubes were shaken at 38 °C for 12 to 15 hours, and then 13.5 µl of chloroform was added. The tubes were vortexed and RNAse A and DNAse I were each added to a final concentration of 50 µg/ml. The tubes were incubated at 37°C for 30 minutes. Sodium chloride was added to a final concentration of 1 M. After mixing, the solution was centrifuged at 5,921 x g for 20 minutes and at 4°C. The supernatant containing the phage particles was saved, and the pellet containing bacterial debris was discarded. The supernatant was treated with 1.5 ml of 40 % polyethylene glycol (PEG), and stored on ice for 1 hour. The phage was then pelleted with the PEG by spinning at 12,085 x g for 20 minutes at 4 °C. The phage-PEG pellet was resuspended in 1 ml of SM medium. The solution was divided into two-1.5 ml microfuge tubes and extracted with an equal volume of chloroform twice to remove the PEG. The upper aqueous layer obtained after chloroform extraction was transferred to a fresh tube and added with 2.7 µl 0.5 M EDTA, 10 µl of a 10 % (w/v) SDS, and 1.25 µl of a 20 mg/ml stock of proteinase K. The tubes were incubated at 65°C for 30 minutes. After incubation, the proteinase K digest was subjected to extraction once with phenol, extraction with phenol:chloroform twice and extraction with chloroform 3 times, and then ethanol precipitated using sodium acetate as the salt.

N. *In situ* Plaque Hybridizations and Southern Blots

Recombinant phages containing the cDNA library was plated with *E coli* strain C600 hflA while phages containing the genomic library was plated with *E coli* strain LE392.

When the plaques were about 0.2 mm, the plates were transferred to 4 °C for an hour. The plaques formed were allowed to transfer to Nytran membranes for 2 minutes for the first filter and for 5 minutes for the duplicate filter. The phage protein coat was removed by denaturation in 1.5 M Tris, 0.5 M NaOH for 2 minutes, and then neutralized in 1.5 M NaCl, 0.5 M Tris for 5 minutes. After neutralization, the filters were allowed to soak for 2 minutes in twice concentrated Saline sodium citrate solution (SSC). The filters were air dried for 15 minutes and then transferred to 60 °C oven for 20 minutes.

The dry filters were then transferred to 11" x 12" heat sealable pouches (Kapak Co., Minneapolis, Minn), and wetted with prehybridization buffer. The bag was incubated in a water bath at 42 °C, for 6 hrs. After prehybridization, all the solution was drained from the bag and replaced with a similar solution except for the presence of the labeled nucleotide in the hybridization mix. Labeled PCR product or primers containing 4 x 10^7 cpm/μg DNA to 2 x 10^8 cpm/μg DNA were used. Hybridization was allowed to proceed at 42 °C for 36 hours. The filters were washed initially with 6x SSC at 37 °C, then 2X SSC, at 42 °C, for first screening, and then at higher stringency during plaque purification steps.

O. Subcloning of the Genomic Fragments

For further investigations the fragments from the genomic clones that hybridized with the PCR product were subcloned into M13mp18 (Yanisch-Perron *et al.*,1984) as follows: 100 ng (5.2 picomoles) of M13mp18 digested with Eco R1 were ligated with 0.4 µg Eco R1 digest each of clones #24 and 29 or a ratio of 1:2 moles insert to vector ratio.

Similarly, 833 ng Bam H1 digested M13mp18 were ligated with 3 µg Bam H1 digest of clone # 15. Ligations were done at 0 °C overnight, using T4-DNa ligase. Recombinants were selected on H medium plates and H top agar supplemented with 100 µl of 100 mmol/l solution of IPTG and 50 µl of a 2 % (w/v) solution of X-gal in dimethyl formamide.

P. Cloning of the PCR Product

The PCR product was cloned into pBluescript KS II (+) for sequencing. The PCR products were first pooled from the tubes and ethanol precipitated. The contaminating mineral oil from the reaction mixture was removed first extracting the solution twice with 500 µl hexane, and twice with chloroform before addition of the salt and ethanol. After ethanol precipitation, the PCR product was electroeluted from 4 % agarose gels. Alternatively, the bands of interest was visualized after electrophoresis and were cut out from the gels and recovered by the phenol freeze extraction method as reported by Bewsey *et al.* (1991). The PCR product

was blunt ended with the Klenow fragment of DNA polymerase 1 before ligating with Sma 1 digested phagemid vector. Since the recovered PCR products were very low in concentration, the pooled products from 10 PCR reaction tubes were ligated to 2 µg Sma 1 digested vector, after subjecting the restricted vector to an alkaline phosphatase catalyzed reaction. The recombinant phagemids were selected on LB plates to which 100 µg/ml ampicillin, IPTG and X-gal were added as above.

Q. Subcloning of the cDNA Inserts from cDNA Clones

Since it was not possible to release the cDNA inserts from λ gt10 using Eco R1, an alternative approach to characterizing the putative clones was used. It consisted of double digesting the cDNA clones with Bgl II and Hind III, two restriction sites flanking the Eco RI site in the vector λ gt 10, and ligating the digest with M13mp18 digested with Bam H1 and Hind III.

A 200 ng aliquot of the M13 vectors M13mp18 and M13mp19 were double digested with Bgl II and Hind III. The double digests were phenol extracted and reprecipitated, and then treated with alkaline phosphatase. The DNA was then ligated to double digests of the cDNA clones (approximately 500 ng of each). The ligation reaction mixtures used to transform *E. coli* DH5α and the transformed bacteria were plated in H medium modified with ampicillin, X-gal and IPTG. The plates were incubated at 37 °C overnight and white colonies appearing on the plates were isolated.

R. Bacterial Transformation

E. coli strains LE392, C600, C600 hfl and DH5α were made competent for transformation as in the method of Mandel and Higa (1970). For DH5α, five ml of LB medium was inoculated with a single bacterial colony and grown for 5 hours at 37 °C. For LE392, the cells were grown in 5 ml TB medium supplemented with 50 µl of 1 M $MgSO_4$ and 50 µl of 20 % maltose, and the

incubation was done overnight at 35 °C. For C600 and C600 hfl, the cells were grown in LB medium supplemented with 50 µl 1 M $MgSO_4$. The cells were transferred to 7 ml falcon tubes, chilled on ice for 5 minutes and centrifuged at 4000 x g for 5 minutes, at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in 2.5 ml sterile solution of 50 mM $CaCl_2$. The resuspended bacterial cells were stored on ice for 15 minutes, and again centrifuged as above. The pellet was resuspended in 300 µl of 50 mM $CaCl_2$ and stored at 4 °C overnight before use.

S. DNA Sequencing

Sequencing of both the PCR clones and M13 subclones was done using the Sequenase kit from U.S. Biochemical Corporation, Cleveland, Ohio.

The following steps were carried out for DNA sequencing. Approximately 1-2 µg of single stranded M13 DNA dissolved in 7 µl of water was mixed with 1 µl of M13 universal primer (5'GTAAAACGACGCCAGT-3', 0.5 pmol/µl), and 2 µl of concentrated sequenase buffer. The annealing reaction mixture was heated to 65 °C for 3 minutes and then allowed to cool slowly to room temperature. For double stranded DNA sequencing of the pBluescript KSII + clones, the template was first denatured by adding 1/10th volume of 2M NaOH, 2mM EDTA and then incubating at 37 °C for 5 minutes. The mixture was neutralized by adding 0.4 volumes of 3 M sodium acetate, pH 5.2, and then precipitated with 4 volumes of 95 % ethanol. Precipitation was done at -70 °C

for 30 minutes. The DNA was pelleted by centrifugation at 12,000 x g 10 minutes. The pellet was washed in 70 % ethanol and then dried *in vacuo*. The dried pellet was redissolved in 7 µl water.

To the annealed template and primer (10 µl total volume), the following were added: 1 µl DTT (0.1M), 2 µl labeling mix diluted 5 times, 0.5 µl of [α-^{35}S] dATP (10 µCi/µl), and 2 µl of sequenase buffer diluted 8 times. The solution was mixed thoroughly while avoiding bubble formation. The extension reaction was allowed to proceed at room temperature for 5 minutes.

To terminate the reaction, 3.5 µl of the reaction mixture was aliquoted into prewarmed termination reaction tubes labeled G, A, T, and C, containing 2.5 µl of the dideoxy termination mixes. termination reaction was allowed to proceed for 5 more minutes at 37 °C, and then stopped by adding 4 µl of stop solution (95 % formamide, 20 mM EDTA 0.05% bromophenol blue, 0.05% xylene cyanol).

The reaction vessels were heated to 95°C for 5 minutes and then immersed on ice prior to loading into 6 % acrylamide sequencing gels in a 27 cm x 55 cm Biorad Sequencing apparatus. The gels were electrophoresed at constant power of 55 watts from Biorad power source 3000 XI.

Acrylamide sequencing gels were prepared and casted as follows: A 40 % solution of acrylamide-bisacrylamide was prepared by adding 38 g acrylamide and 2 g bisacrylamide to double distilled water. After dissolving the crystals, the volume was

adjusted to 100 ml. The solution was filtered through a #4 Whatman filter paper. Nine ml of the 40 % acrylamide solution was added with 25.2 g urea. The solution was stirred at room temperature and the volume adjusted to 60 ml with distilled water. The bottom of the sequencing gel sandwich was sealed by adding 240 µl of 25 % ammonium persulfate and 240 µl TEMED to initiate and catalyse the polymerization, and the solution was poured into the casting tray, with the apparatus in vertical position. Polymerization was observed within 5 minutes. After sealing the bottom of the gel sandwich, the apparatus was brought to an angle of about 45^{o}. Another 60 ml of the 6 % acrylamide gel was prepared, and added with 120 µl 25 % ammonium persulfate and 80 µl TEMED. This solution was applied between the plates with the use of a 50 ml syringe. The acrylamide gel was allowed to polymerize for at least 2 hours.

The gels were electrophoresed on a TBE buffer for about an hour at 55 watts constant power to bring the temperature up to 55 °C before the samples were loaded.

Immediately after electrophoresis, the gel was washed on a 10 % methanol-10 % acetic acid solution for 10 minutes, and then rinsed twice with distilled water. The gels were dried at 80°C for 2 hours and Fuji X-ray film was exposed on the gel overnight. After exposure, the films were soaked on Kodak X-ray developer for 5 minutes, washed in water for 1 minute and soaked in Kodak X-ray fixer for 1 minute, before washing extensively with water.

V. RESULTS

The object of the study was to investigate the molecular genetics of the cardiotonic polypeptides anthopleurins a and b, produced by the sea anemone, *Anthopleura xanthogrammica*.

In order to begin the study, high molecular weight DNA from the animal was needed.

A. Isolation of high molecular weight DNA

Since there has been no previous reports on isolation of DNA from this animal, two methods were used. The two methods were those of Kaiser and Murray (1985), and a modification of the method reported by Maniatis *et al.* (1982).

The DNA obtained from the two methods was electrophoresed on 0.5 % agarose gel and the patterns are shown in Figs. 1 and 2. Fig. 1 shows that the DNA obtained using the method of Kaiser and Murray (1985) was highly sheared, and that the distribution of the fragments of DNA ranged from less than 43 kb to less than 3.5 kb. On the other hand, the DNA obtained using the method of Maniatis *et al.* (1982) had a higher proportion molecules whose size is greater than 43 kb as shown in Fig. 2.

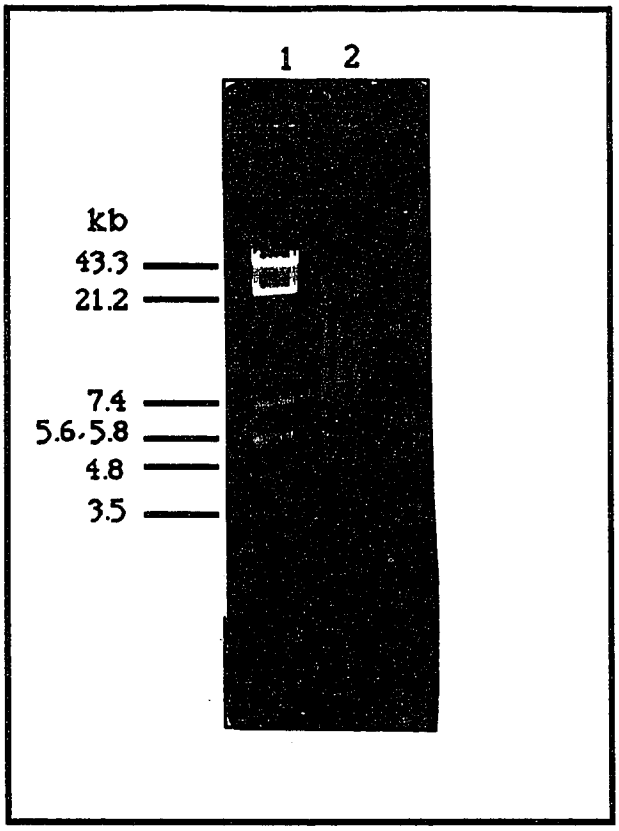


Fig. 1. Agarose gel of genomic DNA isolated from *Anthopleura xanthogrammica* using the method of Kaiser and Murray (1985). From left lanes 1) uncut λ and Eco RI-λ molecular weight marker, 2) aliquot of *Anthopleura xanthogrammica* DNA

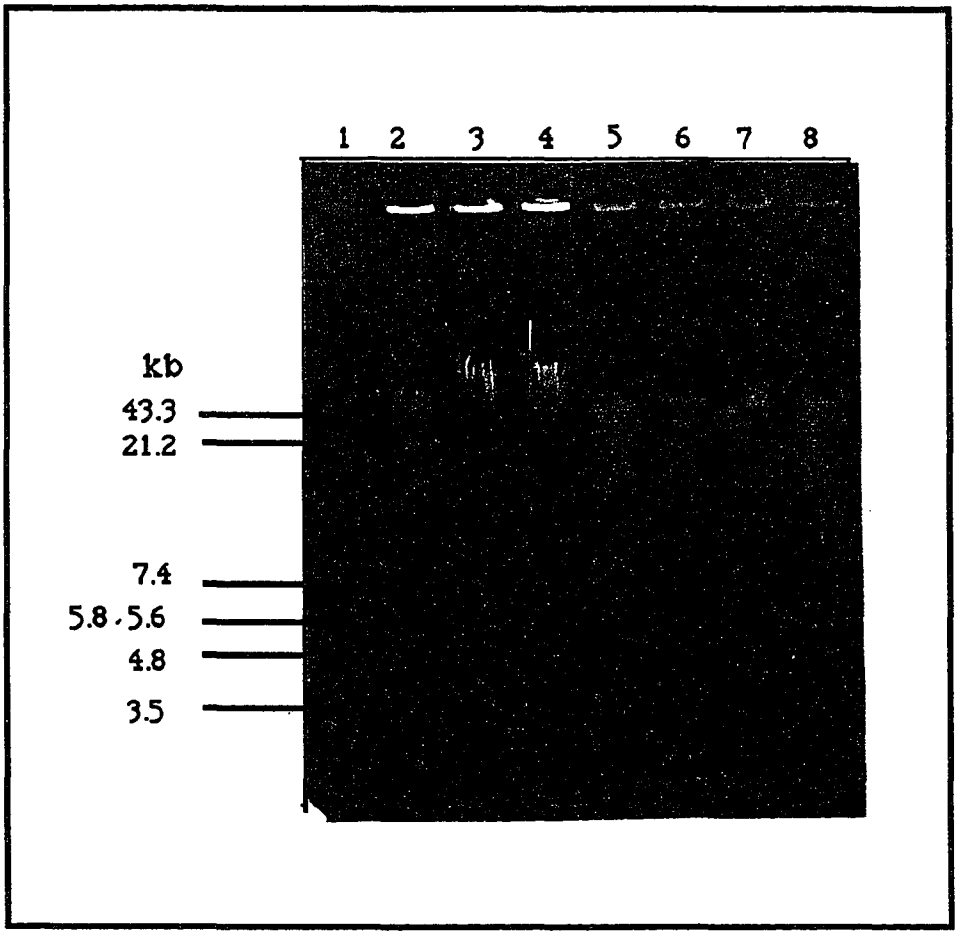


Fig. 2. Agarose gel of genomic DNA isolated from *Anthopleura xanthogrammica* using the method of Maniatis *et al.*, (1982) 1) uncut λ and Eco RI-λ molecular weight marker, 2-8) aliquots from different tubes.

B. Construction of the Genomic Library

Since the DNA obtained by the method of Maniatis *et al* (1982) which employed proteinase K digestion and several phenol extractions had a greater percentage of high molecular weight DNA, it was used to construct the genomic library of the animal. The DNA was partially digested with Sau 3A and subjected to sucrose density gradient centrifugation. Aliquots of the two gradients were electrophoresed on 0.5 % agarose gel. Fig. 3 shows that the DNA fragments were distributed along the gradient according to mass, and tubes 4 and 5 from the first gradient tube, and 5 and 6 from the second gradient tube contained the fragments that are closest to 20 kb in size.

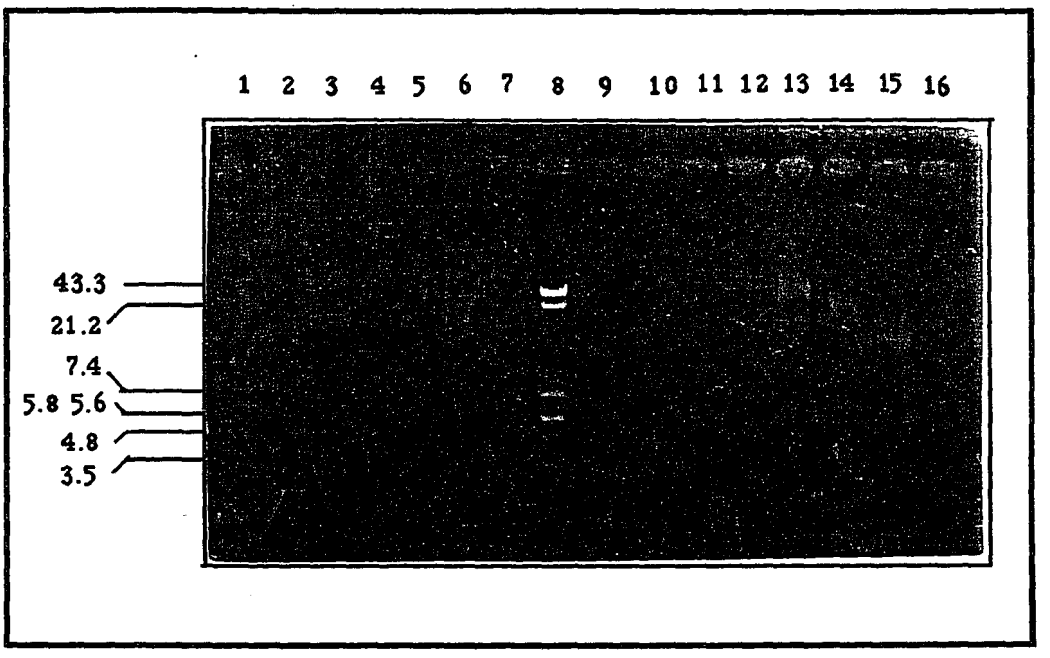


Fig. 3. Agarose gel of aliquots of partial Sau 3A genomic DNA digest after sucrose density gradient centrifugation. Lanes 1-7) aliquots from 1 gradient tube, 8) λ -Eco RI molecular weight marker, 9-15) aliquots from a similar gradient. Each well was loaded with a 8 µl aliquot from a 1. 0 ml sample.

The gradient fraction consisting mainly of the 17-20 kb fragments were pooled and reprecipitated and again electrophoresed on a 0.5 % gel for sizing. Fig. 4 shows that the partial digest that had not been subjected to sucrose gradient centrifugation ranged in size from > 43.3 kb to < 3.5 kb, whereas the pooled fractions consisted mainly of the desired 17-20 kb fragment size.

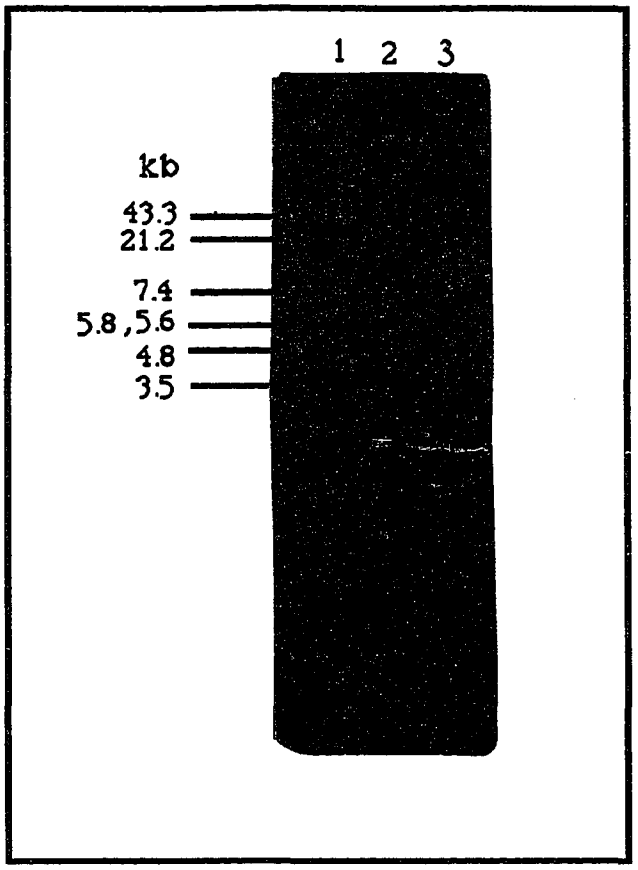


Fig. 4. Agarose gel of partial Sau 3A digest of *Anthopleura xanthogrammica* DNA after reprecipitation following sucrose density centrifugation. Lane 1) Eco RI- λ + uncut λ molecular weight marker, 2) aliquot of parial Sau 3A digest of *Anthopleura xanthogrammica* before sucrose density gradient centrifugation, 3) aliquot of partial Sau 3A digest after sucrose gradient centrifugation and reprecipitation.

The pooled fractions consisting mainly of the 17-20 kb fragments were inserted into the Bam HI site of λ replacement vector EMBL3 as shown in Fig. 5.

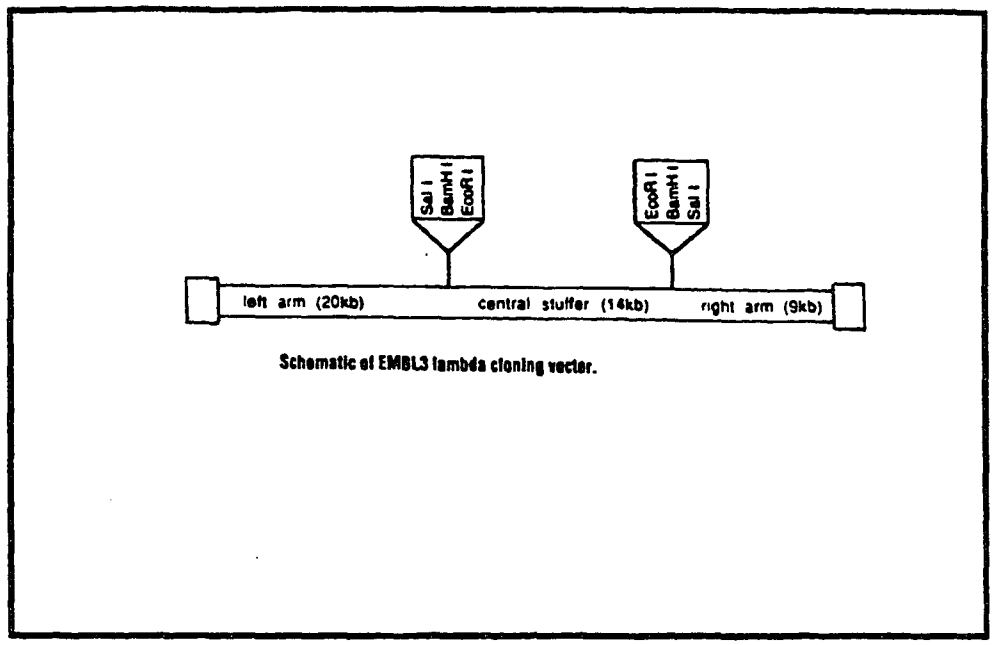


Fig. 5. Schematic representation of λ EMBL3 cloning vector (Frischauf *et al.*, 1983)

There were 543,000 clones in the unamplified library. Based on an average insert of 17 kb and an extrapolated value of 8×10^8 base pairs for invertebrates, the library consisted of a total fragment size greater than 10 times the size of the genome of the animal. The genomic library was amplified and titered on *E. coli* strain LE392.

C. Construction of the cDNA Library

To construct the cDNA library, total RNA was isolated from fresh tissues. The poly-adenylated mRNA was separated from ribosomal RNA and tRNA by affinity chromatography, using an oligo dT column.

About 0.6 mg total RNA was isolated from 1 g fresh tissue, and from this total RNA, 18 μg poly-adenylated mRNA was isolated from oligo dT column. From 2.4 μg poly-adenylated mRNA, 4.66 μg double stranded cDNA was obtained. The double stranded cDNA was ligated into Eco RI adaptors and inserted into the single Eco RI site of λ gt10 as shown in Fig. 6.

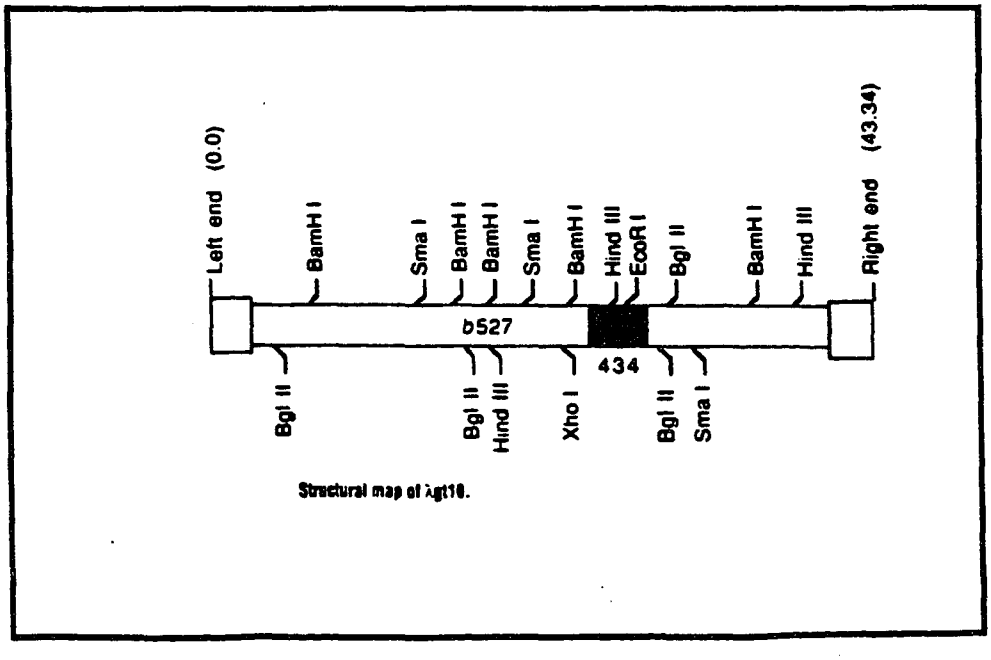


Fig. 6. Schematic representation of λ cloning vector λ gt 10 (Murray *et al*, 1977)

The Eco RI adaptor ligation system that was used is shown in Fig. 7.

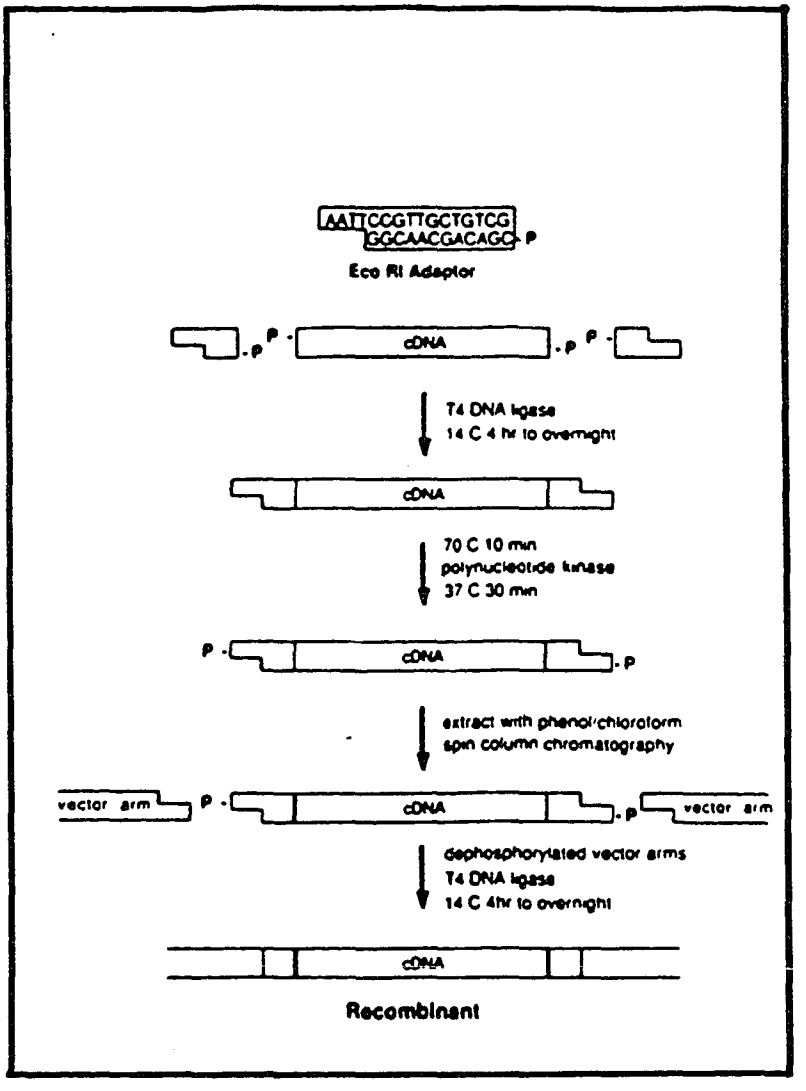


Fig. 7. Schematic representation of Eco-RI adaptor ligation system used in constructing the cDNA library

From 1 μg of cDNA ligated to 3 μg of the vector, 325,000 cDNA clones were obtained.

D. Polymerase Chain Reaction

Screening of either the genomic or the cDNA library with two sets of synthetic oligomeric probes constructed from the back translation of AP-B yielded inconsistent results. Plaques isolated using these four primers did not show signals upon subsequent purification steps. The failure to detect clones harboring the message for the peptide was probably due to the lack of sufficient data to create an oligomer that most closely resembles the nucleic acids coding for the amino acids.

Since screening of the libraries with the synthetic probes did not result in the isolation of clones that harbor the AP-A or AP-B gene, the polymerase chain reaction was deemed an alternative approach.

Two sets of highly degenerate oligonucleotide primers were constructed based on the backtranslation coding for the two peptides. These primers were used to amplify the message using genomic and cDNA as templates. Fig. 8 shows an agarose gel when the products of the polymerase chain reaction were electrophoresed on a 4% agarose gel. Three distinct bands were present in aliquots of reactions that received cDNA as the template as shown in lanes 1, 2, 14, and 15. These bands are also present in aliquots of reactions which received genomic DNA as template as shown in lanes 3, 4, 11, 12 and 13. In contrast, the aliquot from the control reaction which received the primers alone show only two bands, the larger probably representing the dimerization product of the two primers.

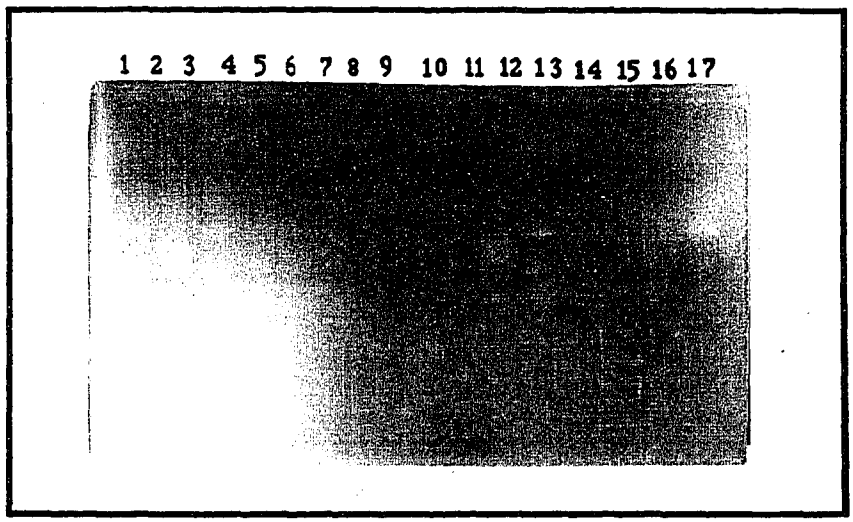


Fig. 8. Agarose gel of polymerase chain reaction products from different templates. From left lanes 1 and 2, cDNA as template, 3 and 4, genomic DNA as template, lanes 5-8, cDNA as template, lane 9, primers alone, lane 10, cDNA as template, lanes 11-13, genomic DNA as template, lanes 14 and 15, cDNA as template. Each lane was loaded with 25 µl aliquot from a total PCR reaction tube of 100 µl.

To ascertain whether the product obtained from the poymerase chain reaction is the fragment which is of interest, aliquots of the tubes that showed a third band were run on another 4% agarose gel for sizing. In order to be certain that the larger band is not an artifact, nor a polymerization product of the primers alone, double the enzyme concentration was added to the control reactions receiving only the primers as in the previous reactions. The tubes were again loaded to the thermal cycler for another round of chain reaction.

Fig. 9 shows that a 144-147 base pair product was obtained from the polymerase chain reaction, and that the control reactions did not show another band after 2 rounds of chain reaction.

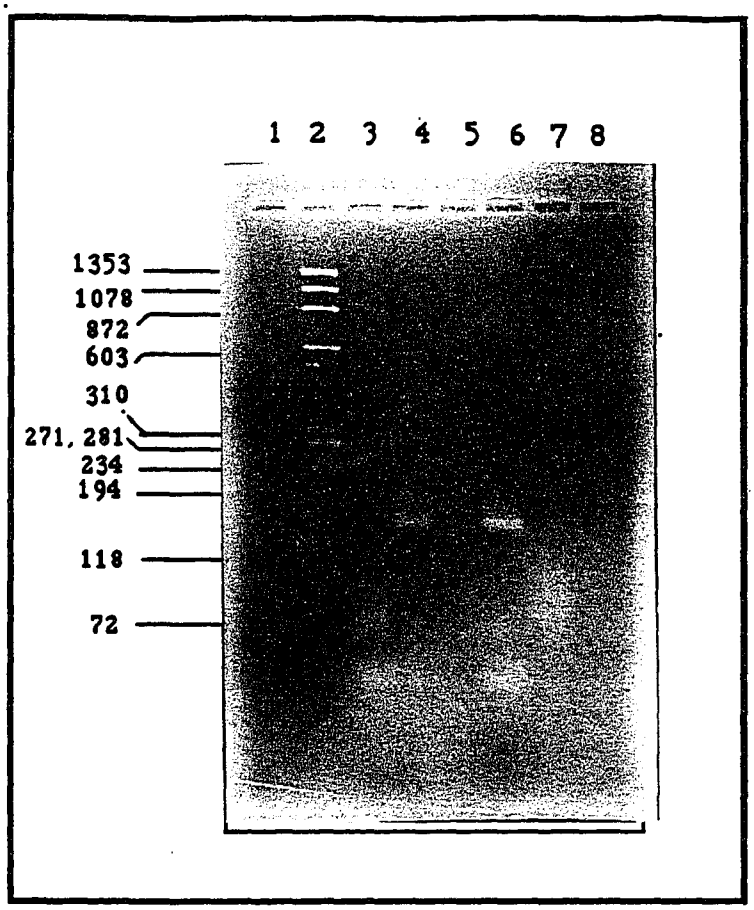


Fig. 9. Polymerase chain reaction products sized on a 4 % agarose gel Lane 1) dye, 2) phi-x174 Hae III molecular weight marker ,500 ng, 3) 30 µl of control reaction amplified twice, 4) 15 µl of PCR product using cDNA as template, 5) 15 µl of PCR product using genomic DNA as template, 6) same as 4, 7) same as 3.

Since the size of the product of the polymerase chain reaction corresponded to the expected size of DNA for the message of the anthopleurin peptides, the next step was to sequence the product. In order to sequence the product without subcloning, either primer should be able to catalyze a polymerase chain reaction and generate the correctly sized product when present in excess. The principle is to use each primer to enrich for one strand, and then use the other primer for the sequencing reaction.

In addition, if the message for the peptides anthopleurins a and be are present in the message isolated, such primers should be able to catalyze the second strand synthesis of cDNA. Thus, another set of polymerase chain reaction was initiated, using varying ratios of the primers and either mRNA or cDNA as template.

Fig. 10 shows that the primers are effective in generating the desired product from the cDNA template as shown in lanes 7-13, for as little as a 10:1 ratio of each. Further, either primer can be used to prime the second strand synthesis of cDNA as shown in lanes 4 and 6.

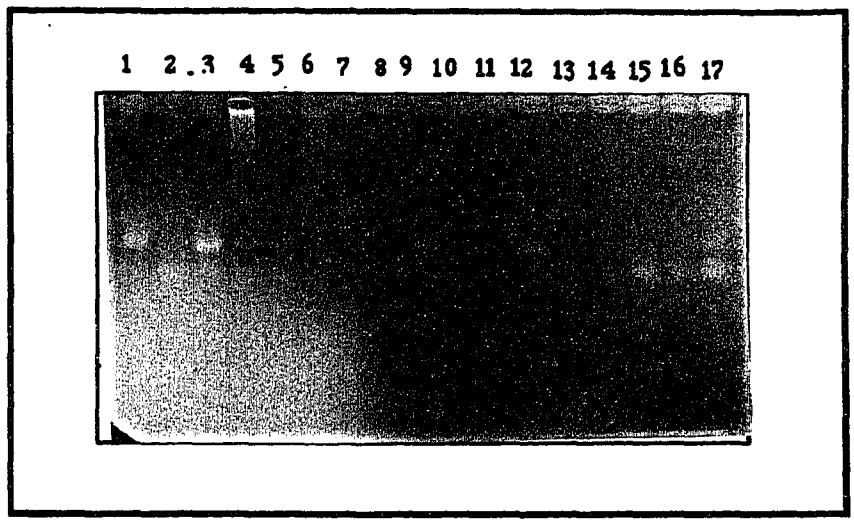


Fig. 10. Agarose gel of polymerase chain reaction products from different templates and varying primer ratios. From left, lane 1) both primers without a template, 2) KY-1 alone; 3) KY-2 alone; 4) mRNA as template, 1:10 KY-1:KY-2 ratio; 5) mRNA template 1:1 ratio of KY-1:KY-2; 6) mRNA template, 10:1 KY-1:KY-2 ratio; 7) cDNA template, 1:1 ratio of KY-1:KY-2; 8) cDNA template 10: 1 ratio KY-1:KY-2; 9) cDNA template 1: 10 ratio KY-1:KY-2 ; 10) cDNA template 1.25:1 KY-1:KY-2 ratio 11) same as 8; 12) same as 9; 13) same as 8; 14) 0 template, 10:1 ratio KY-1:KY-2; 15) 0 template 1:1 ratio KY-1:KY-2; 16) KY- alone 17) KY-2 alone

Since previous attempts to isolate genomic or cDNA clones harboring the message for the anthopleurin peptides using synthetic oligomeric probes yielded negative results, and since the product from the polymerase chain reaction yielded the correctly sized fragment, it was deemed possible to use the product from the PCR to screen the libraries.

The product from the polymerase chain reaction was first separated from the remaining primers after the reaction by concentration on Centricon filters. The concentrated product was then electroeluted from a 4 % gel. Fig. 11 shows that although the correctly sized band was prominent, the concentrated product has a large range of fragment sizes, whereas the electroeluted product consists mainly of the desired fragment.

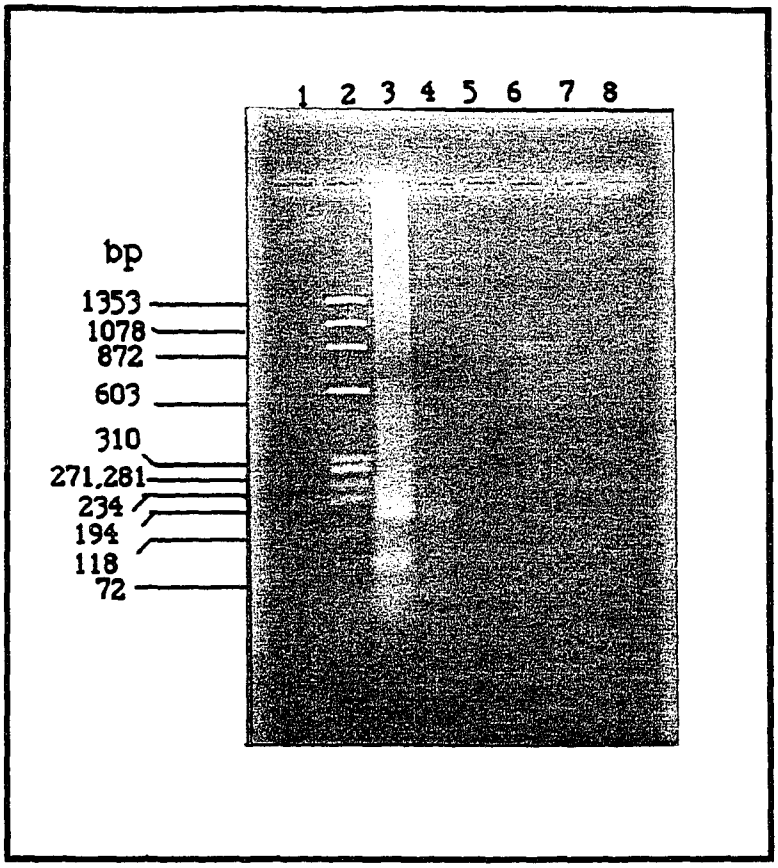


Fig. 11. The product from the polymerase chain reaction after concentration on centricon filters and after electroelution from a 4 % agarose gel. Lane 1) phi-X 174 Hae III molecular weight marker, 2) aliquot of product after concentration in centricon filter, 3) electroeluted PCR product.

E. Screening of genomic and cDNA libraries with PCR product

To determine whether the product can be used to screen the libraries, the PCR product was first used to probe genomic DNA digested with different restriction enzymes. Genomic DNA was digested with 6 base cutters Eco RI, Bam HI and Hind III, and the digests were electrophoresed on a 1% agarose gel overnight. Fig. 12 shows the complete digest of the *Anthopleura xanthogrammica* DNA as seen on the agarose gel. The range of the fragments generated was from 23 kb to less than 125 base pairs.

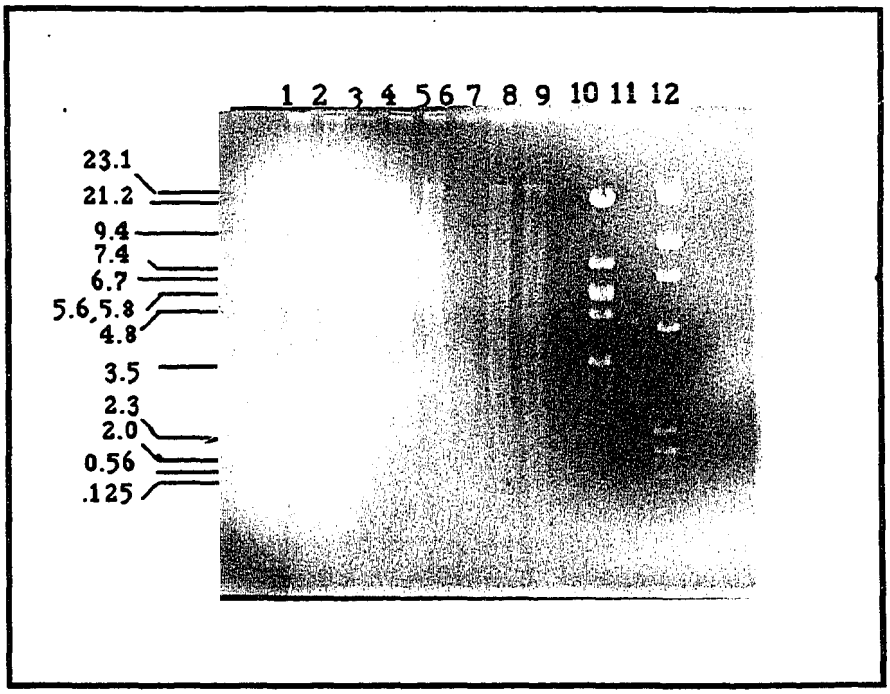


Fig. 12. Agarose gel of Genomic DNA isolated from *Anthopleura xanthogrammica* after complete digestion with Eco RI, Bam HI and Hind III. Lanes 1 and 2, genomic DNA digested with Hind III, lanes 4 and 5 genomic DNA digested with Eco RI ; lanes 7 and 8 genomic DNA digested with Bam HI; lane 10, Eco- RI λ, lane 12, Hind III λ. Each lane was loaded with either 5.6 μg genomic digest or 500 ng each of the molecular weight marker.

The DNA from the gel was allowed to transfer to a nylon membrane, and probed with the product from the polymerase chain reaction.

Fig. 13 shows the Southern blot of genomic DNA digested with three restriction enzymes and probed with the labeled product from the polymerase chain reaction.

The Eco RI digest showed 4 prominent bands corresponding to 8, 6.6, 4.1 and 3.6 kb. The Bam HI digest showed bands corresponding to 8 and 6 kb, and the Hind III digest showed 7.4 and 6.6 kb bands.

The presence of the bands that hybridized tightly with the radiolabeled probe indicates that the amplified product from the cDNA has sequence homology with genes present in the DNA of the animal. Furthermore, the presence of several bands in each of the digest suggests one of two things, that the gene is present in more than one copy, or that the digestion has not gone to completion. Alternatively, the labeled PCR product could be binding to genomic DNA sequences that have some sequence similarity with the anthopleurin peptide messages.

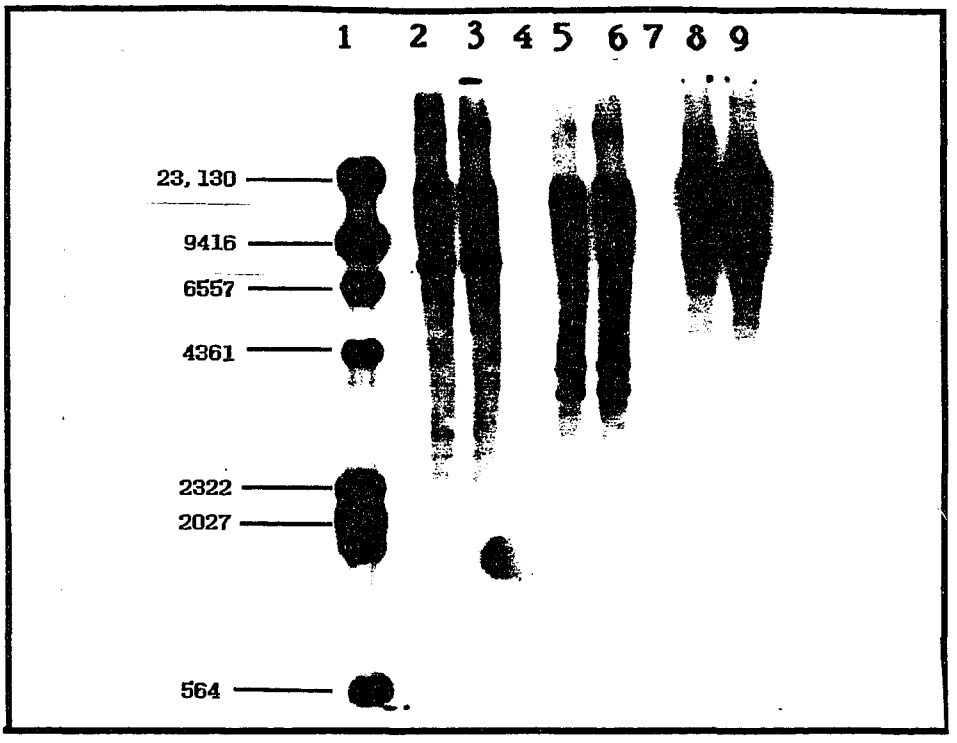


Fig. 13. Southern blot of genomic DNA isolated from *Anthopleura xanthogrammica* after complete digestion with Eco RI, Bam HI and Hind III, probed with the PCR product. Lanes 1) λ Hind III molecular weight marker, 2 and 3, genomic DNA digested with Hind III, lanes 5 and 6 genomic DNA digested with Eco RI ; lanes 8 and 9 genomic DNA digested with Bam HI; lane 10, Eco- RI λ, lane 12, Hind III λ. Each lane was loaded with either 5.6 μg genomic digest or 500 ng each of the molecular weight marker.

Using the radiolabeled product from the polymerase chain reaction as the probe, the amplified genomic library was screened for clones harboring the gene for the anthopleurin peptides.

Fig. 14 shows an autoradiogram of one of filters used in screening the library. The presence of many positive clones probably indicates amplification of similar clones. Alternatively, it could also reflect the heterogeneity of the PCR products. Thirty one genomic clones were isolated from the genomic library. Clones were confirmed positive if the filters showed an exact match upon alignment with the master plate.

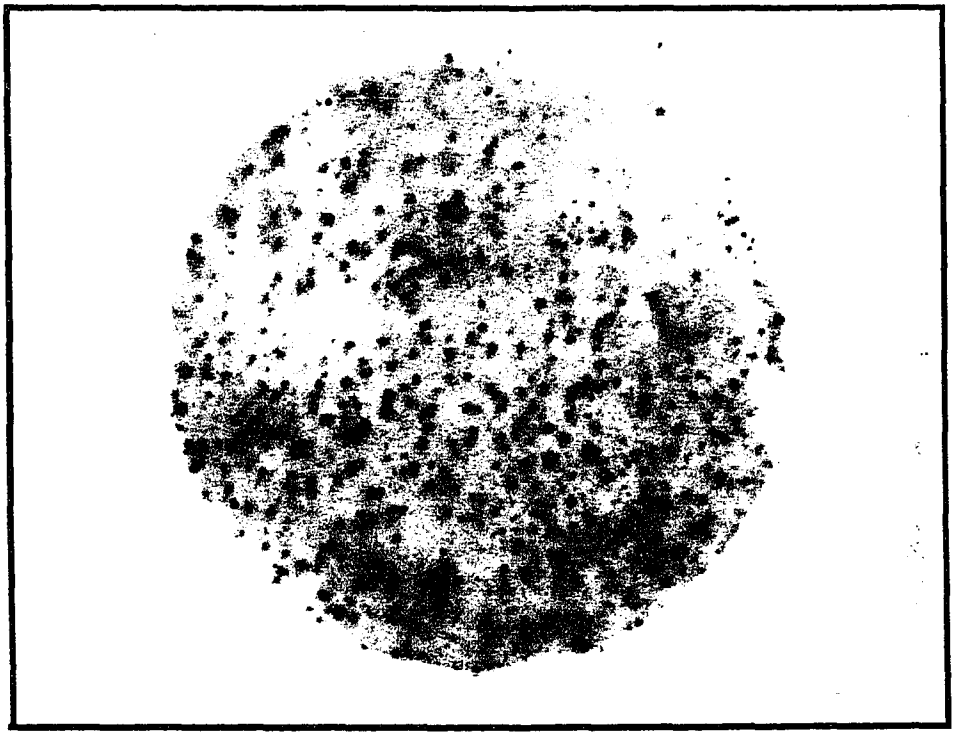


Fig 14. Autoradiogram of a filter used in *in situ* plaque hybridization of the amplified genomic library probed with the labeled PCR products

Fourteen of the 31 genomic clones isolated were investigated further. The phages were grown in *E. coli* strain LE392 and the DNA isolated. The clones were digested with Eco RI, and the digests were electrophoresed on 1% agarose gel for Southern blot. Fig. 15 shows that instead of distinct bands, a smear was obtained, the smear probably representing contamination by bacterial DNA. However, since the probe to be used for Southern blots was obtained by labeling purified products from the polymerase chain reaction and should not at all hybridize with bacterial DNA, the DNA from the digests were transferred to nylon membranes for Southern blotting.

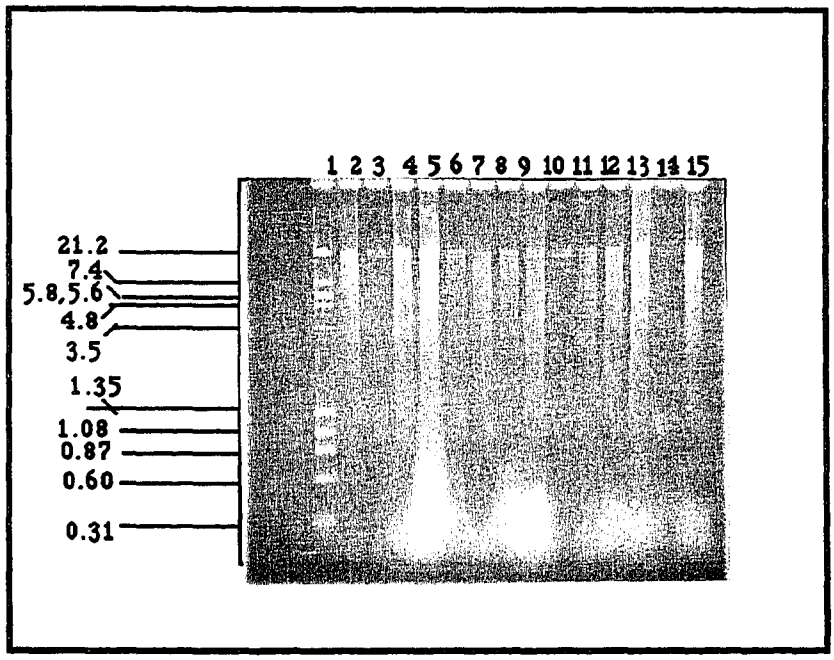


Fig. 15. Agarose gel 14 genomic clones digested with Eco RI. Lanes 1) λ-Hind III + phi- x174 molecular weight markers, 1 μg, 2) g1, 3) g2, 4) g3, 5) g11, 6) g13, 7) g15, 8) g17, 9) g18, 10) g19, 11) g22, 12) g23, 13) g24, 14) g27, 15) g29

When probed with the PCR product, clones # 24 and 29 showed a 2.8 kb Eco RI band hybridizing strongly with the labeled PCR product as shown in lanes 2 and 4 of Fig. 16. Since the autoradiograms were developed after only an overnight exposure of the film, the data strongly suggests that the clones 24 and 29 harbor the gene for the anthopleurin peptides. The presence of a bigger band that corresponds to the vector probably indicates some molecules of the 2.8 kb fragment trapped in the vector fragment.

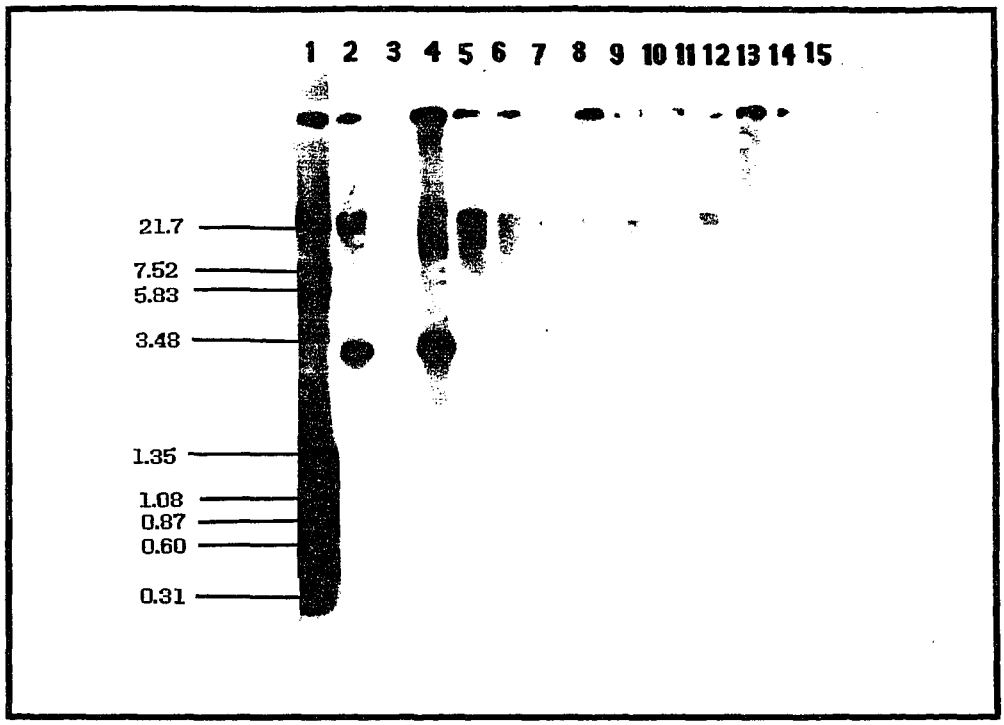


Fig. 16. Southern blot of Eco RI digests of 14 genomic clones using PCR product as probe. Lanes 1) λ-Hind III + phi X-174, 2) g29, 3) g27, 4) g24, 5) g23, 6) g22, 7) 19, 8) g18, 9) g17, 10) g15, 11) g13, 12) g11, 13) g3, 14) g2, 15) g1.

Because previous digestion of the genomic DNA with Eco RI did not show a 2.8 kb band, another set of digestion of the genomic DNA with the same set of restriction enzymes was done. Similarly, λ clones g24 and g29 were subjected to restriction enzyme digestion and were electrophoresed alongside of the genomic DNA digests, in order to verify the result of the previous Southern blot. Fig. 17 shows that λ clones were releasing the inserts. The Bam HI digest pattern of the two clones appear similar as shown in lanes 8 and 9, but the Hind III digests as shown in lanes 11 and 12 were different suggesting that the two clones are distinct from one another.

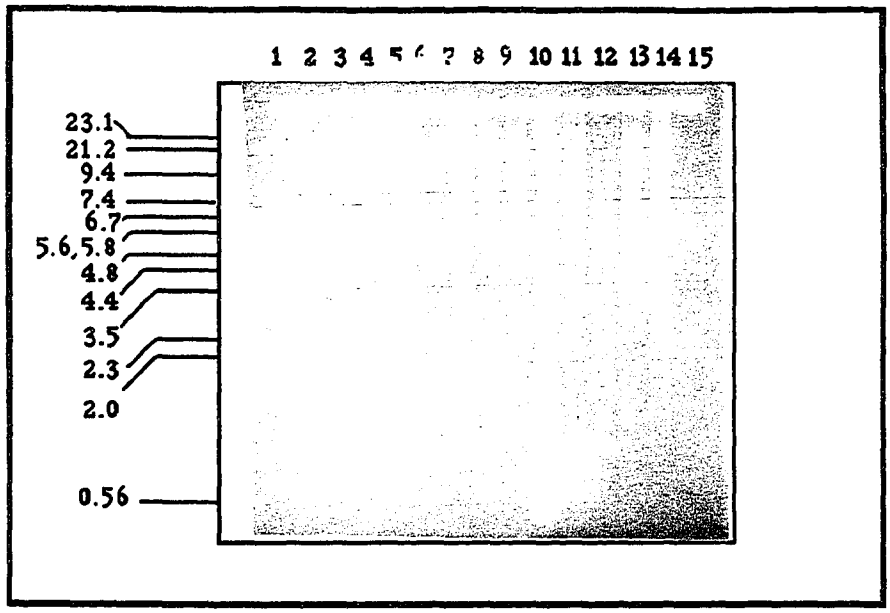


Fig. 17. Comparison of genomic DNA and genomic clones digested with Eco RI, Bam HI and Hind III electrophoresed on a 1% agarose gel. Lane 1) Eco RI-λ; 2) Hind III-λ; 3) g24 Eco RI digest, 4) g29 Eco RI-digest, 5) genomic DNA Eco RI-digest, 6) Eco RI-Bam HI double digest of clone 24, 7)genomic DNA Bam HI- digest, 8) g24 Bam HI digest, 9) g29 Bam HI digest, 10) genomic DNA Hind III digest, 11) g24 Hind III-digest, 12) g29 Hind III digest, 13) g1 Eco RI digest; 14) g3 Eco RI digest. Ten μg of the genomic digest and 6μg of the genomic clone digests were loaded in each well.

When the different digests of the two clones were probed with the radiolabeled PCR product, the autoradiogram shown in Fig. 18 was obtained.

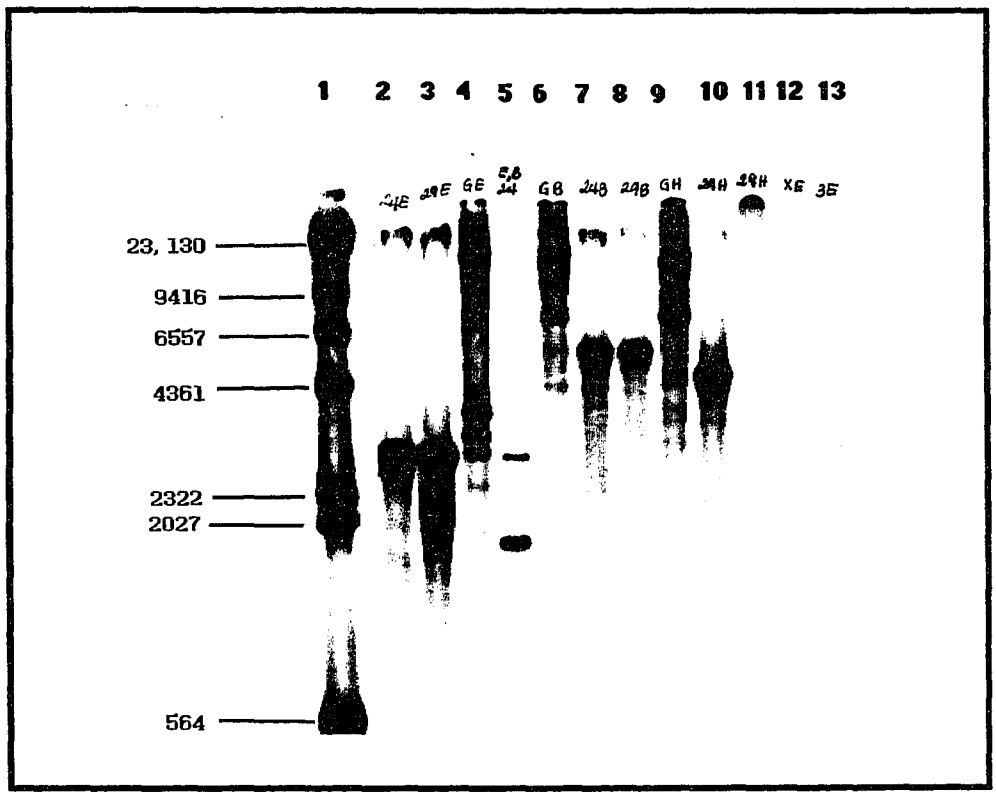


Fig. 18. Southern blot of genomic DNA and genomic clones digested with Eco RI, Bam HI and Hind III, using labeled PCR product as the probe. Lane 1) Eco RI-λ; 2) Hind III-λ; 3) g24 digested with Eco RI; 4) g29 digested with Eco RI; 5) genomic DNA digested with Eco RI; 6) Eco RI-Bam HI double digest of clone 24; 7)genomic DNA Bam HI digest; 8) g24 Bam HI digest; 9) g29 Bam HI digest; 10) genomic DNA Hind III digest; 11) g24 Hind III digest; 12 g29 Hind III digest; 13) g1 Eco RI digest; 14) g3 Eco RI digest.

Genomic clones # 24 and # 29 were digested with Eco RI, Bam HI and Hind III, and redigestion of the genomic DNA was also done. When reprobed with the labeled PCR product, the autoradiogram of genomic DNA digested with Eco RI showed 4 strong bands corresponding to 4.1, 4.0, 3.6 and 2.8 kb. The 6.6 kb band previously present (Fig. 15) was more faint, suggesting that it was being split into the 2.8 and 4 kb bands. In comparison with the previous digestion (Fig. 13), the strong 8 kb band and a more faint 5.8 and 4.4 kb bands are present in the genomic DNA digested with Bam HI. On the other hand, digestion of the genomic DNA with Hind III gave a strong 7.4 kb band, and in place of the 6.6 kb band, 4 fainter bands corresponding to 5.0, 4.4, 3.8 and 3.4 kb again suggesting that the previous digestion had not gone to completion. Hybridization of the 2 clones digested with Eco RI with the labeled PCR product yielded a strong 2.8 kb band, whereas digestion with Bam HI gave different results. In both clones, a 5.8 kb Bam HI fragment was present, consistent with the presence of a faint 5.8 kb Bam HI fragment on the Bam HI restricted genomic DNA. In addition to the strong 5.8 kb band present in clone # 24, there is also a fainter signal corresponding to 4.4 kb, indicating that the clones are different from each other and that the signals obtained represent similar inserts that are homologous to the PCR product.

Double digestion of clone #24, first with Eco RI and then with Bam HI resulted in a decrease of the 2.8 kb signal and the

appearance of a 1.8 kb band, suggesting that the 1.8 kb fragment is nested within the 2.8 kb Eco RI fragment.

In order to determine which other clones contain an insert that hybridizes to the PCR product, 8 genomic clones were digested with Bam HI and Hind III and subjected to agarose gel electrophoresis. The results are shown in Fig 19.

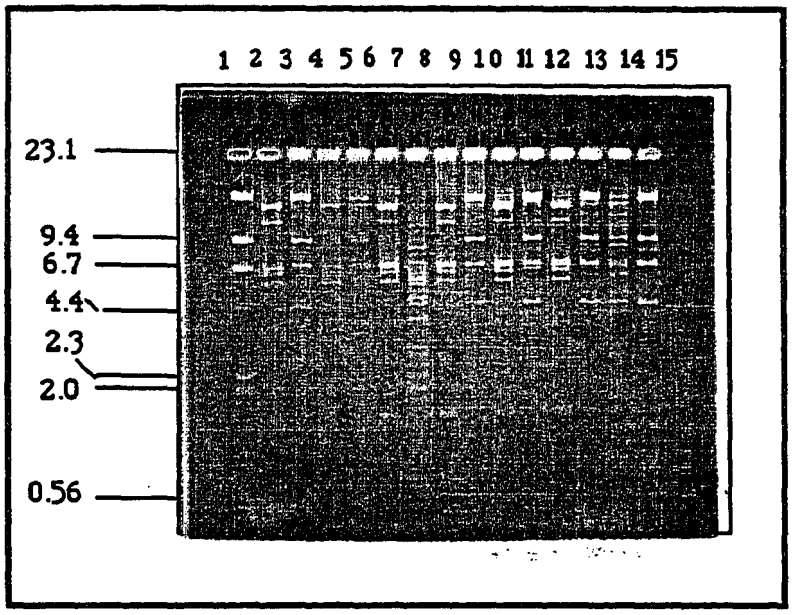


Fig. 19. Agarose gel of 8 genomic clones digested with Bam HI and Hind III. Lane 1) λ -Hind III marker; 2) g1 Bam HI- digest, 3) g1, Hind III-digest, 4) g11, Bam HI digest;, 5) g11, Hind III digest, 6) g15, Bam HI digest; 7) g15, Hind III digest, 8) g16, Bam HI digest, 9) g16, Hind III digest, 10) g18, Bam HI digest, 11) g18, Hind III digest, 12) g19, Bam HI digest, 13) g19, Hind III digest, 14) g24 Hind III digest, 15) g29, Hind III digest

The agarose gel patterns (Fig. 19) show that genomic clones #1, 11, 15, 16, 18, 19, 24 and 29 have inserts which average 17 kb. Hybridization with radiolabeled PCR product (Fig. 20) shows a 8kb, a 5.8 kb and a 1.8 kb Bam HI fragments which hybridize strongly with the labeled PCR product. These fragments were common to 5 of the 8 clones, and these fragments probably contain the whole gene or a part of it for the anthopleurin peptides. The absence of the 1.8 kb fragment from the genomic DNA restricted with Bam HI (Fig. 18) can be explained by the complete lack of a 4.4 kb Bam HI fragment from the clones. This observation suggests that there is a Sau 3A site within the 4.4. kb fragment that was cut, and was inserted into the vector.

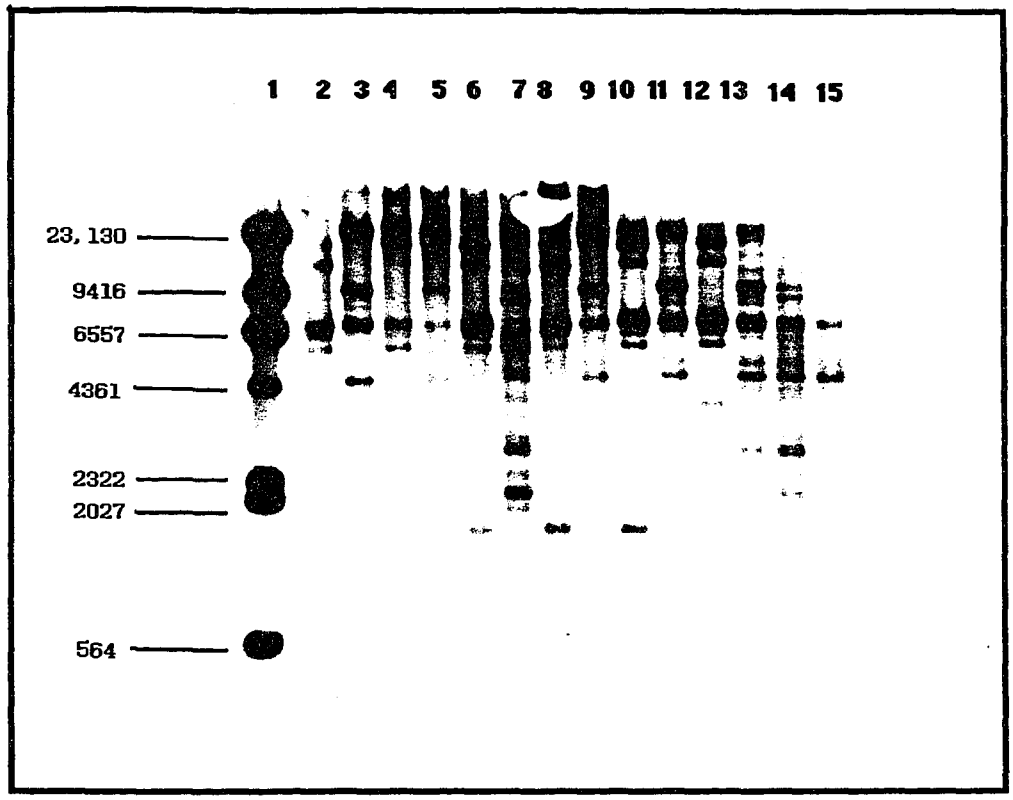


Fig. 20. Southern blot of 8 genomic clones digested with Bam HI and Hind III, probed with labeled PCR product. Lane 1) λ -Hind III marker; 2) g 1, Bam HI digest; 3) g 1, Hind III digest; 4) g 11, Bam HI digest; 5) g 11, Hind III digest; 6) g 15, Bam HI digest; 7) g 15, Hind III digest; 8) g 16, Bam HI digest; 9) g 16, Hind III digest; 10) g 18, Bam HI digest; 11) g 18, Hind III digest, 12) g 19, Bam HI digest; 13) g 19, Hind III digest, 14) g 24 Hind III digest, 15) g 29, Hind III digest

F. Cloning of the 2. 8 kb Eco RI and 1.8 kb Bam HI fragments

The 2.8 kb Eco RI fragment present in both genomic clones g24 and g29 were subcloned in the Eco RI site of M13mp18. When the subclones were digested with Eco RI, some of the subclones showed 10 and 4.4 kb inserts as shown in lanes 12, 13, 14, and 15 of Fig. 21, while the rest had a 4 and 2.8 kb inserts.

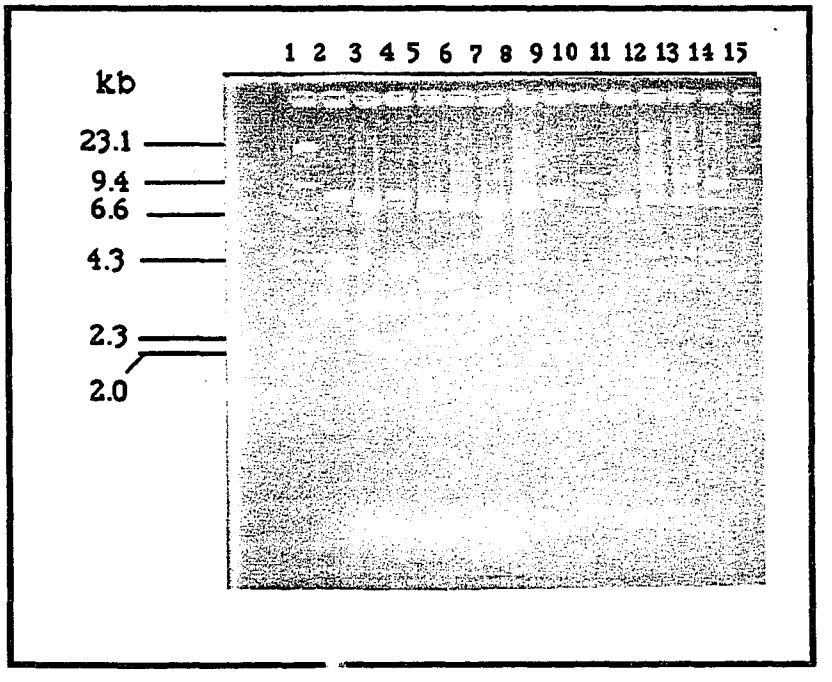


Fig. 21 . Agarose gel of Eco RI digest of the M13mp18 subclones from g24. Lanes 1) λ-Hind III molecular weight marker, 2-15) subclones 1-14.

When the digests were blotted to nylon membranes and probed with the PCR product, the autoradiogram shown in Fig. 22 was obtained. The PCR product bound to the 4 kb and 2.8 kb Eco RI fragments, but in addition, the product also bound to the M13 vector from where these fragments were released. If the PCR product in fact binds to the M13 vector by itself, all the vector lanes should show hybridization with the probe. However, this is true only of subclones 1 to 7 (lanes 2-8), which harbored the 4 kb and 2.8 kb bands. Therefore, the binding of the labeled PCR product to the vector only represents some 2.8 kb fragments tha got trapped with the vector. Alternatively, such binding could be due to some undigested M13 subclones, that being supercoiled, comigrated with the linear vector band.

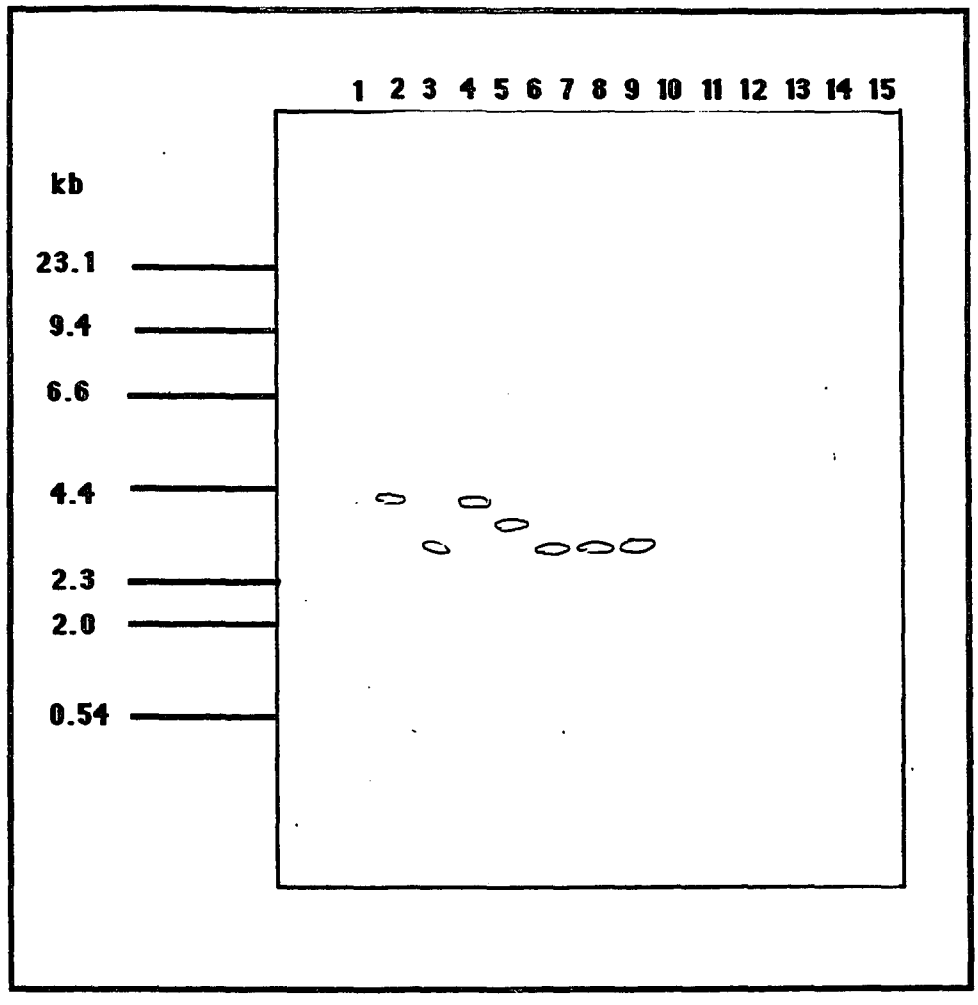


Fig. 22. Southern blot of Eco RI digest of M13 mp 18 subclones from g24. Lane 1) λ-Hind III molecular weight marker, 2-15) subclones 1-14

Since a 1.8 kb Bam HI fragment was present in 5 of the 14 genomic clones examined and since this 1.8 kb fragment seems to be nested within the 2.8 kb Eco RI fragment, the 1.8 kb fragment was subcloned into M13 mp18.

Fig. 23 shows the agarose gel pattern when the M13mp18 subclones were digested with Bam HI. Subclones 1, 4, and 5 which are subclones from g16, and 8 which is a subclone of g18 (lanes 2, 5, 6 and 9 respectively) had the expected 1.8 kb Bam HI fragments.

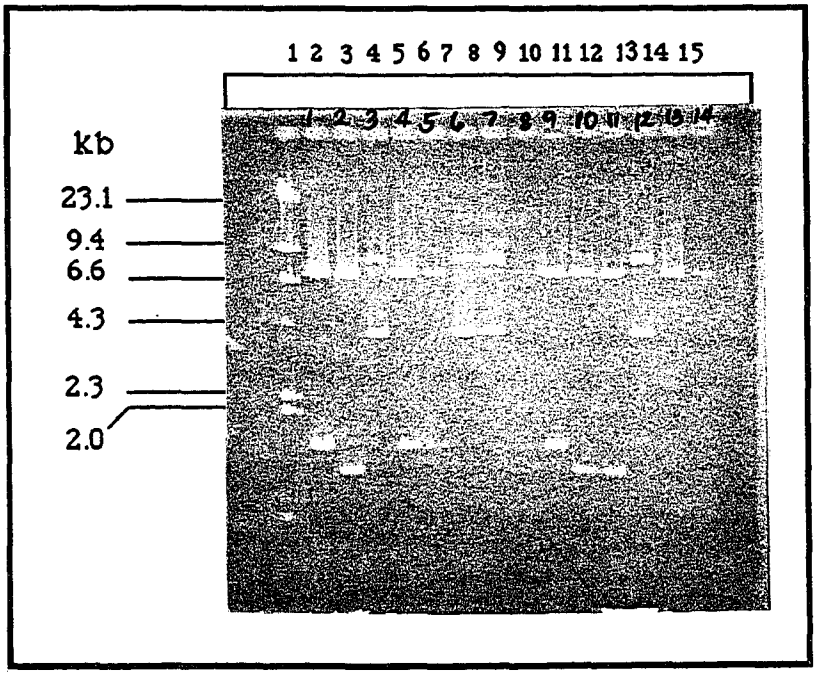


Fig. 23. Agarose gel pattern of M13mp18 subclones from g16 and g18. From left, lane 1) Hind III -λ molecular weight marker, 2-15) subclones 1-14

When the Bam HI digests were subjected to Southern blots, using the PCR product as the probe, the 1.8 kb fragment hybridized with the PCR product as shown in Fig. 24.

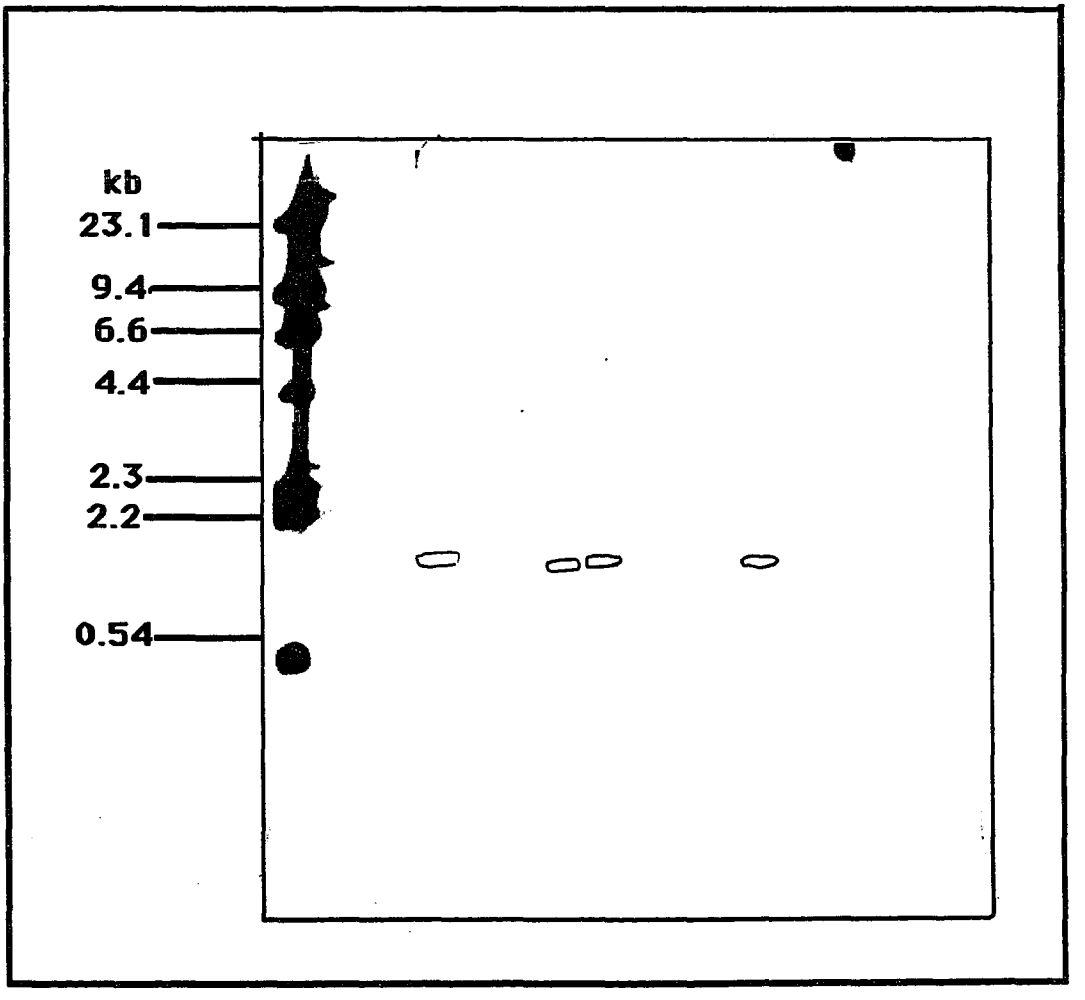


Fig. 24. Southern blot of M13mp18 subclones showing the 1.8 kb fragment hybridizing with the PCR product.

The 1.8 kb fragment present in 5 of the genomic clones was already shown to hybridize with the PCR product. To further prove that this fragment contains the gene or part of it coding for the anthopleurin peptides is its binding to the primers by themselves. Hence, another set of subclones from genomic clone 15 were digested with Bam HI. Fig. 25 shows that of the 14 subclones, there were only 2 (lanes 5 and 7) that did not contain the desired 1.8 kb fragment.

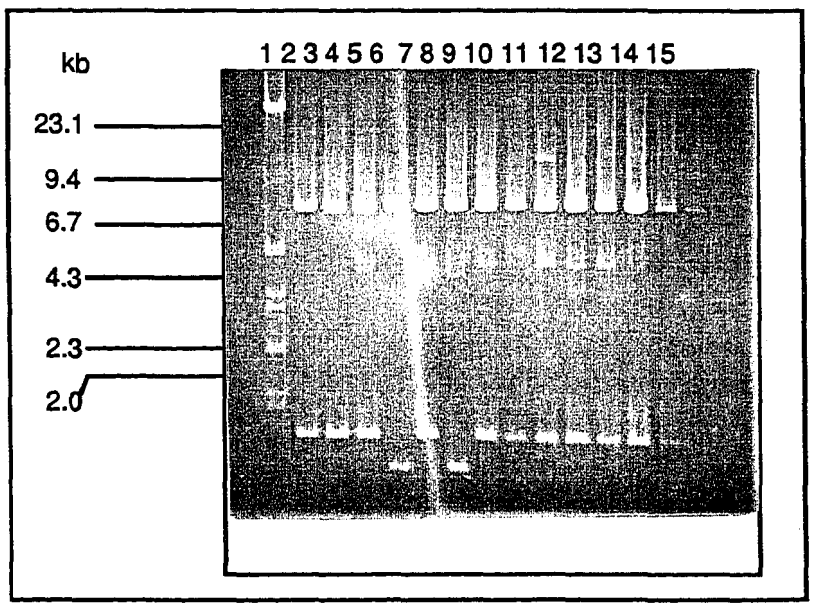


Fig. 25. Agarose gel pattern of 14 M13mp18 subclones from g15 . From left, lane 1, Hind III 1 -molecular weight marker; lanes 2-15) M13mp18 subclones 1-14

When subjected to Southern blot, using the labeled primers as the probe, the 1.8 kb Bam HI fragment from the subclones hybridized with the primers. Further, the labeled primers did not at all bind to the M13mp18 vector.

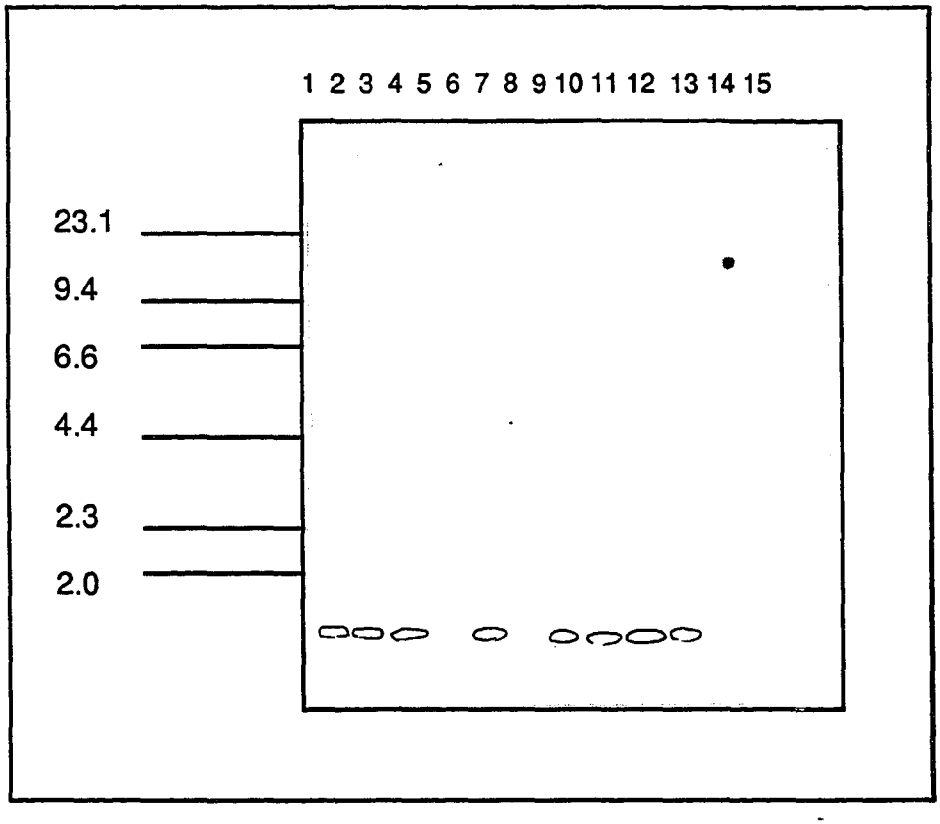


Fig. 26. Southern blot of the M13mp18 subclones probed with the labeled primers.

G. Screening of the cDNA clones with the PCR product

The PCR product was also used to screen the unamplified cDNA library, and 7 positive clones were identified.

Digestion of the cDNA clones with Eco RI resulted in the splitting of the cDNA subclones into 2 large fragments as shown in Fig. 27.

This could be due to one of three possibilities; that one or both of the Eco RI sites flanking the insert has been destroyed, or that the insert has an Eco RI site that the enzyme preferentially digested, or that there are two adjacent Eco RI sites in the insert, releasing a very small fragment that migrated very fast in the 1 % agarose gel used.

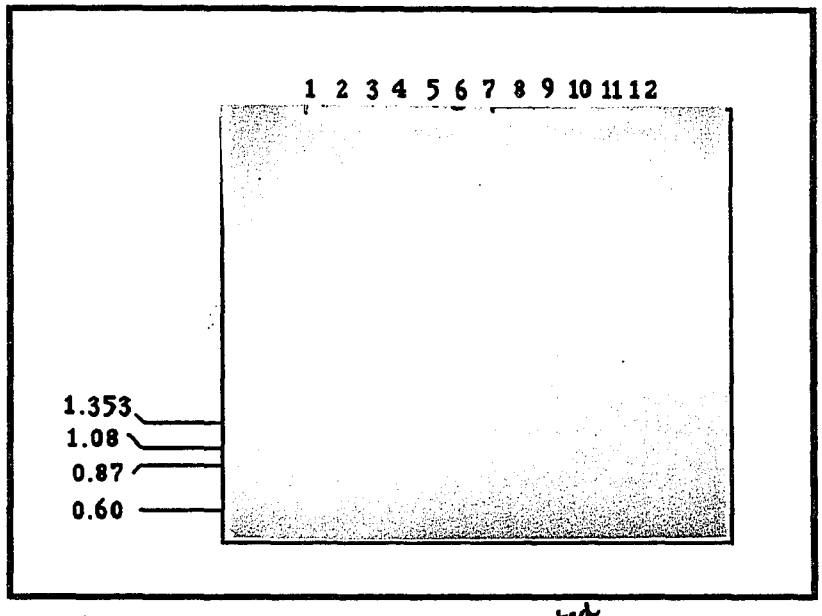


Fig. 27. Agarose gel of cDNA clones digested with Eco RI. Lanes 1) phi X174-Hae III molecular weight marker, 2) blank, 3) c1, 4) c2, 5) c3, 6) c5, 7) c6, 8) c17.

When the digest was subjected to Southern blot, both fragments hybridized with the PCR product as shown in Fig. 28, indicating that in both fragments, there are sequences homologous to the PCR product.

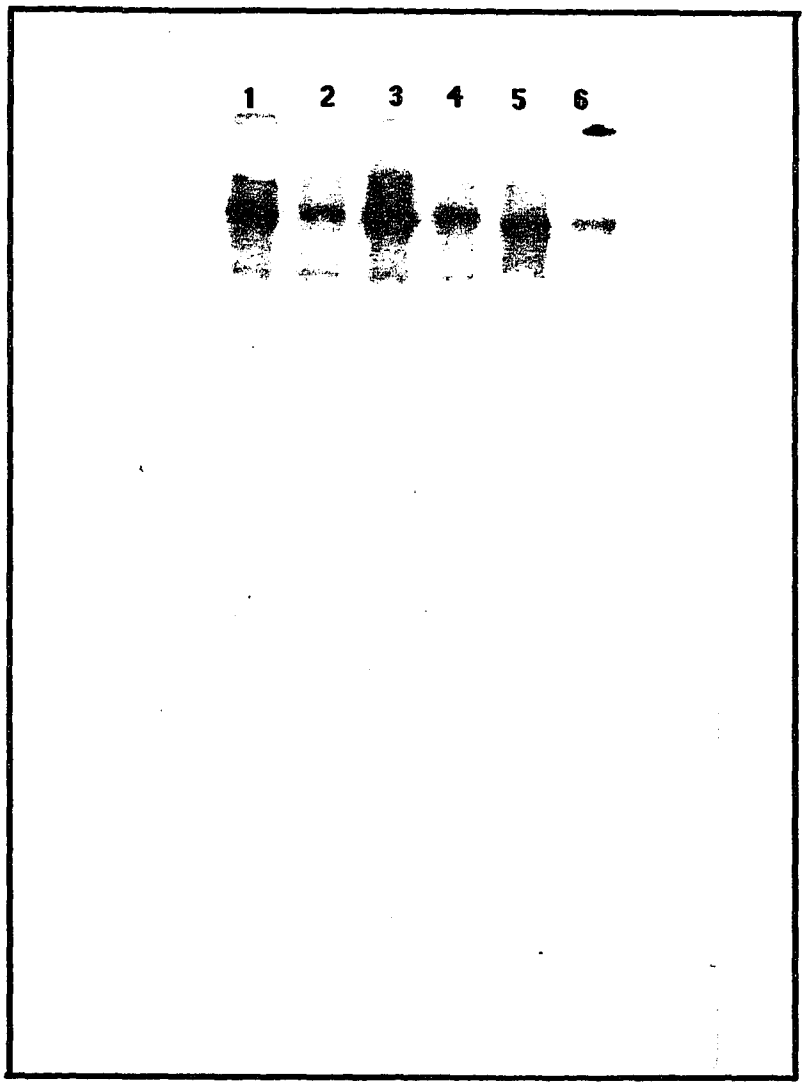


Fig. 28. Southern blot of the cDNA clones digested with Eco RI, using the PCR product as probe. From left, lanes 1) c1; 2) c2; 3) c3; 4) c5; 5) c6; 6)c17.

H. Subcloning of the cDNA inserts from λ gt10 into M13mp18 and M13mp19

Since attempts to release the insert from the cDNA clones using Eco RI proved difficult, another approach to sequencing the inserts was done. This involved digesting the cDNA clones with restriction enzymes flanking the Eco RI site, and subcloning the digests in M13 cloning vectors M13mp18 and M13mp19. The two enzymes used were Bgl II and Hind III.

In order to be certain that none of the λ vector arms will not be subcloned, the M13 vectors were digested with Bam HI and Hind III and subjected to phospatase treatment before ligation.

Of the 5 cDNA clones double digested with Bgl II and Hind III and ligated with the M13 vectors digested with Bam HI and Hind III, one M13 isolate showed an insert. Upon subsequent digestion of this clone with Eco RI, the apparent the vector released fragments that are respectively 0.9 kb, 0.8 kb, 0.48 kb and 0.3 kb, as shown in Fig. 29, indicating that the cDNA insert was about 2.5 kb.

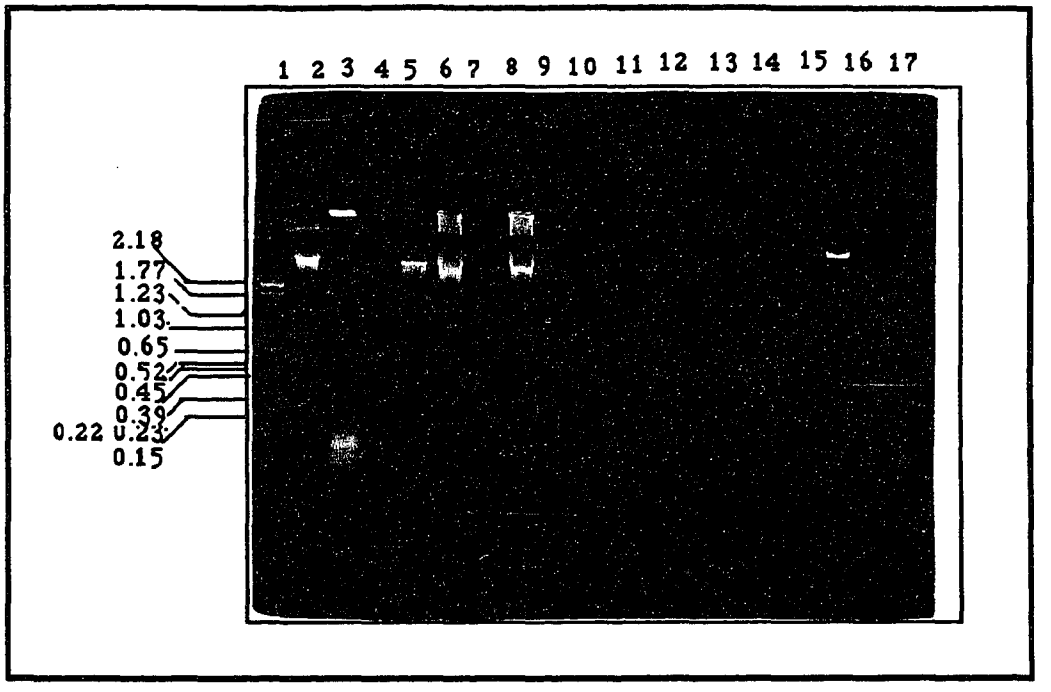


Fig. 29. Agarose gel of the M13mp18-cDNA subclones digested with Eco-RI. From left, 1) molecular weight marker, 2) uncut c3-18, 3) aliquot of PCR product after concentration in Centricon filter, 4) aliquot of PCR product after phenol freeze extraction 5) c3-18 Eco RI digest, 6) c6-19 Eco RI digest, 7) c5-19 Eco RI digest, 8) c1-18 Eco RI digest

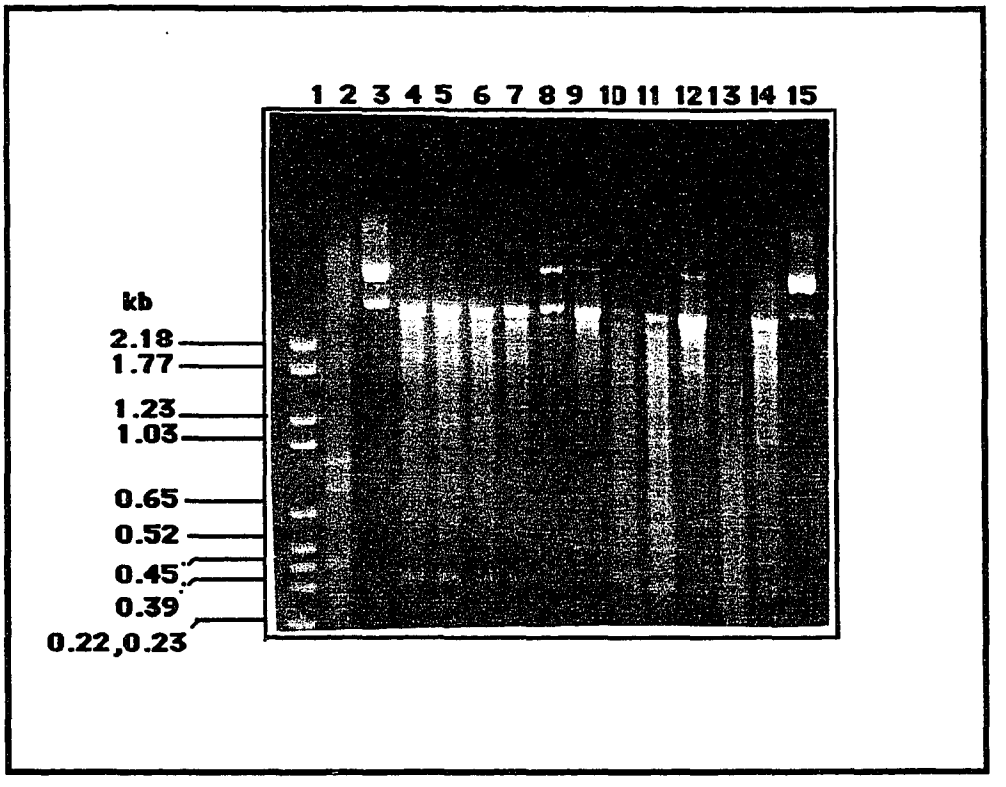


Fig. 30. Agarose gel of M13mp18 cDNA subclone and PCR product pBS subclones with Eco RI

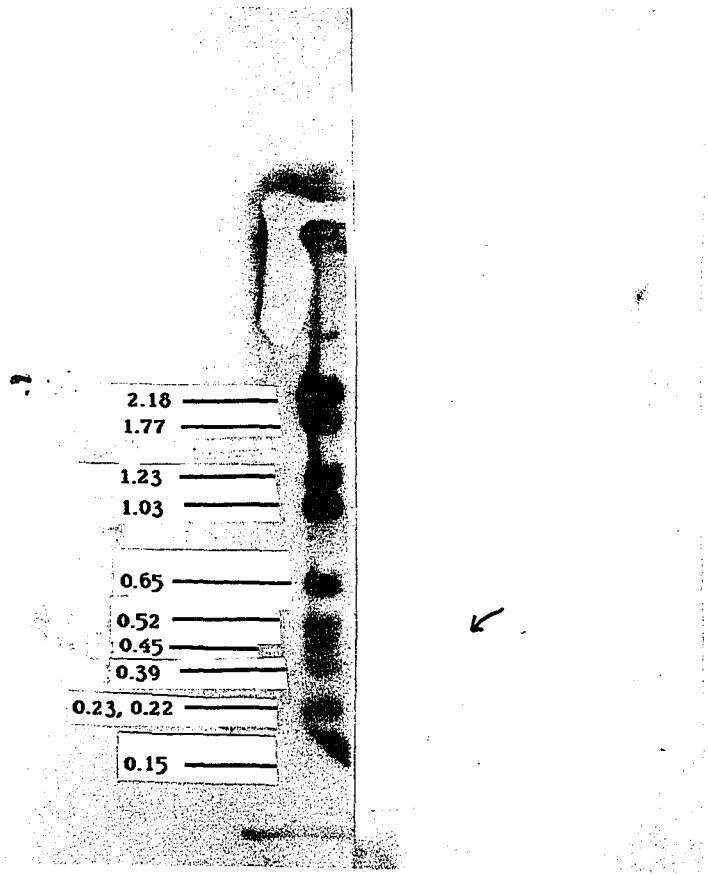


Fig. 31. Southern blot of M13mp18-cDNA subclones, using KY-1 and KY-2 as the probe.

I. Cloning and sequencing of the PCR product

Since direct sequencing of the PCR product using the primers yielded negative results, another approach was used.

The PCR product was cloned into the phagemid vector pBluescript KS II+, and the recombinants were selected in LB plates modified with ampicillin, Xgal and IPTG. In these plates only those bacteria that harbor the phagemid will grow, and and those bacteria harboring the phagemids whose b galactosidase gene has been interrupted will be colorless.

Four white colonies were randomly isolated from the plates and sequenced. To determine the length of time necessary to electrophorese the sequencing gels, the first clone pBS1 was electrophoresed for two hours initially. The sequence showed that this electrophoresis time was sufficient to read close to the priming site

Clone pBS #1 which was sequenced using 2, 3 and 4 hour electrophoretic runs showed the sequence of the vector close to the priming site (Appendix 2), as well as a 237 base pair sequence (Appendix 1), that is not at all homologous to the vector. When this 237 base pair sequence was subjected to sequence analysis and compared to the ambiguous sequence of the peptides AP-A and AP-B, there is some sequence similarity with the ambiguous sequences of both toxins (Appendix 3 and 4). The data showed that the consensus sequence of the clone has a 57 % similarity and 22 % identity with that reported for the ambiguous sequence of AP-A (Appendix 3) and a 53 % similarity with that reported for AP-B.

However upon translation into all 6 reading frames , the consensus sequence for clone pBS1 showed only a 24 % identity over a 25 amino acid overlap with the peptide anthopleurin A (Appendix 5) and 67 % identity over a 6 amino acid overlap with anthopleurin B (appendix 6)

When the 3 other clones pBS3, pBS7 and pBS8 were sequenced, they all showed sequence homology with the vector (Appendix 7).

VI. DISCUSSION

The toxins anthopleurins a and b are of interest because they bind to the region of the voltage gated sodium channel responsible for its closing. In addition, the toxins could have a therapeutic value in the treatment of heart disorders. In order to utilize these peptides to probe the sodium channel, and in order to further explore their value as therapeutic heart stimulants, the peptides should be readily available. The first step in obtaining large amounts of the toxins is cloning of the genes that code for them. Since there had been no nucleic acid studies done previously on the anthopleurin peptides, this study was undertaken to gain information on the nucleic acids coding for anthopleurins a and b.

Southern blotting of the genomic DNA with labeled PCR products. The genomic digest of the DNA of the sea anemone, *Anthopleura xanthogrammica*, was probed with the labeled PCR product. Strong bands were obtained (Fig. 13) indicating that it is indeed the animal that is producing the peptides and not the algal symbionts.

Genomic and cDNA Libraries. High molecular weight DNA was first isolated using two methods, and the modified method of Maniatis et al (1982) yielded DNA which was suitable for genomic library construction. Since there has been no published protocols for such isolation, the method reported here can be used to isolate DNA from other sea anemones. The method which employed proteinase K digestion and several phenol extractions was

probably the crucial factor in releasing the chromosomal DNA without too much shearing.

The genomic library of *Anthopleura xanthogrammica* was constructed in the *Bam* HI site of the λ cloning vector EMBL3. The primary library consisted of 543,000 clones. Based on the extrapolated value for an invertebrate genome of 8.1 $x10^8$ base pairs (Britten and Davidson, 1971) and an average insert size of 17 kb, the library consisted of DNA fragments totalling more than 10 times the putative size of the animal's genome, and hence even a single copy gene will be represented in this library. In addition to it's value in the study of the anthopleurins, this library can be used in studies of the other toxins whose amino acid structure is similar to those of the anthopleurins, among them the 47 amino acid toxin II from *Anemonia sulcata.*

The *E. coli* host strain LE392 was chosen for titering the λ EMBL3 because it lacks the *E. coli* K restriction system and will not modify inserted DNA. It is a rec+ strain and thus will allow recombination of the phages. It is also a permissive host and allows both parental and recombinant phages to grow, but since the vector arms were predigested and treated with alkaline phosphatase, it was not necessary to select against parental phages (Huynh *et al*, 1985).

The cDNA library was constructed in the unique *Eco* RI site of the cI gene in λ gt10. Insertion at this site causes inactivation of the λ repressor. Consequently, the recombinant phages are lytic and produce clear plaques, whereas parental phages are lysogenic

and produce turbid plaques. When grown in a permissive host like C600, both parental and recombinant phages grow, but when grown in a restrictive host like C600 hfl (high frequency lysogeny) as was used here the lytic phage grows in preference to the lysogenic phage.

The unamplified cDNA library consisted of 325,000 clones, which translates to a transformation efficiency of 3.25 $x10^5$ plaque forming units (pfu) /μg of the cDNA. Since λ gt10 packages from 0-7 kb of DNA, it was not possible to determine the number of messages cloned. However, since the library was titered in the non-permissive host C600 hfl, the clones must represent a large spectrum of messages that the animal expresses at the time of RNA extraction. Furthermore, both anthopleurins a and b can be extracted at any time in the life cycle of the animal, thus indicating that the peptides are constitutively produced. These facts taken together made it highly probable that the cDNA library contains the messages for both the peptides.

Screening of the amplified genomic library and the cDNA using the first two sets of synthetic oligonucleotides constructed from backtranslation of the anthopleurin B peptide yielded only spurious spots, probably because of a lack of information as to the codon usage for the amino acids comprising the peptides. Consequently, two degenerate primers spanning the first and last 10 amino acids of the peptides were constructed to determine whether the message could be amplified via the polymerase chain reaction (Saiki *et al*, 1988) using cDNA as the template.

Polymerase Chain Reaction. The polymerase chain reaction is a highly sensitive method to amplify DNA, as long as two primers that will bind the two strands of the DNA of interest can be obtained. The method was first used by Saiki *et al.* (1986) in amplifying human β globin gene. The key component of the reaction is the Taq polymerase, an enzyme isolated from *Thermus aquaticus*, which is not heat denatured up to temperatures of 95°C. The optimum temperature for activity of the enzyme is 70°C. This characteristic allows for a continuous cycle consisting of denaturation of the DNA sample at 94°C, annealing of template DNA and primer at 50°C and extension of the primer at 70°C.

Of the two oligonucleotides that were constructed for use as primers for the PCR reaction, KY-1 is a mixture of 29-mers constructed by backtranslating the first 10 amino acids of the AP-B peptide while KY-2 is a mixture of 30-mers constructed by obtaining the complement of the backtranslated amino acids 40-49 of AP-A. Both primers represent all the possible codons for the constituent amino acids. As a consequence, KY-1 is comprised of a combination of 16,384 possible sequences, while KY-2 is comprised of 12,288 possible sequences. Hence it was expected that both the AP-B and AP-A messages would be isolated, and in addition, messages related to the two peptides.

Using the two degenerate oligonucleotides as primers for the PCR reaction, a 144-147 base pair PCR product was obtained (Fig. 9). This size is that expected as both AP-A and AP-B are 49 amino acid peptides, and the uninterrupted message should be a

sequence of 147 base pairs. It is interesting to note that even when genomic DNA was used as the template, a similarly sized product was obtained (Fig. 8), suggesting that either or both of the peptides are encoded for by a single exon.

The PCR product was separated from the primers by concentration on a Centricon filter, labeled by random priming and used to probe the genomic DNA, and to probe the genomic DNA and to screen both the genomic and cDNA libraries.

Screening of the genomic library with the product from the polymerase chain reaction. When complete digests of the genomic DNA were probed with the PCR product, strong signals were obtained, indicating that the genomic DNA of *Anthopleura xanthogrammica* contains sequences that are similar to that of the amplified product (Fig. 13).

The *Eco* RI digest of the genomic DNA showed 4 prominent bands that hybridized tightly with the radiolabeled PCR product. The bands corresponed to 8, 6.6, 4.1 and 3.6 kb. The *Bam* HI digest showed bands corresponding to 8 and 6 kb bands, while the Hind III digest showed 7.4 kb and 6.6 kb bands.

Upon screening the genomic and cDNA libraries with the PCR product, 31 genomic clones and 7 cDNA clones were isolated. When reprobed with the PCR product, these clones consistently showed strong signals indicating that they contained sequences that are similar to those of the PCR product.

The genomic and cDNA clones were examined for the presence of sequences that could contain all or part of the gene for the anthopleurin peptides.

Previous digestion of the genomic DNA with *Eco* RI (Fig. 13) showed 4 prominent bands corresponding to 8, 6.6, 4.1 and 3.6 kb. Subsequent digestion of the genomic DNA with *Eco* RI as shown in Fig. 19 shows that the 6.6 kb band that was previously present became more faint, and a 4.0 and 2.8 kb fragments appeared, indicating that the 6.6 kb fragment was being split into the 4.0 and 2.8 kb fragments, and that each fragment contained sequences that are homologous to the sequence of the PCR product, indicating that the gene was being split.

Of the 14 genomic clones examined 3 clones contained a 2.8 kb *Eco* RI fragment that hybridized strongly with the PCR product (Fig. 18) and 5 contained a 1.8 kb *Bam* HI fragment that also gave a strong signal with the labeled probe (Fig. 20).

These fragments were subsequently subcloned into M13 and probed with the primers used in the PCR reaction, and found to hybridize also with the primers alone (Fig. 22 and 26), suggesting that there are sequences in the clones that are similar to those of the primers. These clones could contain part or all of the gene for the peptides of interest. Fig. 23 and 24 show that when the recombinant M13mp18 were subjected to restriction enzyme digestion, the 2.8 kb fragment was released, and and it also hybridized with the PCR product (Fig. 24)

Digestion of genomic clone #24 first with *Eco* RI and then with *Bam* HI (Fig. 18) showed that there was a marked reduction of the 2.8 kb signal and a consequent appearance of a strong 1.8 kb band, indicating that there is at least one *Bam* HI site nested within the 2.8 kb *Eco* RI fragment, and that this fragment contains the sequences of interest, since the remaining 1 kb fragment did not show a signal with the labeled probe.

Since the1.8 kb *Bam* HI fragment appears to be nested within the 2.8 kb *Eco* RI fragment, and since the 1.8 kb fragment seems to be common to the 5 clones, it was subcloned into M13mp18 for future sequencing.

M13 was used as a subcloning vehicle mainly for ease of generating a single-stranded template for sequencing. M13 is a single-stranded filamentous phage which replicates inside the host, first by forming a double stranded circular DNA termed the replicative form (RF). When about 200 copies of RF are formed inside the host, it starts to package and extrude the single stranded form of DNA. The reason for this is that once the host has made about 200 copies of the RF, the protein product of gene V accumulates at high levels and binds the + strand of the phage DNA, thus preventing further formation of RF. The + strand assembles with coat proteins to form a single stranded mature phage.

This DNA is extruded into the medium, and can therefore be collected without lysing the host. The RF form can be obtained by alkaline lysis of the host. This property of M13 allows it to be

used as a cloning vector for sequencing. The RF can be digested with restriction enzymes. The DNA of interest can then be ligated with the RF form, if the insert was generated using the same enzyme.

The M13 derived vectors contain the 5' regulatory region coding for the first 145 amino acids of the *E. coli* lac operon (Messing 1977). These sequences code for the α peptide of the enzyme β galactosidase. The polylinker cloning site is so arranged that the correct reading frame is maintained. When expression of this gene is induced, for example by IPTG, in a host which lacks a functional α peptide but produces the wild type ω peptide, the two polypeptides assemble to form a functional enzyme, and galactose is utilized as a carbon source.

The insertion of the DNA of interest disrupts the β galactosidase gene, and when bacteria transfected with the phage are plated in a medium containing X-gal (5-bromo-4 chloro-3-indolyl-β-galactoside), a chromogenic dye, the plaques generated by phages containing an insert will appear colorless. In contrast, those phages which do not have an insert will appear blue as complementation between the host and the phage will allow the β galactosidase gene to be expressed, and a blue product of the reaction is formed. This allows for a simple screening procedure.

The white colonies can then be screened for the right sized insert easily, and upon finding the putative recombinants of interest, sequenced.

Since the single stranded form is extruded into the medium, it allows for an easy method of obtaining template for sequencing.

The PCR product was also used to screen the cDNA library, and seven cDNA clones were isolated. Since the cDNA was cloned in the single *Eco* RI site of the cloning vector λ gt10, the clones were digested with *Eco* RI in an attempt to release the insert.

When the seven cDNA clones were digested with *Eco* RI to release the insert, there were only two bands that could be obtained, both of which hybridized strongly with the labeled PCR product. The two fragments released were very large, which probably corresponded to the vector sizes. This observation suggests several explanations: that the insert could not be released because the *Eco* RI sites were destroyed when the adaptor was ligated into the blunt ended cDNA; and that there is an *Eco* RI site within the insert that is cleaved leaving part of the insert with the arms of the vector λ gt10. Another possibility is that there is more than one *Eco* RI site in the insert, and that the fragment released was too small in comparison with the arms, to be seen in gels. If the latter were true, the released small fragment could not be detected in the Southern blots because it would migrate very fast in the 1.0 % gels used for the Southern blots. Indeed such resistance to restriction enzyme digestion after cloning into the *Eco* RI site of both λ gt10 and the expression vector λgt 11 have been previously reported (Helms *et al*, 1985; Ziai *et al*, 1988).

Since it was not feasible to sequence the cDNA clones directly, another approach was attempted, this time involving double digestion of the cDNA clones with two enzymes flanking the *Eco* RI site, and subcloning the digest in an M13 vector. Using this method one subclone was isolated containing a 480 basepair *Eco* RI fragment that hybridized with the primers (Fig. 31), suggesting that this subclone harbors part of the message for either AP-A or AP-B. Similarly, sequencing the PCR product directly was attempted but yielded negative results presumably because the primers used were too degenerate. As noted previously, KY -1, the mixture of 30 -mers of synthetic oligonucleotides coding for the first 10 amino acids of the anthopleurin peptides represents 16,384 possible sequences, while KY-2 represents 12,288 possible sequences. This means that in order to compensate for the degeneracy of the primers, the primer concentration should be raised, however, doing so could effectively inhibit the enzyme as the primers could chelate the $MgCl_2$.

Other problems with sequencing the PCR is that small linear molecules rapidly reanneal, preventing annealing of the desired primer with the template. In addition, depending on the base composition of the DNA, small linear DNA molecules may exhibit regions of strong secondary structures that impede the progress of the polymerase along the template. The problem of formation of the secondary structure can be alleviated by raising the reaction temperature but polymerases such as AMV reverse transcriptase,

the Klenow fragment of DNA polymerase I, and T7 DNA polymerase are rapidly deactivated at elevated temperatures.

Since the PCR product could not be sequenced directly the product was cloned into pBluescript KS II+ and 4 white colonies were chosen randomly and sequenced.

A sample of Clone pBS #1 was electrophoresed at three different lenghts of time. The two hour sequence showed regions that are close to the priming site (Appendix 2), right at the region of the Sma I cloning site, and was therefore used as the basis for electrophoresing the samples of other PCR subclones.

The three other clones that were sequenced pBS3, pBS7, and pBS8 showed homology with each other, as well as to the vector (Appendix 7). All clones showed homology with the vector at regions different from the Sma 1 cloning site as it was in pBS1. This indicates that the color selection was not perfect. It is known that the absence of a single nucleotide in β galactosidase gene is enough to offset color selection.

A possible explanation could be the presence of nuclease activity in the calf intestinal phosphatase used to dephosphorylate the vector (David Haymer, personal communication) which could have degraded the vector. This is in fact evident in the gaps shown in the comparisons.

There was no region of overlap when clone pBS1 was sequenced at 3 different lengths of times were compared to each other which suggests that clone pBS1 was a composite of more than one PCR product which ligated to each other. This is possible

since individual comparisons of the three 1-hour electrophoresis runs showed that each of the three has sequence homology with the anthopleurin peptides (Appendix 1). This being the case, the three sequences were aligned to each other to give maximum overlap. The results showed that there was correspondence with the anthopleurin peptides at amino acids 1, 5, 10, 11, 12, 19, 22, 24 and 25. The sequence that was cloned does not translate to the original peptide since the PCR product that was generated by using very degenerate primers is a heterogeneous mixture, and the clone that was sequenced was not the correct one.

When a comparison of the 2, 3 and 4 hour sequences of pBS1 were done against the ambiguous and unambiguous sequences of the backtranslation of the two peptides using the codon for prokaryotes, drosophila and yeast, the data obtained showed that glycine is preferentially coded for by GGC, cysteine by TGA, proline by CCT, leucine by CTC, and arginine by CGA. Such information can be used in designing a more specific primer for screening the libraries of the sea anemone *Anthopleura xanthogrammica* or, in designing a better primer for amplifying cDNA from the animal.

VII. SUMMARY AND CONCLUSIONS

Genomic and cDNA libraries of the sea anemone, *Anthopleura xanthogrammica* were constructed in lambda replacement vectors, in order to investigate the 49 amino acid peptide toxins, anthopleurins a and b at the nucleic acid level. The libraries were screened, first with synthetic nucleotides, and then with a PCR product.

However, the use of primers in screening the cDNA and genomic libraries of the sea anemone *Anthopleura xanthogrammica* was not effective in isolating clones that harbor the message for the peptides anthopleurins a and b.

The use of degenerate primers in priming both the cDNA and genomic DNA for the polymerase chain reaction generates the correctly sized PCR product. The degeneracy of the primers used effectively eliminated their further use in directly sequencing the PCR product.

The 144-147 base pair PCR product was used in isolating the putative cDNA clones that may harbor the anthopleurin peptide messages. A 480 base pair cDNA fragment that hybridized with the primers was isolated from one of the cDNA clones, and subcloned into M13mp18 vector.

The PCR product was also used to isolate genomic clones that may harbor the gene for the peptide toxins.

A 2.8 kb Eco RI and a 1.8 kb *Bam* H1 genomic DNA fragments that hybridized with the PCR product and the primers were subcloned into M13mp18 vector.

Cloning and sequencing the PCR product generated offers an alternative way of characterizing the message for the small peptides anthopleurins a and b. However, the use of degenerate primers leads to the generation of multiple heterogeneous PCR products, some of which have limited homology to the desired sequence.

The data obtained from sequencing the PCR products can be used to design less degenerate primers, to prime cDNA.

APPENDIX 1. Comparison of the sequence of the clone pBS1 with the ambiguous and unambigous sequences of the AP-A and AP-B sequences. N=A,T,G, or C;Y=T or C; S=G or C; H=T or C;M=C or A;W=T or A or C; R=A or G.

Amino Acid	Ap-A Amb	Ap-b Amb	pBS1 2hr	pBS1 3hr	pBS1 4hr	Ap-B Prok	Ap-B Dros	Ap-B Yeast	Ap-A Prok	ApA Yeast	Ap-A Dros	
	GLY	GLY	GLY			GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	G			G	G	G	G	G	G	+
1	G	G	G			G	G	G	G	G	G	+
	N	N	C			T	C	T	T	T	C	+
	VAL	VAL	GLU			VAL	VAL	VAL	VAL	VAL	VAL	
	G	G	G			G	G	G	G	G	G	+
2	T	T	A			T	T	T	T	T	T	-
	N	N	A			T	G	T	T	T	G	+
	SER	PRO	LEU	TYR		PRO	PRO	PRO	SER	SER	SER	
	W	W	T	T		C	C	C	T	T	T	+
3	S	S	T	A		C	C	C	C	C	C	-
	N	N	G	T		G	C	A	C	T	C	+
	CYS	CYS	GLU	ALA		CYS	CYS	CYS	CYS	CYS	CYS	
	T	T	G	G		T	T	T	T	T	T	-
4	G	G	A	C		G	G	G	G	G	G	-
	Y	Y	G	T		C	C	T	C	T	C	+
	LEU	LEU	LEU	GLU		LEU	LEU	LEU	LEU	LEU	LEU	
	Y	Y	C	G		C	C	C	C	C	C	+
5	T	T	T	A		T	T	T	T	T	T	+
	N	N	C	G		G	G	G	G	G	G	+
	CYS	CYS	HIS	STOP		CYS	CYS	CYS	CYS	CYS	CYS	
	T	T	C	T		T	T	T	T	T	T	+
6	G	G	A	G		G	G	G	G	G	G	+
	Y	Y	C	A		C	C	T	C	T	C	+
	ASP	ASP	ARG	TYR		ASP	ASP	ASP	ASP	ASP	ASP	
	G	G	C	T		G	G	G	G	G	G	-
7	A	A	G	A		A	A	A	A	A	A	+
	Y	Y	C	T		C	C	C	C	C	C	+
	SER	SER	GLY	PRO		SER	SER	SER	SER	SER	SER	
	W	W	G	C		T	T	T	T	T	T	-
8	S	S	G	C		C	C	C	C	C	C	+
	N	N	T	C		C	C	T	C	T	C	+

Amino Acid	Ap-A Amb	Ap-b Amb	pBS1 2hr	pBS1 3hr	pBS1 4hr	Ap-B Prok	Ap-B Dros	Ap-B Yeast	Ap-A Prok	ApAAp-A Yeast	Dros	
	ASP	ASP	GLY	GLY		ASP	ASP	ASP	ASP	ASP	ASP	
	G	G	G	G		G	G	G	G	G	G	+
9	A	A	G	G		A	A	A	A	A	A	-
	Y	Y	C	T		C	C	C	C	C	C	+
	GLY	GLY	GLY	TYR		GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	G	T		G	G	G	G	G	G	-
10	G	G	G	A		G	G	G	G	G	G	+
	N	N	A	C		T	C	T	T	T	C	+
	PRO	PRO	ARG	PRO		PRO	PRO	PRO	PRO	PRO	PRO	
	C	C	C	C		C	C	C	C	C	C	+
11	C	C	G	C		C	C	C	C	C	C	+
	N	N	C	T		G	C	A	G	A	C	+
	SER	ARG	SER	ARG		ARG	ARG	ARG	SER	SER	SER	
	W	M	T	C		C	C	A	T	T	T	+
12	S	G	C	G		G	G	G	C	C	C	+
	N	N	T	A		T	C	A	C	T	C	+
	VAL	PRO	GLU	GLY	ILE	PRO	PRO	PRO	VAL	VAL	VAL	
	G	C	A	G	A	C	C	C	G	G	G	+
13	T	C	G	G	T	C	C	C	T	T	T	+
	N	N	A	T	C	G	C	A	T	T	G	+
	ARG	ARG	ILE	GLY	LEU	ARG	ARG	ARG	ARG	ARG	ARG	
	M	M	A	G	T	C	C	A	C	A	C	+
14	G	G	T	G	T	G	G	G	G	G	G	+
	N	N	A	A	G	T	C	A	T	A	C	+
	GLY	GLY	VAL	ARG	ILE	GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	G	C	A	G	G	G	G	G	G	+
15	G	G	T	G	T	G	G	G	G	G	G	+
	N	N	G	A	C	T	C	T	T	T	C	+
	ASN	ASN	GLY	ARG	THR	ASN	ASN	ASN	ASN	ASN	ASN	
	A	A	G	C	A	A	A	A	A	A	A	+
16	A	A	A	G	C	A	A	A	A	A	A	+
	Y	Y	T	A	C	C	C	C	C	C	C	+
	THR	THR	PRO	PRO	SER	THR	THR	THR	THR	THR	THR	
	A	A	C	C	T	A	A	A	A	A	A	-
17	C	C	C	C	C	C	C	C	C	C	G	+
	N	N	C	G	A	C	C	C	C	C	C	+

Amino Acid	Ap-A Amb	Ap-b Amb	pBS1 2hr	pBS1 3hr	pBS1 4hr	Ap-B Prok	Ap-B Dros	Ap-B Yeast	Ap-A Prok	ApA Yeast	Ap-A Dros	
	LEU	LEU	PRO	THR	GLY	LEU	LEU	LEU	LEU	LEU	LEU	
	Y	Y	C	A	G	C	C	T	C	T	C	+
18	T	T	C	C	G	T	T	T	T	T	T	-
	N	N	G	G	G	G	G	G	G	G	G	+
	SER	SER	VAL	SER	ALA	SER	SER	SER	SER	SER	SER	
	W	W	G	A	G	T	T	T	T	T	T	-
19	S	S	T	G	C	C	C	C	C	C	C	+
	N	N	A	C	C	C	C	T	C	T	C	+
	GLY	GLY	PRO	GLU	ASP	GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	C	G	G	G	G	G	G	G	G	+
20	G	G	C	A	A	G	G	G	G	G	G	-
	N	N	C	G	C	T	C	T	T	T	C	+
	THR	ILE	TYR	ARG	VAL	ILE	ILE	ILE	THR	THR	THR	
	A	A	T	C	G	G	G	G	G	G	G	+
21	C	T	A	G	T	G	G	G	G	G	G	+
	N	H	C	A	C	T	C	T	T	T	C	+
	LEU	LEU	VAL	GLY	LEU	LEU	LEU	LEU	LEU	LEU	LEU	
	Y	Y	G	G	C	C	C	T	C	T	A	+
22	T	T	T	G	T	T	T	T	T	T	C	+
	N	N	T	A	T	G	G	G	G	G	C	-
	TRP	TRP	LEU	ASP	LYS	TRP	TRP	TRP	TRP	TRP	TRP	
	T	T	T	G	A	T	T	T	T	T	T	+
23	G	G	T	A	A	G	G	G	G	G	G	-
	G	G	G	T	G	G	G	G	G	G	G	+
	LEU	PHE	LEU	LEU	LEU	PHE	PHE	PHE	LEU	LEU	LEU	
	Y	T	C	C	C	T	T	T	C	T	A	+
24	T	T	T	T	T	T	T	T	T	T	C	+
	N	Y	C	T	A	C	C	C	G	G	C	+
	TYR	TYR	ARG	ASP	TYR	TYR	TYR	TYR	TYR	TYR	TYR	
	T	T	C	G	T	T	T	T	T	T	G	+
25	A	A	G	A	A	A	A	A	A	A	C	+
	Y	Y	T	T	T	C	C	C	C	C	T	+
	PRO	PRO	LEU	HIS	SER	PRO	PRO	PRO	PRO	PRO	PRO	
	C	C	T	C	T	C	C	C	C	C	C	+
26	C	C	T	A	C	C	C	C	C	C	C	+
	N	N	A	C	G	G	C	A	G	A	C	+

Amino Acid	Ap-A Amb	Ap-b Amb	pBS1 2hr	pBS1 3hr	pBS1 4hr	Ap-B Prok	Ap-B Dros	Ap-B Yeast	Ap-A Prok	ApA Yeast	Ap-A Dros	
	SER	SER	VAL	LEU	ASN	SER	SER	SER	SER	SER	SER	
	W	W	G	C	A	T	T	T	T	T	T	+
27	S	S	T	T	A	C	C	C	C	C	C	-
	N	N	C	A	T	C	C	T	C	T	C	+
	GLY	GLY	SER	THR	CYS	GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	T	A	T	G	G	G	G	G	G	+
28	G	G	C	C	G	G	G	G	G	G	G	+
	N	N	G	G	C	T	C	T	T	T	C	+
	CYS	CYS	GLY	GLY	TYR	CYS	CYS	CYS	CYS	CYS	CYS	
	T	T	G	G	T	T	T	T	T	T	T	+
29	G	G	G	G	A	G	G	G	G	G	G	+
	Y	Y	C	C	T	C	C	T	C	T	C	+
	PRO	PRO	LEU	LEU	GLY	PRO	PRO	PRO	PRO	PRO	PRO	
	C	C	C	C	G	C	C	C	C	C	C	+
30	C	C	T	T	G	C	C	C	C	C	C	-
	N	N	T	C	C	G	C	A	G	A	C	+
	SER	SER	ASN	ARG	THR	SER	SER	SER	SER	SER	SER	
	W	W	A	C	A	T	T	T	T	T	T	-
31	S	S	A	G	C	C	C	C	C	C	C	+
	N	N	T	A	A	C	C	T	C	T	C	+
	GLY	GLY	SER	THR	CYS	GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	T	A	T	G	G	G	G	G	G	-
32	G	G	C	C	G	G	G	G	G	G	G	+
	N	N	G	G	C	T	C	T	T	T	C	+
	TRP	TRP	LEU	ILE	MET	TRP	TRP	TRP	TRP	TRP	TRP	
	T	T	C	A	A	T	T	T	T	T	T	-
33	G	G	T	T	T	G	G	G	G	G	G	-
	G	G	A	A	G	G	G	G	G	G	G	+
	HIS	HIS	ALA	GLN	GLU	HIS	HIS	HIS	HIS	HIS	HIS	
	C	C	G	C	G	C	C	C	C	C	C	+
34	A	A	C	A	A	A	A	A	A	A	A	+
	Y	Y	C	G	G	C	C	C	C	C	C	+
	ASN	ASN	ARG	LEU	LEU	ASN	ASN	ASN	ASN	ASN	ASN	
	A	A	C	C	C	A	A	A	A	A	A	-
35	A	A	G	T	T	A	A	A	A	A	A	-
	Y	Y	C	T	C	C	C	C	C	C	C	+

Amino Acid	Ap-A Amb	Ap-b Amb	pBS1 2hr	pBS1 3hr	pBS1 4hr	Ap-B Prok	Ap-B Dros	Ap-B Yeast	Ap-A Prok	ApA Yeast	Ap-A Dros	
	CYS	CYS	PHE	GLU	ASP	CYS	CYS	CYS	CYS	CYS	CYS	
	T	T	T	G	G	T	T	T	T	T	T	+
36	G	G	T	A	A	G	G	G	G	G	G	-
	Y	Y	C	G	T	C	C	T	C	T	C	+
	LYS	LYS	GLY	ARG		LYS	LYS	LYS	LYS	LYS	LYS	
	A	A	G	A		A	A	A	A	A	A	+
37	A	A	G	G		A	A	A	A	A	A	-
	R	R	G	A		A	G	G	G	G	G	+
	ALA	ALA	LEU	LEU		ALA	ALA	ALA	ALA	ALA	ALA	
	G	G	C	C		G	G	G	G	G	G	-
38	C	C	T	T		C	C	C	C	C	C	-
	N	N	C	A		T	C	T	T	T	C	+
	HIS	HIS	ASN	STOP		HIS	HIS	HIS	HIS	HIS	HIS	
	C	C	A	T		C	C	C	C	C	C	-
39	A	A	A	A		A	A	A	A	A	A	+
	Y	Y	T	A		C	C	C	C	C	C	+
	GLY	GLY	HIS	VAL		GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	C	G		G	G	G	G	G	G	+
40	G	G	A	T		G	G	G	G	G	G	-
	N	N	T	T		T	C	T	T	T	C	+
	PRO	PRO	ARG	ALA		PRO	PRO	PRO	PRO	PRO	PRO	
	C	C	C	G		C	C	C	C	C	C	+
41	C	C	G	C		C	C	C	C	C	C	+
	N	N	G	A		G	C	A	G	A	C	+
	ASN	THR	TYR	ILE		THR	THR	THR	ASN	ASN	ASN	
	A	A	T	A		A	A	A	A	A	A	+
42	C	A	A	T		A	A	A	C	C	C	+
	N	Y	T	A		C	C	C	C	T	C	+
	ILE	ILE	SER	ASP		ILE	ILE	ILE	ILE	ILE	ILE	
	A	A	A	G		A	A	A	A	A	A	+
43	T	T	G	A		T	T	T	T	T	T	-
	H	H	C	C		C	C	T	C	C	C	+
	GLY	GLY	CYS	TYR		GLY	GLY	GLY	GLY	GLY	GLY	
	G	G	T	T		G	G	G	G	G	G	-
44	G	G	G	A		G	G	G	G	G	G	+
	N	N	C	T		T	C	T	T	T	C	+

Amino Acid	Ap-A Amb	Ap-b Amb	pBS1 2hr	pBS1 3hr	pBS1 4hr	Ap-B Prok	Ap-B Dros	Ap-B Yeast	Ap-A Prok	ApA Yeast	Ap-A Dros	
	TRP	TRP	PHE	GLY		TRP	TRP	TRP	TRP	TRP	TRP	
	T	T	T	G		T	T	T	T	T	T	+
45	G	G	T	G		G	G	G	G	G	G	+
	G	G	T			G	G	G	G	G	G	+
	CYS	CYS	PRO			CYS	CYS	CYS	CYS	CYS	CYS	
	T	T	C			T	T	T	T	T	T	-
46	G	G	C			G	G	G	G	G	G	-
	Y	Y	T			C	C	T	C	T	C	+
	CYS	CYS	VAL			CYS	CYS	CYS	CYS	CYS	CYS	
	T	T	G			T	T	T	T	T	T	+
47	G	G	T			G	G	G	G	G	G	+
	Y	Y	G			C	C	T	C	T	C	+
	LYS	LYS	STOP			LYS	LYS	LYS	LYS	LYS	LYS	
	A	A	T			A	A	A	A	A	A	-
48	A	A	G			A	A	A	A	A	A	-
	R	R	A			A	G	G	G	G	G	+
	GLN	LYS	ASN			LYS	LYS	LYS	GLN	GLN	GLN	
	C	A	A			A	A	A	C	C	C	+
49	A	A	A			A	A	A	A	A	A	+
	R	R	T			A	G	G	G	A	G	-

```
SCORES      Init1: 164 Initn: 295 Opt: 212
             98.2% Identity in 57 bp overlap

                                                    10          20          30
Pbs1.S                                              GGGCGAATTGGAGCTCCACCGCGGTGGCGG
                                                    ||||||||||||||||||||||||||||||
Blues. GGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGG
            620         630         640         650         660         670

                 40          50          60          70          80
Pbs1.S CCGCTCTAGAA-TAGTGGATCCCCCGGTACCCTACGTTTTGCTCCGTTTAGTCGAGGGCC
       |||||||||||| ||||||||||||||||
Blues. CCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATAC
            680         690         700         710         720         730
```

Appendix 2. Comparison of two hr sequence of pBS1 with pBluescript KSII+

```
 GAP of: Blue.Consensus  check: 2409  from: 1  to: 212


 to: Apa.Amb  check: 4637  from: 1  to: 147

 BACKTRANSLATE of: : Naxa  check: 3375  from: 1  to: 49
P1;NAXA - Anthopleurin A - Giant green sea anemone
C;Species: Anthopleura xanthogrammica (giant green sea anemone)
C;Accession: A01794
R;Tanaka, M., Hanlu, M., Yasunobu, K.T., and Norton, T.R.
Biochemistry 16, 204-208, 1977 (Complete sequence) . . .

 Symbol comparison table: Gencoredisk:[Gcgcore.Data.Rundata]Nwsgapdna.Cmp
 CompCheck: 6876

        Gap Weight:  5.000      Average Match:  1.000
     Length Weight:  0.300   Average Mismatch:  0.000

           Quality:   78.4             Length:    212
             Ratio:  0.533               Gaps:      1
 Percent Similarity: 57.143   Percent Identity: 22.449

 Blue.Consensus x Apa.Amb  June 3, 1992  09:27  ..

      .         .         .         .         .
  1 GATCTTGATCACCTCAGGGGCCGACGTCCTTAAGCTATATTCGAATAGCT 50
                  ||:  ::::| ::|: |: |:: :     : |:
  1 .................GGNGTNWSNTGYYTNTGYGAYWSNGAYGGNCCN 33
      .         .         .         .         .
 51 ATGGCACATGCATGGAGCTCGATATCAACGTTATCGATACCGATCTCGCT 100
    : :| :: :| :   | :: :: :  :||::|:| | |  : |:| : ::
 34 WSNGTNMGNGGNAAYACNYTNWSNGGNACNYTNTGGYT..NTAYCCNWSN 81
      .         .         .         .         .
101 AGCTCGACTAGGGCCGTACCAGCTTGTCCTTACCCTTTAGTGAGGGTTAA 150
     |:|   |::::| :   |    :||:     :| :  :  :| : |:
 82 GGNTGYCCNWSNGGNTGGCAYAAYTGYAARGCNCAYGGNCCNACNATHGG 131
      .         .         .         .         .
151 TTTGCGATCCCTTGAGGTCCAATCATGGTCATAGCTGTTTTCCTGTGAAA 200
    :| | | | :    |:
132 NTGGTGYTGYAARCAR................................ 147
                .
```

Appendix 3. Gap analysis of the consensus sequence of the pBS1 insert against the ambiguous sequence obtained by backtranslating anthopleurin A.

```
GAP of: Blue.Consensus  check: 2409  from: 1  to: 212


to: Apb.Amb  check: 3012  from: 1  to: 147

 BACKTRANSLATE of: : Naxab  check: 2355  from: 1  to: 49
P1;NAXAB - Anthopleurin B - Giant green sea anemone
C;Species: Anthopleura xanthogrammica (giant green sea anemone)
C;Accession: A01795
R;Reimer, N.S., Yasunobu, C.L., Yasunobu, K.T., and Norton, T.R.
J. Biol. Chem. 260, 8690-8693, 1985 (Sequence) . . .

 Symbol comparison table: Gencoredisk:[Gcgcore.Data.Rundata]Nwsgapdna.Cmp
 CompCheck: 6876

        Gap Weight:  5.000      Average Match:  1.000
     Length Weight:  0.300   Average Mismatch:  0.000

           Quality:   78.0            Length:    212
             Ratio:  0.531              Gaps:      0
Percent Similarity: 53.061   Percent Identity: 23.129

Blue.Consensus x Apb.Amb  June 3, 1992  09:33  ..

      1 GATCTTGATCACCTCAGGGGCCGACGTCCTTAAGCTATATTCGAATAGCT 50
                                                  |  :
      1 ..............................................GGN 3

     51 ATGGCACATGCATGGAGCTCGATATCAACGTTATCGATACCGATCTCGCT 100
        |: |:  :  :||   ::::     : |:  : |:: :  :| : |::|
      4 GTNCCNTGYYTNTGYGAYWSNGAYGGNCCNMGNCCNMGNGGNAAYACNYT 53

    101 AGCTCGACTAGGGCCGTACCAGCTTGTCCTTACCCTTTAGTGAGGGTTAA 150
        : :: |: |:  :  || : | | :  :  :  :| :: : |: ||   |
     54 NWSNGGNATHYTNTGGTTYTAYCCNWSNGGNTGYCCNWSNGGNTGGCAYA 103

    151 TTTGCGATCCCTTGAGGTCCAATCATGGTCATAGCTGTTTTCCTGTGAAA 200
         :||: |  |:    |: |:||:  :||:    |:||:
    104 AYTGYAARGCNCAYGGNCCNAAYATHGGNTGGTGYTGYAARAAR..... 147
```

Appendix 4. Gap analysis of the consensus sequence of the pBS1 insert against the ambiguous sequence obtained by backtranslating anthopleurin B.

```
C;Species: Anthopleura xanthogrammica (giant green sea anemone)
C;Accession: A01794
R;Tanaka, M., Haniu, M., Yasunobu, K.T., and Norton, T.R.
Biochemistry 16, 204-208, 1977 (Complete sequence)
R;Yasunobu, K.T.
unpublished results, cited by Norton, T.R., Fed. Proc. 40, 21-25, 1981 . . .

SCORES     Frame: (2) Init1: 18 Initn: 18 Opt: 26
            24.0% identity in 25 aa overlap

                                10        20        30        40
Naxa                    GVSCLCDSDGPSVRGNTLSGTLWLYPSGCPSGWHNCKAHGPTIGWC
                              | : : :| :::|::: :  | :||
Blue.C ILITSGADVLKLYSNSYGTCMELDINVIDTDLASSTRAVP-ACPYPLVRVNLRSLEVQSW
                10        20        30        40          50
```

Appendix 5. Comparison between the amino acid sequence obtained by translating the consensus sequence of clone pBS1 and the anthopleurin A peptide.

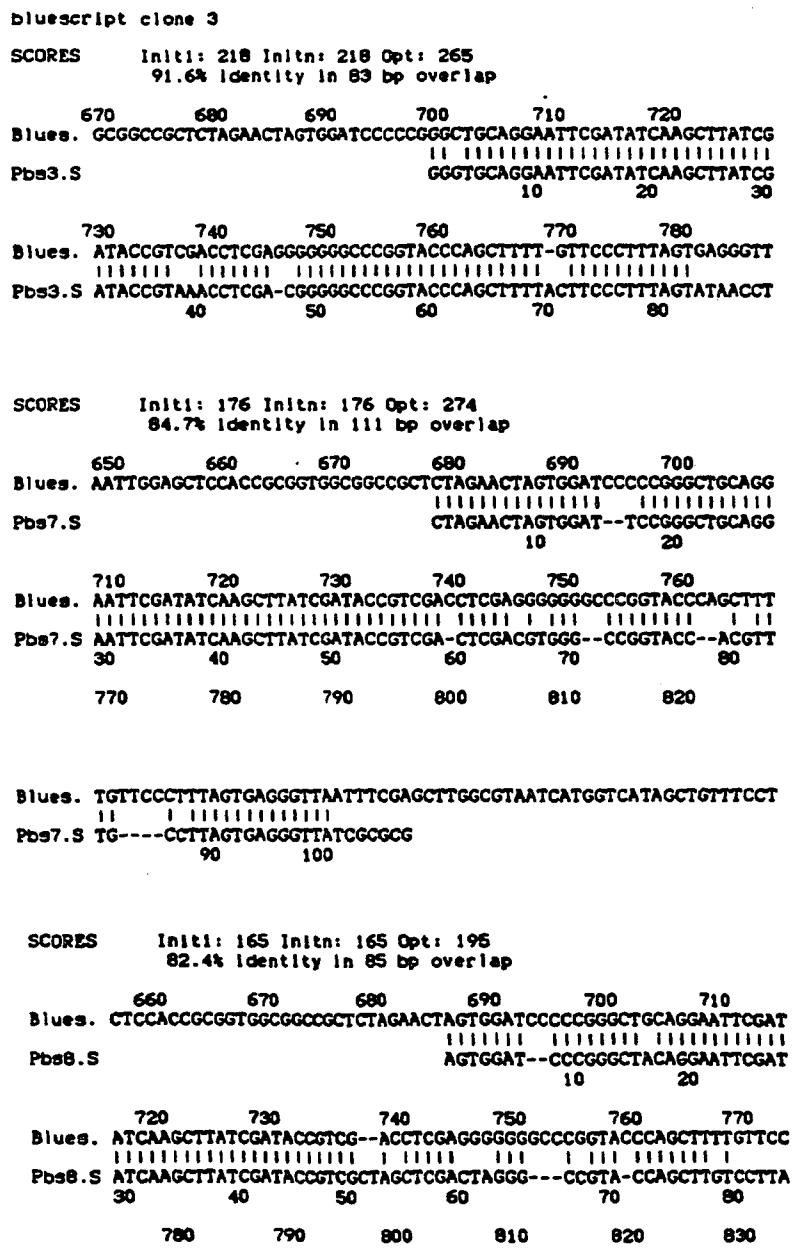
```
C;Species: Anthopleura xanthogrammica (giant green sea anemone)
C;Accession: A01795
R;Reimer, N.S., Yasunobu, C.L., Yasunobu, K.T., and Norton, T.R.
J. Biol. Chem. 260, 8690-8693, 1985 (Sequence)
A;Three disulfide bonds are present.
R;Bennett, C.D. . . .

SCORES     Frame: (4) Init1: 18 Initn: 18 Opt: 37
            66.7% identity in 6 aa overlap

                                                10        20        30
Naxab                                        GVPCLCDSDGPRPRGNTLSGILWFYPSGCPSG
                                               ||:|:|
Blue.C ANXPSLKGKDKLVRPXSSXRDRYRXRXYRAPCMCHSYSNIAXGRRPLRXS
      20        30        40        50        60

             40
Naxab  WHNCKAHGPNIGWCCKK
```

Appendix 6. Comparison between the amino acid sequence obtained by translating the consensus sequence of clone pBS1 and the anthopleurin B peptide.



Appendix 7. Comparison between the sequences of clones pBS3, pBS7, and pBS8 with the cloning vector pBluescript KSII+.

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