A Study of Heat Shock Protein 90 from the Filarial Nematode, *Brugia pahangi*

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

by

Alexis Cunliffe Cockroft

Department of Veterinary Parasitology
Veterinary School
Bearsden Road
Glasgow

January 1999

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Lymphatic filariasis has been reported as the second leading cause of permanent and long term disability world wide. The most important filarial nematodes with respect to lymphatic filariasis are *Wuchereria bancrofti* and *Brugia malayi*. *Wuchereria* is host-specific, but the sub-periodic strain of *Brugia malayi* and the closely related parasite *Brugia pahangi* infect a wide range of hosts.

Heat shock proteins (HSPs) have been studied in vector-borne parasites, like *Brugia*, the life cycle of which involves a compulsory transition from ambient temperature to mammalian body temperature. The role of various HSPs remains controversial, but the expression of these proteins may confer a survival advantage in the parasite following transmission to the mammalian host or in the face of an immune response.

Brugia pahangi hsp90 has high homology to *hsp90* clones from *B. malayi* and the predicted amino acid sequence contains conserved domains present in HSP90s from other species. Southern blot analyses suggested that *hsp90* was a single copy gene and that related genes were not present in the *B. pahangi* genome. A 1.2kb upstream region of *B. pahangi hsp90* was analyzed and putative transcription factor binding sites were identified. The (major) transcriptional start site of *hsp90* was calculated using a modified 5′ RACE protocol. A 0.54kb region of the *hsp90* "promoter", containing the transcriptional start site, a TATA box, five heat shock elements, a GC box and a CCAAT box, induced the expression of a reporter gene in an heterologous transfection system.

Northern blot analysis revealed that *hsp90* is heat shock inducible, consistent with a heat shock protein gene and that *hsp90* mRNA is enriched in mf maintained at 37°C when compared to adults at 37°C and mf at 28°C. Polyclonal antiserum raised *B. pahangi* HSP90, and a monoclonal antibody raised against heterologous HSP90s, reacted with an 85kDa protein in extracts from mf and adults. The anti-HSP90 antiserum also immunoprecipitation an 85kDa protein from extracts of *B. pahangi* adults maintained at 37°C and heat shocked at 41°C for 2 hours. Comparison of the ^{35}S labelled protein profiles from mf maintained at 37°C and at 28°C confirmed the differential expression of HSP90, observed by Northern blotting.

This thesis is dedicated to my Mum and Dad, my Gran and Grandad and my brother for their love and for their belief in me.

The research reported in this thesis is my own original work, except where otherwise stated and has not been submitted for any other degree.

Alexis C. Cockroft

Acknowledgements

I would like to thank my supervisor, Eileen Devaney and also Fiona Thompson for reading and commenting on my thesis as well as Eileen Devaney, Fiona Thompson and Sarah Hunter for considerable practical assistance. Certain other members of staff and students (past and present) at the Department of Veterinary Parasitology and the Wellcome Unit of Molecular Parasitology have also made valuable contributions towards my area of research, for which I would like to acknowledge their help. Thank you also to Julie Nybo for non-scientific help. My work was generously supported by a Medical Research Council studentship.

My good friends have given support and advice throughout my PhD and include Aoibhinn, Geoffrey, George, Kerry, Linda, Lucy, Rosie, Toseef, Trudy and Jon, Vicky, Zoe, and others too numerous to mention. Thank you to Mike Hayes for help and for words of wisdom. Special thanks to Jo for putting up with me under her roof for so long! Last but not least, thanks to my Mum for her time and assistance, to my father and brother for their technical wizardry, to Alex for his patience and to my grandparents for nourishment.

Here are a few words from "The Importance of Being Earnest" by Oscar Wilde that somehow seem appropriate:

"I am sick to death of cleverness. Everybody is clever nowadays. You can't go anywhere without meeting clever people. The thing has become an absolute public nuisance. I wish to goodness we had a few fools left."

"We have."

"I should extremely like to meet them. What do they talk about?"

"The fools? Oh! About the clever people of course."

"What fools."

Abbreviations

ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
Ci	Curie
cpm	counts per minute
ddH_2O	double deionized water
dNTPs	deoxyribonucleotides
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DOC	deoxycholate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
ES	excretory/secretory
EST	expressed sequence tag
FCS	foetal calf serum
g	gram
g	gravitational force
HBSS	Hanks balanced salts solution
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
IEF	isoelectric focusing
kb	kilobase
kDa	kilodalton
λ	lambda
ℓ	litre
mA	milliamperes
mAb	monoclonal antibody
mf	microfilariae
mg	milligram

mM	millimolar
mRNA	messenger RNA
MS	multiple sclerosis
NaCl	sodium chloride
ng	nanogram
nm	nanometres
°C	degree centigrade
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pg	picogram
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase
SDS	sodium dodecylsulphate
SL	spliced leader
SLE	systemic lupus erythematosus
T.E.	Tris-EDTA buffer
TEMED	N,N,N′,N′-tetramethylenediamine
Tris	Tris (hydroxymethyl)methylamine
μg	microgram
μl	microlitre
μM	micromolar
UTR	untranslated region
U.V.	ultraviolet radiation
V	volts
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-2-galactopyranoside

List of figures

List of tables

Contents

1.0 Introduction

1.1 Lymphatic filariasis - the parasites

The term filaria is described as, "any parasitic nematode worm of the family Filariidae, living in the blood and tissues of vertebrates and transmitted by insects: cause of filariasis" [Collins English Dictionary, 1994].

The most important filarial worms with respect to human disease are *Wuchereria bancrofti* and *Brugia malayi*, which cause lymphatic filariasis and *Onchocerca volvulus* which causes river-blindness and severe skin disease. In 1995 the World Health Organisation (WHO) reported that lymphatic filariasis was the second leading cause of permanent and long term disability world wide. There are an estimated 120 million people in 73 countries infected with lymphatic filariae and the majority of these cases are caused by *W. bancrofti* [Ottesen *et al* 1997]. Regional variations in the prevalence of Bancroftian filariasis vary from an estimated 9.0% in sub-Saharan Africa to 0.1% in regions of Latin America and the Caribbean [Michael and Bundy 1997].

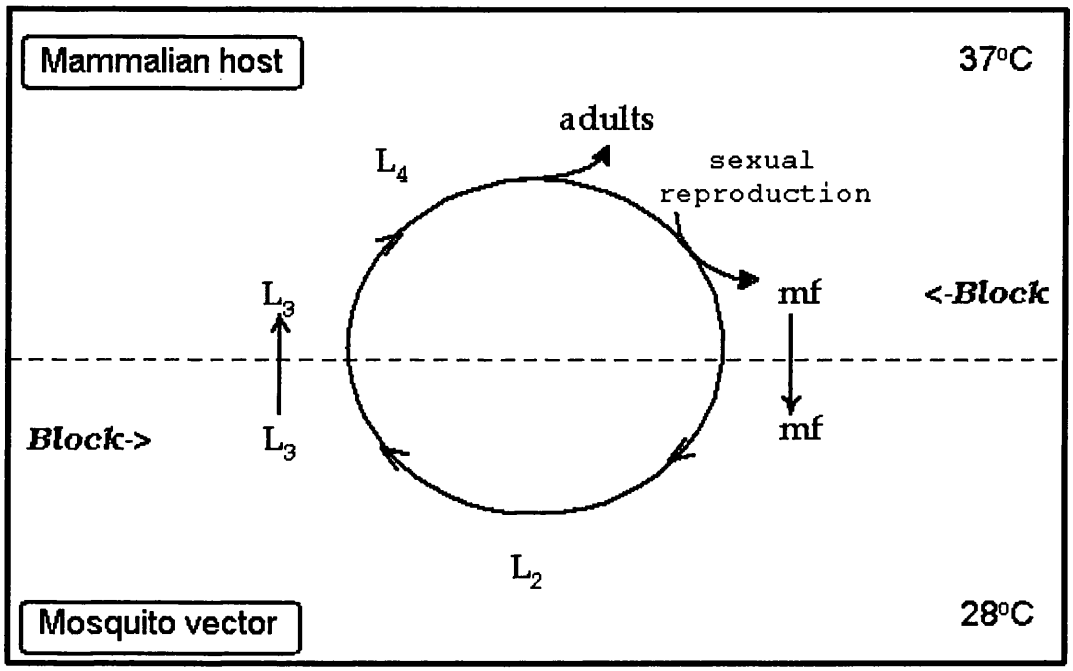
The principal vectors of Brugian filariasis are *Mansonia* and *Anopheles* mosquitoes but Bancroftian filariasis can be transmitted by up to forty-eight different species of mosquito. Since the number of appropriate vectors and the distribution of the vectors govern the geographic distribution of lymphatic filariasis, it is apparent why *W. bancrofti* accounts for 90% of infections ranging through tropical and subtropical countries. *B. malayi* is mainly limited to Southern and South East Asia and Indonesia. Another species, *Brugia timori* was identified on the islands of Timor and Flores.

1.1.1 The life cycle of filarial nematodes

The life cycle of filarial worms begins with the bite of an infected mosquito carrying infective third stage larvae (L_3) in the head and mouthparts. The L_3 move from the surface of the skin, through the puncture wound and into the mammalian host. The larvae localize to the lymphatic system and through a series of moults, develop into the fourth stage larvae (L_4) and then the adult stage. Mature adults sexually reproduce and the females release hundreds of microfilariae (mf) which circulate in the bloodstream. Mf remain viable for months in the mammalian host but are developmentally arrested. Development is resumed when a female mosquito ingests a blood meal containing mf.

Once inside the mosquito, the mf travel to the thoracic flight muscles where they moult and develop to L_2 and finally to L_3 which migrate to the head and mouthparts. The optimal temperature for development in the mosquito vector is 28°C. As with the mf in the bloodstream, the L_3 are developmentally arrested in the mosquito vector. Development is resumed when the mosquito takes a bloodmeal and the L_3 enter the mammalian host.

Figure 1.1 : The filarial life cycle



1.1.2 The laboratory model

Wuchereria and *Onchocerca* are extremely host-specific and no convenient animal models exist for these species. In contrast, the sub-periodic strain of *Brugia malayi* and the closely related parasite *Brugia pahangi* infect a wide range of hosts including the jird, *Meriones unguiculatus*. In this laboratory, the life cycle of *B. pahangi* is maintained by passage through a susceptible laboratory strain of *Aedes aegypti* (Refm) and the jird. Mosquitoes are fed rabbit blood mixed with *B. pahangi* mf. The mf develop normally through to L_3 and they are then harvested by disrupting the mosquito bodies in medium. Viable worms actively move through layers of material and are collected. Jirds are infected by intra-peritoneal injection of L_3. The parasite develops to L_4 and finally to adult worms in the peritoneal cavity. Adults sexually reproduce and mf

are produced approximately three months after initial infection. Mf and adult stages are collected by peritoneal lavage and mf can then be used to infect mosquitoes to complete the life cycle.

1.1.3 The disease – lymphatic filariasis

Lymphatic filariasis is often termed a "spectral" disease and the clinical manifestations may be divided into five groups.

1) Endemic Normals

This category describes individuals in the endemic region who have no clinical or parasitological evidence of infection but show a considerably higher response to parasite antigen than uninfected individuals in a non-endemic area. Endemic normals may have developed protective immunity to infection. In one study of 19 adults, diagnosed as endemic normals, all were free from *W. bancrofti* infection 17 years later, supporting this hypothesis [Steel *et al* 1996]. However, this classification also includes individuals who have sub-threshold or pre-patent infections and discrimination between these two types of individuals may be possible with the recent advances in sensitive diagnostic techniques. These techniques include a rapid immunochromatographic test (ICT) [Weil *et al* 1997], antigen detection by ELISA [Nicolas 1997] and PCR assays [Williams *et al* 1996, Abbasi *et al* 1996]. The first two of these tests identify filarial antigens in blood samples while the latter detects parasite DNA from blood or sputum.

2) Asymptomatic Microfilaremics

These individuals contain mf in their bloodstream but do not appear to suffer from overt clinical pathology. Recent evidence indicates that abnormalities of the renal and lymphatic systems are found in some apparently asymptomatic individuals [Dreyer 1992]. As a group, the asymptomatic microfilaremics are characterized by a profound defect in proliferative responses to filarial antigen [King *et al* 1993]. The hyporesponsiveness of this group appears to reflect a parasite-induced down-regulation of the immune response [Maizels and Lawrence 1991]. Not all immune responses are suppressed by the parasite. Th1 responses are down regulated, but Th2 responses appears to be up-regulated in microfilaremics [Maizel and Lawrence 1991].

3) Filarial Fevers

Filarial fevers describe episodes of lymphadenitis, fever and occasional lymphangitis in response to parasitic infection. The symptoms last approximately five days and may recur two to six times each year. During this time, an increase in circulating immune complexes is observed, perhaps reflecting the release of filarial antigen into the circulation [Kar *et al* 1993]. However, it has been suggested that the fever corresponds with a clearance of mf from the bloodstream. Accordingly, this group is more immunoresponsive to filarial antigen than the asymptomatic microfilaremics.

4) Lymphatic Pathology

This term relates to the repeated damage to the lymphatic vessels, resulting in fibrosis and finally to obstruction, which leads to disfiguring pathology. Gross swelling of the limbs is observed due to lymphoedema and in Bancroftian filariasis hydrocoele may also occur. Reduced circulation may result in secondary infections by fungal or bacterial agents which often increase the immune-related pathology [Ottesen 1994]. Products from live lymphatic-dwelling worms and the host's immune response to the adults are both implicated in inflammation and injury to the lymphatic system [Case *et al* 1991]. Unfortunately, diethylcarbamazine citrate (DEC) treatment of these individuals does not have an effect on existing lymphatic pathology [Freedman *et al* 1995].

5) Tropical Pulmonary Eosinophilia (TPE)

Although less common than the other conditions, the clinical features of this group are the most distinct and are restricted to *W. bancrofti* infections. Individuals experience nocturnal coughing, asthmatic wheezing, fever and interstitial mottling on chest radiogram [Ottesen and Nutman 1992]. Mf in the lungs appear to promote an allergic response resulting in eosinophilia and enhanced IgE production. If untreated, fibrosis can occur in the lungs causing reduced pulmonary function and eventual pulmonary failure. Mf are not found in blood samples, which may imply that they are killed in the lungs before reaching the peripheral blood. The hyperactive immune response may therefore be directed towards antigen released from dead mf. It has been reported that TPE is more likely to occur in individuals who have no immunity to the parasite, such as visitors to an endemic area, than in the endemic population [Ong and Doyle 1998].

Children born from infected mothers appear to have a higher susceptibility to infection than those children born from amicrofilaremic mothers [Lammie *et al* 1991]. Paternal infection status does not correlate with susceptibility of children to filariasis, so it is probable that neonatal tolerance is involved. A study of 17-19 year old Polynesians, free of *W. bancrofti* infection, identified a reduced immune response to mf antigen in juveniles born of microfilaremic mothers compared to those born of infection-free mothers, implying *in utero* acquired tolerance in the former group [Steel *et al* 1994]. While it is thought that the transplacental transfer of *W. bancrofti* mf is a rare phenomenon [Campello *et al* 1993], filarial antigens or excretory/secretory (ES) products may cross the placenta.

1.1.4 Chemotherapy

There is no vaccine for lymphatic filariasis and since perpetuation of the infection requires transfer of mf to a feeding mosquito, a reduction in the incidence of the disease involves reducing the mf density and prevalence within the population. A recent bulletin from WHO (1997) described a programme to be implemented for the treatment of lymphatic filariasis. This involved the use of three drugs: the piperazine derivative, DEC, also known as Heterazan, Banocide, and Notezine; the avermectin derivative, ivermectin (22,23-dihydroavermectin B1), also known as Mectizan and the benzimidazole derivative, albendazole, known as Albenza.

The traditional 12-day treatment of filariasis with DEC succeeded in reducing circulating mf, but did not always kill adult worms. Side effects include fever, vomiting, lymphadenitis and headaches and are due to the rapid destruction of mf, followed by the release of mf antigen. Individuals with Brugian filariasis suffer more extreme effects when treated with DEC than those with Bancroftian filariasis. These unpleasant side effects result in the poor adherence of the infected population to a course of treatment. More recently, six monthly doses of DEC to the whole population have been tried and/or addition of DEC to the cooking/table salt to ensure daily consumption [Simonsen *et al* 1995]. Both procedures are more cost effective than the 12-day regime and appear more acceptable to the population [Michael *et al* 1996].

Ivermectin was originally investigated for the treatment of onchocerciasis but was discovered to be an effective microfilaricide for most filarial species. Although DEC is

reported to cause more damage to adult worms and a greater reduction in mf than ivermectin, high doses of ivermectin appear to suppress mf for much longer [Richards *et al* 1991]. Ivermectin is thought to cause the release of γ-aminobutyric acid (GABA) and increase the permeability of parasite chloride channels [Brownlee *et al* 1997]. Albendazole has been used to treat protozoa, tapeworm and intestinal nematode infections and more recently lymphatic filariasis. The drug is thought to inhibit tubulin polymerization resulting in a reduction in parasite cytoplasmic microtubules [Jimenezgonzalez *et al* 1991].

A four month study of *W. bancrofti* infected school children given a single dose of albendazole or ivermectin or dual therapy reported a much greater reduction in circulating mf as a result of co-administration compared with either drug alone [Addiss *et al* 1997]. The introduction of single yearly doses of combined antifilarial drugs, ivermectin with DEC or albendazole has been reported to reduce blood mf by 99% for a year, compared to a single dose of DEC or ivermectin which results in a 90% reduction [Ottesen *et al* 1997]. However there is evidence that living adult worms persist despite treatment with DEC and/or ivermectin [Eberhard *et al* 1997].

1.1.5 Controlling disease transmission

The breeding sites of mosquitoes are an obvious target for disease control and strategies include spraying areas with appropriate biocides and overlaying the water source with polystyrene beads [Maxwell *et al* 1990]. The prevention of biting is also important and aerosol sprays of insecticides are used as well as insecticide impregnated bednets. The organochloride D.D.T. (permitted for limited use), and organophosphates such as pyrethroids are utilized as insecticides but problems have arisen firstly from the development of resistant mosquitoes [Hemingway *et al* 1997] and from the potential human and environmental hazard the chemicals pose. However, Higgs *et al* (1998) genetically engineered *Aedes aegypti* mosquitoes which were refractory to yellow fever viral strains. It may therefore be possible to incorporate resistance to other pathogens, such as filarial worms, into the genetic repertoire of insects in endemic areas.

1.2 Heat shock proteins

A small number of proteins are preferentially expressed in response to conditions that stress the cell, such as a heat shock. These proteins, referred to as heat shock proteins (HSPs), are also present under normal conditions but during cellular stress accumulate in high concentrations [Tissieres *et al* 1974, Schlesinger *et al* 1982]. The protein families were named according to their approximate molecular weight, the large HSPs, HSP100, HSP90, HSP70 and HSP60 and small HSPs (including the α-crystallins) which have a greater diversity of mass. On closer examination, HSPs vary in size depending on the species from which they are isolated, for example the cytoplasmic HSP90s actually range in size from 80kDa to 90KDa. Alterations in the expression of heat shock proteins are observed in diverse organisms both in the presence and absence of cellular stress. In addition to their protective role during a heat shock, they have also been implicated in a wide range of developmental and pathological processes [Lindquist 1986]. Heat shock proteins which are expressed under non-stress conditions are sometimes referred to as heat shock cognates (hscs), for example yeast HSC82 which is expressed under normal conditions at a higher level than yeast HSP82 [Lindquist 1991].

1.2.1 HSP70

HSP70 and the *E. coli* homologue, DNAK, are the most highly conserved of the HSPs and have been extensively studied. Heat shock induction of HSP70 confers thermoprotection to the cell and this process is thought to involve the binding of HSP70 to unfolding proteins which subsequently prevents the formation of insoluble protein aggregates [Schröder *et al* 1993]. Under normal cellular conditions, members of the HSP70 family are implicated in the folding of nascent polypeptide chains. For example DNAK and DNAJ (a homologue of HSP40) have been observed to bind to newly synthesized polypeptide chains and it was suggested that by binding to the hydrophobic regions of the proteins, these regions were shielded from the aqueous environment thus reducing their propensity to aggregate with other unfolded proteins. Upon dissociation (mediated by an apparently unique *E. coli* protein, GRPE) the newly synthesized polypeptide chains are thought to either fold into a native conformation, to undergo more cycles of binding and release or to be transferred to another set of molecular chaperones [Hartl and Martin 1995]. HSP70s appear to contain an ATP-binding site in the N terminal domain, which has weak ATPase activity and a C terminal domain

capable of binding peptides [Gething and Sambrook 1992]. Evidence for the involvement of the carboxyl terminus was obtained by the use of a DNAK mutant lacking the majority of the C-terminus. When a plasmid containing the truncated *dnak* gene was used to transform *E. coli*, the expressed (mutant) protein was unable to support the folding of a protein substrate *in vivo*. This was believed to be due to inefficient substrate binding [Thomas and Baneyx 1996]. Georgopoulus and Welch (1993) proposed that the release of polypeptides from the DNAK/DNAJ/GRPE complex requires the hydrolysis of ATP. However this point is controversial since Vidal *et al* (1996) reported that the release of substrate from a member of the HSP70 family, BiP, did not require the hydrolysis of ATP; rather he hypothesized that ATP hydrolysis was necessary for recycling BiP molecules. In this role, the energy released from ATP hydrolysis would be used to change the conformation of BiP to a form competent for binding new substrate.

A possible extension to this folding and assembly activity is the proposal that HSP70 may regulate tubulin polymerization. Before its recognition as a heat shock protein, HSP70 was previously characterized as a microtubule associated protein. HSP70 was reported to interact with the carboxyl terminal end of microtubules [Liang and MacRae 1997]. In the ciliated protozoan, *Tetrahymena thermophila,* three homologues of HSP70 were found to be associated with high molecular weight tubulin-containing complexes during a heat shock. Although in this case HSP70 was thought to be part of a protective assemblage, rather than a normal intermediate, the protein was subsequently observed in association with ciliated and cortical microtubules under normal conditions. HSP70 was therefore suggested to have a direct role in the *in vivo* assembly and/or function of microtubules [Williams and Nelsen 1997].

1.2.3 HSP60

The *E. coli* homologue of HSP60, GroEL has been extensively studied and is probably the most characterized chaperone. GroEL forms an intricate double ring structure sometimes called a "double doughnut", consisting of 14 subunits (each of 60kD) each of which is divided into three domains [Braig *et al* 1994]. A second chaperonin, GroES, which is 10kDa, associates and disassociates with GroEL (analogous to a lid) during a folding cycle. A protein substrate binds within the enclosed space of the GroEL/GroES complex concomitant with the hydrolysis of ATP to ADP and causes the dissociation of

GroES from GroEL. ATP then binds to GroEL resulting in a conformational change which finally releases the folded protein [Roseman *et al* 1996]. During a heat shock, unfolded proteins accumulate and an elevated level of HSP60 is thought to facilitate the refolding of denatured proteins and to prevent protein aggregates forming [Martinus *et al* 1995].

In eukaryotes, HSP60 is found in mitochondria and in the chloroplasts of plants [Hartl *et al* 1994]. Indeed HSP60 is believed to be important in the formation and regeneration of mitochondria and this is thought to explain the prominence of rat HSP60 in highly replicating cells such as the epithelium of the intestine and keratinizing cells of the oesophagus [Mobius *et al* 1997]. A specifically adapted HSP60 homologue, known as CCT (chaperonin containing TCP-1) or TRiC (TCP ring complex) is present in the cytosol of eukaryotes where it is important in the folding of actins and tubulins [Gao *et al* 1992, Yaffe *et al* 1992]. When the promoter from *C. elegans cct-1* was used to "drive" the reporter gene, β-galactosidase, expression was observed in muscle, neuronal and hypodermal cells, consistent with a need for actin and tubulin folding in these tissues [Leroux and Candido 1997].

1.2.2 Small HSPs

Small heat shock proteins (sHSPs) are molecular chaperones and can suppress the aggregation of cellular proteins which occurs at elevated temperatures [Jakob *et al* 1993]. The sizes of sHSPs range from 15kDa to 30kDa, and whereas yeast only appears to have one sHSP, *Drosophila*, for example, has four sHSPs [Voellmy *et al* 1981] and plants often have close to 20 [Jakob and Buchner 1994]. The α-crystallin proteins, which are present in the eye, are homologues of the small HSPs and have a role in maintaining lens stability and transparency [Merck *et al* 1993]. Small HSPs appear to be interchangeable (with the exception of the α-crystallins); loss of one sHSP can be compensated for by another [Buchner 1996]. Small HSPs from different species share little identity when compared to the homology between larger HSPs and regions of conserved amino acids are restricted to the carboxyl terminus.

Over-expression of human HSP27 was observed to confer thermotolerance to rodent cells suggesting a protective role for this protein [Landry *et al* 1989]. Small HSPs have a tendency to form large homo-oligomeric complexes and during a heat shock, sHSP

complexes concentrate in the nucleus where they have been detected in association with RNA [Lindquist 1986]. In *E. coli* sHSP homologues are observed to be associated with inclusion bodies, resulting from the overexpression of non-native protein, which may suggest a role for sHSPs in protein folding [Allen *et al* 1992]. When HeLa cells were exposed to heat shock temperatures, the rate of recovery of heat-induced nuclear protein aggregates paralleled HSP27 levels, suggesting that thermoprotection may involve more rapid protein disaggregation [Stege *et al* 1995].

1.2.4 Other HSPs

HSP47

HSP47 was identified as a collagen binding protein in chicken embryo fibroblasts and its presence in the endoplasmic reticulum (ER) was revealed by immuno-localization studies. HSP47 associates with procollagen chains in the ER immediately after translation; the procollagen is processed to mature collagen, transported to the Golgi by HSP47 which then dissociates before the protein is secreted [Satoh *et al* 1996]. During a heat shock, there is a significant increase in the level of chicken HSP47, which has been proposed to have a role in preventing the secretion of aberrantly folded procollagen [Nagata *et al* 1996].

However, HSP47 also appears to be developmentally regulated. For example, during mouse ocular development, HSP47 is expressed strongly in early embryonic ocular tissue and is thought to have a role in corneal morphogenesis [Tanaka *et al* 1996]. HSP47 induction during development may be explained by its function as a molecular chaperone for collagen, since in developing zebrafish, the expression of *hsp47* mRNA closely correlates with the expression of type II collagen mRNA in tissues such as the notochord and developing fins [Lele and Krone 1997].

HSP100

Proteins of 100-110kDa (HSP100s) are synthesized by most cells in response to heat, with the possible exception of *Drosophila* [Lindquist and Craig 1988]. The HSP100 homologue in yeast, HSP104, is only present at very low levels in the absence of cellular stress and HSP104 deletion mutants are viable under normal growth conditions suggesting that its function is dispensable. However these cells are highly susceptible to hyperthermia suggesting that HSP104 is important for thermotolerance [Craig *et al*

1993]. The function of the HSP100 family appears to be to re-solubilize protein aggregates and to direct the proteolytic degradation of proteins which cannot be renatured [Schirmer *et al* 1996].

1.3 HSP90

1.3.1 HSP90 as a molecular chaperone

HSP90, like other heat shock proteins, shows increases in concentration as a result of a heat shock but is also present in many cells at a relatively high level under normal conditions. It has been proposed that HSP90 is a more specific chaperone and targets certain proteins which are difficult to fold and/or are inherently unstable. Furthermore, it was suggested that HSP90 enhances the rate of reactivation of heat-unfolded proteins but does not have a general protective role during a heat shock [Nathan *et al* 1997]. However, HSP90 has been reported by other researchers to have a protective function similar to that observed for other HSPs such as HSP70. For example, heat treatment of purified mitochondrial citrate synthase resulted in the formation of protein aggregates detected by an increase in light scatter. The addition of a twofold excess of purified bovine HSP90 inhibited aggregate formation by approximately 50% whilst an eightfold molar excess completely prevented protein aggregation. Similar results were obtained when a Fab fragment was used as a substrate, but in this case the optimum ratio of HSP90 to substrate was between 1:1 and 1:2 [Wiech *et al* 1992]. It was suggested that HSP90 specifically bound to early unfolding intermediates of citrate synthase and prevented them from interacting with each other, thus reducing the rate of irreversible aggregation reactions [Jakob *et al* 1995a]. Similarly, Yonehara *et al* (1996) observed that when heated to temperatures higher than 46°C, purified HSP90 behaved as a molecular chaperone and could prevent the irreversible thermal denaturation of the protein substrate, firefly luciferase. In this case, it was proposed that HSP90 bound to proteins undergoing thermal unfolding and maintained their folding competent state. Furthermore, the incubation of HSP90-associated substrates with GroEL/GroES complexes resulted in ATP-dependent folding of the proteins to their native conformation [Yonehara *et al* 1996].

Mammalian HSP90 has been reported to bind to the multicatalytic proteinase (MCP) (or 20S proteasome) and to protect it from oxidative damage. The proteosome is believed to

have a role in the degradation of damaged proteins thus reducing their level in the cell. Depleting the HSP90 concentration in a human cell line resulted in increased susceptibility of MCP to oxidative inactivation of its trypsin-like activity [Conconi *et al* 1998]. The ability of HSP90 to maintain non-native protein intermediates in a state capable of refolding (during a heat shock) was proposed to be unique to this chaperone and was not evident for HSP70, for example [Freeman and Morimoto 1996]. Another intriguing feature of HSP90 was reported by Schneider *et al* (1996) who observed that prolonging the interaction of HSP90 with protein substrates (using ansamycin antibiotics) increased the rate of degradation of the target molecules. It was therefore concluded that a balance existed between protein refolding and degradation and this balance was affected by altering the interaction of proteins with HSP90.

1.3.2 Evolutionary conservation of the structure and function of HSP90

There is a high degree of homology between HSP90s from different species and this is reflected in the report that *Trypansoma cruzi* HSP83 can functionally complement yeast. Yeast and *Trypanosoma* HSP90s share 63% identity, whereas the *E. coli* homologue of HSP90 (HtpG), which cannot complement yeast, is 42% identical to these proteins [Palmer *et al* 1995]. *Sacchromyces cerevisiae* has two *hsp90* genes, *hsp82* and *hsc82* and disruptions of both genes renders the organism inviable, but yeast can survive with one of these gene disrupted [Borkovich *et al* 1989]. However, *E. coli* HtpG is neither essential for bacterial function nor is it able to replace the function of eukaryotic HSP90s.

The induction temperature of HSP90 (and HSP70) does not appear to be strictly genetically predetermined, but appears to be influenced by environmental conditions. For example, in the fish, *Gillichthys mirabilis*, the temperature that the fish had previously been exposed to affected the threshold for heat shock induction: a lower threshold was observed with fish kept at lower temperatures [Dietz 1994].

1.3.3 HSP90α and HSP90β

In vertebrates, two forms of HSP90 exist and these are termed HSP90α and HSP90β (HSP84 and HSP86 in the mouse). The homology between HSP90α sequences from different species is higher than the homology between HSP90α and HSP90β from the same species. Differences in the expression patterns of HSP90α and HSP90β have been

reported. For example, an investigation into the expression pattern of chicken *hsp90α* revealed an accumulation of *hsp90α* mRNA after cells were stimulated with serum or insulin. Furthermore, *hsp90α* was suggested to be a secondary response gene since an increase in the transcription of this gene occurred between mid G_1 and the G_1/S transition of the cell cycle, thus resulting in a maximum level of hsp90α preceding DNA synthesis [Jerome *et al* 1993]. However, the expression of *hsp90β* was not observed to alter in this way during the cell cycle. Additionally, in the mouse, HSP86 expression was observed to increase during postnatal development whilst HSP84 expression decreased, which suggested a differential regulation of these two members of the HSP90 family during embryogenesis [Sarge and Cullen 1997].

1.3.4 Endoplasmic reticulum and plastid homologues of HSP90

Non-cytoplasmic homologues of HSP90 include the glucose-regulated protein, GRP94, which was thought to be present in the lumen of the endoplasmic reticulum (ER) [Sorger and Pelham 1987]. A study of porcine GRP94 revealed that the native protein appeared to be a soluble lumenal protein, which was glycosylated and whose mobility was consistent with a dimer [Wearsch and Nicchitta 1996]. Murine ERp99 has high homology to GRP94, but is a trans-membrane protein that spans the ER membrane once and has both a cytoplasmic and luminal domain. It was suggested that ERpp99 might be involved in the translocation of nascent proteins through the ER [Mazzarella and Green 1987]. Other homologues include an ER-located protein from the Madagascar periwinkle, *Catharanthus roseus*, which has higher homology to GRP94 than HSP90, but appeared to be regulated neither by glucose nor significantly induced by heat or other stresses. The *C. roseus* HSP90 homologue was not detected in extracts of the plant but was observed at high levels in cultured cells. Since the cell cultures had a high rate of growth and division, it was suggested that this HSP90 may have a role in the synthesis of secreted protein such as components of the cell wall [Schroder *et al* 1993]. In rye, *Secale cereale*, a member of the HSP90 family, which is located within plastids of the leaves, has been identified. Schmitz *et al* (1996) reported that this protein, cpHSP82, was moderately heat inducible and contained an amino terminal transit peptide sequence, which was not present in other HSP90s, but was characteristic of proteins encoded by the nucleus and transported from the cytoplasm into an organelle. It

was proposed to be a chaperone involved in the folding and protection of proteins synthesized in the plastid.

1.4 Specialized *in vivo* functions of HSP90

1.4.1 HSP90 and steroid hormone receptors

HSP90 was first identified as a 90kDa phosphoprotein that associated with non-transformed steroid hormone receptors (SHRs). Extracts of chick oviducts contained two forms of receptor complexes, differentiated by their sedimentation coefficients: the 8S form did not have DNA-binding activity but the 4S form contained transformed receptors capable of binding to steroid response elements. The 8S non-transformed receptor complex contained a 90kDa protein that could be dissociated from the receptor by high salt treatment or addition of the steroid hormone ligand [Joab *et al* 1984]. The receptor, free of bound 90kDa protein, had DNA-binding activity suggesting that the 90kDa protein had a role in blocking DNA-binding. Peptide mapping of the 90kDa protein against purified HSP90 indicated that the two proteins were identical [Catelli *et al* 1985]. Immunoprecipitation experiments using antibodies raised against HSP90, identified the 90kDa protein from non-transformed steroid receptor complexes. This led to the hypothesis that HSP90, by binding to SHRs, inhibits receptor activation. Consistent with the inhibitory hypothesis, a constitutively active glucocorticoid receptor (GR) derivative, unable to bind HSP90, was not affected by varying the concentration of HSP90 [Picard *et al* 1990].

Although dissociation of HSP90 from the non-transformed receptor (aporeceptor) complex transformed the receptor to a DNA-binding state, the resulting receptor conformation had severely reduced hormone-binding ability [Bresnick *et al* 1898]. This implied that HSP90 had more than just an inhibitory role in the function of steroid receptors. Indeed, studies on a yeast mutant carrying plasmids encoding mammalian steroid hormone receptors and a regulatable *hsp90* gene revealed a reduction in the hormonal activation of expressed mammalian steroid hormone receptors (glucocorticoid receptor and oestrogen receptor, ER) with a reduction in HSP90. These findings are consistent with a role for HSP90 in maintaining the viability of receptors [Picard *et al* 1990].

However, a discrepancy had to be addressed. Unlike the GR, which is found in the cytoplasm in its non-transformed state, the progesterone and oestrogen receptors have a predominantly nuclear location whilst HSP90 appears to be a cytoplasmic protein. In HeLa cells, the amount of HSP90 in the nucleus doubles from ~7% to 14% during a heat shock and this correlates with a paralysis in the response to oestrogen [Sabbah *et al* 1996]. Smith (1993) demonstrated that HSP90 is required for high affinity hormone binding by progesterone receptors (PR) at physiological temperatures, but proposed that the interaction was dynamic with a half-life of approximately 5 minutes which would explain the frequent immunoprecipitation of HSP90-free PR and also HSP90-free ER [Denis *et al* 1989]. It was therefore surmised that HSP90 had a general role in the function of steroid hormones.

1.4.2 Studies utilizing stabilized HSP90-SHR complexes

To study the activity of HSP90 related to receptor function, the ability to manipulate the association and dissociation of the HSP90 multi-protein complex (8S) was invaluable. An endogenous metal anion with properties indistinguishable from molybdate was observed to stabilize the 8S complex and this stabilization could also be achieved by adding molybdate. Exogenous molybdate is thought to bind to the site for the endogenous anion and stabilizes the association of HSP90 with the receptor [Meshinchi *et al* 1988]. Molybdate stabilization severely restricted hormone-dependent nuclear import of GR·HSP90 and both hormone-dependent and hormone-independent nuclear import of PR·HSP90 [Yang and DeFranco 1996]. Therefore, HSP90 may help maintain PR and GR in an inactive state *in vivo*. Indeed, a PR receptor lacking the site of HSP90 interaction was constitutively active and insensitive to molybdate [Carson-Jurica *et al* 1989].

The stoichiometry of HSP90 association was studied and utilizing molybdate to stabilize HSP90-GR association, a ratio of two HSP90 molecules to one receptor molecular was deduced. However, it was evident that other more loosely bound components were also associated with the GR·HSP90 complex [Bresnick 1990]. HSP90 sedimented in glycerol density gradients at a size (7S) indicating it existed *in vivo* as a dimer [Sullivan and Toft 1993]. Deletions of two regions that affected dimer formation of HSP90 created mutants that interacted with GR but the associated receptor appeared

to lack hormone-binding activity [Cadepond *et al* 1993]. Consequently HSP90 was thought to be functionally active as a dimer.

Tungstate ions also stabilize non-transformed steroid receptor complexes, but this differs from molybdate stabilization. The release of HSP90 from non-transformed receptor complexes is inhibited more strongly by tungstate, such that high salt treatment does not dissociate HSP90 from the complex. Nevertheless, the loss of a protein called p59 was observed due to high salt concentration and appeared to bind to the complex via HSP90 [Renoir *et al* 1990]. Immunoadsorption of HSP90 from cell lysates co-immunoprecipitated p59 (also called HSP56) and a protein called Cyp-40, (Cyp-40 and p59 are immunophilins which have peptidyl prolyl isomerase activity). Evidence indicated at least two types of non-transformed GR·HSP90 complexes: one which contained p59 and another which contained Cyp-40. The activity of the immunophilin was suggested to be required for proper folding of the ligand binding domain by a HSP90-associated system [Owens-Grillo *et al* 1995].

It has been proposed that HSP70 and other members of a "foldosome" interact with steroid hormone receptors thus creating a receptor conformation capable of binding steroid ligand. Some components such as HSP90, the immunophilins p59 (HSP56) and Cyp-40 are thought to have a more prolonged association with the receptor and remain bound after dissociation of the "foldosome" but others, such as HSP70, are assumed to have a more transient interaction [Smith 1993, Dittmar *et al* 1998]. ***Figure 1.2*** shows a proposed cycle of glucocorticoid aporeceptor activation, deactivation and reconstitution of the receptor complex, which involves the binding of HSP90, HSP56 and HSP70 [Bohen and Yamamoto 1994].

Figure 1.2 : The interaction of HSP90 with the glucocorticoid receptor

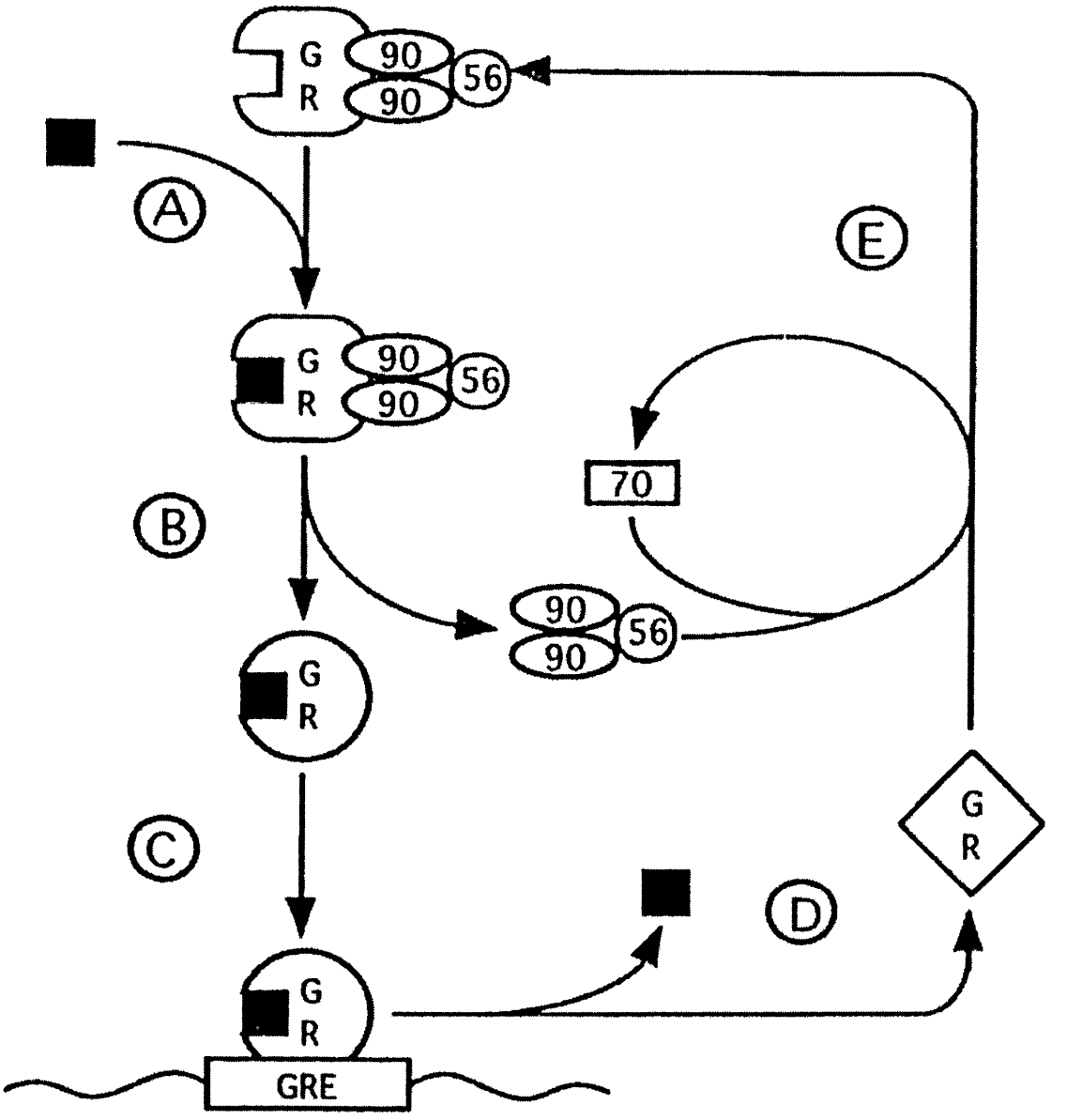


Figure 1.2 :

A The ligand (■) binds to the aporeceptor complex forming a transient aporeceptor complex. **B** HSP90 and HSP56 dissociate from the complex and the resulting activated complex undergoes a conformational change. **C** The activated receptor binds to glucorticoid response elements (GREs) and modulates transcription from nearby promoters. **D** The ligand dissociates from the GREs. **E** The deactivated aporeceptor re-associates with HSP90 and HSP56 in the cytoplasm and reconstitutes the aporeceptor complex. HSP70 transiently associates with the aporeceptor during the assembly of the complex.

1.4.3 The interaction of HSP90 with vitamin and dioxin receptors

There are examples of non-steroid hormone receptors that also appear to require HSP90. The vitamin receptors, like the steroid hormone receptors are members of the intracellular receptor superfamily. Mammalian and avian retinoid receptors were transfected into a yeast strain where endogenous HSP90 could be repressed to 1/20 of the normal level. Under normal levels of expression of yeast HSP90, immunoprecipitation of the retinoic acid receptor did not co-precipitate HSP90 implying a lack of association between the receptor and HSP90. However activation of the receptor was severely compromised by repression of HSP90 expression [Holley and Yamamoto 1995].

Another example, the dioxin receptor (AHR), is not a steroid hormone receptor but a member of a family of basic helix-loop-helix regulatory proteins. The mammalian AHR can be activated by environmental pollutants such as dioxins but the endogenous ligand has not been identified [Henry and Gasiewicz 1993]. HSP90 appears to be involved in the maintenance of an AHR conformation capable of ligand binding and also repression of receptor DNA-binding activity [Whitelaw *et al* 1993]. Deletion of the AHR region involved in HSP90 association resulted in constitutive dimerization of AHR with a partner factor, Arnt, which is involved in nuclear translocation of the receptor [Antonsson *et al* 1995]. A homologue of the vertebrate AHR has been identified in *C. elegans* and this receptor was observed to tightly bind rabbit HSP90. However native ligands that associate with *C. elegans* AHR have not been found [Powell-Coffman *et al* 1998].

1.4.4 Binding sites for HSP90

Site-directed mutagenesis of the HSP90 molecule has been used to study regions which may be important in interacting with steroid hormone receptors and/or dimer formation. Removal of the amino terminal half of HSP90 had only a modest effect on HSP90 association with the PR suggesting that the carboxyl terminal half was important in this function [Sullivan and Toft 1993]. In addition, the removal of the final 30 a.a. from chicken HSP90α caused the disruption of HSP90 dimers and implied that the carboxyl terminal region was also necessary for homodimer formation [Meng *et al* 1990].

A highly charged region (chicken HSP90α 221-290a.a.) is thought to form a mainly α-helical structure (and thus has a probable surface location) and has been implicated in a possible ionic protein to protein interaction [Cadepond *et al* 1993, Binart *et al* 1989]. Although HSP90 interaction with GR is disrupted by a deletion in this region, the same deletion mutant still associates with ER [Cadepond *et al* 1993, Meng *et al* 1990]. Binding of dioxins to the AHR receptor only appears to require a region in chicken HSP90α from amino acids 230-421, which is in contrast to the much larger (carboxyl) regions required for efficient ligand binding to steroid hormone receptors. This may indicate a difference in the role of HSP90 in the two-receptor complexes [Whitelaw *et al* 1993].

1.4.5 HSP90 and kinases

A 90kDa protein observed to bind to PRs was also found to associate with oncogenic members of the tyrosine kinase family, namely $pp60^{v\text{-}src}$ from Rous sarcoma virus and $pp85^{gag\text{-}fes}$ and $pp83^{gag\text{-}fgr}$ from two strains of Feline sarcoma virus. This 90kDa protein was identified as HSP90 [Ziemiecki *et al* 1986]. The transition metal oxyanions, molybdate, vanadate and tungstate prolonged the binding of HSP90 with the tyrosine kinase $pp60^{v\text{-}src}$ and these oxyanions were thought to bind to a site within an HSP90 complex normally occupied by an endogenous cytosolic metal ion, similar to the proposed stabilization of steroid hormone receptors heterocomplexes [Hutchison *et al* 1992]. The formation of HSP90 heterocomplexes does not appear to be restricted to tyrosine kinases (or steroid hormone receptors). Stancato *et al* (1993) also detected the cellular serine/threonine kinase, c-Raf, in association with HSP90 but this interaction was less stable than complexes containing $pp60^{v\text{-}src}$. The nature of the interaction between the molecular chaperone, HSP90 and these kinases was suggested to be a conformational change, which produced a viable enzyme. This mechanism was also described for the heme-regulated eukaryotic initiation factor 2α (eIF-2α) kinase since disruption of the binding between HSP90 and eIF-2α kinase inhibited the acquisition of competent kinase activity [Uma *et al* 1997]. Nadeau *et al* (1993) observed that purified yeast and mammalian HSP90 were able to bind to and retain the heat shock transcription factor (HSF), but the relevance of this interaction was not apparent. However, the reported binding of HSP70 to HSF was suggested to result in negative regulation of the transcription factor [Abravaya *et al* 1992]. It was therefore proposed

that HSP90 may have a role in maintaining HSF in an inactive state [Nadeau *et al* 1993].

1.4.6 HSP90 and structural proteins

The observation that some HSP90 localized to membrane ruffles in human, mouse and rat cultured cells led to the hypothesis that this chaperone may interact with actin filaments [Koyasu *et al* 1986]. Kellermayer and Csermely (1995) reported that the binding of HSP90 to filamentous actin prevented its association with tropomyosin and in addition, an increase in ATP concentration induced the dissociation of HSP90 from F-actin. It was suggested that ATP-binding induced a conformational change in the actin-chaperone complex resulting in the loss of HSP90 and since the interaction of actin with tropomyosin is required for muscle contraction, HSP90 was proposed to interfere with the motility of the actomyosin complex [Kellermayer and Csermely 1995]. HSP90 was also reported to associate with other structural proteins. The *in vitro* binding of HSP90 to tubulin dimers was studied using purified porcine proteins. The polymerization of tubulin was inhibited by HSP90 in a buffer containing a low concentration of magnesium. The stoichiometry of association of HSP90 to tubulin dimers was confirmed by a sedimentation assay to be one. It was therefore suggested that under heat shock conditions, HSP90 sequesters tubulin dimers resulting in the inhibition of tubulin polymerization and thus cell division ceases [Garnier *et al* 1998]. Williams and Nelsen (1997) also suggested a direct role for HSP90 (and HSP70) in the *in vivo* assembly and/or function of cytoplasmic and mitotic microtubules. Using antibodies to tubulin and HSP90, co-localization of HSP90 with microtubules in cultured cells was observed. A possible role for HSP90 in microtubule-based movement was also proposed [Liang and MacRae 1997]. As with HSP70, in the ciliated protozoan, *Tetrahymena thermophila*, the association of an HSP90 homologue with tubulin was identified by immunoprecipitation studies.

1.4.7 HSP90, nitric acid synthase and reverse transcriptase

HSP90 binds to a multitude of other proteins, for example García-Cardeña *et al* (1998) reported the binding of HSP90 to nitric oxide synthase in mammalian endothelial cells (eNOS). Production of endothelium-derived nitric oxide (NO) can be induced by specific growth factors, calcium-mobilizing agonists and shear stress. It was proposed that association with HSP90 resulted in the activation of eNOS via a conformational

change and thus HSP90 may be considered as an agonist in the signalling cascade resulting in NO release [García-Cardeña *et al* 1998]. HSP90 was detected in association with duck hepatitic virus and was proposed to facilitate the interaction between reverse transcriptase (RT) and the RNA pre-genome [Hu and Seeger 1996]. A chaperone complex, containing HSP90, was suggested to maintain RT in a conformation competent to bind to a short RNA sequence located at the 5′ end of the viral pre-genomic RNA [Hu and Seeger 1997].

1.5 Transcriptional control of *hsps*

Transcriptional activation of *hsps* by hyperthermia involves the activation of the heat shock transcription factor (HSF) and the subsequent binding of an HSF trimer to transcriptional control elements, heat shock elements (HSEs), upstream of the gene [Morimoto *et al* 1992]. Zarzov *et al* (1997) identified a yeast mutant with a defect in the *hsf* gene, which resulted in a dramatic reduction in HSP82 and HSC82 expression. In addition, the deletion or substitution of HSE1, one of three HSEs in the promoter region upstream of yeast *hsp82*, resulted in more than a twofold reduction in both the basal and heat-induced transcription of the gene [Gross *et al* 1993]. These studies confirmed that yeast HSF was required for HSP expression and that HSEs were involved in the transcription of *hsp* genes. The heat shock response also corresponds with an inhibition in the transcription of other genes and this in turn has been proposed to involve HSF either directly or indirectly [Westwood *et al* 1991]. Consistent with this hypothesis was the observation that, during a heat shock, human HSF1 concentrated at a number of nuclear foci which were distinct from *hsp* transcription sites and may have represented active genes [Jolly *et al* 1997].

1.5.1 Multiple HSFs

In most cell types, there is more than one HSF, for example, there are two human HSFs [Sistonen *et al* 1993] and three tomato [Scharf *et al* 1990] and chicken HSFs [Nakai and Morimoto 1993]. Screening a tomato (*Lycopersicon peruvianum*) expression library with an HSE DNA-ligand isolated three cDNA clones, which contained different *hsf* genes. One of the genes appeared to be constitutively expressed whilst the other two were heat shock inducible [Scharf *et al* 1990]. Three *hsf*s were also isolated from chicken cDNA libraries screened with mouse *hsf1* and *hsf2* probes. Chicken HSF2

bound constitutively to a heat shock element sequence, but HSF1 and HSF3 did not bind to the same sequence *in vitro*. The DNA-binding activity of HSF1 was heat shock inducible, but HSF3 was not induced by these conditions [Nakai and Morimoto 1993]. The functional diversification of HSF has also been suggested for vertebrate species. The activation of human HSF2 was observed during the differentiation of erythroleukemia cells and resulted in the increased expression of heat shock genes including *hsp90*, *hsp70* and *grp78*. This process appeared to be induced by hemin and not stress [Sistonen *et al* 1992]. It was thus proposed that human HSF1 and HSF2 are differentially regulated, HSF1 being activated by heat shock and other cellular stresses and HSF2 activated under certain non-stress conditions such as differentiation and development [Sistonen *et al* 1994].

1.5.2 Yeast HSF

Yeast HSF does not appear to be activated by trimerization, but has been reported to bind constitutively to DNA as a trimer. Heat shock induces the phosphorylation of the protein and this appears to result in increased gene expression. However, Giardina and Lis (1995) reported that HSF bound to HSEs within the *hsp82* promoter in non-heat-shocked cells but during a heat shock the transcription factor bound to additional (weak) HSEs. Unlike *Drosophila* or vertebrate HSFs, the affinity of the transcription factor for DNA does not increase due to heat shock, but the nature of the interaction of yeast HSF with the promoter may alter, activating transcription [Sorger *et al* 1987]. Nevertheless, the conservation of the heat shock response is exemplified by the following observations of yeast and *Drosophila*. HSF was purified from *Saccharomyces* and from *Drosophila* and their ability to bind to HSE sequences was investigated. Remarkably, the HSFs appeared to be identical both in size and also in their DNA binding properties. The transcription factors bound not only to their own HSE but also to an HSE sequence from the other species [Wiederrecht *et al* 1987].

1.5.3 HSF and the heat shock response

HSF1 appears to have the major role in the heat shock-induced expression of HSPs [Sarge *et al* 1991, Sistonen *et al* 1993]. For example, as with the higher vertebrate HSFs, in *Xenopus* oocytes, heat shock resulted in an increase in the HSE-binding activity of HSF1, but the binding activity of HSF2 appeared to be unaltered [Gordon *et al* 1997]. Targeted disruption of mouse *hsf1* resulted in a drastic reduction in the

transcription of heat shock genes during a heat shock and abolished the thermotolerance of murine cells, which was observed as an increase in heat-induced apoptosis [McMillan *et al* 1998].

The activation of human HSF1 requires trimerization, but under normal (non-stress) conditions this process is inhibited and HSF1 exists as monomers [Shi *et al* 1995]. Zuo *et al* (1994) suggested that HSF forms intra-molecular interactions via two leucine zipper regions and that an increase in temperature disrupts these internal leucine zippers in favour of forming inter-molecular interactions. However Zuo *et al* (1995) also proposed that HSF1 trimerization was only part of the activation process and that an additional conformational change occurred when the trimer translocated to the nucleus which resulted in the unmasking of the transcriptional activation domain. The *Drosophila* HSF trimer was observed to bind to inverted repeats of a five base motif, NGAAN [Perisic *et al* 1989], but fine structure analysis of the binding of *Drosophila* and *Saccharomyces* HSF revealed that there was a preference for adenine at position one [Fernandes *et al* 1994]. The three monomeric units within an activated HSF trimer have the potential to fully interact with an HSE that contains three inverted repeats. ***Figure 1.3*** shows a diagram of the binding of activated HSF with a heat shock element that contains two, three and four inverted repeats of NGAAN. It highlights the ability of HSF monomeric units to interact with the 5-bp units arranged in either orientation [Fernandes *et al* 1994].

Xiao *et al* (1991) suggested that cooperativity between HSF trimers, bound to a heat shock element, was required for the binding of HSF at heat shock temperatures. At 37°C, the *in vitro* binding of *Drosophila* HSF to one fragment containing a single site of three 5-bp units and to a second fragment containing six 5-bp units was studied. It was revealed that the relative binding affinity of HSF to the latter fragment was over 10^6-fold greater than to the former [Xiao *et al* 1991]. A study of *Drosophila* HSF revealed that HSF bound to the regulatory region of *hsp83* with an affinity 4-fold higher than to the *hsp23*, *hsp26*, *hsp27* and *hsp70* regulatory regions.

Figure 1.3 : Model for the interaction of HSF trimers with a heat shock element

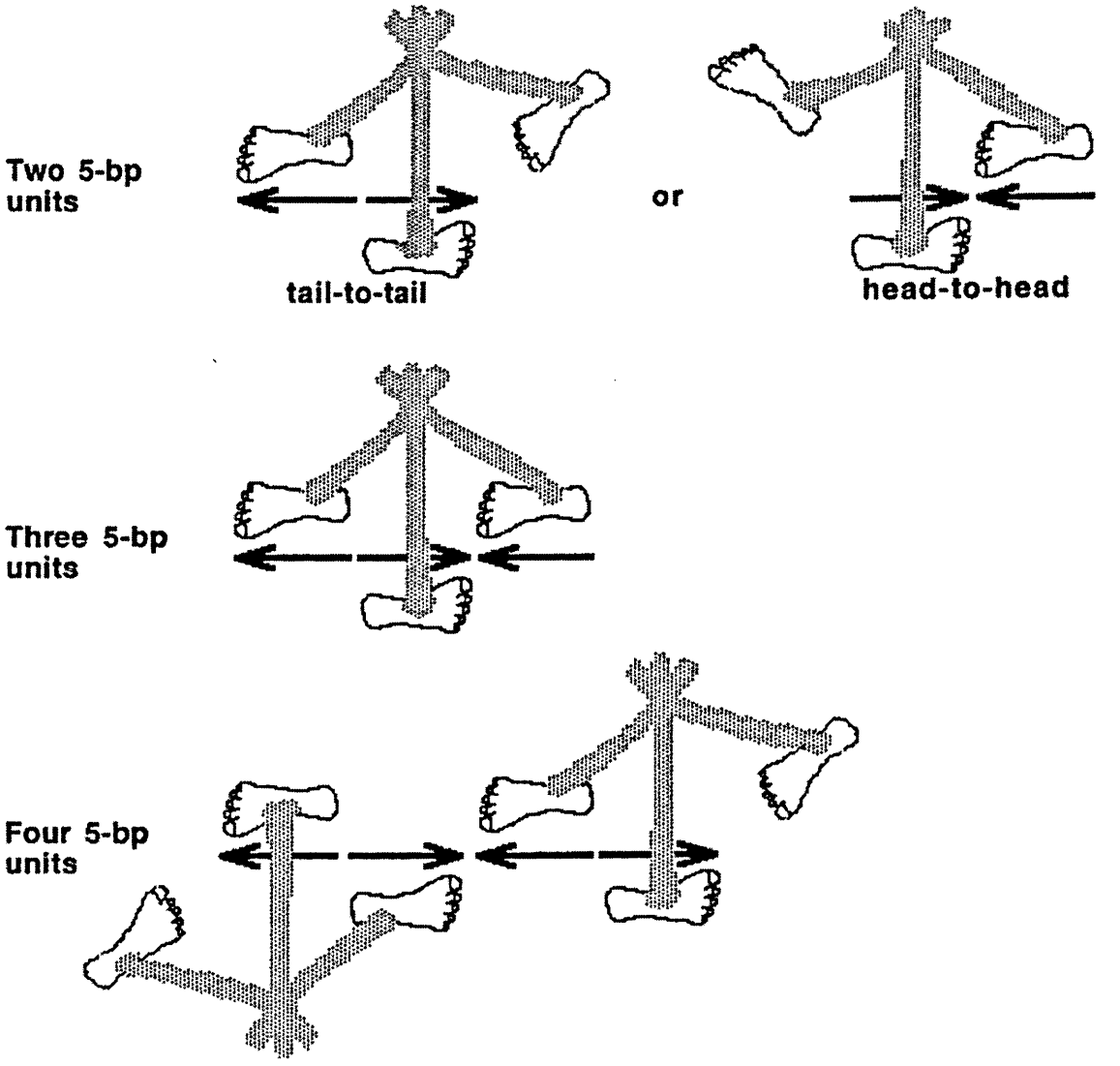


Figure 1.3 :

Arrows designate the 5-bp units and their relative orientation. The foot represents the DNA-binding domain of HSF and the legs join the subunits of the trimer. The HSF trimer can interact fully with a heat shock element that contains three 5-bp units. The original schematic was created by Lis *et al* (1990).

The promoter of *hsp83* has an HSE which contains a single array of eight 5-bp units whilst the promoters of the other *hsp*s have more HSEs but less 5-bp repeats within the HSEs. It was therefore suggested that there is direct protein-protein interaction between the HSF trimers bound to the *hsp83* HSE, which results in high affinity binding. Thus, there is a higher level of expression of *hsp83* under mild heat shock conditions, when compared to other *hsp*s [Fernandes *et al* 1995]. The affinity of mouse HSF1 or HSF2 for a particular HSE appeared to be different and in addition to this, HSF1 showed a higher degree of cooperative interaction with adjacent trimers than HSF2, possibly reflecting a major role for HSF1 during a heat shock [Kroeger and Morimoto 1994]. However, HSF2 trimers do exhibit cooperativity. Electron microscopy was used to study the binding of human HSF2 with fragments of DNA, which contained two HSEs, separated by ~900bp. Loops of DNA were visible and resulted from the distant HSEs being brought together as HSF2 trimers bound cooperatively [Wyman *et al* 1995].

1.5.4 Non-heat shock induced HSF binding

The rapid binding of HSF to HSEs is not restricted to heat-induced cellular stress, but also occurs due to other factors, for example, HSF2 activation by hemin as mentioned in 1.5.1. Becker *et al* (1990) observed that treating *Drosophila* and mouse cells with hydrogen peroxide resulted in the activation of HSE binding. Increased HSF-HSE binding was also observed when non-stressed cell extracts were treated with hydrogen peroxide *in vitro* [Becker *et al* 1990]. The cyclopentenone prostaglandins (PGs) have potent anti-proliferative effects and cause a block in cell cycle progression in the G_1 phase. Addition of PGs to HeLa S3 cells resulted in the transient expression of two HSP70 proteins [Ohno *et al* 1988]. Synthesis of an HSP70 was also observed when human K562 erythroleukemia cells were treated with PGs [Santoro *et al* 1989]. The accumulation of HSP70s was suggested to be associated with an inhibition in cell proliferation [Santoro *et al* 1989] and a regulatory role for HSP70 was proposed in normal cell cycle progression [Ohno *et al* 1988]. Induction of HSP expression resulted from PGs activating the binding of HSF [Rossi *et al* 1997]. Exposure of HeLa S3 cells to arachidonate, a precursor of prostaglandins, also resulted in the activation of HSF-DNA binding and a subsequent increase in the expression of HSP70 and HSP90. Gel mobility shift assays, using antibody perturbation, revealed that arachidonate induced the DNA-binding of HSF1, but not HSF2 [Jurivich *et al* 1994].

1.5.5 HSP expression via other transcriptional control elements

Possibly the most widely quoted example of the control of *hsp* genes via transcriptional control elements other than HSEs, is the expression of the small *hsp*s in *Drosophila.* The expression of *D. melanogaster hsp22, hsp23, hsp26* and *hsp27* can be induced by the steroid hormone, ecdysterone [Ireland *et al* 1982]. The ecdysterone inducibility of *hsp27* has been localized to a region in the promoter of the gene, bases –553 to –527, which is proposed to contain ecdysterone response elements (EREs) [Riddihough and Pelham 1987]. Furthermore, deletion of the promoter segment between –181 and –242, upstream of *hsp23*, yielded mutant promoters that were heat-inducible but were no longer under ecdysterone control [Mestril *et al* 1986]. Distinct regulatory regions within the promoter appear to be involved in the activation of transcription during the heat shock response (HSEs) and in the response to ecdysterone (EREs) [Mestril *et al* 1986].

Engelberg *et al* (1994) reported that the activation of Ras proteins, triggered by the depletion of cAMP in the growth medium, resulted in the transcription of *hsp70* and small *hsp* genes. The induction of *hsp* transcription did not appear to be due to the binding of HSF to the promoter region of these genes. Therefore the Ras pathway was suggested to involve a different transcription factor and transcriptional control elements [Engelberg *et al* 1994]. Although *hsps* appear to share the common feature of having HSEs in their promoter regions, mechanisms exist which can result in the differential expression of HSPs.

1.6 HSP expression and development

HSPs have been implicated in a wide range of developmental processes, for example, a specific role for HSPs has been proposed in mouse spermatogenesis. Biggiogera *et al* (1996) used immunocytochemistry to investigate the location of HSP90 and HSP27 in the mouse testes. HSP90 and HSP27 were detected mainly in the cytoplasm of Sertoli cells, spermatogonia, spermatocyctes and spermatids. However these HSPs were observed in the cytoplasm of all testis cell types during mouse spermatogenesis [Biggiogera *et al* 1996]. Transgenic mice (hsp70-2$^{-/-}$) with disruptions in *hsp70-2* were used to study the requirement of this molecular chaperone for the activity of CDC2 kinase. Spermatogenesis does not progress through G_2/M of meiosis I in these mice. HSP70-2 was proposed to assist in CDC2/cyclin B1 complex formation in mouse spermatocytes, by maintaining CDC2 in the correct conformation [Zhu *et al* 1997].

Another HSP, 73T (a member of the HSP70 family), is also thought to have a role in spermatogenesis. This protein appears to be germ cell-specific, as it was not detected in mutant mice devoid of germ cells [Sarge and Cullen 1997].

The technique of whole mount *in situ* hybridization analysis has been used to examine the pattern of tissue-specific mRNA expression in zebrafish embryos. Constitutive expression of *hsp90α* mRNA was observed in a small subset of cells within the pre-somatic paraxial mesoderm, somites and pectoral fin buds of developing embryos, the locations of muscle progenitor cells [Sass *et al* 1996]. MyoD is a myogenic regulatory factor and the location of *myoD* mRNA was compared to that of *hsp90α*. The *hsp90α*-expressing cells of the somites and pectoral fin buds also expressed *myoD*, suggesting a role for the proteins in early myogenesis [Krone *et al* 1997]. Similarly, an examination of *hsp90α* expression in chicken embryos also suggested the involvement of this gene in myogenesis. *Hsp90α* mRNA was highly expressed in developing somites and as with the zebrafish, appeared to be restricted to myogenic cells [Sass and Krone 1997]. Santacruz *et al* (1997) reported an enrichment of transcript for the heat shock cognate gene, *hsc70*, in the developing central nervous system (CNS) of zebrafish embryos and this HSP was also suggested to be involved in the process of differentiation.

HSPs have been observed to correlate with cell differentiation processes in mammals. Chiesa *et al* (1997) analyzed protein extracts from the lens and other tissues of Sprague-Dawley rats. During lens cell differentiation, the synthesis of α-crystallins A and HSP25 in lens cells increased. Changes in the phosphorylation of HSP25 also occurred, resulting in the accumulation of phosphorylated HSP25 in mature fibre cells. However in mice, an increase in α-crystallin B (HSP22) also appears to correlate with the activation of myogenesis during embryogenesis and a high level of HSP22 expression is associated with embryonic heart development [Benjamin *et al* 1997]. This is consistent with the suggestion by Loones *et al* (1997) that the expression of rat HSP25 is related to the innervation of muscles.

The small HSPs have also been implicated in the control of cell proliferation. In Ehrlich ascites tumour (EAT) cells, the expression of the murine small HSP, HSP25 appears to show a negative correlation with growth; for example cells in the exponential growth phase express only low levels of the protein. Transfection of EAT cells with a construct containing HSP25 and subsequent over-expression of this protein results in inhibition of proliferation [Knauf *et al* 1992]. Hashizume *et al* (1997) reported that during the mouse hair cycle, the levels of HSP27, HSP60 and HSP72 increase significantly during anagen-catagen transformation, suggesting their involvement in keratinocyte terminal differentiation and/or apoptosis.

1.7 HSPs and disease

HSPs have been implicated in a variety of different disease processes. It has been proposed that antibodies to HSPs, derived from infectious organisms, may cross-react with host components and give rise to autoimmune disease. Serum antibodies from patients suffering from tuberculosis or leprosy recognize a range of mycobacterial proteins. The genes encoding twelve antigens have been cloned and analyzed and three *Mycobacterium leprae* antigens and two *Mycobacterium tuberculosis* antigens were identified as homologues of HSP70, HSP60 (GroEL) and small HSPs. Reactivity to these antigens, with significant homology to self-proteins, may occasionally result in an autoimmune response [Young *et al* 1988]. Birk *et al* (1996) reported that in a non-obese diabetic (NOD) strain of mice, anti-HSP60 antibodies correlated with the destruction of β-cells. Furthermore, treating pre-diabetic mice with an epitope of HSP60 appeared to lower the incidence of diabetes and death of the mice, leading to the hypothesis that diabetes is modulated by anti-HSP60 T-cells.

Certain HSPs have also been implicated in other autoimmune diseases. For example, Noort *et al* (1995) used samples of myelin proteins from the CNS of multiple sclerosis (MS) and healthy control subjects to prime peripheral blood mononuclear cells (PBMCs). Differences in T cell responses to proteins from healthy and MS-affected tissues were observed and a 23kDa protein, identified as αB-crystallin, was detected as an immunodominant antigen present in MS samples. The expression level of αB-crystallin was up-regulated in oligodendrocytes and astrocytes in MS lesions and it was suggested that this small HSP may be involved in the development of MS. A role for

HSP47 has been suggested in the pathology of rheumatoid arthritis. The serum from patients suffering from this condition recognizes a 47kDa antigen identified as an HSP47 homologue. This protein was implicated in the process of cartilage destruction, perhaps due to the disorder of collagen resulting in the disruption of articular chondrocytes [Hattori *et al* 1998].

Clinical studies suggested that HSP90 may have a role in the autoimmune condition, systemic lupus erythematosus (SLE) since in patients with active disease there is a significant increase in the HSP90 concentration in PBMCs when compared to normal controls [Dhillon *et al* 1993]. An anomalous surface localization of HSP90 has also been observed on cells from SLE patients, which are over-expressing this protein [Latchman and Isenberg 1994]. In addition to the elevated level of HSP90 detected in serum from patients with active SLE, a higher than normal level of the cytokine IL-6 is also evident. A study utilizing a chloramphenicol acetyltransferase reporter gene, under the control of the human *hsp90*β promoter, to transfect a human hepatoma cell line showed the induction of gene expression by IL-6. Furthermore, a similar response was obtained by over-expressing the IL-6-induced transcription factors NF-IL-6 and NF-IL-6β [Stephanou *et al* 1997]. This suggests that high HSP90 levels may be a result, not only of induction by HSF, but also of the binding of other transcription factors to the gene promoter. Antibodies to self-HSP90 have also been observed in approximately 50% SLE patients and may be responsible for some of the symptoms, since these autoantibodies were not observed in normal individuals or in a group of rheumatoid arthritis patients that were tested [Minota *et al* 1988]. Conroy *et al* (1994) proposed that an elevated level of HSP90 autoantibodies correlated with active renal disease in SLE patients. There may also be a genetic predisposition to producing anti-HSP90 autoantibodies since there also appeared to be a link between the HLA haplotype of the patients and the elevation of these antibodies [Conroy *et al* 1994].

In humans, *hsp70* is located on chromosome 6, within the class III region of the human major histocompatibility complex (MHC), between the complement and tumour necrosis factor genes [Leung and Gershwin 1991]. This observation would be consistent with the suggestion that HSP70, as well as HSP90 and GRP94, may be primordial host defense molecules [Srivastava *et al* 1998]. HSP70 was previously identified as a tumour

rejection antigen and although HSP70 isolated from tumour cells differs antigenically from HSP70 isolated from normal cells, no mutations in the protein sequence were observed. Vaccination of mice with an HSP70 preparation from Meth A fibrosarcoma cells resulted in tumour-specific immunity to a subsequent challenge with Meth A cells [Udono and Srivastava 1993]. This result can be explained by the observation that endogenous tumour-derived peptides can bind within the carboxyl terminus groove of HSP70 and this is thought to result in the subsequent presentation of the tumour peptide via MHC class I molecules on the surface of the cell. An immune response is thus raised against the peptide and it has been suggested that this process could be manipulated in the development of vaccines to trigger the appropriate T cell response [Srivastava *et al* 1998, Zihai 1997].

1.8 HSPs and parasites

As in other eukaryotes, protozoa and helminth parasites express a full range of HSPs, many of which have been the focus of study. HSPs have been studied particularly in vector-borne parasites, the life cycle of which involves a compulsory transition from ambient temperature to mammalian body temperature. Although the role of HSPS in the transition between hosts remains controversial, the expression of these proteins may confer a survival advantage in the parasite following transmission to the mammalian host or in the face of an immune response [Polla *et al* 1991].

1.8.1 HSPs and protozoa

Much of the work on HSPs in parasitic protozoa has demonstrated a correlation between up-regulation of HSP expression and the *in vitro* culture conditions that induce differentiation of the parasite. The differentiation of *Leishmania mexicana* promastigotes to amastigotes *in vitro* requires a shift in the temperature from 24°C to 34°C. A comparison of promastigotes growing at 24°C and those undergoing differentiation at 34°C revealed an increase in the synthesis of seven polypeptides, by the differentiating *Leishmania*. These proteins had molecular weights similar to the seven HSPs produced by *Drosophila* during a heat shock [Hunter *et al* 1984]. However, Brandau *et al* (1995) reported that promastigotes from *Leishmania* contained constitutively high steady-state levels of the HSPs, HSP70, HSP83 (HSP90) and HSP100 which were not significantly altered by shifting the temperature from 25°C to 37°C. Any elevation in *Leishmania* HSPs appeared to result from an increase in

translation but not in transcription [Hunter *et al* 1984, Brandau *et al* 1995]. Nuclear run-on analysis of *L. amazonensis hsp83* transcription in promastigotes cultured at 26°C and at 35°C showed that the elevated temperature had little or no effect on nascent transcription. Transfection of a CAT reporter gene construct, containing the flanking regions of *hsp83,* into promastigotes demonstrated that the 5′ and 3′ intergenic regions (both approximately 1.5kb) were responsible for temperature-dependent control of mRNA stability [Argaman *et al* 1994]. Indeed, when these regions were replaced with intergenic regions from a non-heat shock gene, *cat* mRNA stability was unaffected by elevated temperatures [Aly *et al* 1994].

In *L. donovani*, HSP70 and HSP90 were observed to be transiently expressed, by the parasite, 24 hours after phagocytosis by human macrophages and were suggested to have a role in the conversion of the promastigote to the amastigote stage [Streit *et al* 1996]. Additionally, a role for HSP100 has been proposed in *L. donovani* amastigote development. Although HSP100 was barely detectable in unstressed promastigotes, (but increased significantly during heat stress), it was abundant in amastigotes isolated from infected lymph nodes, in the absence of cellular stress [Hubel *et al* 1998]. Disruption of *hsp100* impaired the expression of the A2 gene family, which are stage-specific marker proteins, and has been shown to significantly reduce *L. donovani* virulence in infected hosts. HSP100 may therefore have a developmental role in the parasite life cycle but it may also be required in the context of a heat shock protein to protect *L. donovani* in the hostile environment of its host [Krobitsch *et al* 1998].

HSPs may also be involved in the virulence of *Leishmania*. Salotra *et al* (1995) detected an increase in the expression of HSP70 (and also HSP90 and HSP60 homologues), in virulent but not avirulent *L. donovani* promastigotes, which had been exposed to human tumour necrosis factor (TNF). HSP70 was proposed to confer protection from the stress experienced in the macrophage and thus surviving promastigotes would be able to differentiate into amastigotes. Experimental infections of mice with *L. major* mutants, which had both alleles of ClpB (*hsp100*) replaced, resulted in a delay in lesion development when compared to wild-type *L. major*. Furthermore, overexpression of exogenous ClpB genes partially restored virulence to the mutants, which indicated a role for HSP100 in initial stages of mammalian infection [Hubel *et al* 1997].

Two isoforms of *L. major* HSP60, 65kDa and 67kDa, were observed to be expressed at a high level in infected macrophages, reaching a maximum at 24 hours post-infection [Rey-Ladino and Reiner 1993]. Polyclonal antiserum raised against *Cyanobacteria* HSP60 was used to investigate the sub-cellular localization of *L. major* HSP60, which was identified in the mitochondrial-enriched fraction of an extract of promastigotes. This is consistent with the proposed role of HSP60 in eukaryotes in the formation and regeneration of mitochondria [Hartl *et al* 1994]. A fusion protein was produced from the sequence of *L. major hsp60* (*hsp65*). The recombinant protein was used to examine the sera of patients with leishmaniasis for antibodies against HSP60. The sera from infected individuals strongly reacted with *Leishmania* HSP60 in contrast to the control sera, which showed little reactivity to this antigen. Furthermore, a humoral immune response against *L. major* HSP60 did not appear to be protective against infection with the parasite [Rey-Ladino *et al* 1997]. Immunization of BALB/c mice with a protein containing an HSP70 homologue from *Leishmania infantum* fused to the maltose-binding protein (MBP), produced a stronger humoral and cellular immune response than with an inoculation of only MBP. Similar to the proposed role of mammalian HSP70 in antigen-presentation [Udono and Srivastava 1993, Srivastava 1998], *L .infantum* HSP70 was suggested to have an adjuvant effect with respect to the covalently attached MBP protein [Rico *et al* 1998].

A role for HSPs in the development and virulence of other protozoan parasites, such as *Toxoplasma,* has also been proposed. Weiss *et al* (1998) observed the induction of a 72kDa homologue of HSP70 from *T. gondii* during bradyzoite development. Immunofluorescence studies demonstrated the co-localization of the HSP70 protein with bradyzoite-specific antigens. Addition of quercetin, which inhibits the synthesis of HSP70, HSP90 and HSP27 caused the suppression of bradyzoite development and it was proposed that HSP70 may be important in bradyzoite differentiation [Weiss *et al* 1998]. However, Lyons and Johnson (1995) suggested that an increase in HSP70 expression may be required to protect *T. gondii* from the immune response of the host. Avirulent and virulent strains of *T. gondii* appeared to express a different profile of proteins upon entering murine host macrophages. The virulent strain of *T. gondii* expressed high levels of an HSP70 homologue whilst this protein was not observed either in the avirulent strain or in the virulent strain in an immuno-compromised host [Lyons and Johnson 1995]. In mice, protection against an infection with the *T. gondii*

correlates with host expression of HSP65 (an HSP60 homologue) within and on macrophages [Hisaeda *et al* 1995]. More specifically, HSP65 appears to prevent the apoptosis of infected macrophage and this increases host defense against the parasite [Hisaeda *et al* 1997].

Other examples of the possible functional roles for HSPs in parasitic protozoa include a potential function for HSP70 in the assembly/disassembly of the protozoan cytoskeleton, as observed in *Plasmodium knowlesi*. An attempt to obtain proteins from merozoites, involved in actin polymerization, identified an HSP70 homologue (HSC70) as an F-actin binding protein. Two proteins of 32kDa and 34kDa co-eluted with HSP70 from F-actin columns and complexes containing all three proteins were also observed using gel filtration chromatography. Addition of the HSP70 complex to rabbit skeletal muscle inhibited the formation of actin filaments and it was suggested that in *P. knowlesi* regulation of actin filament growth by the complex facilitates movement of the merozoite into the host cell [Tardieux *et al* 1998]. A mitochondrial homologue of HSP70 in the Trypansomatidae, *Trypanosoma cruzi*, *T. brucei* and *L. major* was observed to be concentrated in the kinetoplast (within the parasite) and, like *P. knowesi* HSP70, may be involved in the assembly/disassembly of the cytoskeleton. Klein *et al* (1995) suggested that the mitochondrial HSP70 protein had a specific role either in the maintenance of the submitochondrial structure, or alternatively, in processes involving the kDNA housed within. HSP90 may also have a structural role in *P. falciparum*, as this HSP was reported to be associated with the parasitophorous vacuole, the space that surrounds the parasite within the erythrocyte [Bonnefoy *et al* 1994].

1.8.2 HSPs and helminths

Anti-sera from helminth-infected individuals frequently recognizes antigens identified as heat shock protein homologues. For example HSP70 homologues from the trematode parasites, *Schistosoma japonica* and *Schistosoma mansoni*, provoke an immunodominant response in infected individuals, but do not appear to result in a reaction to host HSP70 [Hedstrom *et al* 1988]. Similarly, the serum from individuals suffering from Brugian filariasis strongly reacted with a fusion protein obtained from a *B. pahangi* cDNA expression library, which was identified as HSC70, a heat shock cognate homologue of HSP70 [Selkirk *et al* 1989].

Caenorhabditis elegans

Although *C. elegans* is not a parasitic species it is an excellent model for many parasitic nematodes, hence the literature on HSPs and *C. elegans* has been included in this section. As in other organisms, heat shock induces a full range of HSPs in *C. elegans.* The ability to manipulate *C. elegans* with transfection constructs has led to a greater understanding of the role of HSPs in this nematode compared to others.

Lithgow *et al* (1996) identified mutants of *C. elegans* that had a longer than normal life span. These mutants were intrinsically thermotolerant and also resistant to oxidative stress and U.V. radiation. It was proposed that by reducing cellular damage resulting from stress, heat shock proteins may have the capacity to slow the ageing process. Transfection and over-expression of a member of the *hsp70* family, *hsp70A,* from the free-living nematode *C. elegans* in the entomopathogenic nematode, *Heterorhabditis bacteriophora* Hp88, resulted in enhanced thermotolerance of the transgenic parasite. This confirmed that like other HSP70s, the nematode gene product had a role in protecting the organism from heat-induced cellular damage [Hashmi *et al* 1998].

Russnak *et al* (1983) studied the profile of ^{35}S-labelled polypeptides from *C. elegans* adult worms under control conditions (22°C) or heat shock conditions (35°C). Heat shock proteins, HSP25, HSP18 and HSP16 were observed only in the samples from heat shock treated adults. Furthermore, *hsp16* transcripts were not detected in control (22°C) worms by northern blot analysis. However, the heat shock-induced expression of HSP16 was affected by development. In a strain transformed with HSP16, HSP16 fusion proteins were not observed during gametogenesis and early embryogenesis and first became heat inducible in gastrulating embryos. A possible explanation for this is that maternal *hsp16* genes are not inducible during gametogenesis and early embryogenesis, and zygotic genes are not transcribed until gastrulation [Stringham *et al* 1992].

Transcripts for *Sec-1*, a *C. elegans* gene with homology to small *hsp*s, were only observed in oocytes and developing embryos. The rapid synthesis of SEC-1 correlated with mitogenesis during early embryogenesis and a role for this protein, during development, was proposed [Linder *et al* 1996]. Snutch *et al* (1988) suggested that two members of the *C. elegans hsp70* family may be developmentally regulated. *Hsp70*A

and *hsp70*C mRNA were most abundant in the first stage (L_1) larvae but decreased by 50% and 15% respectively in the adult stage. The dauer larvae of *C. elegans* is an alternative, resilient and developmentally arrested life cycle stage which is induced by unfavourable environmental conditions, such as a lack of food or overcrowding. Dalley and Golomb (1992) reported that while myosin, actin and histone mRNAs were significantly depleted, and the proportion of *hsp70* mRNA was equivalent to other life cycle stages, *hsp90* mRNA was greatly enriched in dauer larvae. Furthermore, re-initiation of development of the larvae resulted in a rapid decline of *hsp90* transcript levels. This led to the proposal that *hsp90* may have a role in the developmental arrest which occurs in the dauer larvae [Dalley and Golomb 1992]. The ability of the dauer larvae to survive for long periods of time without further development and the observation that the mf of filarial nematodes can survive for many months in the mammalian host, may indicate similarities in the processes which maintain the developmental blocks in these life cycle stages.

Filarial nematodes

As with the protozoan parasites, much of the study of HSPs in helminths has been focused on species which are vector-borne. In some such parasites for example, *Brugia sp.*, HSPs appear to be expressed under non-stress conditions. In filarial nematodes, the mf and the L_3 are developmentally arrested in one host and only resume development after transfer to the next host (as described in 1.1.1). One possible cue for the cessation of the developmental block is the temperature shift experienced by the mf and L_3 as a result of the transfer. Christensen and Hollander (1978) studied the effect of different environmental temperatures (18.5°C, 22.5°C, 26.5°C and 34.5°C) on the development of *Dirofilaria immitis* in the mosquito vector, *Aedes trivittatus*. The optimal temperature range for the development of *D. immitis* mf in *Ae. trivittatus* was reported to be 22.5°C-26.5°C and higher temperatures resulted in reduced yields of L_3. Similarly, the development of *B. pahangi* mf in *Ae. aegypti* was studied at 28°C and 32-37°C. At the higher temperatures larvae development was inhibited [Devaney and Lewis 1993].

Differences in the profiles of newly synthesized polypeptides were observed when L_3 and mf were cultured at 28°C and 37°C. Devaney *et al* (1992) observed the constitutive expression of two small HSPs (22-24kDa and 18kDa) in extracts from mf cultured at 37°C but not at 28°C. Unlike a true heat shock response, no significant reduction in the

overall rate of protein synthesis, resulting from the temperature shift (28°C to 37°C), was observed. To investigate these observations further, a small *hsp* gene, *Bphsp7*, was cloned from *B. pahangi*. The presence of two potential start codons in the cDNA sequence indicated that two gene products of 21kDa and 18kDa may be expressed from a single transcript. The sizes of the gene products were consistent with the sizes of the ^{35}S-labelled polypeptides observed in mf cultured at 37°C. Northern blot analysis of *B. pahangi* mRNA revealed that *Bphsp7* mRNA was detectable in mf cultured at 37°C and in mf and adult worms heat shocked at 41°C. However, *Bphsp7* transcripts were not detected in RNA from mf cultured at 28°C or adults cultured at 37°C [Thompson *et al* 1996]. It appears that the expression of *Bphsp7* is not strictly heat inducible and may also be developmentally regulated. Furthermore, the small HSPs, 22-24kDa and 18kDa, may have a role in the process of developmental arrest that occurs in mf circulating in the mammalian bloodstream. Transient expression of the two small HSPs was observed in L_3 shifted from 28°C to 37°C and it was suggested that the expression of the HSPs reflected a classical heat shock response as the L_3 moves from mosquito vector to mammalian host [Jecock and Devaney 1992].

In contrast to the results described above, in the filarial nematode, *D. immitis*, a small HSP homologue, termed p27 was not up-regulated by exposure to 43°C. Antiserum to the recombinant p27 was used to localize this protein to the hypodermal tissue of L_3 and L_4 of *D. immitis* and the L_3 of *Onchocerca volvulus*. Western blotting analysis, revealed that p27 was expressed constitutively throughout the L_3 to L_4 moult [Lillibridge *et al* 1996]. In *B. malayi* an HSP70 homologue was identified, which under non-stress conditions, localized to the somatic musculature, hypodermis, lateral chords, alimentary tract and reproductive structures of the L_4 and adult worms. However, culturing mosquito-derived L_3 at 37°C for 24 hours resulted in a classical heat shock response and HSP70 was observed in all tissues [Schmitz *et al* 1996].

Parasitic nematodes, like other organisms, appear to respond to elevated, non-physiological temperatures with the rapid synthesis of a small number of (HSP) proteins. For example Selkirk *et al* (1989) compared the profile ^{35}S-methionine labelled polypeptides from mf, L_3 and adult male worms that had been cultured at 26°C, 37°C and 43°C. A heat shock of 43°C resulted in the almost exclusive expression of five polypeptides of 18.5kDa, 22.5kDa, 62kDa, 70kDa and 85kDa. Similarly, when the

parasitic nematodes *Trichinella spiralis* and *Trichinella pseudospiralis* were exposed to a heat shock temperature of 43°C, the transcription and expression of proteins identified as major HSPs were induced [Ko and Fan 1996].

Other nematodes, trematodes and cestodes

A developmental role for HSP70 was proposed in the life cycle of the trematode, *Schistosoma mansoni*, where an *hsp70* gene is constitutively transcribed in the miracidia, sporocyst and adult but not in the cercariae. The transient accumulation of high levels of HSP70 was observed during the *in vitro* cercariae/schistosomula transformation and was thought to be a stress response due to the shift in temperature (from 23°C to 37°C) and osmotic stress (from water to isotonic medium). It was also speculated that *in vivo* transcription of *hsp70* in the cercariae is inhibited by signals from the tail section, since removal of the tail is required for the induction of the heat-induced expression of *hsp70* genes in schistosomula [Neumann *et al* 1993].

It is possible that an increase in the concentration of chaperones, such as the small HSPs, is required to assist the folding of structural proteins expressed during the moulting process. For example, in the intestinal nematode, *Nippostrongylus brasiliensis*, northern blot analysis was used to study transcripts for the small *hsp*, Nb*hsp*20 in different life cycle stages. Nb*hsp20* mRNA was first observed 4 days post-infection, corresponding to the period of the final moult to adults, and peaked at 9 days post-infection [Tweedie *et al* 1993].

HSPs and the immune response

More than 90% of *Schistosoma mansoni*-infected individuals were observed to elicit a strong immune response to a 40kDa antigen (p40) from *S. mansoni* eggs and miracidia. Analysis of the antigen revealed that it was composed of four separate proteins originating from a multi-gene family. The cloning and sequencing of two of these genes identified their homology with small *hsp*s and α-crystallins [Nene *et al* 1986]. Cai *et al* (1996) demonstrated that r38, an *S. mansoni* fusion protein identical to p40, induced chronic T-cell-mediated granulomatous tissue responses in mice. This suggested that small HSP homologues from *S. mansoni* eggs may contribute to the immunopathology observed in infected individuals.

Wales and Kusel (1992) suggested that the induction of protective immunity by irradiated larvae of the parasitic helminth, *S. mansoni*, may relate to an inhibition in the synthesis of HSPs and the subsequent accumulation of aberrant proteins induced by irradiation. Furthermore, alterations in antigen conformation and presentation may result in effective immunity. However this hypothesis was not supported by the gastrointestinal parasitic nematode, *Heligmosomoides polygyrus*, since few differences in protein synthesis were observed between γ-irradiated and unirradiated larvae. Heat shocking both irradiated and unirradiated larvae at 42°C induced the expression of a specific set of proteins i.e. HSPs. At 42°C the synthesis or secretion of certain excretory/secretory (ES) proteins were markedly depressed in irradiated parasite. It was thus proposed that prevention of maturation may explain the efficacy of a vaccine of radiation attenuated parasites [Pleass and Bianco 1996]. The effect of irradiation on protein synthesis in the filarial nematode, *B. pahangi*, has also been investigated. As with *H. polygyrus* no differences were observed in newly synthesized polypeptides as a result of irradiation [Devaney *et al* 1993].

Kumari *et al* (1994) utilized rabbit antiserum raised against *B. malayi* ES antigens to screen a *B. malayi* cDNA expression library and isolated a recombinant antigen, which was identified as a member of the HSP90 family. The identification of HSP90 as an ES protein may be artifactual, due to leakage from damaged worms, but anti-serum from patients suffering from Brugian filariasis, ascariasis, onchocerciasis and loiasis reacted with the *B. malayi* fusion protein, suggesting that HSP90 is a major antigen in these infections. The sera from some patients infected with *Trichinella spiralis* recognizes parasite HSP60 but this was also suggested to result from the leakage of this abundant protein from dead or damaged worms [Allegretti *et al* 1997].

In an elaborate investigation, mRNA from *S. mansoni* adults and eggs was translated *in vitro* and the products were immunoprecipitated with sera from rabbits inoculated with schistosomula or soluble egg antigen, from mice inoculated with cercariae and sera from human patients infected with *S. mansoni*. All sera tested reacted with proteins of 100kDa, 86kDa and 28kDa [Taylor *et al* 1983]. In an attempt to identify surface located antigens, anti-schistosomula antisera was incubated with formalin-fixed and live larvae and then used to immunoprecipitate *in vitro* translation products. The 86kDa protein

was not precipitated suggesting it was located on the parasite surface [Cordingley *et al* 1986]. A cDNA encoding the 86kDa antigen was isolated, sequenced and identified as an HSP90 homologue. Antisera from humans infected with the parasite recognized the protein. The original identification of the 86kDa (HSP86) protein as a surface antigen in schistosomula was proposed to have resulted from possible damage to the tegumental membrane, (similar to the explanation for the recognition of *T. spiralis* HSP60 by the serum of infected individuals). It was suggested that *S. mansoni* HSP86 may confer protection to the parasite and/or have a role in host immunity or pathology [Johnson *et al* 1989].

Ernani and Estes (1993) showed, by drug inhibition studies and western blot analysis, that HSP70 and HSP60 were actively exported when *Mesocestoides corti* was shifted from room temperature to 37°C. HSP60 and HSP70 were therefore thought to have a role in the immune response to *M. corti*. Furthermore, the stimulation of splenocytes, from infected mice, with *M. corti* HSP70 resulted in the expansion of an unusual cell type, similar to large granular lymphocytes [Estes *et al* 1993].

These examples of parasite *hsp* transcription and translation reveal the possible involvement of the major heat shock proteins in development, virulence and survival. In addition, host immune responses to parasite HSPs have been implicated in both protective immunity and immunopathology.

1.8.3 HSP expression in pathogenic fungus

An immunodominant 47kDa protein from the pathogenic fungus, *Candida albicans*, was identified and antibodies against this protein were reported to protect against candidiasis. Studies by Swoboda *et al* (1995) identified this 47kDa protein as being a carboxyl terminal fragment encoded by the *C. albicans hsp90* gene. It was suggested that HSP90 is multi-functional, important in fungal growth, stress and morphogenesis. In the life cycle of the pathogenic fungus, *Paracoccidioides brasiliensis*, morphological changes occur during temperature shift. The yeast stage is observed at 36°C whilst mycelium are observed at 26°C. Changes in protein synthesis occur during these transitions and stage-specific proteins include HSP70, which is only detected in yeast cells [Dasilva *et al* 1994]. A substantial quantity of *hsp70* and *hsp80* mRNA was observed in germinating spores, from the plant pathogenic fungus, *Leptosphaeria*

maculans, in the absence of stress, but these transcripts were not detectable in dormant spores. These *hsp*s were therefore proposed to have some role in the process of germination [Patterson and Kapoor 1995]. During fungal development, HSPs may act as molecular chaperones to assist the folding of newly synthesized proteins, however it is possible that they also have a more specific role in developmental processes.

1.9 Aims of the project

As summarized above, HSPs are amongst the best studied of all proteins and many of their defined functions have been elucidated, mostly in *in vitro* studies using cell lines. Their roles in development *in vivo* remain more controversial. Although numerous studies have implicated HSPs in a variety of developmental processes, few have formally proven that HSPs are essential.

As stated in 1.8.2 previous work on HSPs from *Brugia* had shown a close correlation between small HSP expression and the developmental block experienced by the mf in the mammalian host. Similarities between the developmentally arrested *C. elegans* dauer larvae and mf were also described and the accumulation of *hsp90* mRNA in dauer larvae may also indicate a role for this *hsp* in the developmental block in *B. pahangi* mf. Therefore, the aim of this project was to isolate and characterize *hsp90* from the parasitic nematode, *Brugia pahangi*.

2.0 Materials and Methods

2.1 Maintenance of parasite life cycle

The *B. pahangi* life cycle was maintained by passage through a susceptible (Ref^m) strain of *Aedes aegypti*, the mosquito vector and the Mongolian jird, *Meriones unguiculatus* as the mammalian host.

2.1.1 Maintaining the mosquitoes

Mosquitoes were kept at 28°C in an insectary and the relative humidity was maintained at 70-80%. Adults were confined in netted cages, fed on sugar cubes and supplied with water from moist cotton wool pads. Larvae and pupae were kept in plastic trays filled with water and were fed on yeast tablets. Pupae were separated from the larvae each day and placed in small pots of water inside netted cages, where they emerged as adults. Stock mosquitoes were starved of sugar for 24 hours and then fed on bovine blood via a membrane feeding system. The blood was maintained at 37°C during feeding. Eggs were collected on moist filter paper placed in the bottom of the cages. The papers were then dried and stored until required. To hatch the eggs, a filter paper was placed in trays of water containing a yeast tablet.

2.1.2 Maintaining the parasites

Jirds were infected, by injection, with 250 L_3 into the peritoneal cavity. After 3 months jirds were sacrificed by CO_2 anaesthesia and exsanguinated by cardiac puncture. Adults and mf were obtained by flushing the peritoneal cavity with Hanks Balanced Salt Solution (HBSS, Gibco BRL) at 37°C and pH 7.2-7.4. Adults were transferred with a glass hook to a cryovial and stored in liquid nitrogen until required. The mf were washed again with HBSS and then resuspended in rabbit blood at a density of 350-450 mf per 20µl blood. Adult mosquitoes were fed the mf-infected blood via a membrane feeding system, which kept the blood at 37°C during feeding.

2.2 The polymerase chain reaction

PCR [Mullis and Faloona 1987] was used to obtain fragments of *B. pahangi hsp90* from genomic DNA and cDNA. Although the precise reaction conditions were modified depending on certain requirements, the general procedure was as follows:

1-5µl of template (genomic DNA, 1st strand cDNA, plasmid or PCR product)

4.5µl of 11.1x PCR buffer A

1.25 units of AmpliTaq (Perkin Elmer)

100 pmoles (2µM) of forward primer

100 pmoles (2µM) of reverse primer

adjusted to 50µl with filter sterilized double deionized water (ddH_2O)

Control samples had no template, and/or one or both of the primers omitted. The samples were then overlaid with a small quantity of mineral oil (Sigma) and subjected to the relevant PCR programme, usually:

Denaturation at 95°C for 2 minutes		1 cycle
94°C for 1 minute	⎫	
55°C* for 1 minute	⎬	30 cycles
72°C for 3 minutes	⎭	
Extension at 72°C for 10 minutes		1 cycle

* A different annealing temperature was sometimes utilized.

2.2.1 PCR and cloning of *hsp90* fragments

Internal fragments of *B. pahangi hsp90* were amplified from *B. pahangi* adult genomic DNA and from first strand cDNA, prepared from RNA from L_3 isolated from jirds 24 hours post-infection. The primers hsp90f2 and hsp90r2 were utilized and since these are heterologous primers, differences between the primer and template sequences were anticipated and therefore a lower annealing temperature (50°C) was utilized in an attempt to compensate for mismatches. The product, hsp90f2-r2, amplified from genomic DNA was 1.3kb and hsp90f2-r2 amplified from cDNA was 0.92kb. The reactions are described in 3.2.2 and 3.2.3 and the PCR products are shown in ***Figures 3.2*** and ***3.3***.

The 5′ end of *hsp90* was obtained by using the spliced leader (SL1) primer and the hsp90r3 primer (designed from new sequence information), to amplify first strand cDNA prepared from RNA from mf cultured for 2 hours at 37°C. Hsp90r3 and SL1 are homologous primers and therefore an annealing temperature of 55°C could be utilized.

The product, hsp90SL1-r3, was 1.6kb. Hsp90SL1-r3 was digested with *Eco*RI and the released fragment of 0.76kb was sub-cloned (sc90*e*slr3). In addition, hsp90SL1-r3 was amplified with hsp90f2 and hsp90r3 and the PCR product, sc90f2-r3 (0.68kb) was cloned and sequenced. These procedures are described in 3.2.5 and the PCR products are shown in ***Figure 3.4***.

Fragments of the 3'end of *hsp90* were amplified from first strand mf cDNA. Only one primer, hsp90f4, was used in a PCR and the product hsp90f4end (1.1kb) was amplified (see ***Figure 3.6***). A run of thymidine nucleotides on the sense strand prevented sequencing of the anti-sense strand. Hsp90f4end was digested with *Sph*I to remove a 0.39kb fragment from the 3' end and with *Hind*III to remove a 0.37kb fragment. The plasmid was then re-ligated to produce scf4*sph*Ir (0.71kb) and scf4*Hind*IIIr (0.73kb), which were sequenced. The procedures are detailed in 3.2.6 to 3.2.8. Hsp90f4end was truncated and therefore the primer hsp90f6, and the adapted dT primer were used to amplify a second product, hsp90f6dt (0.61kb), from first strand mf cDNA (see 3.2.9).

All the PCR products were purified from agarose gels (see 2.5), cloned and sequenced. ***Table 3.2*** contains information about the plasmids used in the cloning of the PCR products. The *hsp90* fragments were assembled into the complete sequence (see ***Figures 3.7*** and ***3.8***) using the "gelassemble" programme (described in 2.25). Primer sequences are shown in ***Table 2.1***.

Reagents :

PCR buffer A (11.1x) (Dr. A.J. Jeffreys, University of Leicester)

	Final concentration in reaction (1x)
334µl of 2M Tris.HCl pH 8.8	45mM
166µl of 1M ammonium sulphate	11mM
67µl of 1M magnesium chloride	4.5mM
7.2µl of 100% β-mercaptoethanol	6.7mM
6.8µl of 10mM EDTA pH 8.0	4.4µM
150µl of 10mM dATP (Pharmacia)	1mM
150µl of 10mM dCTP	1mM
150µl of 10mM dGTP	1mM

150µl of 10mM dTTP	1mM
170µl of 10mg/ml BSA (Boehringer Mannheim)	113µg/ml
100µl aliquots stored at -20°C	
(diluted in PCR reaction to 1 x)	

2.3 Library screening for *hsp90*

2.3.1 Screening a genomic library for *B. pahangi hsp90*

An *hsp90* genomic clone was obtained by screening an adult *B. pahangi* genomic library in EMBL3 [Frischauf *et al* 1983] using the protocol supplied with the Lambda Dash® II/*Bam*HI Vector Kit (Stratagene #247211, #247611, #247711). The library, acquired as a gift from Prof. Gird Hobom at the University of Geissen, was constructed from DNA from adult, mixed sex *B. pahangi* which had been partially digested with *Mbo*I and size fractionated to restrict the inserts to 9-23kb. Two probes were generated by PCR from adult genomic DNA: a product of 1.3kb (hsp90f2-r2) using the primers hsp90f2 and hsp90r2 and a product of 0.43kb (hsp90f5-r5) using the primers hsp90f5 and hsp90r5. Hsp90f5-r5 corresponds to a region near the 5′ end of *hsp90* and hsp90f2-r2 corresponds to a central region of *hsp90*. These PCR products were run on 1% TAE agarose gels and the bands excised and Spin X purified, as described in 2.5.

In the primary round, eight 150mm plates, each with 5 x 10^4 pfu were screened, a total of 4 x 10^5 pfu. The agar plates were chilled at 4°C for 2 hours and then overlaid with Hybond-N filters (Amersham). The filters were orientated using needle pricks through the filter and into the agar and then lifted off. They were submerged for 2 minutes in denaturing solution, then for 5 minutes in neutralizing solution and finally rinsed briefly in rinsing solution. The DNA was fixed onto the filters by exposing them to 150mjoules of U.V. radiation in a U.V. cross-linker (BioRad). DNA probes were denatured and then radiolabelled, by random primed DNA labelling, using High Prime (Boehringer Mannheim) with ^{32}P-dCTP (Amersham 3000Ci/mmol). The first lift was probed with hsp90f5-r5 and the second with hsp90f2-r2. The filters were washed to 0.2 x SSC, 0.1% SDS, and then rinsed with 2 x SSC and exposed to film. Fourteen plaques were identified which hybridized to both probes and seven of these, chosen randomly, were taken to a secondary screen. After the secondary screen, five duplicate positives were plated out as single pfu and probed with hsp90f2-r2 and hsp90f5-r5 to confirm that the

plaques were homogeneous. Lambda preparations (Promega) were made of the five clones (see 4.2.7 to 4.2.11).

2.3.2 Screening a cDNA library for *hsp90*

A *B. pahangi* cDNA library was screened using the genomic *hsp90* fragment, hsp90f2-r2 in an attempt to obtain a full-length *hsp90* cDNA clone. The protocol outlined in the Uni-ZAP™ XR Cloning kit instruction manual (Stratagene) [Chauthaiwale *et al* 1992] was used to screen a Uni-Zap library constructed from cDNA from mf heat shocked at 41°C [Thompson *et al* 1996]. Five plates were screened, each with 5 x 10^4pfu, a total of 2.5 x 10^5pfu and duplicate filter lifts were taken. Approximately 25ng of the PCR fragment, hsp90f2-r2, was radiolabelled and the filters were hybridized with the probe overnight at 65°C. The filters were then washed at 65°C to 0.2 x SSC, 0.1% SDS.

a) Bacterial strain used for screening an adult *B. pahangi* genomic library in Lambda Dash® :

XL1-Blue MRA

Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, gyrA96, relA1, lac

b) Bacterial strains used for screening a mf cDNA library in Uni-Zap™ XR :

XL1-Blue MRF′

Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac, [F′ *proAB, lacI*^{q}*ZΔM15*, Tn*10* (tet^r)]

SOLR

e14$^-$ *(mcrA), Δ(mcrCB-hsdSMR-mrr) 171, sbcC, recB, recJ, umuC::*Tn*5*(kan^r)*, uvrC, lac, gyrA96, relA1, thi-1, endA1,* λ^R, [F′ *proAB, lacI*^{q}*ZΔM15*] Su$^-$ (nonsuppressing)

Reagents :

L-broth

10g Bactotryptone (Difco)

5g yeast extract (Difco)

L-agar

7.5g agar (Difco)

adjust to 500ml with L-broth

10g NaCl
dissolve and sterilize by autoclaving
dissolve in 1ℓ ddH_2O
cool to 45°C before pouring plates (add antibiotics if required)
aliquot into 100ml bottle and autoclave
store agar plates at 4°C before use
store at room temperature

Top agarose
0.7% agarose in L-broth
dissolve and sterilize by autoclaving
cool to ~48°C before use
store at room temperature

Ampicillin stock (100mg/ml)
1g ampicillin (Sigma)
add sterile ddH_2O to 10ml
store at -20°C
(use at 100μg/ml in agar plates)

SM buffer
5.8g NaCl
2.0g $MgSO_4 \cdot H_2O$
50ml of 1M Tris.HCl pH 7.5
dissolve and sterilize by autoclaving
5.0ml of 2% (w/v) gelatin
adjust to 1ℓ with ddH_2O
autoclave and store at room temperature
(50mM Tris-HCl, 0.1M NaCl, 8mM $MgSO_4$, 0.01% gelatin)

NZY plates
add 15g agar to:
1ℓ of NZY broth (Difco)
dissolve and sterilize by autoclaving
cool to 55°C and pour plates
store at 4°C

Denhardt's solution (50x)
1% Ficoll (type 400)
1% polyvinyl pyrollidone
1% bovine serum albumin fraction 5
adjust to 100ml with ddH_2O
aliquot and store at -20°C

20 x SSC
3M NaCl (175.3g)
0.3M Sodium citrate (88.2g)
800ml ddH_2O
adjust to pH7.0 with conc. NaOH
adjust to 1ℓ with ddH_2O
store at room temperature

Tetracyclin stock (12.5mg/ml)
To 125mg tetracyclin (Sigma) add 10ml ethanol and store at -20°C

Kanamycin stock (50mg/ml)
To 0.5g kanamycin (Sigma) add sterile ddH_2O to 10ml and store at -20°C

IPTG stock

100mM isopropyl B-D-thiogalactopyranoside (Sigma) in sterile ddH_2O, store at 4°C.

Denaturing solution

1.5M NaCl

0.5M NaOH

adjust to 1ℓ with ddH_2O

store at room temperature

Neutralizing solution

1M Tris base

1.5M NaCl

adjust to pH 7.4 with conc. HCl

adjust to 1ℓ with ddH_2O

store at room temperature

Pre-hybridization solution

6.25ml 20 x SSC

2.5ml Denhardt's solution

1.25ml 10% SDS

15ml ddH_2O

heat to hybridization temperature

add 50µl heat denatured salmon sperm DNA (10mg/ml)

use immediately

Wash solutions

2.5-150ml 20 x SSC

5ml 10% SDS

adjust to 500ml with ddH_2O

store at room temperature

pre-heat before use

Rinsing solution

50ml of 20 x SSC, 100ml of 1M Tris.HCl pH 7.5, adjust to 500ml with ddH_2O

store at room temperature

2.4 Preparation of lambda clones

The method followed was that outlined in the protocol supplied with the Wizard™ Lambda Preps DNA Purification System (Promega A7290). A single phage plaque was picked from an agar plate using a borosilicate Pasteur pipette. The agar plug was dislodged into a 1.5ml microcentrifuge tube containing 100µl of Phage Buffer (supplied with the kit) and placed at 4°C overnight to allow the phage to diffuse into the solution. A single colony of XL1-Blue MRA′ cells was used to inoculate 5ml of LB broth supplemented with 50µl of 20% maltose and 50µl of 1M $MgSO_4$ and this was incubated overnight at 37°C in a horizontal shaker. A 500µl aliquot of the culture was added to a tube containing 20µl of the phage solution and the tube was incubated at 37°C for 20 minutes. The infected culture was then transferred to a 250ml Erlenmeyer flask

containing 100ml of pre-warmed (37°C) LB medium supplemented with 1ml of 1M $MgSO_4$. The flask was incubated at 37°C in a horizontal shaker until the cells were lysed by the phage (typically 5 hours) and the medium appeared clear. Next 500µl of chloroform was added and the cell lysate was shaken for a further 15 minutes at 37°C. Cellular debris was removed by centrifugation at 8000g for 10 minutes and the supernatant was transferred to a sterile 50ml tube. This preparation was stored at 4°C and was viable for up to 6 months.

2.4.1 Purification of the lambda clone

A 10ml aliquot of the lysate preparation was transferred to a 15ml centrifuge tube, mixed with 40µl of Nuclease Mixture (supplied with the kit) and incubated at 37°C for 15 minutes. The contents were then transferred to a 15ml Beckman polycarbonate centrifuge tube. The tube was then placed on ice, 4ml of Phage Precipitant (supplied with the kit) was added and the contents were incubated for 30 minutes. The phage were pelleted by centrifugation at 10000g for 10 minutes. The supernatant was decanted, the phage were resuspended in 500µl of Phage Buffer and the solution was transferred to a 1.5ml microcentrifuge tube. Proteinase K was then added to a concentration of 0.5mg/ml and the phage solution was incubated at 37°C for 5 minutes. Insoluble particles were removed by centrifuging at 12000g for 10 second and transferring the supernatant to a fresh tube. The supernatant was then mixed with 1ml of Purification Resin (supplied with the kit) and was added to a 3ml disposable syringe barrel which was attached to the top of a Wizard™ Minicolumn. The resin solution was drawn into the column under vacuum and 2ml of 80% isopropanol was then flushed through the column. The Minicolumn was transferred to a microcentrifuge, spun at 12000g for 20 seconds to remove residual isopropanol and was then transferred to a fresh tube. To elute the lambda DNA, 100µl of sterile deionized water, pre-heated to 80°C, was applied to the column and it was immediately spun at 12000g for 20 seconds. The column was then discarded and the purified lambda DNA was stored at –20°C.

Reagents :

Nuclease Mixture : 0.25mg/ml RNase A, 0.25mg/ml DNase, 150mM NaCl, 50% glycerol

Phage Buffer : 150mM NaCl, 40mM Tris-HCl pH 7.4, 10mM $MgSO_4$

Phage Precipitant : 33% polyethylene glycol (PEG-8000), 3.3M NaCl

2.5 Purification of DNA from agarose gels

If possible, 0.8% gels were used for this procedure since the yield of DNA decreased with increasing agarose concentration. The DNA fragment was run an appropriate distance on the gel, visualized briefly on a U.V. transiluminator and the band was carefully excised using a sterile scalpel. The agarose slice was then transferred to a microcentrifuge tube and subjected to two rounds of freeze/thawing at -70°C for 15 minutes and at 42°C for 15 minutes. The contents of the tube were then transferred to a 0.22μm cellulose acetate (Spin X) column (Costar) within a microcentrifuge tube and T.E. buffer was added to increase the volume to 200μl. The column/tube was spun in a microcentrifuge at 13000g for 15 minutes, 200μl T.E. buffer was added to the top of the column and agarose adhering to the cellulose membrane was dislodged into the solution. The column/tube was spun again and then the column was discarded. The DNA was salt precipitated by adding 40μl of 3M sodium acetate and 880μl of ethanol. Salts were removed by washing with 1ml 70% ethanol and the purified DNA was resuspended in an appropriate volume of ddH_2O. The quantity of DNA recovered was assessed by running an aliquot alongside a known quantity of λ*Hind*III markers.

2.6 Random (High Prime) labelling and purification of DNA probes

(The method followed was essentially that outlined in the Boehringer Mannheim High Prime labelling leaflet 1585 592, Feinberg and Vogelstein 1983). In brief, purified double stranded DNA (25ng in 11μl) was denatured in boiling water for 10 minutes and then kept on ice for 5 minutes. High Prime mix (4μl), (containing random oligonucleotides, 1unit/μl Klenow polymerase, 0.125mM dATP, 0.125mM dGTP, 0.125mM dTTP) and 50μCi of ^{32}P-dCTP (5μl of 3000mCi/ml) were added to the DNA and the sample was incubated at 37°C for 30-60 minutes. The reaction was terminated with 2μl 0.2M EDTA. A Nick™ (Sephadex G-50) column (Pharmacia) was used to separate free nucleotides from the radiolabelled probe. The column was equilibrated with T.E. buffer and the probe solution was added to the pre-wet column. The column was flushed with 400μl of T.E.(see 2.7) and then the radiolabelled probe was eluted

with a further 400µl of T.E. A 1µl aliquot of the probe (400µl total) was tested to ensure an activity >6 x 10^6cpm/ml. Before addition to the pre-hybridization solution, the probe was denatured in boiling water for 10 minutes.

2.7 Genomic DNA isolation

The procedure followed was modified from "A Protocol for Isolating High-Molecular-weight DNA from Mouse Tails" [Sambrook *et al* 1989]. Approximately 100 adult worms were transferred from liquid nitrogen to a 1.5ml microcentrifuge tube containing 700µl of TENS buffer and the worms were chopped up using small stainless steel scissors. Next, 35µl of 10mg/ml Proteinase K was added and the tube was incubated at 55°C overnight. The tube was allowed to cool, 20µl of 13µg/ml RNase (Sigma) was added and it was incubated at 37°C for 1-2 hours. The tube was then filled with buffered saturated phenol at pH 8 (Gibco) and placed on a vertical rotator for 1 hour. The two phases were separated by centrifugation at 13000g for 10 minutes. The end of a yellow Gilson tip (200µl) was removed with sterile scissors and the aqueous phase (including the interface) was transferred to a fresh 1.5ml tube using this tip. The tube was then filled with 1:1 phenol/chloroform (Sigma), the contents were mixed by inversion and the phases were separated as above. The aqueous phase (including the interface) was transferred to fresh tube, using a blunt-ended tip and the tube was filled with 24:1 chloroform/isoamyl alcohol. The contents of the tube were mixed by inversion and the phases were separated by centrifugation. The aqueous phase (but not the interface) was transferred to a fresh tube and this tube was filled with isopropanol. The end of a thin glass capillary tube was sealed in a Bunsen flame and allowed to cool. The capillary tube was dipped into the isopropanol solution and the precipitated DNA was coiled around the tube by gently stirring the solution. The DNA (attached to the glass tube) was dipped into 70% ethanol, then into 100% ethanol and allowed to air dry. A diamond-tipped cutter was used to score the capillary tube and the end (plus DNA) was dropped into a 1.5ml microcentrifuge tube containing 500µl of T.E. buffer pH 7.4. The tube was then placed on a horizontal rotator at 30-60rpm overnight and the glass tip was removed using sterile forceps. An aliquot of DNA was diluted 1/100 and used to assess the purity of the DNA by calculating the ratio of DNA (absorbance) to protein (absorbance at 280nm), which should be between 1.8 and 2.0. The concentration of the

genomic DNA was calculated by the formula: (absorbance at 260nm) x 50 x (dilution factor) = μg/μl of DNA.

Reagents :

TENS buffer

50mM Tris.HCl pH 8, 100mM EDTA, 100mM NaCl, 1% SDS

T.E. buffer

10mM Tris.HCl pH 7.6, 1mM EDTA

2.8 Southern blotting

For a genomic Southern blot, 10μg of *B. pahangi* high molecular weight adult genomic DNA was digested with 50 units of an appropriate restriction endonuclease, for example *Bgl*II, *Eco*RI, *Bam*HI, *Hind*III, *Kpn*I, or *Pst*I (GibcoBRL). The restriction digests were incubated in a volume of 400μl at 37°C overnight and were precipitated with 3M sodium acetate pH 5.2 (40μl) and ethanol (880μl). The salts were removed with a 70% ethanol wash (1ml) and the DNA was resuspended at 37°C, for several hours, in 25μl ddH_2O. DNA loading buffer (10x, 2.23.1) was added to each sample and the fragments were separated on a 0.8% agarose gel (TAE). DNA markers, usually *Hind*III digested λ DNA (GibcoBRL), were also added to facilitate size estimations. After the bromophenol dye front had migrated 2/3 the length of the gel, the gel was stained for 30 minutes in ddH_2O containing 1.5μg/ml ethidium bromide, viewed on a U.V. transilluminator and photographed (beside a ruler) using Polaroid high speed, black and white film, type 667. Gels were depurinated in 0.25M HCl for 30 minutes to improve transfer of high molecular weight fragments. They were then soaked in denaturing solution for 30 minutes and in neutralizing solution for 30 minutes. The gels were rinsed in ddH_2O between each solution. For PCR blots, the PCR products and DNA markers were separated on a 1% TAE agarose gels containing 0.5μg/ml ethidium bromide and photographed on a trans-illuminator. The gel was then treated as for a genomic DNA gel.

2.9 RNA extraction

TRIzol Reagent (GibcoBRL, Chomczyski 1993), a mono-phasic solution of phenol and guanidine isothiocyanate, was used to isolate total RNA from mf and adults. The protocol from the TRIzol leaflet (form 3796) was used with the modifications stated below.

Mf or adult worms were quickly transferred from liquid nitrogen to pre-heated microcentrifuge tubes containing 200µl lysis buffer and 12.5µl of 10mg/ml proteinase K. The tubes were incubated at 65°C for 15 minutes and were frequently mixed by inversion. Aliquots of 1ml of TRIzol in RNAse-free 1.5ml microcentrifuge tubes were pre-heated to 65°C, the TRIzol was then added to each sample and the tubes were incubated for 20 minutes at 65°C. To separate the aqueous and organic phases, 0.2ml chloroform was added, the tubes were vigorously shaken for 15 seconds, incubated for 2-3 minutes at room temperature and then spun at 12000g for 15 minutes (at 4°C). The aqueous phase was then transferred to a fresh RNAse-free tube and 0.5ml isopropyl alcohol (2-propanol, Sigma) was added to precipitate the RNA. The samples were incubated for 10 minutes at room temperature, spun at 12000g for 10 minutes (at 4°C) and the supernatant was removed by aspiration. To remove salts from the sample, 1ml of 75% ice-cold ethanol (25% DEPC treated ddH2O) was added, the tube was spun at 12000g for 15 minutes (at 4°C) and the supernatant was removed. The RNA pellet was briefly air-dried, an appropriate volume of DEPC treated ddH_2O was added and the sample was incubated at 55°C until the RNA dissolved. To quantify the RNA, an aliquot was run on an agarose-formaldehyde gel alongside a known quantity of RNA markers. For storage, 0.3M RNAse-free sodium acetate and 2 volumes of 100% ethanol were added and the samples were stored at -70°C.

Reagents :

Lysis buffer (10ml)

(0.1M Tris.HCl, 0.2M NaCl, 2% SDS, 0.2M EDTA)

1ml of 1M Tris.HCl pH 8.0

1ml of 2M NaCl

2ml of 10% SDS

4ml of 0.5M EDTA pH 8.0

2ml of DEPC ddH_2O

prepared fresh for each RNA extraction

Proteinase K solution

proteinase K (Sigma) dissolved in ddH_2O to 10mg/ml

store at -20°C

DEPC ddH_2O

0.01% diethylpyrocarbonate (Sigma) in ddH_2O, leave at room temperature overnight and then autoclave. Store at room temperature.

2.10 Northern blotting

Total *B. pahangi* RNA (1-2μg) was used for Northern blots. RNA was diluted with DEPC ddH_2O, formamide was added to 50% (v/v) and ethidium bromide to 50ng/ml. DNA loading dye (10x) was added to the samples which were denatured at 65°C for 10 minutes and loaded onto a 1.2% agarose gel (MOPS) containing 17% formaldehyde. RNA markers (GibcoBRL, 0.24-9.5kb ladder) were also loaded alongside the samples and the gel was photographed before blotting.

Reagents :

10x MOPS : 0.2M 3-morpholinopropanesulfonic acid, 50mM sodium acetate, 10mM EDTA pH 5.6

2.11 Transfer of DNA and RNA to charged nylon membrane

A DNA gel was treated as described in 2.8 and a gel containing RNA was immersed in denaturing solution for 30 minutes, rinsed and immersed in neutralizing solution for 30 minutes. The capillary transfer of DNA and RNA was by the standard method of Southern (1975). DNA and RNA was cross-linked and immobilized onto Magna Charge nylon membrane using 150 mjoules of U.V. radiation.

2.12 Washing probed filters

In order to remove radiolabelled probe, which was non-specifically associated with a hybridized filter, the filter was washed in buffers of increasing stringency, usually at the same temperature as used for hybridization. The hybridizing solution which contained

the probe was carefully poured away and approximately 50ml of 6 x SSC, 0.1% SDS was added to the hybridization cylinder. The filter was then briefly washed with this solution (1 minute) and the buffer was poured away. Typically the next buffer was 3 x SSC, 0.1% SDS: 50ml was added and the filter was incubated in the buffer at the hybridization temperature for 20 minutes. The buffer was poured away, 50ml of 2 x SSC, 0.1% SDS was added and the filter was incubated again for 20 minutes. If a heterologous probe had been utilized, a second 2 x SSC, 0.1% SDS buffer wash was often used, but for a homologous probe, a 1 x or 0.2 x SSC, 0.1% SDS buffer was added and the filter was incubated for 20 minutes. Finally the filter was rinsed in 2 x SSC for 30 seconds at room temperature, the filter was blotted on 3MM (Whatman) paper and sealed in plastic. It was then autoradiographed.

2.13 First strand cDNA synthesis

A sample was prepared containing 1-2μg *B. pahangi* RNA, 1μl of 10x DNase buffer (GibcoBRL) and 1μl of DNase (GibcoBRL) in a volume of 9μl. (DEPC ddH_2O was used to adjust the sample volume). The sample was incubated at room temperature for 15 minutes to digest contaminating genomic DNA. Next, 1μl of 20mM EDTA (GibcoBRL) was added and the sample was incubated at 70°C for 10 minutes, to inhibit enzyme activity. The temperature was then lowered to 65°C, 200ng of oligodT primer (T_{17}) was added and the sample was slowly cooled to room temperature.

Reverse transcription reaction

1μl RNase inhibitor (Promega)

4μl of 5x reverse transcriptase buffer (Promega)

2μl of 10mM dNTPs

1μl of (AMV) reverse transcriptase (5-10units/μl, Promega)

1μl of ddH_2O

Reverse transcription was carried out for 1 hour at 42°C and the sample was then heated to 56°C for 30 minutes. If required, the sample was diluted to 200μl with ddH_2O.

2.14 Modified 5′ RACE protocol for amplifying *hsp90* pre-mRNA

The protocol outlined in the 5′ RACE System for Rapid Amplification of cDNA Ends instruction manual GibcoBRL 18374-025 was used and the protocol was modified where appropriate, as described below. Total RNA from *B. pahangi* mf cultured at 37°C for 3 hours was utilized. First strand cDNA was made from 1μg, 2μg and 3μg of RNA using the *hsp90*-specific primer, 90per. The cDNA was then salt precipitated by adding ddH_2O to 400μl, 40μl of 3M sodium acetate and 880μl of ethanol. Salts were removed with a 70% ethanol wash (1ml) and the cDNA was resuspended in 12.8μl DEPC ddH_2O. The cDNA was then dC-tailed using recombinant terminal deoxynucleotidyl transferase, TdT (GibcoBRL).

TdT tailing of cDNA

cDNA sample (12.8μl)

5μl of 5x TdT buffer (Gibco)

3.75μl of 10mM $MgCl_2$

2.5μl of 2mM dCTP

The solution was incubated for 2-3 minutes at 94°C and then chilled for 1 minute on ice. The contents of the tube were collected by centrifugation at 13000g for 1 minute and the tube was placed on ice. Next, 1μl of TdT (15 units/μl) was added and the solution was incubated for 10 minutes at 37°C. The TdT was heat inactivated at 65°C for 10 minutes and the tube was centrifuged at 13000g for 1 minute and placed on ice. The dC-tailed cDNA was purified by adding 75μl of ddH_2O and 100μl chloroform, spinning at 14000g in a microcentrifuge for 5 minutes and precipitating the aqueous phase with sodium acetate and ethanol (as previously described). The samples were then resuspended in 10μl of sterile ddH_2O.

PCR 1

Reaction mix

200μm of each dNTP

1 x (Cetus) PCR buffer (Perkin Elmer)

(150μM Mg^{2+} from buffer)

0.4μM of each primer

adjust with sterile ddH_2O to 45μl

Hot start mix

12.5 units/μl of AmpliTaq

1 x (Cetus) PCR buffer (with Mg^{2+})

In the first PCR reaction, 1µl of dC-tailed cDNA was amplified with the anchor primer, which contains a string of consecutive guanine nucleotides and the *hsp90*-specific primer, hsp90r5. As a control to ensure the dC-tailing had been successful, 1µl of dC-tailed cDNA was amplified with SL1 and hsp90r5. Control cDNA which had not been dC-tailed was also used. The samples were overlaid with mineral oil (Sigma).

The samples were as follows:

1	sample from 1µg of RNA	⎫	
2	sample from 2µg of RNA	⎪	20 pmoles of anchor primer
3	sample from 3µg of RNA	⎬	and
4	control mf cDNA from 1µg of RNA	⎪	20 pmoles of hsp90r5
5	no template	⎭	
6	sample from 1µg of RNA	⎫	
7	sample from 2µg of RNA	⎪	20 pmoles of SL1
8	sample from 3µg of RNA	⎬	and
9	control mf cDNA from 1µg of RNA	⎪	20 pmoles of hsp90r5
10	no template	⎭	

PCR : denaturing at 95°C for 1 minute, followed by 30 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes and then a final extension at 72°C for 10 minutes. During the denaturing step, 5µl of hot start mix, containing AmpliTaq, was added. This was to prevent aberrant priming. A 10µl aliquot of each PCR sample was run on a 1% agarose gel. The results are presented in 5.2.4 and in ***Figure 5.7B***. The remaining 40µl was purified by salt precipitation with sodium acetate and ethanol, washed with 1ml of 70% ethanol and the PCR products resuspended in 10µl of ddH_2O. In the second PCR, 1µl of (PCR 1) sample was used per reaction.

PCR 2

The reaction mix was made up as for PCR1 and the samples were as follows:

PCR	template (1µl)	primer 1 (18 pmoles)	primer 2 (20 pmoles)
1	none	adapter	prex1

2	Sample 1 (PCR1)	|	|
3	Sample 2 (PCR1)	|	|
4	Sample 3 (PCR1)	|	|
5	Sample 4 (PCR1)	v	v
6	none	adapter	prex2
7	Sample 1 (PCR1)	|	|
8	Sample 2 (PCR1)	|	|
9	Sample 3 (PCR1)	|	|
10	Sample 4 (PCR1)	v	v
11	none	adapter	hsp90r5
12	Sample 1 (PCR1)	|	|
13	Sample 2 (PCR1)	|	|
14	Sample 3 (PCR1)	|	|
15	Sample 4 (PCR1)	v	v

PCR : denaturing at 95°C for 1 minute, followed by 25 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute and then an extension at 72°C for 10 minutes. A 10µl aliquot of each PCR sample was run on a 1% agarose gel, see ***Figure 5.7C***.

PCR samples 3 and 4 were pooled, salt precipitated and the purified products were ligated into pCR2.1 (Invitrogen). The remaining 40µl from samples 8 and 9 were run on a second 1% agarose gel and a minor band of approximately 300bp was excised from the gel and purified using a Spin X 22µm column (Costar). The purified products were ligated into pCR2.1. A 3µl aliquot, from each ligation reaction, was used to transform 20µl of INVαF′ cells (Invitrogen). Mini-preps were made of transformed colonies which had been grown overnight in L-broth. A 1µl aliquot was then digested with 10 units of *Eco*RI for 1 hour at 37°C. The digested samples were run on a 1% agarose gel and since *Eco*RI releases inserts from pCR2.1, the presence of a band of approximately 0.3kb identified clones which contained inserts. Plasmid preps containing cloned inserts were sequenced using fluorescent M13rev primer and either T7 or M13 primer. The sequence information was aligned using the GCG gelassemble programme (Wisconsin Sequencing Analysis Package).

2.15 Transient transfection of COS-7 with a region upstream of *hsp90*

2.15.1 Plasmid constructs for transfection

pCAT12 (3.6kb) which contains the bacterial *cat* gene [Kirby and Vapnek 1979] fused with the Herpes simplex virus immediate early 5 gene polyadenylation sequence [Spandidos and Riggio 1986]. In pCAT12, *cat* is not under the control of a promoter and this plasmid was therefore used as a negative control for transfection.

pLW2 (3.5kb) contains the *cat* gene (transposon-9) [Alton and Vapnek 1979], and the polyadenylation signal and a 210bp fragment from the HSV-2 IE gene 4/5 promoter [Gaffney *et al* 1985]. Plasmid pLW2 contains a TATA box and two inverted copies of the GC box, the binding site for the Sp1 family of transcription factors. Transfection of pLW2 into a eukaryotic cell line results in the constitutive expression of CAT at a high level and this plasmid was used as a positive control for transfection.

HSE1-pCAT12

Primers, 210pf1 which contains a *Sst*I site at the 5′ end and p1pr1 which contains a *Bam*HI site at the 3′ end were used to amplify a region of 0.24kb (-474 → -236) from the *hsp90* upstream region. The PCR product, HSE1, contains a putative TATA box, GC box, three HSEs and an inverted CCAAT box and has 38 bases downstream of the TATA box. HSE1 was digested with *Sst*I and *Bam*HI and cloned into pre-cut pCAT12, creating HSE1-pCAT12.

HSE2-pCAT12

Primers, tranf which contains a *Sst*I site at the 5′ end and tranr which contains a *Bam*HI site at the 3′ end, were designed to amplify a 0.54kb region upstream from *hsp90* (-781 → -241 in ***Figure 5.1***). The PCR product, HSE2, contains the elucidated major transcriptional start site, a TATA box, two inverted CCAAT boxes, a GC box and five HSEs. HSE2 was digested with *Sst*I and *Bam*HI and cloned into pre-cut pCAT12, creating HSE2-pCAT12.

Unless otherwise stated, the cell culture reagents were supplied by GibcoBRL and solutions were prepared using aseptic techniques. The plasmids for transfection were prepared by large scale alkaline lysis using CsCl/EtBr equilibrium centrifugation as

described by Sambrook *et al* (1989). Purified plasmid was resuspended in sterile ddH_2O, a 10µl aliquot was diluted to 1ml with ddH_2O, the O.D. was measured at 260nm wavelength and the concentration of the plasmid solution was calculated using the formula :

(1000 ÷ volume of aliquot in µl) x $O.D._{260}$ x 50 = DNA concentration in µg/µl

A 1µg/µl stock was used in the transfection experiments.

2.15.2 Culturing COS-7 cells

Cells were passaged twice a week. The medium was removed and the cells were rinsed by adding 12.5ml of rinsing solution. The rinsing solution was aspirated, 5ml of trypsinizing solution was added and the flasks were incubated at 37°C for 2 minutes. Knocking the sides of the flasks dislodged the remaining adherent cells. The trypsinizing solution was diluted with 25ml of culture medium and the cells were transferred to a sterile 50ml centrifuge tube and pelleted at 200g for 5 minutes. The medium was removed by aspiration and the cells were resuspended in 30ml culture medium. A 50µl aliquot was mixed with 150µl of 0.4% trypan blue solution (Sigma) and the cells were counted using an haemocytometer, to determine the concentration of the cells. Tissue culture flasks ($175cm^2$, Nunc) were seeded with 1 x 10^6 cells and the volume of culture medium in the flask was adjusted to 35ml. For transient transfection experiments, 4 x 10^5 cells were added to 60mm Petri dishes (Nunc). Any remaining cells were pelleted and aliquots of 1-2 x 10^7 cells were resuspended in freezing solution and transferred to 1.5ml cryovials (Nunc). The vials were surrounded by cotton wool, placed at -70°C overnight and then stored in liquid nitrogen. The tissue culture flasks were reused twice and the cells could be passaged for a maximum of 12 times.

Reagents :

Culture medium

DMEM medium plus:
2mM glutamine
10 units/ml penicillin/streptomycin
10% heat inactivated foetal calf serum
store at 4°C and use within 2 weeks

Cell rinsing solution

phosphate buffered saline (PBS)
0.68mM EDTA pH 8.0
store at 4°C

Cell freezing solution

culture medium plus 10% v/v dimethyl sulphoxide (Sigma)

Trypsinizing solution

Trypsin solution 2.5% (Gibco) diluted 1/10 with PBS pH 7.4

2.15.3 Transient transfection using LipoTAXI

The protocol stated in the LipoTAXI™ Mammalian Transfection kit leaflet 204110 (Stratagene) was followed [Alam and Cook 1990]. Briefly, in preliminary experiments, different volumes of the liposome reagent, LipoTAXI™, ranging from 35-100µl were tested using 10µg of pLW2, pCAT12 and HSE1-pCAT12. The volume of LipoTAXI™ had only a marginal effect on the efficiency of transfection and therefore 35µl was used in subsequent experiments (see 5.2.3). DMEM (serum free) was used to prepare the transfection solution (containing LipoTAXI and CAT plasmid, 2.5ml total), the COS-7 cells were transfected for 6 hours and then the transfection solution was inactivated by the addition of 2.5ml culture medium, which contained 10% FCS. After 24 hours the medium was replaced by fresh culture medium. A comparison of transfected cells lysed after 48 and after 72 hours revealed that the level of CAT was not significantly different and 48 hours was therefore used for further experiments. If samples were to be heat shocked, the Petri dishes were moved to a pre-heated container in an incubator set at 41°C and were left to heat shock for 1 hour. Before being lysed, the cells were allowed to cool to 37°C for 10 minutes, to reduce the number of cells that dislodged from the plates during the harvest. Cells were washed three times with 5ml of pre-cooled PBS (4°C) and then overlaid with 1ml of 1 x lysis solution (Boehringer Mannheim, MOPS-buffered saline containing Triton® X-100). After lysis, the supernatant was removed from the Petri dishes and transferred to a 1.5ml microcentrifuge tube. The cell debris was collected by centrifugation for 1 minute at 12000g (at 4°C) and the soluble supernatant was transferred to a fresh tube and stored at –70°C until tested. The chloramphenicol acetyltransferase (CAT) ELISA kit supplied by Boehringer Mannheim was used to quantify the reporter gene product.

2.15.4 Transient transfection using Lipofectin

The protocol stated in the Lipofection reagent leaflet 18292-011 (GibcoBRL) was followed. In brief, a volume of 15µl of the liposome reagent, Lipofectin [Felgner *et al*

1987] and 4μg of plasmid (pLW2, pCAT12, HSE1-pCAT12, HSE2-pCAT12) were used per transfection. OptiMEM reduced serum medium (GibcoBRL) was used to prepare the transfection solution i.e. for each samples:

4μg plasmid 200μl OptiMEM	⎫ ⎭	**A**	(needs no incubation)
15μl Lipofectin 200μl OptiMEM	⎫ ⎭	**B**	incubate for 40 minutes at room temp.

The cells were rinsed with 5 ml of OptiMEM before transfection. Solutions **A** and **B** were combined and incubated for 15 minutes at room temperature. The medium was removed from the cells, 1.8ml of OptiMEM was added to solution **A/B** and this was added drop-wise to the cells. The cells were returned to a 37°C incubator and were exposed to the transfection solution for 7 hours, after which time it was replaced with culture medium. The transfected cells were then incubated for 48 hours at 37°C. If samples were to be heat shocked, the appropriate Petri dishes were moved to a pre-heated container in an incubator set at 41°C and were heat shocked for 1 hour. Before being lysed, the cells were allowed to cool to 37°C, to reduce the number of cells that dislodged from the plates during the harvest. The protocol for the preparation of cell extracts from adherent cells suggested by Boehringer Mannheim (CAT ELISA booklet 1363727) was utilized. Cells were washed three times with 5ml of ice cold PBS and then cells were overlaid with 1ml of 1 x lysis solution (Boehringer Mannheim, MOPS-buffered saline containing Triton® X-100). After lysis, the solution was removed from the Petri dishes and transferred to a 1.5ml microcentrifuge tube. To remove solid cell debris, the samples were spun at 12000g (at 4°C) for 1 minute and the supernatants transferred to fresh tubes. The samples were stored at -70°C until tested.

2.15.5 Protein assay

The protein concentration of the samples was determined using in-house prepared Bradford reagent [Bradford 1976]. An aliquot was diluted with ddH_2O (typically $^1/_{10}$) and 20μl of this was added in duplicate to the wells of a flat-bottomed 96-well plate. Standards were prepared using dilutions of BSA (e.g. 10μg/ml to 200μg/ml) and 20μl of each standard was added in duplicate to the plate. Next 180μl of Bradford reagent

[Bradford 1976] was added to each well, the samples were carefully mixed and read on a micotiter plate reader at 595nm wavelength after approximately 10 minutes. The protein concentrations of the CAT samples were determined by utilizing a protein standard curve (linear) taking into account the dilution factor. Samples were then diluted to 250μg/ml in sample buffer (Boehringer Mannheim) and the CAT concentrations were determined using a pre-coated ELISA plate as per instructions.

Reagents :

Bradford reagent

0.01% Coomassie® Blue G-250

4.7% ethanol

8.5% phosphoric acid

dissolve in sterile ddH_2O

2.15.6 CAT ELISA

ELISA plates pre-coated with a sheep polyclonal antibody to CAT were used (Boehringer Mannheim CAT ELISA 1363 727). Test samples were tested in duplicate or in triplicate and duplicate standard samples were measured. To each test well, 200μl of sample was added (equivalent to 50μg in each well). CAT standards, from CAT supplied by Boehringer Mannheim, were prepared in sample buffer at concentrations generally in the range of 31.25-1200ng/ml and 200μl of each was added to wells on the plate. The plate was covered with plastic film and was incubated at 37°C for 1 hour. The solution was then discarded and the wells were rinsed 6 times with 250μl of 1 x PBS containing Tween® 20 (also from the kit). The wells were then allowed to dry briefly before 200μl of 2μg/ml sheep anti-CAT-digoxigenin polyclonal antibody was added to each well. The plate was incubated for 1 hour at 37°C, the solution was then discarded and the wells were rinsed out as before. Next 200μl of 150mU/ml sheep anti-digoxigenin-peroxidase polyclonal conjugated antibody was added to each well. The plate was incubated for 1 hour at 37°C, the solution was then discarded and the wells were rinsed out as above. For the colorimetric detection of CAT, 200μl of POD (ABTS®) substrate was added, the colour of the standard wells allowed to reach a suitable optical density (typically 10-40 minutes) and the samples were read on a Dynatech microtiter plate (ELISA) reader at 405nm wavelength (reference wavelength

490nm). The concentration of CAT was calculated automatically from the standard samples.

2.16 Pe90 cloning, expression and purification

2.16.1 Producing pe90

Primers pef90 and per90 were designed from the available *hsp90* cDNA sequence. A *Bam*HI site was added to the forward primer, pef90 and a *Kpn*I site to the reverse primer, per90. A plasmid (p902), which contained the carboxyl half of *hsp90*, was used in a PCR reaction with pef90 and per90 to produce the fragment pe90. The sequence of pe90 (see ***Figure 6.4***) corresponds to amino acids 479-717 of the translation of *B. pahangi hsp90* cDNA (accession no. AJ005784) and the stop codon from the cDNA sequence is also present. However, the first amino acid, which is glutamic acid in the cDNA clone, was changed to glycine (**G**) in pe90 to create a *KpnI* site.

Amplification of pe90

1µl (100ng) of plasmid containing the *hsp90* carboxyl fragment
1.75µl of AmpliTaq (5units/µl, Cetus)
31.5µl of 11.1x PCR buffer A
302µl of sterile ddH_2O

Aliquots of 48µl were added to seven 0.5ml GeneAmp tubes (Perkin Elmer). To four of the tubes, 100pmoles (1µl) of primers pef90 and per90 was added. The three remaining tubes were controls for the PCR. The thermal cycling reaction were:

95°C for 3 minutes	1 cycle
94°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes	1 cycle
94°C for 1 minute, 65°C for 1 minute and 72°C for 3 minutes	29 cycles
72°C for 10 minutes	1 cycle

The amplified products were separated on 1% agarose gel and the bands corresponding to pe90 were excised and purified using Spin X columns. Pe90 was then digested with *Bam*HI and *Kpn*I:

Restriction digestion with *Kpn*I and *Bam*HI

pe90 resuspended in 25µl of ddH_2O

3µl of 10x buffer 4 (GibcoBRL)

1µl (10units) of *Bam*HI

1µl (10units) of *Kpn*I

The digestion was carried out at 37°C for 4 hours, following which the DNA was salt precipitated at –70°C, washed with 70% ethanol and resuspended in 5µl ddH_2O. An aliquot (2µl) was run on a 1% TAE agarose gel to determine the concentration of pe90. Approximately 5ng (1µl) was used in a ligation with pQE30.

2.16.2 Cloning a fragment of *B. pahangi hsp90* into pQE30

The expression plasmid, pQE30 (Qiagen, Stuber *et al* 1990) codes for a stretch of 6 histidine residues positioned at the N-terminus of a fusion protein. It has a multiple cloning site and *Bam*HI (5′) and *Kpn*I (3′) were chosen as appropriate restriction sites for the insertion of the *hsp90* fragment. A plasmid miniprep of pQE30-transformed bacteria (30µl total in ddH_2O) was utilized. The plasmid was digested firstly with *Kpn*I :

Restriction digestion with *Kpn*I

10µl of pQE30 mini prep.

1µl (10units) of *Kpn*I (GibcoBRL)

2µl of 10x reaction buffer (GibcoBRL)

8µl of ddH_2O

The sample was digested at 37°C for 4 hours, run on a 0.8% agarose gel, excised from the gel and purified using 0.22µm Spin X cellulose acetate columns (Costar). The linearized plasmid was resuspended in 17µl of ddH_2O and then digested with *Bam*HI:

Restriction digestion with *Bam*HI

*Kpn*I cut pQE30 resuspended in 17µl of ddH_2O

2µl of 10x buffer (GibcoBRL)

1µl (10units) of *Bam*HI (GibcoBRL)

The sample was digested at 37°C for 4 hours, salt precipitated using sodium acetate, washed with 70% ethanol and resuspended in 5µl ddH_2O. An aliquot (2µl) was run on a 1% TAE agarose gel to determine the concentration of the plasmid. Approximately 5ng (1µl) was used in a ligation with the pe90.

Ligating pe90 and pQE30

5ng of pQE30

5ng of pe90

2µl of 5x ligase buffer (GibcoBRL)

0.5µl of 5mM DTT/0.5mM ATP

ddH_2O to 9.5µl

Add 0.5µl (2 units) of (HC) T4 DNA ligase (GibcoBRL)

Incubated at 16°C overnight

The competent cells used for protein expression were M15[pREP4] containing the pREP4 plasmid which codes for the *lac* repressor protein and allows efficient regulation of fusion protein expression. A 200µl aliquot of M15[pREP4] cells was transformed with 6µl of the ligation reaction. The protocol outlined for the transformation of DH5α cells (GibcoBRL) was followed i.e. the cells (in a 1.5ml microcentrifuge tube) were defrosted briefly on ice and 6µl of ligation mix was added. The tube was incubated on ice for 30 minutes, followed by a heat shock at 42°C for 45 seconds. The cells were then placed back on ice for 2 minutes. Next, 1ml of L-broth was added and the tube was incubated for 1 hour at 37°C. The cells were collected by spinning for 30 seconds at 13000g (microcentrifuge) and were gently resuspended in 100µl of L-broth. The sample was spread over two LB plates which contained 100µg/ml of ampicillin (to select for pQE30) and 25µg/ml of kanamycin (to select for pREP4) and the plates were incubated overnight at 37°C.

2.16.3 Selecting pe90-pQE30 clones

There is no colour selection with the pQE system, therefore 24 white colonies were randomly picked, plasmid minipreps were made and digested with *Kpn*I and *Bam*HI. Three plasmids contained inserts of the correct size and were sequenced fully to confirm that they contained pe90. Bacterial stocks containing pe90-pQE30 were frozen

at -70°C (200µl of bacterial culture in L-broth containing ampicillin and kanamycin and 800µl of sterile (autoclaved) glycerol.

2.16.4 Pe90 fusion protein expression

Protocols from the "QIAexpressionist" handbook, March 1997 (Qiagen) were followed for both small scale and larger scale protein expressions. The pe90 protein (approximately 30kD) was observed when a culture (at 37°C) was induced with 1mM IPTG for 4 hours, but was not observed in the absence of IPTG. A 1ℓ culture was therefore induced with 1mM IPTG for 4 hour, the cells collected by spinning at 4000g for 20 minutes and resuspended in lysis buffer and digested with 1mg/ml lysozyme. Then the cells were lysed on ice by sonication, centrifuged at 10000g for 20-30 minutes (at 4°C) and the supernatant transferred to a fresh tube. Nickel agarose (Ni-NTA) resin was used to purify pe90 [Hochuli *et al* 1987, Hochuli *et al* 1988]. An approximate ratio of 1ml of 50% Ni-NTA slurry per 4ml of cell lysate was used. Protein preparations were carried out either under native conditions or in the presence of 1-2M urea. Pe90 was eluted from the Ni-NTA column with 0.1-0.5M imidazole. Imidazole was removed from a pe90 protein solution by dialysis against PBS, using dialysis tubing (a semi-permeable membrane). The protein, which was in approximately 15ml of buffer, was carefully transferred to dialysis tubing, sealed with plastic clips and submerged in 2 litres of PBS in a cold room (at 4°C). The PBS was circulated using a magnetic flea, it was replaced twice and after 2 days the dialysed pe90 solution was transferred to 5ml sterile bijou tubes.

2.16.5 Producing anti-serum to pe90

Two rabbits (95FP and 43FT) were initially vaccinated with 130µg of pe90 fusion protein, (as determined by Bradford reagent [Bradford 1976]), was mixed with adjuvant. The ratio of protein solution to Freund's incomplete adjuvant was approximately 1:1 in a total volume of 0.25ml. Boosts were carried out using fusion protein which had been additionally purified by separation on a 12.5% gel by standard SDS-PAGE (as described in 2.24.1), due to contamination of pe90 with small amounts of *E. coli* proteins. The gel was stained with Coomassie® blue and the bands corresponding to pe90 were excised and stored at -20°C. For a vaccination, the gel slices were extensively washed in sterile deionized water and then homogenized with

0.2ml of ddH_2O and emulsified with 0.5ml of Freund's incomplete adjuvant. The rabbits received approximately 200μg per boost. Bleeds were taken before the first vaccination and after the first and second boosts. Blood was allowed to clot at 4°C, following which red blood cells were sedimented by centrifugation and the serum harvested. The red blood cells were spun down at 12000g for 1 minute (at 4°C), the serum was transferred to fresh microcentrifuge tubes and were stored at -20°C. Western blotting with 95FP and 43FT antiserum showed that 95FP is more specific for HSP90 than 43FT.

2.17 Metabolically labelling microfilariae and adults

Mf and adults were recovered from the host by peritoneal lavage as described in 2.1.2. If red blood cells contaminated the mf preparation, 1ml of sterile deionized water was used to lyse cells and the volume was then made up to 4ml with HBSS. Mf were separated from contaminating peritoneal cells using a Lymphoprep™ Sigma 1077 gradient [Boyum 1964]. For labelling, mf and adults were washed with HBSS before being transferred to sterile 1.5ml screwtop microcentrifuge tubes and resuspended in 0.5ml of culture medium. The parasites were allowed to reach thermal equilibrium (mf at 28°C, 37°C or 41°C and adults at 37°C or 41°C) for 10 minutes in a water bath and then 50μCi of ^{35}S-methionine (15mCi/ml, >1000Ci/mmol Amersham SJ 234) was added. Metabolic labelling was carried out for 2 or 3 hours and then the radiolabelled methionine was rinsed from the worms. Adult worms were carefully transferring with a glass hook to a 60mm Petri dish filled with fresh culture medium. Mf were collected in the bottom of the tube by centrifugation at 6500g for 1 minute and were resuspended in fresh culture medium. This procedure was repeated twice with the mf. The adults were transferred to a fresh tube. Excess medium was removed from mf and adults and they were frozen at -20°C. Protein was extracted from the samples using IEF lysis buffer (see 2.19), DOC (see 2.18) or SDS sample cocktail (see 2.24.1.1). Samples were analyzed by SDS-PAGE (as described in 2.24.1) and autoradiography.

Reagents :

Unless otherwise stated, the culturing reagents were supplied by GibcoBRL.

Culture medium

1% glucose (Sigma)

25mM HEPES

4mM glutamine

1% non-essential amino acids

100μg/ml gentamycin (Sigma)

make up in DMEM (minus methionine)

Filter (Costar) under vacuum into sterile container

5% foetal calf serum

store at 4°C

2.18 DOC extract

Adult worms were homogenized on ice with a ground glass homogenizer (Jencons). Metabolically labelled mf were not DOC extracted, but DOC extracts were prepared from unlabelled mf. Mf were resuspended in 0.25ml of extraction buffer and sonicated (200-300W) on ice (10 seconds on 30 seconds off) until visibly fragmented. Sodium deoxycholate was added to a final concentration of 1% and the sample was left to extract for 1 hour at room temperature. The sample was then spun (in a microcentrifuge) at 14000g for 30 minutes (at 4°C) to pellet the insoluble components. The soluble supernatant was transferred to a fresh tube and both tubes were stored at –20°C. For use in western blotting, the soluble supernatant was mixed with an equal volume of SDS sample cocktail and 0.25ml of SDS sample cocktail was added to the insoluble pellet as described in 2.13.1.

Reagents :

Extraction buffer (10ml)

10mM Tris.HCl pH 8.3

1 Complete™ Mini protease inhibitor cocktail tablet (Boehringer Mannheim)

(each tablet contains both reversible and irreversible inhibitors for a broad spectrum of serine, cysteine and metalloproteases and calpains).

2.19 IEF lysis buffer extract

Mf were left to extract in IEF buffer for 2 hours at room temperature. The extracts were transferred to a microcentrifuge tube and residual material was washed out of the homogenizer with 100µl of fresh lysis buffer. The samples were spun at 13000g to pellet insoluble components and the supernatant was transferred to a fresh tube and stored at -20°C.

Reagents :

IEF lysis buffer

(9.5M urea, 2% NP-40, 2% Ampholines pH 3.5-10, 50mM dithiothreitol)

5.5g of urea

2ml of 10% NP-40

0.5ml of Ampholines (Pharmacia)

80mg of DTT

adjust to 10ml with ddH_2O

store as 0.5ml aliquots at -70°C

2.20 TCA precipitation

To determine the amount of ^{35}S-methionine incorporation in a parasite extract, a trichloroacetic acid precipitation (TCA) was utilized. A 2.5µl aliquot of the ^{35}S-labelled extract was mixed with 10µl of rabbit serum, 1ml of ice cold 10% TCA (Sigma) was added and the solution was incubated on ice for 10 minutes. A conical vacuum flask was used to filter the TCA-precipitated proteins onto Whatman glass fibre filter paper disks. The TCA solution was filtered though the disk and the vial was rinsed out with 1ml of TCA, which was also dropped onto the disk. Next 1ml of ethanol was filtered through, followed by 1ml of acetone. The protein-impregnated disks were then transferred to scintillation vials, 3ml of OptiScint scintillation fluid (EG + G Wallac) was added and the ^{35}S activity was measured for 60 seconds (cpm) in a Beckman scintillation counter.

2.21 Immunoprecipitation of *hsp90* using 95FP

DOC extracts of adults metabolically labelled with ^{35}S-methionine at 37°C or 41°C, were produced. For each sample, 250000 TCA precipitable counts per minute (cpm)

were mixed with 20µl of antiserum (collected after the second boost) from rabbit 95FP or 20µl of a pre-bleed from 95FP. The samples were incubated at room temperature for 2 hours, 20µg of Protein A Sepharose (Pharmacia) was then added and they were incubated for a further hour at room temperature. The Sepharose was pelleted by centrifugation at 6500g for 2 minutes and was washed twice and then washed overnight in low strength washing buffer. Next, 50µl of SDS sample cocktail was added to the tube containing the adult extract and the sample was boiled for 3 minutes and spun at 13000g to pellet the insoluble components. The supernatant was run on a SDS PAGE gel, stained, destained, incubated in Amplify (Amersham) for 30 minutes on a rocker and dried using a vacuum gel-drier (BioRad). The metabolically labelled proteins were visualized by autoradiography at –70°C.

Reagents :

Low strength washing buffer
150mM NaCl
5mM EDTA pH 8.0
50mM Tris.HCl pH 7.4
0.1% Triton X 100
0.01% SDS

2.22 Attempts to obtain *B. pahangi hsf*

2.22.1 Screening for *B. pahangi hsf* using a tomato *hsf* probe

In an attempt to obtain a cDNA clone containing a *B. pahangi* heat shock factor gene (*hsf*), a heterologous screening protocol was utilized. A partial *Lycopersicon peruvianum* (tomato) *hsf* clone termed T26 (bp 330-1113, x55347) [Scharf *et al* 1990] was kindly donated by K.D. Scharf. The 790bp insert was excised from the plasmid by digesting with *Not*I (Gibco), purified from a 1% agarose gel using a 0.22µm SpinX column (Costar) and 25ng of DNA was radiolabelled. The probe was used to screen four 140mm plates containing 2 x 10^5 plaque forming units (pfu) from the heat shocked mf cDNA library. The agar plates were chilled at 4°C for 2 hours before overlaying filters. The filters were orientated using needle pricks through the filter and into the agar. The filters were removed and immediately submerged for 2 minutes in denaturing

solution, then for 5 minutes in neutralizing solution and finally rinsed briefly in rinsing solution. The filters were pre-hybridized for 4 hours in pre-hybridization solution, pre-heated to 50°C. The probe was denatured and then added, the filters were hybridized at 50°C overnight, washed at 50°C to 2 x SSC, 0.1% SDS and then rinsed in 2 x SSC. The filters were sealed in plastic, exposed to film for 3 days and 6 days and any duplicate positives were identified.

2.22.2 South-western screening for *hsf*

The protocol described by Singh *et al* (1988) was followed. The aim of the screen was to isolate a cDNA clone containing *B. pahangi* heat shock factor (*hsf*). Fusion proteins, expressed from a *B. pahangi* cDNA library, were probed with a duplex containing a heat shock element, the binding site for HSF. The double stranded probe (HSE duplex) utilized in the screen, was designed from the sequence of the HSB probe described by Scharf *et al* (1990), which was used to isolate three *hsf* genes from tomato.

HSE duplex:

5′-TCGAGGATCCTAGAAGCTTCCAGAAGCTTCTAGAAGCAGATC-3′
3′-CCTAGGATCTTCGAAGGTCTTCGAAGATCTTCGTCTAGAGCT-5′

2.22.2.1 Annealing and end labelling the HSE probe

The two oligonucleotides designed for the HSE duplex were synthesized in-house (T. MacPherson, Dept. of Pathology) and were diluted with T.E. buffer to 1μg/μl. An aliquot of 99μl of TEN buffer was mixed with 0.5μg (0.5μl) of each oligonucleotide. The solution was heated to 70°C for 10 minutes and was then allowed to cool slowly to room temperature. This was achieved by transferring 50ml of water from the water bath (70°C) to a container, standing the tube in this water and leaving the container at room temperature to cool. The annealed duplex was precipitated at –70°C by the addition of 0.3M sodium acetate and 200μl of ethanol to the sample. After 30 minutes, the tube was spun at 13000g for 15 minutes and the supernatant was discarded. Salts were removed by adding 1ml of with ice cold 70% ethanol, spinning at 13000g for 15 minutes and discarding the supernatant. The duplex was resuspended in 10μl of ddH_2O to give a concentration of approximately 0.1μg/μl. To create a probe, 1μl of the duplex solution, 38.5μl of ddH_2O and 5μl of 10 x kinase buffer (Promega) were added to a 1.5ml screwtop microcentrifuge tube. Next 5μl (50μCi) of γ-^{32}P-ATP (Amersham

>5000Ci/mmol, 10mCi/ml) and 0.5µl (5 units) of T4 Polynucleotide Kinase (Promega) were added. The sample was incubated at 37°C for 30 minutes. Unincorporated nucleotides were removed using a NucTrap column (Stratagene). The NucTrap column was pre-wet with 70µl of 1 x STE, before 20µl of STE was added to the sample and the sample was loaded onto the top of the column. The sample was gradually forced into the column and the HSE duplex probe was eluted with 70µl of STE.

2.22.2.2 Screening with the HSE duplex

XL1-Blue MRF' cells were prepared as described in the Uni-ZAP™ XR Cloning kit instruction manual and a *B. pahangi* mixed sex adult stage cDNA expression library [Cox-Singh *et al* 1994] was titered as described in the Gigapack®II Packaging Extract instruction manual (Stratagene). Hybond-C (Amersham) 132mm nitrocellulose filters were used and the expression of the library was induced by soaking filters in 10mM IPTG for 30 minutes and gently laying them onto each plate. The plates were incubated at 37°C for 3½ hours, then the filters were removed, placed protein-side-up into BLOTTO and were left rocking for 1 hour at room temperature or overnight at 4°C. They were then washed twice (for 1-5 minutes) in TNE-50. The filters were screened by hybridization at room temperature in TNE-50 containing ~0.1nM of the HSE duplex and ~10µM of the non-specific probe, poly(dI-dC)·poly(dI-dC) (Sigma). After 60 minutes, the filters were washed four times at room temperature with TNE-50, blotted dry and autoradiographed at –70°C. Four plates with 5 x 10^4 pfu (2 x 10^5 pfu in total) were screened with the HSE duplex and duplicate filter lifts were taken from each plate.

Reagents :

BLOTTO

5% Marvel skimmed milk powder
50mM Tris.HCl pH 7.5
50mM NaCl
1mM EDTA

TNE-50

10mM Tris.HCl pH 7.5
50mM NaCl
1mM EDTA

add freshly prepared dithiothreitol to a final concentration of 1mM before use.

TEN buffer	**STE buffer (1 x)**
0.1M NaCl	0.1 NaCl
10mM Tris.HCl pH 8.0	20mM Tris.HCl pH 7.5
1mM pH 8.0	10mM EDTA

2.22.3 PCR using an heterologous primer, M6

A primer, M6, was designed from a 21bp region of *Drosophila hsf* (m60070) with 100% homology to 5 other *hsf* genes (m65217, x61754, m64673, x61753, l06098, l36924) as presented in ***Figure 5.13***. This primer was used with the T3 primer in an attempt to amplify a region of *B. pahangi hsf* from an adult *B. pahangi* cDNA library in λUni-Zap (see 5.3.5) and with the SL1 primer in an attempt to amplify a product from first strand adult cDNA (see 5.3.6). The PCR procedure in 2.22 was utilized, but an annealing temperature of 50°C was used for both reactions.

2.23 General molecular biology methods

2.23.1 Agarose gel electrophoresis

Gels containing 0.8%-2% agarose were used to separate and analyze DNA molecules. Typically, 0.8g-2g of agarose was mixed with 100ml 1x TAE buffer and dissolved by boiling briefly. The solution was allowed to cool for 10 minutes before adding 0.5μg/ml ethidium bromide. However, ethidium bromide was not added to gels used for genomic Southern blots. Gels were cast in GibcoBRL horizontal tanks using an appropriately sized comb to form wells. When set, sufficient 1x TAE buffer was added to the tank to submerge the gel. Aliquots of samples and DNA markers (usually λ*Hind*III) containing 1% DNA loading dye were carefully loaded into the wells and allowed to settle.

For the electrophoresis of genomic DNA, gels were run at 25V and as the DNA migrated into the gel and the voltage was then increased to 35V. The gel was removed after the bromophenol blue dye front had migrated two thirds of the length of the gel.

For other DNA samples, the voltage was set at 50V and the DNA was run until an appropriate separation was achieved.

Reagents :

TAE buffer (50x stock)

242g Tris base

57.1ml glacial acetic acid

100ml of 0.5M EDTA pH 8.0

adjust to 1ℓ with ddH_2O

store at room temperature

DNA loading dye (10x)

25% Ficoll (Sigma)

0.4% bromophenol blue (Sigma)

0.4% xylene cyanole FF (Kodak)

store at room temperature

1 x TAE : 40mM Tris-acetate, 1mM EDTA

Ethidium bromide stock (10mg/ml)

One 100mg ethidium bromide tablet (Sigma) dissolved in 10 ml ddH_2O

2.23.2 Ligation and transformation

Fragments of DNA for cloning either had overhanging adenine nucleotides (PCR-derived) or had "sticky ends" due to restriction digestion with endonucleases. DNA was ligated into either a TA vector (pCR®2.1 (Invitrogen), pTAg (R + D systems) or pT7blue (Novagen)), or with pBluescript SK^{II} (Stratagene) or pQE30 (Qiagen). Typically the ligation reaction was:

25-50ng plasmid

10-50ng DNA fragment

2μl of 5x ligase buffer (GibcoBRL)

0.5μl (2 units) of (HC) T4 DNA ligase

Incubated overnight at 16°C

Competent cells such as DH5α cells (GibcoBRL) were transformed with an aliquot of the ligation reaction. Commonly, 20μl of DH5α cells and 2μl of ligation reaction was used. The protocol outlined in the GibcoBRL leaflet for DH5α cells was followed. Cells were spread onto LB plates containing an appropriate antibiotic (for example 100μg/ml ampicillin) and left to grow overnight at 37°C. For inserts cloned into plasmids within the β-galactosidase gene, colonies could be selected with respect to their ability to hydrolyse the substrate analogue 5-bromo-4-chloro-3-indolyl-2-galactopyranoside (X-gal). Positive colonies have no β-galactosidase activity and therefore contain inserts.

2.23.3 Colony hybridization

Colonies were sub-cultured onto duplicate LB/antibiotic plates containing grid markings. One plate additonally contained a Hybond-N, nylon (gridded) filter and bacteria were inoculated directly onto the filter. The second plate was a stock of the colonies. Colonies were grown overnight at 37°C and the stock plate was stored at 4°C. The filter was carefully lifted from the agar plate and placed for 2 minutes on filter paper soaked with DNA denaturing solution. Next the nylon filter was transferred for 2 minutes to filter paper soaked with neutralization solution and finally was floated (bacteria upwards) for 30 seconds on rinsing solution (0.2M Tris.HCl pH 7.5, 2x SSC). DNA was cross-linked to the nylon membrane by U.V. irradiation (150mjoules in a Bio-Rad cross-linker). Inserts were screened by hybridization using an appropriate radiolabelled probe.

2.23.4 Screening colonies by PCR

After transformation (see 2.23.2), single bacterial colonies were touched with a 10µl disposable Gilson tips, dipped into a 0.5ml PCR tube containing 10µl of PCR mix and then the tip was used to streaked the colony onto another agar plate (contained suitable antibiotics). The PCR mix contains 1x PCR buffer A, 2µM of appropriate forward and reverse primers and 0.25units of AmpliTaq (Perkin Elmer). The PCR samples were overlaid with 10µl of mineral oil (Sigma). Normally, the PCR programme in 2.2 was utilized, but sometimes an annealing temperature of 60°C was used and when short products were being amplified, an extension time (at 72°C) of 1 minute was used rather than 3 minutes. DNA loading dye was added to each sample (to 1x) and the whole reaction was run on a 1% TAE agarose gel and studied on a U.V. transilluminator to identify transformants. These colonies (duplicated on an agar plate) were then used to inoculate 3ml of LB broth (plus antibiotics), the plasmid was extracted by mini-prep. and was sequenced.

2.23.5 Automated sequencing

Two types of sequencing were utilized: Li-cor, which uses fluorescently labelled oligonucleotide primers and ABI (Stretch 373A and the ABI 377) which uses fluorescently labelled dideoxynucleotides. (For ABI sequencing, a service is provided at

the Anderson College (University of Glasgow) and only the preparation of samples was required as sequencing technicians performed the rest).

2.23.5.1 Plasmid preparation for sequencing: plasmid miniprep

Transformed bacteria were grown overnight in 3ml of L-broth containing 100μg/ml of ampicillin (or other appropriate antibiotic), half the culture was transferred to a 1.5ml microcentrifuge tube and the cells spun at 13000g for 1 minute. The supernatant was removed, the remaining culture was added to the tube, the cells were spun down as previously and the supernatant was discarded. The cells were resuspended in 200μl of resuspension solution until the solution was homogeneous and then 200μl of cell lysis solution was added. The tube was inverted several times to ensure that all cells were lysed, 200μl of neutralization solution was added and the sample was mixed again by inversion. Spinning the sample at 13000g for 15 minutes pelleted the insoluble cellular debris. To precipitate the DNA, 0.5ml of the soluble supernatant was transferred to a fresh microcentrifuge tube and 1ml of ethanol was added. The sample was thoroughly mixed by inversion and then the DNA pelleted by spinning at 13000g for 15 minutes. The supernatant was removed and 400μl of ddH_2O, 40μl of 3M sodium acetate (pH 5.2) and 880μl of ethanol was added. The sample was left at -70°C for 30 minutes or overnight at -20°C before spinning at 13000g for 15 minutes which pelleted the DNA again. Salts were removed by washing with 70% ethanol and the DNA was resuspended in 10μl of ddH_2O. The concentration of the plasmid solution was calculated by running 1μl of the preparation on a 1% agarose gel alongside a known quantity of λ*Hind*III markers.

Reagents :

Cell resuspension solution

50mM Tris.HCl pH 7.5, 10mM EDTA, 100μg/ml RNase A

Cell lysis solution

0.2M NaOH, 1% SDS

Neutralization solution

1.32M potassium acetate pH 4.8

(the pH of the potassium acetate solution was adjusted with acetic acid)

2.23.5.2 Preparation of sequencing samples (ABI)

The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit reagents and protocol (Perkin Elmer P/N 402078) was utilized [Sanger and Coulson 1975, Prober *et al* 1987]. By arrangement sequencing data could be received via electronic mail.

Reaction mix

0.3-0.5μg of DNA (plasmid) template

3.2pmoles of DNA primer

8.0μl Terminator Ready Reaction Mix (Perkin Elmer)

ddH_2O to 20μl

2.23.5.3 Sequencing on the Model 4000 Automated (Li-cor) DNA Sequencer

Gels were poured as described in the Li-cor sequencing bulletins for the Model 4000 Automated DNA Sequencer. The reagents and protocol were as described in the SequiTherm EXCEL™ II Long-Read™ DNA Sequencing Kits-LC product information (Epicentre Technologies). For each sample, four 0.5ml microcentrifuge tubes were prepared, each containing 2μl of either SequiTherm EXCEL™ II Termination Mix A, T, C or G (which contained a mixture of deoxy- and dideoxynucleotides). The reaction mix was then equally divided between the four tubes (4μl to each), the samples were overlaid with mineral oil and placed in a Perkin Elmer thermocycler (PCR machine).

Reaction mix

0.4-1.0μg DNA (plasmid) template

2pmoles of IRD41 labelled primer

5μl of 5x SequiTherm EXCEL™ II Sequencing buffer

1μl of SequiTherm EXCEL™ II DNA Polymerase

ddH_2O to 17μl

Cycle sequencing

95°C for 5 minutes		1 cycle
95°C for 30 seconds	⎫	
60°C for 30 seconds	⎬	30 cycles
70°C for 1 minute	⎭	

On completion, 4µl of SequiTherm™ Stop Solution was added. Before loading on the sequencing gels, the DNA samples were denatured at 95°C for 5 minutes and then rapidly placed on ice. Typically 1µl of the sample was loaded for sequencing. The sequence data was captured automatically and was downloaded and transferred to the local UNIX system for analysis using the Wisconsin Sequencing Analysis Package (GCG).

Reagents :

TBE buffer (10x)
(890mM Tris-borate, 20mM EDTA)
107.8g Tris base
55.0g boric acid
7.4g disodium dihydrated EDTA
adjust to 1ℓ with ddH_2O
store at room temperature for up to 2 weeks

Sequencing gel solution (6%)
21g urea (Boehringer Mannheim)
6ml of 10x TBE buffer
6ml Long Ranger™ gel concentrate
ddH_2O to 50ml
stir until solution is clear
filter to remove solid particles

2.24 General protein methods

2.24.1 SDS polyacrylamide gel electrophoresis (PAGE)

The Hoefer vertical electrophoresis system was used with either longer length mini gels (10.1 x 10.6cm) or standard size (17.9 x 16cm) slab gels according to requirements. Electrophoresis was carried out at 20mA per gel for mini gels and at 30-40mA (or at 12mA overnight) for larger gels.

2.24.1.1 Casting gels

To polymerize, 100µl of freshly prepared 10% ammonium persulphate (APS) and 20µl of N,N,N′,N′-tetramethylenediamine (TEMED) was added to the gel solution (see **Reagents**). When the gel solution reached approximately one inch from the top of the

plates (large gels), a layer of water-saturated butanol was carefully added to form a flat surface. The gel was left to set for 0.5-1 hour. The butanol was rinsed off the top of the separating gel with ddH_2O and thin strips of 3MM filter paper were used to remove water from near the gel surface. Next, 50µl of APS and 10µl of TEMED were added to the stacking gel solution, which was then carefully poured between the plates and an appropriate comb was inserted. Typically, more solution was required as the gel settled. When the stacking gel had set, the wells were rinsed out with ddH_2O and then with 1x running buffer. Protein samples were mixed with an equal volume of SDS sample cocktail, heated in boiling water for 5 minutes, spun at 13000g for 1 minute and an aliquot of the soluble components loaded into the wells of the gel.

Reagents :

1.5M Tris.HCl pH 8.8

36.3g of Tris base
ddH_2O to 150ml
stir until dissolved
adjust to pH 8.8 with conc. HCl
adjust to 200ml with ddH_2O
store at 4°C

0.5M Tris.HCl pH 6.8

3g of Tris base
ddH_2O to 30ml
stir until dissolved
adjust to pH 6.8 with conc. HCl
adjust to 50ml with ddH_2O
store at 4°C

Running buffer pH 8.3 (10x)

(0.25M Tris, 1.92M glycine, 0.1% SDS)
151.15g of Tris base
721g of glycine
ddH_2O to 4ℓ
stir until dissolved
50g of SDS
adjust to pH 8.3
ddH_2O to 5ℓ
store at room temperature

SDS Sample cocktail

a) 25ml 0.5M Tris.HCl pH 6.8
40ml of 10% SDS
20ml glycerol
ddH_2O to 100ml
aliquot and store at room temp
b) 0.01g of bromophenol blue[1]
2.3g of dithiothreitol[2]
ddH_2O to 10ml
aliquot and store at -20°C
for use: mix 650µl of **a** and 100µl of **b**

(0.108M Tris, 3.5%SDS, 17.3% glycerol, 0.013% BPB[1], 0.2M DTT[2])

Coomassie® blue stain (5ℓ)

5g of Coomassie® blue

2250ml of methanol

stir until dissolved

2250ml of ddH_2O

500ml of glacial acetic acid

filter through Whatman No.1

store at room temperature

gel destain (5ℓ)

350ml of glacial acetic acid

1ℓ of methanol

3650ml of ddH_2O

drying reagent

100ml of glacial acetic acid

10ml of glycerol

ddH_2O to 1ℓ

12.5% separating gel

12.5ml of 30% acrylamide solution (Scotlab)

11.2ml of 1.5M Tris.HCl pH 8.8

6.2ml of ddH_2O

de-gas the solution under vacuum for 5 minutes

add 0.3ml of 10% SDS

5% stacking gel

1.67ml of 30% acrylamide

1.25ml of 0.5M Tris.HCl pH 6.8

7.0ml of ddH_2O

0.1ml of 10% SDS

(30% acrylamide solution contains 2.7% N,N'-methylene-bis-acrylamide)

Low molecular weight markers (Bio-Rad) : 14.4-97.4kD

Contains: lysozyme (14.4kD), trypsin inhibitor (21.5kD), carbonic anhydrase (31.0kD), ovalbumin (45.0kD), serum albumin (66.2kD), phosphorylase B (97.4kD).

High molecular weight markers (Bio-Rad) : 45-200kD

Contains: ovalbumin (45.0kD), serum albumin (66.2kD), phosphorylase B (97.4kD), β-galactosidase (116.25kD), myosin (200kD).

For use, protein standards were diluted 1:20 in SDS sample cocktail.

2.24.2 Western blotting

Protein samples and markers (Bio-Rad, Laemmli 1970) were run on a SDS-polyacrylamide gel (12.5%). The proteins were then transferred onto nitrocellulose membrane using the Hoefer mini-blotting system at a constant current of 200mA for 1 hour. The blot was stained for 5 minutes with Ponceau S, positioning markings were added and the protein lanes were cut into strips. To block non-specific sites, the sample strips were soaked in TBS/Tween 20/5% BSA for 1 hour. The strips were then rinsed

with TBS/Tween 20 and were incubated for 1 hour at room temperature with test rabbit serum or control rabbit serum at dilutions ranging from $^1/_{100}$ to $^1/_{600}$. Monoclonal antibodies (SPA-830, SPA-835 and SPA-845 StressGen) were also used and these were diluted to $^1/_{500}$ to $^1/_{2000}$. The strips were rinsed with TBS/Tween 20 and then incubated for 1 hour at room temperature with the secondary antibody. Goat anti-rabbit alkaline phosphatase conjugate (ICN) diluted $^1/_{6000}$ was used in conjunction with the primary rabbit anti-serum and an anti-mouse or rat anti-IgG alkaline phosphatase conjugate (PharMingen, Serotec) diluted at $^1/_{4000}$ or $^1/_{8000}$ was used in conjunction with the monoclonal antibodies. The strips were rinsed again and the blot was developed by incubating the strips in BCIP/NPT substrate (Dynatech) until adequate staining occurred. Rinsing thoroughly with ddH2O stopped the reaction. The blot was then reformed by carefully re-aligning the strips using the positioning markings and adhering them to paper.

Reagents :

Transfer buffer

6.05g of Tris base

28.2g of glycine

400ml of methanol

ddH_2O to 2ℓ

(0.025M Tris, 0.19M glycine)

Tris buffered saline (TBS)

12.1g of Tris base

43.75g of NaCl

adjust to pH 7.4 with conc. HCl

ddH_2O to 5ℓ

(0.05M Tris, 0.15M NaCl)

TBS/Tween 20

0.05% Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma) in TBS

Ponceau S Stain

Dissolve 0.2g Ponceau S (Sigma) in 100ml of 3% trichloroacetic acid solution

2.25 Biocomputing

The Wisconsin Sequencing Analysis Package (GCG) on the local UNIX system was used for many of the sequence analyses including primer design ("prime") the assembly of DNA fragments ("gelassemble"), the alignment of two sequences ("bestfit"), and multiple sequence alignments ("pileup"). GCG was used to create an HSP90 cladogram.

In this case, 30 amino acid sequences obtained from the protein databanks and translations of DNA sequences in the DNA databanks were aligned using "pileup". "Lineup" was then used to make all the sequences in the multiple sequence alignment the same length, allowing the use of the "distances" programme which calculated the difference of each HSP90 sequence (Kimura protein distances). The "growtree" programme could then be used on the protein distances. The data for the HSP90 phylogenetic tree was viewed on a PC with Treeview (copyright© Roderic D.M. Page 1997).

Analysis software on the world wide web was also utilized, such as at Sanger Centre (www.sanger.ac.uk) and NCBI (www3.ncbi.nlm.nih.gov), ExPASy (expasy.hcuge.ch) and sequence interpretation tools at the Tokyo genome centre (www.tokyo-center.genome.ad.jp/SIT/SIT.html).

Table 2.1 : Oligonucleotide primers

SL1	5′ GCCGGAATTCGGTTTAATTACCCAAGTTTGAG 3′
oligodT	5′ GCCGCTCGAGTTTTTTTTTTTTTTTT 3′
adap.dT	5′ GTCAGATCTACGCGTCGACCTCGAGTTTTTTTTTTTTTTTT 3′
adapter	5′ GTCAGATCTACGCGTCGACCTCGAG 3′
anchor	5′ GTCAGATCTACGCGTCGACCTCGAGGGIGGGIIGGGIIG 3′
hsp90f2	5′ CGGCGAATTCGAAGAATATGCTGAGTTCTACAAG 3′
hsp90r2	5′ CGGCGAATTCCGCTCCATGTTAGCGGACCA 3′
hsp90r3	5′ CTGTTGCACACAATACTCGTCTATCGG 3′
hsp90f4	5′ GTATCCATGAAGATTCAACCAATCG 3′
hsp90f5	5′ GAATGGTGAAACTTTTGCGTTTCAG 3′
hsp90r5	5′ GCTTTAGTCATACCAATTCCCGTGTC 3′
hsp90f6	5′ AACATCTCGAAATCAACCCTGACC 3′
hsp90r6	5′ TCAAGCGAAAAACCAGAAGAAAGC 3′
aeed	5′ GCCGAGGAAGATGCATCGAGGATG 3′
210pf1	5′ CGGAGCTCCCGGAACATCGTACAATGC 3′
p1pr1	5′ CGGGATCCCATCACTAGTGTCCCAAGG 3′
prex1	5′ GCACACGAAAGCTGAAATGTGAG 3′
prex2	5′ CATTGTTGCTAGTCTTATTCCGATTG 3′
upf1	5′ ATCGCTGCTGCCTGCAAGAACC 3′
tranf	5′ CGGAGCTCCGTGGATATCGGTCGTATTG 3′
tranr	5′ CGGGATCCCTAGTGTCCCAAGGTTTCCG 3′
90pef	5′ CTGGTGGATCCAGGGAAGCTGTCGCC 3′
90per	5′ CCATGGTACCTTAATCAACTTCTTCCATCC 3′
m6 (HSF)	5′ GTTCGTGTTGTTGTACCGGTCCTTAAGGCCG 3′
T3	5′ AATTAACCCTCACTAAAGGG 3′
T3*	5′ AATTAACCCTCACTAAAG 3′
T7*	5′ TAATACGACTCACTATAGGG 3′
M13rev*	5′ CAGGAAACAGCTATGAC 3′
M13* (-40)	5′ GTTTTCCCAGTCACGAC 3′

* Fluorescent (IRD41 labelled) primers corresponding to these sequences were used for LI-COR automated sequencing. Primers were synthesized by Cruachem and MWG.

3.0 Cloning and Characterization of a *Brugia pahangi* cDNA Homologue of *hsp90*

3.1 Introduction

As discussed in the Introduction, *hsp90* can have a wide range of roles in eukaryotic cells. Many *hsp90* sequences have been submitted to databases such as Genbank, perhaps reflecting the relative abundance of this protein. At the start of this project, *hsp90* sequences were available for the parasitic helminth *S. mansoni* and for the free-living nematode *C. elegans* and these were used for the design of primers for PCR. The transcription of *hsp90* has been studied and in *C. elegans* it is interesting to speculate on the possible role of *hsp90* in this nematode, in which reverse genetics has elucidated functional roles for many different genes.

In *C. elegans*, *hsp90* mRNA has an intriguing expression pattern - the studies of Dalley and Golomb (1992) used nuclear run-ons to investigate transcription of several genes during the dauer pathway. This is an alternative developmental pathway, initiated at the L_2 stage by stresses such as overcrowding or lack of food. Dauer larvae are developmentally arrested, have a reduction in transcription of most genes and are very resistant to environmental stresses. These studies demonstrated that *hsp90* mRNA was 15 fold enriched in the dauer stage. Upon recovery from the dauer stage, *hsp90* transcripts declined sharply within 75 minutes. The ability of HSP90 to interact with steroid hormone receptors [Catelli *et al* 1985, Ziemiecki *et al* 1986] and various regulatory proteins such as kinases [Uma *et al* 1997, Inanobe *et al* 1994] suggests a number of ways in which HSP90 may have a role in dauer larvae. The *hsp90* gene of *C. elegans* has been identified as a member of the daf (dauer formation) gene family, *daf21*. DAF21 has a specific role in chemosensation and is also required for the function of other sensory neurones. It was hypothesized that HSP90 functions in the negative regulation of a transmembrane guanyl cyclase via interaction with a protein kinase-like domain [Malone and Thomas 1996]. This would imply that DAF21 has a role in a dauer signal transduction pathway and may prevent a cyclic GMP stimulated cascade.

The first approach to isolating the *B. pahangi hsp90* homologue was to design primers based on sequences from closely related species and to amplify a product initially from genomic DNA and then from cDNA. The *C. elegans* sequencing projects have created a

large amount of both genomic and cDNA sequence data. Cosmid and expressed sequence tag (EST) sequences with homology to *hsp90* have been entered into the Genbank database. In addition, a partial cDNA sequence, which encodes 1365bp corresponding to the carboxyl terminus of a *S. mansoni hsp90* homologue, has been sequenced and this sequence was also available [Johnson *et al* 1989].

3.2 Results

3.2 1 Alignment of *S. mansoni* and *C. elegans* partial *hsp90* sequences for primer design

Three primers were designed by comparing the sequence from *S. mansoni* and that of three *C. elegans* ESTs (see ***Table 3.1*** and ***Figure 3.1***). The regions, which had high sequence homology between these two species, were chosen for primer design. The *C. elegans* codon bias was used for these primers since this nematode is a closer relative to *Brugia* than the trematode, *S. mansoni*. A GC clamp and *Eco*RI site were added to the primer to permit efficient restriction digestion of the product and aid in the cloning of a product. In addition, the GC clamp increases the stability of interaction between primer and template after an initial amplification.

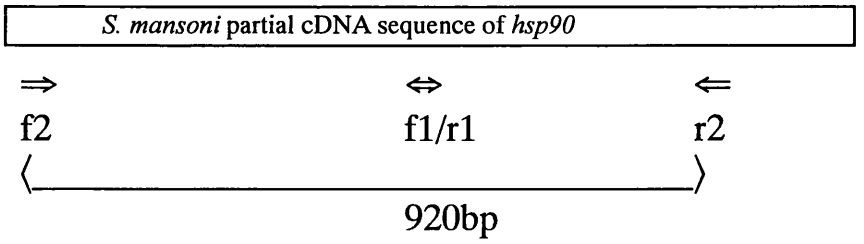
Hsp90f2 corresponds to sequence near the 5′ end of the *S. mansoni* clone and hsp90r2 to sequence 920bp downstream from hsp90f2. Hsp90f1 and hsp90r1 were designed as additional primers that could be used with hsp90f2 and hsp90r2 to produce smaller PCR products. They correspond to sequence within the region between hsp90f2 and hsp90r2 and have the same sequence, but in the forward and reverse directions respectively. The largest product should therefore be obtained using the hsp90f2 and hsp90r2 primers, with an estimated size of 920bp based on the *S. mansoni* sequence, (see ***Figure 3.1***).

Table 3.1 : Designing primers from *C. elegans* EST sequences

C .elegans EST	Size of EST	Primer Name	Primer Sequence
m75850	319bp	hsp90f1	cggcgaattc**caagaagttctacgagcaattc**
		hsp90r1	cggcgaattc**gaattgctcgtagaacttcttg**
t01565	504bp	hsp90f2	cggcgaattc**gaagaatatgctgagttctacaag**
t00844	368bp	hsp90r2	cggcgaattc**cgctccatgttagcggacca**

The accession numbers of the ESTs are shown in the table. These contain regions with high homology to the *S. mansoni hsp90* sequence and these regions were used to design the above primers. The *hsp90* sequence from *C. elegans* is in **bold**. The GC clamp is dotted underlined, the *Eco*RI site is double underlined and provides a convenient site for cloning the PCR product.

Figure 3.1 : Heterologous *hsp90* primers aligned to *S. mansoni hsp90*



The primers hsp90f2 and hsp90r2 should amplify a cDNA product of 920bp based on the position of the primers with respect to the sequence of the *S. mansoni* j04017 clone. This clone encodes the carboxyl terminal of an *hsp90* gene (*hsp86*) and the product hsp90f2-r2 is thus an internal *hsp90* fragment. The primers hsp90f1 and hsp90r1 are within the 920bp region and should amplify products of a similar size: hsp90f2-r1, 410bp and hsp90f1-r2, 530bp. Sequence alignments of *C. elegans* ESTs and *S. mansoni hsp86*, which includes the regions (in bold) used for primer design, are shown below. The GC clamp is underlined (dotted) and the *Eco*RI site is double underlined.

```
                    271                                                 320
C. elegans : t01565 GAAGAATATGCTGAGTTCTACAAGAGCTTGTCCAATNACTGGGGAGNTCA
                    ||||| ||||| ||||| |||||| || | |||:| |||| |: ||
S. mansoni hsp86    GAAGAGTATGCAGAGTTTTACAAGTCGTTAACTAATGATTGGGAGGACCA
                    50                                                  99

hsp90f2 :           5' CGGCGAATTCGAAGAATATGCTGAGTTCTACAAG 3'

                    151                                                 200
C. elegans : t00844 ACTTCCGAGTACGGATGGTCCGCTAACATGGAGCGCATCATGAAAGCTCA
                    ||||| |||| ||| ||||| |||||||||||| | |||||||| || ||
S. mansoni hsp86    ACTTCAGAGTTCGGTTGGTCTGCTAACATGGAGAGGATCATGAAGGCACA
                    935                                                 984

hsp90r2 :           5' CGGCGAATTCCGCTCCATGTTAGCGGACCA 3'

                    151                                                 200
C. elegans : m75850 GACAAAGACAACTTCAAGAAGTTCTACGAGCAATTCGGAAAGAATCTCAA
                    || ||||| |||| |||||||||||| ||||||||| ||| | | | ||
S. mansoni hsp86    GATAAAGAAAACTACAAGAAGTTCTATGAGCAATTCTCAAAAAGTATAAA
                    422                                                 471

hsp90f1 :           5' CGGCGAATTCCAAGAAGTTCTACGAGCAATTC 3'

hsp90r1 :           5' CGGCGAATTCGAATTGCTCGTAGAACTTCTTG 3'
```

3.2.2 PCR procedure

The heterologous primers were used in a PCR with *B. pahangi* genomic DNA. An annealing temperature of 50°C was used to allow for possible mismatches between the primer and the true sequence. The primers hsp90f2 and hsp90r2 amplified a product of 1.3kb (see ***Figure 3.2***). No product was observed using combinations of the hsp90f1 or hsp90r1 primers.

The genomic PCR product was digested with *Eco*RI and cloned into *Eco*RI-cut pBluescript SK^{II}. This enzyme cut the product into two fragments (0.5kb and 0.8kb) revealing the presence of an *Eco*RI site within the hsp90f2-r2 sequence. The two inserts were sequenced, which confirmed their homology to *hsp90* at the DNA level. The *B. pahangi* sequence appeared to have introns, consistent with the observation that the product amplified from *B. pahangi* (1.3kb) was larger than estimated from the *S. mansoni* cDNA sequence (920bp). Indeed a comparison of the *S. mansoni* cDNA and *B. pahangi* genomic fragments identified four probable introns in the *B. pahangi* sequence.

3.2.3 Amplifying an internal *hsp90* cDNA product

Attempts were made to amplify a cDNA fragment from *B. pahangi* first strand cDNA prepared from RNA isolated from different life cycle stages. The same primers (hsp90f2 and hsp90r2) that were used to amplify the genomic fragment were used in a variety of PCRs carried out on different first strand cDNAs : 37°C mf; 41°C adults; 24 hour post-infective L_3. A product of 920bp was only obtained from L_3 harvested from the mammalian host at 24 hours post infection. ***Figure 3.3*** shows the cDNA product, hsp90f2-r2. The product was cloned into a TA vector and sequenced. A comparison of the genomic and cDNA sequences confirmed four introns in the region between the hsp90f2 and hsp90r2 primers.

3.2.4 Screening a mf cDNA library

A mf cDNA library available in this laboratory [Thompson *et al* 1996] was screened with the hsp90f2-r2 genomic PCR product. The λ-Unizap library was made by RT-PCR using SL1 and oligodT primers on RNA extracted from mf heat shocked at 41°C. It should therefore be enriched for heat shock protein cDNA. Five plates were screened, each with 5×10^4pfu, giving a total of 2.5×10^5pfu.

Figure 3.2 :

Amplification of an *hsp90* fragment from genomic DNA using hsp90f2 and hsp90r2

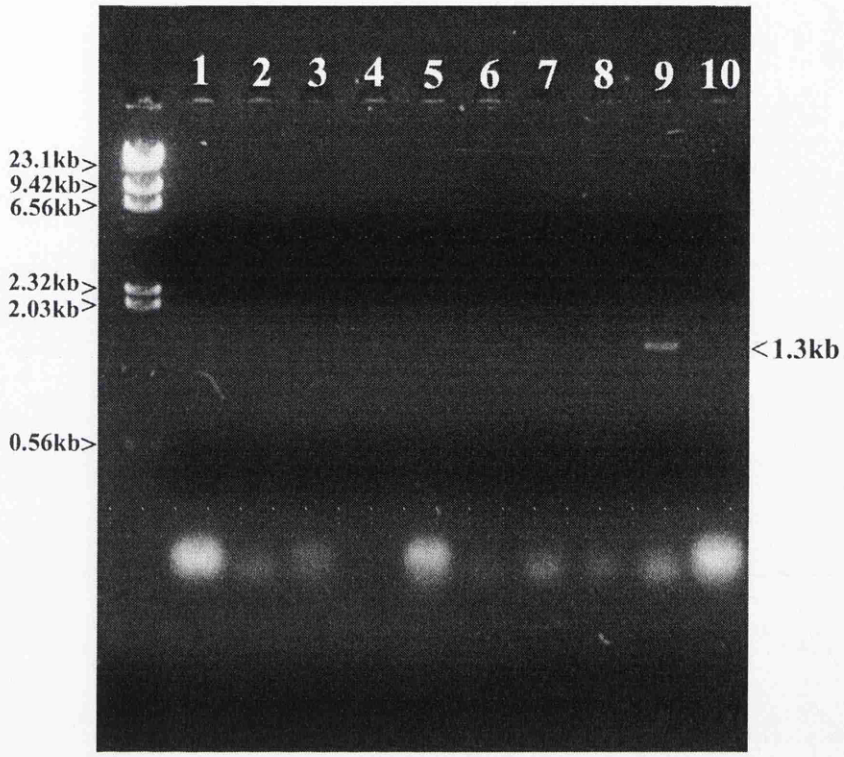


Figure 3.2 :

Approximately 100 pmoles of the primers hsp90f2 and hsp90r2 were used in a PCR with 18.5ng of *B. pahangi* adult genomic DNA. The PCR products were run on a 1% agarose gel containing ethidium bromide. The sizes of the λ*Hind*III markers are shown and their positions are indicated by arrows on the left hand side of the image. A PCR product (hsp90f2-r2) of 1.3kb is indicated by an arrow on the right hand side of the image. The PCR reactions were as follows:

1	hsp90f1/hsp90r2	6	hsp90f2 only
2	hsp90f1	7	hsp90f1/hsp90r2
3	hsp90r1	8	hsp90r2
4	no primers	9	hsp90f2/hsp90r2
5	hsp90f2/hsp90r1	10	no template, all primers

Figure 3.3 : Amplification of a fragment of *hsp90* from cDNA using hsp90f2 and hsp90r2

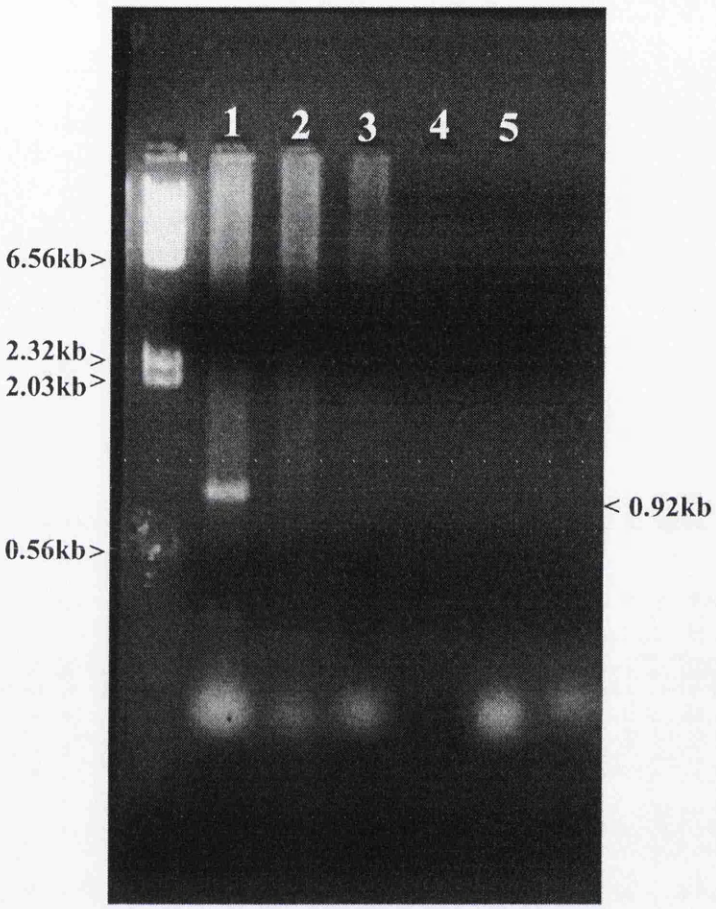


Figure 3.3 :

Approximately 100 pmoles of the primers hsp90f2 and hsp90r2 were used in a PCR with 1µl of first strand cDNA from *B. pahangi* 24 hours post-infective L_3. The PCR products were run on a 1% agarose gel containing ethidium bromide. The sizes of the λ*Hind*III markers are shown and their positions are indicated by arrows on the left-hand side of the image. A PCR product (hsp90f2-r2) of 0.92kb is indicated by an arrow on the right hand side of the image. The PCR reactions were as follows:

1	hsp90f2/hsp90r2	4	no primers
2	hsp90f2	5	no template, both primers
3	hsp90r2		

Duplicate filter lifts were taken from each plate and to decrease the probability of artifacts, only plaques positively identified from both filters were chosen. Six duplicate positives were isolated from the first screen but in the second round of screening only two of these original plaques strongly hybridized to the probe. Two positive clones were rescued and the plasmids sequenced. The inserts were all identical, 280bp in length and represented internal fragments of *hsp90*. Although no new sequence information was obtained, since the insert corresponded to part of the hsp90f2-r2 probe, sequencing of these clones did confirm the veracity of the sequence already available.

3.2.5 Obtaining the 5′ end

In *Ascaris suum* greater than 80% of mRNAs appear to possess a 22 base pair spliced leader sequence on the 5′ terminus, (SL1). *Trans*-splicing of pre-mRNA occurs in nematodes and also in trypanosome parasites [Nilsen 1993]. With this in mind, amplification of cDNA was attempted using a primer corresponding to SL1 and an *hsp90*-specific reverse primer designed from the newly acquired sequence information. The SL1 primer is 32 bases in length and contains the SL1 spliced leader sequence (bold), a GC clamp (dotted underlined) and an *Eco*RI site (double underlined).

SL1 : 5′ GCCGGAATTC**GGTTTAATTACCCAAGTTTGAG** 3′

The gene-specific primer consists of 27 bases corresponding to the amino acids: PIDEYCVQQ.

Hsp90r3 : 5′ **CTGTTGCACACAATACTCGTCTATCGG** 3′

The primers were used in a PCR reaction on first strand cDNA prepared from mf incubated at 37°C. SL1 can bind to the cDNA from many different genes and therefore only the hsp90r3 primer is specific to *hsp90*. An annealing temperature of 55°C was thus chosen to ensure some degree of specificity.

After 30 cycles a product of 1.6kb was obtained and this was cloned into a TA vector. Approximately 500bp was sequenced from either direction. To obtain more sequence information hsp90SL1-r3 was amplified with the hsp90f2 and hsp90r3 primers at an annealing temperature of 50°C and this gave a product (sc90f2-r3) of 0.68kb, (see ***Figure 3.4***). This was cloned and sequenced.

Figure 3.4 : Amplification of sc90f2-r3 from hsp90SL1-r3 using hsp90f2 and hsp90r3

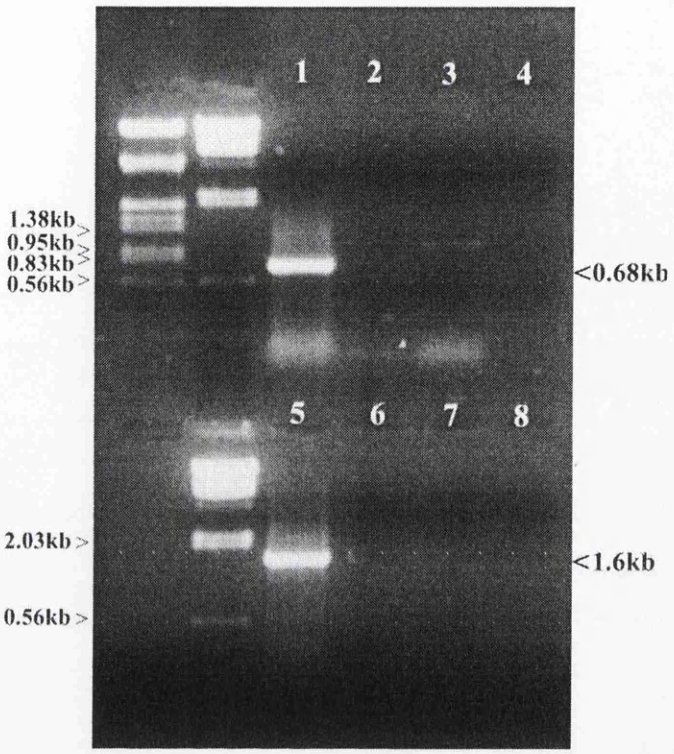


Figure 3.4 :

Approximately 100 pmoles of the primers hsp90f2 and hsp90r3 were used in a PCR reaction with plasmid (pTAg) containing hsp90SL1-r3. For each reaction approximately 15ng of hsp90SL1-r3 insert was utilized. The PCR products were run on a 1% agarose gel containing ethidium bromide. Markers λ*Hind*III/*Eco*RI and λ*Hind*III were run in upper half of the gel and λ*Hind*III in the lower half. The sizes of four of the λ*Hind*III/*Eco*RI marker bands are shown for the upper gel half and the sizes of two of the λ*Hind*III marker bands are shown for the lower gel half. Their positions are indicated by arrows on the left hand side of the image. PCR products sc90f2-r3 (0.68kb) and hsp90SL1-r3 (1.6kb) are indicated by arrows on the right hand side of the image. The PCR reactions were as follows:

1	hsp90f2/hsp90r3	5	SL1/hsp90r3
2	hsp90f2	6	SL1
3	hsp90r3	7	no template, hsp90f2/hsp90r3
4	no primers	8	no template, SL1/hsp90r3

The final sequence data was obtained by digesting the hsp90SL1-r3 product with *EcoRI* and cloning a 0.76kb fragment into pBluescript SK^{II} (sc90*e*slr3). ***Figure 3.5*** describes the relationship between the various PCR products obtained by this point.

Comparison of the sc90f2-r3 cDNA product with the original genomic hsp90f2-r2 product revealed a single intron in the cDNA (intron 8 in genomic sequence), while the corresponding region in the genomic sequence contained three introns. This may imply that the template amplified by the SL1 primer and hsp90r3 originated from an mRNA species which had not been completely spliced to remove all the introns.

3.2.6 Obtaining the 3′end

At this stage the 5′ cDNA sequence of *hsp90* was complete, but the 3′ end had not been obtained. In order to facilitate isolation of the 3′ end an homologous primer was designed from the hsp90f2-r2 cDNA product

hsp90f4 : 5′-GTATCCATGAAGATTCAACCAATCG-3′

Adapted oligodT primer :

5′-**GTCAGATCTACGCGTCGACCTCGAG**TTTTTTTTTTTTTTTTT-3′
〈_____Adapter primer_____〉〈oligodT_____〉

First strand cDNA was prepared from 37°C mf using the adapted oligodT primer (shown above) in the reverse transcription. The 5′ end of the adapted oligodT primer is GC rich and a corresponding adapter primer, which anneals to this region, can be used in subsequent PCR reactions. Since oligodT can prime non-specifically to adenine rich sequences [Joshua and Hsieh 1995], the adapter primer is used to reduce the amplification of products resulting from the mis-priming by oligodT. The hsp90f4 primer was used in a PCR amplification of the mf cDNA together with either the oligodT primer or the adapter primer. A very faint product was obtained in the single primer lane in which mf cDNA was amplified with hsp90f4 alone. Only low molecular weight bands were observed in the PCR with hsp90f4 and oligodT or hsp90f4 and the adapter primer.

Figure 3.5 : Schematic of *hsp90* overlapping fragments

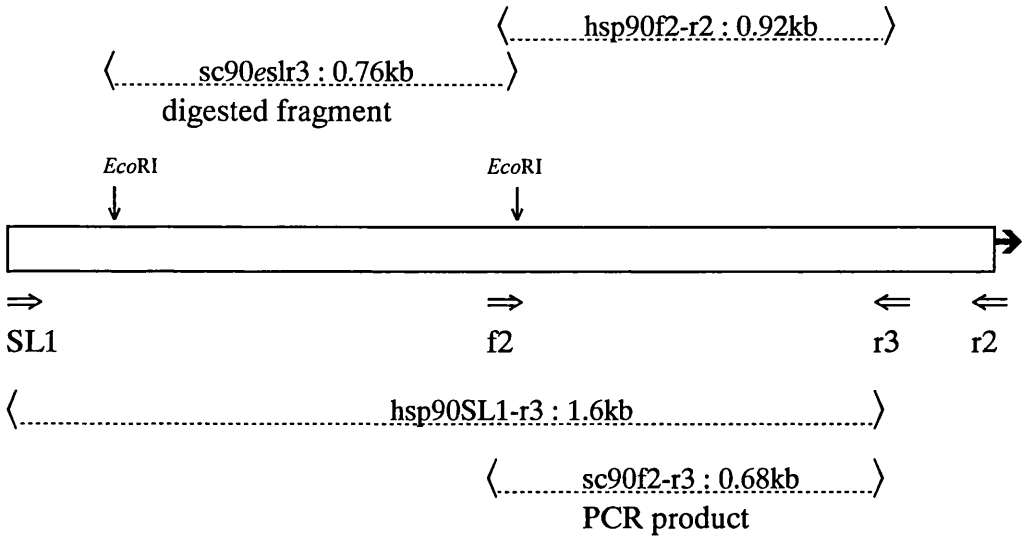


Figure 3.5 :

The diagram shows the relative positions of hsp90f2-r2, hsp90SL1-r3 and sub-clones sc90*e*slr3 and sc90f2-r3. The sub-clone sc90eslr3 was produced by digesting hsp90SL1-r3 with *Eco*RI and the sub-clone sc90f2-r3 was produced by PCR amplification of hsp90SL1-r3 with the primers hsp90f2 and hsp90r3. The sequence of hsp90SL1-r3 covers most of the sequence of the product hsp90f2-r2.

3.2.7 Isolating the minor product

The faint product obtained in the single primer PCR was excised from a low melting point Seaplaque gel and an "in-gel" ligation was attempted using the pT7blue vector. Transformations with the ligation reaction gave no colonies with inserts. The ligation reaction was then diluted and used as the template in a second PCR with only the hsp90f4 primer. A product of the correct estimated size, 1.1kb was amplified and cloned into pT7blue. ***Figure 3.6*** is an image of the products obtained in the PCR reaction on the ligated product, both the 1.1kb band and a smaller band are visible. The smaller band may be due to non-specific priming by hsp90f4. The insert was sequenced in one direction, but sequencing from the opposite direction was not successful due to a string of consecutive thymidines on the sense strand, which prevented the polymerase from efficiently reading the template.

3.2.8 Sub-cloning the insert

The 1.1kb fragment was digested with a range of enzymes. The digests revealed a *Hind*III and an *Sph*I site within the insert. Fragments of 0.39kb and 0.37kb were released from the plasmid by digesting with *Sph*I and *Hind*III respectively. These fragments contained a string of thymidines, which had hindered sequencing. The remaining vector was re-ligated creating inserts of 0.71kb (*Sph*I) and 0.73kb (*Hind*III) which were sequenced in both directions. ABI sequencing on the original insert (hsp90f4end) with a new primer produced sequence information for the 3′ end. However, although there was a stop codon in the insert, there was no polyA tail or polyadenylation signal in the 3′ UTR, implying that the product was truncated. ***Figure 3.7*** shows the position of the PCR products and sub-clones in relation to the complete sequence of the cDNA.

3.2.9 Amplifying a new 3' product

The truncated product, hsp90f4end, did not confirm the presence of a polyadenylation signal so an additional primer, hsp90f6, was designed from newly acquired sequence information. As can be seen from ***Figure 3.7***, this primer is closer to the 3′ end than hsp90f4 and a smaller product may be more readily amplified in the reaction.

Hsp90f6 : 5′-AACATCTCGAAATCAACCCTGACC-3′

Figure 3.6 : Amplification of hsp90f4end

Approximately 100pmoles of the adapter primer and hsp90f4 were used to amplify the components of a ligation reaction. A product, faintly visible on an agarose gel, had previously been obtained using hsp90f4 to amplify first strand cDNA prepared from mf RNA. The product had been excised from the gel and used in a ligation reaction with a TA vector (pT7blue). The transformation was unsuccessful and so the ligation mix was used as a template for PCR to re-amplify the product. The total volume of the ligation mix was 10µl and 1µl was used for each reaction. The sizes of λ*Hind*III markers are shown on the left hand side and their positions are indicated with arrows. The sizes of two of the λ*Hind*III/*Eco*RI marker bands are shown on the right hand side and their positions are also indicated with arrows. Products of 1.1kb and 0.5kb were obtained using only the hsp90f4 primer and the 1.1kb band was excised, purified and cloned into pT7blue. The PCR reactions were as follows:

1	hsp90f4/adapter	4	no primers
2	hsp90f4	5	no template
3	adapter		

Figure 3.7 : The complete *hsp90* cDNA assembled from the amplified fragments

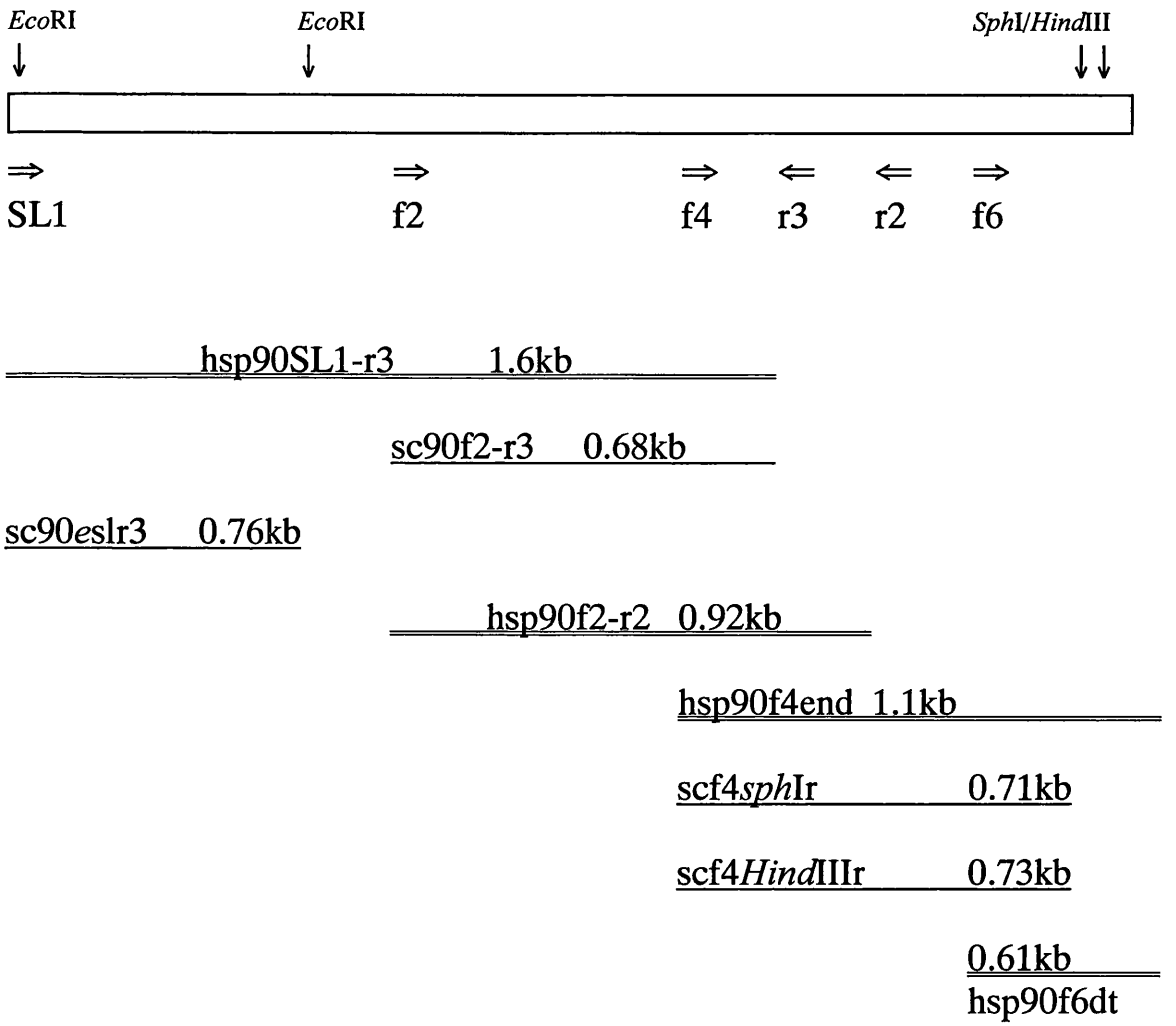


Figure 3.7 :

The original PCR products are denoted by double lines and sub-clones of these products are shown as a single line. Six primers, SL1 and five *hsp90*-specific primers were utilized in the PCR amplification of the cDNA sequence of *hsp90*. In addition, the restriction enzymes *Eco*RI, *Sph*I and *Hind*III were used to sub-clone PCR products and facilitate sequencing.

Amplification of first strand mf cDNA using hsp90f6 and the adapted oligodT primer produced a fragment of 0.61kb. The cDNA product, called hsp90f6dT, was cloned and sequenced. A string of thymidines (the polyA tail) prevented sequencing in one direction. The final confirmation of the extreme 3´end was by ABI sequencing using a primer (aeed) designed from amino acids near the end of the open reading frame.

3.2.10 The completed cDNA sequence

The Wisconsin Sequencing Package (or GCG) contains the program "gelassemble" which can be used to align and edit fragments of sequence information. The various fragments that contribute to the final sequence of the *B. pahangi hsp90* cDNA are listed in ***Table 3.2***. The PCR products were thus condensed into the consensus sequence in ***Figure 3.8***.

The spliced leader, SL1, is at the start of the sequence, 70 bp from the first ATG. There are two possible translational start sites but the first one has been used in the calculation of the protein size and sequence. The sites for the three restriction enzymes, *Eco*RI, *Hind*III and *Sph*I which were used to sub-clone sections of the cDNA can be seen in the sequence. The stop codon (TAA) is immediately after the five residues MEEVD and the polyadenylation signal (PAS), is 270 bp downstream from the stop codon.

3.2.11 Comparison of *B. pahangi hsp90* with partial cDNA sequences from *B. malayi*

Four *B. malayi* ESTs, with homology to *hsp90*, have been sequenced from the *Brugia* sequencing project: three from an L_3 cDNA library and one from an adult cDNA library. The inserts in these libraries were originally amplified from first strand cDNA using SL1 and oligodT primers, consistent with the proposal that *hsp90* is a gene which undergoes *trans*-splicing. A comparison of the *B. malayi* ESTs with the *B. pahangi* cDNA revealed high homology between the sequences, as may be expected for two species with a close evolutionary relationship. Indeed there are only a few base changes evident for the regions of comparison. The percentage similarity is 98% between the EST, aa273170, and *B. pahangi hsp90*. The high homology may imply that the *B. malayi* EST sequences correspond to the homologue of the *B. pahangi hsp90* gene.

Table 3.2 : Summary of the sequencing of *hsp90* cDNA

Clone Name	Vector Name	Insert size	Sequencing Primers		Sequencer
hsp90f2-r2	pTAg	0.92kb	$M13_{for}$	T7	Li-cor
hsp90SL1-r3	pTAg	1.6kb	$M13_{for}$	T7	Li-cor
⌊sc90f2-r3	pT7blue	0.68kb	$M13_{for}$	T7	Li-cor
⌊sc90*e*slr3	$pBScSK^{II}$	0.76kb	T3	T7	Li-cor
hsp90f4end	pT7blue	1.1kb	$M13_{for}$		Li-cor
hsp90f4end	pT7blue	1.1kb	hsp90f6	aeed	ABI
⌊scf4*sph*Ir	$pBScSK^{II}$	0.71kb	T3	T7	Li-cor
⌊scf4*hind*IIIr	$pBScSK^{II}$	0.73kb	T3	T7	Li-cor
hsp90f6dt	pCR2.1	0.61kb	$M13_{rev}$	T7	Li-cor
hsp90f6dt	pCR2.1	0.61kb	aeed		ABI

The symbol ⌊ denotes the sub-clone of the above clone.

The *hsp90* cDNA was sequenced at least twice (generally once in each direction) to ensure the accuracy of the final sequence. ABI sequencing was utilized where Li-cor sequencing was not possible due to a string of consecutive nucleotides (hsp90f4end and hsp90f6dt). In these cases, *hsp90*-specific forward primers (aeed and hsp90f6) were used to sequence towards the runs of adenine/thymidine.

Figure 3.8 :

The sequence was compiled from all the amplified fragments of the cDNA. Specific points of interest are the spliced leader at the 5′ end which is underlined, two possible translational start sites (**ATG**), restriction sites which aided with sub-cloning (these are shown in bold italics), the termination codon (**TAA**) and polyadenylation signal (**AATAAA**). The sequence which codes for the terminal five amino acids is shown in bold. There is an ambiguity (N) in the 3′ UTR due to the difficulty in sequencing this region.

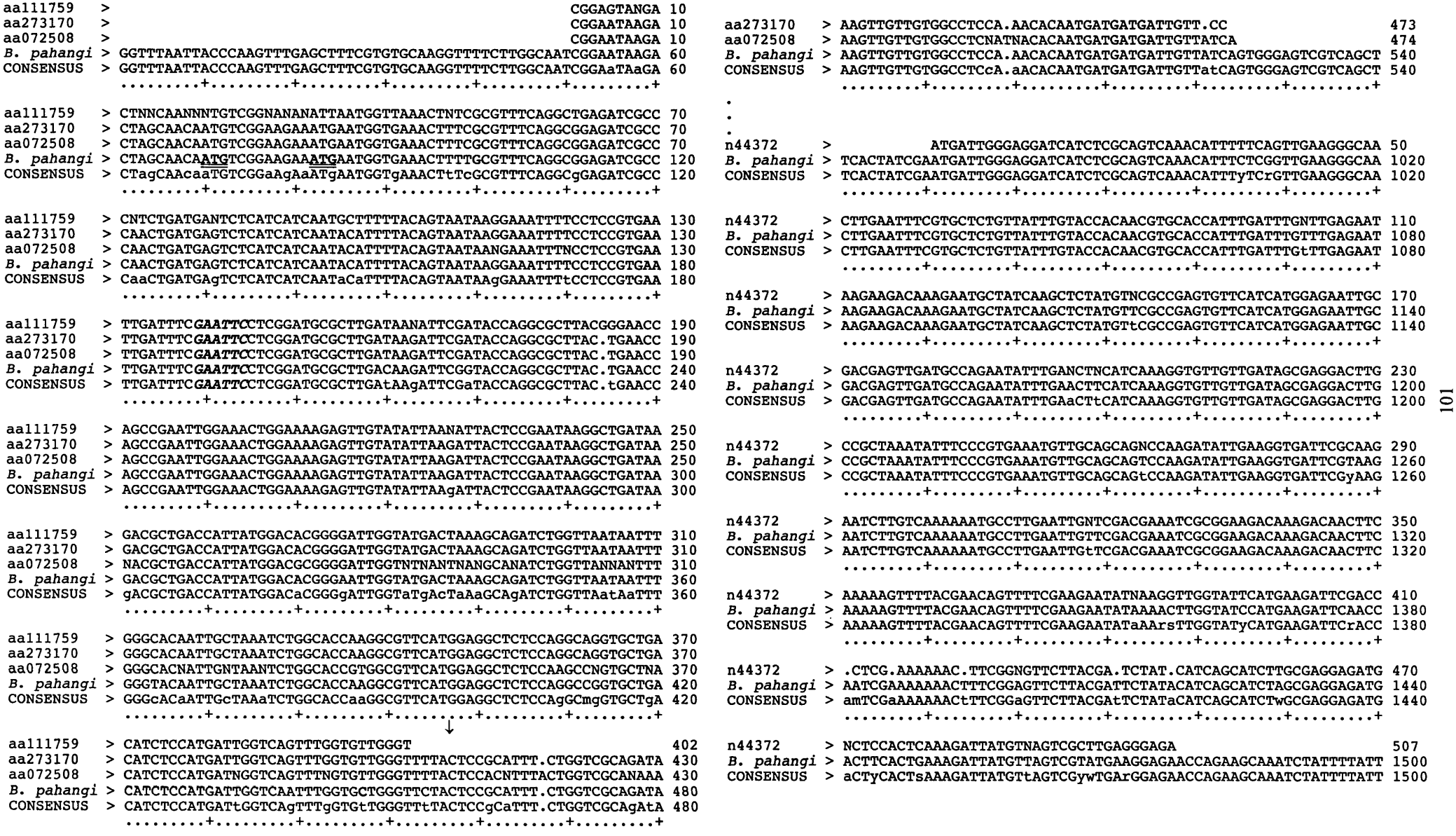
Figure 3.8 : Complete cDNA sequence of the *B. pahangi hsp90* homologue

```
   1  GGTTTAATTA CCCAAGTTTG AGCTTTCGTG TGCAAGGTTT TCTTGGCAAT
  51  CGGAATAAGA CTAGCAACAA TGTCGGAAGA AATGAATGGT GAAACTTTTG
 101  CGTTTCAGGC GGAGATCGCC CAACTGATGA GTCTCATCAT CAATACATTT
 151  TACAGTAATA AGGAAATTTT CCTCCGTGAA TTGATTTCGA ATTCCTCGGA
 201  TGCGCTTGAC AAGATTCGGT ACCAGGCGCT TACTGAACCA GCCGAATTGG
 251  AAACTGGAAA AGAGTTGTAT ATTAAGATTA CTCCGAATAA GGCTGATAAG
 301  ACGCTGACCA TTATGGACAC GGGAATTGGT ATGACTAAAG CAGATCTGGT
 351  TAATAATTTG GGTACAATTG CTAAATCTGG CACCAAGGCG TTCATGGAGG
 401  CTCTCCAGGC CGGTGCTGAC ATCTCCATGA TTGGTCAATT TGGTGCTGGG
 451  TTCTACTCCG CATTTCTGGT CGCAGATAAA GTTGTTGTGG CCTCCAAACA
 501  CAATGATGAT GATTGTTATC AGTGGGAGTC GTCAGCTGGA GGCTCATTCA
 551  TTATTCGACA GGTGAATGAT CCAGAGCTTA CACGTGGCAC CAAAATTACC
 601  CTGTACATCA AGGAGGATCA GACTGACTAT CTTGAAGAGC GTCGCATCAA
 651  GGAGATTGTG AAGAAGCACT CACAGTTTAT CGGGTATCCG ATTAAACTTA
 701  CTGTAGAGAA AGAGCGTGAT AAAGAAGTTT CTGATGATGA AGCGGAAGAA
 751  GAAAAGAAGG ACGAAGATAA GGAAAAGAAG GAAGGTGAGA TTGAGGATGT
 801  TGGAGAAGAT GAAGAAGAAG ATAAGAAGGA TAAAGACAAG AAGAAGAAAA
 851  AGATCAAGGA GAAGTACCAT GAAGATGAAG AACTGAACAA GACAAAGCCC
 901  ATTTGGACAC GTAATCCTGA TGATATAAGC AATGAAGAAT ATGCTGAATT
 951  CTACAAGTCA CTATCGAATG ATTGGGAGGA TCATCTCGCA GTCAAACATT
1001  TCTCGGTTGA AGGGCAACTT GAATTTCGTG CTCTGTTATT TGTACCACAA
1051  CGTGCACCAT TTGATTTGTT TGAGAATAAG AAGACAAAGA ATGCTATCAA
1101  GCTCTATGTT CGCCGAGTGT TCATCATGGA GAATTGCGAC GAGTTGATGC
1151  CAGAATATTT GAACTTCATC AAAGGTGTTG TTGATAGCGA GGACTTGCCG
1201  CTAAATATTT CCCGTGAAAT GTTGCAGCAG TCCAAGATAT TGAAGGTGAT
1251  TCGTAAGAAT CTTGTCAAAA AATGCCTTGA ATTGTTCGAC GAAATCGCGG
1301  AAGACAAAGA CAACTTCAAA AAGTTTTACG AACAGTTTTC GAAGAATATA
1351  AAACTTGGTA TCCATGAAGA TTCAACCAAT CGAAAAAAAC TTTCGGAGTT
1401  CTTACGATTC TATACATCAG CATCTAGCGA GGAGATGACT TCACTGAAAG
1451  ATTATGTTAG TCGTATGAAG GAGAACCAGA AGCAAATCTA TTTTATTACT
1501  GGTGAATCCA GGGAAGCTGT CGCCAGTTCT GCATTTGTGG AGCGTGTCAA
1551  GAGACGCGGT TTCGAGGTTA TTTACATGAC TGATCCGATA GACGAGTATT
1601  GTGTGCAACA GCTGAAAGAA TATGATGGCA AAAAGCTGGT CTCAGTAACT
1651  AAGGAAGGTC TTGAACTGCC TGAAAGTGAG GAGGAGAAGA AGAAATTTGA
1701  GGAAGACAAA GTTAAATTTG AGAATTTGTG CAAGGTCATG AAGGACATTT
1751  TGGAGAAGAA GGTTGAGAAA GTTGCTGTAT CAAATCGATT GGTCTCATCT
1801  CCTTGTTGCA TTGTAACATC TGAATATGGA TGGTCTGCCA ATATGGAGCG
1851  AATTATGAAA GCGCAGGCAC TGCGGGATTC TTCTACAATG GGATATATGG
1901  CTGCCAAAAA ACATCTCGAA ATCAACCCTG ACCATTCTGT TATCAAAGCG
1951  CTGCGAGAGC GTGTCGAGGC AGACAAAAAC GATAAGACTG TGAAAGATTT
2001  AGTGGTTTTG CTCTTTGAAA CTGCCTTGCT TTCTTCTGGT TTTTCGCTTG
2051  AAGATCCGCA GTTGCATGCA TCAAGGATAT ACCGCATGAT TAAGCTTGGG
2101  CTTGATATTA CGGAGGATGA GGAAGAAGAA GCAATTGCAT CCGTTTCTGG
2151  TGAGAAAGAC GAATGTGTGC CAAACTTAGT TGGTGCCGAG GAAGATGCAT
2201  CGAGGATGGA AGAAGTTGAT TAATTCTCTC ATTAAATTTT AATAATGTTT
2251  CAATTCAATA TGAGTTAAAT TGTAGTAAAA TTAAGTGGAA ATTGCTCAAG
2301  CAGTGTTTCC GTGATGATTT TTCATTTGAC GTCGAACTTG TGCGTTTTTG
2351  TTGATCTCGT GTTGAGTTGT TCAATCTTTA TAATGAGGAA GCAAAATTGT
2401  TAAAAGTGCA TCAATATGCC ACGCGTTTTT TAATGTTTTT TTTCTTTTTG
2451  AATGTGTTTT GTGTTATTTC TATGTTTCTG TACTATCAAT AAAATTTTCT
2501  TTGAAGNGTT AAAAAAAAAA AAAAAAA
```

Figure 3.9 :

The ESTs with accession numbers aa111759, aa273170, aa072508 are from a *B. malayi* L_3 cDNA library and n44372 is from a *B. malayi* adult cDNA library. Both *B. malayi* libraries were constructed using PCR amplification with SL1 and oligodT. The two possible start codons in the *B. pahangi* sequence (in bold and underlined) are also present in the *B. malayi* sequences. An *Eco*RI site near the start of the open reading frame is in bold italics. The *Eco*RI site shown was utilized in the sub-cloning of the *B. pahangi* product hsp90SL1-r3 and is conserved in the *B. malayi* EST sequences. The nucleotide (at position 447 of the *B. pahangi* sequence) in bold, and indicated by an arrow, is a possible PCR error as discussed later, since it results in an amino acid difference between the *B. pahangi* and *B. malayi* sequences.

Figure 3.9 : Alignment of the *B. malayi* ESTs with homology to *B. pahangi hsp90*



3.2.12 Translation of the *B. pahangi* cDNA sequence

The cDNA consensus sequence was translated with the GCG program "translate". The theoretical mass of the protein (unmodified) is 83 kD which is consistent with the observation that cytoplasmic *hsp90* genes range in size from 80 kD to 90 kD. ***Figure 3.10*** shows an alignment or "pileup" of HSP90 from different species. This "pileup" was used in the construction of a cladogram and includes the HtpG sequence from *E. coli* and from a range of eukaryotes.

There is a high degree of homology between all HSP90s as is evident from the alignment. However the N-terminal region of the protein has much higher homology between proteins of different species than the C-terminal region which appears to have greater diversity [Johnson *et al* 1989]. The N and C-terminal regions are separated by a domain with a high concentration of charged residues (residues 230-320 on the "pileup"), which varies in length between species; for example this region is 85 amino acids long in *P. falciparum* but is absent in *E. coli* [Bardwell and Craig 1987]. The charged domain is hypothesized to adopt a conformation, which mimics double stranded DNA [Gupta 1995]. This conformation is thought to facilitate the interaction of certain DNA-binding proteins with HSP90 [Binart *et al* 1989].

There are two highly immunogenic regions high-lighted in the alignment (Regions I and II). Region I includes the aforementioned highly charged domain and is thought to be exposed at the surface of the protein [Nemato *et al* 1997] thus it may be a target for an antibody-mediated response to the protein. The C-terminus is required for the association of HSP90 to steroid hormone receptors such as the progesterone receptor [Sullivan and Toft 1993]. The final 200 residues of this region are necessary for the formation of an HSP90 homodimer and are thought to contain a leucine zipper [Nemato *et al* 1995].

The two putative ATP-binding sites are hypothetical, due to their homology with a domain known to bind ATP in *E. coli* gyrase [Bergerat *et al* 1997]. A domain, which includes the putative ATP-binding sites, is thought to regulate the conformation of HSP90 thus regulating the association of HSP90 with other proteins [Grenert *et al* 1997].

Figure 3.10 : Alignment of HSP90 sequences

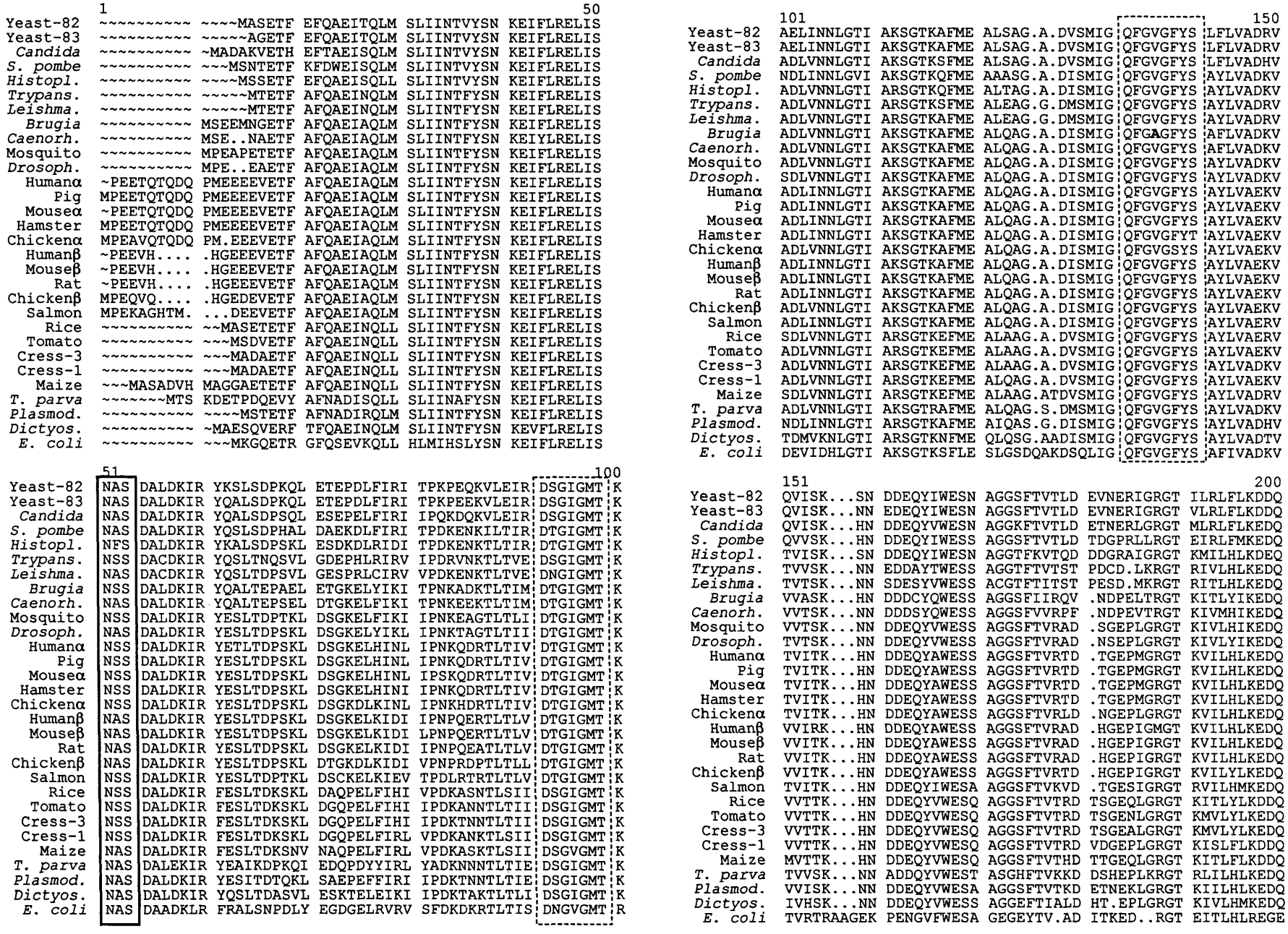


Figure 3.10 : Alignment of HSP90 sequences

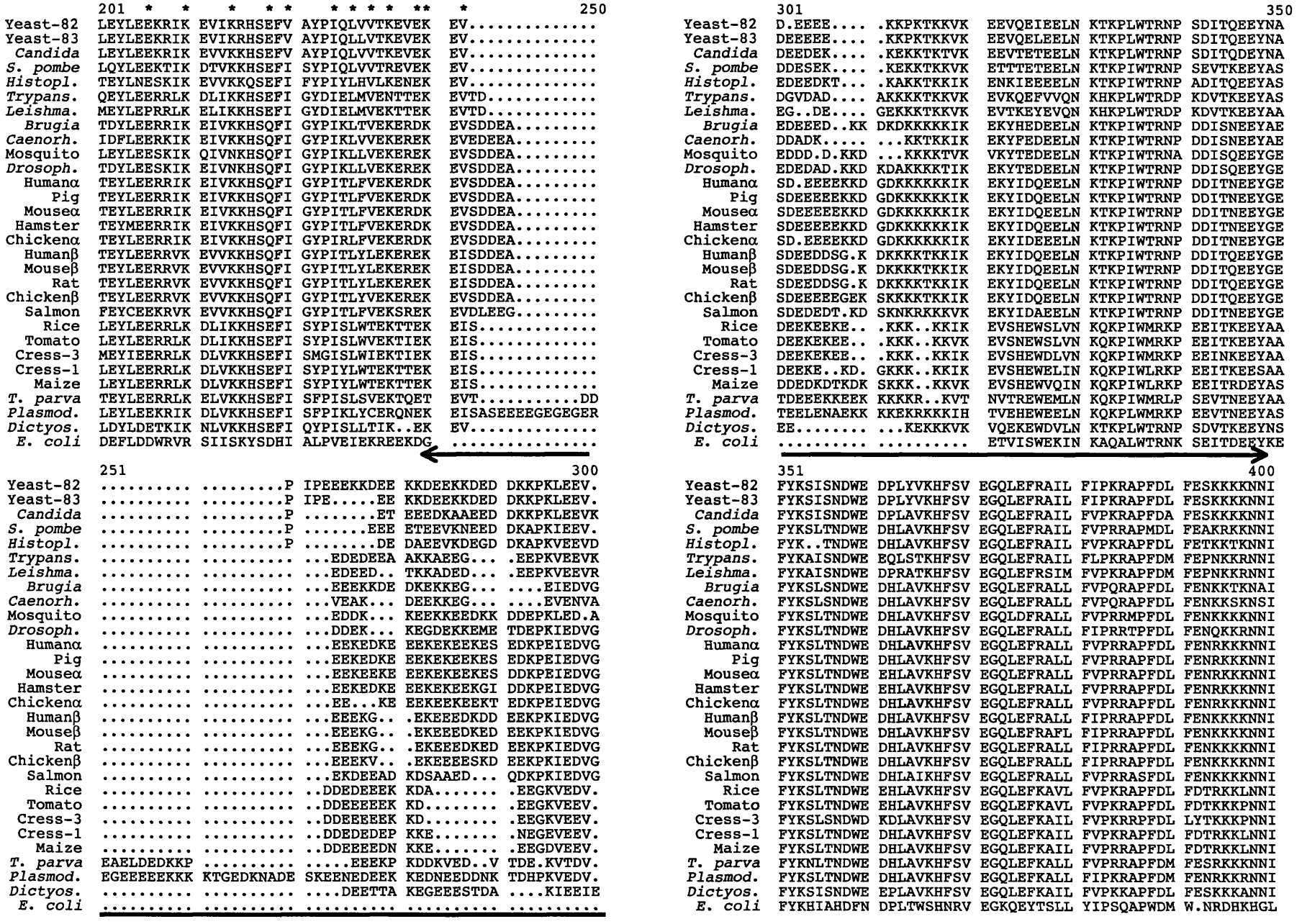


Figure 3.10 : Alignment of HSP90 sequences

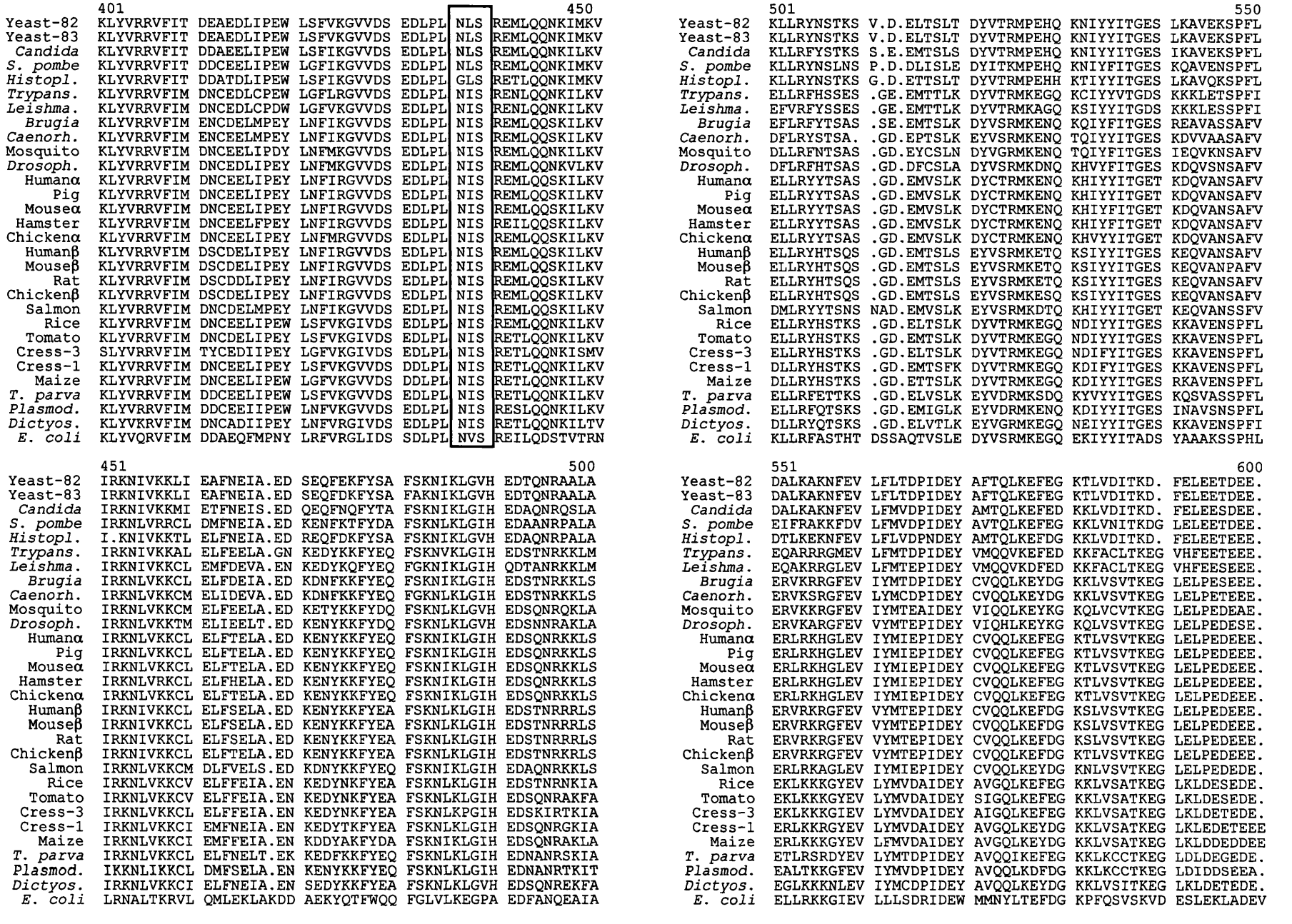


Figure 3.10 : Alignment of HSP90 sequences

```
                601                                                650
Yeast-82    .KAEREKEIK EYEPLTKALK EILGDQVEKV VVSYKLLDAP AAIRTGQFGW
Yeast-83    .KAEREKEIK EYEPLTKALK DILGDQVEKV VVSYKLLDAP AAIRTGQFGW
 Candida    .KAAREKEIK EYEPLTKALK DILGDQVEKV VVSYKLVDAP AAIRTGQFGW
S. pombe    .KAAREKLEK EYEEFAKQLK TILGDKVEKV VVSNKIVGSP CLLTTGQYGW
Histopl.    .KKDREAEEK EYEGLAKSLE NILGDKVEKV VVSHKLIGSP CAIRTGQFGW
Trypans.    .KKQREEEKA SYERLCKAMK EVLGDKVEKV VVSDRLATSP CILVTSEFGW
Leishma.    .KQQREEEKA ACEKLCKTMK EVLGDKVEKV IVSECLSTSP CILVTSEFGW
  Brugia    .KKKFEEDKV KFENLCKVMK DILEKKVEKV AVSNRLVSSP CCIVTSEYGW
Caenorh.    .KKKFEEDKV AYENLCKVIK DILEKKVEKV GVSNRLVSSP CCIVTSEYGW
Mosquito    .KKKREEDKA KFENLCKVMK SVLESKVEKV VVSNRLVDSP CCIVTSQYGW
Drosoph.    .KKKREEDKA KFESLCKLMK SILDNKVEKV VVSNRLVDSP CCIVTSQFGW
  Humanα    .KKKQEEKKT KFENLCKIMK DILEKKVEKV VVSNRLVTSP CCIVTSTYGW
    Pig     .KKKQEEKKT KFENLCKIMK DILEKKVEKV VVSNRLVTSP CCIVTSTYGW
  Mouseα    .KKKQEEKKT KFENLCKIMK DILEKKVEKV VVSNRLVTSP CCIVTSTYGW
 Hamster    .KKKQEEKKT KFENLCKIMK DILEKKVEKV VVSNRLVTSP CCIVTSTYGW
Chickenα    .KKKQEEKKA KFENLCKIMK DILEKKVEKV VVSNRLVTSP CCIVTSTYGW
  Humanβ    .KKKMEESKA KFENLCKLMK EILDKKVEKV TISNRLVSSP CCIVTSTYGW
  Mouseβ    .KKKMEESKA KFENLCKLMK EILDKKVEKV TISNRLVSSP CCIVTSTYGW
    Rat     .KKKMEESKA RFENLCKLMK EILDKKVEKV TISNRLVSSP CCIVTSTYGW
Chickenβ    .KKNMEESKA KFETLCKLMK EILDKKVEKV TISNRLVSSP CCIVTSTYGW
  Salmon    .KKKQEELNT KFENLCKTMK DILDKKIEKV SVSNRLVSSP CCIVTSTYGW
    Rice    .KKRKEELKE KFEGLCKVIK EVLGDKVEKV VVSDRVVDSP CCLVTGEYGW
  Tomato    .KKKQEELKE KFEGLCKVMK DVLGDKVEKV IVSDRVVDSP CCLVTGEYGW
 Cress-3    .KKKKEELNE KFEGLCKVIK DVLGDKVEKV IVSDRVLDSP CCLVTGEYGW
 Cress-1    .KKKREEKKK SFENLCKTIK EILGDKVEKV VVSDRIVDSP CCLVTGEYGW
   Maize    AKKRREERKK RFEELCKVIK DILGDRVEKV VVSDRIVDSP CCLVTGEYGW
T. parva    .KKSFEALKE EMEPLCKHIK EVLHDKVEKV VCGTRFTDSP CALVTSEFGW
Plasmod.    .KKDFETLKA EYEGLCKVIK DVLHEKVEKV VVGQRITDSP CVLVTSEFGW
Dictyos.    .KKKAEQDKA ANEELLKQVK DVLGDKVEKV VLSTRLANSP CVLVTSEYGW
 E. coli    DESAKEAEKA .LTPFIDRVK ALLGERVKDV RLTHRLTDTP AIVSTDADEM

                651                                                700
Yeast-82    SANMERIMKA QALRDSSMSS YMSSKKTFEI SPKSPIIKEL KKRVDEGGAQ
Yeast-83    SANMERIMKA QALRDSSMSS YMSSKKTFEI SPKSPIIKEL KKRVDEGGAQ
  p46598    SANMERIMKA QALRDTTMSS YMSSKKTFEI SPSSPIIKEL KKKVETDGAE
S. pombe    SANMERIMKL KPSRDTSMSA YMSSRKTFEI NPKSPIIAEL KKKVEENGAE
Histopl.    SANMERIMKA QALRDTSMSS RRSSRPT... .......... .......EGN
Trypans.    SAHMEQIMRN QALRDSSMSA YMMRKKTMEI NTTHAIVKEL KRRVEAD.EN
Leishma.    SAHMEQIMRN QALRDSSMAQ YMMSKKTMEL NPRHPIIKEL RRRVDAD.EN
  Brugia    SANMERIMKA QALRDSSTMG YMAAKKHLEI NPDHSVIKAL RERVEAD.KN
Caenorh.    SANMERIMKA QALRDSSTMG YMAAKKHLEI NPDHAIMKTL RDRVEVD.KN
Mosquito    SANMERIMKA QALRDSSAMG YMAGKKHLEI NPDHAIIETL RQRAEAD.KN
Drosoph.    SANMERIMKA QALRDTATMG YMAGKKQLEI NPDHPIVETL RQKADAD.KN
  Humanα    TANMERIMKA QALRDNSTMG YMAAKKHLEI NPDHSIIETL RQKAEAD.KN
    Pig     TANMERIMKA QALRDNSTMG YMAAKKHLEI NPDHSIIETL RQKAEAD.KN
  Mouseα    TANMERIMKA QALRDNSTMG YMAAKKHLEI NPDHSIIETL RQKAEAD.KN
 Hamster    TANMERIIKA QALRDNSTMG YMAAKKHLEI NPDHSIIETL RQKAEAD.KN
Chickenα    TANMERIMKA QALRDNSTMG YMAAKKHLEI NPDHSIIETL RQKAEAD.KN
  Humanβ    TANMERIMKA QALRDNSTMG YMMAKKHLEI NPDHPIVETL RQKAEAD.KN
  Mouseβ    TANMERIMKA QALRDNSTMG YMMAKKHLEI NPDHPIVETL RQKAEAD.KN
    Rat     TANMERIMKA QALRDNSTMG YMMAKKHLEI NPDHPIVETL RQKAEAD.KN
Chickenβ    TANMERIMKA QALRDNSTMG YMMAKKHLEI NPDHPIVETL RQKADAN.KN
  Salmon    TANMERIMKS QALRDNSTMG YMTAKKHLEI NPTHPIVETL REKAEAD.KN
    Rice    TANMERIMKA QALRDSSMAG YMSSKKTMEI NPENAIMEEL RKRADAD.KN
  Tomato    TANMERIMKA QALRDSSMAG YMSSKKTMEI NPENSIMDEL RKRADAD.KN
 Cress-3    TANMERIMKA QALRDSSMGG YMSSKKTMEI NPENLIMDEL RKRADAD.KN
 Cress-1    TANMERIMKA QALRDSSMSG YMSSKKTMEI NPDNGIMEEL RKRAEAD.KN
   Maize    TANMERIMKA QALRDSSMSA YMSSKKTMEI NPDNGIMEEL RKRAEAD.RN
T. parva    SANMERIMKA QALRDSSITS YMLSKKIMEI NPRHSIMKEL KTRAAND.KT
Plasmod.    SANMERITKA QALRDNSMTS YMLSKKIMEI NARHPIISAL KQKADAD.KS
Dictyos.    SANMERIMKA QALRDSSMSS YMSSKKTLEL NPDHPIVRDL AKKA.AE.KS
 E. coli    STQMAKLFAA AGQKVPEV.. ....KYIFEL NPDHVLV... ..KRAADTED

                701                                                750
Yeast-82    DKTVKDLTKL LYETALLTSG FSLDEPTSFA SRINRLISLG LNIDED.EET
Yeast-83    DKTVKDLTNL LFETALLTSG FSLEEPTSFA SRINRLISLG LNIDED.EET
 Candida    DKTVKDLTTL LFDTALLTSG FTLDEPSNFA HRINRLIALG LNIDDDSEET
S. pombe    DRSVKDLATI LYETALLSSG FTLHDPSAYA QRINRLISLG LSIDE..EEE
Histopl.    DRTVKSITQL LFETSLLVSG FTIEEPSGFA GRIHKLVSLG LNIDEDAE..
Trypans.    DKAAKDLIFL LFDTSLLTSG FTLDDPTAYA DRIHRMIKLG LSLDDDAEEE
Leishma.    DKAVKDLVFL LFDTSLLTSG FQLEDPT.YA ERINRMIKLG LSLDEEEEVV
  Brugia    DKTVKDLVVL LFETALLSSG FSLEDPQLHA SRIYRMIKLG LDITEDE...
Caenorh.    DKTVKDLVVL LFETALLASG FSLEEPQSHA SRIYRMIKLG LDIGDDE...
Mosquito    DKAVKDLVIL LFETALLSSG FSLDEPGTHA ARIYRMVKLG LGIDDDEPMT
Drosoph.    DKAVKDLVIL LFETSLLSSG FSLDSPQVHA SRIYRMIKLG LGIDEDEPMT
  Humanα    DKSVKDLVIL LYETALLSSG FSLEDPQTHA NRIYRMIKLG LGIDEDDP.T
    Pig     DKSVKDLVIL LYETALLSSG FSLEDPQTHA NRIYRMIKLG LGIDEDDP.T
  Mouseα    DKSVKDLVIL LYETALLSSG FSLEDPQTHA NRIYRMIKLG LGIDEDDP.T
 Hamster    DKSVKDLVIL LYETALLSSG FSLEDPQTHA NRIYRMIKLG LGIDEDDP.T
Chickenα    DKSVKDLVIL LYETALLSSG FSLEDPQTHA NRIYRMIKLG LGIDEDDT.A
  Humanβ    DKAVKDLVVL LFETALLSSG FSLEDPQTHS NRIYRMIKLG LGIDEDEV.A
  Mouseβ    DKAVKDLVVL LFETALLSSG FSLEDPQTHS NRIYRMIKLG LGIDEDEV.T
    Rat     DKAVKDLVVL LFETALSSLA SHFRRPKTHS NRIYRMIKLG LGIDEDEV.T
Chickenβ    DKAVKDLVVL LFETALLSSG FSLEDPQTHS NRIYRMIKLG LGIDEDEV.I
  Salmon    DKAVKDLVIL LFETALLSSG FTLDDPQTHA NRIYRMIKLG LGIDGDDS.A
    Rice    DKSVKDLVLL LFETALLTSG FSLDDPNTFG SRIHRMLKLG LSIDEDETAE
  Tomato    DKSVKDLVLL LFETALLTSG FSLEEPNTFG NRIHRMLKLG LSIDE.ESGD
 Cress-3    DKSVKDLVLL LFETALLTSG FSLDEPNTFG SRIHRMLKLG LSIEEDDAVE
 Cress-1    DKSVKDLVML LYETALLTSG FSLDEPNTFA ARIHRMLKLG LSIDEDENVE
   Maize    DKSVKDLVLL LFETALLTSG FSLDDPNTFA ARIHRMLKLG LNIDEDAAAD
T. parva    DKTVKDLVWL LYDTALLTSG FNLDEPTQFG NRIYRMIKLG LSLDDEEHVE
Plasmod.    DKTVKDLIWL LFDTSLLTSG FALEEPTTFS KRIHRMIKLG LSIDEEEN..
Dictyos.    .KTFKDFVYL LYETALLTSG FSLDEPSSFA SRIHRMIKLG LSIQDDSSST
 E. coli    EAKFSEWVEL LLDQALLAER GTLEDPNLFI RRMNQLLVS~ ~~~~~~~~~~

                751                                                786
Yeast-82    ETAPEASTAA PV........ ...EEVPADT E MEEVD
Yeast-83    ETAPEASTEA PV........ ...EEVPADT E MEEVD
 Candida    AVEPEATTTA ST........ ...DEPAGES A MEEVD
S. pombe    APIEEISTES VA........ ...AENNAES K MEEVD
Histopl.    .TSEEKEADT VV........ ...AEAPADS D MEEVD
Trypans.    EAQAPVA..A AANSS..... .......TGAS G MEEVD
Leishma.    AAEATVAETA PAEVT..... .......AGTS S MEQVD
  Brugia    .EEEAIASVS GEKDECVPNL VGAEEDA..S R MEEVD
Caenorh.    .IEDSAVPSS CTAE...AKI EGAEEDA..S R MEEVD
Mosquito    TDEVSGAGAP TTAAGDAPPL VDDSEDL..S H MEEVD
Drosoph.    TDDAQ..... ..SAGDAPSL VEDTEDA..S H MEEVD
  Humanα    ADDTSAAVTE .....EMPPL EGD.DDT..S R MEEVD
    Pig     ADDSSAAVTE .....EMPPL EGD.DDT..S R MEEVD
  Mouseα    VDDTSAAVTE .....EMPPL EGD.DDT..S R MEEVD
 Hamster    VDDTSAAVTE .....EMPPL EGD.DDT..S R MEEVD
Chickenα    AEEASPAVTE .....EMPPL EGD.DDT..S R MEEVD
  Humanβ    AEEPNAAVPD .....EIPPL EGD.EDA..S R MEEVD
  Mouseβ    AEEPSAAVPD .....EIPPL EGD.EDA..S R MEEVD
    Rat     AEEPSAAVPD .....EIPPL EGD.EDA..S R MEEVD
Chickenβ    AEESSIAPPD .....EIPPL EGD.EDT..S R MEEVD
  Salmon    VEEILQPSED .....DMPVL EGD.DDT..S R MEEVD
    Rice    AD........ ....TDMPPL ED...DAGES K MEEVD
  Tomato    AD........ ....ADMPAL EDPEADAEGS K MEEVD
 Cress-3    AD........ ....AEMPPL ED.DADAEGS K MEEVD
 Cress-1    ED........ ....GDMPEL EEDA..AEES K MEEVD
   Maize    ED........ ....ADMPAL DEGA..AEES K MEEVD
T. parva    ED........ ....SSMPPL DEP.V..VDS K MEEVD
Plasmod.    ND........ ....IDLPPL EET.VDATDS K MEEVD
Dictyos.    TEES....TN TTTSDDIPPL EENDE...PS E MEKVD
 E. coli    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~ ~~~~~~
```

Figure 3.10 :

Species names have been abbreviated and in some cases common names have been used, e.g. *Leishma.* is *Leishmania*, *Caenorh.* is *Caenorhabditis elegans*, Cress is *Arabidopsis thaliana*, Mosquito is *Anopheles albimanus*. A full list of the species plus accession numbers is included with ***Appendix I*** since the above alignment was utilized in the construction of a cladogram. The residue at position 138 in all the aligned HSP90 sequences is valine, but the *B. pahangi* cDNA codes for alanine at that position. This may represent a PCR error during the amplification of *B. pahangi hsp90* since *B. malayi* ESTs of *hsp90* have a single base difference at this position when compared with the *B. pahangi* sequence, resulting in a valine codon.

Two HSP90 sequences were used for *A. thaliana*, *S. cerevisiae* and some vertebrate species. The vertebrate proteins are denoted α and β, *S. cerevisiae* as 82 and 83 (HSP82 and HSC82) and *A. thaliana* as 1 and 3 (HSP81-1 and HSP81-3).

- highly conserved residues which form a putative peptide clamp are indicated by an asterisk above the residues.
- the charged domain of HSP90 (residues 235-320) is shown between two solid lines and has a high concentration of acidic and basic residues.
- two motifs in dashed boxes (93-99 and 135-142) are putative ATP-binding domains.
- two regions which confer most of the immunogenicity of HSP90 are underlined and denoted with arrows.
    - these regions are at positions 233-349 for region I and at positions 747-767 for region II.
- the residues boxed with a narrow solid boundary (51-53 and 436-438) are putative N-glycosylation sites and have the consensus sequence N-X-T/S.
- the serine/threonine residue at position 237 in many of the HSP90 sequences, may be a target for phosphorylation.
- the C-terminal five residues (MEEVD) are also boxed with a solid boundary and with two exceptions show complete sequence conservation.

3.2.13 Cladogram using HSP90 as a common feature

As seen in the alignment, the complete amino acid sequences from 30 HSP90 homologues were obtained from the Swissprot and Owl databases, or from the translation of complete *hsp90*s in the databank. These represented various species but also different copies of *hsp90* from the same organism. The alignment of these sequences was used to generate a distance matrix (Kimura protein distance), (see Appendix I), from which a cladogram was constructed from the protein distances (***Figure 3.11***). The HSP90 homologue from *E. coli* (HtpG) was used as the outgroup to root the tree since all the other sequences are from eukaryotes and have a much lower homology to HtpG than to each other. Indeed the *E. coli* sequence appears to be truncated in comparison to other HSP90 sequences and does not possess the conserved five terminal amino acids, MEEVD, which are the hallmark of the cytoplasmic HSP90 family [Bardwell and Craig 1987].

As would be expected, the vertebrates group together (**d**), but the HSP90α and HSP90β sequences from different species show greater homology to each other than do HSP90α or HSP90β from one species [Gupta 1995]. The difference is particularly pronounced in chicken where HSP90β shows no (or very little) increase in response to heat shock in contrast to HSP90α which rapidly accumulates when stimulated by the same stress [Meng *et al* 1993]. This may be explained by the duplication of an ancestral *hsp90* which occurred before the emergence of the vertebrates and resulted in two genes with divergent code [Gupta 1995].

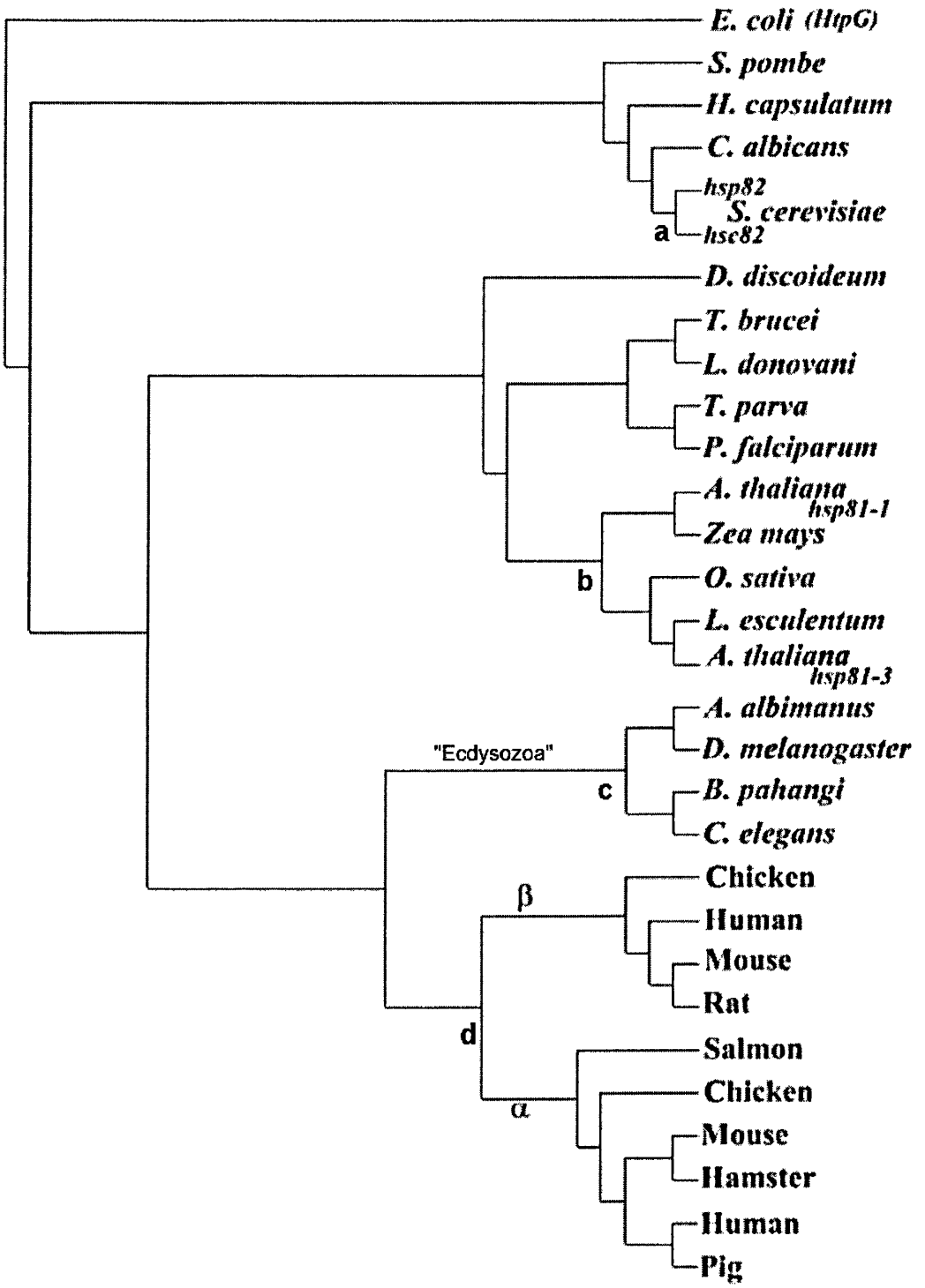
Plant HSP90s group together (**b**) but the two proteins from *Arabidopsis*, HSP81-1 and HSP81-3 are in different clades. HSP81-1 has higher homology with maize HSP82 than with HSP81-3 and HSP81-3 has greater homology with tomato (*Lycopersicon esculentum*) HSC80 than to HSP81-1. This is not altogether surprising since both HSP81-1 and maize HSP82 are mainly responsive to heat shock [Yabe *et al* 1994], but HSP81-3 and tomato HSC80 are regulated in a tissue specific manner [Marrs *et al* 1993]. This may be another example of the duplication of an *hsp90*, similar to the previously described gene duplication and may have occurred before or at the emergence of the Angiospermae to create more than one HSP90.

Figure 3.11 :

A cladogram was constructed using HSP90 sequences from a variety of species. Different HSP90 homologues from *Saccharomyces cerevisiae* and certain plants and vertebrates were also utilized. The clade containing the two HSP90 homologues from yeast is indicated by **(a)**, the clade containing plant HSP90s is indicated by **(b)**, the clade containing the "ecdysozoa" HSP90s is indicated by **(c)** and the clade containing the vertebrates HSP90s is indicated by **(d)**.

The term "ecdysozoa" is used for the clade containing the mosquito, *Anopheles albimanus*, the fruit fly, *Drosophila melanogaster*, and the nematodes *Brugia pahangi* and *Caenorhabditis elegans* and refers to their collective characteristic of moulting [Aguilaldo *et al* 1997]. The symbols α and β denote the two forms of HSP90 present in vertebrates and show that HSP90αs (and HSP90βs) from different species are more similar to each other than to HSP90α and HSP90β from the same species.

Figure 3.11 : Cladogram using HSP90 as a common feature



Finally, the two proteins of yeast (**a**), HSC82 and HSP82 also appear to have arisen independently from a duplication event [Gupta 1995]. The heat shock cognate gene product, HSC82, is constitutively expressed and has higher homology with the second yeast *hsp90* gene product, HSP82, which is highly heat shock inducible, than with heat shock cognate or heat shock inducible HSP90s from other species.

The cladogram also reveals that the Nematoda and the Arthropoda group together (**c**) which may merely reflect the extensive differences between these groups and other members of the tree. However, it is interesting to note that a clade which includes nematodes and arthropods has been proposed [Aguinaldo *et al* 1997] and that the analysis utilized rDNA sequence data and not protein information. The clade, Ecdysozoa, is so called since it contains moulting animals.

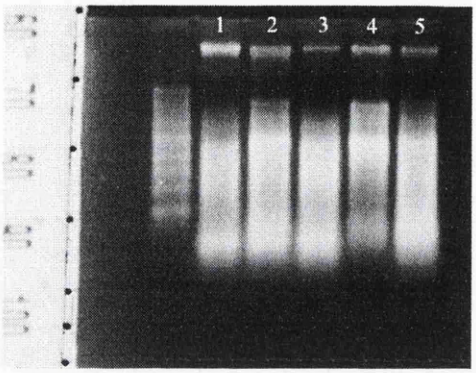
3.2.14 Northern analysis of *hsp90*

The isolation of an *hsp90* homologue from cDNA implied active transcription of this gene in the parasite. Northern analysis was used to confirm the presence of transcripts for *hsp90* in RNA extracted from *B. pahangi* (mf and adults) and to obtain the size of the mature transcript. This experiment was carried out on a number of occasions with similar results. In the experiment shown in ***Figure 3.12***, total RNA was extracted from adults cultured at 37°C and 41°C and mf cultured at either 28°C, 37°C or 41°C for two hours. A northern blot of this material was probed with hsp90f2-r2. ***Figure 3.12*** **A** is a photograph of an ethidium bromide stained gel to demonstrate the approximately equal quantities of RNA loaded. ***Figure 3.12*** **B** shows the autoradiograph, exposed for 4½ hours.

This experiment (***Figure 3.12B*** and ***Figure 3.12C***) shows that *hsp90* transcripts are barely detectable in RNA from adult parasites cultured at 37°C (lane 1), in contrast to the high level of transcripts detected in RNA from mf at 37°C (lane 2). The heat inducibility of the *hsp90* transcripts at 41°C is clearly observed in the adults (compare lanes 1 and 3) but there is little difference in *hsp90* transcripts between mf at 41°C and mf at 37°C (lanes 2 and 4). *Hsp90* mRNA was also barely detectable in mf cultured at 28°C (the optimal temperature for development in the mosquito), even in ***Figure 3.12C***, where the film was exposed for 24 hours.

Figure 3.12 : Northern blot analysis of *hsp90* mRNA in mf and adult worms

A

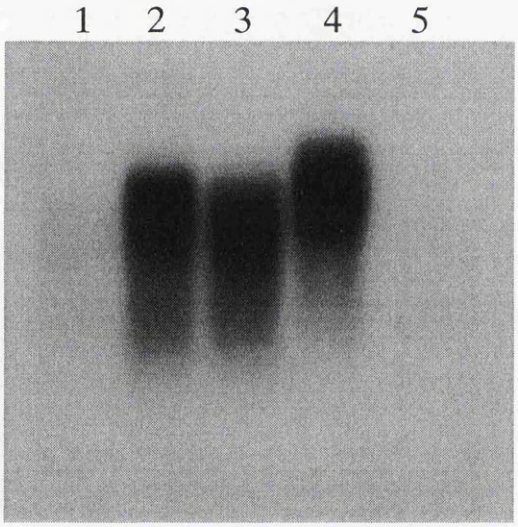


Approximately 2μg of total RNA and 1μg of RNA markers were loaded onto a 1.2% formaldehyde gel and stained with ethidium bromide as shown in **A**.

RNA was extracted from mf and adults that had been incubated at the following temperatures for 2 hours:

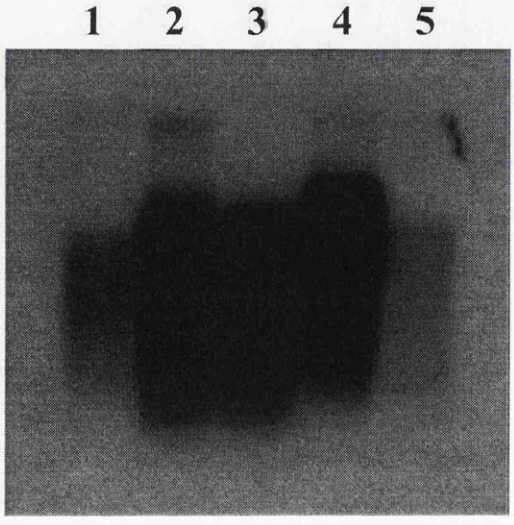
1	Adults at 37°C
2	Mf at 37°C
3	Adults at 41°C
4	Mf at 41°C
5	Mf at 28°C

B



The mf and adult RNA samples were probed with an *hsp90* fragment, hsp90f2-r2 and washed at 65°C using 1 x SSC, 0.1% SDS. The autoradiograph was exposed for 4½ hours (see **B**) and for 24 hours (see **C**)

C



Examination of the ethidium bromide stained gel in ***Figure 3.12A*** suggests that the RNA from mf cultured at 37°C and 41°C (lanes 2 and 4) has a higher proportion of larger transcripts than the corresponding sample from adults cultured at the same temperatures (lanes 1 and 3). On the autoradiograph shown in ***Figures 3.12B*** the transcripts do not resolve into sharp bands, but there does appear to be a difference in the size of hybridizing bands in mf at 37°C compared to adults at 37°C. In addition, the transcript size appears to be increased further between mf cultured at 37°C and at 41°C (compare lanes 2 and 4). The approximate size of the *hsp90* transcript (2.6kb) was determined from a separate northern blot (not shown), by comparison with a band of previously calculated size, and is consistent with the size predicted from the cDNA sequence.

3.13 Discussion

HSP90 appears to have an essential and conserved function in eukaryotes (as presented in the Introduction). This is emphasized by the high degree of sequence homology of this gene between diverse species. The approach used in this study to clone an *hsp90* homologue from *B. pahangi* was based on PCR using primers initially designed from ESTs for *hsp90* from the nematode *C. elegans*. A genomic fragment of *B. pahangi hsp90* was initially obtained and subsequent attempts were made to isolate a cDNA product using the same primers on reverse transcribed RNA from different life cycle stages. Surprisingly, a cDNA fragment could only be obtained from p.i. L_3, despite later evidence from northern analysis that the mRNA is abundantly transcribed in the mf life cycle stage (at 37°C). Attempts to obtain a cDNA fragment from mf cultured at 37°C or 41°C and from adults cultured at 41°C were not successful. The reason for the efficient PCR of an *hsp90* cDNA fragment from p.i. L_3 may have had little to do with the parasite stage used and more to do with the quality of the template.

As the L_3 experiences a heat shock as part of its life cycle during transition from the mosquito to the mammalian host, the up-regulation of HSPs during this process may constitute a true heat shock response. In a previous study in which the pattern of ^{35}S-labelled proteins was compared in L_3 cultured at 28°C (vector temperature) or 37°C (mammalian temperature), a protein of approximately 83kD was observed to be up-regulated at 37°C and this was thought to be HSP90. However analysis of the small HSPs revealed that these proteins were only synthesized for a limited time in the L_3,

upon transfer to the mammalian host, and were not detected 24 hours post-infection [Jecock and Devaney 1992]. HSP90 is required for many cellular processes under normal growth conditions, therefore transcripts for *hsp90* would be expected to be present, at least at a low level, in all life cycle stages of *B. pahangi*. Indeed *hsp90* mRNA appears to be present in *B. malayi* L_3 as is evident from the three *hsp90* clones which were obtained from an L_3 cDNA library (see *Figure 3.9*). If *hsp90* is expressed in all life cycle stages, RT-PCR could be used to confirm the presence of *hsp90* mRNA in samples of *B. pahangi* cDNA.

A cDNA library, which had previously been constructed from RNA prepared from mf enriched in heat shock transcripts and amplified with the primers SL1 and oligodT, was screened in an attempt to obtain more of the *hsp90* sequence. However only small fragments were isolated in the screen. There are a number of possible explanations for these results but the explanation most consistent with the data is that the fragments may have originated not from *hsp90* mRNA, but from genomic DNA or pre-mRNA. As proposed in ***Figure 3.13***, the oligodT primer, used to amplify the original mRNA template, may have primed in intron 6 of contaminating genomic DNA or incompletely spliced *hsp90* mRNA. In addition to this, an *Eco*RI site in the next intron (intron 7) in the *hsp90* genomic sequence may have permitted the cloning of small fragments of *hsp90* in the library. These 280 bp fragments correspond to a region within the hsp90f2-r2 probe and were therefore identified in the screen. A 3′ fragment of the hsp90f2-r2 product could have been used in a second attempt to isolate a clone that contained more sequence information, as this would not hybridize to the 280bp fragments under stringent conditions. However, due to the *Eco*RI sites in the *hsp90* cDNA and the use of this restriction enzyme in the construction of the library, a full-length gene would not have been obtained.

Figure 3.13 : Schematic of the genomic and cDNA fragments

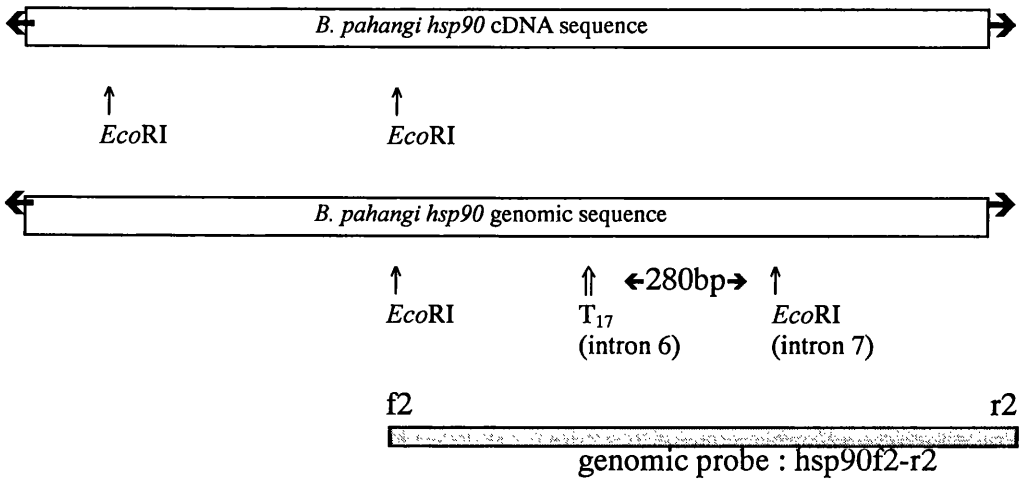


Figure 3.13 :

There are two *Eco*RI sites in the cDNA and genomic and their positions are indicated with arrows. Reverse transcription of mRNA with oligodT should produce copies of *hsp90*, which have been polyadenylated, but may also amplify a region of (contaminating) genomic DNA by priming from a string of adenine nucleotides within intron 6. The primers SL1 and oligodT are often used for the amplification of first strand products and there are restriction sites within these primers (SL1: *Eco*RI, oligodT: *Xho*I). Products can be cloned into pre-cut lambda arms by digesting them with *Eco*RI and *Xho*I. However if *hsp90* cDNA was digested with *Eco*RI, it would create three fragments of *hsp90*. The same digest on the genomic *hsp90* PCR product would create one fragment (280bp), between the mis-priming oligodT and an internal *Eco*RI site. This may explain why small fragments of *hsp90* were present in the heat shocked mf cDNA library.

Attempts were next made to obtain the 5′ end of the *hsp90* cDNA using SL1. The SL1 primer is a useful tool in nematode molecular biology since the 5′ region of genes, which are trans-spliced, can be amplified with one gene-specific primer and SL1. The use of SL1 in a PCR reaction eliminates the need for 5′ RACE (Rapid Amplification of cDNA Ends) which can be time consuming and technically difficult. The percentage of *Brugia* genes which are *trans*-spliced is not known but in *Ascaris* more than 80% of genes are thought to be trans-spliced [Nilsen 1993] and the *C. elegans* sequencing project predicts approximately 70% of genes are trans-spliced in this nematode [Blumenthal and Speith 1996]. *Brugia* PCR libraries constructed using SL1 and oligodT have been used to produce a large and varied amount of sequence information for the ongoing EST sequencing projects. A second spliced leader, SL2, has been identified in *C. elegans* [Huang and Hirsh 1989] although to date no such variant has been identified in *Brugia* [Blaxter *et al* 1996]. Studies of SL2 *trans*-splicing suggests that this spliced leader may be involved in the splicing of polycistronic transcription units, a process which may or may not be peculiar to *C. elegans* [Speith *et al* 1993, Blumenthal and Speith 1996]. Recently, a variant of SL1 has been found in the plant parasitic nematode genus, *Meloidogyne.* The spliced leader, called SL1M, has a G to A single base substitution three nucleotides from the 3′ end compared to SL1 [Koltai *et al* 1997]. No such variant has been documented in *Brugia.*

The 5′ end of the cDNA was isolated (hsp90SL1-r3) but appeared to originate from an incompletely spliced transcript since a single intron remained. Other examples of similar phenomenon include the isolation of a cDNA clone (gp100-in4) that contained an intron, from a human cDNA library. The transcript corresponding to this clone could be detected by RT-PCR but not by northern blot analysis, which lead to the conclusion that it was a rare transcript [Robbins *et al* 1997]. However, investigations into insulin mRNA in mouse cells revealed that some mRNA retained intron 1 (of two) and that the transcripts entered the cytoplasm and constituted 2-10% of insulin mRNA. Furthermore, partially spliced insulin mRNA was relatively stable in the cytoplasm, with a half-life similar to the completely spliced form [Wang *et al* 1997].

It is unlikely that the *hsp90* PCR product was amplified from genomic DNA since the corresponding genomic sequence contains three introns. In addition, hsp90SL1-r3

contains a splice leader, which would not be present in a genomic copy of the gene. Total RNA was used for first strand cDNA synthesis so partially spliced transcripts from the nucleus may contaminate the preparation. However there should be a much higher level of fully spliced mRNA present, reducing the probability of amplification from a partially spliced *hsp90* transcript. The large number of introns in *hsp90* pre-mRNA may increase the probability of isolating a partially spliced transcript compared to genes with fewer intervening sequences. However the amplification of a fragment of *hsp90* which contains one remaining intron is probably a chance occurrence.

The difficulties in obtaining the 3′ end using a combination of an *hsp90*-specific primer and oligodT may relate to the propensity of oligodT to anneal to adenine rich regions in the genome. This has previously been reported with *B. pahangi* [Martin *et al* 1996] and with *A. cantonensis* [Joshua and Hsieh 1995]. The genome of *B. pahangi* is 28% G+C [Hammond 1994] and concentrated regions of A and T include the 3′ UTR. The low molecular weight products visible in PCR reactions with oligodT and hsp90f4 may represent products amplified by oligodT priming from adenine rich regions on the sense and anti-sense strands. Indeed, one such product was cloned during this project, a *B. pahangi* homologue of 25S RNA which contained the oligodT sequence at both 5′ and 3′ end of the cloned fragment (data not shown).

The amplification of first strand cDNA with hsp90f4 (in a single primer reaction) may have been due to small amounts of contaminating oligodT carried over from the reverse transcription reaction. A low concentration of primer would increase the specificity of priming and this may have enabled the amplification of a larger product from hsp90f4 and oligodT. Two PCR reactions were necessary to sufficiently increase the quantity of the *hsp90* product but a single PCR with a higher concentration of oligodT produced only small products. It was therefore necessary to use only hsp90f4 in the second round of amplification. If the initial product was a result of oligodT present in the cDNA it is possible that oligodT adhered to and co-migrated with the product that was excised from the gel for the in-gel ligation and was thus introduced to the second PCR reaction. Another hypothesis would be priming of hsp90f4 in both the forward and reverse direction, which would therefore not require a second primer for amplification.

However sequence with sufficient homology for the priming of hsp90f4 was not observed on the anti-sense strand of *hsp90*.

The hsp90f6 primer and adapted oligodT were used to amplify a product that contained the complete 3′ end. Previous attempts at amplification using hsp90f6 and adapter primer had not been successful so PCR with the adapted oligodT primer was attempted. Adapted oligodT primer is able to hybridize to the same sequences as oligodT but due to the GC rich 5′ end, a higher annealing temperature can be used (after the first round of PCR), thus increasing the specificity of the primer/template interaction.

The complete cDNA sequence enabled the elucidation of the protein code for *B. pahangi* HSP90 to be determined. The protein sequence was analyzed to identify motifs common to the HSP90 family. The alignment of HSP90 from a variety of species highlights the high degree of homology between these proteins.

Cytoplasmic HSP90 has an almost completely conserved terminal five amino acids with the exception of some members of the trypanosomatidae which have a single substitution, e.g. *L. donovani* where MEEVD→MEQVD [Shapira and Pedraza 1990] and *D. discoideum* where MEEVD→MEKVD [Boves *et al* unpublished]. However, these terminal residues do not appear to be essential for the function of the protein as indicated by their absence in HtpG and by truncation experiments on HSP90 [Bardwell and Craig 1987, Louvion *et al* 1996]. The residues EEVD are highly conserved in HSP70 and one hypothesis is that these terminal amino acids may allow for preferential translation of this heat shock gene during a heat shock [Denisenko and Yarchuk 1989, Marrs *et al* 1993]. Removal of more of the C-terminus results in HSP90 monomers, demonstrating that residues involved in dimerization are located in the C-terminal region of HSP90. Indeed, a leucine zipper motif has been proposed in this region [Shaknovich *et al* 1992 and Yabe *et al* 1994].

Experiments carried out on an N-terminal fragment of yeast HSP90 revealed a highly ordered secondary structure which consisted of eight β-sheets flanked by α-helices. The role of this conformation in the native protein was hypothesized to be a mechanism for relaying information between the N-terminal and C-terminal regions of the protein, i.e. creating coordination between the separated domains [Prodromou *et al* 1997].

Studies on the immunogenicity of HSP90 utilized monoclonal antibodies to human HSP90α and HSP90β to map the sites of antibody recognition [Nemato *et al* 1997]. The two main regions which were identified as being "hotspots" for antibody recognition were also regions of greater amino acid diversity. These include the charged domain and part of the carboxyl terminal section. One explanation is the accessibility of residues to interacting antibodies and consistent with this theory is the observation that Region I (charged domain) appears to be located on the surface of HSP90 [Nemato 1997]. However there is another hypothesis for the antibody response being concentrated in a region of greater sequence diversity. Autoantibodies to HSP90 have been identified in patients suffering from an auto-immune disease, systemic lupus erythromatosis [Conroy *et al* 1994]. It has been proposed that HSP90 molecules from an infecting organism may initiate an antibody response which results in the recognition of "self HSP90" [Minota *et al* 1987]. It should be noted that exposed domains of molecules, which may be targets for antibodies, are thought to be under greater pressure to diversify and this may also explain the sequence divergence observed in the antigenic regions of HSP90. For example the hook protein, FlgE, from *Campylobacter jejuni* was observed to have a high degree of variability in the surface exposed domains of the protein, thought to be a result of the pressure for variation [Luneberg *et al* 1998]

HSP90 sequences from 25 different species were used to construct a cladogram and one of the clades contained sequences from the diptera *D. melanogaster*, *A. albimanus*, *B. pahangi* and *C. elegans*. This is consistent with a recent analysis that grouped nematodes and arthropods in a single new clade called the Ecdysozoa, so called because both organisms undergo moults during development [Aguinaldo *et al* 1997]. While the moulting process in insects is well described, very little is known about moulting in nematodes. In insects, moulting is controlled by the steroid hormone ecdysterone and previous workers have hypothesized a similar hormonal control in nematodes, including *Brugia* [Barker *et al* 1991]. Indeed the addition of ecdysterone to *D. immitis* L_3 stimulated premature moulting when compared to control larvae [Warbrick *et al* 1993].

Although protozoa [Clark *et al* 1996, Bonnefoy *et al* 1994], invertebrates, including *C. elegans* [ACeDb] and most fungi [Swoboda *et al* 1995] only appear to have one HSP90 (which is sometimes coded for by multiple copies of *hsp90* [Mottram *et al* 1989]) there

are two (or more) distinct type of HSP90 present in many species including the lower eukaryote, *S. cerevisiae* and members of the vertebrates and plants, (e.g. human and *Arabidopsis*). As previously discussed, *S. cerevisiae*, HSC82 is expressed under non-stress conditions, in contrast to HSP82 which is highly heat inducible but is not expressed under normal growth conditions. Yeast remains viable after the deletion of either one of the loci for *hsp90* but no double deletion mutants can be isolated emphasizing the absolute requirement for HSP90 [Borkovich *et al* 1989].

The divide between constitutive and heat-inducible expression is also observed in plants where one HSP90 (e.g. maize HSP82 [Marrs *et al* 1993]) is particularly important during stress, but another (e.g. tomato HSC80 [Konig *et al* 1992]) has a more important role in the development of the plant. In vertebrates, an extreme example of the difference between HSP90 expression is evident in chicken. The HSP90α and HSP90β proteins from mammalian species are not as markedly different in expression but do appear to show some tissue specific differences. Indeed, the difference in the sensitivity of certain organs to steroid hormone may relate to both the total concentration of HSP90 and the levels of the isoforms present [Vamvakopoulos 1993].

Although duplication and divergence of *hsp90* was suggested to have arisen from three independent events (3.2.13), the common result is the acquisition of two differentially expressed proteins. However, there does not appear to be more than one type of *hsp90* in invertebrates and there is no evidence from studies presented in this thesis that *B. pahangi* contains more than one *hsp90*. The function of HSP90 in *B. pahangi* may therefore be modified by the association of different co-chaperones with HSP90 which increase the functional repertoire of a single protein, as described by Scheibel *et al* [1998].

The transcription of *hsp90* was confirmed by Northern analysis. The size of the mature mRNA was estimated to be 2.6kb which is consistent with the size calculated from the cDNA sequence. Transcripts for *hsp90* are enriched in adults exposed to a heat shock temperature (41°C) confirming the inducibility of *hsp90* by heat shock. However, *hsp90* mRNA is barely detectable in adults cultured at 37°C whilst mf cultured at the same temperature have a higher level of these transcripts; this difference cannot be attributed

to heat shock, but may imply a developmental role for the gene. It is possible that culturing the parasite *in vitro* may create artifacts, resulting in the transcription of genes that would not be induced at the same temperature *in vivo*. Mf and adults were cultured under the same conditions and the high concentration of *hsp90* mRNA in mf cultured at 37°C may still indicate the differential transcription of *hsp90* in these two life cycle stages. Another possible explanation for the differential expression of *hsp90* in mf and adults at 37°C, is that mf may have a lower heat shock threshold than adult parasites. The level of transcripts for *hsp90* is dramatically different in mf at 37°C compared to 28°C, at which temperature virtually no transcripts were detected. The pattern of expression of *hsp90* is therefore very similar to that reported previously for small *hsp* mRNA [Thompson *et al* 1996]. The correlation between *hsp* expression and the developmental block is interesting to note. Further circumstantial evidence that *hsp* expression may have a role in parasite development comes from the work in which *B. pahangi* infected mosquitoes were maintained at elevated temperatures. The optimal temperature for development in the mosquito is 28°C, but mosquitoes maintained at 37°C did not support development of the mf [Devaney and Lewis 1993].

The larger sized transcripts observed in the RNA from mf heat shocked at 41°C (see ***Figure 3.12***) may be due to the disruption of RNA splicing at this temperature. *Hsp90* pre-mRNA is predicted to be 3.6kb (see ***Figures 3.12*** and ***5.1***). Previous studies on a small *hsp* in *B. pahangi* demonstrated that the mRNA is *trans*-spliced [Thompson *et al* 1996] and that introns are present in the genomic sequence [Thompson personal communication]. The expression of this mRNA was studied in mf and adults cultured at different temperatures. In mf at 37°C, a transcript of 1.6kb was identified (the expected size of the mature mRNA). However in mf cultured at 41°C an additional transcript of 2.6kb was also observed. This larger transcript was suggested have resulted from a disruption in RNA processing, causing an accumulation of larger precursor mRNA [Thompson *et al* 1996]. In contrast to the observation in mf, adults cultured at 41°C contained only the smaller transcript of 1.6kb. However, increasing the temperature of the culture to 43°C resulted in the appearance of the larger transcript in the adult parasite. It was speculated that the appearance of the larger transcript in mf at 41°C correlated with the greater thermotolerance of this stage. These observations may also support the hypothesis that the two different life cycle stages have different heat shock thresholds.

Defects in 3′ processing have resulted in the accumulation of larger transcripts upon heat shock. In *Drosophila*, heat shock was reported to induce the accumulation of larger transcripts for two small *hsp*s, with 3′ ends extensions of approximately 1.5kb [Berger *et al* 1985]. This was suggested to result from an alteration in the normal transcription termination process causing longer transcripts [Pauli *et al* 1988]. Differences in the length of the polyA tail may also explain the appearance of larger transcripts on the northern blot. Under normal cellular conditions, polyA tails ranging from 12 to 60 nucleotides have been detected on *Xenopus* vitellogenin mRNA, in contrast to albumin mRNA, which was reported to have a consistently short polyA tail (12-17nt.) [Rao *et al* 1996]. Furthermore, differences in the polyA tail length of *Drosophila* small *hsp*s transcripts have also been reported [Berger *et al* 1985].

The pattern of expression of both the small *hsp* transcripts [Thompson *et al* 1996] and *hsp90* transcripts appears to be similar in the RNA samples from mf cultured for different times at 28°C and 37°C. The level of transcripts for the small *hsp* and *hsp90* are dramatically different in mf at 37°C compared to mf at 28°C, a temperature at which no transcripts were detected. This was of particular interest since mf are developmentally arrested in the mammalian host and only resume development upon transfer to the mosquito vector.

Previous studies on *B. pahangi* demonstrated that mf at 37°C synthesized small HSPs whilst mf at 28°C did not synthesize these proteins [Devaney *et al* 1992] and this is consistent with the Northern analysis reported by Thompson *et al* (1996). The difference in the level of transcripts for *hsp90* in adults cultured at 37°C compared to mf at 37°C and in mf cultured at 37°C compared to mf cultured at 28°C may indicate differences in transcription in the two life cycle stages and at the two different temperatures. However it is also possible that under normal conditions *hsp90* (and small *hsp*) is transcribed at a relatively high level but that the transcripts are rapidly degraded, resulting in a low steady state concentration of mRNA and (also protein). In mf (but not adults), mammalian body temperature may inhibit the degradation of *hsp90* transcripts and thus result in a higher concentration of HSP90. A repression of small HSP synthesis has been observed in mf, which were exposed to 28°C and then shifted back to 37°C [Devaney *et al* 1992]. Hypothetically, the inhibition of small *hsp* mRNA degradation in

mf would cease at 28°C and small *hsp* mRNA would be rapidly degraded. When mf were transferred to 37°C, a delay may occur in the resumption of degradation-inhibition and the level of sHSP expression would therefore be lower in these mf than in mf continually exposed to 37°C. Post-translational control of *hsp90* has been observed in the parasitic protozoa, *Leishmania*, where an increase in HSP90 concentration during heat shock results not from an increase in the transcription of the gene, but from an apparent increase in *hsp90* mRNA stability [Argaman *et al* 1994]. However, a combination of transcriptional and translational control mechanisms are perhaps more likely in the control of HSP90 expression. Indeed in *Drosophila* cells, the production of HSP70 increases in response to increased stress. After sufficient HSP70 has accumulated, the expression of HSP70 appears to be limited both by the repression of mRNA synthesis and the destabilization of transcripts [DiDomenico *et al* 1982].

In summary

- An *hsp90* cDNA clone was obtained by PCR and sequenced
- *B. pahangi hsp90* has high homology to *hsp90* clones from *B. malayi*
- The predicted amino acid sequence contains conserved domains present in HSP90s from other species

Northern blot analysis revealed that:

- *hsp90* was heat shock inducible, consistent with a heat shock protein gene
- *hsp90* mRNA was enriched in mf cultured at 37°C when compared to adults cultured at 37°C and mf cultured at 28°C

4.0 Cloning and sequence analysis of an *hsp90* genomic clone

4.1 Introduction

This chapter describes the isolation of a genomic clone corresponding to *hsp90*. The isolation of a genomic clone would allow the confirmation of the cDNA sequence and identification of any introns. In addition, by analyzing sequence upstream of the coding region it was hoped that information would be obtained on the putative promoter region.

Sorger (1991) reported that the heat shock response in eukaryotes (with the possible exception of protists) is controlled at the transcriptional level by the interaction of the heat shock transcription factor (HSF) with regulatory elements, termed heat shock elements (HSEs), upstream of the transcriptional start site of heat shock genes. Heat shock elements are highly conserved between diverse species and have the consensus sequence NGAAN (in many cases AGAAN) arranged in inverted repeats [Fernandes *et al* 1994]. Heat shock factor is activated by a conformational change that results in trimers and an alteration in the phosphorylation state of the protein [Zuo *et al* 1994, Sorger 1991]. One monomeric subunit of HSF is capable of interacting with a single five base pair unit in an HSE, therefore total binding requires three inverted repeats [Perisic *et al* 1989].

During a stress response, the transcriptional activation of heat shock genes occurs not only in preference to the transcription of other genes, but expression of non-heat inducible genes is also strongly inhibited [Schlesinger 1994]. This results in the rapid accumulation of HSPs, which provide protective functions in the cell, such as the prevention of protein aggregation and the accelerated degradation of aberrant polypeptides [Stege *et al* 1995]. The expression of HSPs is not restricted to a heat shock or other cellular assaults, as some also have important functions in normal cellular processes. For example, HSP90 is required for the viability of steroid hormone receptors [Georgopoulos and Welch 1993] (see 1.4.1). It is not clear whether the transcription of *hsp* genes under normal cellular conditions utilizes HSEs or whether other regulatory elements are involved [Bienz 1985, Engelberg *et al* 1994]. In *Drosophila,* the insect moulting hormone, ecdysterone, binds to ecdysone receptors which are activated and bind to specific transcriptional elements in the promoter region of small heat shock protein genes (*shsps*). These are distinct from HSEs, thus allowing hormonal regulation

of these genes [Riddinough and Pelham 1987, Mestril *et al* 1986]. However, steroid receptor binding elements have not been identified in the promoters of other *hsp*s or even *shsp*s from species other than *Drosophila*.

Regulation of HSPs also occurs at the level of translation and mRNA stability in eukaryotes. At higher temperatures the *hsp* transcripts are more stable and appear to be preferentially translated [DiDomenico *et al* 1982]. The 5′ and 3′ untranslated regions (UTRs) of *hsp*s have been implicated in the selective degradation of *hsp* mRNA at normal cellular temperatures [Aly *et al* 1994] and control elements thought to be involved in specific translation of *hsp* mRNA, have been identified in the 5′ UTR of these transcripts [Hultmark *et al* 1986].

The disruption of RNA processing due to the presence of intervening sequences in *hsp*s can result in a reduction in gene product during extreme heat shock. Indeed it has been hypothesized that there is pressure for a lack of introns in *hsp*s [Lindquist 1986] but there are several examples of *hsp*s which contain intervening sequences, many of which are members of the *hsp90* family [Minchiotti *et al* 1991, Brandon *et al* 1989, Rebbe *et al* 1989]. This may relate to the fact that many *hsp90*s are expressed under non-heat shock conditions.

4.2 Results

4.2.1 The *B. pahangi* genomic library

An EMBL3 genomic library was acquired as a gift from Prof. Gird Hobom at the University of Geissen. The library was constructed from DNA from adult, mixed sex *B. pahangi* which had been partially digested with *Mbo*I and size fractionated to restrict the inserts to 9-23kb. The aim of screening the genomic library was to obtain both the coding region of *hsp90* and sequence upstream of the start methionine. To achieve this, two *hsp90* probes were made, one from a central region of the gene and one from the 5′ end. Hybridizing duplicate filters separately with two different regions of the gene should decrease the number of non-specific clones, while using a region near the start of the open reading frame increases the probability of isolating a fragment which contains sequence upstream of the *hsp90* coding region.

4.2.2 Creating a 5′ probe

Using the cDNA sequence, with the aid of the GCG Prime program, two primers were designed. The forward primer hsp90f5 starts at nt. 15 of the putative open reading frame. The size of the cDNA fragment amplified by hsp90f5 and the reverse primer hsp90r5 is 258bp. However when these primers were used on genomic DNA a product of 430bp was amplified. This was subsequently explained when cloning and sequencing of the product (hsp90f5-r5) revealed two introns that split the coding sequence into three exons.

4.2.3 Creating a central probe

The heterologous primers hsp90f2 and hsp90r2 were used to amplify the genomic product hsp90f2r2 mentioned in 3.2.3. This fragment corresponds to a region in the centre of the gene. Plaques recognized by both probes should contain most, if not all, of the *hsp90* gene. ***Figure 4.1*** is a schematic showing the PCR products and the position of the primers on the cDNA.

4.2.4 Screening the library

The size of the *B. pahangi* genome is approximately 100Mb and the theoretical size of the smallest insert in the genomic library is 9kb. If the genome was equally divided into 9kb fragments, there would be a total of approximately 1.1×10^4 such fragments. Eight plates, each with 5×10^4 pfu were screened. Theoretically, within the 4×10^5 pfu screened there should be an estimated minimum of 36 copies of a single copy gene ($4 \times 10^5 \div 1.1 \times 10^4 = 36$). Since some of the copies of *hsp90* may be fragmented in the construction of the library, less than 36 pfu are expected to have inserts which hybridize to both probes. However, the library was amplified soon after being received and this will affect the ratios of the constituent genes.

Duplicate filter lifts were taken from the plates. The first lift was probed with hsp90f5-r5 and the second with hsp90f2r2. Fourteen plaques were identified which hybridized to both probes and seven of these, chosen randomly, were taken to a secondary screen. Finally, five duplicate positives were plated out as single pfu, probed to confirm homology and then isolated.

Figure 4.1 : Relative positions of the probes hsp90f5-r5 and hsp90f2-r2

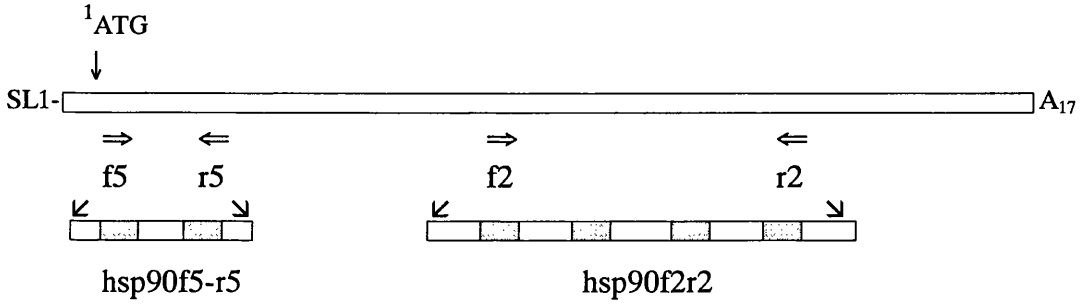


Figure 4.1 :

Diagram of the cDNA which codes for *B. pahangi hsp90*. The top (open) bar represents the *B. pahangi hsp90* cDNA and the lower two checked bars represent two probes produced by PCR from *B. pahangi* genomic DNA, which were used to screen a genomic library. The arrows show the positions of the primers on the cDNA which were used to create the genomic probes. The coding region is represented by an open box and the grey sections are introns.

4.2.5 Southern analysis of isolated clones

It was important to know if these five lambda clones represented the same gene or a number of *hsp90* homologues. The purified clones, designated 111, 311, 512, 513 and 821, were digested with three enzymes. *Kpn*I, *Sal*I and *Sst*I were used in single digests and a double digest using *Sal*I and *Sst*I was also performed. The fragments were separated by electrophoresis, blotted and probed with the cDNA product, hsp90f4end (see the sub-clone list in Table 3.2). ***Figure 4.2*A** and ***Figure 4.2*B** are photographs of the restriction digests of the five lambda clones, **C** and **D** show the autoradiographs of these clones probed with hsp90f4end.

When digested with each of the restriction enzymes and with both *Sal*I and *Sst*I, all five clones, viewed by ethidium bromide staining, had the same restriction patterns (see ***Figure 4.2A*** and ***B***). In addition, hsp90f4end hybridizes to the same number and size of fragments for each of the clones. This implied that 111, 311, 512, 513 and 821 were identical. The double bands observed in the *Sst*I digests shown in ***Figure 4.2*** **C** and **D** are due to the presence of an *Sst*I site in the region of the genomic clone which corresponds to the sequence of the probe.

To further investigate whether the lambda clones were identical, they were also subjected to PCR with the primers hsp90f5 and hsp90r5. As shown in ***Figure 4.1***, there are two introns in the sequence hsp90f5-r5; it was unlikely that a different homologue of *hsp90* would contain two intervening sequences of identical size and position. Therefore, if the clones all contain *hsp90* but represent different homologues, a PCR product of varying size would be expected. ***Figure 4.3*** is a picture of an ethidium bromide stained gel of all five PCR reactions and a negative control. The amplified band is the same size for all the clones, approximately 400bp (the size of the genomic product hsp90f5-r5), which implies the presence of introns in the sequence. No product was observed using the primers in the absence of template.

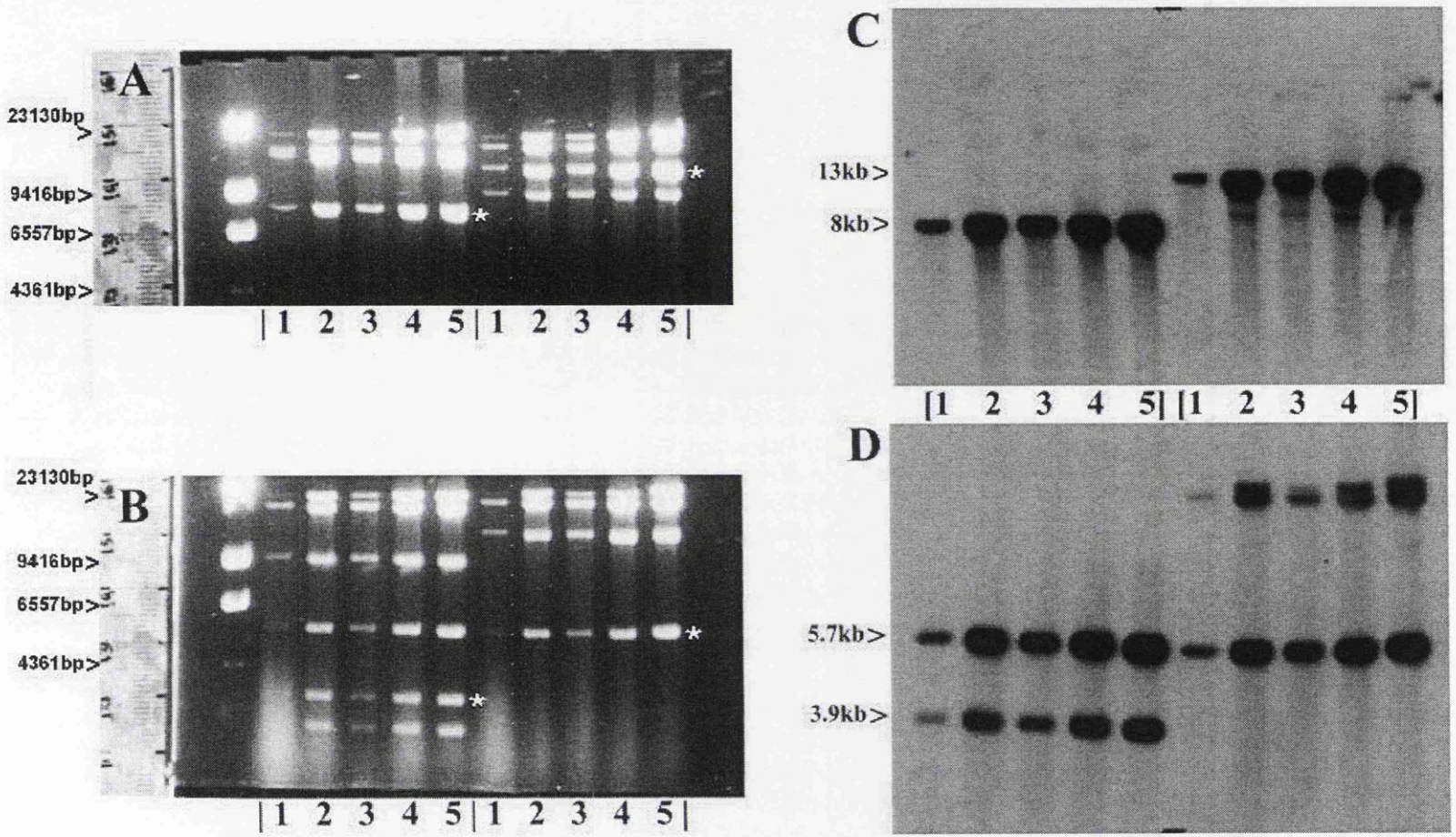
Figure 4.2 :

- **A** and **B** are the ethidium bromide stained gels of the restriction digest of five lambda clones. λ*Hind*III markers are in the first lane of both gels and the sizes of these markers are indicated on the left hand side.
- **C** and **D** show the corresponding autoradiographs for **A** and **B** respectively which have been probed with the cDNA probe hsp90f4end. The filters were hybridized at 65°C and washed to 0.2 x SSC, 0.1% SDS at 65°C.
- The sizes of the fragments which hybridize to hsp90f4end are shown in **C** and **D** and the bands in **A** and **B**, which correspond to these fragments are indicated by an asterisk.
- In **A** and **C**, the first five lambda clones are digested with *Kpn*I and the last five clones are digested with *Sal*I.
- In **B** and **D**, the first five lambda clones are digested with *Sal*I and *Sst*I and the last five clones are digested with *Sst*I only.

The lambda clones are :

1 111
2 311
3 512
4 513
5 821

Figure 4.2 : Analysis of five lambda clones isolated from the genomic library



4.2.6 Mapping the genomic clone

As the preliminary analysis suggested that all five lambda clone were identical, one of the clones (513) was subjected to further analysis. *Sal*I sites flank the EMBL3 cloning site and digesting clone 513 with this enzyme released a 13kb fragment. Clone 513 was mapped to determine the orientation of the *hsp90* gene with respect to the lambda backbone. In addition it was important to identify within the 13kb insert, the restriction fragments which contain *hsp90* sequence. *Kpn*I, *Sph*I, *Sst*I and *Sal*I were used to digest 513 in single or double digests. The fragments were blotted and hybridized with two *hsp90* probes, hsp90f5-r5 (see 4.2.2) and a new product, hsp90f6-r6. Hsp90f6-r6 was amplified from genomic DNA and corresponds to 215bp near to the 3′ end of the coding region. There is one intron in the hsp90f6-r6 sequence and it contains an *Sst*I site.

Hybridization with the probe, hsp90f4end, identified an *Sst*I site in *hsp90* near the 3′ end, (probing an *Sst*I digest of the lambda clones produced two bands). The use of two probes, one positioned close to the start (hsp90f5-r5) and one positioned close to the end (hsp90f6-r6) of the open reading frame, increased the chance of identifying restriction fragments which contained *hsp90*. Since after digestion, fragments which do not contain the sequence corresponding to hsp90f5-r5 may contain sequence corresponding to hsp90f6-r6 or vice versa, more *hsp90* fragments can be visualized using both probes.

Figure 4.4 shows the ethidium bromide stained gel of the restriction fragments of clone 513 (**A**) and the autoradiograph of these fragments probed with both hsp90f5-r5 and hsp90f6-r6 (**B**) to identify fragments which contain *hsp90*. Digesting with either *Sal*I or *Kpn*I/*Sal*I resulted in only one visible fragment which hybridized to the probes. The *Sal*I fragment observed on the autoradiograph represents the whole insert and as mentioned previously is an estimated 13kb. Other fragments, which hybridize to hsp90 probes, are summarized in ***Table 4.1***.

Figure 4.3 : PCR on the five lambda clones

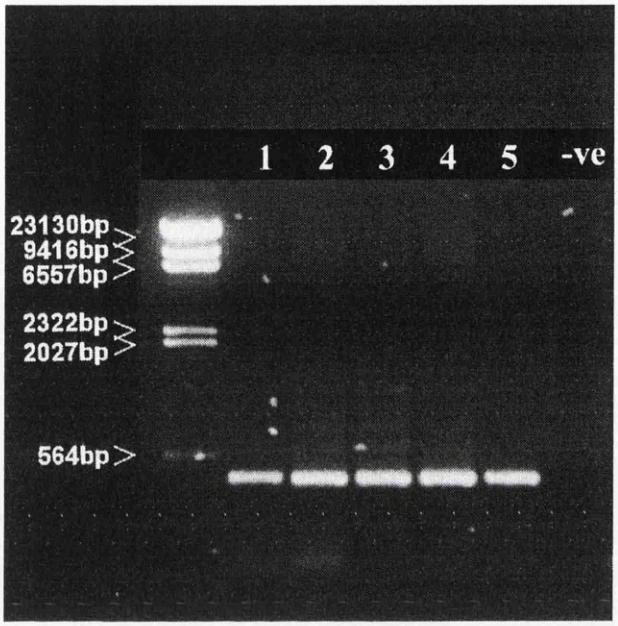


Figure 4.3 :

An ethidium bromide stained gel showing the PCR products that were amplified using the primers hsp90f5 and hsp90r5 with 1μl of each lambda clone (lanes 1-5). The negative control (-ve) is also indicated and this PCR reaction contained both primers with no template. The size of the λ*Hind*III markers are indicated and the PCR products in lanes 1-5 are all approximately 400bp.

The lambda clones are :

1 111

2 311

3 512

4 513

5 821

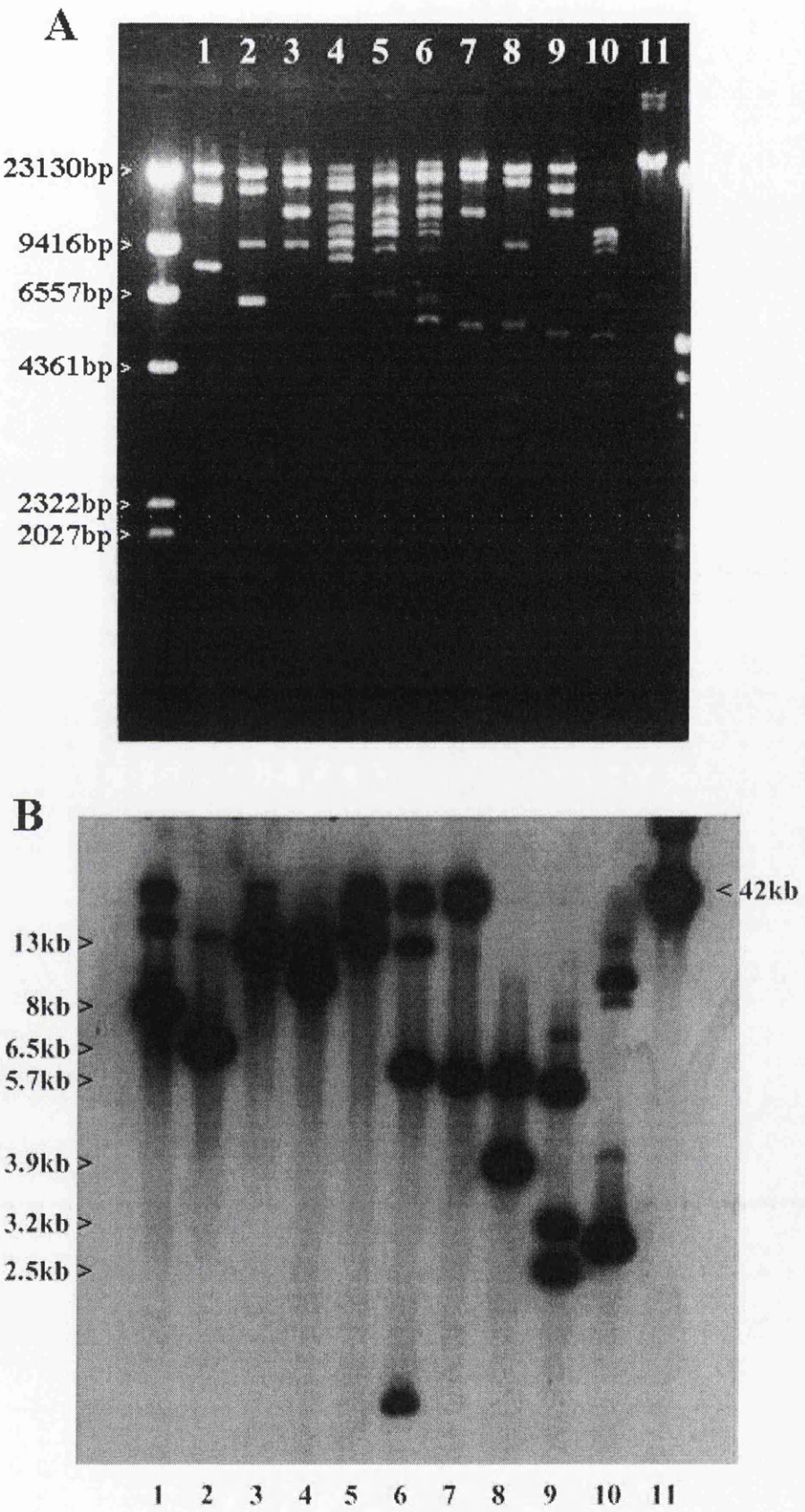
***Figure 4.4* :**

A, an ethidium bromide stained gel showing restriction digests of the lambda clone, 513. The λ*Hind*III markers were used to calculate the size of the restriction fragments. **B,** an autoradiograph showing the hybridization of the restriction fragments with two probes, hsp90f5-r5 and hsp90f6-r6. Hsp90f5-r5 contains a *Kpn*I site and hsp90f6-r6 contains an *Sst*I site. The filters were hybridized at 65°C and washed to 0.2 x SSC, 0.1% SDS at 65°C. The film was exposed for 5 minutes. The arrow on the right side of **B** indicates the size of the uncut clone and the arrows on the left hand side indicate the sizes of some of the fragments which contain *hsp90*.

The digests are as follows :

1	*Kpn*I	2	*Kpn*I/*Sal*I
3	*Sal*I	4	*Sal*I/*Sph*I
5	*Sph*I	6	*Sph*I/*Sst*I
7	*Sst*I	8	*Sst*I/*Sal*I
9	*Sst*I/*Kpn*I	10	*Kpn*I/*Sph*I
11	513 uncut		

Figure 4.4 : Restriction mapping of clone 513



Hsp90f5-r5 contains a *Kpn*I site and hsp90f6-r6, an *Sst*I site so two fragments are expected to hybridize to the same probe when either of these enzymes are used. However, digesting with *Kpn*I/*Sal*I appeared to only produce one fragment to which the probe hybridized, but an analysis of the other restriction digest patterns suggested the presence of two fragments of the same size. With this information, a restriction map was proposed in ***Figure 4.5***.

4.2.7 Sub-cloning the lambda insert

The lambda clone contained a large insert and was therefore sub-cloned to allow easier manipulation of the clone. The purified lambda clone was digested with *Sst*I, which released a 5.7kb fragment, and with *Sst*I/*Sal*I which released a 3.9kb fragment. These fragments were cloned into pBluescript SK^{II}. The newly constructed plasmids were used to transform *E. coli* and transformants were screened by hybridization to identify those containing *hsp90*.

Figure 4.6 shows autoradiographs from the colony screens. Filter **A** was probed with hsp90f5-r5 and identified two clones (23 and 5). Sequencing the inserts of sub-clones of 23 and 5 (called *Sst*I90) confirmed that they were identical and contained the *hsp90* sequence. The 3′ end of the *hsp90* genomic sequence was not contained within *Sst*I90 due to an *Sst*I near the 3′ end of the gene which resulted in the excision of this region during cloning. Filter **B** was probed with a *Hind*III fragment from the product hsp90f4end which contains the extreme 3′ end of the cDNA sequence. This 3′ probe was used to identify sub-clones that contained the remaining *hsp90* sequence, absent in clones 23 and 5. A sub-clone, which contained the 3.9kb SstI/SalI fragment, was identified. The *Sst*I/*Sal*I sub-clone (called *Sst*I*Sal*I90) contained the end of the coding region of *hsp90*, the 3′ untranslated region and included a polyadenylation signal.

Figure 4.5 :

Diagram showing the restriction fragments which are identified by the two probes hsp90f5-r5 and hsp90f6-r6. The *Kpn*I site in hsp90f5-r5 and the *Sst*I site in hsp90f6-r6 facilitated in mapping the orientation of *hsp90* with respect to EMBL3. The 13kb insert is released from EMBL3 by digestion with *Sal*I, which does not cut within *hsp90*. Digesting with *Sst*I/*Sal*I produced three fragments, two of which contain the *hsp90* sequence: an *Sst*I fragment (5.7kb) and an *Sst*I/*Sal*I fragment (3.9kb). The gene is orientated such that the 5′ end is closest to the small (9.2kb) lambda arm.

Table 4.1 : Restriction mapping the lambda clone

Restriction digest	Size of restriction fragments
*Sal*I	13kb
*Sst*I	>20kb, 5.7kb
*Kpn*I	~16kb, 8kb
*Sal*I/*Kpn*I	6.5kb
*Sst*I/*Sal*I	5.7kb, 3.9kb
*Sst*I/*Kpn*I	5.5kb, 3.2kb, 2.5kb

Figure 4.5 : Diagram of the lambda clone containing *hsp90*

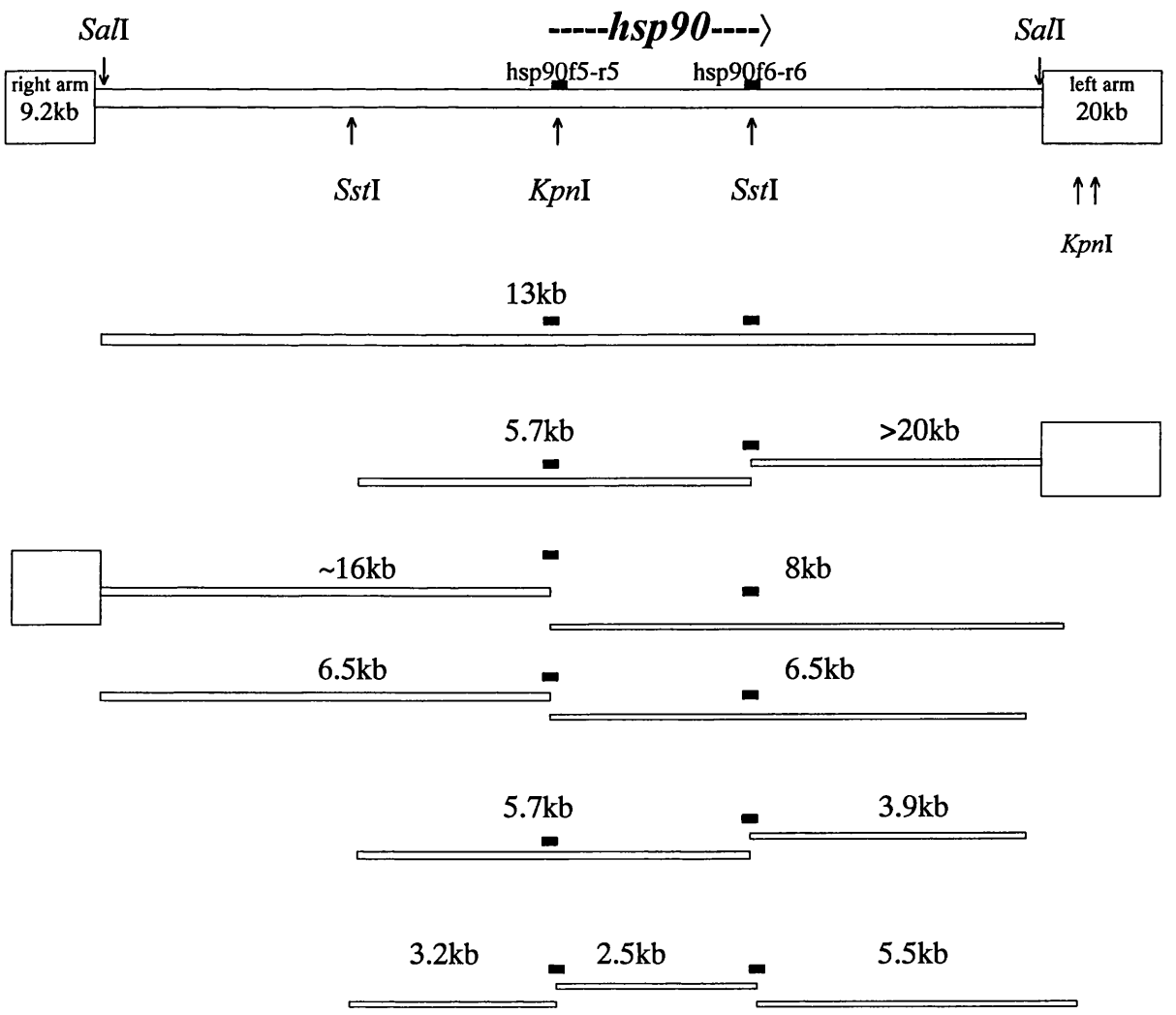


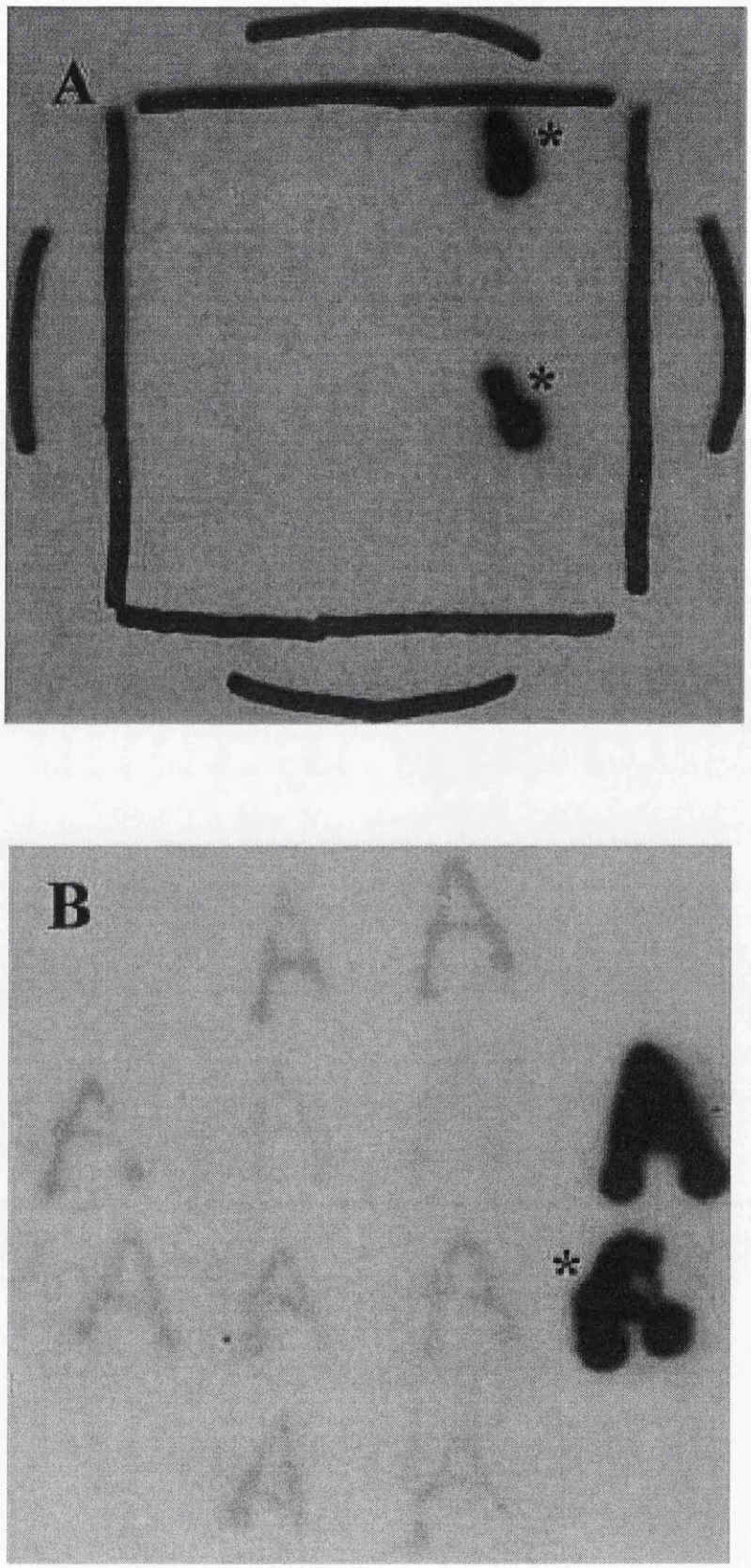
Figure 4.6 :

Two colony hybridizations are shown above. Colonies transformants were streaked onto a gridded membrane, which was probed and onto a duplicate plate, which was maintained as a stock of the colonies. The filters were hybridized at 65°C and washed to 0.2 x SSC, 0.1% SDS at 65°C.

Firstly, clone 513 was digested with *Sst*I and the fragments were cloned into pBluescript SK^{II}. **A** shows an autoradiograph of the transformants screened with the genomic probe, hsp90f5-r5. Two sub-clones were identified (23 and 5). Sequencing sub-clones 23 and 5 with T7 revealed that the *hsp90* insert was truncated due to an *Sst*I site close to the 3′ end of the gene. The sub-clones were identical and were called *Sst*I90.

To obtain the remaining *hsp90* sequence, an *Sst*I/*Sal*I digestion of 513 was cloned into pBluescript SK^{II} and the transformants were screened with the 3′ end of the cDNA probe, hsp90f4end (contained within an *Hind*III fragment). **B** shows the autoradiograph of the filter probed with this fragment. One of the hybridizing transformants (sub-clone 13) was analyzed and the plasmid had an insert of 3.9kb, which contained the 3′ end of the gene. The sub-clone was called *Sst*I/*Sal*I. Transformants containing the sub-clones *Sst*I90 (23 and 5) and sub-clone *Sst*I/*Sal*I90 are marked with an asterisk.

Figure 4.6 : Identifying the sub-cloned fragments from clone 513



4.2.8 Further sub-cloning

Due to the position of *hsp90* in the lambda clone, sequence information for *hsp90* could only be obtained from one direction. Further sub-cloning was necessary to complete the sequencing of the *hsp90* genomic clone. Digesting *Sst*I90 with *Kpn*I released a fragment of 3.2kb from the plasmid. This removed a region upstream of the gene and a small amount of the 5′ end of the gene. The plasmid was re-ligated and the insert size was thus reduced to 2.5kb and the sub-clone was named *KpnISst*I90. The 3.2kb fragment was sub-cloned cloned into pBluescript SK^{II} to create a new sub-clone, KU90 which contained 2.8kb of sequence upstream from the first ATG. ***Figure 4.7*** summarizes the restriction digests and sub-cloning of *Sst*I90.

4.2.9 Sub-cloning *KpnISst*I90

Sub-clone *KpnISst*I90 was sequenced from either side of the insert but additional information could only be gained with further sub-cloning. A map of the cDNA highlighted potentially useful restriction sites. ***Figure 4.8*** shows the positions and spacing of the restriction sites for *Bgl*II, *Cla*I and *Eco*RI. Using *Bgl*II with *Eco*RI (830bp), *Eco*RI alone (540bp) and *Eco*RI with *Cla*I (710bp) three sub-clones were produced. All the inserts were cloned into pre-cut pBluescript SK^{II} and sequenced. Sequence corresponding to the 3′ UTR was confirmed by ABI sequencing using the primer designated aeed (Table 3.2).

4.2.10 Sub-cloning KU90

A restriction map of the *hsp90* cDNA facilitated sub-cloning of the *KpnISst*I fragment. However, little information was available about restriction sites in the upstream region. Digesting KU90 with various enzymes identified a *Pst*I site which divides the insert into two fragments of approximately equal sizes, 1.4kb and 1.8kb. The smaller fragment, (PKU90) was sub-cloned and sequenced. Additional sequence information at the 3′ end revealed a *Bgl*II site 265bp into the insert. In addition to *Bgl*II, restrictions sites in the multiple cloning site were used to further sub-clone a product of 1.1kb, HBPKU90. ***Figure 4.9*** summarizes the sub-cloning process.

Figure 4.7 : Sub-cloning *Sst*I90

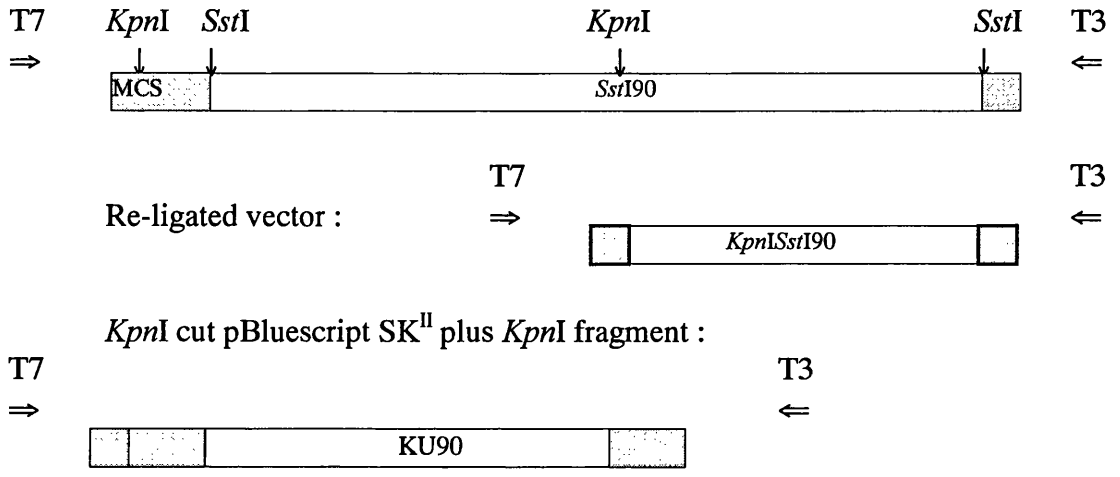


Figure 4.7 :

The open boxes represent *B. pahangi* genomic DNA inserts. The grey regions represent multiple cloning sites (MCS) from pBluescript. The arrows show the orientation of the insert with respect to the T3 and T7 primer sites of the plasmid. *Sst*I90 was digested with *Kpn*I, which released a 3.2kb fragment, and the plasmid was re-ligated to produce *Kpn*I*Sst*I90 (2.5kb). The *Kpn*I fragment was sub-cloned to produce KU90.

Figure 4.8 : Schematic diagram of the restriction fragments from *Kpn*I*Sst*I90

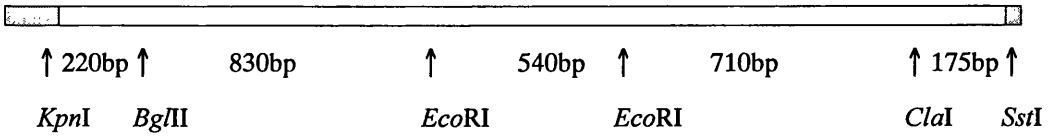


Figure 4.8 :

The open box represents the *B. pahangi* insert (2.5kb) which contains most of the *hsp90* coding region. The grey regions represent multiple cloning sites from pBluescript. *Kpn*I*Sst*I90 was sub-cloned with the restriction enzymes shown and the size of the resulting inserts are marked.

4.2.11 Assembling the sequence of *hsp90* plus putative promoter

The whole genomic sequence was assembled using the GCG gelassemble program, thus creating a consensus sequence of 4.66kb. The sequence contains a region of 1.2kb 5′ to the putative start codon and the coding region covers 3.18kb. The remaining 0.28kb corresponded to the 3′ UTR between the stop codon and the polyadenylation signal, see ***Figure 4.10.***

4.2.12 Comparison of the cDNA and genomic sequences for *hsp90*

There are five bases which differ between the open reading frame of the cDNA and the corresponding genomic sequence. Four of these base differences represent silent changes but one base change converts an alanine in the cDNA to a valine in the genomic sequence (^{445}GCT→GTT). One of the silent changes (at position 192 cDNA) removes an *Eco*RI site from the genomic clone.

4.2.13 Introns interrupt the coding region

A comparison of the cDNA and genomic sequences revealed 11 introns which divide the coding region into 12 exons. The 5′ UTR is identical in the genomic sequence up to the acceptor site, AG, for the spliced leader. In addition, the 3′ UTR is identical up to the site of polyadenylation on the cDNA. The introns all vary in size but none are greater than 200bp and the total amount of non-coding sequence is approximately 1kb. ***Figure 4.11*** is a comparison of the *hsp90* cDNA and genomic sequences. Introns are identified by a gap in the cDNA homology and the presence of conserved splice junctions.

4.2.14 The sequence of the 11 introns from *B. pahangi hsp90*

In ***Figure 4.12***, the junctions of the introns have been aligned and a consensus for *B. pahangi hsp90* introns has been deduced. The splice junctions of the eleven introns from *B. pahangi hsp90* are shown in order of occurrence and the consensus sequence for these junctions has been calculated. The numbers superscripted to the nucleotides indicate the frequency of that base calculated as a percentage of the whole for *hsp90*, e.g. in position 1 adenine occurs 6/11 times = 54%.

Figure 4.9 : Restriction sites used to sub-clone KU90

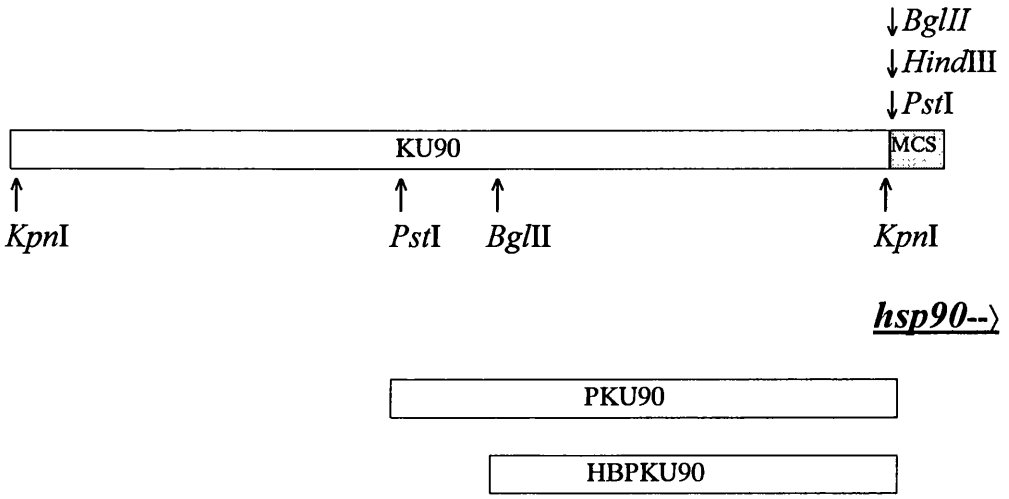


Figure 4.9 :

PKU90 (1.4kb) and HBPKU90 (1.1kb) are sub-clones of KU90. PKU90 was produced by digesting KU90 with *PstI* and HBPKU90 was produced by digesting PKU90 with *BglII*. The direction of the *hsp90* coding region is shown (arrow), approximately 0.2kb of which is contained in the sub-clones. The multiple cloning site of pBluescript SK^{II} is indicated (MCS).

Figure 4.10 : Schematic of the completed sequence for the *B. pahangi hsp90*

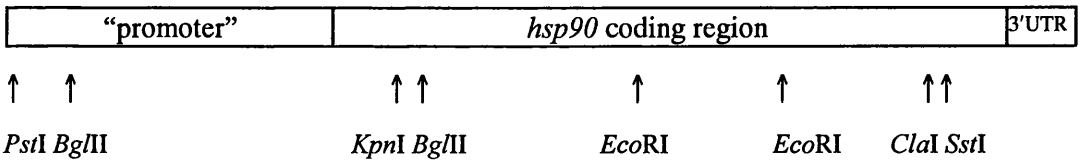


Figure 4.10 :

The restriction enzymes used to clone *hsp90* and "promoter" are shown and the position of the restriction sites are indicated by arrows.

Figure 4.11 : Alignment of the *hsp90* cDNA and genomic sequences

```
 883 CTGAACAAG.............intron 5.............................. 889
     |||||||||
2339 CTGAACAAGGTAGGTTCGAAGACTTAAGAGGTTGTATATGGTTAATGCTTTGGAATAATA 2398

 890 .....................................ACAAAGCCCATTTGGACACGTAA 914
                                          ||||||||||||||||||||||||
2399 TATGCACTTCTGTATTACGCGATTGAATTATTTTTAGACAAAGCCCATTTGGACACGTAA 2458

 915 TCCTGATGATATAAGCAATGAAGAATATGCTGAATTCTACAAGTCACTATCGAATGATTG 974
     ||||||||||||||||||||||||||||||*|||||||||||||||||||||||||||||
2459 TCCTGATGATATAAGCAATGAAGAATATGCGGAATTCTACAAGTCACTATCGAATGATTG 2518

 975 GGAGGATCATCTCGCAGTCAAACATTTCTCGGTTGAAGGGCAACTTGAATTTCGTGCTCT 1034
     ||||||||||||||||||||||||||||||*|||||||||||||||||||||||||||||
2519 GGAGGATCATCTCGCAGTCAAACATTTCTCAGTTGAAGGGCAACTTGAATTTCGTGCTCT 2578

1035 GTTATTTGTACCACAACGTGCACCATTTGATTTGTTTGAGAATAAGAAGACAAAGAATGC 1094
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2579 GTTATTTGTACCACAACGTGCACCATTTGATTTGTTTGAGAATAAGAAGACAAAGAATGC 2638

1095 TATCAAG.............intron 6.............................. 1099
     |||||||
2639 TATCAAGGTCTGTTGGATTCATCAGGCTCTATATGGTTTTACTAGAACTTCTGGAGCTTC 2698

1100 ..................................................CTCTAT 1107
                                                      ||||||
2699 TGCAAAGTCTCGCATATTTCAGCCGTCTGACGCTGTTTTTTTTTTTTTTTTAGCTCTAT 2758

1108 GTTCGCCGAGTGTTCATCATGGAGAATTGCGACGAGTTGATGCCAGAATATTTGAACTTC 1167
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2759 GTTCGCCGAGTGTTCATCATGGAGAATTGCGACGAGTTGATGCCAGAATATTTGAACTTC 2818

1168 ATCAAAGGTGTTGTTGATAGCGAGGACTTGCCGCTAAATATTTCCCGTGAAATGTTGCAG 1227
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2819 ATCAAAGGTGTTGTTGATAGCGAGGACTTGCCGCTAAATATTTCCCGTGAAATGTTGCAG 2878

1228 CAGTCCAAGATATTGAAGGTGATTCGTAAGAATCTTGTCAAAAAATGCCTTGAATTGTTC 1287
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2879 CAGTCCAAGATATTGAAGGTGATTCGTAAGAATCTTGTCAAAAAATGCCTTGAATTGTTC 2938

1288 GACGAAATCGCGGAAGACAAAGACAACTTCAAAAAGTTTTACGAACAGTTTTCGAAGAAT 1347
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2939 GACGAAATCGCGGAAGACAAAGACAACTTCAAAAAGTTTTACGAACAGTTTTCGAAGAAT 2998
```

```
1348 ATAAAA.............intron 7.............................. 1353
     ||||||
2999 ATAAAAGTATTGTTTCCTGTCAGTTTAAATTTTTAAGAGGTGAATTCTATAATCAGAAAT 3058

1354 .....................................CTTGGTATCCATGAAGATTCAACC 1377
                                          ||||||||||||||||||||*|||
3059 GTTCCGTTGTTTCCGCTTGTACACGGCTAATTGCAGCTTGGTATCCATGAAGATTCGACC 3118

1378 AATCGAAAAAAACTTTCGGAGTTCTTACGATTCTATACATCAGCATCTAGCGAGGAGATG 1437
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3119 AATCGAAAAAAACTTTCGGAGTTCTTACGATTCTATACATCAGCATCTAGCGAGGAGATG 3178

1438 ACTTCACTGAAAGATTATGTTAGTCGTATGAAGGAGAACCAGAAGCAAATCTATTTTATT 1497
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3179 ACTTCACTGAAAGATTATGTTAGTCGTATGAAGGAGAACCAGAAGCAAATCTATTTTATT 3238

1498 ACTGGTGAATCCAGGGAAGCTGTCGCCAGTTCTGCATTTGTGGAGCGTGTCAAGAGACGC 1557
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3239 ACTGGTGAATCCAGGGAAGCTGTCGCCAGTTCTGCATTTGTGGAGCGTGTCAAGAGACGC 3298

1558 GGTTTCGAG.............intron 8.............................. 1564
     |||||||||
3299 GGTTTCGAGGTTTGTCAGACAGTCGGATTTATGTCAATCTCTTTTATTTTCCACAATAGA 3358

1565 .................GTTATTTACATGACTGATCCGATAGACGAGTATTGTGTGCAA 1608
                     ||||||||||||||||||||||||||||||||||||||||||||
3359 ATGTTGCAATTTTTACAGGTTATTTACATGACTGATCCGATAGACGAGTATTGTGTGCAA 3418

1609 CAGCTGAAAGAATATGATGGCAAAAAGCTGGTCTCAGTAACTAAGGAAGGTCTTGAACTG 1668
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3419 CAGCTGAAAGAATATGATGGCAAAAAGCTGGTCTCAGTAACTAAGGAAGGTCTTGAACTG 3478

1669 CCTGAAAGTGAGGAGGAGAAGAAGAAATTTGAGGAAGACAAAGTTAAATTTGAGAATTTG 1728
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3479 CCTGAAAGTGAGGAGGAGAAGAAGAAATTTGAGGAAGACAAAGTTAAATTTGAGAATTTG 3538

1729 TGCAAGGTCATGAAGGACATTTTGGAGAAGAAG..... 1759
     |||||||||||||||||||||||||||||||||
3539 TGCAAGGTCATGAAGGACATTTTGGAGAAGAAGGTACATTAAGCGATTTATAATGCATGA 3598

1760 ..... 1760

3599 GTTTAGATAGCGGCTGCTCAATTCGGTTTGGAATACCAGAAATTTGATATAAAGACGTTG 3658
```

Figure 4.11 : Alignment of the *hsp90* cDNA and genomic sequences

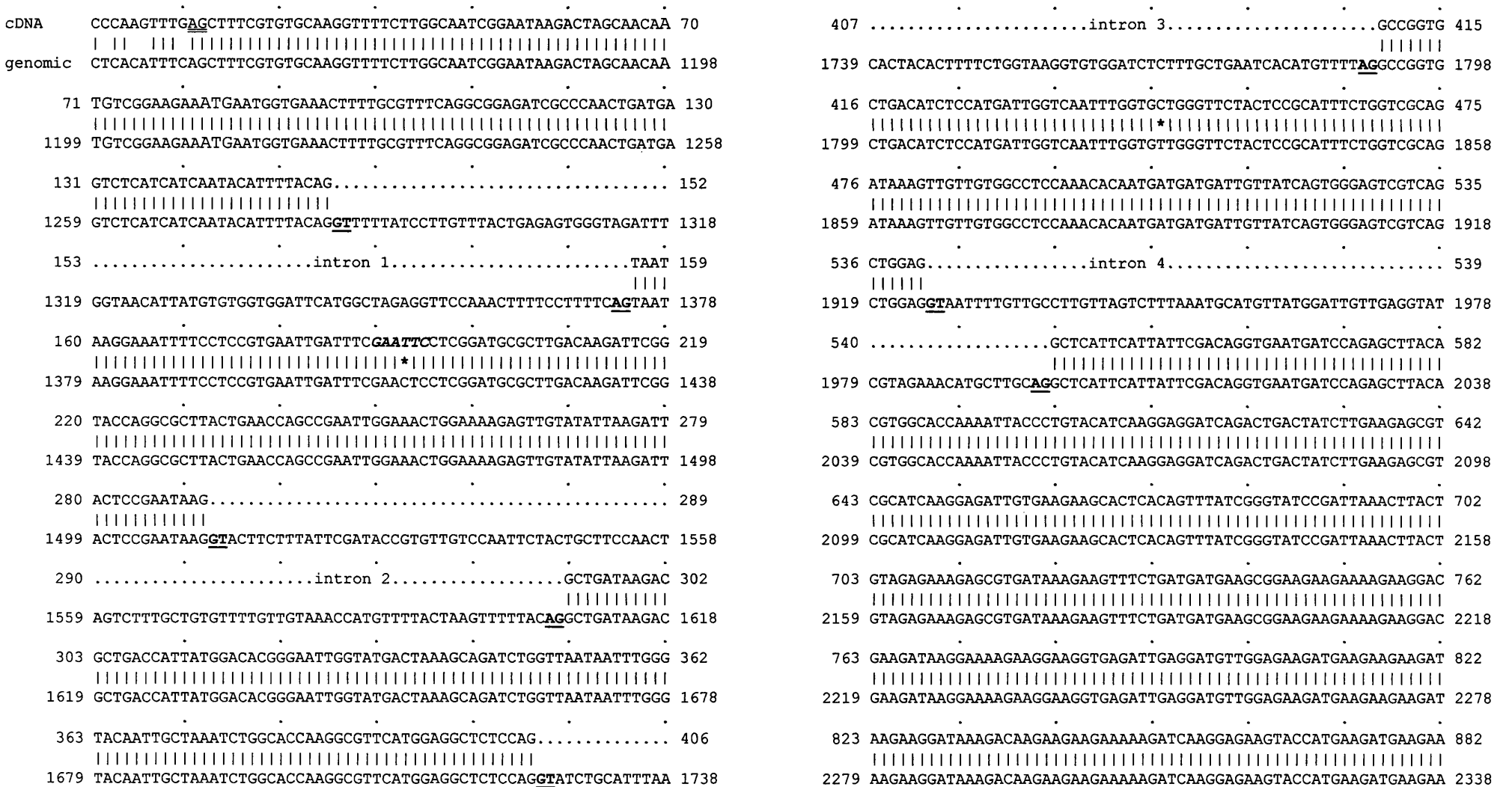


Figure 4.11 :

Base changes between the genomic and cDNA sequences are indicated by an asterisk (*) Three *Eco*RI sites are in bold, italic (***GAATTC***): note the loss of a site in the genomic sequence (nt.1411) and an additional genomic site in intron 7. The SL1 *trans*-splice site is indicated (AG) and the genomic and cDNA sequences do not align prior to this due to the addition of the mini-exon to the cDNA sequence. Donor and acceptor splice sites of introns are in bold and underlined (**GT**/**AG**) Two possible start methionines are in larger text (ATG) at nts.70 and 82, the stop codon is in bold (**TAA**) at nt. 2221 and the polyadenylation signal is in bold (**AATAAA**) at nt. 2488.

Figure 4.11 : Alignment of the *hsp90* cDNA and genomic sequences

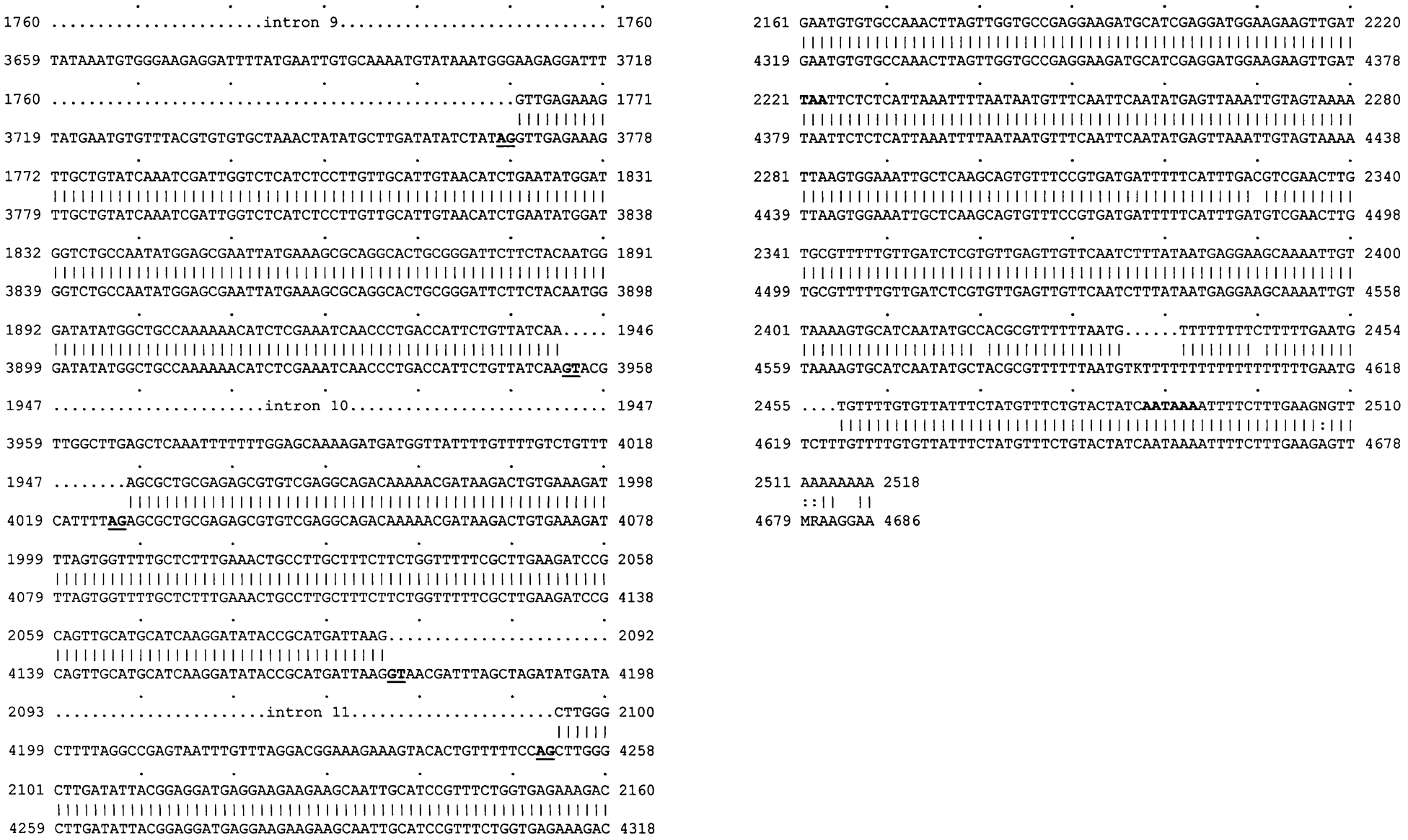


Figure 4.12 : Comparing the splice junctions of the introns in *hsp90*

Brugia pahangi hsp90 :

Exon	Donor	Acceptor	Exon
C A G	**G T** T T T T	T T T C **A G**	T
A A G	**G T** A C T T	T T A C **A G**	G
C A G	**G T** A T C T	T T T T **A G**	G
G A G	**G T** A A T T	T T G C **A G**	G
A A G	**G T** A G G T	T T T T **A G**	A
A A G	**G T** C T G T	T T T T **A G**	C
A A A	**G T** A T T G	T T G C **A G**	C
G A G	**G T** T T G T	T T A C **A G**	G
A A G	**G T** A C A T	C T A T **A G**	G
C A A	**G T** A C G T	T T T T **A G**	A
A A G	**G T** A A C G	T T C C **A G**	C

HSP90 CONSENSUS :

$A^{54}A^{100}G^{82}\,|\,G^{100}T^{100}A^{73}T^{45}\begin{matrix}T^{36}\\ \\ G^{36}\end{matrix}T^{82}\quad T^{91}T^{100}T^{45}\begin{matrix}C^{55}\\ A^{100}G^{100}\\ T^{45}\end{matrix}\,|\,G^{45}$

Figure 4.13 The splice junctions in *B. malayi, Bmhs1*

Brugia malayi Bmhs1 (hsp70) :

Exon	Donor	Acceptor	Exon
A A G	**G T** A C A A	T T G C **A G**	G
T C G	**G T** A A A T	T T T C **A G**	A
A A G	**G T** G C T T	T T A C **A G**	G
G A G	**G T** A T G T	C A G G **A G**	A
G A G	**G T** A C A T	T T C C **A G**	T
G A A	**G T** G A G T	T T G A **A G**	G
C A G	**G T** T T G T	A T T C **A G**	G
A A G	**G T** A A T T	T T C C **A G**	T
G A G	**G T** A A T G	T T T C **A G**	G

JOINT CONSENSUS :

$A^{45}A^{95}G^{85}\,|\,G^{100}T^{100}A^{70}T^{35}\begin{matrix}G^{35}\\ \\ T^{35}\end{matrix}T^{80}\quad T^{85}T^{95}T^{40}C^{65}A^{100}G^{100}\;G^{50}$

The splice junctions of *B. pahangi hsp90* were compared with those of another *Brugia hsp*, the *B. malayi* gene, *Bmhs1*. This gene is a member of the *hsp70* family and has the highest homology to *hsc70* genes, heat shock cognates which are expressed constitutively. The splice junctions for the nine introns from *Bmhs*1 are presented in ***Figure 4.13*** in order of occurrence in the sequence. The joint consensus utilizes the information for both *B. pahangi hsp90* and *B. malayi Bmhs1*, 20 introns in total (see ***Figure 4.13***).

To identify any discrepancies in the splice site sequences from *hsp90* and *Bmhs*1, the joint consensus sequence can also be compared to that calculated for *Brugia* species by Hammond (1994) who used 53 introns from *Brugia* genes to create a splice junction consensus. This is shown below.

$$\begin{matrix} A^{40} & & & & & & & & & & C^{58} & & \\ & A^{69}\ G^{75} & | & G^{100} & T^{100} & A^{56} & A^{54} & G^{54} & T^{69} & T^{77} & T^{92}\ T^{60} & A^{100} & G^{100} & | & G^{60} \\ C^{27} & & & & & & & & & & T^{34} & & \end{matrix}$$

Both the consensus from the *B. pahangi hsp90* splice junctions and the joint consensus for the splice junctions from the two heat shock genes closely correlate with the consensus calculated, by Hammond (1994), for *Brugia* spp. Indeed the GT/AG boundaries are completely conserved. The seventh nucleotide of the Hammond consensus is stated as a 54% occurrence of "A" but in the *hsp90/Bmhs1* consensus suggests a T at this position. However the preference for "T" is weak (7/20 nucleotides) and adenine is represented at this position in some of the introns (6/20 nucleotides).

4.2.15 Features of the 11 introns in *hsp90*

In ***Figure 4.14***, the intron sequences are shown in full. They vary in length from 67bp to 197bp. One interesting observation is an almost identical repeat within intron 9. These two regions are shown in bold in ***Figure 4.14***. This may represent a duplication of part of the intron which occurred in the evolution of the species. The repeat is shown below :

TGTATAAATGTGGGAAGAGGATTTTATGAATTGTG
TGTATAAATG-GG-AAGAGGATTTTATGAAT-GTG

Figure 4.14 : The 11 introns of *B. pahangi hsp90*

1 GTTTTTATCCTTGTTTACTGAGAGTGGGTAGATTTGGTAACATTATGTGTGGTGGATTCATGGCTAGAGGTTCCAAACTTTTCCTTTTCAG

2 GTACTTCTTTATTCGATACCGTGTTGTCCAATTCTACTGCTTCCAACTAGTCTTTGCTGTGTTTTGTTGTAAACCATGTTTTACTAAGTTTTTACAG

3 GTATCTGCATTTAACACTACACTTTTCTGGTAAGGTGTGGATCTCTTTGCTGAATCACATGTTTTAG

4 GTAATTTTGTTGCCTTGTTAGTCTTTAAATGCATGTTATGGATTGTTGAGGTATCGTAGAAACATGCTTGCAG

5 GTAGGTTCGAAGACTTAAGAGGTTGTATATGGTTAATGCTTTGGAATAATATATGCACTTCTGTATTACGCGATTGAATTATTTTTAG

6 GTCTGTTGGATTCATCAGGCTCTATATGGTTTTACTAGAACTTCTGGAGCTTCTGCAAAGTCTCGCATATTTCAGCCGTCTGACGCTGTTTTTTTTTTTTTTTTTAG

7 GTATTGTTTCCTGTCAGTTTAAATTTTTAAGAGGTGAATTCTATAATCAGAAATGTTCCGTTGTTTCCGCTTGTACACGGCTAATTGCAG

8 GTTTGTCAGACAGTCGGATTTATGTCAATCTCTTTTATTTTCCACAATAGAATGTTGCAATTTTTACAG

9 GTACATTAAGCGATTTATAATGCATGAGTTTAGATAGCGGCTGCTCAATTCGGTTTGGAATACCAGAAATTTGATATAAAGACGT**TGTATAAATGTGGGAAGAGGATTTTATGAATTGTG**

CAAAA**TGTATAAATGGGAAGAGGATTTTATGAATGTG**TTTACGTGTGTGCTAAACTATATGCTTGATATATCTATAG

10 GTACGTTGGCTTGAGCTCAAATTTTTTTGGAGCAAAAGATGATGGTTATTTTGTTTTGTCTGTTTCATTTTAG

11 GTAACGATTTAGCTAGATATGATACTTTTAGGCCGAGTAATTTGTTTAGGACGGAAAGAAAGTACACTGTTTTTCCAG

Figure 4.14 :

The introns from *B. pahangi hsp90* are numbered according to order of appearance in the sequence. The regions of intron 9 in bold are almost identical and may represent a duplication of a section of the intron. All 11 introns have the donor (GT) and acceptor (AG) sites completely conserved.

Figure 4.15 : An alignment of intron 10 from *B. pahangi hsp90* and intron 3 from *C. elegans hsp90*

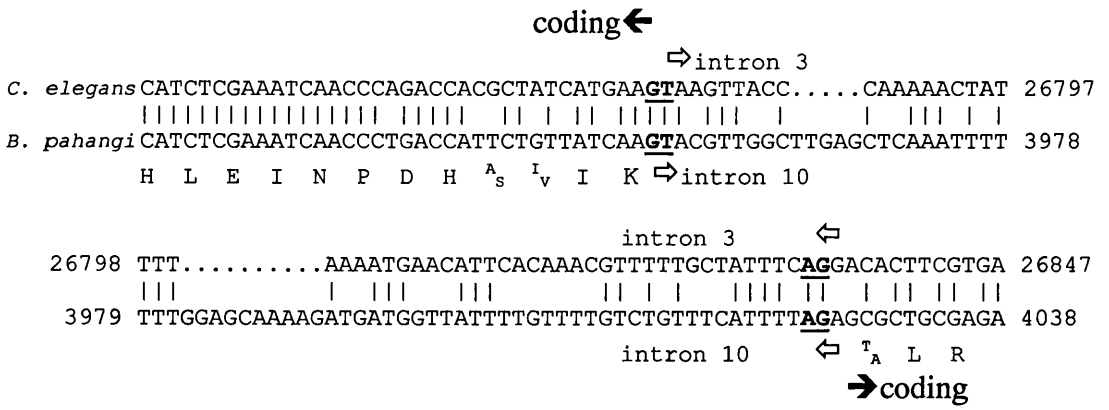


Figure 4.15 :

The alignment of a region of *C. elegans* z75530 (*hsp90*) and *B. pahangi hsp90* is shown and highlights the conserved position of an intervening sequence. The corresponding amino acid sequence has been added for clarity and a divergence of the sequence is denoted by the use of superscript for *C. elegans* residues and subscript for *B. pahangi* residues. The splice donor (GT) and acceptor (AG) are in bold, underlined. The open arrows show the exon/intron boundary and the filled arrows show the position of coding regions.

Figure 4.16 : The three introns from *C. elegans hsp90*

Exon	Donor	Acceptor	Exon
C A G	**G T** T T G T	T T T T **A G**	G
A A G	**G T** A T T T	T T T C **A G**	A
G A A	**G T** A A G T	T T T C **A G**	G

Brugia pahangi hsp90 splice junction consensus :

$$A^{54}A^{100}G^{82}\,|\,G^{100}T^{100}A^{73}\begin{matrix}T^{45}\\T^{36}\\G^{36}\end{matrix}\;T^{82}\qquad \begin{matrix}C^{55}\\T^{91}T^{100}T^{45}\\T^{45}\end{matrix}\;A^{100}G^{100}\,|\,G^{45}$$

The *C. elegans* cosmid C47E8 (z75530), which contains *hsp90*, was aligned with the *B. pahangi* genomic *hsp90* sequence. There are three introns which interrupt the *C. elegans* coding region, the third of which corresponds to intron 10 of the *Brugia* gene. ***Figure 4.15*** is an alignment of intron 10 and the corresponding region in *C. elegans.* This non-coding region has not been completely conserved but sequence similarities are evident. Indeed, the fact that these two nematodes have an intron at the same position may highlight the similarity of the two species. However, it may also indicate that the intron has a role in controlling the expression of the gene. In ***Figure 4.16***, the splice junctions from the three *C. elegans hsp90* introns have been aligned and the consensus sequence from *B. pahangi hsp90* has been added for comparison. The *C. elegans* splice junctions also adhere to the consensus sequence.

4.3 Southern analysis of *B. pahangi hsp90*

In other species, two or more genes have been identified which code for *hsp90*. In *L. donovani* multiple copies of *hsp90* have been identified, adjacent to each other in the genome. However, in vertebrates, such as the mouse, two types of *hsp90* gene exist which code for different proteins (HSP90α and HSP90β). To investigate whether *B. pahangi* contained multiple copies of *hsp90* and/or more than one type of *hsp90* gene is coded for in the parasite genome, the lambda clone of *hsp90* (513) and *B. pahangi* genomic DNA were subjected to Southern analysis.

Two restriction digests were carried out on the lambda clone and on *B. pahangi* genomic DNA, the first using *Cla*I and the second using both *Cla*I and *Kpn*I. In addition, *Bam*HI and *Kpn*I were used, separately, to digest genomic DNA. When *Cla*I and *Kpn*I were both utilized on either 513 or on genomic DNA, a fragment of 2.4kb was produced. The filters were hybridized with a cDNA probe (pe90) corresponding to the last 714bp of the open reading frame. The probe contains an internal *Cla*I site (see ***Figure 4.17***).

Figure 4.17 : The probe : pe90

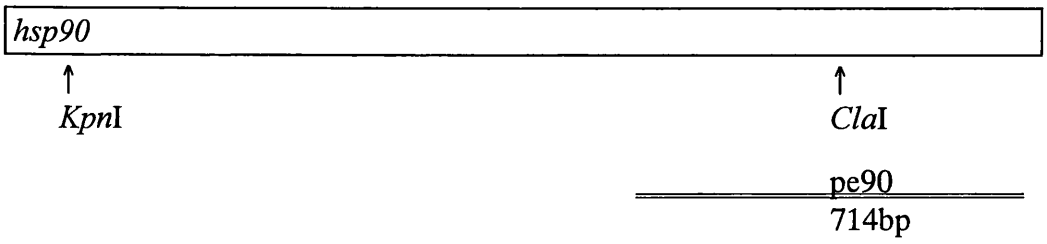


Figure 4.18*A** shows an agarose gel containing digested genomic DNA, which has been stained with ethidium bromide and is visible as a smear. *Figure 4.18B,** lane 5 shows seven major bands which were produced by digesting clone 513 with *Cla*I and one minor band, which may possibly be the result of partial digestion. In lane 6, digesting with *Cla*I and *Kpn*I resulted in the loss of two major bands (7-9kb) observed in the single digest with *Cla*I and the appearance of six additional bands.

***Figure 4.18*C,** lanes 1 and 4 shows that probing the *Bam*HI and *Kpn*I digests of *B. pahangi* genomic DNA with pe90 resulted in single bands. In lanes 2 and 3, two bands are visible as a result of hybridizing the *Cla*I and *Cla*I/*Kpn*I digests of genomic DNA. In lane 2, the band of higher molecular weight is quite faint and a second band of approximately 4.5kb appears to be the same size as a band observed in lane 3. A band of approximately 2.4kb is observed is lane 3, but is not visible in lanes 1, 2 or 4. In lanes 5 and 6, the probe hybridizes to two bands of similar sizes (7-9kb) in the *ClaI* digest of 513 and also to two bands in the *Cla*I/*Kpn*I digest of 513. The bands in lane 5 are larger in size than those visible in lane 6. In lanes 3 and 6, pe90 hybridizes to a 2.4kb band in the *Cla*I/*Kpn*I digests both of genomic DNA and of 513.

In *B. pahangi* genomic DNA, *Kpn*I cuts at a position 5′ to that of the *Cla*I site in *hsp90.* A restriction digest using *Kpn*I/*Cla*I and probed with pe90, identified two fragments, one of which is smaller (2.4kb) than both fragments obtained from a *Cla*I only digest. In addition, the smallest *Cla*I/*Kpn*I fragment originates from the larger of the two *Cla*I fragments. However the presence of a *Cla*I fragment (~5kb) in both single and double digest lanes implies that *Kpn*I does not cut between the *Cla*I site in *hsp90* and the next *Cla*I site downstream.

Figure 4.18 :

Panel **A** shows an ethidium bromide stained gel containing adult *B. pahangi* genomic DNA digested with the following enzymes:

1 *Bam*HI

2 *Cla*I

3 *Cla*I/*Kpn*I

4 *Kpn*I

Panel **B** shows an ethidium bromide stained gel containing lambda clone 513, which has been digested with the following enzymes:

5 *Cla*I

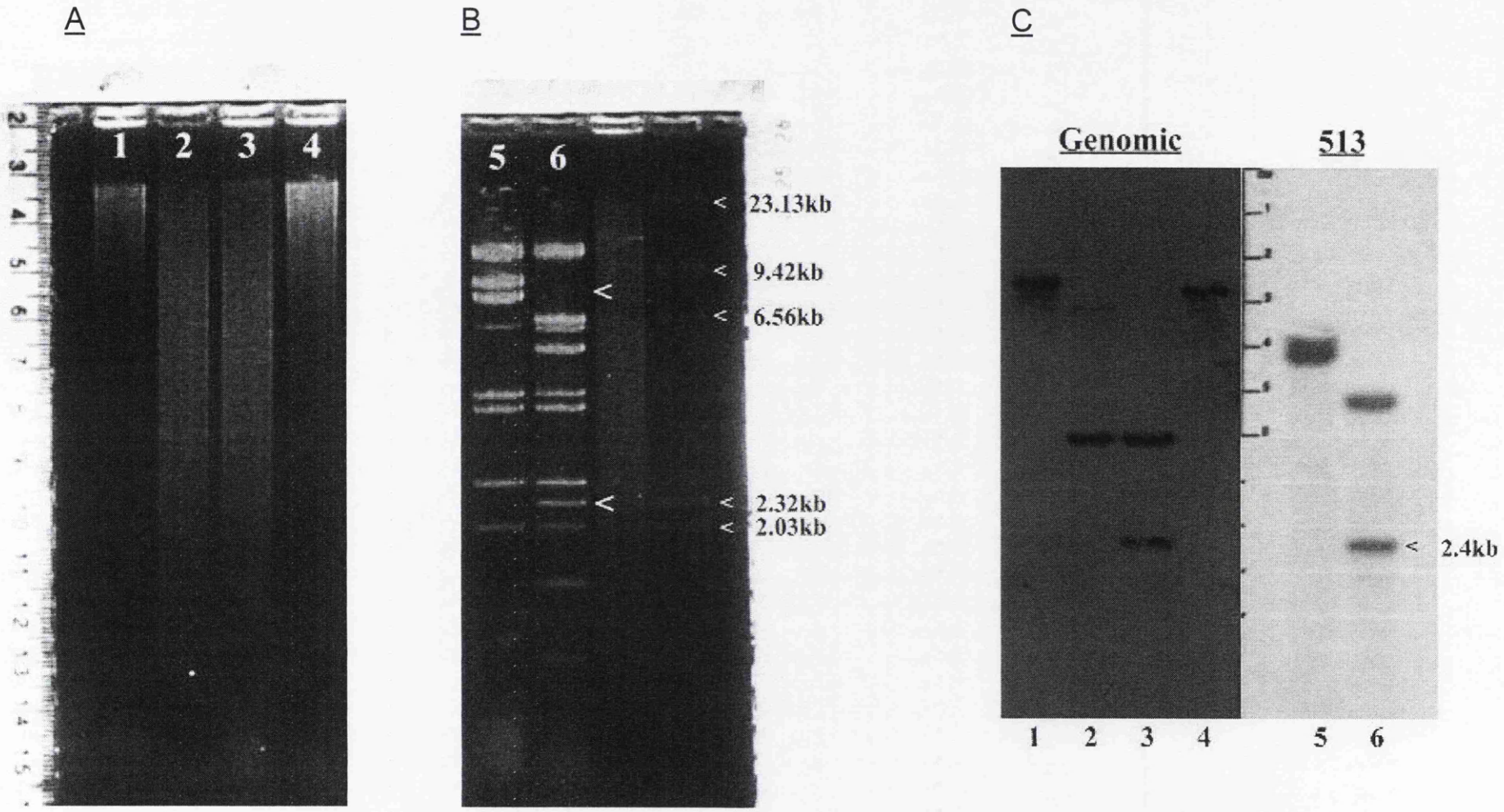
6 *Cla*I/*Kpn*I

The right hand lane in **B** contains λ*Hind*III markers, indicated with arrows.

Panel **C** shows corresponding autoradiographs for **A** (genomic) and **B** (513), which have been probed with pe90. The filters were hybridized at 65°C, washed to 0.2 x SSC, 0.1% SDS at 65°C and exposed to film.

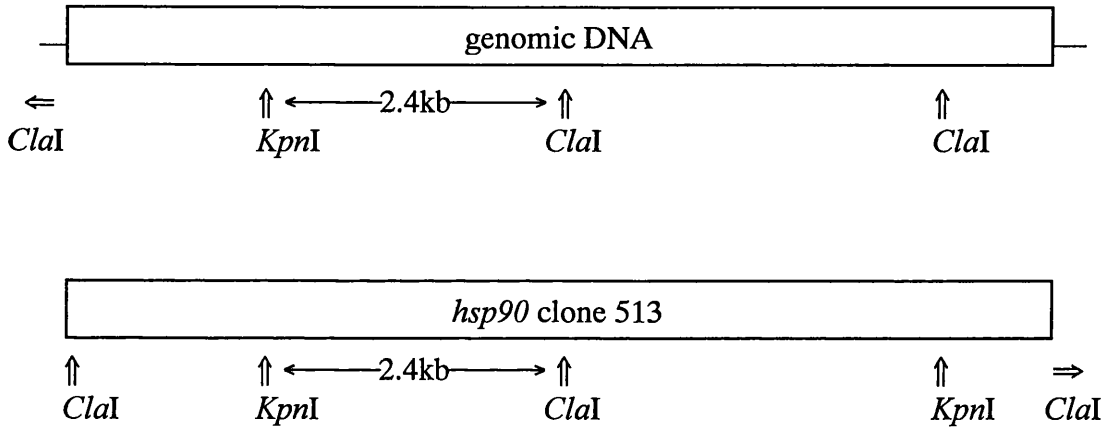
Large white arrows in **B** indicate two bands of 7-9kb in lane 5, which are not present in lane 6, and a band of 2.4kb in lane 6 which is observed in **C** (lanes 3 and 6). This 2.4kb band, present in both the genomic DNA and λ513 Southern blots, is also indicated with an arrow in **C**.

Figure 4.18 : Southern analysis of clone 513 and genomic DNA



During the construction of the *B. pahangi* genomic library, 3′ *Cla*I sites may have been removed from the genomic DNA fragment containing *hsp90*. This would explain why pe90 hybridized to bands of different sizes in the *Cla*I digest of clone 513 compared to the *Cla*I digest of genomic DNA (see ***Figure 5.19***).

Figure 4.19 : Schematic of the *Cla*I and *Kpn*I sites in genomic DNA and clone 513



There appears to be one copy of *hsp90* in the *B. pahangi* genome, since two or more copies of the same gene would result in more than two bands in a *Cla*I digest. The pe90 probe can hybridize to *hsp90* fragments immediately upstream and downstream from this restriction site. A second gene would result in one or two additional bands depending on the presence or absence of a *Cla*I site between the two *hsp90* copies.

4.3.1 Southern analysis to investigate the presence of a related *hsp90* homologue in the *B. pahangi* genome

While the analyses carried out so far did not suggest that a second *hsp90* gene identical to the first was present in the *B. pahangi* genome, it would not have permitted the identification of a related gene, as all hybridizations were subject to high stringency. In order to further investigate the possible presence of a related gene, an additional series of Southern analyses were carried out at moderate stringency. Genomic DNA was digested with *Eco*RI, *Hind*III, *Kpn*I and *Pst*I and probed with a fragment (hsp90f5-r3) containing 2.21kb of the *hsp90* genomic sequence. Hsp90f5-r3 starts in the middle of the second potential start codon and covers most of the coding region. There are two

*Eco*RI sites in the probe one of which is in an intron. However, the corresponding cDNA sequence has an *Eco*RI site which is absent in the genomic fragment (as discussed in 4.2.12) due to a single base substitution. A *Hind*III site is present in the cDNA clone but intron 11 disrupts this site in the genomic sequence.

***Figure 4.20*a** is a photograph of an ethidium bromide stained gel showing *B. pahangi* genomic DNA which has been digested with *Pst*I (lane 1), *Kpn*I (lane 2), *Hind*III (lane 3) and *Eco*RI (lane 4). The sizes of λ*Hind*III markers are shown.

Panel **b** is the autoradiograph of the genomic Southern blot (**a**) probed with hsp90f5-r3 and washed to moderate stringency. Panel **c** is the same Southern blot washed again to high stringency. Single bands were observed in the *Pst*I, *Kpn*I and *Hind*III digests (lanes 1-3) on both autoradiographs (compare panels **b** and **c**) but multiple bands are visible in the *Eco*RI digested samples (lane 4).

In lane 5, a second genomic Southern blot has been probed with hsp90f5-r3. This additional lane shows the *Eco*RI digested *B. pahangi* genomic DNA washed to high stringency and exposed for 6 days. Five bands are clearly visible and have been labelled A-E for convenience.

The single bands in the *Pst*I (lane 1), *Kpn*I (lane 2) and *Hind*III (lane 3) digests do not support the hypothesis that an additional gene with moderate homology is present in the genome. The pattern of hybridization obtained with the *Eco*RI digest is more complex. Panel **d** shows an autoradiograph of the Southern blot (**a**) probed with hsp90f5-r5. This fragment covers the *Eco*RI site near the 5′ end of the *hsp90* cDNA (not present in the genomic clone) but does not contain the two *Eco*RI sites further downstream. Lanes 1-3 contain single bands but three bands are observed in the *Eco*RI digest (lane 6).

Figure 4.20 :

Panel **a** shows an ethidium bromide stained gel containing genomic DNA digested with the following enzymes:

1 *Pst*I

2 *Kpn*I

3 *Hind*III

4 *Eco*RI

There are λ*Hind*III markers in the first and last lanes and their sizes are shown on the left-hand side. Panel **c**, lane 5 is from a different Southern blot of adult *B. pahangi* genomic DNA and this sample was digested with *Eco*RI. Panel **d**, lane 6 corresponds to panel **a**, lane 4.

Hybridization was carried with hsp90f5-r3 :

b The blot was washed to low stringency, (2 x SSC, 0.1% SDS), at 55°C. After being exposed to film for 6 days, the filter was washed to high stringency (**c**).

c The blot was washed to 0.2 x SSC, 0.1% SDS at 65°C and exposed to film for 14 days. Lane 5 (an *Eco*RI digest from a different blot), was treated in the same way.

Hybridization was carried out with hsp90f5-r5 :

d The blot was washed to high stringency, (0.2 x SSC, 0.1%SDS), at 65°C and exposed to film for 14 days.

The hybridizing bands, visible in the *Eco*RI lanes (4, 5 and 6), have the following sizes :

A 0.55kb

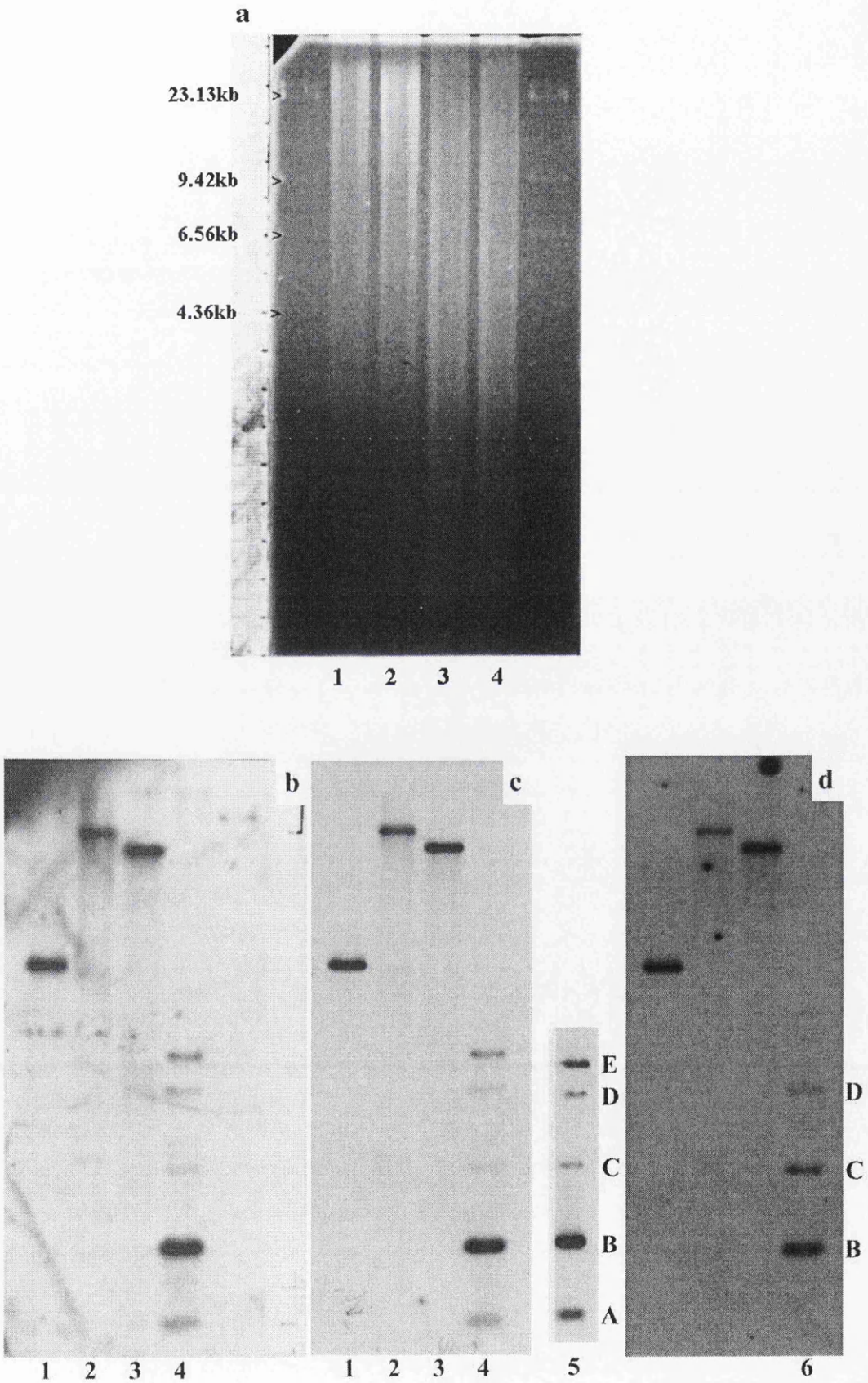
B 1.08kb

C >1.26kb, <2kb

D >1.41kb, <2kb

E >2.49kb, <4.1kb

Figure 4.20 : Genomic Southern analysis at moderate and high stringency



4.4 Discussion

Five lambda clones were isolated which hybridized to probes designed from both a 5′ and an internal region of *hsp90*. Restriction digests of these clones and subsequent hybridization with an *hsp90* probe was consistent with five identical inserts. It should be noted that the method of screening would bias for the identification of a genomic copy of the previously isolated cDNA clone. Although the two probes were amplified from genomic DNA, the primers for hsp90f5-r5 were designed from the cDNA sequence and the genomic hsp90f2-r2 corresponded to the cDNA product, hsp90f2-r2. Since only duplicate positives were chosen, the likelihood of isolating an homologous gene was reduced, especially at the high stringency used.

A comparison of the cDNA and genomic sequences for *hsp90* revealed five base differences. Two of these base changes were of particular interest. There is a single amino acid substitution in the cDNA which may be due to a PCR error during amplification of this region (valine GTT→alanine ^{445}GCT). Valine is the most probable amino acid since the *C. elegans* cosmid C47E8 containing *hsp90* and three *B. malayi* ESTs coding for *hsp90* have the same codon, coding for valine, at this position. In addition, the *P. falciparum hsp90* [Bonneyfoy *et al* 1994] codes for valine here, as do many other HSP90s (see ***Figure 3.10***). Thus it is reasonable to hypothesize that the substitution in the *B. pahangi* cDNA sequence was due the mis-incorporation of a nucleotide during PCR amplification. Although the PCR product was sequenced on a number of occasions, this highlights the necessity to sequence more than one PCR product.

The second base change, an *Eco*RI site present in the cDNA clone, is absent in the genomic sequence due to a base substitution (gaa^{192}Ttc→gaa^{1411}Ctc, ***Figure 4.11***). An *Eco*RI site is present at this position in three *B. malayi* ESTs. The restriction site is not present in *C. elegans hsp90* but there is sequence divergence around this region. As the *Eco*RI site is also present in *hsp90* from a closely related species it is less likely that the sequence divergence is due to a PCR error during the amplification of the cDNA. However, the *B. malayi* cDNA library from which the comparable *hsp90* sequences were obtained was constructed using a PCR-based method, so the possibility of PCR

error cannot be ruled out. A further possible explanation is a polymorphism of *hsp90* genes in a population of *B. pahangi*. Polymorphism in the *hsp70*A gene between *Heterorhabditis* species were identified by RFLP analyses [Hashimi *et al* 1997]. In addition, Snutch and Baillie (1984) compared the 5′ and 3′ flanking regions of *hsp70* from the Bristol and Bergerac strains of *C. elegans* and reported a higher than normal accumulation of mutations in these regions. In *P. falciparum*, one cDNA coding for *hsp90* was observed to contain an extra two codons when compared with a second *hsp90* cDNA cloned from the same species [Su and Wellems 1994]. These genes were cloned from two different strains of *P. falciparum*. Furthermore, the genomic hsp90f5-r5 PCR product (see 4.2.2), which was amplified from *B. pahangi* genomic DNA from *B. pahangi* adult worms available in the laboratory, was sequenced and an *Eco*RI site was identified at position 1411. The genomic library, from which the *hsp90* clone was isolated, was constructed from a strain of *B. pahangi* maintained at Geissen, Germany and it is possible that there are polymorphisms between the Giessen strain and the strain maintained in Glasgow.

A comparison of the intron splice junctions in the eleven intervening sequences of *hsp90* revealed a complete conservation of donor and acceptor splice sites. In addition, the nucleotides adjacent to these sites are consistent with the previously described consensus sequence for *B. pahangi* species [Hammond 1994]. As mentioned, one of the three *C. elegans hsp90* introns coincides with intron 10 in *B. pahangi hsp90*. The intron sequences are not identical but do have some homology, especially near the splice junctions. The position of this intron, in *hsp90,* is therefore conserved between two different nematodes.

The presence of multiple intervening sequences in *B. pahangi* is not unique for a *Brugia* gene. For example, *B. malayi* myosin heavy chain gene has 14 introns and *B. pahangi* β-tubulin has 8 introns [Hammond and Bianco 1992]. Furthermore, multiple introns are not uncommon in *hsp90* genes from other species. Human *hsp90*α and *hsp90*β have 10 and 11 introns respectively [Brandon *et al* 1989, Rebbe *et al* 1989]. However *hsp90* from *C. elegans* has three introns, while *hsp90* genes from *Drosophila* and *Anopheles* have only one. In addition the only intron positions which relate to those of *B. pahangi*

hsp90 are from chicken *hsp90*β, one intron of which coincides with intron 3 and *C. elegans hsp90* the third intron of which coincides with intron 10. It is interesting that genes from species as diverse as chicken and nematode have an intervening sequence at the same position and may imply a role for intron in controlling the expression of the gene.

There are two hypotheses for the presence of introns. One is the "introns-late" theory and the second is the "introns-early" theory [Long *et al* 1995]. The first theory proposes that the primordial genes had no intervening sequences and with time introns were inserted either randomly or partially randomly [Palmer and Logsdon 1991]. The second theory proposes that the primordial genes were created by mini-exons linked together to form functional genes and that the introns which acted as "linkers", were lost with time [Gilbert and Glynias 1993].

Drosophila [Yost and Lindquist 1986], *Anopheles* [Benedict *et al* 1996], *C. elegans* [ACeDb] and *B. pahangi* are all members of the controversial Ecdysozoa clade [Aguinaldo *et al* 1997] but the *hsp90* gene from *B. pahangi* has many more introns than *hsp90* from the other three species. *B. pahangi hsp90* does however have one intron position in common with *C. elegans hsp90*. In addition, it shares an intron position with a vertebrate *hsp90* (chicken) and has a similar number of intervening sequences to two mammalian *hsp90*s [Hickey *et al* 1989, Rebbe *et al* 1993]. This may imply a model (introns-early) where the primordial gene had many introns and lost these during evolution. If an insertion event in *hsp90* at the emergence of the Nematoda resulted in intron 3 (*B. pahangi*)/intron 10 (*C. elegans*), this would explain why both species possess this intervening sequence. A further 10 insertions may then have occurred in *B. pahangi*, after the divergence of the two species. However, a combination of insertion and intron removal may have given rise to the introns in *hsp90*.

It is possible that *B. pahangi* and other species do not have an absolute requirement for newly translated HSP90 during extreme cellular insult. Indeed *D. melanogaster* HSP83 is expressed at high levels during a moderate heat shock (33-35°C) but during an extreme heat shock (37°C) there is a much lower level of expression. The genomic sequence of *hsp83* contains an intron and although there is a high concentration of

hsp83 transcripts during extreme heat stress, disruption of RNA splicing in *Drosophila* appears to result in incorrect protein translation [Yost and Lindquist 1986]. However, extreme heat shock does not always result in disruption of splicing, HSP82 from *Histoplasma capsulatum* is expressed at high levels during a severe heat shock (40-42°C). Two introns present in *hsp82* are properly spliced under these conditions [Minchiotti *et al* 1991].

Hsp90 from *B. pahangi*, human [Hickey *et al* 1989, Rebbe *et al* 1989] and chicken [Meng *et al* 1993] and the homologue of *hsp70*, *Bmhs1* [Rothstein and Rajan 1991] from *B. malayi* contain multiple introns which may result in the disruption of protein expression during an extreme heat shock. As mentioned in 3.13, larger than normal transcripts of a *B. pahangi* small *hsp* are observed when mf are heat shocked at 41°C for 2 hours and this may result from the disruption of RNA splicing under these conditions. Most of the *hsp90* genes appear to be regulated by (moderate) heat induction but they are also induced by non-heat stimuli. Indeed, chicken *hsp90*β is only marginally induced by heat, suggesting an additional role in the absence of heat shock [Meng *et al* 1993] *B. pahangi hsp90* is differentially expressed in adult worms and mf at mammalian body temperature (see 3.2.14). However, the expression of *hsp90* mRNA after a heat shock (41°C) is induced in both life cycle stages. The gene may therefore be regulated both by stress and by a developmental factor. In *D. melanogaster*, the heat shock and developmental regulation of *hsp83* has been proposed to involve different regions of the promoter [Xiao and Lis 1989].

Due to the existence of *hsp90* genes, which contain introns, including the above examples, it is feasible that the presence of intervening sequences has a function in the expression of the protein. Indeed, the first intron of the human gene for platelet derived growth factor (PDGF) appears to interact with the promoter region and negatively or positively regulates transcription depending on the cell type used for expression [Franklin *et al* 1991]. Similarly, the first intron of the Bruton's tyrosine kinase gene (*Btk*) contains two transcriptional control elements, Sp1 binding sites, which positively regulate the expression of the gene plus a second region, required for cell-type specific negative regulation of transcription [Conley 1998].

Introns do not only appear to affect transcription. Okkema *et al* (1993) observed that an intron near the 5′ end of the *C. elegans* myosin heavy chain gene, *unc-54* stimulated expression and that this effect was independent of intron sequence. A study of chicken *zfp-36* which encodes tristetrapolin (TTP), a putative zinc finger protein, also revealed that the presence of an intron in the gene resulted in a higher level of expression of TTP when compared with an equivalent intron-less construct. The control of expression appeared to be post-transcriptional implying a role for the intron in the rate of translation of *zpf-36* transcripts [Lai *et al* 1998]. Researchers have reported an apparent translational silencing of *in vivo* transcribed mRNA of the intron-less gene, histone H1 in *Xenopus* oocytes in contrast to the efficient translation of transcripts for the intron-containing TFIIIA gene [Matsumoto *et al* 1998]. It was also observed that *hsp70*, which lacks introns, was also translationally repressed in oocytes [Bienz 1984].

A lack of introns in *hsp*s, which should ensure translation of these HSPs during a severe heat shock, may, (like the intron-less *Xenopus* histone H1 gene) result in minimal expression under normal conditions. Indeed at normal temperatures, *Drosophila* HSP70 is almost undetectable [Lindquist 1986]. The advantage of introns in *hsp90* genes may be to facilitate the accumulation of HSP90, which is required at a much higher concentration under normal conditions than HSP70.

It is not only the presence of introns in *hsp*s that is thought to have a role in the control of HSP expression. The 5′ and 3′ UTRs of *hsp* mRNA are implicated in the stability and preferential translation of the transcripts. In *Drosophila*, preferential translation of *hsp22* during heat shock requires specific sequences in the 5′ UTR [Hultmark *et al* 1986]. In addition, an investigation of *hsp70* mRNA indicates that at higher temperatures the minimal secondary structure observed in the 5′ UTR is crucial for efficient translation [Hess and Duncan 1996]. *Hsp70* mRNA is inherently unstable at normal temperatures and it is proposed that a mechanism operates through the 3′ UTR to rapidly degrade transcripts. This mechanism is thought to be inactivated by heat shock thus permitting *hsp* mRNA to accumulate to higher levels [Yost *et al* 1990]. In *Leishmania*, *hsp83* mRNA is rapidly degraded at normal temperatures but increases in stability with an increase in temperature, thus resulting in rapid HSP83 accumulation

during a heat shock [Argaman *et al* 1994]. Temperature dependent decay of *hsp83* mRNA appears to be controlled by both the 5′ and 3′ UTRs. Truncations of either UTR that abolished temperature dependent degradation of the *hsp83* mRNA also eliminated the preferential translation of *hsp83* at elevated temperatures [Aly *et al* 1994]. A comprehensive study by Joshi and Nguyen (1995) of the 5′ UTRs from 140 *hsp* genes, from a variety of eukaryotes, suggested a motif which may function as an internal ribosome entry site for the selective translational initiation of the mRNA during heat shock. However, such a motif is not evident in the 5′ UTR of the *B. pahangi hsp90*. The sophisticated expression of *B. pahangi* HSP90 may involve a combination of transcriptional and translational control mechanisms including a requirement for such a seemingly large number of introns.

The analysis of the *B. pahangi hsp90* lambda clone and genomic DNA was consistent with the cloned *hsp90* homologue being a single copy gene. This was demonstrated by a restriction digest with *Cla*I and *Kpn*I, sites for which are present in the *hsp90* lambda clone (513). When 513 and genomic DNA were digested with the two enzymes and were then probed with pe90, an identical band of 2.4kb was observed for both samples. In addition, hybridization and washing at moderate stringency implies that there are no other *hsp90* homologues in the *B. pahangi* genome. The *C. elegans* sequencing project has to date only identified a single locus for a sequence with high homology to cytoplasmic *hsp90*s, (see ***Figure 4.21***).

The analysis of *B. pahangi* genomic DNA digested with *Eco*RI and probed for *hsp90* resulted in an unexpected and complex pattern of hybridizing bands (***Figure 4.20***, lanes 4-6). One possible explanation of these findings originates from the fact that a number of worms are used to make genomic DNA. As mentioned in 4.2.12, there is single base difference between the cDNA and genomic clones, which results in the removal of an *Eco*RI site from the genomic sequence of *hsp90*. Putative polymorphisms between the Giessen strain and the Glasgow strains of *B. pahangi* (inter-strain variation) were mentioned previously, but it is also possible that differences may exist between individuals within a population of worms (intra-strain variation). The majority of individuals in the Giessen strain may have *hsp90*s that lack the *Eco*RI, suggesting why

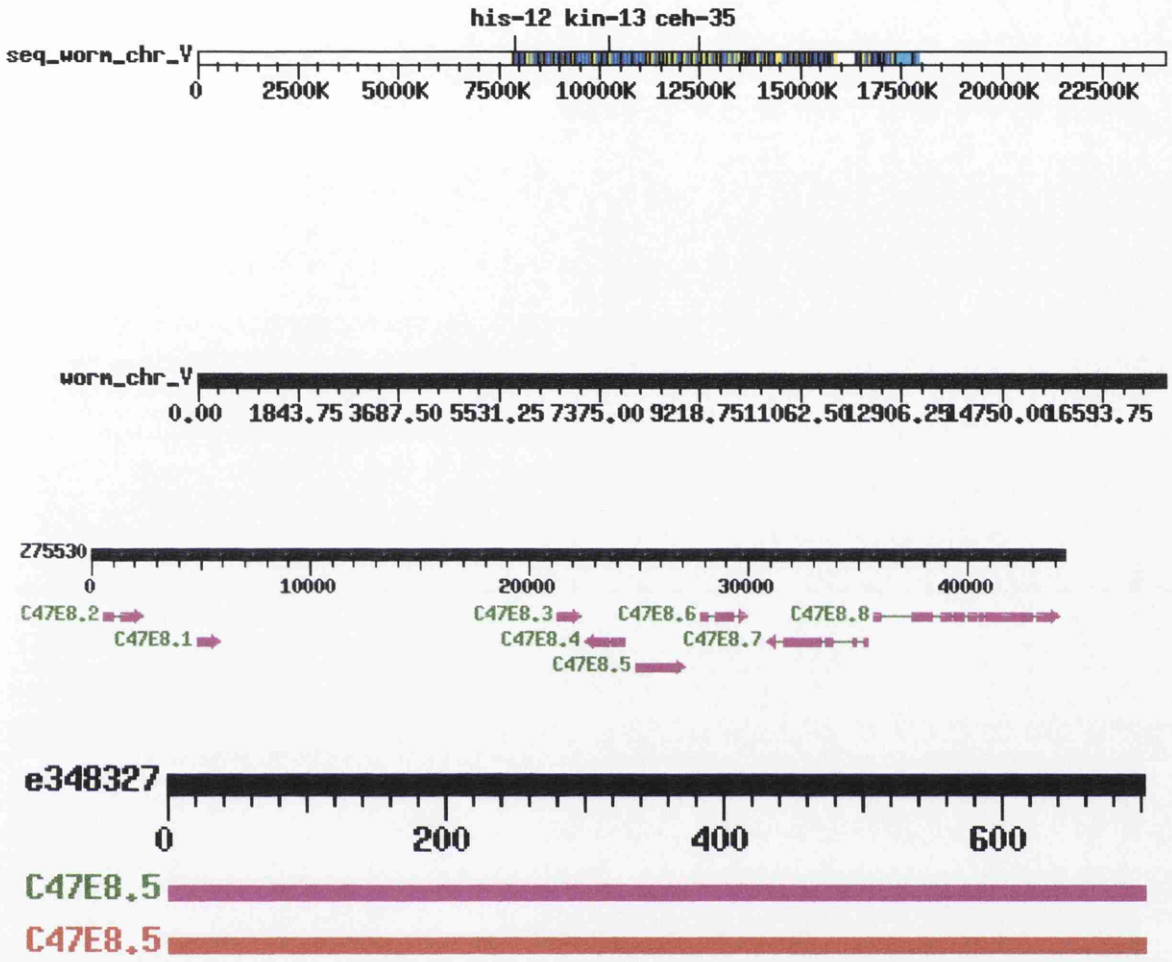
the *hsp90* clone isolated from the *B. pahangi* library lacked this restriction site, whilst the majority of individuals in the *B. pahangi* strain, from Glasgow, may have *hsp90*s with the *Eco*RI site at position 1411. However, in both strains, there may some *B. pahangi* individuals with and some without the *Eco*RI site. The resulting fragments from an *Eco*RI restriction digest of genomic DNA would therefore be polymorphic. A diagrammatic representation of this hypothesis showing the fragments which may hybridize to *Eco*RI digests of *B. pahangi* genomic DNA probed with hsp90f5-r3 and hsp90f5-r5 is shown in ***Figure 4.22.*** However it is possible that the bands observed in the Southern blots are a result of partial digestion of genomic DNA by *Eco*RI.

The results presented in this chapter confirm the sequence data presented in Chapter 3 and revealed the organization of the genomic clone. The upstream sequence of the gene was next used to make a reporter gene construct to investigate this region as a putative heat shock promoter.

In summary

- a genomic clone of *hsp90*, was isolated from a *B. pahangi* library. The gene and a 1.2kb upstream region were sub-cloned and sequenced
- comparison of the cDNA and genomic sequences revealed the presence of 11 introns and five base changes
- Southern blot analyses suggested that *hsp90* was a single copy gene and that related genes were not present in the *B. pahangi* genome

Figure 4.21 : *C. elegans hsp90* is located on chromosome V




```
LOCUS       1418460       702 aa                              23-JAN-1998
DEFINITION  C47E8.5.
ACCESSION   1418460
PID         g1418460
DBSOURCE    EMBL: locus CEC47E8, accession Z75530
SOURCE      Caenorhabditis elegans.
FEATURES             Location/Qualifiers
     source          1..702
                     /organism="Caenorhabditis elegans"
                     /db_xref="taxon:6239"
                     /chromosome="V"
                     /clone="C47E8"
     Protein         1..702
                     /product="C47E8.5"
     CDS             1..702
                     /note="Similarity to Human heat shock protein HSP-90
        1 msenaetfaf qaeiaqlmsl iintfysnke iylrelisna sdaldkiryq altepseldt
       61 gkelfikitp nkeektltim dtgigmtkad lvnnlgtiak sgtkafmeal qagadismig
      121 qfgvgfysaf lvadkvvvts knndddsyqw essaggsfvv rpfndpevtr gtkivmhike
      181 dqidfleerk ikeivkkhsq figypiklvv ekerekeved eeaveakdee kkegevenva
      241 ddadkkktkk ikekyfedee lnktkpiwtr npddisneey aefykslsnd wedhlavkhf
      301 svegqlefra llfvpqrapf dlfenkkskn siklyvrrvf imenceelmp eylnfikgvv
      361 dsedlplnis remlqqskil kvirknlvkk cmelidevae dkdnfkkfye qfgknlklgi
      421 hedstnrkkl sdflrystsa gdeptslkey vsrmkenqtq iyyitgeskd vvaasafver
      481 vksrgfevly mcdpideycv qqlkeydgkk lvsvtkegle lpeteeekkk feedkvayen
      541 lckvikdile kkvekvgvsn rlvsspcciv tseygwsanm erimkaqalr dsstmgymaa
      601 kkhleinpdh aimktlrdrv evdkndktvk dlvvllfeta llasgfslee pqshasriyr
      661 miklgldigd deiedsavps sctaeakieg aeedasrmee vd
```

Figure 4.22 : Proposed fragments created by an *Eco*RI digest of *hsp90*

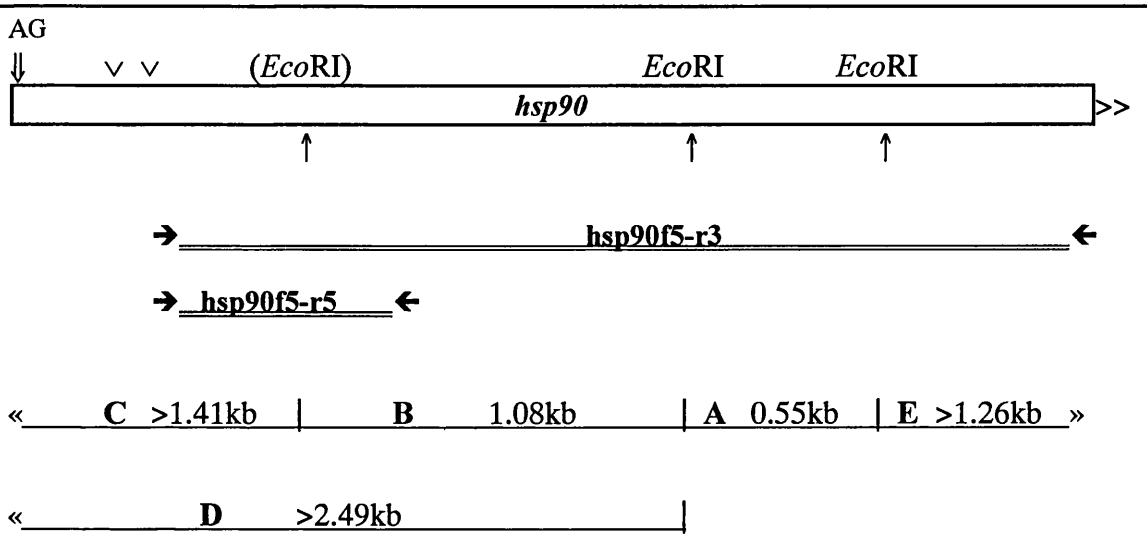


Figure 4.22 :

The *trans*-splice site (AG) is shown (⇓) and the two possible start codons are also indicated (∨). The sequence of the probe, hsp90f5-r3 starts in the middle of the second ATG and is 2.21kb long. Hsp90f5-r5 spans the *Eco*RI site of interest. The five fragments (**A-E**) which hsp90f5-r3 could theoretically have hybridized to are shown above. The sizes of **A** and **B** can be calculated from the genomic sequence. Note the region covered by **D** would be divided into **B** and **C** by the presence of the controversial *Eco*RI site. Hsp90f5-r5 could hybridize to **B**, **C** and **D** if present.

5.0 Analysis of the putative *B. pahangi hsp90* promoter

5.1 Introduction

The differential expression of *B. pahangi hsp90* observed in mf and adult worms appears to be controlled at least partially at the level of transcription as is evident from the Northern blot analysis of the mRNA. Therefore it was of interest to investigate the upstream region of the gene to define potential regulatory elements. The *Brugia malayi* sequencing project is providing considerable amounts of new data on cDNA clones expressed at different life cycle stages. However at the present time very little is understood of how gene expression is controlled in parasitic nematodes. One way to explore this particular area further is to isolate genomic sequences and analyze upstream regions for putative transcription factor binding sites.

In *C. elegans*, putative promoter regions may be analyzed by the use of transfection. The sequence of interest is ligated to a reporter gene and the promoter-driven expression is proportional to the rate of accumulation of the reporter gene product. Stable transfection requires the micro-injection of embryonic stages of the worm. In addition to the studies on *hsp16* in *C. elegans*, the *C. elegans hsp16* gene fused to *E. coli* β-galactosidase has been successfully transfected, by micro-injection, into the entomopathogenic nematode, *Heterorhabditis bacteriophora* [Hashmi *et al* 1995]. In filarial nematodes, it is not yet possible to culture worms *in vitro* from the first stage larvae through a series of developmental moults to adults. This means that stable transfection of these parasites is not a viable option.

However, mammalian cells have been transiently transfected with reporter gene constructs containing putative helminth promoters. For example, Levy-Holtzmann and Schechter (1995) investigated the *S. mansoni hsp70* promoter by ligating the putative promoter to chloramphenicol acetyl transferase (CAT), a protein which is not expressed naturally by eukaryotic cells and transfecting Chinese hamster ovary (CHO) cells with the construct. The level of CAT expression was studied after a series of treatments including heat shocking the cells at 42°C. The heat shock induction of CAT expression, demonstrated that the mammalian transcriptional apparatus was capable of initiating transcription from the *S. mansoni* heat shock promoter. The stress response appeared to

be sufficiently conserved between species for the trematode heat shock promoter to increase the expression of CAT upon activation by CHO transcription factors.

5.2 Results

The genomic clone of *hsp90* (513) contained 1.2kb of sequence upstream from the open reading frame (ORF) referred to in 4.2.11 and shown in ***Figure 4.10***. This region was sequenced on both strands and the sequence was analyzed for putative regulatory elements. An on-line sequence interpretation tool, "TFSEARCH", was utilized for this purpose (pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). An example of the output from the analysis is shown in *Appendix II.* The number of transcription factor binding sites indicated is dependent upon the threshold parameter (i.e. the sequence divergence permitted between the regulatory elements from the TRANSFAC database and the input sequence).

5.2.1 Putative regulatory elements identified in the *hsp90* "promoter"

Analysis of the *hsp90* upstream sequence identified putative binding sites for transcriptional control factors. ***Figure 5.1*** shows the upstream sequence of *hsp90* with putative transcriptional control elements indicated. These include three putative TATA boxes, which are homologous to the prokaryotic Pribnow box [Dale 1998] and may be involved in the initiation of transcription [McKnight and Kingsbury 1982].

-892 → -884	G**TATA**AAAG
-875 → -867	G**TATA**AAGT
-275 → -267	A**TATA**AAAG
TATA consensus	S**TATA**AAWR

Another regulatory element, the CCAAT box, the binding site for the NY-1 transcription factor, which functions both in the forward and reverse orientation was also identified. The *hsp90* "promoter" contains two putative inverted CCAAT sequences (-617 → -609, -340 → -332) homologous to the consensus for the cellular and viral CCAAT boxes [Bucher 1990]. Three CCAAT boxes were identified in the *S. mansoni hsp70* promoter [Neumann *et al* 1992] and also in the *S. mansoni* glutathione-S-transferase promoter [Serra *et al* 1996]. NY-1, like the TATA box binding protein, is thought to have a role in the transcription of a range of genes, but has also been

implicated in the recruitment of additional regulatory factors to a promoter [Wright *et al* 1994].

-617 → -609	AT**CCAAT**CC
-340 → -332	AA**CCAAT**GA
CCAAT consensus	RR**CCAAT**SA

There is a putative Sp1-binding site (inverted GC box) which is homologous to the consensus for the GC box elements, [Bucher 1990].

-320 → -308	GT**GGGCGG**TGCTT
GC box consensus	RG**GGGCGG**GGCNK

There are two putative inverted GATA elements (-1159 → -1151, -808 → -800) which have a high homology to the consensus sequence of thirteen elements from *C. elegans* and *C. briggsae* vitellogenin genes [Zucker-Aprison and Blumenthal 1989].

-1159 → -1151	ACT**GATA**AA
-808 → -800	TAT**GATA**AG
Vit consensus	ACT**GATA**AG

5.2.2 Putative heat shock factor binding elements in the *B. pahangi hsp90* "promoter"

Heat shock elements are a characteristic feature of the promoters of eukaryotic heat shock genes and consist of inverted repeats of the pentanucleotide NGAAN. A HSF monomer can bind to one pentanucleotide of an HSE. Thus three inverted repeats permit maximum interaction of an HSF trimer. [Perisic *et al* 1989]. The region of *B. pahangi hsp90* sequenced has five HSEs, which have completely conserved pentanucleotide inverted repeats. ***Figure 5.2*** shows the *B. pahangi* HSEs aligned with HSEs from the upstream region of *hsp* genes from *C. elegans*, *S. mansoni* and *B. malayi* and also with an HSE from another heat shock gene, a small *hsp* from *B. pahangi, Bphsp7*. All these HSEs adhere to the NGAAN consensus. Analysis of the incidence of nucleotides at positions in the helminth heat shock elements identified a preference for adenine in the first position (AGAAN) which is consistent with a previous analysis of HSEs from *Drosophila* and *Saccharomyces* species [Fernandes *et al* 1994].

Figure 5.1 : Putative transcriptional control regions upstream of *B. pahangi hsp90*

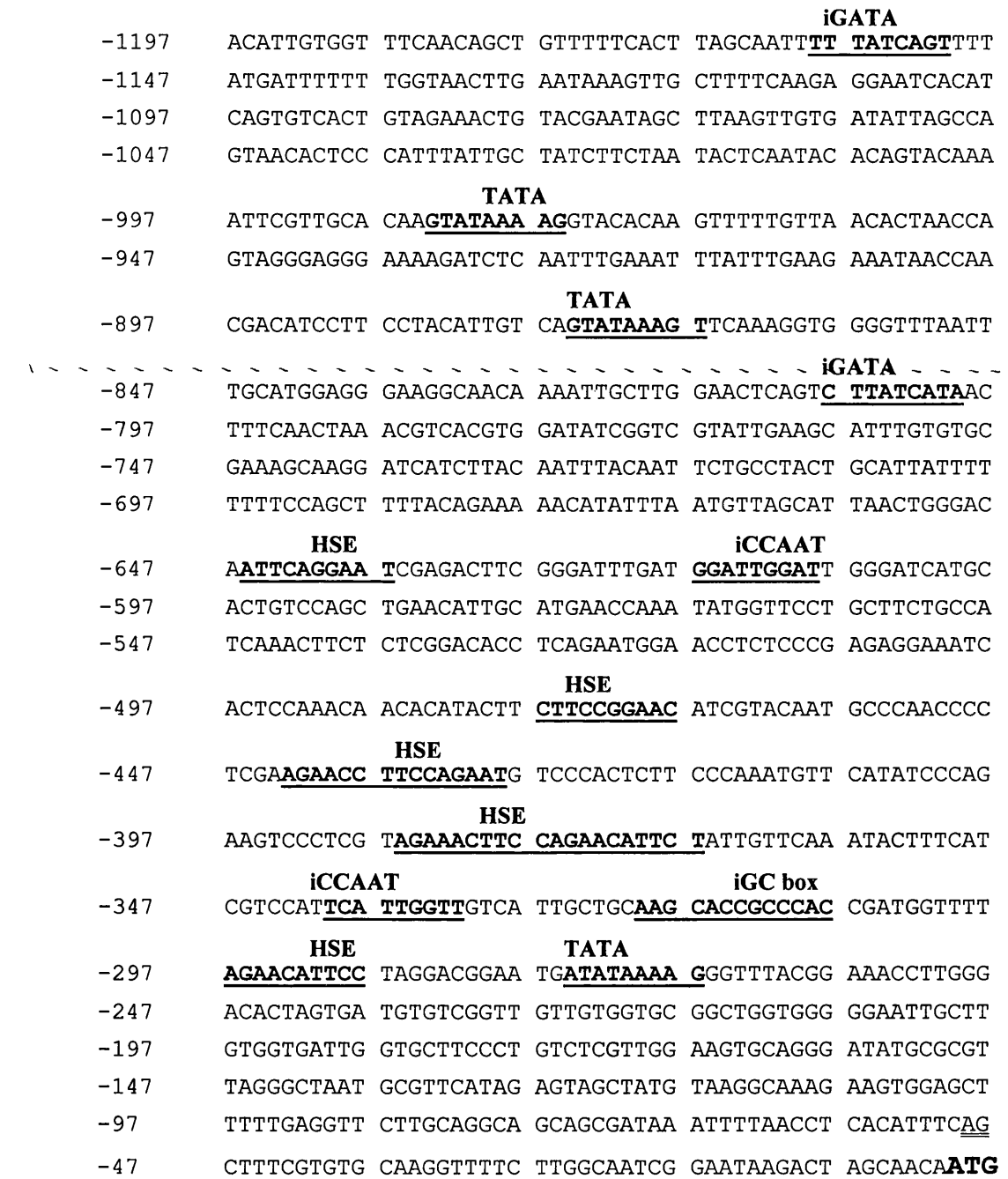


Figure 5.1 :

Putative transcription factor binding elements (shown in bold and underlined) include three TATA boxes, five HSEs, two inverted (i) CCAAT boxes, two inverted GATA boxes and an inverted GC box. The *trans*-splice site, indicated by a comparison of the *hsp90* genomic and cDNA clone, is double underlined. Numbering of the upstream region is based on the first methionine of *hsp90* (shown in bold, larger text).

Figure 5.2 : Heat shock elements from helminth heat shock protein promoters

Smhsp70 (*S. mansoni*)	AGAAAGTTCT	
Cehsp70A (*C. elegans*)	CGAACATTCT	
	CGAACATTCT	
	CGAATTTTCTAGAAT	
Cehsp16 (*C. elegans*)	AGAATGTTCTAGAAG	
	CGAATGTTCTAGAAA	
Bmhs1 (*B. malayi*)	GTTCCAGAACATTCT	
(*hsp70*)	GTTCTAGAACATTCT	
	TGAACATTCT	
	CTTCCAGAACATTCG	
Bphsp7 (*B. pahangi*)	GGAAAATTCC	
Bphsp90 (*B. pahangi*)	ATTCAGGAAT	distal
	CTTCCGGAAC	|
	AGAACCTTCCAGAAT	|
	AGAAACTTCCAGAACATTCT	↓
	AGAACATTCC	proximal

NGAAN = 21

position 1 :	A 13/21	C 4/21	G 3/21	T 1/21
position 5 :	A 4/21	C 10/21	G 1/21	T 6/21

NTTCN = 20

position 1 :	A 10/20	C 4/20	G 5/20	T 1/20
position 5 :	A 1/20	C 7/20	G 1/20	T 11/20

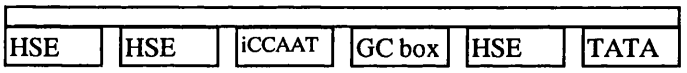
Figure 5.2

There is a preference for adenine at position 1 (and thymidine at position 5 in the reverse orientation). There also appears to be a preference for cytidine at position 5 for the NGAAN orientation, but in contrast there is a preference for adenine at position 1 in the NTTCN orientation. The consensus for the above heat shock elements could therefore be modified to : AGAAN with a possible C biasing at position 5.

5.2.3 Designing a reporter construct controlled by a *B. pahangi* "promoter"

In promoters from the genes of higher eukaryotes the TATA box is usually situated 25 to 30 bases from the transcription initiation site. The transcriptional start site has also been mapped for some nematode genes, for example, in the vitellogenin genes from *C. elegans* and *C. briggsae*, the transcription initiation sites map approximately 30 bases (and all less than 35 bases) from the TATA box (see ***Figure 5.9***). Primers, 210pf1 and p1pr1, were designed from the available *B. pahangi hsp90* upstream sequence to amplify a compact region of 0.24kb which contained: the putative TATA box; GC box; three HSEs and an inverted CCAAT box. The amplified region, verified by sequencing, also contained sequence 38 bases downstream of the TATA box designed to include a transcriptional start site.

The HSE1 fragment :



Two CAT plasmids were available in the laboratory: pCAT12, which contains the bacterial *cat* gene [Kirby and Vapnek 1979] fused with the Herpes simplex virus immediate early 5 gene polyadenylation sequence [Spandidos and Riggio 1986] and pLW2, which contains the *cat* gene (transposon-9), polyadenylation signal and a 210bp fragment from the HSV-2 IE gene 4/5 promoter [Gaffney *et al* 1985]. Plasmid pLW2 (3.5kb) contains a TATA box and two inverted copies of the GC box (binding site for the Sp1 family of transcription factors). Transfection of pLW2 into an eukaryotic cell line results in the constitutive expression of CAT at a high level and thus the plasmid is useful as a positive control of transfection. In contrast, CAT is not under the control of a promoter in pCAT12 and therefore this plasmid may be utilized as a negative control to assess the level of non-promoter driven CAT expression. To permit the formation of a *B. pahangi* promoter-reporter gene construct, an *Sst*I site was added to the forward primer and a *Bam*HI site added to the reverse primer to allow the directional cloning of the "promoter" region upstream of a CAT reporter gene within the plasmid, pCAT12. The *B. pahangi* construct was designated HSE1-pCAT12.

COS-7 (monkey kidney) cells were transiently transfected with the constructs. Cells (~4 x 10^5) were grown for 24 hours and then transfected. The cells were grown for a further

72 hours and were lysed directly on the plates using a hypotonic lysis buffer. The COS-7 cell lysates were diluted to 250μg/ml, before being assayed by ELISA for CAT concentration (see ***Figure 5.3***).

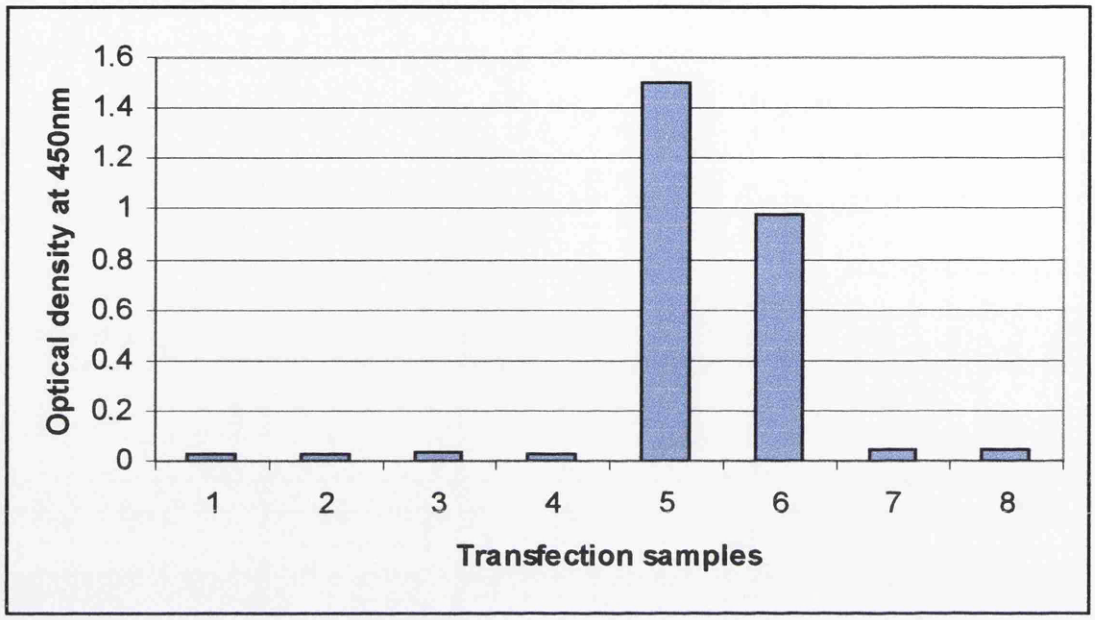
After the initial experiment with LipoTAXI (Stratagene), problems occurred with subsequent transfection experiments resulting in no detectable CAT or a very low concentration being detected. Although many of the conditions such as the amount of transfection reagent added, the time of harvest after transfection and the number of cells used were modified, substantial quantities of the reporter gene product were not obtained. For example, ***Figure 5.4*** shows the results from an experiment where the harvest time was changed from 72 hours to 48 hours after transfection. The ELISA plates required a 24-hour incubation at 4°C before CAT could be detected in the positive control samples (pLW2). Eventually a different transfection reagent, Lipofectin (Gibco BRL) was employed, which resulted in an efficient transfection measured by the CAT activity in the pLW2 transfected samples (see ***Figure 5.5***).

Since the positive control gene gave a good level of CAT expression, it was possible to assess whether or not the region, HSE1, of the *B. pahangi* promoter was active in driving CAT expression. From the results presented in ***Figure 5.5***, it is evident that the HSE1-pCAT12 construct did not significantly increase the expression of CAT protein, when transfected into COS-7 cells, even when the cells were heat shocked for an hour at 41°C. Indeed the concentration of CAT detected in the HSE1-pCAT12 samples was similar to the level detected in the negative control, pCAT12, consistent with a lack of promoter-driven transcription.

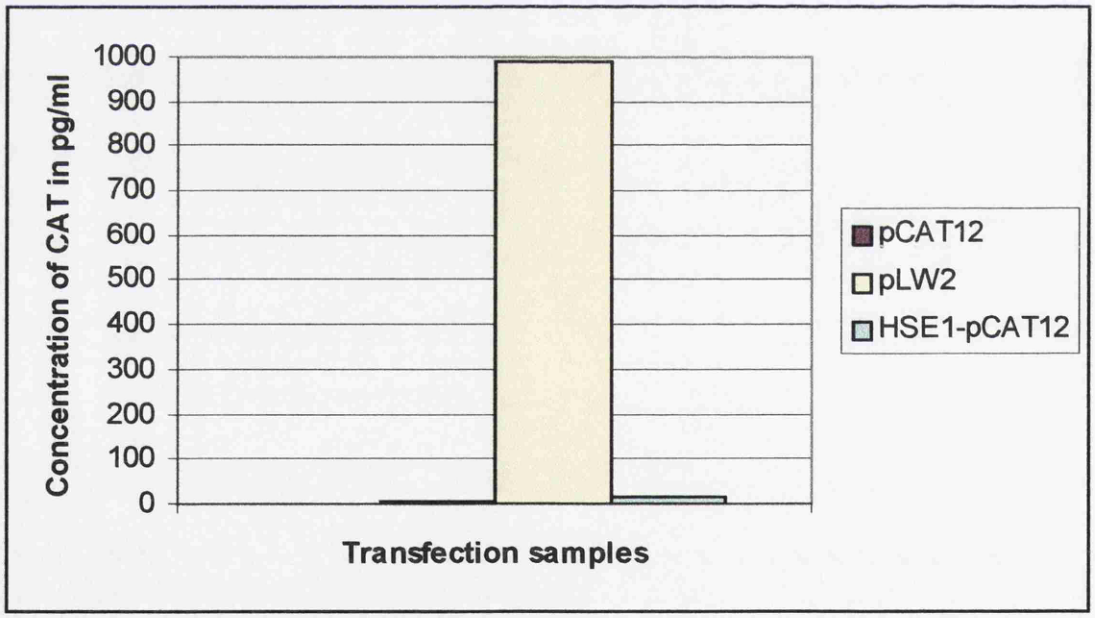
Further examination of the HSE1-pCAT12 sequence, suggested that an ATG codon at the extreme 3′ terminus of the HSE1 fragment may cause initiation of translation upstream from the actual CAT start codon, producing nonsense transcripts which could not be translated to CAT. A further possibility was that the transcriptional start site may be further downstream from the putative TATA box and therefore not included in the HSE1-pCAT12 construct. The decision was therefore made to map the *B. pahangi hsp90* transcriptional start site and to use this information to amplify an appropriate section of the *hsp90* "promoter" and to create a second reporter gene construct.

Figure 5.3 :

A



B



In graph **B** the mean values of CAT concentration have been calculated from the following samples in graph **A**:

pCAT12	3 and 4
pLW2	5 and 6
HSE1-pCAT12	7 and 8

Figure 5.3 : Transfection of CAT reporter gene constructs using LipoTAXI

50µg of lysate (250µg/ml)		Optical density at 450nm			conc. in pg/ml	
Sample		O.D.1	O.D.2	Mean	[CAT]	Mean
1	control	0.024	0.029	0.027	2	
2		0.024	0.028	0.026	2	2
3	pCAT12	0.031	0.040	0.035	9	
4		0.028	0.027	0.027	2	6
5	pLW2	1.539	1.466	1.502	1204*	
6		0.875	1.074	0.974	774	989
7	HSE1-pCAT12	0.040	0.041	0.041	14	
8		0.037	0.052	0.044	16	15

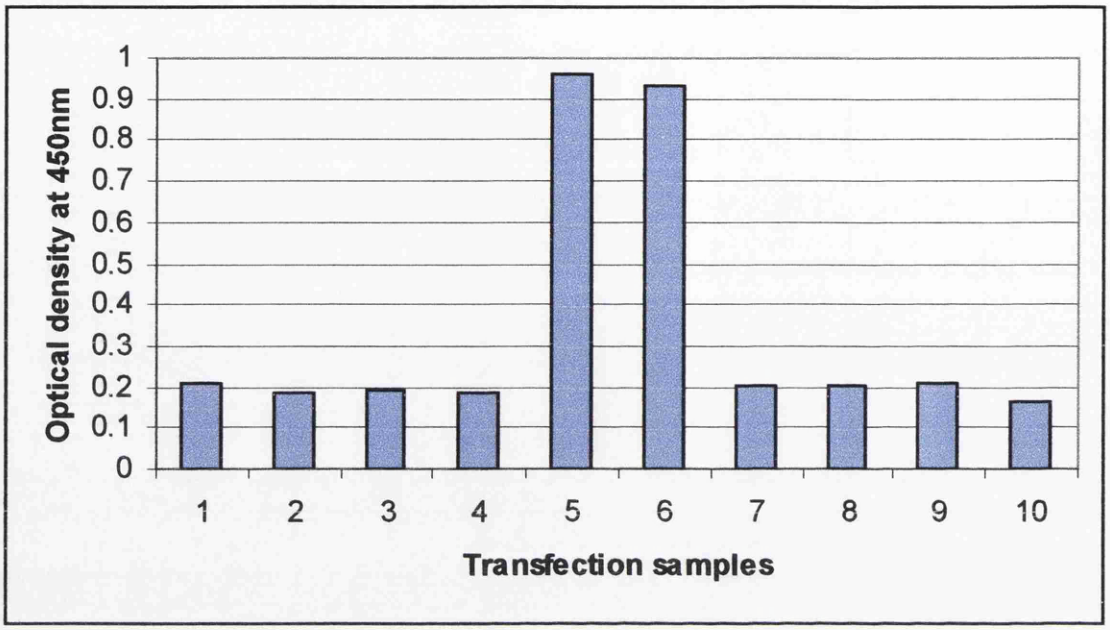
* CAT concentrations extrapolated from the standard plot.

Figure 5.3 :

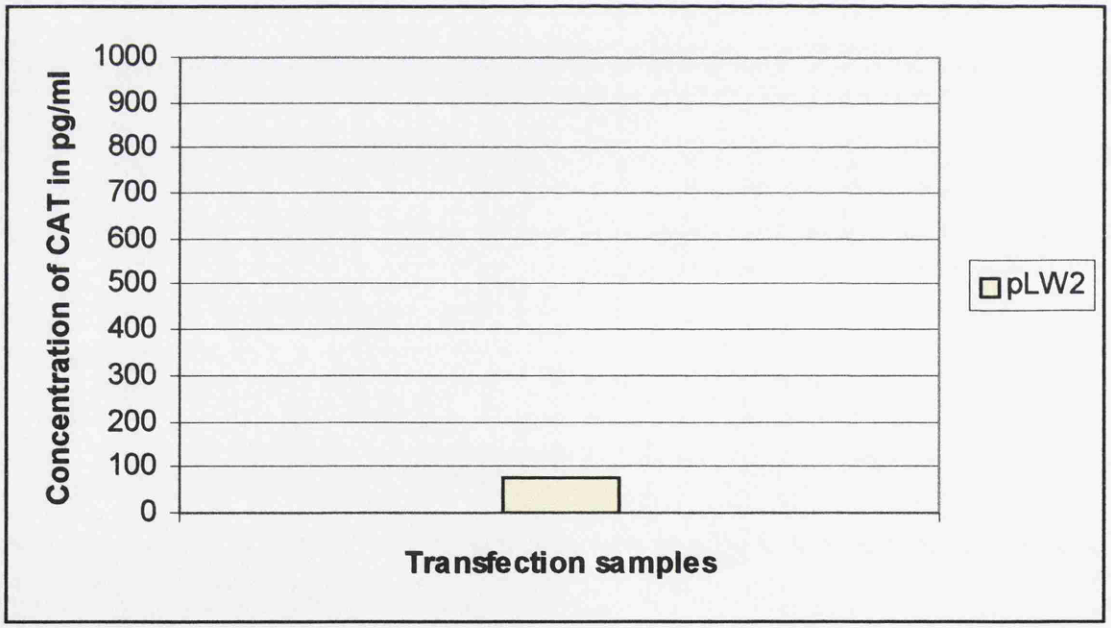
Samples containing 4 x 10^5 cells were transfected, in duplicate, with the above constructs using the LipoTAXI transfection agent. The cells were harvested 72 hours after transfection, as described in 2.15.3, and levels of CAT assayed by ELISA. The optical densities (O.D.s), O.D.1 and O.D.2, refer to duplicate aliquots from the transfected samples. The mean O.D.s are presented in graph **A**. The mean CAT concentrations of the samples are presented in graph **B**.

Figure 5.4 :

A



B



In graph **B** the mean value of CAT concentration has been calculated from the following sample in graph **A**:

pLW2 5 and 6

Figure 5.4 : Low transfection efficiency obtained by using LipoTAXI

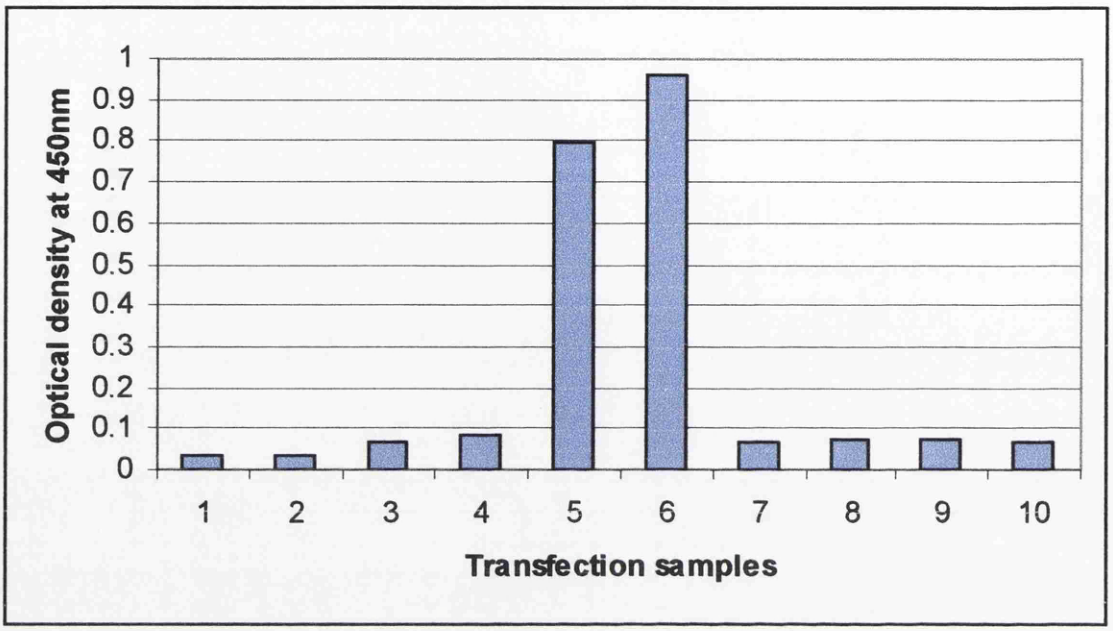
50μg of lysate (250μg/ml)		Optical density at 450nm			conc. in pg/ml	
Sample		O.D.1	O.D.2	Mean	[CAT]	Mean
1	control	0.213	0.202	0.208	N.D.	
2		0.178	0.189	0.184	N.D.	-
3	pCAT12	0.212	0.171	0.192	N.D.	
4		0.186	0.185	0.186	N.D.	-
5	pLW2	0.968	0.958	0.963	79.244	
6		0.984	0.885	0.935	76.078	77.661
7	HSE1-pCAT12 (37°C)	0.207	0.202	0.205	N.D.	
8		0.215	0.190	0.203	N.D.	-
9	HSE1-pCAT12 (41°C)	0.204	0.213	0.209	N.D.	
10		0.162	0.161	0.162	N.D.	

Figure 5.4 :

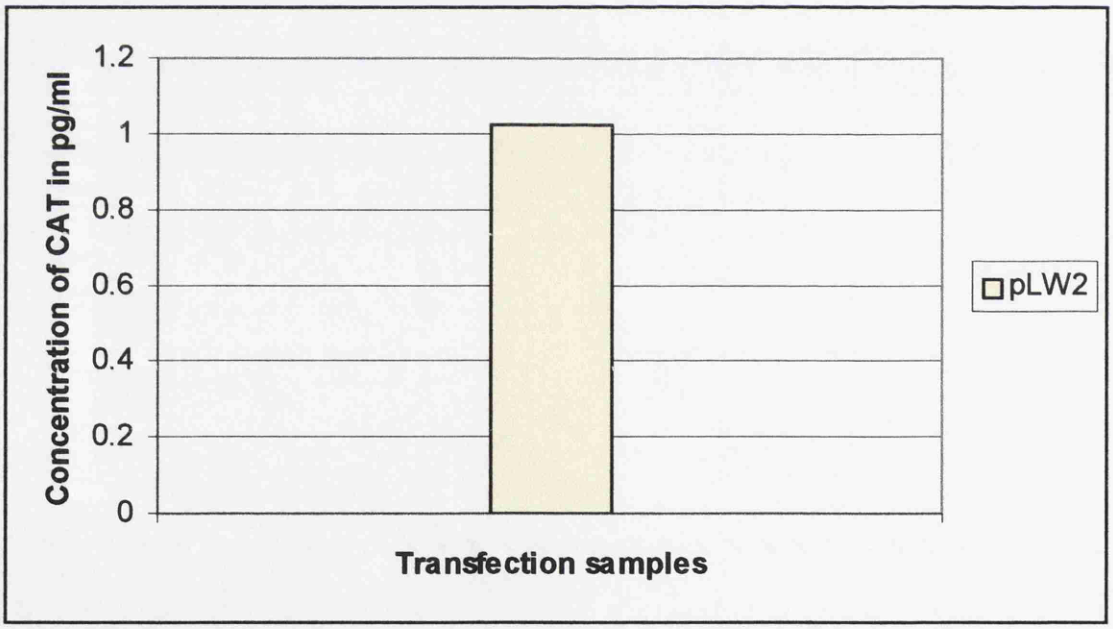
Samples containing 4 x 10^5 cells were transfected, in duplicate, with the above constructs using the LipoTAXI transfection agent. The cells were harvested 48 hours after transfection and levels of CAT assayed by ELISA. Before harvesting, samples 9 and 10 were heat shocked at 41°C for 1 hour as described in 2.15.3. The optical densities (O.D.s), O.D.1 and O.D.2, refer to duplicate aliquots from the transfected samples. The mean O.D.s are presented in graph **A**. The mean CAT concentrations of the samples are presented in graph **B**. The same scale has been used for graph **B** as used for ***Figure 5.3***, graph **B**, to show the reduced CAT activity in the pLW2 (positive control) samples.

Figure 5.5 :

A



B



In graph **B** the mean value of CAT concentration has been calculated from the following samples in graph **A**:

pLW2 5 and 6

Figure 5.5 : Transfection of CAT reporter gene constructs using Lipofectin

50µg of lysate (250µg/ml)		Optical density at 450nm			conc. in pg/ml	
Sample		O.D.1	O.D.2	Mean	[CAT]	Mean
1	control	0.032	0.035	0.034	N.D.	
2		0.031	0.038	0.035	N.D.	-
3	pCAT12	0.070	0.070	0.070	N.D.	
4		0.086	0.083	0.085	N.D.	-
5	pLW2	0.810	0.789	0.799	928	
6		0.988	0.930	0.959	1123	1026
7	HSE1-pCAT12 (37°C)	0.067	0.074	0.070	N.D.	
8		0.070	0.076	0.073	N.D.	-
9	HSE1-pCAT12 (41°C)	0.070	0.078	0.074	N.D.	
10		0.063	0.074	0.068	N.D.	-

Figure 5.5 :

Samples containing 4 x 10^5 cells were transfected, in duplicate, with the above constructs using the Lipofectin transfection agent. The cells were harvested 48 hours after transfection and levels of CAT assayed by ELISA. Before harvesting, samples 9 and 10 were heat shocked at 41°C for 1 hours as described in 2.15.4. The optical densities (O.D.s), O.D.1 and O.D.2, refer to duplicate aliquots from the transfected samples. The mean O.D.s are presented in graph **A**. The mean CAT concentrations of the samples are presented in graph **B**.

5.2.4 5′RACE to acquire the start of transcription

In 3.2.5 a primer based on the sequence of the spliced leader was utilized in the amplification of the 5′ end of a mature *hsp90* mRNA. One disadvantage to the *trans*-splicing of transcripts is the difficulty in identifying the start of transcription. Only the pre-mRNA of a gene that is normally *trans*-spliced, will contain the original bases of the extreme 5′ termini. Since splicing is an efficient process, pre-mRNA will constitute a small percentage of total transcripts. In an attempt to identify the transcriptional start site of *B. pahangi hsp90*, two primers, prex1 and prex2, were designed for use in a 5′ RACE protocol. One primer, prex1, amplifies preferentially from pre-mRNA, while the second primer, prex2 can amplify from either spliced or unspliced *hsp90* templates (see ***Figure 5.6***). ***Figure 5.7A*** shows the procedure used to amplify products from *hsp90* transcripts. First strand cDNA was prepared by reverse transcription of total RNA using the primer, per90, which is specific to *hsp90*. Cytosine nucleotides were added to first strand cDNA to form a terminus to the anchor primer, a guanine-rich primer, could anneal. Two PCR reactions were performed, the first reaction to amplify all *hsp90* templates (see ***Figure 5.7B***) and the second reaction either to specifically amplify *hsp90* pre-mRNA, using prex1, or again to amplify all *hsp90* templates, using prex2, (see ***Figure 5.7C***). The size of the product obtained using the adapter primer and hsp90r5, (***Figure 5.7C***. lanes 13 +14), is consistent with the size calculated from the *hsp90* cDNA clone, 0.34kb, (from the *trans*-spliced site to the region corresponding to hsp90r5), and indicates that no introns are present. A PCR was also carried using the anchor primer, prex1, prex2 and hsp90r5, to ensure that these primers did not amplify genomic DNA. A product was only obtained in the control reaction using hsp90f5 and hsp90r5 (data not shown).

After the final PCR, the products were purified, cloned into a TA vector (pCR2.1) and sequenced at least once on both strands. Small PCR products from *trans*-spliced templates were isolated, but products were not identified which had been amplified from the region upstream of the first TATA box. An alignment of the PCR products is shown in ***Figure 5.8***. The sequence obtained by 5′ RACE coincides with the sequence of the *hsp90* genomic clone immediately after a polyguanine track and these nucleotides (shown double underlined) are assumed to represent the start of the *hsp90* pre-mRNA. However, one single nucleotide is not clear as the 5′ terminus of the transcript.

Figure 5.6 : Design of primers for 5′ RACE of *hsp90*

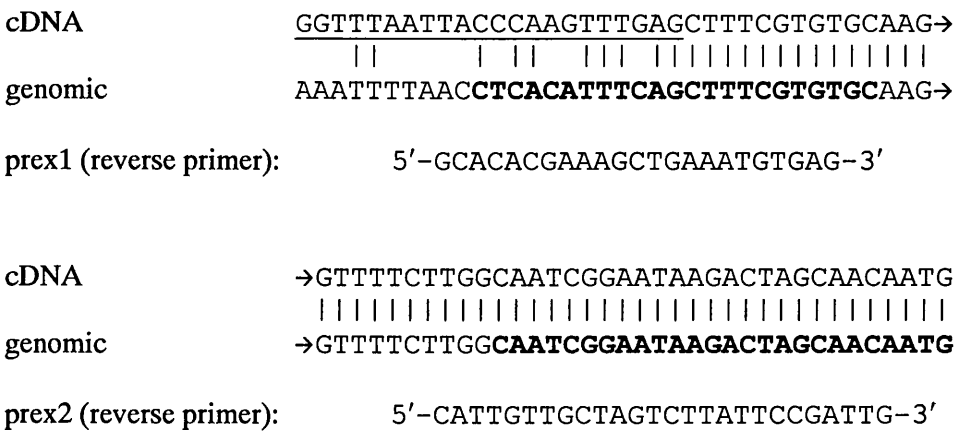


Figure 5.6 :

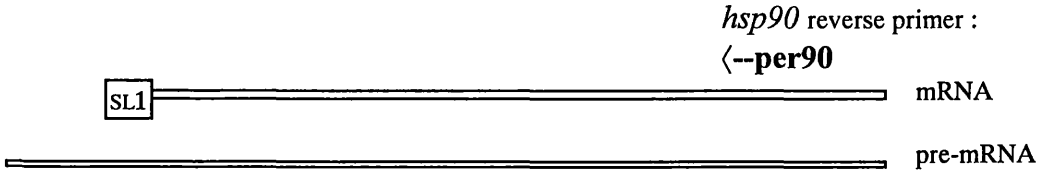
Prex1 was designed from the genomic sequence which spans the SL1 *trans*-splice site and there are three mismatches between the sequence of the primer and the corresponding cDNA sequence. PCR at 60°C with prex1 and a universal adapter primer should preferentially amplify *hsp90* cDNA from transcripts which have not been *trans*-spliced. Therefore PCR products from *hsp90* pre-mRNA should be obtained. Prex2 was designed from the 5′ UTR and the first methionine codon and PCR with this primer and the adapter primer should amplify both a short product (<100bp) from a mature mRNA template and longer products from a pre-mRNA template.

Figure 5.7A :

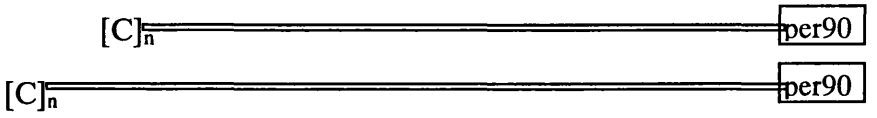
Total RNA from mf cultured at 37°C was reverse transcribed, using the primer, per90, which is specific to *hsp90* (1). Cytodine nucleotides were added to the (*hsp90*) cDNA, using terminal transferase, to create dC-tailed cDNA (2). In the first PCR (3), the guanine-rich primer, anchor, and hsp90r5 (an *hsp90*-specific primer), were used to amplify the dC-tailed cDNA. In the second PCR (4), the adapter primer, whose sequence is contained within the anchor primer, and the *hsp90*-specific primers prex1 and prex2 were used to amplify the product of the first PCR.

Figure 5.7A : The 5′ RACE Procedure

1. Reverse transcription using an *hsp90*-specific primer :



2. Addition of cytidine using terminal transferase :



3.

PCR 1 :
anchor primer
and hsp90r5 primer
30 cycles (annealing
temperature, 55°C)

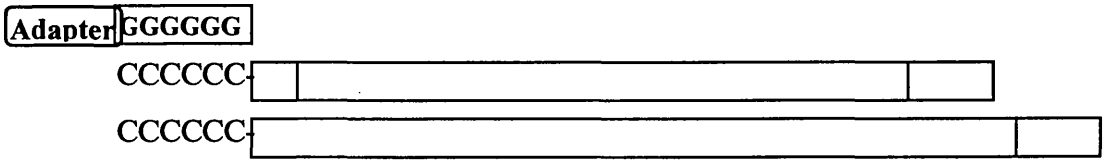
→

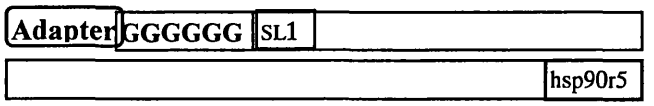
4.

PCR 2 :
adapter primer and
prex1 or prex2 primer
25 cycles (annealing
temperature, 60°C)

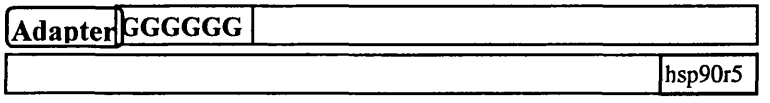
3.

anchor primer---〉 **〈--hsp90r5**





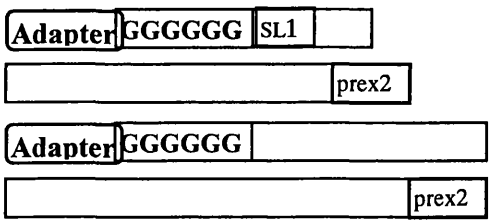
from mature mRNA



from pre-mRNA

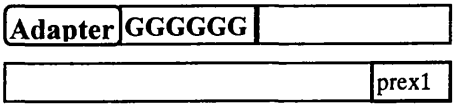
4.

adapter--〉 **〈--prex2**



adapter--〉 **〈--prex1**

no product from the mature mRNA using the adapter primer and prex1 primer



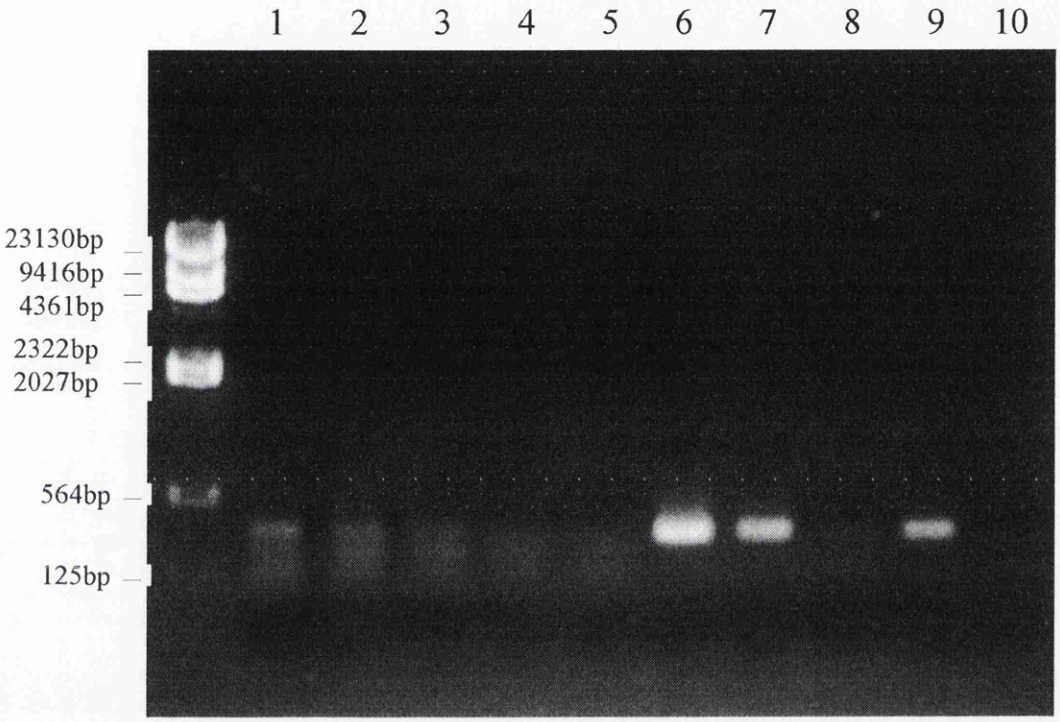
Figures 5.7B and ***5.7C***

Aliquots of 10μl from PCR 1 and PCR 2 were separated on 0.8% agarose gels. The remaining volume (40μl) from PCR 1 was purified and used as the template for PCR 2.

Figure 5.7B

SL1 and hsp90r5 were used in the first PCR (lanes 6, 7 and 9). The products from this reaction were compared to the products from the anchor primer and hsp90r5 reactions (lanes 1 and 2). The sizes of the products, resulting from the anchor primer and SL1 reactions are consistent with the assumption that the majority of the products are from *trans*-spliced templates. In addition, it indicates that the products from the anchor primer reactions do not contain introns (since two introns are present in the corresponding region, in the genomic sequence of *hsp90*).

Figure 5.7B : Amplification of first strand cDNA coding for *hsp90*, (PCR 1)



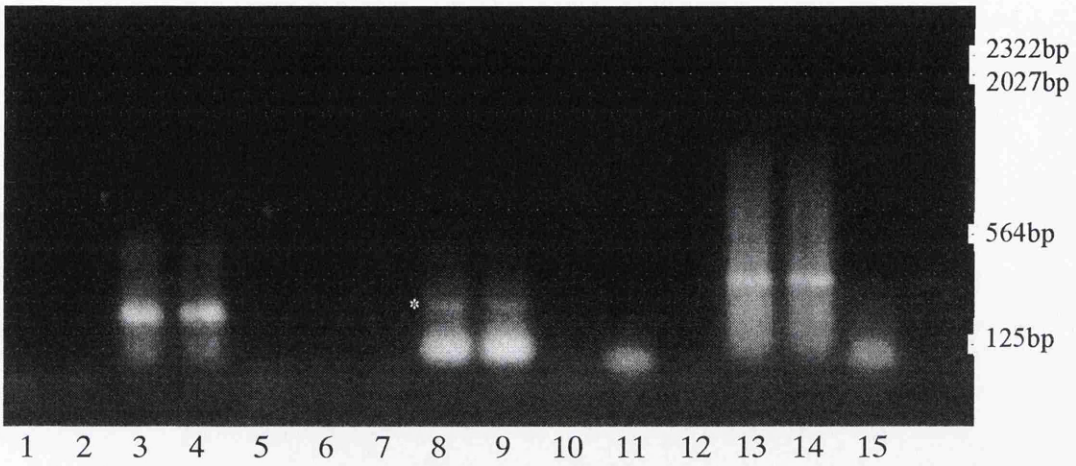
	template (dC-tailed cDNA)		primers
1	sample from 1µg of mf RNA	⎫	
2	sample from 2µg of mf RNA	⎪	20 pmoles of anchor primer
3	sample from 3µg of mf RNA	⎬	and
4	control mf cDNA from 1µg of RNA*	⎪	20 pmoles of hsp90r5
5	no template	⎭	
6	sample from 1µg of mf RNA	⎫	
7	sample from 2µg of mf RNA	⎪	20 pmoles of SL1
8	sample from 3µg of mf RNA	⎬	and
9	control mf cDNA from 1µg of RNA*	⎪	20 pmoles of hsp90r5
10	no template	⎭	

* not dC-tailed

Figure 5.7C

Prex1 was designed to amplify products originating from pre-mRNA and these products (lanes 3 and 4) were cloned and sequenced. A minor band, indicated with an asterisk (*), was produced using prex2 (lanes 8 and 9). This band, assumed to be amplified from templates not containing SL1, was excised from the gel and the products were cloned and sequenced. The sequences from both the prex1 and prex2 clones were used to deduce the start of transcription. The size of the major band using hsp90r5 (lanes 13 and 14), is consistent with the size of a product calculated for the *trans*-spliced *hsp90* mRNA. It indicates that no introns are present, since as mentioned earlier, two introns are present in the corresponding region of genomic DNA and it is therefore unlikely that the products from the second PCR, resulted from genomic DNA.

Figure 5.7C : PCR amplification of 5′ RACE products using prex1 and prex2 (PCR 2)



	template (1µl)	primer 1 (18 pmoles)	primer 2 (18 pmoles)
1	none	adapter	prex1
2	Sample 1 (PCR1)	|	|
3	Sample 2 (PCR1)	|	|
4	Sample 3 (PCR1)	|	|
5	Sample 4 (PCR1)	v	v
6	none	adapter	prex2
7	Sample 1 (PCR1)	|	|
8	Sample 2 (PCR1)	|	|
9	Sample 3 (PCR1)	|	|
10	Sample 4 (PCR1)	v	v
11	none	adapter	hsp90r5
12	Sample 1 (PCR1)	|	|
13	Sample 2 (PCR1)	|	|
14	Sample 3 (PCR1)	|	|
15	Sample 4 (PCR1)	v	v

Figure 5.8 :

The sequences from 6 different clones containing the region amplified by 5′ RACE are aligned. Clones 3, 7 and 31 were sequenced twice on each strand whilst clones 6 and 11 were sequenced once on each strand. Clones 3,6,7 and 11 are products amplified with prex1 and the adapter primer, whilst clone 31 is a PCR product amplified with prex2 and the adapter primer. The boundary with the plasmid (pCR2.1) is indicated with a horizontal arrow. The polyguanine track (resulting from the addition of cytidine with terminal transferase) is shown in bold. The nucleotides which appear to be at the start of the *hsp90* unspliced transcripts, are shown double underlined and the adenine nucleotides in this region are indicated with vertical arrows. The sequence of the prex1, prex2 and adapter primers are underlined.

Figure 5.8 : Alignment of the *hsp90* 5′ RACE products

```
clone       primer
                  ⇐pCR2.1
31A         -prex2                                                GGGGGG.........GG
7A          -prex1                                            CGAGGGGGGGGGGG   GGGG
7B          -prex1   GAATTCGGCTTKTMARATCTACCCGTCGACMTCAAANGNGNAACGR.....GG
6           -prex1   GAATTCGGCTTGTCAGATCTACCCGTCGACCTCGAGGGGGGGGGG.......GG
3A          -prex1   GAATTCGGCTTGTCAGATCTACCCGTCGACCTCGAGGKGGGGGGG.....GGG
3B          -prex1   GAATTCGGCTTGTCAGATCTACGCGTCGACCTCGAGGGGGGGGGGG....GGG
11          -prex1   GAATTCGGCTTGTCAGATCTACCCSTCGACCTCGAGGGGRAMNNNVAMVMGGG
31B         -prex2   GAATTCGGCTTGTCAGATCTACSCGTCGACCCCGAGGGGGGRVVGGGGGKKKG
CONSENSUS   -        GAATTCGGCTTGTCAGATCTACCCGTCGACCTCGAGGGGGGGGGG....GGG

                 ⇓ ⇓  ⇓
4           <        NAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGRNNAATTGCTTGTGGTGA
31A         < GGACACTAGTGATGTTGCCGGTTGTTGTGGTGCGGCTGGTGGGGNNATTGCTTGTGGTGW
7A          < GG..ACTAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGGGSHATTGCTTGTGGTGA
7B          < DDDAACTAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGRVMMATTGCTTGTGGTGA
6           < GGGSACTAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGGGSMATTGCTTGTGGTGA
3A          < GG.BACTAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGGAGAATTGCTTGTKGTGA
3B          < GGAC.CTAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGGAGAATTGCTTGTGGTGA
11          < GGVC.CGAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGGAVAATTGCTTGTGGTGA
31B         < D.RCACTAGTGATGTTGCCGGTTGTTGTGGTGCGGCTGGTGGGGNNDTTGCTTGTGGTGA
CONSENSUS   > GGACACTAGTGATGTTGTCGGTTGTTGTGGTGCGGCTGGTGGGGGAATTGCTTGTGGTGA


4           < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGWTCAT
31A         < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATCCGTTCAT
7A          < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
7B          < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
6           < TTGGTGCTTCCTGTCTCGTTGGAAGTGBAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
3A          < TTGGTGCTTCCTGTCTCGTTGGAAGTGBAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
3B          < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
11          < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
31B         < TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGTTCAT
CONSENSUS   > TTGGTGCTTCCTGTCTCGTTGGAAGTGCAGGGATATGCGCGTTAGGGCTAATGCGTTCAT


4           < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGWTCTTGCAGGCAGCAGCGAT
31A         < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
7A          < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
7B          < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
6           < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGWTCTTGCAGGCAGCAGCGAT
3A          < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
3B          < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
11          < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
31B         < AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT
CONSENSUS   > AGAGTAGCTATGTAAGGCAAAGAAGTGGAGCTTTTTGAGGTTCTTGCAGGCAGCAGCGAT


4           < AAATTTTAACCTCACATTTCAGCTTTCGTGTGC
31A         < AAATTTTAACCTCACATTTCAGCTTTCGTGTGCAAGGTTTTCTTGGCAATCGGAATAAGA
7A          < AAAAATTAACCTCACATTTCAGCTTTCGTGTGC
7B          < AAATTTTAACCTCACATTTCAGCTTTCGTGTGC
6           < AAATTTTAACCTCACATTTCAGCTTTCGTGTGC
3A          < AAATTTTAACCTCACATTTCAGCTTTCGTGTGC
3B          < AAATTTTAACCTCACATTTCAGCTTTCGTGTGC
11          < AAATTTTAACCTCACATTTCAGCTTTCGTGTGC
31B         < AAATTTTAACCTCACATTTCAGCTTTCGTGTGCAAGGTTTTCTTGGCAATCGGAATAAGA
CONSENSUS   > AAATTTTAACCTCACATTTCAGCTTTCGTGTGCAAGGTTTTCTTGGCAATCGGAATAAGA


                          pCR2.1⇒
31A         < CTAGCAACAATG
31B         < CTAGCAACAATG
CONSENSUS   > CTAGCAACAATG
```

5.2.5 Transcription initiation upstream of *hsp90*

Two PCR cycles were utilized to elucidate the start of the *hsp90* pre-mRNA due to the predicted low level of these transcripts within total RNA pool. However, the number of cycles of amplification (a total of 55) may explain the ambiguity of the terminal nucleotide. There are three adenine nucleotides, which may represent the start of transcription. Alternatively it is possible that all three adenines are utilized *in vivo*. With the information from the sequences of both the *hsp90* genomic clone and the 5′ RACE, the position of the (major) start of transcription, downstream from the proximal TATA box, was assessed. The bases with the highest homology to other nematode transcriptional start site sequences are aligned in ***Figure 5.9A.*** The region containing the other two putative TATA boxes have also been added for comparison.

The regions from the nematode gene promoters have been numbered from the thymidine of the TATA box and the number at the end of each region is the position of the first adenine of transcription. For vertebrates, transcription usually starts between 25 and 30 bases from the TATA box (if one is present), whereas transcription initiation in yeast can occur much further from the TATA box. The nematode sequences appear to fit more closely to the vertebrate model, however, the number and variety of nematode promoters analyzed does not permit a more stringent analysis.

Figure 5.9B and ***Figure 5.9C*** are tables showing the occurrence of specific nucleotides for the TATA box, from 389 cellular and viral TATA box elements and the cap signal, from 303 eukaryotic cap sites. The consensus sequence for each of these elements is also shown. The tables were obtained from TFMATRIX (transcription factor binding site profile database). The nematode TATA boxes and transcriptional start sites in ***Figure 5.9A*** are all consistent with the consensus from diverse species. A putative TATA box and putative transcriptional initiation sequence from the region upstream of the *B. pahangi* small *hsp* gene, *Bphsp7*, has also been included for comparison. The sequences of the three TATA boxes from *B. pahangi hsp90* appear to equally fit the consensus and have a region an appropriate distance from the TATA box which may be a site for transcriptional initiation. In the case of the proximal TATA box, transcription of *hsp90* probably occurs close to or at the adenine highlighted.

Figure 5.9 :

In **A**, regions of nematode gene promoter have been aligned to highlight the TATA box sequences (shown boxed) and the transcriptional start sites, the first nucleotides (adenines) of which are shown in bold and indicated with an arrow. The numbers on the right hand side are the distances between the TATA box and the (putative) transcriptional start site. The sequences of a putative TATA box from the upstream region of *B. pahangi Bphsp7*, (a small *hsp*) and all three putative TATA boxes from the upstream region of *B. pahangi hsp90*, have also been included in the alignment. For comparison, tables for the consensus sequences of 389 cellular and viral TATA box elements (**B**) and 303 eukaryotic cap sites (**C**) are shown [Bucher 1990].

Figure 5.9 : An alignment of the transcription start sites of nematode genes

A.

					↓		
C. elegans:					+1		
vit-1	G	TATA	TAAGGT	TACCTGTGAAGAGGAAATT	CATT	GTCCA	30
vit-2	G	TATA	TAAAGG	TGCACTGAAAACAAGCCAAT	CACG	GTTCA	31
vit-4	G	TATA	AATAGA	AACGCTGGAAAGGGAATAAT	CACT	CTCGC	31
vit-5	C	TATA	AAAAGG	GTAACGGAGGAACCATAGT	CACT	CTCGC	30
vit-6	G	TATA	AAAGGA	CACGAGCTTCATGTATTCTT	CACT	CGGTC	31
hsp16$_1$	G	TATA	AATACA	GTGACAAAACCGAAC	CAAA	CAACA	26
hsp16$_{48}$	G	TATA	TAAGCC	AATCGTGTTCAGAGG	AAAC	CAATA	26
Cefkh-1	G	TATC	AAGAGA	CCTAACAAAATCACC	CAAC	CCCAG	26
C. briggsae:							
vit-1	G	TATA	ATAGGG	ATGCCTTGACAGCAGTATATT	CACT	ATTCA	32
vit-2	G	TATT	TAAGGG	AGCCGTAGAGACAGAGAATA	CAGG	TTTCA	31
vit-4	G	TATA	AAAGGG	GTGCCTGGAACTGAAATAAT	CACT	CTCGC	31
vit-5	G	TATA	AAAGGG	GTGCTTGGAACTGAAATAAT	CACT	CTCGC	31
vit-6	G	TATA	AAAGAG	CACGAGCTCGACATACTCTT	CACT	CGGTT	31
B. pahangi:							
bphsp7	C	TATA	TAAGAG	AGACTGCGATGGATG	CACT	GACCA	26
hsp90	A	TATA	AAAGGG	TTTACGGAAACCTTGGGA	CACT	AGTGA	29
	G	TATA	AAGTTC	AAAGGTGGGGTTTAATTTG	CATG	GAGGG	30
	G	TATA	AAAGGT	ACACAAGTTTTTGTTAA	CACT	AACCA	28

B.

cellular and viral TATA box elements

A	C	G	T	consensus
61	145	152	31	**S**
16	46	18	309	**T**
352	0	2	35	**A**
3	10	2	374	**T**
354	0	5	30	**A**
268	0	0	121	**A**
360	3	20	6	**A**
222	2	44	121	**W**
155	44	157	33	**R**
56	135	150	48	**N**
83	147	128	31	**N**

Bucher 1990

C.

cap signal for transcriptional initiation

A	C	G	T	consensus
49	48	69	137	**N**
0	303	0	0	**C**
288	0	0	15	**A**
26	81	116	80	**N**
77	95	0	131	**N**
67	118	46	72	**N**
45	85	73	100	**N**

5.2.6 Constructing HSE2-pCAT12

Primers, tranf and tranr, were designed to amplify a 0.54kb region (-781 → -241 in ***Figure 5.1***) upstream from *hsp90* which did not include the ATG present in HSE1-pCAT12, but did include three additional putative transcription regulatory elements compared to the original construct. The sequence corresponding to the region between the two primers contains the likely major transcriptional start site (TSS), a TATA box, two inverted CCAAT boxes, a GC box and five HSEs but not two additional putative TATA boxes further upstream. These distal TATA boxes were deliberately omitted during the primer design in an attempt to prevent potential problems from transcriptional initiation at more than one site in the promoter. Multiple sites of transcriptional initiation may interfere with the efficient expression of the CAT reporter gene. The "promoter" region of *B. pahangi hsp90* was cloned into pCAT12, a plasmid containing the *cat* gene. The construct was sequenced to confirm the correct orientation of the "promoter".

The HSE2 fragment :

5.2.7 Transfection using HSE2-pCAT12

COS-7 cells were transfected with pCAT12, pLW2 or HSE2-pCAT12, using Lipofectin, and cultured at 37°C for 48 hours. Before harvesting, the cells were either heat shocked at 41°C for 1 hour or maintained at 37°C (see ***Figure 5.10***). The cell samples were treated in the following way:

- cells were not treated with Lipofectin or any of the CAT plasmids (sample 1).
- cells were treated with pLW2 in the absence of Lipofectin (sample 2).
- cells were transfected with the negative control, pCAT12 (sample 3).
- cells were transfected with the positive control, pLW2 (sample 4).
- cells were transfected with HSE2-pCAT12 (sample 5).
- cells were transfected with HSE2-pCAT12 and heat shocked at 41°C for 1 hour before harvesting (samples 6+7).

CAT was not detected in the control samples (1 and 2) therefore there does not appear to be any endogenous CAT in the COS-7 cells and no transfection occurring in the absence of the transfection reagent, Lipofection. Minimal amounts of CAT were detectable in the pCAT12 sample (3), therefore only a low level of CAT expression occurred in the absence of an upstream promoter. A high concentration of CAT was detected in the pLW2 sample (4), confirming the transfection, transcription and translation of the CAT construct in the COS-7 cells and also the efficiency of the herpes simplex viral promoter in stimulating the expression of the downstream reporter gene. The *B. pahangi* heat shock "promoter" resulted in a high level of CAT expression both in the sample from transfected cells cultured under normal physiological conditions (5) and in the sample from heat shocked cells (6 and 7). There is a 1.7-fold difference in CAT concentrations between sample 5 and the mean of samples 6 and 7.

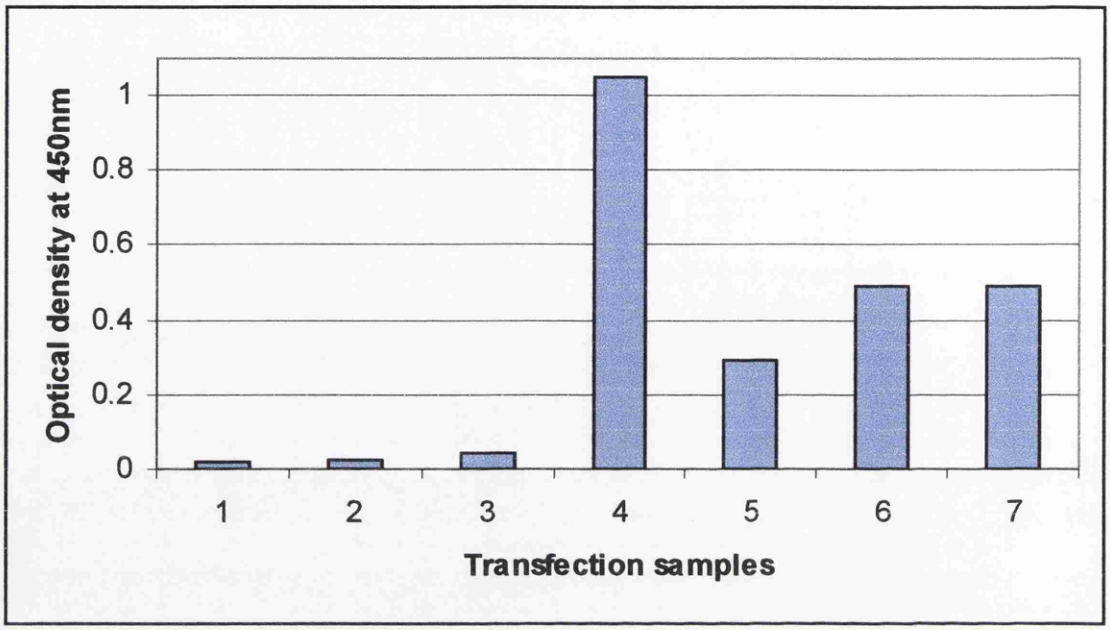
5.2.7.1 The activity of transfected plasmids at 37°C and after a heat shock at 41°C

In the next experiment, the effect of a heat shock on the expression of CAT from the control plasmid, as well as from HSE2-pCAT12, was investigated. Duplicate plates of COS-7 cells were transfected with either pLW2, pCAT12 or HSE2-pCAT12. After 48 hours half of the cell samples were heat shocked at 41°C for 1 hour and the remainder were maintained at 37°C. Three aliquots of each cell lysate were tested for CAT concentration, by ELISA, to increase accuracy of the mean values (see ***Figure 5.11***). Samples 1-6 are from cells, which were maintained at 37°C, while samples 7-12 are from heat shocked cells. Consistent with results of the previous transfection, the expression of CAT from pCAT12 (1, 2, 7, 8) is undetectable and expression of CAT from pLW2 (3, 4, 9, 10) is high, both at 37°C and after a heat shock. There is a slight reduction in the mean quantity of CAT obtained from pLW2 samples from heat shocked cells (9, 10) compared to cells not exposed to 41°C (3, 4), but the levels are not significantly different.

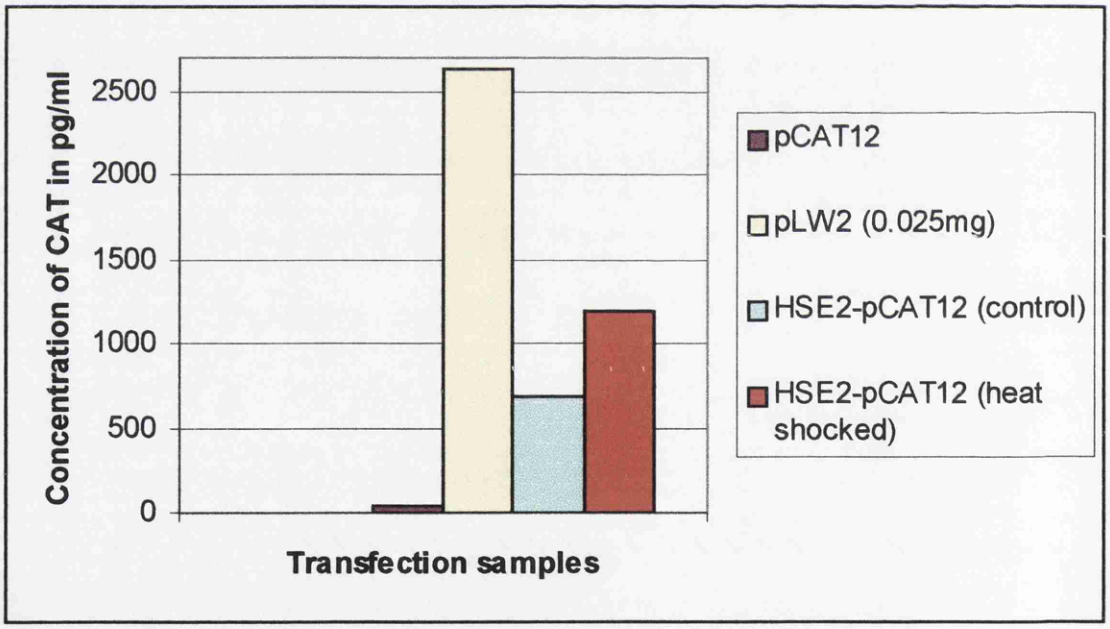
In this experiment, the level of CAT production from the *B. pahangi* promoter was significantly less than in the previous experiment. However, there was expression of CAT under the control of the HSE region both after heat shock (11 and 12) and under physiological conditions (5 and 6).

Figure 5.10 :

A



B



In graph **B** the values of CAT concentration have been calculated from the following samples in graph **A**:

pCAT12	3
pLW2	4
HSE2-pCAT12 (37°C)	5
HSE2-pCAT12 (41°C)	6

Figure 5 10 : Transfection of COS-7 cells with CAT reporter gene constructs

50μg of lysate (250μg/ml)		Optical density at 450nm			conc. in pg/ml
Sample		O.D.1	O.D.2	Mean	[CAT]
1	control	0.021	0.020	0.021	N.D.
2	no Lipofectin	0.022	0.021	0.022	N.D.
3	pCAT12	0.041	0.048	0.045	46.438
4	pLW2 (25μg, 125μg/ml)*	0.981	1.125	1.053	2627.713 *
5	HSE2-pCAT12(37°C)	0.280	0.304	0.292	680.233
6	HSE2-pCAT12(41°C)	0.475	0.505	0.490	1185.989 *
7	HSE2-pCAT12(41°C)	0.577	0.401	0.489	1184.708 *

* ∴ the O.D. and therefore the [CAT] in 50μg of pLW2 lysate would be twice the above value.

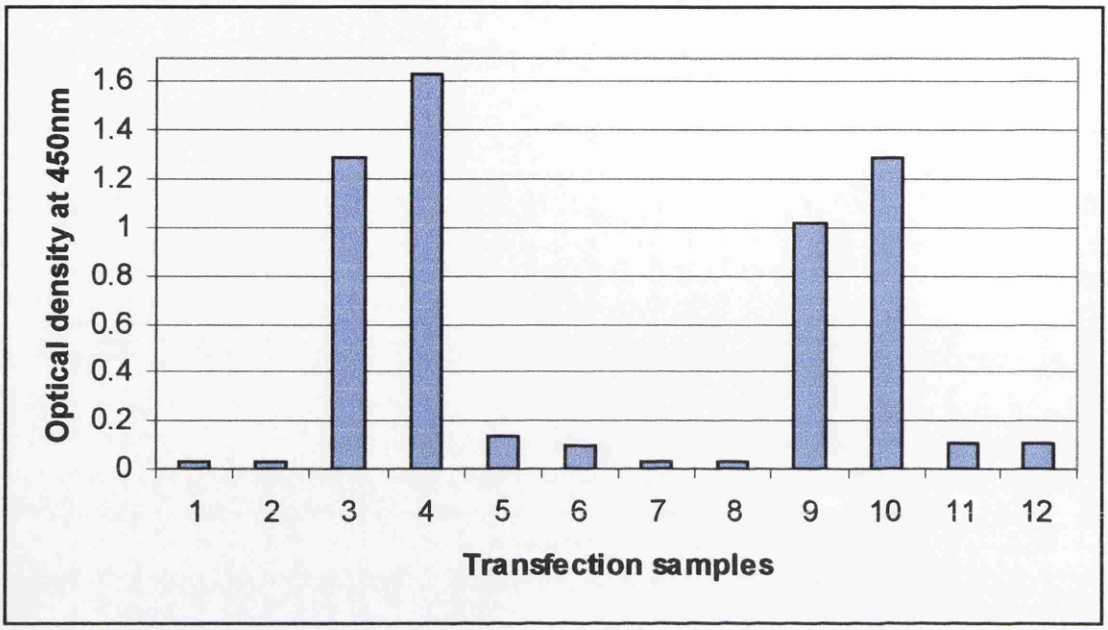
* These values were extrapolated from the standard plot (concentration range from 31.25 to 1000pg/ml).

Figure 5.10 :

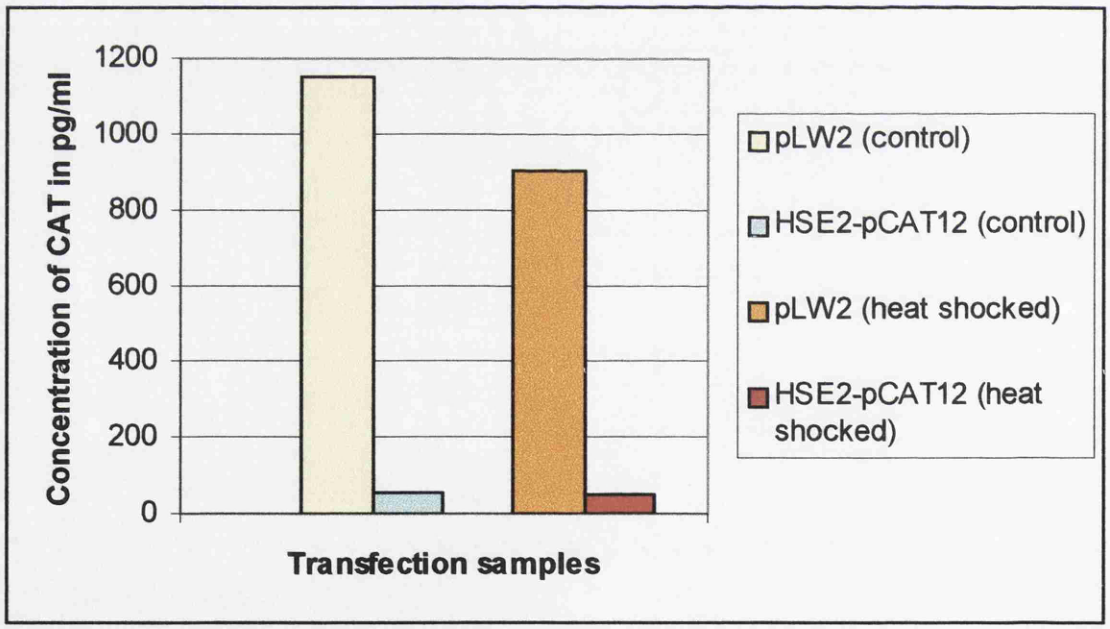
Samples containing 4 x 10^5 cells were transfected with the above constructs using the Lipofectin transfection agent. The cells were harvested 48 hours after transfection and levels of CAT assayed by ELISA. Before harvesting, samples 6 and 7 were heat shocked at 41°C for 1 hours as described in 2.15.4. The optical densities (O.D.s), O.D.1 and O.D.2, refer to duplicate aliquots from the transfected samples. The mean O.D.s are presented in graph **A**. The CAT concentrations of the samples are presented in graph **B**.

Figure 5.11 :

A



B



In graph **B** the values of CAT concentration have been calculated from the following samples in graph **A**:

pLW2 (37°C)	3 and 4
HSE2-pCAT12 (37°C)	5 and 6
pLW2 (41°C)	9 and 10
HSE2-pCAT12 (41°C)	11 and 12

Figure 5.11 : The activity of CAT reporter gene constructs under control and heat shock conditions

50µg of lysate		Optical density at 450nm					conc. in ng/ml	
Sample		O.D.1	O.D.2	O.D.3	Mean	S.D.	[CAT]	Mean
1	pCAT12	0.031	0.031	0.029	0.030	0.001	N.D.	
2	pCAT12	0.032	0.030	0.030	0.031	0.001	N.D.	-
3	pLW2	1.330	1.244	1.298	1.291	0.043	1012.410	
4	pLW2	1.633	1.633	1.632	1.633	0.001	1290.289	1151.350
5	HSE2-pCAT12	0.136	0.131	0.133	0.133	0.003	72.063	
6	HSE2-pCAT12	0.096	0.091	0.088	0.092	0.004	38.208	55.136
7	pCAT12	0.031	0.031	0.031	0.031	0.000	N.D.	
8	pCAT12	0.032	0.035	0.032	0.033	0.002	N.D.	-
9	pLW2	1.049	1.010	1.004	1.021	0.024	793.303	
10	pLW2	1.341	1.227	1.293	1.287	0.057	1009.431	901.367
11	HSE2-pCAT12	0.104	0.107	0.114	0.108	0.005	51.750	
12	HSE2-pCAT12	0.102	0.103	0.103	0.103	0.001	47.146	49.448

Figure 5.11 :

Samples containing 4 x 10^5 cells were transfected with the above constructs using the Lipofectin transfection agent. The cells were either maintained at 37°C throughout the experiment (1-6) or were exposed to 41°C for 1 hour prior to harvesting (7-12). The cells were harvested 48 hours after transfection and levels of CAT assayed by ELISA as described in 2.15.4. The optical densities (O.D.s), O.D.1, O.D.2 and O.D.3 refer to triplicate aliquots from the transfected samples. The mean O.D.s are presented in graph **A**. The mean CAT concentrations of the samples are presented in graph **B**.

The mean concentration of CAT from non-heat shocked cells is higher than from heat shocked cells. However, the duplicate samples for heat shocked cells contain similar levels of CAT (47pg/ml and 52 pg/ml) whereas one of the cell lysates from 37°C contained a lower concentration of CAT (38pg/ml) than in the heat shocked samples and the duplicate contained a higher concentration of CAT (72 pg/ml). Therefore the level of CAT expression from HSE2-pCAT12 under physiological and heat shock conditions are not significantly different.

5.3 Attempts to clone an *hsf* homologue from *B. pahangi*

It was of interest to attempt to clone a *B. pahangi* homologue of the heat shock factor (*hsf*), since this transcription factor may be involved in the expression of *B. pahangi hsp*s. Although the sequences of cloned *hsf*s are highly divergent, the presence of a gene(s) for this transcription factor appears to be conserved between eukaryotic species, as is evident from the numerous *hsf* sequences available in the sequence databases. It was therefore hypothesized that the *B. pahangi* genome would also contain an *hsf* gene. An electromobility shift assay (EMSA) or "gel shift assay" was used by Levy-Holtzman *et al* (1994) to study extracts from the trematode, *S. mansoni*. In that investigation, ^{32}P-labelled synthetic oligonucleotides were utilized, which were designed from putative HSEs from the promoter region of *S. mansoni hsp70*. Proteins present in the parasite extract bound to the HSE oligonucleotides which contained motifs with high homology to the NGAAN consensus, demonstrating the presence of a putative HSF in *S. mansoni*.

5.3.1 South-western screening of a *B. pahangi* cDNA library

The first approach adopted was to use the technique of South-western screening. This method employs a double stranded oligonucleotide probe to screen an expression library. The procedure was used by Scharf *et al* (1990) to isolate three tomato *hsf*s. A probe containing an HSE, which consisted of five inverted repeats of the NGAAN motif, was utilized. The conserved nucleotides of the motif are shown in ***Figure 5.12***, in bold text.

Figure 5.12 : The synthetic HSE duplex probe

5′-TCGAGGATCCTA**GAA**GC**TTC**CA**GAA**GC**TTC**TA**GAA**GCAGATC-3′
3′-CCTAGGATCTTCGAAGGTCTTCGAAGATCTTCGTCTAGAGCT-5′

The 42bp HSE duplex was used to screen a *B. pahangi* adult cDNA expression library [Cox-Singh *et al* 1994]. Four plates with 5 x 10^4 pfu (2 x 10^5 pfu in total) from the expression library (in Uni-Zap XR^{TM}) were screened with the double stranded probe. Duplicate filter lifts were taken from each plate. Five positive clones were identified in the primary screen, although none of these were duplicates. No increase in the number of plaques was observed in a secondary screen and no duplicate positives were visible in a tertiary screen.

It had been suggested that concatenation of probes, which contain transcription factor binding elements, could increase the association of expressed transcription factors with the double stranded target [Scharf *et al* 1990]. The high density of protein binding sites allows one DNA duplex to be tethered at more than one site to the DNA-binding protein. Therefore, the HSE probe was subjected to ligation reactions, but concatenated products were not obtained.

5.3.2 Heterologous screening of a *B. pahangi* cDNA library with a tomato HSF probe

Another approach was to utilize an *hsf* gene, from a different species, as an heterologous probe for screening a *B. pahangi* library. A partial *Lycopersicon peruvianum* (tomato) *hsf* clone termed T26 (bp 330-1113 of x55347) [Scharf *et al* 1990] was obtained from Dr. K. Scharf. The 790bp plasmid insert was used to probe Southern blots of *B. pahangi* adult genomic DNA. The genomic blots were hybridized at 40°C and washed to low stringency (2 x SSC, 0.1% SDS) either at 45°C or at 50°C. Probing with the tomato HSF fragment did not produce clear bands under either condition. Nevertheless, a cDNA library, constructed from RNA from mf heat shocked for 2 hours at 41°C, was screened using the tomato *hsf*. Four plates containing 2 x 10^5 pfu were screened with the T26 probe. Twelve positives were chosen from the primary screen, but non-specific hybridization of T26 to the plaques in the secondary screen prevented further screening.

5.3.3 Heterologous screening of a *B. pahangi* cDNA library with a *Drosophila* HSF probe

The next approach adopted was to probe the adult *B. pahangi* cDNA library with a different heterologous probe. For this purpose *Drosophila hsf* (m60070) [Clos *et al*

1990] was obtained from Dr. J. Clos, as in evolutionary terms *Drosophila* is more closely related to *Brugia*. To investigate whether the *Drosophila* sequence would bind to a *B. pahangi* gene, a *B. pahangi* genomic Southern blot was probed with a 638bp *Pvu*II fragment of *hsf* (*Pvu*II-pHSF15), which contained the conserved DNA-binding domain. The DNA-binding region should have the greatest sequence conservation between the two species, therefore increasing the chance of the probe hybridizing to *B. pahangi* genomic DNA. A Southern blot containing *B. pahangi* genomic DNA was hybridized at 50°C with *Pvu*II-pHSF15, washed to 2 x SSC, 0.1% SDS and exposed to film for 5 days. A second Southern blot was also utilized, which contained two lanes of *B. pahangi* genomic DNA and two lanes of *D. melanogaster* genomic DNA, as a control. This blot was also probed at 50°C, washed to 2 x SSC, 0.1% SDS and was exposed to film for 2, 4 and 14 days. However, although bands were observed in the lanes containing *D. melanogaster* DNA, clear bands were not observed in the lanes containing *B. pahangi* DNA (data not shown).

5.3.4 PCR amplification using an heterologous *hsf* primer

*Hsf*s from different species were aligned and analyzed to identify any regions of sequence homology which may be used to design primers for PCR. At this time, an *hsf* gene had not been cloned from a helminth and *Drosophila* was assumed to be the closest evolutionary relation from which an *hsf* sequence was available. There appeared to be only one region with sufficiently significant sequence homology between *hsf* genes and a primer was designed from this region using the *D. melanogaster hsf* sequence. The 21bp region is identical between the *hsf* cDNA sequences from *Drosophila*, *Xenopus* and Chicken. This region (corresponding to bp 504-524 of m60070) is within the conserved DNA-binding domain of HSF, which is observed to have the highest homology between *hsf*s from different species. ***Figure 5.13*** shows an alignment of this region between seven *hsf* sequences from five different species. M6 was designed as a reverse primer and contains the conserved 21bp sequence with the addition of an *Eco*RI site and a GC clamp.

Figure 5.13 : Alignment of a conserved region from seven *hsf* genes

Species	Accession no.	Sequence
Drosophila	m60070	-CAAGCACAACAACATGGCCAG-
Human	m65217 (HSF2)	-CAAGCACAAtAAtATGGCaAG-
Mouse	x61754 (HSF2)	-CAAaCACAAtAACATGGCgAG-
Human	m64673 (HSF1)	-CAAGCACAACAACATGGCCAG-
Mouse	x61753 (HSF1)	-CAAGCACAACAACATGGCtAG-
Chick	l06098 (HSF3)	-CAAGCACAACAACATGGCCAG-
Xenopus	l36924 (HSF1)	-CAAGCACAACAACATGGCCAG-
consensus		CAAGCACAACAACATGGCCAG
M6 primer		3'-**GTTCGTGTTGTTGTACCGGTC***CTTAAG*GCCG-5'

Figure 5.13 :

A 21bp region from a number of *hsf* gene was aligned. A high degree of homology is apparent and any base changes are shown in lower case. A reverse primer, m6, was designed from the *Drosophila* HSF and is shown in bold. A GC clamp (underlined) and an *Eco*RI site (in italics) were also added to aid in cloning.

5.3.5 PCR amplification of a *B. pahangi* cDNA library

The M6 primer was used with the universal T3 primer in a PCR on an adult *B. pahangi* cDNA library. A single band was amplified and this was purified and cloned into pCR2.1. However, the band contained three products. Comparing the sequences of the three clones, with entries in sequence databanks, suggested that none of the products corresponded to an *hsf* cDNA. One product cloned contained only λUni-Zap vector sequence, the other two clones (designated 2611 and 2612) had homology to helminth sequences. Clone 2611 contained an insert of 142bp, whilst clone 2612 contained an insert of 82bp. Clones 2611 and 2612 appeared to be products from the same cDNA sequence and both had homology to three *B. malayi* adult male cDNA clones (see ***Figure 5.14***).

5.3.6 PCR amplification of *B. pahangi* adult first strand cDNA

Without information on the *B. pahangi hsf* it was not possible to determine whether the gene would be *trans*-spliced with SL1. Since cDNA templates from *trans*-spliced transcripts can be amplified using a primer corresponding to SL1 and a second gene-specific primer, preliminary PCR reactions were carried out on first strand adult cDNA to observe whether *hsf* PCR products could be obtained with SL1 and M6. A product of 0.5kb, clone 12, was amplified, cloned and sequenced. The sequence of clone 12, which contained an SL1 spliced leader, was compared to entries in the sequence databanks, but had no homology with *hsf* genes. However, the amino acid translation of the insert had 35% identity to the translated sequence of a *C. elegans* clone (z68319) and both the *B. pahangi* and *C. elegans* sequences had homology to mammalian heterogeneous nuclear ribonucleoproteins (e.g. p52597 and p31943). The *B. pahangi* cDNA clone may be truncated since the *C. elegans* protein from z68319 is predicted to be 351 amino acids in length (see ***Figure 5.15***).

Figure 5.15 : The translated sequence of *B. pahangi* clone 12

```
1    MSWIIRLQRL PLSANAADIR SFFAGLRIPD GAVHIVGGPD GDAFIGFATD
51   EDARQAMRFD NRRIHDQRVR LLLSSRVEMD AVIAKARAGD LSVVGVASAT
101  SVAAPSLRRD SAPAARPGGV QVIRFYSG*
```

Since the *B. pahangi* hsf gene may not be present in the cDNA library, PCR was also carried on a genomic library using the universal primers T3 and T7, (present in the flanking regions of the library), and M6. Multiple products were observed using these primers, but none have as yet been cloned. A Southern blot of the PCR products, probed with *Drosophila hsf* and washed to low stringency (2 x SSC, 0.1% SDS) at 50°C, did not produce visible bands.

5.4 Discussion

The putative *B. pahangi hsp90* promoter has a similar structure to the promoter described for other heat shock genes, with five HSEs, two CCAAT boxes and a GC box in addition to at least one TATA box. These sites were also found in the rat *hsp70* promoter [Lisowska *et al* 1994], murine *hsp70* promoter [Bevilacqua *et al* 1997] and murine *hsp84* promoter [Dale *et al* 1996]. In the mouse system, the TATA box has a role in the initiation of transcription and the CCAAT box binding factor, NF-Y, has been implicated in the recruitment of additional transcriptional factors to the promoter elements [Wright *et al* 1994]. In addition, the basal regulation of the human heat shock gene, *hsp27*, was localized to a GC box region [Oesterreich *et al* 1996].

The role of heat shock elements in the heat shock response appears to be conserved throughout eukaryotes with the possible exception of the trypanosomatidae [Shapira and Pedraza 1990]. HSF from yeast is able to bind to heat shock elements in human (HeLa) cells emphasizing the conservation of the HSF-HSE interaction between diverse species. However, in yeast the HSF appears to be bound to HSEs in the absence of cellular stress; the activation of yeast heat shock genes is dependent upon the phosphorylation of HSF, which results in its activation and a consequent increase in the transcription of the corresponding heat shock gene [Sorger *et al* 1987]. In contrast, activation of HSF in higher eukaryotes requires trimerization and the HSF trimer binds to HSEs enhancing gene transcription [Sorger 1991].

The binding of HSF to HSEs in the upstream regions of heat shock genes enhance levels of transcription during heat shock. However many *hsp*s are also transcribed at a basal level in the absence of cellular stress and other more general transcription factors are implicated in this process. They include the GC box binding factor, stimulatory protein (Sp1) and the CCAAT box binding factor, NY-1 both of which have been observed in the promoter regions of a diverse range of genes including human aldehyde dehydrogenase 2 [Stewart *et al* 1996] and calreticulin [McCauliffe *et al* 1992] as well as murine presenilin-1 [Mitsuda *et al* 1997].

An analysis of the murine *hsp84* promoter, a region of 627bp upstream from the transcriptional start site, identified putative regulatory elements. These included a heat shock element (beginning at -440bp), an AP1-binding site (-178bp), four stimulatory protein-1 (Sp1)-binding sites (-16bp, -103bp, -108bp, -145bp), a cyclic AMP response element (-117bp), and a TATA box (-26bp) [Dale *et al* 1996]. A similar analysis of the chicken *hsp90*β promoter identified three putative Sp1-binding sites (-45bp, -91bp, -109bp) and a TATA box (-26bp). An HSE was also observed, but much further upstream at -1949bp. The transcriptional control of chicken *hsp90*β is of particular interest since heat shock does not appear to stimulate transcription of the gene [Meng *et al* 1995]. The more proximal positions of the *B. pahangi hsp90* HSEs may indicate that this gene, unlike chicken *hsp90*β, is heat inducible. The upstream region of the *C. elegans hsp70A* has been described and contains three putative HSEs (-132bp, -199bp, -363bp), three CCAAT boxes (-125bp, -169bp, -205bp) and a TATA box (-113bp) [Snutch *et al* 1988]. A putative Sp1-binding site is also evident at -109bp. The bases were numbered in respect to the initiating codon as the start of transcription has not been determined.

Morgan *et al* (1987) reported than the basal activity of a human *hsp70* promoter was primarily dependent on a CCAAT box sequence located 65bp upstream from the transcriptional start site. In addition, a human CCAAT-binding factor was identified that activated transcription from the *hsp70* promoter [Lum *et al* 1990]. The *S. mansoni hsp70* promoter, (numbered with respect to the initiator codon), contains three putative inverted CCAAT boxes (-138bp, -170bp, -381bp), two HSEs (-101bp, -229bp) and a

TATA box (-75bp) [Neumann *et al* 1992]. Similarly, the upstream region of *Bmhs1* (*hsp70*) from *B. malayi* has also been analyzed and contains a putative CCAAT box (-377bp) four potential HSEs (-235bp, -250bp, -398bp, -450bp) and a TATA box (-209bp) with respect to the first methionine codon [Rothstein and Rajan 1991]. *Bmhs1*, like *B. pahangi hsp90* is *trans*-spliced and the distance from the putative TATA box in the upstream region to the first methionine codon of *hsp90* is a comparable distance (274bp for *hsp90* cf. 209bp for *Bmhs1*). The transcription of *B. pahangi hsp90* may involve both the binding of a *B. pahangi* CCAAT-binding factor and an HSF to regulatory elements in the *hsp90* promoter region.

Two GATA boxes were identified upstream of *B. pahangi hsp90* and interestingly, the GATA family of transcription factors appear to be important for differentiation, development and tissue-specific expression of genes [Simon 1995]. A GATA element has also been described in a promoter from the rice *Tungro bacilliform* virus where this element is involved in the tissue specificity of the virus, which is limited to the phloem of an infected plant [Yin *et al* 1997]. In *C. elegans*, deletion of tandem GATA sites from the promoter for *ges-1*, which is normally expressed in the embryonic gut, results in expression of the gene in the embryonic pharynx and tail [Fukushige *et al* 1996]. In addition, regulation of the temporal and spatial expression of the *C. elegans cpr-1* (cysteine protease) gene, also normally expressed in gut cells, appears to involve two GATA motifs in the *cpr-1* promoter. Studies on the promoter region revealed that the deletion of both GATA elements caused the complete loss of expression. Furthermore, a concatemer, containing six GATA motifs, which was placed upstream of a reporter gene, resulted in expression specifically in the gut cells of a transformed *C. elegans* embryo [Britton *et al* 1998]. In *B. pahangi*, a GATA binding transcription factor may be involved in the stage-specific expression of *hsp90* and further analysis of these elements may provide more evidence for their involvement.

A *B. pahangi* "promoter"-CAT reporter gene construct, HSE1-pCAT12 was created by cloning a product, amplified from a region upstream from *hsp90*. This was designed as an initial construct and it was anticipated that other deletions or extensions of the promoter region would eventually be utilized to elucidate the function of the constituent regulatory elements. However, significant quantities of CAT were not detected from the

samples of COS-7 cells transfected with HSE1-pCAT12. Possible explanations for the lack of detectable CAT were: the requirement for the presence of an additional two HSEs and an inverted CCAAT box, which were omitted from the construct; the incompatibility of the simian transcription factors and the *B. pahangi* elements; the presence of as yet undescribed negative regulatory (silencing) elements. Additionally, since the transcripts of the gene were *trans*-spliced in *B. pahangi*, appropriate information regarding the transcription initiation site could not be acquired from the mature mRNA (cDNA clone). The transcription initiation site was postulated to be approximately 30bp downstream of the putative TATA box. As the first construct, HSE1-pCAT12, was not successful at driving the expression of CAT, it was decided to attempt to identify the transcriptional start site for *B. pahangi hsp90* and to utilize the information to produce a second CAT reporter gene construct.

A modified 5′ RACE protocol was used to identify the start (or major start) of transcription by preferentially amplifying pre-mRNA *hsp90* templates. The first nucleotide of the pre-mRNA was ambiguous but a comparison of the first few nucleotides, from a collection of PCR products, with the known transcriptional start sites from other nematode genes indicated the most probable *hsp90* start site (-275 → -267). Additionally, the distance from the *hsp90* putative (proximal) TATA box to this site is consistent with the distances observed for other nematode gene promoters (26-32bp from the TATA box), shown in ***Figure 5.9*** Two TATA boxes upstream from the first may also be utilized *in vivo*, since they also conform to the TATA consensus sequence and have a region with homology to the cap consensus, situated at a distance consistent with the other aligned promoters including the other *B. pahangi hsp90* TATA regions. With this information, it is evident that transcripts from HSE1-pCAT12 would include a codon for ATG. Therefore, translation may have been initiated from this codon rather than from the first *cat* codon, which is in a different frame. As mentioned in 5.2.3, the lack of significant CAT expression in cells transfected with HSE1-pCAT12 may be due to this 5′ methionine codon.

It is possible that these additional TATA boxes may provide a level of transcriptional control by the use of alternative promoter regions. Ayoubi and van de Van (1996) reviewed genes which contained alternative promoters and suggested that multiple

promoters gave an organism additional flexibility in the control of expression of the gene. In addition, it was proposed that the use of multiple promoters could guarantee the expression of a gene, in every cell type, even with a different pool of transcription factors present. Therefore, *B. pahangi hsp90* may utilize all three TATA boxes, and the use of a particular TATA box may depend on the cell type or the life cycle stage, which is expressing *hsp90*. Confirmation or rejection of such a hypothesis would require further investigation. However, it should be noted that larger products, which may have originated from pre-mRNA transcribed from other transcriptional start sites, were not observed in the 5′ RACE (see ***Figure 5.7C***).

In the present study, a mammalian transfection system was utilized in order to begin the analysis of the *B. pahangi hsp90* promoter. A CAT reporter gene was used for the transfection of COS-7 cells. An investigation of a region of the *hsp90* putative promoter in COS-7 cells produced interesting results. It was not clear whether the mammalian transcriptional apparatus of the cells would be able to initiate transcription from a nematode promoter. However, the upstream region, HSE2, from *B. pahangi hsp90* did result in the expression of a significant level of reporter gene product in transfected COS-7 cells. Importantly, transcription from the *B. pahangi* heat shock promoter also occurred after a 1 hour heat shock at 41°C but whether there was an increase in comparison to non-stress conditions was not conclusively proven.

It is possible that the 1-hour heat shock at 41°C was not severe enough to cause a marked difference in the expression of CAT from HSE2-pCAT12. Further experiments could utilize higher heat shock temperatures or other cellular stresses to investigate the promoter-driven expression of CAT. It should also be noted that the protocol for obtaining a cell lysate includes washing the intact cells three times with ice cold buffer. This exposure to a lower temperature may constitute a "cold shock" and may result in the induction of heat shock genes and thus the expression of CAT under the control of the *hsp90* upstream region. Cold shock induction of HSPs has been described in human HeLa cells exposed to 4°C [Liu *et al* 1994]. This may explain the high level of CAT detected in the HSE2-pCAT12 transfected cells that did not receive a heat shock. Protocols with and without the 4°C step could be investigated to study the effect of an exposure to this low temperature on the expression of CAT from HSE2-pCAT12.

It is not however, unusual for HSP90 to be expressed under normal conditions, in the absence of cellular insult. There are examples of the developmental regulation of HSP90, for example during embryogenesis (see 1.6). The interaction of transcription factors in the COS-7 cells with transcriptional regulatory elements in the upstream region of *B. pahangi hsp90* may result in constitutive gene expression at normal physiological temperatures. As mentioned previously, a CCAAT-binding factor and Sp-1 transcription factor may be involved in basal transcription from HSE2-pCAT12. Other transcription factors, which are present in the nematode, may not be present in the monkey, or may share only limited homology. These *B. pahangi* factors could also be involved in *hsp90 in vivo.* For example, a *B. pahangi* member of the GATA transcription factor family could induce *hsp90* transcription in mf. Negative regulatory factors also be involved in *hsp90* expression and may also only have limited sequence homology between species. Liu *et al* (1993) described a rodent transcription factor that bound to a heat shock promoter and negatively regulated the heat shock gene.

Only minimal reporter gene expression was observed at 37°C when the *S. mansoni hsp70* promoter was studied in CHO cells, in comparison to the high level of CAT expression as a result of heat shocking the cells at 42°C [Levy-Holtzman and Schechter 1995]. However, *B. pahangi hsp90* may not be as strictly heat inducible as indicated by a high concentration of *hsp90* mRNA in mf cultured at 37°C. It may be possible to dissect the functions of the transcription factor binding sites by producing deletion mutations of HSE2-pCAT12. However, additional regulatory elements may exist further upstream from HSE2 and may be required for efficient heat shock induction of *hsp90.* Reporter gene constructs, which contain a larger fragment of the *hsp90* upstream region, could be produced to study the promoter. Problems may arise from having more than one TATA box present, since transcription may be initiated from multiple sites. DNA footprinting could alternatively be used to provide information about transcription factor binding sites in the *hsp90* promoter. It may also be possible to use nuclear extracts from *C. elegans* for gel retardation assays. However, Levy-Holtzman *et al* (1995) used total extracts from *S. mansoni* to perform gel retardation assays on the *S. mansoni hsp70* promoter and therefore total extracts from *B. pahangi* could be used in a similar way with the *hsp90* promoter.

***B. pahangi* HSF**

The *C. elegans* sequencing project has identified a cDNA clone (yk347g12.5) with homology to *Drosophila hsf* (m60070). The DNA sequence homology is 61% over 276bp, corresponding to bp. 363-588 of m60070 and this includes the 21bp region used to design the M6 primer (see ***Figure 5.16***). There are five base mismatches between the sequence of M6 and the *C. elegans* sequence. If the sequence of *B. pahangi hsf* is homologous to the *C. elegans* sequence, then mismatches between M6 and the actual sequence may explain the difficulty in amplifying a *B. pahangi hsf* product.

With sequence information available for a nematode *hsf* gene, it would be possible to make heterologous primers, for example, from the highly conserved DNA-binding domain and to use these primers to amplify *B. pahangi* genomic DNA. If an internal fragment of a *B. pahangi hsf* gene was amplified and sequenced, this new sequence could be used to design homologous primers to obtain the 5′ and 3′ ends of the gene. Alternatively, the PCR fragment could be utilized to screen a *B. pahangi* library to isolate a clone or clones that contained the *B. pahangi hsf* gene(s).

Sequences in the upstream region of *B. pahangi* genes, including *hsp90*, have provided information about putative *B. pahangi* heat shock elements. It would be possible to using a *B. pahangi*-specific double stranded DNA probe to repeat the South-western screen and to isolate an *hsf* homologue. For example, a 110bp region (***Figure 5.1*** -477 → -367) has three consecutive HSEs, with a total of nine NGAAN inverted repeats. During a south-western screen this HSE probe could theoretically be "tethered" by three immobilized HSF trimers, expressed from a *B. pahangi* cDNA library, and this may increase the probability of obtaining an *hsf* cDNA clone. In addition, *B. pahangi* HSF may have greater affinity for HSEs from a nematode (*B. pahangi*) promoter than to the HSE duplex in ***Figure 5.12***. Although there are not many differences between the 5-bp units from the synthetic HSE probe and the 5-bp units from the upstream region of nematode genes, the two NGAAN units of the synthetic probe have guanine (G) at the fifth position, but only 1 of 21 nematode NGAAN units have G in this position (see ***Figure 5.2***). This difference may have reduced the affinity of *B. pahangi* HSF in the South-western screen.

Figure 5.16 : *C. elegans* cDNA clone with homology to *Drosophila hsf*

yk347g12.5 :

ACCGAATCGACCACCACGGCAGAATCATCAAAATGGGGCAATTGGTGGCAAAAA
GTCATCGGTTACTATACAAGAAGTTCCGAATAATGCGTATTTGGAGACCTTGAA
CAAATCCGGAAACAACAAAGTTGACGACGACAAGCTTCCAGTATTTCTGATAAA
ATTGTGGAATATCGTAGAAGATCCAAATCTTCAATCAATTGTTCATTGGGATGA
TTCTGGTGCAAGCTTTCATATTTCTGATCCATATTTATTTGGGAGAAATGTTTT
GCCGCATTTTTT**CAAGCATAACAATATGAATAG**CATGGTCAGACAGTTGAATAT
GTNCGGCTTCCGAAAGATGACTCCACTGTCCCAAGGTGGTCTAACTCGAACA

Figure 5.16 :

The complete sequence of clone yk347g12.5 is shown above. The region corresponding to the M6 primer is indicated in bold and the bases that mismatch with M6 (and thus with the *Drosophila* sequence) are underlined.

In summary

- A 1.2kb region upstream from *B. pahangi hsp90* was analyzed and putative transcription factor binding sites were identified
- The (major) transcriptional start site of *hsp90* was identified using a modified 5′ RACE protocol
- A region of the *hsp90* "promoter" was demonstrated to induce the expression of a reporter gene in an heterologous transfection system

6.0 Cloning, expression and analysis of the carboxyl terminus of *B. pahangi hsp90*

6.1 Introduction

Interest in HSP90 in the field of parasitology was increased by the identification of this HSP as a major antigen in a variety of different infectious diseases. For example, immune responses directed against HSP90 were detected in serum from individuals infected with *Candida albicans* [Swoboda *et al* 1995], *Leishmania braziliensis* [Skeiky *et al* 1995] *Schistosoma mansoni* [Johnson *et al* 1989] and *B. malayi* [Kumari *et al* 1994]. Bonnefoy *et al* (1990) reported that vaccinating squirrel monkeys with a 90-110kDa protein fraction from *Plasmodium falciparum* resulted in the protection of three out of five monkeys from a challenge with this parasite. Furthermore, serum from the protected monkeys reacted strongly against purified *P. falciparum* HSP90 indicating that this protein may have elicited the protective response [Bonnefoy *et al* 1994]. A 47kDa carboxyl terminal fragment of *C. albicans* HSP90 was also thought to immunoprotective [Swoboda *et al* 1995].

A possible role of HSP90 in parasite development has been studied in protozoan parasites. Shifting the culture temperature of *Leishmania* flagellated promastigotes from 24°C to 34°C results in their differentiation to non-flagellated amastigotes and also in the over-expression of an 83kDa protein [Hunter *et al* 1984], which was subsequently identified as HSP90 [Shapira and Pedraza 1990]. However, in this organism, increased translation of HSP90 hardly altered the steady state level of the protein [Shapira and Pedraza 1990]. The differential expression of an 87kDa homologue of HSP90 was observed in *Theileria parva*. HSP90 was detected in schizont and sporozoite stages but not in the piroplasm stage [Gerhards *et al* 1994].

Selkirk *et al* (1989) observed a highly expressed 85kDa protein in extracts from *B. pahangi* mf, metabolically labelled at 43°C which was predicted to be HSP90 Furthermore shifting the temperature of *B. pahangi* L_3 from 28°C to 37°C also appeared to increase the synthesis of an 87kDa protein [Jecock and Devaney 1992]. In this chapter, confirmation of the identity of the *B. pahangi* 85kDa/87kDa protein and its expression in mf and adult worms is presented.

6.2 Results

6.2.1 Is HSP90 differentially expressed in mf at 37°C compared to mf at 28°C?

In 3.2.14 it was observed that the expression of *hsp90* transcripts was elevated in mf cultured at 37°C in comparison to mf cultured at 28°C and adult worms cultured at 37°C. It was of interest to determine whether the difference in mRNA levels was reflected in a difference in the level of HSP90 protein in mf cultured at 37°C and at 28°C. To investigate differences in protein synthesis, mf were cultured at 28°C or 37°C for 3 hours in the presence of ^{35}S-methionine and the profiles of newly synthesized polypeptides were compared by SDS-PAGE and autoradiography (see ***Figure 6.1***).

A comparison of the protein profiles from the mf cultured at the two temperatures indicated that most ^{35}S-labelled polypeptides were expressed in both samples. However, at 37°C (lane 2) proteins of approximately 85kDa, 70kDa, 23kDa and 19kDa appeared to be upregulated. The 85kDa protein, up-regulated in mf at 37°C may be the *B. pahangi* homologue of HSP90, as it is in the correct size range, predicted to be 83kDa from the translation of the *hsp90* cDNA clone.

6.2.2 Western blot analyses using the anti-HSP90 monoclonal antibody, SPA835

To confirm the identity of the 85kDa protein as an HSP90 homologue, commercially available anti-HSP90 monoclonal antibodies (mAbs) were tested against *B. pahangi* protein extracts to investigate whether the mAbs would cross-react with *B. pahangi* HSP90 and to determine whether differences in HSP90 concentration could be observed in mf cultured at different temperatures.

As is evident from ***Figure 3.10***, there is a high degree of homology between the amino acid sequences of HSP90s from diverse species. The mAb, SPA835, was raised against human HSP90 and is documented to cross-react with HSP90 from canine, rat, murine, bovine, *Drosophila* and cotton (StressGen 1997).

Figure 6.1 : Metabolically labelling mf with ^{35}S-methionine

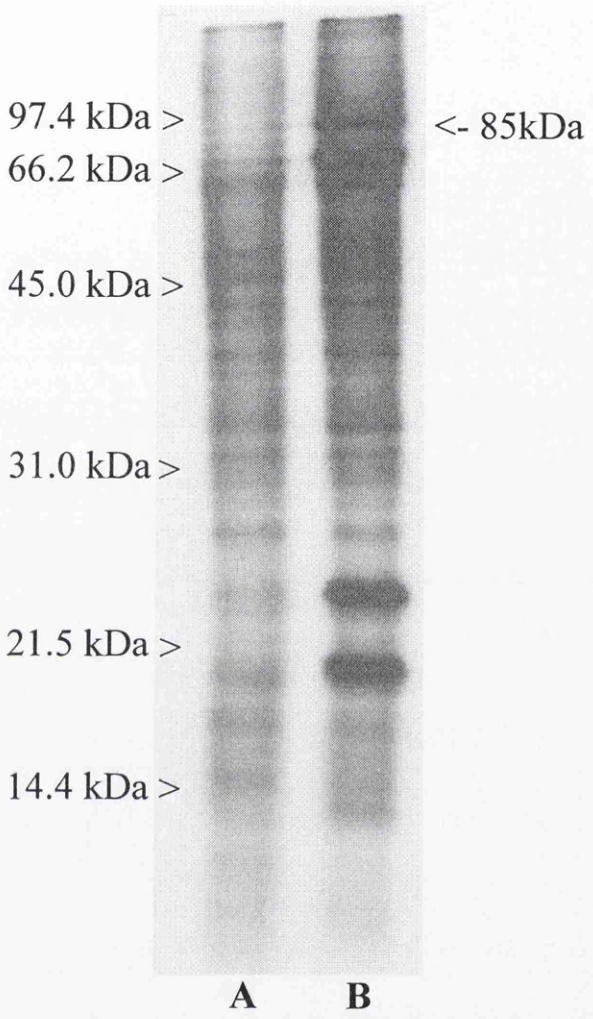


Figure 6.1

An autoradiograph from an experiment in which mf were metabolically labelled, in mammalian medium (DMEM containing 5% FCS, 1% glucose, 25mM HEPES, 4mM glutamine, 1% non-essential amino acids, 100μg/ml gentamycin), in the presence of ^{35}S-methionine at 28°C (**A**) or 37°C (**B**) for 3 hours. The mf were extracted into isoelectric focusing lysis buffer and extracts equivalent to 1 x 10^5 TCA-precipitable cpm were mixed with SDS-sample cocktail. The radiolabelled polypeptides were separated by SDS-PAGE (12.5% gel) and visualized by autoradiography. The sizes of protein molecular weight markers are indicated on the left hand side, by arrows. An 85kDa protein, thought to HSP90, is indicated on the right hand size with an arrow and is up-regulated in mf at 37°C (**B**).

Mf were cultured at 28°C, 37°C and 41°C for 2 hours and protein was extracted using SDS-sample cocktail. The extracted proteins were separated by SDS-PAGE and the samples were then compared (data not shown). Little if any difference was apparent in the total protein profiles at the three different temperatures. However, an increase in the expression of HSP90, over 2 hours, will probably not alter the steady state level of HSP90 sufficiently for a difference to be visible on a Coomassie blue stained gel. Mf do not survive at 41°C for more than 2 hours, so a longer exposure time was not possible.

The protein extract from mf cultured at 37°C was subject to western blot analysis with SPA835. The blot shown in ***Figure 6.2*** was carried out with a total protein extract of mf solubilized in SDS sample cocktail. In this experiment bovine HSP90 was used as a positive control. SPA835 strongly bound to a single band of approximately 90kDa corresponding to bovine HSP90 (lanes 13 and 14) and also recognized a band of approximately the same size in the mf samples, which was not recognized by control serum. However, a number of other bands are also visible in the lanes incubated with SPA835 which are not present in the control serum lanes. These bands range in size from ~100kDa to ~35kDa and are much fainter in the mf samples probed with 1/2000 SPA835 (lanes 5 and 11) compared to mf samples probed with a higher concentration of the mAb. However the 90kDa band is also fainter in lanes 5 and 11.

6.2.2.1 Further analysis using SPA835

In most organisms, HSP90 proteins terminating with the amino acids, MEEVD are located in the cytoplasm and may therefore be expected to be present in a soluble extract of mf. Separation of DOC soluble and insoluble components may reduce the cross-reactivity observed in the western blot. In this experiment, mf were cultured at 37°C or 41°C, disrupted by sonication and extracted with 1% deoxycholate (DOC). After recovery of the supernatant, the remaining DOC insoluble material was extracted using SDS-sample cocktail. DOC soluble and SDS sample cocktail soluble samples were then subjected to western blot analysis (data not shown). A band of approximately 85kDa was observed in DOC soluble mf extracts incubated with SPA835 but not in samples incubated with control serum or in the residual SDS-sample cocktail soluble mf extracts incubated with SPA835.

Figure 6.2 :

Western blot of total protein extracts from mf probed with the mAb, SPA835

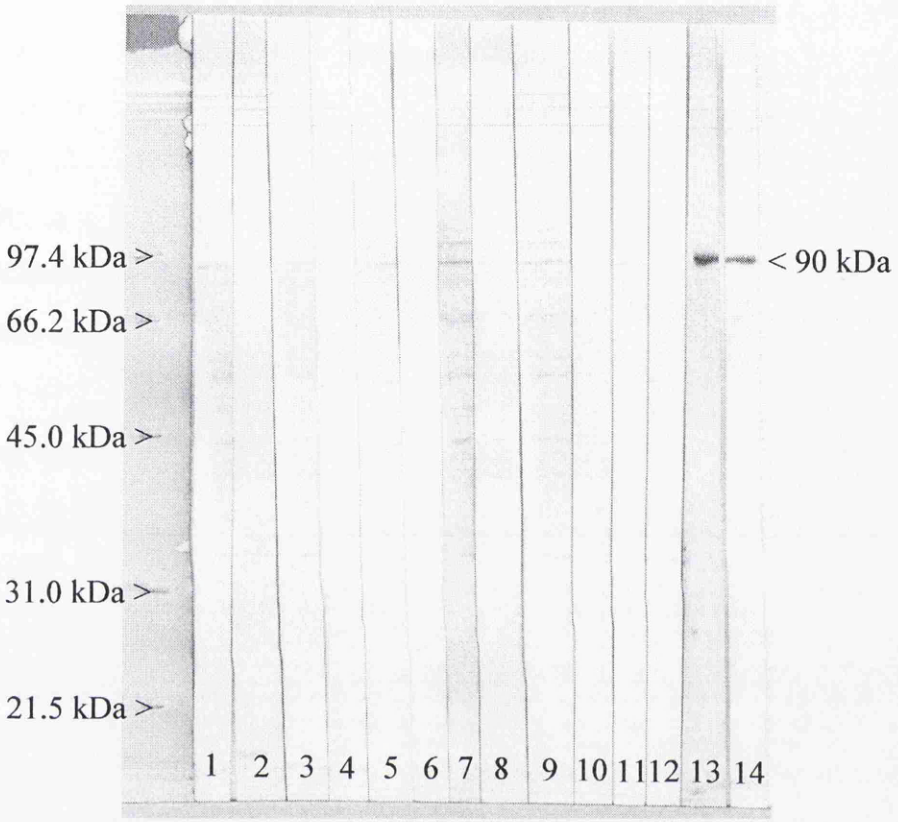


Figure 6.2 :

A western blot probed with the mAb, SPA835, at dilutions of 1/500, 1/1000 and 1/2000, followed by an anti-rat IgG alkaline phosphatase conjugate at dilutions of 1/4000 and 1/8000. The blot was developed with BCIP/NBT. Total protein extracts in SDS-sample cocktail of mf cultured at 37°C (lanes 1-12) were incubated with the anti-HSP90 mAb, SPA835 (lanes 1,3,5,7,9,11) or control mouse IgG (lanes 2,4,6,8,10,12). The secondary antibody was used at a dilution of 1/4000 (lanes 1-6) or 1/8000 (lanes 7-12). In addition, purified bovine HSP90 was reacted with the anti-HSP90 mAb at a dilution of 1/1000 and the secondary antibody at a dilution of 1/4000 (lane 13) or 1/8000 (lane 14) and the 90 kDa band corresponding to bovine HSP90 is indicated with an arrow on the right hand side. The sizes of protein molecular weight markers are indicated on the left hand side, by arrows.

It was hoped that by using a mild treatment with detergents to solubilize the 85kDa protein, fewer proteins that cross-reacted with the mAb would be extracted from mf. However, as observed with the first western blot, additional bands were also visible.

6.2.3 Western analyses using the anti-HSP90 monoclonal antibody, SPA830

It is possible that the epitope of human HSP90 recognized by SPA835 is only partially conserved in *B. pahangi* HSP90, resulting in a low binding affinity of this mAb for *B. pahangi* HSP90. A second anti-HSP90 mAb, SPA830 raised against HSP90 from the water mold, *Achyla ambisexualis* was therefore tested. The epitope recognized by this mAb (also called AC88) was located within amino acids 604-697 of human HSP90α [Nemato *et al* 1997]. Below is an alignment of this region in HSP90α (residues 604-697, on the top) and the corresponding region in *B. pahangi* HSP90 (residues 587-680, on the bottom). Identical residues are indicated and it is evident that the *B. pahangi* sequence is highly homologous to the human sequence within this region. Therefore, since SPA830 recognizes human HSP90α, it is probable that it will recognize the *B. pahangi* homologue.

⇐ residues 604-697 ⇒

```
GWTANMERIMKAQALRDNSTMGYMAAKKHLEINPDHSIIETLRQKAEADKNDKSVKDLVILLYETALLSSGFSLEDPQTHANRIYRMIKLGLGI
|| |||||||||||||| |||||||||||||||||||| | ||  ||||||| ||||| || |||||||||||||| || |||||||||| |
GWSANMERIMKAQALRDSSTMGYMAAKKHLEINPDHSVIKALRERVEADKNDKTVKDLVVLLFETALLSSGFSLEDPQLHASRIYRMIKLGLDI
```

⇐ residues 587-680) ⇒

Mf were cultured at 28°C, 37°C and 41°C and adult worms were maintained at 37°C or 41°C for 2 hours. Protein was extracted from mf and adults using SDS-sample cocktail. The protein samples were analyzed by western blotting (see ***Figure 6.3***). The mAb identified a faint band of 85kDa in the mf and adult extracts (lanes 2,4,6,8 and 10) but the same band was not observed in the *B. pahangi* samples incubated with control serum (lanes 1,3,5,7 and 9). In addition, a 50kDa band, which possibly represents non-specific binding of the mAb, was observed in these lanes. No obvious difference in the intensity of the 85kDa band was observed between mf cultured at 28°C, 37°C or 41°C and adults cultured at 37°C and 41°C. However, different amounts of HSP90 may not be observed by this western blotting protocol. Nevertheless, the western blot was encouraging since less reactivity of SPA830 with other proteins was observed than with SPA835 and the 85kDa band was clearly defined.

Figure 6.3 : Western blot of mf and adult extracts probed with the mAb, SPA830

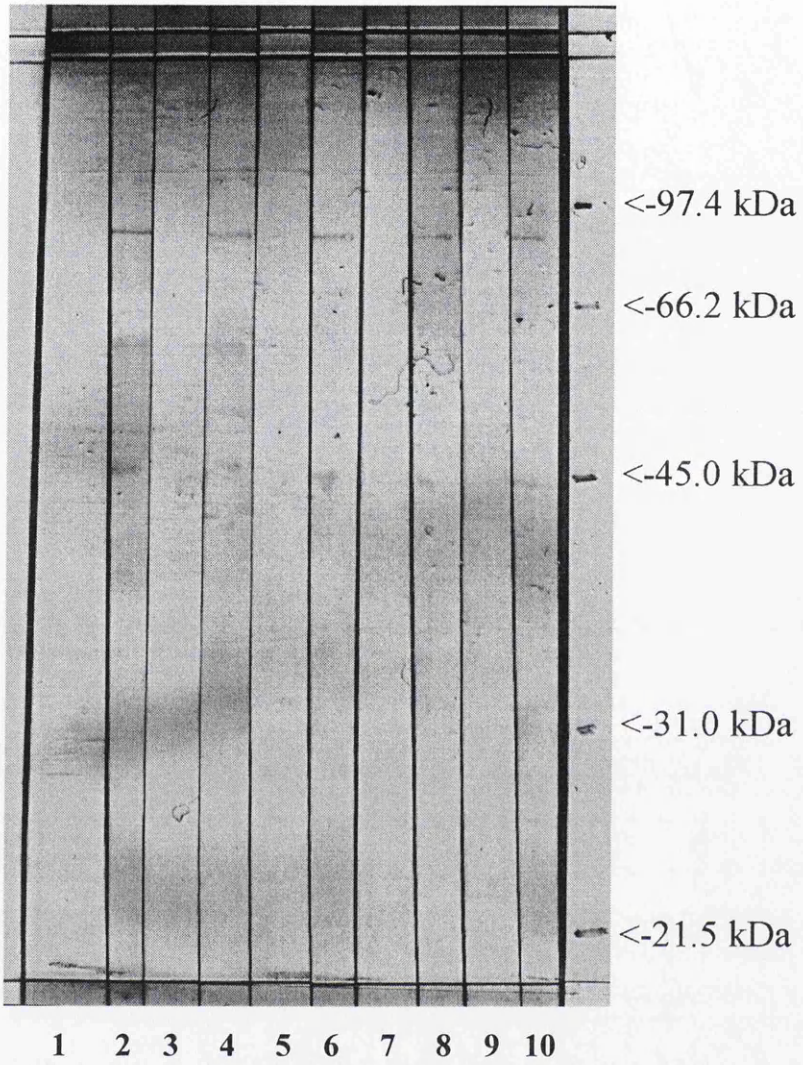


Figure 6.3 :

A western blot probed with mAb SPA830 at a dilution of 1/1000, followed by an anti-mouse IgG alkaline phosphatase conjugate at a dilution of 1/4000 and developed with BCIP/NBT. Total SDS-sample cocktail soluble extracts from mf cultured at 28°C (lanes 1 and 2), 37°C (lanes 3 and 4) and 41°C (lanes 5 and 6) and from adults cultured at 37°C (lanes 7 and 8) and 41°C (lanes 9 and 10) were incubated with SPA830 (lanes 2,4,6,8) or control serum (lanes 1,3,5,9). The sizes of protein molecular weight markers are indicated on the right hand side, by arrows. A band of 85kDa (*B. pahangi* HSP90) is visible in lanes 2,4,6,8 and 10. A band of approximately 45kDa is also faintly visible in these lanes.

6.2.4 Producing a polyclonal antiserum against *B. pahangi* HSP90

The two commercial anti-HSP90 antibodies identified a protein of 85kDa, which is present in total protein extracts from mf and adults. In addition, the 85kDa band was identified in a DOC soluble extract from mf (6.2.2.1). To further investigate the *B. pahangi* HSP90, a polyclonal antiserum was raised in rabbits against an expressed fragment of the *B. pahangi hsp90* cDNA clone. To increase the probability of raising antibodies specific to *B. pahangi* HSP90, a fragment corresponding to the carboxyl terminus of the HSP90 sequence was chosen. This region contains the most divergent sequence between HSP90s from different species (see ***Figure 3.10***) and therefore a fusion protein, which contains a carboxyl terminal fragment of *B. pahangi* HSP90, should have a greater chance of eliciting antibodies to *B. pahangi*-specific epitopes.

6.2.4.1 Cloning a fragment of the *B. pahangi hsp90* gene into an expression vector

Primers were made that would amplify the final 714bp (238 residues) of the open reading frame to create a fragment referred to as pe90. The primers were designated 90pef and 90per and contained restriction sites at the 5′ end to facilitate the cloning of the fragment into the expression vector (pQE30) (see ***Figure 6.4***). This vector results in the expression of a fusion protein with a histidine-tag at the N-terminus, which permits purification of the protein using nickel-agarose resin (Ni-NTA). The predicted size of pe90 is 27kDa and the additional histidine tag increases the size of the recombinant protein by approximately 1kD.

The pe90 fragment was over-expressed in bacterial cells and purified using Ni-NTA. Pe90 co-purified with other proteins under non-denaturing conditions (data not shown). Therefore to reduce association of *E. coli* proteins with pe90 it was necessary to utilize urea as a denaturing agent. Pe90 was subsequently purified under partially denaturing conditions (2M urea) to reduce the specific and non-specific interaction of other proteins with pe90 (see ***Figure 6.5***). However, it was necessary to further purify the fusion protein by SDS-PAGE due to trace amounts of contaminants. The fusion protein was excised from the Coomassie Blue stained gel and used to vaccinate a rabbit as described in the material and methods. After the second boost, the polyclonal antiserum was collected and utilized as described below.

Figure 6.4 : Primers designed for the amplification of the fragment, pe90

			*Bam*HI				
90pef (forward):	5′-CTGGTG**G**ATCCAGGGAAGCTGTCGCC-3′						
cDNA sequence :	CTGGTG**A**ATCCAGGGAAGCTGTCGCC						
Translation :	G/E	S	R	E	A	V	A
			*Kpn*I	**stop**			
90per (reverse):	5′-CCATGGTACC**TTA**ATCAACTTCTTCCATCC-3′						
cDNA sequence :	TTAATCAACTTCTTCCATCC						
Translation :	M	E	E	V	D	*	

Sequence of pe90 :

GSREAVASSAFV ERVKRRGFEV IYMTDPIDEY CVQQLKEYDG KKLVSVTKEG
LELPESEEEK KKFEEDKVKF ENLCKVMKDI LEKKVEKVAV SNRLVSSPCC
IVTSEYGWSA NMERIMKAQA LRDSSTMGYM AAKKHLEINP DHSVIKALRE
RVEADKNDKT VKDLVVLLFE TALLSSGFSL EDPQLHASRI YRMIKLGLDI
TEDEEEEAIA SVSGEKDECV PNLVGAEEDA SRMEEVD

Figure 6.4 :

The sequence of the primers is shown aligned with the corresponding sequence from the *hsp90* cDNA clone. A single base substitution in 90pef compared to the cDNA sequence, generated a *Bam*HI site. The base change causes a substitution of glycine for glutamic acid in the fusion protein, pe90. Five bases 5′ to the *Bam*HI site in 90pef were retained to improve the efficiency of cleavage. A *Kpn*I site and four additional bases were added 5′ to the sequence for *hsp90* in 90per, which includes a stop codon (in bold). The amino acid sequence that corresponds to the primer sequence is shown below the respective primer. The sequence of pe90 is also shown and the substituted glycine residue is indicated in bold. The *Bam*HI and *Kpn*I sites were used to clone the PCR product into the expression vector, pQE30.

Figure 6.5 : Nickel-agarose resin purification of the pe90 fusion protein

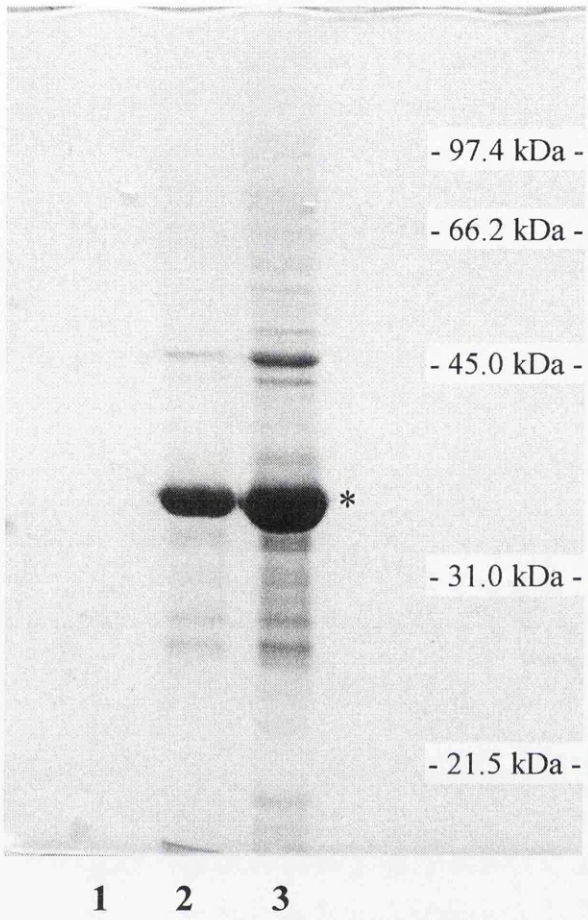


Figure 6.5 :

A Coomassie Blue stained SDS polyacrylamide gel (12.5%) loaded with the pe90 fusion protein. Pe90 was expressed in bacterial cells and the cells were disrupted by sonication. The fusion protein was then purified, in the presence of 2M urea, from the cell lysate by affinity binding with Ni-NTA. Imidazole solutions of increasing molar strength were added to the column, 5ml at a time, to elute the fusion protein by displacement. The sizes of protein molecular weight markers are indicated on the right hand side by arrows and the fusion protein is indicated with an asterisk (*). The larger than predicted size of pe90 is due to the anomalous migration of histidine-tagged protein with SDS-PAGE [Qia*expressionist* booklet March 1997]. The following elution buffers were used :

1 50mM imidazole

2 100mM imidazole

3 500mM imidazole

6.2.5 Western blot analysis of mf and adult extracts using anti-HSP90 polyclonal serum

Western blots were carried out with the anti-HSP90 (pe90) antiserum in order determine whether the immunization protocol had resulted in an antibody which recognized *B. pahangi* HSP90 and to compare the size of the recognized band with the ^{35}S-labelled 85kDa protein observed in mf at 37°C ***(Figure 6.1)***. Protein extracts were made from mf cultured at 28°C, 37°C and 41°C and from adult worms cultured at 37°C and 41°C using SDS-sample cocktail. Firstly, the protein samples were compared by SDS-PAGE and then subjected to western blot analysis (see ***Figure 6.6***).

There are no visible differences between the mf extracts cultured at 28°C, 37°C and 41°C or between adult extracts cultured at 37°C and 41°C. However, some differences are evident between mf extracts and adult extracts, but these do not include proteins around 85kDa. The same samples were then run and blotted and probed with rabbit polyclonal anti-HSP90 anti-serum. A band of approximately 85kDa was identified by the anti-HSP90 antiserum in the mf (lanes 2, 4, 6) and adult extracts (lanes 8 and 10) at all temperatures, but was not identified by the pre-immune serum. There is very little difference in the intensity of this band from the different extracts. An additional band of approximately 70kDa also reacted with the immune serum (lane 8).

6.2.6 Immunoprecipitation of protein extracts from metabolically labelled adults

Western blot analysis confirmed the presence of *B. pahangi* HSP90 in total protein from mf and adults. To further investigate the biosynthesis of HSP90 in the parasite, adult worms were metabolically labelled in the presence of ^{35}S-methionine for 2 hours and a DOC soluble protein extract was subjected to immunoprecipitation using the anti-HSP90 antiserum. Mf were not used in this study due to safety considerations. A glass homogenizer can be used to prepare an adult extract, but mf cannot be disrupted this way and must be sonicated. Sonication of ^{35}S-labelled material is undesirable due to the creation of a radioactive aerosol.

Figure 6.6 : Western blot of mf and adults extracts using anti-HSP90 anti-serum

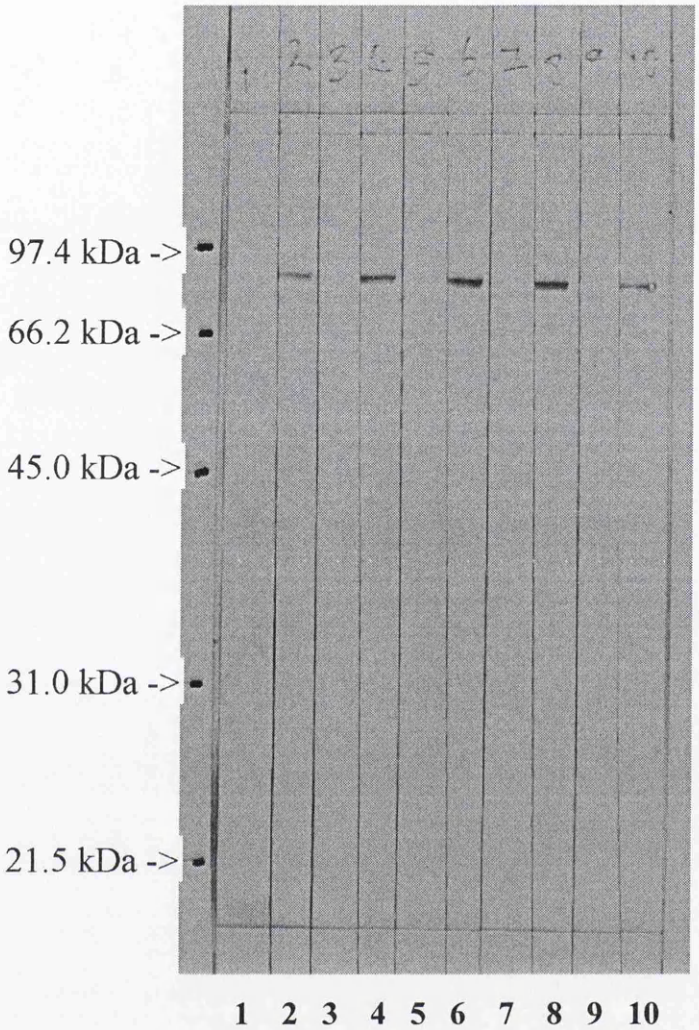


Figure 6.6 :

A western blot probed with the polyclonal anti-HSP90 antiserum or with pre-immune serum from the same rabbit, followed by an anti-rabbit IgG alkaline phosphatase conjugate at a dilution of 1/6000 and developed with BCIP/NBT. Total SDS-sample cocktail extracts from mf maintained at 28°C (lanes 1 and 2), 37°C (lanes 3 and 4) and 41°C (lanes 5 and 6) and from adults maintained at 37°C (lanes 7 and 8) and 41°C (lanes 9 and 10) were incubated with anti-HSP90 antiserum at a dilution of 1/200 (lanes 2,4,6,8,10) or with the pre-immune serum (lanes 1,3,5,7,9) at a dilution of 1/200. The sizes of protein molecular weight markers are indicated on the left hand side, by arrows. A band of 85kDa (*B. pahangi* HSP90) is visible in lanes 2,4,6,8 and 10. A band of approximately 70kDa is faintly visible in lane 8.

In this experiment (see ***Figure 6.7***), the incorporation of ^{35}S-methionine into adults cultured at 37°C was 9932.4cpm/µl which is approximately twice that of the incorporation in adults cultured at 41°C, 4518.6cpm/µl. This may be explained by the general reduction in protein synthesis which results from a heat shock (41°C). Indeed the extract from heat shocked adults (lane 2) shows lower levels of most ^{35}S-labelled polypeptides compared to the extract from 37°C cultured adults, but an increase in the level of polypeptides of 85kDa (HSP90) and 70kDa (HSP70) is evident. Confirmation of the identity of the 85kDa protein was obtained by the immunoprecipitation of the same extracts with anti-HSP90 antiserum.

The ^{35}S-labelled polypeptides from adult worms maintained at 37°C and 41°C were immunoprecipitated using the polyclonal anti-HSP90 anti-serum and precipitated protein was separated by SDS-PAGE and visualized by autoradiography (see ***Figure 6.8***). The anti-HSP90 antiserum precipitated a band of approximately 85kDa in extracts from adults cultured at 37°C and 41°C, but this band was not precipitated with the pre-immune serum. A faint band of approximately 70kDa was also visible in both extracts precipitated with anti-HSP90 antiserum (lanes 2 and 4). A few other faint bands were visible in the extract from adults cultured at 37°C, but these bands were precipitated both by the anti-HSP90 and the pre-immune serum (lanes 1 and 2). The additional bands are probably due to non-specific binding of the polyclonal serum with proteins in the extract, but may also be due to shared epitopes present both on *B. pahangi* HSP90 and on other proteins, resulting in their immunoprecipitation.

6.3 Discussion

Metabolic labelling of mf identified an 85kDa protein, which was up-regulated in mf cultured at 37°C when compared to mf cultured at 28°C. This paralleled the observation of a difference in the level of expression of *hsp90* mRNA in mf cultured at 37°C and 28°C. Previous studies in which mf of *B. pahangi* were heat shocked at 41°C demonstrated the almost exclusive expression of polypeptides of 85kDa, 70kDa, 62kDa, 22.5kDa and 18.5kDa during a heat shock [Selkirk *et al* 1989] suggesting that these proteins were HSPs.

Figure 6.7 : Metabolic labelling of adult worms with ^{35}S-methionine

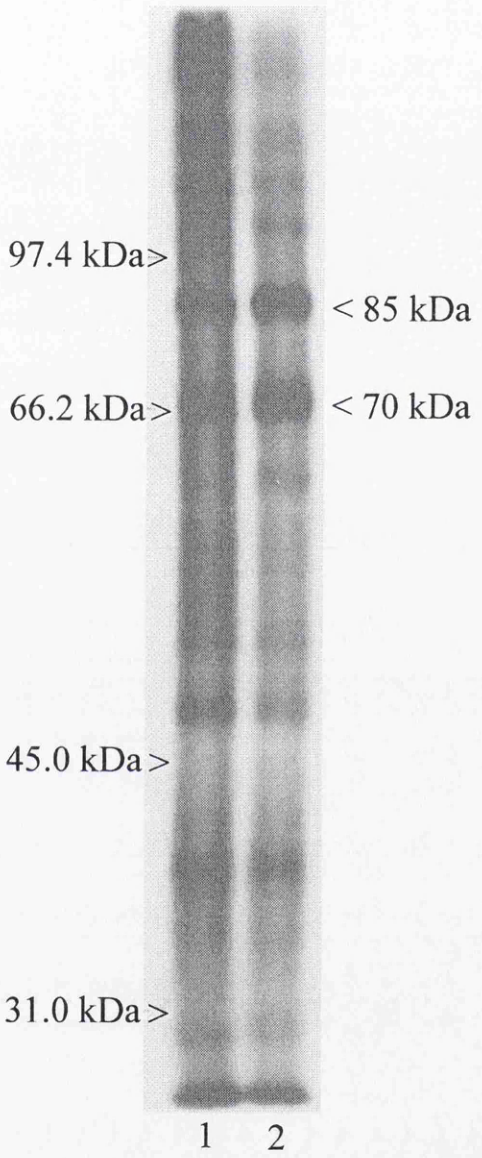


Figure 6.7 :

An autoradiograph of newly synthesized polypeptides labelled with ^{35}S-methionine. Adults were cultured at 37°C (lane 1) and 41°C (lane 2) and DOC extracted proteins were separated by SDS-PAGE on a 10% gel. The sizes of protein molecular weight markers are indicated by arrows on the left hand side and bands of 85kDa (HSP90) and 70kDa (HSP70) are indicated by arrows on the right hand side.

Figure 6.8 : Immunoprecipitation of metabolically labelled adult worms with anti-HSP90 polyclonal anti-serum

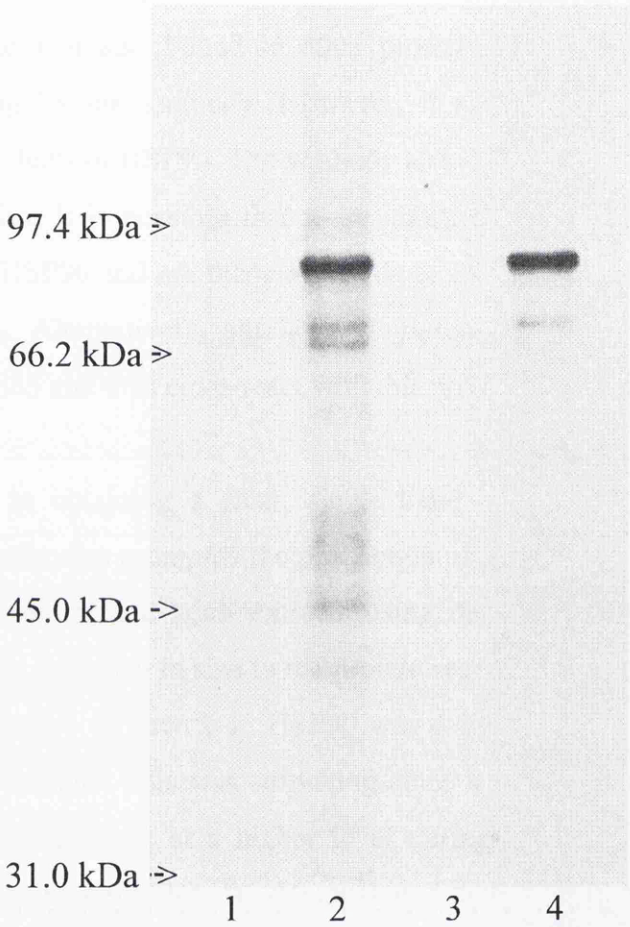


Figure 6.8 :

An autoradiograph showing an immunoprecipitation of ^{35}S-labelled adult with the polyclonal anti-HSP90 antiserum or with the pre-bleed. DOC extracts (2.5 x 10^5 TCA precipitable cpm) from adults maintained at 37°C (lanes 1 and 2) and 41°C (lanes 3 and 4) were mixed with 20µl anti-HSP90 antiserum (lanes 2 and 4) or 20µl pre-immune serum (lanes 1 and 3) followed by precipitation of the immune complexes with Protein A Sepharose. The sizes of protein molecular weight markers are indicated by arrows on the left hand side.

Two commercial mAbs raised against human HSP90 (SPA835) and *Achyla ambisexualis* (SPA830) recognized an 85kDa protein in western blots of extracts from *B. pahangi*. This implied that there was sufficient similarity between the HSP90s of human and *Achyla ambisexualis* and the *B. pahangi* HSP90 for the antibodies to recognize the *B. pahangi* protein. In the first western blot, although SPA835 recognized an 85kDa protein, it also bound to other proteins. This most likely represents non-specific binding by the antibody. However, it is possible that the bands represent degradation products of HSP90. The antibody also bound to a component of greater than 90kDa. Therefore it is possible that these additional bands represent proteins which associate with HSP90 and are recognized due to contaminating HSP90 which is bound to the proteins. Alternatively, unrelated *B. pahangi* proteins may share the epitope bound by SPA835 and thus cross-react with this mAb.

The difficulty in obtaining a clear, single band of reasonable intensity with these heterologous antibodies prompted the production of an anti-serum to *B. pahangi* HSP90. A western blot of mf and adult extracts using the polyclonal antiserum identified a protein of 85kDa, identical in size to the protein recognized by SPA830 (***Figure 6.3*** and ***6.6***). No quantitative difference in HSP90 was evident, even in the extracts from heat shocked mf and adults. This was surprising since it was predicted that this heat shock protein should accumulate at a higher level during a heat shock, but the result was reproducible. The western blot also demonstrated that HSP90 was present in mf at 28°C and adults at 37°C even though *hsp90* mRNA was not observed by Northern blot analysis (***Figure 3.12***). HSP90 is an essential protein for the function of many eukaryotic cells, under normal conditions. For example, it is reported to chaperone the folding of newly synthesized proteins [Wiech *et al* 1992] and is also thought to be involved in the process of actin polymerization [Koyasu *et al* 1986]. It accounts for almost 0.1% cellular protein in mammalian cells [Lindquist and Craig 1988]. The western blot is an analysis of total HSP90 and so the relative difference in HSP90 concentration in mf or adults may be small even after 2 hours at 41°C. Hence, the western blot may not be sufficiently sensitive to detect differences in HSP90 concentration between normal conditions and during a heat shock or importantly between mf cultured at 37°C and mf cultured at 28°C.

The use of a more sophisticated quantitative western blotting protocol may be required to investigate a difference in the concentration of HSP90 in extracts from mf and adults cultured at different temperatures. A technique utilizing a ^{125}I labelled counter-antibody has been described for the quantification of HSP90 [Ning and Sánchez 1996, Bresnick *et al* 1990]. This method utilizes an ^{125}I-conjugated anti-mouse IgG followed by an alkaline phosphatase conjugate. The quantity of HSP90 is determined by excising the stained HSP90 band from the western blot and counting the radioactivity.

Western blotting identifies steady state HSP90 and cannot measure the rapidity of protein turnover. The ^{35}S-methionine metabolic labelling experiments (***Figures 6.1*** and ***6.7***) could be repeated but modified to a pulse and chase protocol by the subsequent addition of "cold" methionine. HSP90 in mf cultured at 28°C, 37°C and 41°C and adults cultured at 37°C or 41°C may have a different biological half-life, which would explain the similarity of HSP90 concentration observed by western blotting. In pulse chase experiments, a rapid turnover of HSP90 would be observed as a diminished signal for HSP90 by autoradiography.

Metabolically labelling adult parasites with ^{35}S-methionine at 37°C and 41°C identified proteins of 85kDa and 70kDa which were up-regulated during a heat shock of 41°C, consistent with their identity as possible heat shock proteins. The apparent difference in the expression of the 85kDa protein (presumed to be HSP90) at 37°C and 41°C was not as pronounced as would be predicted from *hsp90* mRNA levels in adults cultured at these temperatures (see ***Figure 3.12***). Adult females were used in the metabolic labelling and some of the expressed 85kDa protein may originate from embryos within the females. Mouse early embryos synthesize HSP90α and HSP90β at a very high rate during normal development [Loones *et al* 1997]. Furthermore, early embryos of the sea urchin, *Strongylocentrotus purpuratus* express HSP90, but the protein is derived from the translation of maternally-derived mRNA and not from transcription of the embryo gene [Bédard and Brandhorst 1986]. If translational activation of *hsp90* transcripts occurred in *B. pahangi* embryos, then the increased expression of HSP90 would be observed in extracts from adult females, but the level of *hsp90* mRNA would be unaltered.

HSP90 anti-serum precipitated a polypeptide of 85kDa from adult extracts (***Figure 6.8***, lanes 2 and 4) but a polypeptide of this size was not precipitated by pre-immune serum (lanes 1 and 3). This is evidence that the ^{35}S-methionine labelled 85kDa protein observed in adults cultured at 37°C and 41°C is indeed HSP90. Other, fainter bands are also visible in the autoradiograph and these probably represent the non-specific binding of the polyclonal anti-HSP90 antibodies to other proteins. However, it is possible that these proteins were either specifically bound by anti-HSP90 antibodies or were co-precipitated in association with HSP90. HSP70 and HSP90 share the terminal four residue (EEVD) [Boorstein *et al* 1994] and therefore the 70kDa polypeptide also visible in lanes 2 and 4, may be HSP70, immunoprecipitated by the anti-HSP90 polyclonal serum. A receptor (AHR-1) has recently been described from *C. elegans* which appears to be analogous to the dioxin receptors of vertebrates. The endogenous ligand for AHR-1 has yet to be identified, but like steroid hormone receptors and dioxin receptors, the *C. elegans* receptor associates with HSP90 [Powell-Coffman *et al* 1998]. The predicted size of AHR-1 (unmodified) is 68kDa, similar to the size of the band observed in the samples precipitated with anti-HSP90 antiserum and it is possible that a *B. pahangi* homologue of this receptor may have been co-precipitated due to its association with HSP90. However, the presence of detergents in the washing buffer would not have made the conditions favourable for co-immunoprecipitation.

The difference in the molecular weight predicted from the translation of the cDNA (83kDa) and the protein gels (85kDa) may merely reflect an inaccuracy from measuring the size from SDS-PAGE but may also be due to post-translational modifications of the native molecule. For example HSP90 may be phosphorylated *in vivo* and the addition of phosphates may therefore cause an alteration in the migration of the protein on SDS polyacrylamide gel. Both the murine α and β HSP90 isoforms are constitutively phosphorylated by casein kinase II, exclusively on serine residues and within a highly charged region of the protein [Dougherty *et al* 1987]. A serine (position 237 in ***Figure 3.10***) within the corresponding charged region of *B. pahangi* HSP90 may be a possible target for phosphorylation. The phosphorylation of HSP90 has been proposed as a control mechanism for the association/dissociation of HSP90 with oncogenic tyrosine kinase, pp60$^{v\text{-}src}$ [Mimnaugh *et al* 1995]. Additionally, *B. pahangi* HSP90 may be modified by glycosylation since there are two N-glycosylation sites in the sequence of

HSP90 (***Figure 3.10***, 51-53 and 436-438). However, an investigation of mammalian HSP100 and HSP90 reported glycosylation (and association with the Golgi apparatus) of the former but not of the latter [Welch *et al* 1983].

In future experiments to characterize a potential target of *B. pahangi* HSP90 it may be possible to increase the stability of the interaction between HSP90 and an associated protein and facilitate their co-precipitation. The metal oxyanion, molybdate appears to stabilize the interaction of HSP90 with target proteins such as the steroid hormone receptors [Meshinchi *et al* 1988], the tyrosine kinase, $pp60^{v\text{-}src}$ [Hutchinson *et al* 1992c] and the serine threonine kinase, c-Raf [Stancato *et al* 1993] and thus facilitate their co-immunoprecipitation. The immunoprecipitation experiment in ***Figure 6.8*** could be repeated but with the addition of molybdate to the *B. pahangi* adult extracts in an attempt to increase the quantity of HSP90-associated proteins co-precipitating with HSP90. Consequently the precipitated protein may be eluted from an SDS-PAGE gel and the N-terminal sequenced to elucidate its identity.

In summary

- The differential expression of an 85kDa protein was identified by comparing the protein profiles of mf metabolically labelled with ^{35}S-methionine at 28°C and 37°C for 3 hours
- The presence of HSP90 in mf and adults extracts was confirmed by western blotting, using polyclonal antiserum raised against a fusion protein, which contained a carboxyl terminal fragment of *B. pahangi* HSP90. Monoclonal antibodies raised against heterologous HSP90s also reacted with *B. pahangi* HSP90
- Immunoprecipitation using the anti-HSP90 antiserum confirmed that the 85kDa protein, differentially expressed in mf at 28°C and 37°C, is HSP90

7.0 Final discussion

An *hsp90* homologue was isolated from *B. pahangi* and has high homology to *hsp90* genes from other eukaryotic species, especially to a putative *hsp90* homologue from the nematode, *C. elegans*. Southern blotting suggested that there were no other copies of this *hsp90* gene, or of similar genes, in the *B. pahangi* genome. Similarly, one HSP90 sequence [Wormpep database] was identified from the *C. elegans* sequencing project (completed December 1998).

The presence of 11 introns in *B. pahangi hsp90* is interesting since it might be anticipated that genes required as part of a stress response may have evolved to lose introns. This may provide further indirect evidence that the function of *B. pahangi* HSP90 is not only required during cellular stress but also under normal conditions. Consistent with this theory is the observation that human *hsp90*α and *hsp90*β, which have 10 introns [Hickey *et al* 1989] and 11 introns [Rebbe *et al* 1989] respectively, are expressed at normal physiological temperatures. The presence of introns may be required for the efficient translation of *hsp90* mRNA as described in 4.4. Furthermore, HSP90 might not be required during extreme heat shock conditions (as discussed for *Drosophila* HSP83 in section 4.4) and this would remove evolutionary pressure for an intron-less *hsp90* gene.

Transcripts for *B. pahangi hsp90* were detected, by Northern blotting, at higher levels in mf maintained at 37°C when compared to mf at 28°C and adult worms at 37°C. A comparison of the profiles of newly synthesized polypeptides from mf maintained at 28°C and 37°C revealed the increased synthesis of an 85kDa protein in ^{35}S-labelled proteins from mf maintained at 37°C. The 85kDa protein was immunoprecipitated from extracts of adult worms maintained at 37°C and heat shocked at 41°C, using anti-serum (95FP) raised against a fusion protein containing the carboxyl terminal fragment of *B. pahangi* HSP90. Therefore HSP90 is differentially expressed in mf exposed to mammalian body temperature and since this life cycle stage is developmentally blocked in the mammalian host, HSP90 may be implicated in this block. This may also imply that the expression of HSP90 is controlled not only by heat-induction, but also by a developmental cue.

At 37°C, HSF may be activated in mf by a developmental factor, which is not present or active in adults, resulting in the differential expression of HSP90 in these two life cycle stages. This is supported by the observation that the expression of other HSPs, for example two small HSPs, are also up-regulated in mf but not adults at 37°C [Devaney *et al* 1992, Devaney *et al* 1996]. However, other transcription factors may be activated in mf. For example, a *B. pahangi* homologue of GATA, a family of transcription factors which appear to be important for differentiation, development and tissue-specific expression of genes [Simon 1995], could bind to the *hsp90* promoter and induce the transcription of *hsp90*. Alternatively, *hsp90* mRNA may be more stable in mf than in adults, resulting in a higher cellular level of HSP90. This could be due to the presence of an inhibitor acting on a ribonuclease, which degrades *hsp90* mRNA, or to specific *hsp90* mRNA-proteins which increase the stability of the transcripts.

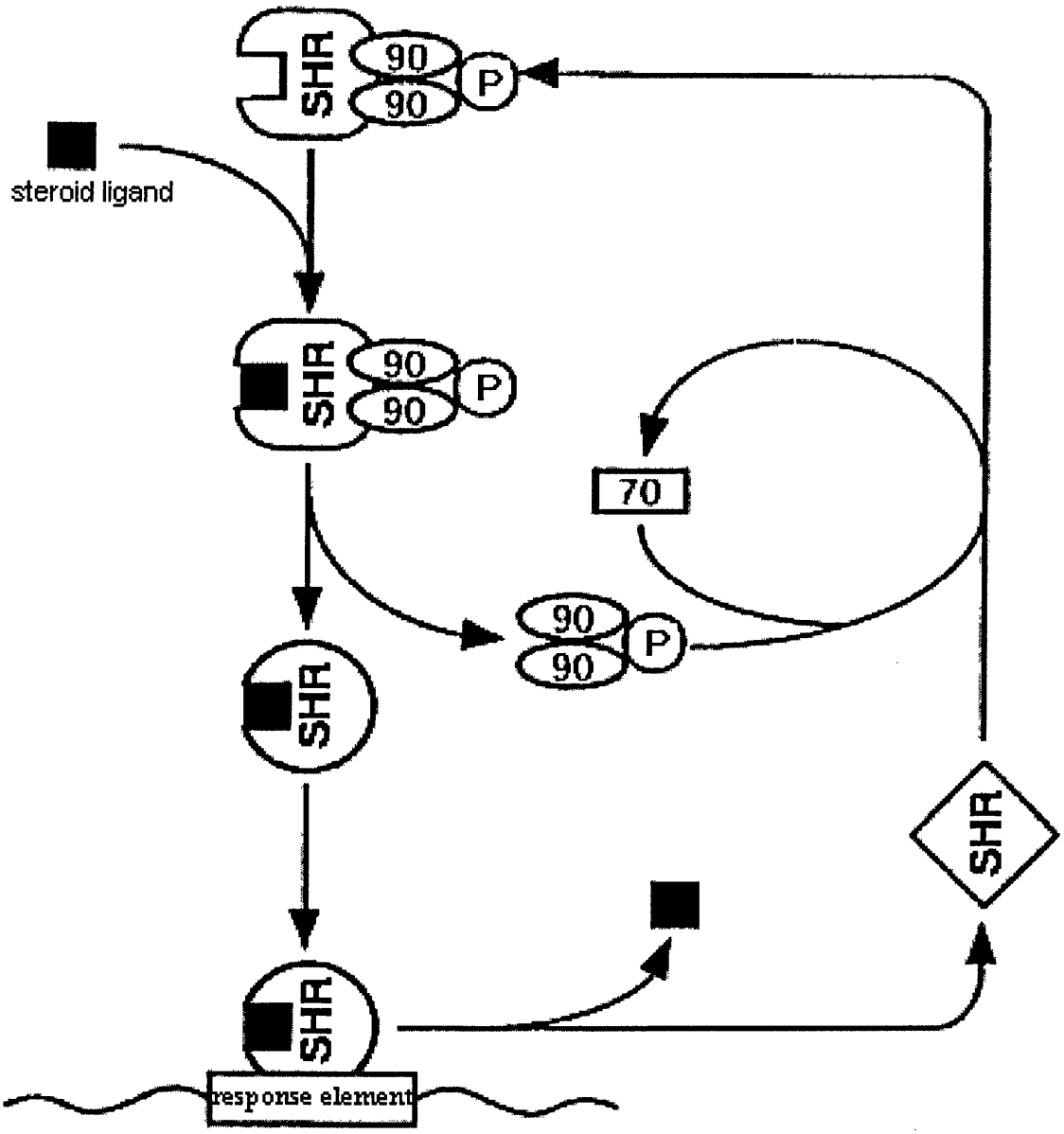
A 1.2kb "promoter" region, upstream from *hsp90*, was sequenced. This region contained a number of putative transcription binding factor sites, which were also observed in the promoters from other *hsp90*s, and from other heat shock protein genes. There are five heat shock elements, which are thought to be required for the heat shock induction of *hsp90* but also CCAAT, GC and GATA boxes, which may have a role in the basal expression of the gene. When a region of the putative *hsp90* promoter was used to control the expression of CAT, (a prokaryotic gene), in mammalian (COS-7) cells, CAT was detected in extracts from cells cultured at a normal physiological temperature (37°C) and after a 1-hour heat shock (41°C). This further supports the proposition that *B. pahangi hsp90* is not strictly heat-inducible and is expressed in the parasite under normal conditions, as well as during a heat shock.

The ability of the *hsp90* promoter region to induce the expression of CAT revealed that transcriptional activators in COS-7 cells were able to bind to and initiate transcription from nematode transcription factor binding elements. This suggests that there is some degree of conservation between the primate and nematode transcription factors, required for the expression of *hsp90*. Similarly the *S. mansoni hsp70* promoter was used to control CAT expression in mammalian (CHO) cells and it was proposed that the heat shock transcription factor (HSF) from the CHO cells bound to the heat shock elements of the trematode promoter and induced transcription [Levy-Holtzman and Schechter 1995]. In addition to HSF, the transcriptional initiation complex from the mammalian

cells would have to bind to and initiate reporter gene transcription from the helminth promoters, suggesting that these proteins are sufficiently conserved between the organisms.

If HSP90 does have a role in the process of developmental arrest in the mf, it may relate to the previously observed association of HSP90 with steroid hormone receptors. In HeLa cells a high concentration of HSP90 both prevents the association of activated oestrogen receptors with oestrogen receptor elements and increases the dissociation of pre-existing complexes thus inhibiting transcriptional activation of target genes [Sabbah *et al* 1996]. Smith (1993) studied steroid hormone receptors from chick oviduct and proposed a model for the interaction of progesterone receptors with HSP90, and other co-chaperones. In this model, the association of HSP90 is dynamic and involves a cycle of association and dissociation that is HSP70 and ATP-dependent. In the period when HSP90 is released, the receptor can bind its ligand and thus enter the activation pathway [Smith 1993]. A model for the hypothetical association of HSP90 with a *B. pahangi* steroid hormone receptor is shown in ***Figure 7.1*** and has been modified from the model by Smith (1993). If HSP90 is present at a high concentration, the receptor may be trapped into constant association with HSP90, even in the presence of the steroid ligand. A steroid hormone receptor may be responsible for the re-initiation of development and the steroid ligand may be prevented from activating the target receptor due to a high concentration of HSP90 in mf circulating in the mammalian bloodstream. In addition, the transcriptional activation, by a *B. pahangi* steroid hormone receptor, of genes required for development to the L_2 may be inhibited by HSP90. After transfer to the mosquito, the repression may cease due to a reduction in HSP90 concentration thus enabling the resumption of development. Devaney and Lewis (1993) reported that elevating the temperature of mf-infected mosquitoes to 37°C resulted in the inhibition of development and that the mf did not develop beyond the late L_1. A theory for this observation is that the increase in temperature may result in a higher cellular concentration of HSP90 in the mf, which may inhibit activation of a steroid hormone receptor involved in the developmental process.

Figure 7.1 : The hypothetical interaction of *B. pahangi* HSP90 with a *B. pahangi* steroid hormone receptor



The *B. pahangi* steroid ligand (■) binds to the steroid hormone receptor (SHR) aporeceptor complex forming a transient aporeceptor complex. HSP90 (and other associated proteins, **P**) dissociate from the complex and the resulting activated SHR complex undergoes a conformational change. The activated receptor binds to response elements and modulates transcription from nearby *B. pahangi* promoters. The ligand then dissociates from the response elements. The deactivated aporeceptor re-associates with HSP90 (and **P**) in the cytoplasm and reconstitutes the aporeceptor complex. HSP70 may transiently associates with the aporeceptor during the assembly of the complex.

The process of moulting is common to both insects and nematodes and it has been suggested that moulting arose once in evolution, a theory supported by the close relationship observed between arthropods, nematodes and other moulting animals by phylogenetic analysis [Aguinaldo *et al* 1997]. Therefore common mechanisms of development might be expected. The steroid ligand hypothesized to bind to a *B. pahangi* SHR and trigger the resumption of *B. pahangi* development, could be ecdysterone, a steroid hormone involved in the moulting of insects. This hormone has been reported to cause the premature timing of the L_3 moult in *D. immitis* [Warbrick *et al* 1993]. The *B. pahangi* SHR involved in mf development could be a homologue of the *C. elegans* hormone receptor, CHR3. The ligand for CHR3 has not been identified, but the receptor is highly homologous to a *Drosophila* ecdysterone-inducible gene product, DHR3. Mutant forms of CHR3 affect *C. elegans* moulting and it was suggested that this receptor might regulate the onset of moulting [Kostrouchova *et al* 1998].

Future work

To investigate the *hsp90* promoter, different fragments of the promoter region could be produced and their effect on CAT expression studied. By creating a battery of fragments which have sequential putative binding elements removed, it may be possible to delineate the heat shock induction and/or basal activation of the promoter. In addition, it may be possible to transfect constructs containing regions of the *B. pahangi* promoters could be transfected into *C. elegans*. The expression of a reporter gene might then be observed in different life cycle stages of *C. elegans* and this expression could be compared to the expression of HSP90 in different life cycle stages of *B. pahangi*. Specifically, it would be interesting to compare the expression of the reporter gene in the dauer larva and *C. elegans* adult compared to the synthesis of *hsp90* mRNA and protein in *B. pahangi* mf and adults. Differential expression due to the *hsp90* promoter could be observed in dauer and adults.

The anti-HSP90 antiserum, 95FP, could be used to confirm the presence of HSP90 in all the life cycle stages. It could also be used to immunoprecipitate *in vitro* translation products, translated from mRNA extracted from mf maintained at 28°C and 37°C and from adults maintained at 37°C. By immunoprecipitation, differences might be observed in the profile of HSP90-associated protein. As described in 1.4.2, molybdate has been reported to stabilize the interaction of HSP90 with steroid hormone receptors and also

with other associated proteins, for example the tyrosine kinase, $pp60^{v\text{-}src}$ [Hutchinson *et al* 1992]. This oxyanion might therefore be useful in increasing the yield of *in vitro* translated proteins immunoprecipitated by 95FP.

More than 200 SHRs have been identified by the *C. elegans* sequencing project and two *B. pahangi* SHRs have also been isolated and sequenced in the laboratory (by Dr. Joyce Moore). These sequences could be used to produce plasmids containing nematode *shr*s and *B. pahangi hsp90*, which could be co-transfected into either a mammalian cell line or into *C. elegans*. The effect of factors, such as ecdysterone, on the interaction with HSP90 with an SHR could then be investigated.

The sequence of *C. elegans hsf* is available and it may therefore be possible to isolate *B. pahangi hsf* by PCR with heterologous primers designed from the *C. elegans* sequence and by screening a *B. pahangi* library with a homologous probe containing a PCR fragment of *B. pahangi hsf*. Alternatively, a repeat of the south-western screening of a *B. pahangi* cDNA expression library could be carried out, using a region of the *hsp90* promoter containing a concentration of heat shock elements. A fusion protein, corresponding to *B. pahangi* HSF, could be isolated and used to make an antiserum for use in gel shift assays. In addition, it may be possible to co-tranfect constructs containing the *B. pahangi hsp90* promoter and *hsf* into COS-7 cells. The promoter-driven expression of CAT could then be observed in relation to the induced expression of *B. pahangi* HSF.

8.0 References

Abravaya, K., Myers, M.P., Murphy, S.P. and Morimoto, R.I. (1992) The Human Heat Shock Protein Hsp70 Interacts with HSF, the Transcription Factor that Regulates Heat Shock Gene Expression. *Genes and Development* **6**, 1153-1164.

Addiss, D.G., Beach, M.J., Streit, T.G., Lutwick, S., LeConte, F.H., Lafontant, J.G., Hightower, A.W. and Lammie, P.J. (1997) Randomised Placebo-controlled Comparison of Ivermectin and Albendazole Alone and in Combination for *Wuchereria bancrofti* Microfilaraemia in Haitian Children. *Lancet* **350**, 480-484.

Adhuna, Salotra, P., Mukhopadhyay, B. and Bhatnagar, R. (1997) Modulation of Macrophage Heat Shock Proteins (HSPs) Expression in Response to Intracellular Infection by Virulent and Avirulent Strains of *Leishmania donovani. Biochemistry and Molecular Biology International* **43**, 1265-1275.

Aguinaldo, A.M.A., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., Raff, R.A. and Lake, J.A. (1997) Evidence for a Clade of Nematodes, Arthropods, and Other Moulting Animals. *Nature* **387**, 489-492.

Alam, J. and Cook, J.L. (1990) Reporter Genes - Applications to the Study of Mammalian Gene-transcription. *Analytical Biochemistry* **188**, 245-254.

Aligue, R., Akhavan-Niak, H. and Russell, P. (1994) A Role for Hsp90 in Cell Cycle Control: Wee1 Tyrosine Kinase Activity Requires Interaction with Hsp90. *EMBO Journal* **13**, 6099-6106.

Allegretti, S., Hambourg, C., Huynh, V.T. and DupouyCamet, J. (1997) An HSP60-63 Homologue is Constitutively Expressed in Infective Larvae of *Trichinella spiralis. Parasite Journal de la Societe Francaise de Parasitologie* **4**, 283-285.

Allen, S.P., Polazzi, J.O., Gierse, J.K. and Eaton, A.M. (1992) Heat Shock Genes Encoding Proteins Produced in Response to Heterologous Protein Expression in *Escherichia coli. Journal of Bacteriology* **174**, 6938-6947.

Alton, N.K. and Vapnek, D. (1979) Nucleotide Sequence Analysis of the Chloramphenicol Resistance Transposon Tn*9*. *Nature* **282**, 864-869.

Aly, R., Argaman, M., Pinelli, E. and Shapira, M. (1993) Intergenic Sequences from the Heat-shock 83-encoding Gene Cluster in *Leishmania mexicana amazonensis* Promote and Regulate Reporter Gene Expression in Transfected Parasites. *Gene* **127**, 155-163.

Aly, R., Argaman, M., Halman, S. and Shapira, M. (1994) A Regulatory Role for the 5' and 3' Untranslated Regions in Differential Expression of Hsp83 in *Leishmania. Nucleic Acids Research* **22**, 2922-2929.

Antonsson, C., Whitelaw, M.L., McGuire, J., Gustafsson, J. and Poellinger, L. (1995) Distinct Roles of the Molecular Chaperone Hsp90 in Modulating Dioxin Receptor Function via the Basic Helix-loop-helix and PAS Domains. *Molecular and Cellular Biology* **15**, 756-765.

Aquino, D.A., Peng, D., Lopez, C. and Farooq, M. (1998) The Constitutive Heat Shock Protein-70 is Required for Optimal Expression of Myelin Basic Protein During Differentiation of Oligodendrocytes. *Neurochemical Research* **23**, 413-420.

Argaman, M., Aly, R. and Shapira, M. (1994) Expression of Heat Shock Protein 83 in *Leishmania* is Regulated Post-translationally. *Molecular and Biochemical Parasitology* **64**, 95-110.

Ayoubi, T.A.Y. and van de Ven, W.J.M. (1996) Regulation of Gene Expression by Alternative Promoter. *FASEB Journal* **10**, 453-460.

Azzaria, M., Goszczynski, B., Chung, M.A., Kalb, J.M. and McGhee, J.D. (1996) A *Fork head*/HNF-3 Homolog Expressed in the Pharynx and Intestine of the *Caenorhabditis elegans* Embryo. *Developmental Biology* **178**, 289-303.

Bardwell, J.C.A. and Craig, E.A. (1987) Eukaryotic M_r 83,000 Heat Shock Protein has a Homologue in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* **84**, 5177-5181.

Barker, G.C., Mercer, J.G., Rees, H.H. and Howells, R.E. (1991) The Effect of Ecdysteroids on the Microfilarial Production of *Brugia pahangi* and the Control of Meiotic Reinitiation in the oocytes of *Dirofilaria immitis*. *Parasitology Research* 77, 65-71.

Barnes, J.A. and Smoak, I.W. (1997) Immunolocalization and Heart Levels of GRP94 in the Mouse During Post-implantation Development. *Anatomy and Embryology* **196**, 335-341.

Becker, J., Mezger, V., Courgeon, A. and Best-Belpomme, M. (1990) Hydrogen Peroxide Activates Immediates Binding of a *Drosophila* Factor to DNA Heat-shock Regulatory Element *in vivo* and *in vitro*. *European Journal of Biochemistry* **189**, 553-558.

Bedard, P. and Brandhorst, B.P. (1986) Translational Activation of Maternal mRNA Encoding the Heat-shock Protein Hsp90 During Sea Urchin Embryogenesis. *Developmental Biology* **117**, 286-293.

Benedict, M.Q., Levine, B.J., Ke, Z.X., Cockburn, A.F. and Seawright, J.A. (1996) Precise Limitations of Concerted Evolution to ORFs in Mosquito Hsp82 Genes. *Insect Molecular Biology* **5**, 73-79.

Berger, E.M., Marino, G. and Torrey, D. (1985) Expression of *Drosophila* Hsp70-CAT Hybrid Gene in *Aedes* Cells Induced by Heat Shock. *Somatic Cell and Molecular Genetics* **11**, 371-377.

Bergerat, A., de Massy, B., Gadelle, D., Varoutas, P., Nicholas, A. and Forterre, P. (1997) An Atypical Topoisomerase II from Archaea with Implications for Meiotic Recombination. *Nature* **386**, 414-417.

Bevilacqua, A., Fiorenza, M.T. and Mangia, F. (1997) Developmental Activation of an Episomic Hsp70 Gene Promoter in Two-cell Mouse Embryos by Transcriptional Factor Sp1. *Nucleic Acids Research* **25**, 1333-1338.

Bienz, M. (1985) Transient and Developmental Activation of Heat-shock Genes. *Trends in Biochemical Sciences* **10**, 157-161.

Biggiogera, M., Tanguay, R.M., Marin, R., Wu, Y., Martin, T.E. and Fakan, S. (1998) Localization of Heat Shock Proteins in Mouse Germ Cells: An Immunoelectron Microscopical Study. *Experimental Cell Research* **229**, 77-85.

Binart, N., Chambraud, B., Levin, J.M., Garnier, J. and Baulieu, E. (1989) A Highly Charged Sequence of Chick Hsp90: A Good Candidate for Interaction with Steroid Receptors. *Journal of Steroid Biochemistry* **34**, 369-374.

Birk, O.S., Elias, D., Weiss, A.S., Rosen, A., van-der Zee, R., Walker, M.D. and Cohen, I.R. (1996) NOD Mouse Diabetes: The Ubiquitous Mouse Hsp60 is a β-cell Target Antigen of Autoimmune T Cells. *Journal of Autoimmunity* **9**, 159-166.

Blagosklonny, M.V., Toretsky, J., Bohen, S. and Neckers, L. (1996) Mutant Conformation of p53 Translated *in vitro* or *in vivo* Requires Functional HSP90. *Proceedings of the National Academy of Sciences USA* **93**, 8379-8383.

Blumenthal, T. and Speith, J. (1996) Gene Structure and Organization in *Caenorhabditis elegans*. *Current Opinion in Genetics and Development* **6**, 692-698.

Bohen, S. (1995) Hsp90 Mutants Disrupt Glucocorticoid Receptor Ligand Binding and Destabilize Aporeceptor Complexes. *Journal of Biological Chemistry* **270**, 29433-29438.

Bonnefoy, S., Attal, G., Langsley, G., Tekaia, F. and Mercereau-Puijalon, O. (1994a) Molecular Characterization of the Heat Shock Protein 90 Gene of the Human Malaria Parasite *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **67**, 157-170.

Bonnefoy, S., Gysin, J., Blisnick, T., Guillotte, M., Carcy, B., da Silva, L.P. and Mercereau-Puijalon, O. (1994b) Immunogenicity and Antigenicity of a *Plasmodium falciparum* Protein Fraction (90-110kDa) Able to Protect Squirrel Monkeys Against Asexual Blood Stages. *Vaccine* **12**, 32-40.

Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J. and Lindquist, S. (1989) Hsp82 is an Essential Protein That is Required in Higher Concentrations for Growth of Cells at Higher Temperatures. *Molecular and Cellular Biology* **9**, 3919-3930.

Bornman, L., Polla, B.S. and Gericke, G.S. (1996) Heat-shock Protein-90 and Ubiquitin - Developmental Regulation During Myogenesis. *Muscle and Nerve* **19**, 574-580.

Boulange, A. and Authie, E. (1994) A 69 kDa Immuno-dominant Antigen of *Trypanosoma (Nannomonas) congolense* is Homologous to Immunoglobulin Heavy Chain Binding Protein (BiP). *Parasitology* **109**, 163-173.

Boyum, A. (1964) Separation of White Blood Cells. *Nature* **204**, 793

Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Analytical Biochemistry* **72**, 248-254.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D.C., Joachimmiak, A., Horwich, A.L. and Sigler, P.B. (1994) The Crystal Structure of the Bacterial Chaperonin GroEL at 2.8 Å. *Nature* **371**, 578-586.

Brandau, S., Dresel, A. and Clos, J. (1995) High Constitutive Levels of Heat-shock Proteins in Human-pathogenic Parasites of the Genus *Leishmania*. *Biochemical Journal* **310**, 225-232.

Brawerman, G. (1987) Determinants of Messenger RNA Stability. *Cell* **48**, 5-6.

Bresnick, E.H., Dalman, F.C., Sanchez, E.R. and Pratt, W.B. (1989) Evidence That the 90-kDa Heat Shock Protein is Necessary for the Steroid Binding Conformation of the L Cell Glucocorticoid Receptor. *Journal of Biological Chemistry* **264**, 4992-4997.

Bresnick, E.H., Dalman, F.C. and Pratt, W.B. (1990) Direct Stoichiometric Evidence that the Untransformed M_r 300,000, 9S, Glucocorticoid Receptor is a Core Unit Derived from a Larger Heteromeric Complex. *Biochemistry* **29**, 520-527.

Britton, C., McKerrow, J.H. and Johnstone, I.L. (1998) Regulation of the *Caenorhabditis elegans* Gut Cysteine Protease Gene *Cpr-1*: Requirement for GATA Motifs. *Journal of Molecular Biology* **283**, 15-27.

Brownlee, D.J.A., HoldenDye, L. and Walker, R.J. (1997) Actions of the Anthelmintic Ivermectin on the Pharyngeal Muscle of the Parasitic Nematode, *Ascaris suum*. *Parasitology* **115**, 553-561.

Bucher, P. (1990) Weight Matrix Description of Four Eukaryotic RNA Polymerase II Promoter Elements Derived from 502 Unrelated Promoter Sequences. *Journal of Molecular Biology* **212**, 563-578.

Buchner, J. (1996) Supervising the Fold: Functional Principles of Molecular Chaperones. *FASEB Journal* **10**, 10-19.

Cadepond, F., Binart, N., Chambraud, B., Jibard, N., Schweizer-Groyer, G., Segard-Maurel, I. and Baulieu, E. (1993) Interaction of Glucocorticoid Receptor and Wild-type or Mutated 90-kDa Heat Shock Protein Coexpressed in Baculovirus-infected Sf9 Cells. *Proceedings of the National Academy of Sciences USA* **90**, 10434-10438.

Cahill, K.M. and O'Brien, W. (1990) Filariasis. In: *Tropical Medicine: A Clinical Text*, 3rd edn. pp. 153-171. Oxford: Heinemann Medical Books

Cai, Y., Langley, J.G., Smith, D.I. and Boros, D.L. (1996) A Cloned Major *Schistosoma mansoni* Egg Antigen with Homologies to Small Heat Shock Proteins Elicit Th1 Responsiveness. *Infection and Immunity* **64**, 1750-1755.

Campello, T.R., Ferreira, R.S., Pires, M.L., Demelo, P.G., Albuquerque, R., Araujo, S. and Dreyer, G. (1993) A Study of Placentas from *Wuchereria bancrofti* Microfilaraemic and Amicrofilaraemic Mothers. *Journal of Tropical Medicine and Hygiene* **96**, 251-255.

Candido, E.P.M., Jones, D., Dixon, D.K., Graham, R.W., Russnak, R.H. and Kay, R.J. (1989) Structure, Organization, and Expression of the 16-kDa Heat Shock Gene Family of *Caenorhabditis elegans*. *Genome* **31**, 690-697.

Carson-Jurica, M.A., Lee, A.T., Dobson, A.W., Conneely, O.M., Schrader, W.T. and O'Malley, B.W. (1989) Interaction of the Chicken Progesterone Receptor with Heat Shock Protein (HSP) 90. *Journal of Steroid Biochemistry* **34**, 1-9.

Case, T., Leis, B., Witte, M., Way, D., Bernas, M., Borgs, P., Crandall, C., Crandall, R., Nagle, R., Jamal, S., Nayar, J. and Witte, C. (1991) Vascular Abnormalities in Experimental and Human Lymphatic Filariasis. *Lymphology* **24**, 174-183.

Catelli, M., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E., Feramisco, J.R. and Welch, W.J. (1985) The Common 90-kd Protein Component of Non-transformed "8S" Steroid Receptors is a Heat Shock Protein. *EMBO Journal* **4**, 3131-3135.

Chauthaiwale, V.M., Therwath, A. and Deshpande, V.V. (1992) Bacteriophage-lambda as a Cloning Vector. *Microbiology Reviews* **56**, 577-591.

Chiesa, R., Noguera, I. and Sredy, J. (1997) Phosphorylation of HSP25 During Lens Cell Differentiation. *Experimental Eye Research* **65**, 223-229.

Chomczynski, P. (1993) A Reagent for the Single-step Simultaneous Isolation of RNA, DNA and Proteins. *Biotechniques* **15**, 532-534.

Christensen, B.M. and Hollander, A.L. (1978) Effect of Temperature on Vector-parasite Relationships of *Aedes trivittatus* and *Dirofilaria immitis*. *Proceedings of the Helminth Society of Washington* **45**, 115-119.

Clark, T.G., Abrahamsen, M.S. and White, M.W. (1996) Developmental Expression of Heat Shock Protein 90 in *Eimeria bovis*. *Molecular and Biochemical Parasitology* **78**, 259-263.

Cohen, I.R. (1997) The Th1/Th2 Dichotomy, Hsp60 Autoimmunity, and Type I Diabetes. *Clinical Immunology and Immunopathology* **84**, 103-106.

Collins English Dictionary (1994), 3rd edn. Glasgow: HarperCollins Publishers.

Conconi, M., Petropoulos, I., Emod, I., Biville, F. and Friguet, B. (1998) Protection from Oxidative Inactivation of the 20S Proteosome by Heat-shock Protein 90. *Biochemical Journal* **333**, 407-415.

Conroy, S.E., Faulds, G.B., Williams, W., Latchman, D.S. and Isenberg, D.A. (1994) Detection of Autoantibodies to the 90 kDa Heat Shock Protein in Systemic Lupus Erythromatosus and Other Autoimmune Diseases. *British Journal of Rheumatology* **33**, 923-926.

Cordingley, J.S., Haddow, W.J., Nene, V. and Taylor, D.W. (1986) Identification by Message Selection of cDNA Clones Encoding Antigens of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **18**, 73-88.

Corell, R.A. and Gross, R.H. (1992) Splicing Thermotolerance Maintains Pre-mRNA Transcripts in the Splicing Pathway During Severe Heat Shock. *Experimental Cell Research* **202**, 233-242.

Cox-Singh, J., Paine, M.J.I., Martin, S.A.M. and Devaney, E. (1994) Stage-specific Differences in Steady-state Levels of Messenger-RNA Encoding the Major Surface Glycoprotein of *Brugia pahangi*. *Tropical Medicine and Parasitology* **45**, 352-354.

Craig, E.A., Gambill, B.D. and Nelson, R.J. (1993) Heat Shock Proteins: Molecular Chaperones of Protein Biogenesis. *Microbiological Reviews* **57**, 402-414.

Csermely, P., Kajtar, J., Hollosi, M., Oikarinen, J. and Somogyi, J. (1994) The 90kDa Heat Shock Protein (Hsp90) Induces the Condensation of the Chromatin Structure. *Biochemical and Biophysical Research Communications* **202**, 1657-1663.

Czar, M.J., Owens-Grillo, J.K., Dittmar, K.D., Hutchison, K.A., Zacharek, A.M., Leach, K.L., Deibel, M.R. and Pratt, W.B. (1994) Characterization of the Protein-protein Interactions Determining the Heat Shock Protein (Hsp90.hsp70.hsp56) Heterocomplex. *Journal of Biological Chemistry* **269**, 11155-11161.

Dale, E.C., Yang, X., Moore, S.K. and Shyamala, G. (1996) Cloning and Characterization of the Promoter for Murine 84-kDa Heat-Shock Protein. *Gene* **172**, 279-284.

Dale, J.W. (1998) Regulation of Gene Expression. In: *Molecular Genetics of Bacteria*, 3rd edn. pp. 69-98. Chichester: John Wiley and Sons Ltd

Dalley, B.K. and Golomb, M. (1992) Gene Expression in the *Caenorhabditis elegans* Dauer Larva: Developmental Regulation of Hsp90 and Other Genes. *Developmental Biology* **151**, 80-90.

Das, A., Syin, C., Fujioka, H., Zheng, H., Goldman, N., Aikawa, M. and Kumar, N. (1997) Molecular Characterization and Ultrastructural Localization of *Plasmodium falciparum* Hsp60. *Molecular and Biochemical Parasitology* **88**, 95-104.

Dasilva, S.P., Felipe, M.S.S., Pereira, M., Azevedo, M.O. and Soares, C.M.D. (1994) Phase Transition and Stage Specific Protein Synthesis in the Dimorphic Fungus *Paracoccidioides brasiliensis*. *Experimental Mycology* **18**, 294-299.

Denham, D.A. and McGreevy, P.B. (1977) Brugian Filariasis: Epidemiology and Experimental Studies. *Advances in Parasitology* **15**, 243-309.

Denis, M., Wilhelmsson, A., Cuthill, S., Poellinger, L. and Gustafsson, J. (1989) Structural Differences Between the Glucocorticoid, Dioxin and Oxysterol Receptors from Rat Cytosol. *Biochemical and Biophysical Research Communications* **163**, 444-451.

Devaney, E., Egan, A., Lewis, E., Warbrick, E.V. and Jecock, R.M. (1992) The Expression of Small Heat Shock Proteins in the Microfilaria of *Brugia pahangi* and Their Possible Role in Development. *Molecular and Biochemical Parasitology* **56**, 209-218.

Devaney, E., Bancroft, A. and Egan, A. (1993) The Effect of Irradiation on the Third Stage Larvae of the Filarial Nematode *Brugia pahangi*. *Parasite Immunology* **15**, 423-427.

Devaney, E., Martin, S.A.M. and Thompson, F.J. (1996) Stage-specific Gene Expression in Lymphatic Filarial Nematodes. *Parasitology Today* **12**, 418-424.

Devaney, E. and Lewis, E. (1993) Temperature-induced Refractoriness of *Aedes aegypti* Mosquitoes to Infection with the filaria *Brugia pahangi*. *Medical and Veterinary Entomology* **7**, 297-298.

Dhillon, V.B., McCallum, S., Norton, P., Twomey, B.M., Erkelleryuksel, F., Lydyard, P., Isenberg, D.A. and Latchman, D.S. (1993) Differential Heat Shock Protein Overexpression and its Clinical Relevance in Systemic Lupus Erythematosus. *Annals of the Rheumatic Diseases* **52**, 436-442.

Dhillon, V.B., McCallum, S., Latchman, D.S. and Isenberg, D.A. (1994) Elevation of the 90 kDa Heat-shock Protein in Specific Subsets of Systemic Lupus Erythematosus. *Quarterly Journal of Medicine* **87**, 215-222.

DiDomenico, B.J., Bugaisky, G.E. and Lindquist, S. (1982) The Heat Shock Response is Self-regulated at Both the Transcriptional and Posttranslational Levels. *Cell* **31**, 593-603.

Dietz, T.J. (1994) Acclimation of the Threshold Induction Temperatures for 70-kDa and 90-kDa Heat Shock Proteins in the Fish *Gillichthys mirabilis*. *Journal of Experimental Biology* **188**, 333-338.

Dittmar, K.D., Banach, M., Galigniana, M.D. and Pratt, W.B. (1998) The Role of DnaJ-like Proteins in Glucocorticoid Receptor.hsp90 Heterocomplex Assembly by the Reconstituted Hsp90.p60.hsp70 Foldosome. *Journal of Biological Chemistry* **273**, 7358-7366.

Dong, J.Z. and Dunstan, D.I. (1996) Characterization of 3 Heat-shock-protein Genes and Their Developmental Regulation During Somatic Embryogenesis in White Spruce [*Picea-glauca (moench) voss*]. *Planta* **200**, 85-91.

Dougherty, J.J., Rabideau, D.A., Iannotti, A.M., Sullivan, W.P. and Toft, D.O. (1987) Identification of the 90 KDa Substrate of Rat-liver Type II Casein Kinase With the Heat Shock Protein Which Binds Steroid Receptors. *Biochimica et Biophysica Acta* **927**, 74-80.

Engelberg, D., Zandi, E., Parker, C.S. and Karin, M. (1994) The Yeast and Mammalian Ras Pathways Control Transcription of Heat Shock Genes Independently of Heat Shock Transcription Factor. *Molecular and Cellular Biology* **14**, 4929-4937.

Ernani, F.P. and Estes, D.M. (1993) Release of Stress Proteins from *Mesocestoides corti* is a Brefeldin A-inhibitable Process - Evidence for Active Export of Stress Proteins. *Infection and Immunity* **61**, 2596-2601.

Estes, D.M., Turaga, P.S.D., Sievers, K.M. and Teale, J.M. (1993) Characterization of an Unusual Cell Type (CD4+CD3-) Expanded by Helminth Infection and Related to the Parasite Stress Response. *Journal of Immunology* **150**, 1846-1856.

Estes, D.M. and Teale, J.M. (1991) Biochemical and Functional Analysis of Extracellular Stress Proteins of *Mesocestoides Corti*. *Journal of Immunology* **147**, 3926-3934.

Feinberg, A.P. and Vogelstein, B. (1983) A Technique for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity. *Analytical Biochemistry* **132**, 6-13.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987) Lipofectin - A Highly Effficient, Lipid-mediated DNA-transfection Procedure. *Proceedings of the National Academy of Sciences USA* **84**, 7413-7417.

Fernandes, M., Xiao, H. and Lis, J.T. (1994) Fine Structure Analyses of the *Drosophila* and *Saccharomyces* Heat Shock Factor - Heat Shock Element Interactions. *Nucleic Acids Research* **22**, 167-173.

Fernandes, M., Xiao, H. and Lis, J.T. (1995) Binding of Heat Shock Factor to and Transcriptional Activation of Heat Shock Genes in *Drosophila*. *Nucleic Acids Research* **23**, 4799-4804.

Fracella, F., Scholle, C., Kallies, A., Haker, T., Schroder, T. and Rensing, L. (1997) Differential HSC70 Expression During Asexual Development of *Neurospora crassa*. *Microbiology UK* **143**, 3615-3624.

Franklin, G.C., Donovan, M., Adam, G.I., Holmgren, L., Pfeifer-Ohlsson, S. and Ohlsson, R. (1991) Expression of the Human PDGF-B Gene is Regulated by Both Positively and Negatively Acting Cell Type-specific Regulatroy Elements Located in the First Intron. *EMBO Journal* **10**, 1365-1373.

Freedman, D.O., Bui, T., Filho, P.J.D., Braga, C., Silva, M.C.M.E., Maciel, A. and Furtado, A.F. (1995) Lymphoscintigraphic Assessment of the Effect of Diethylcarbamazine Treatment of Lymphatic Damage in Human Bancroftian Filariasis. *American Journal of Tropical Medicine and Hygiene* **52**, 258-261.

Freeman, B.C. and Morimoto, R.I. (1996) The Human Cytosolic Molecular Chaperones Hsp90, Hsp70 (Hsc70) and Hdj-1 Have Distinct Roles in Recognition of a Non-native Protein and Protein Refolding. *EMBO Journal* **15**, 2969-2979.

Freitag, D.G., Ouimet, P.M., Girvitz, T.L. and Kapoor, M. (1997) Heat Shock Protein 80 of *Neurospora crassa*, a Cytosolic Molecular Chaperone of the Eukaryotic Stress 90 Family, Interacts Directly with Heat Shock Protein 70. *Biochemistry* **36**, 10221-10229.

Frischauf, A.M., Lehrach, H., Poustka, A. and Murray, N. (1983) Lambda Replacement Vectors Carrying Polylinker Sequences. *Journal of Molecular Biology* **170**, 827-842.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Rapid Production of Full-length cDNAs from Rare Transcripts: Amplification Using a Single Gene-specific Oligonucleotide Primer. *Proceedings of the National Academy of Sciences USA* **85**, 8998-9002.

Fukushige, T., Schroeder, D.F., Allen, F.L., Goszczynski, B. and McGhee, J.D. (1996) Modulation of Gene Expression in the Embryonic Digestive Tract of *C. elegans*. *Developmental Biology* **178**, 276-288.

Gaffney, D.F., McLauchlan, J., Whitton, J.L. and Clements, J.B. (1985) A Modular System for the Assay of Transcription Regulatory Signals: the Sequence TAATGARAT is Required for Herpes Simplex Virus Intermediate Early Gene Activation. *Nucleic Acids Research* **13**, 7847-7863.

Gagliardi, D., Breton, C., Chaboud, A., Vergne, P. and Dumas, C. (1995) Expression of Heat-shock Factor and Heat Shock Protein-70 Genes During Maize Pollen Development. *Plant Molecular Biology* **29**, 841-856.

Galea-Lauri, J., Latchman, D.S. and Katz, D.R. (1996) The Role of the 90-kDa Heat Shock Protein in Cell Cycle Control and Differential of the Monoblastoid Cell Line U937. *Experimental Cell Research* **226**, 243-254.

Gao, Y.J., Thomas, J.O., Chow, R.L., Lee, G.H. and Cowan, N.J. (1992) A Cytoplasmic Chaperonin that Catalyzes Beta-actin Folding. *Cell* **69**, 1043-1050.

Garnier, C., Barbier, P., Gilli, R., Lopez, C., Peyrot, V. and Briand, C. (1998) Heat-shock Protein 90 (Hsp90) Binds *in vitro* to Tubulin Dimer and Inhibits Microtubule Formation. *Biochemical and Biophysical Research Communications* **250**, 414-419.

Georgopoulus, C. and Welch, W.J. (1993) Role of the Major Heat Shock Proteins as Molecular Chaperones. *Annual Review of Cell Biology* **9**, 601-634.

Gerhards, J., Ebel, T., Dobbelaere, D.D.A.E., Morzaria, S.P., Musoke, A.J., Williams, R.O. and Lipp, J. (1994) Sequence and Expression of a 90-kilodalton Heat-shock Protein Family Member of *Theileria parva*. *Molecular and Biochemical Parasitology* **68**, 235-246.

Gething, M.J. and Sambrook, J. (1992) Protein Folding in the Cell. *Nature* **355**, 33-45.

Giardina, C. and Li, J.T. (1995) Dynamic Protein-DNA Architecture of a Yeast Heat Shock Promoter. *Molecular and Cellular Biology* **15**, 2737-2744.

Gilbert, W. and Glynias, M. (1993) On the Ancient Nature of Introns. *Gene* **135**, 137-144.

Gordon, S., Bharadwaj, S., Hnatov, A., Ali, A. and Ovsenek, N. (1997) Distinct Stress-inducible and Developmentally Regulated Heat Shock Transcription Factors in *Xenopus* Oocytes. *Developmental Biology* **181**, 47-63.

Grenert, J.P., Sullivan, W.P., Fadden, P., Haystead, T.A.J., Clark, J., Mimnaugh, E., Krutzsch, H., Ochel, H., Schulte, T.W., Sausville, E., Neckers, L.M. and Toft, D.O. (1997) The Amino-terminal Domain of Heat Shock Protein 90 (Hsp90) that Binds Geldamycin is an ATP/ADP Switch Domain that Regulates Hsp90 Conformation. *Journal of Biological Chemistry* **272**, 23843-23850.

Gross, D.S., Adams, C.C., Lee, S. and Stentz, B. (1993) A Critical Role for Heat Shock Transcription Factor in Establishing a Nucleosome-free Region over the TATA-initiation Site of the Yeast *HSP82* Heat Shock Gene. *EMBO Journal* **12**, 3931-3945.

Gulati, S.C., Kacian, D.L. and Spiegelman, S. (1974) Conditions for Using DNA Polymerase I as an RNA-dependent DNA Polymerase. *Proceedings of the National Academy of Sciences USA* **71**, 1035-1039.

Gupta, R. (1995) Phylogenetic Analysis of the 90 kD Heat Shock Family of Protein Sequences and an Examination of the Relationship Among Animals, Plants, and Fungi Species. *Molecular Biology and Evolution* **12**, 1063-1073.

Hammond, M.P. (1994) Codon Usage and Gene Organization in *Brugia*. *Parasitology Research* **80**, 173-175.

Hammond, M.P. and Bianco, A.E. (1992) Genes and Genomes of Parasitic Nematodes. *Parasitology* **8**, 299-305.

Hartl, F.U., Hloden, R. and Langer, T. (1994) Molecular Chaperones in Protein Folding: The Art of Avoiding Sticky Situations. *Trends in Biochemical Sciences* **19**, 20-25.

Hartl, F.U. and Martin, J. (1995) Molecular Chaperones in Cellular Protein Folding. *Current Opinions in Structural Biology* **5**, 92-102.

Hashizume, H., Tokura, Y., Takigawa, M. and Paus, R. (1997) Hair Cycle-dependent Expression of Heat Shock Proteins in Hair Follicle Epithelium. *International Journal of Dermatology* **36**, 587-592.

Hashmi, G., Hashmi, S., Selvan, S., Grewal, P. and Gaugler, R. (1997) Polymorphism in Heat Shock Protein Gene (Hsp70) in Entomopathogenic Nematodes (Rhabditida). *Journal of Thermal Biology* **22**, 143-149.

Hashmi, S., Hashmi, G. and Gaugler, R. (1995) Genetic Transformation of an Entomopathogenic Nematode by Microinjection. *Journal of Invertebrate Pathology* **66**, 293-296.

Hashmi, S., Hashmi, G., Glazer, I. and Gaugler, R. (1998) Thermal Response of *Heterorhabditis bacteriophora* Transformed with the *Caenorhabditis elegans* Hsp70 Encoding Gene. *Journal of Experimental Zoology* **281**, 164-170.

Hattori, T., Fujisawa, T., Sasaki, K., Yutani, Y., Nakanishi, T., Takahashi, K. and Takigawa, M. (1998) Isolation and Characterization of a Rheumatoid Arthritis-Specific Antigen (RA-A47) from a Human Chondrocytic Cell Line (HCS-2/8). *Biochemical and Biophysical Research Communications* **245**, 679-683.

Hausler, T. and Clayton, C. (1996) Post-transcriptional Control of Hsp70 mRNA in *Trypansoma brucei*. *Molecular and Biochemical Parasitology* **76**, 57-71.

Hedstrom, R., Culpepper, J., Schinski, V., Agabian, N. and Newport, G. (1988) Schistosome Heat-shock Proteins are Immunologically Distinct Host-like Antigens. *Molecular and Biochemical Parasitology* **29**, 275-282.

Hemingway, J., Penilla, R.P., Rodriguez, A.D., James, B.M., Edge, W., Rogers, H. and Rodriguez, M.H. (1997) Resistance Management Strategies in Malaria Vector Mosquito Control. A Large-scale Field Trial in Southern Mexico. *Pesticide Science* **51**, 375-382.

Henry, E.C. and Gasiewicz, T.A. (1993) Transformation of the Aryl Hydrocarbon Receptor to a DNA-binding Form is Accompanied by Release of the 90 kDa Heat-shock Protein and Increased Affinity for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochemical Journal* **294**, 95-101.

Heschl, M.F.P. and Baillie, D.L. (1990) Functional Elements and Domains Inferred from Sequence Comparisons of a Heat Shock Gene in Two Nematodes. *Journal of Molecular Evolution* **31**, 3-9.

Hess, M.A. and Duncan, R.F. (1994) RNA/Protein Interactions in the 5'-Untranslated Leader of HSP70 mRNA in *Drosophila* Lysates. *Journal of Biological Chemistry* **269**, 10913-10922.

Hess, M.A. and Duncan, R.F. (1996) Sequence and Structure Determinants of *Drosophila* Hsp70 mRNA Translation: 5'-UTR Secondary Structure Specifically Inhibits Heat Shock Protein mRNA Translation. *Nucleic Acids Research* **24**, 2441-2449.

Hickey, E., Brandon, S.E., Smale, G., Lloyd, D. and Weber, L.A. (1989) Sequence and Regulation of a Gene Encoding a Human 89-kilodalton Heat Shock Protein. *Molecular and Cellular Biology* **9**, 2615-2626.

Higgs, S., Rayner, J.O., Olsen, K.E., Davis, B.S., Beaty, B.J. and Blair, C.D. (1998) Engineering Resistence in *Aedes aegypti* to a West African and a South American Strain of Yellow Fever Virus. *American Journal of Tropical Medicine and Hygiene* **58**, 663-670.

Hightower, L.E. (1991) Heat Shock, Stress Proteins, Chaperones, and Proteotoxicity. *Cell* **66**, 191-197.

Hisaeda, H., Nagasawa, H., Maeda, K., Maekawa, Y., Ishikawa, H., Ito, Y., Good, R.A. and Himeno, K. (1995) Gamma Delta T cells Play an Important Role in HSP65 Expression and in Acquiring Protective Immune Responses Against Infection with *Toxoplasma Gondii*. *Journal of Immunology* **155**, 244-251.

Hisaeda, H., Sakai, T., Ishikawa, H., Maekawa, Y., Yasutomo, K., Good, R.A. and Himeno, K. (1997) Heat Shock Protein 65 Induced by γδ T Cells Prevents Apoptosis of Macrophage and Contributes to Host Defense in Mice Infected with *Toxoplasma gondii*. *Journal of Immunology* **159**, 2375-2381.

Hisaeda, H. and Himeno, K. (1997) The Role of Host-derived Heat-shock Protein in Immunity Against *Toxoplasma gondii*. *Parasitology Today* **13**, 465-468.

Hochuli, E., Dobeli, H. and Scachter, A. (1987) New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighbouring Histidine Residues. *Journal of Chromatography* **411**, 177-184.

Hochuli, E., Bannwarth, W., Dobeli, H., Gentz, R. and Stuber, D. (1988) Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent. *Bio-technology* **6**, 1321-1325.

Hoffmann, T. and Hovemann, B. (1988) Heat-shock Proteins, Hsp84 and Hsp86, Of Mice and Men: Two Related Genes Encode Formerly Identified Tumour-specific Transplantation Antigens. *Gene* **74**, 491-501.

Holley, S.J. and Yamamoto, K.R. (1995) A Role for Hsp90 in Retinoid Receptor Signal Transduction. *Molecular Biology of the Cell* **6**, 1833-1842.

Hu, J. and Seeger, C. (1996) Hsp90 is required for the Activity of a Hepatitis B Virus Reverse Transcriptase. *Proceedings of the National Academy of Sciences USA* **93**, 1060-1064.

Hu, J. and Seeger, C. (1997) RNA Signals That Control DNA Replication in Hepadnaviruses. *Seminars in Virology* **8**, 205-211.

Hubel, A., Brandau, S., Dresel, A. and Clos, J. (1995) A Member of the Clpb Family of Stress Proteins is Expressed During Heat Shock in *Leishmania spp. Molecular and Biochemical Parasitology* **70**, 107-118.

Hubel, A., Krobitsch, S., Horauf, A. and Clos, J. (1998) *Leishmania major* Hsp100 is Required Chiefly in the Mammalian Stage of the Parasite. *Molecular and Cellular Biology* **17**, 5987-5995.

Hultmark, D., Klemenz, R. and Gehring, W. (1986) Translational and Transcriptional Control Elements in the Untranslated Leader of the Heat-shock Gene *Hsp22*. *Cell* **44**, 429-438.

Hunter, K.W., Cook, C.L. and Hayunga, E.G. (1984) Leishmanial Differential *in vitro*: Induction of Heat Shock Proteins. *Biochemical and Biophysical Research Communications* **125**, 755-760.

Hutchison, K.A., Stancato, L.F., Jove, R. and Pratt, W.B. (1992) The Protein-Protein Complex Between $pp60^{v\text{-}src}$ and Hsp90 is Stabilized by Molybdate, Vanadate, Tungstate, and an Endogenous Cytosolic Metal. *Journal of Biological Chemistry* **267**, 13952-13957.

Hutchison, K.A., Stancato, L.F., Owens-Grillo, J.K., Johnson, J.L., Krishna, P., Toft, D.O. and Pratt, W.B. (1995) The 23-kDa Acidic Protein in Reticulocyte Lysate is the Weakly Bound Component of the Hsp Foldosome That is Required for Assembly of the Glucocorticoid Receptor into a Functional Heterocomplex with Hsp90. *Journal of Biological Chemistry* **270**, 18841-18847.

Imamura, T., Haruta, T., Takata, Y., Usui, I., Iwata, M., Ishihara, H., Ishiki, M., Ishibashi, O., Ueno, E., Sasaoka, T. and Kobayashi, M. (1998) Involvement Heat Shock Protein 90 in the Degradation of Mutant Insulin Receptors by the Proteasome. *Journal of Biological Chemistry* **273**, 11183-11188.

Inanobe, A., Takahashi, K. and Katada, T. (1994) Association of the β-gamma Subunits of Trimeric GTP-Binding Proteins with 90-kDa Heat Shock Protein, Hsp90. *Journal of Biochemistry* **115**, 486-492.

Ireland, R.C., Berger, E., Sirotkin, K., Yund, M.A., Osterbur, D. and Fristrom, J. (1982) Ecdysterone Induces the Transcription of Four Heat-shock Genes in *Drosophila* S3 Cells and Imaginal Discs. *Developmental Biology* **93**, 498-507.

Ireland, R.C. and Berger, E. (1982) Synthesis of Low Molecular Weight Heat Shock Peptides Stimulated by Ecdysterone in a Cultured *Drosophila* Cell Line. *Proceedings of the National Academy of Sciences USA* **79**, 855-859.

Jacquier-Sarlin, M.R., Jornot, L. and Polla, B.S. (1995) Differential Expression and Regulation of Hsp70 and Hsp90 by Phorbol Esters and Heat Shock. *Journal of Biological Chemistry* **270**, 14094-14099.

Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) Small Heat Shock Proteins are Molecular Chaperones. *Journal of Biological Chemistry* **268**, 1517-1520.

Jakob, U., Lilie, H., Meyer, I. and Buchner, J. (1995a) Transient Interactions of Hsp90 with Early Unfolding Intermediates of Citrate Synthase. *Journal of Biological Chemistry* **270**, 7288-7294.

Jakob, U., Meyer, I., Bugl, H., Andre, S., Bardwell, J.C.A. and Buchner, J. (1995b) Structural Organization of Prokaryotic and Eucaryotic Hsp90. *Journal of Biological Chemistry* **270**, 14412-14419.

Jakob, U. and Buchner, J. (1994) Assisting Spontaneity: The Role of Hsp90 and Small Hsps as Molecular Chaperones. *Trends in Biochemical Sciences* **19**, 205-211.

Jecock, R.M. and Devaney, E. (1992) Expression of Small Heat Shock Proteins by the Third-stage Larva of *Brugia pahangi*. *Molecular and Biochemical Parasitology* **56**, 219-226.

Jerome, V., Vourc'h, C., Baulieu, E. and Catelli, M. (1993) Cell Cycle Regulation of the Chicken Hsp90α Expression. *Experimental Cell Research* **205**, 44-51.

Jimenezgonzalez, A., Dearmasserra, C., Criadofornelio, A., Casadoescribano, N., Rodriguezcaabeiro, F. and Diez, J.C. (1991) Preliminary Characterization and Interaction of Tubulin from *Trichinella spiralis* Larvae with Benzimidazole Derivatives. *Veterinary Parasitology* **39**, 89-99.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M., Binart, N., Mester, R. and Baulieu, E. (1984) Common Non-hormone Binding Component in Non-transformed Chick Oviduct Receptors of Four Steroid Hormones. *Nature* **308**, 850-853.

Johnson, K.S., Wells, K., Bock, J.V., Nene, V., Taylor, D.W. and Cordingley, J.S. (1989) The 86-kilodalton Antigen from *Schistosoma mansoni* is a Heat-shock Protein Homologous to Yeast HSP-90. *Molecular and Biochemical Parasitology* **36**, 19-28.

Jolly, C., Morimoto, R.I., Robert-Nicoud, M. and Vourc'h, C. (1997) HSF1 Transcription Factor Concentrates in Nuclear Foci During Heat Shock: Relationship with Transcription Sites. *Journal of Cell Science* **110**, 2935-2941.

Joshi, C.P. and Nguyen, H.T. (1995) 5' Untranslated Leader Sequences of Eukaryotic mRNAs Encoding Heat Shock Induced Proteins. *Nucleic Acids Research* **23**, 541-549.

Joshua, G.W.P. and Hsieh, C.Y. (1995) Stage-specifically Expressed Genes of *Angiostrongylus cantonensis*: Identification by Differential Display. *Molecular and Biochemical Parasitology* **71**, 285-289.

Jurivich, D.A., Sistonen, L., Sarge, K.D. and Morimoto, R.I. (1994) Arachidonate is a Potent Modulator of Human Heat Shock Gene Transcription. *Proceedings of the National Academy of Sciences USA* **91**, 2280-2284.

Kaneko, Y., Nishiyama, H., Nonoguchi, K., Higashitsuji, H., Kishishita, M. and Fujita, J. (1997) A Novel Hsp110-related Gene, Apg-1, That is Abundantly Expressed in the Testis Responds to a Low Temperature Heat Shock Rather Than the Traditional Elevated Temperatures. *Journal of Biological Chemistry* **272**, 2640-2645.

Kar, S.K., Mania, J. and Kar, P.K. (1993) Humoral Immune-Response During Filarial Fever in Bancroftian Filariasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**, 230-233.

Kellermayer, M.S.Z. and Csermely, P. (1995) ATP Induces Dissociation of the 90kDa Heat Shock Protein (Hsp90) from F-actin: Interference with the Binding of Heavy Meromyosin. *Biochemical and Biophysical Research Communications* **211**, 166-174.

King, C.L., Mahanty, S., Kumaraswami, V., Abrams, J.S. and Regunathan, J. (1993) Cytokine Control of Parasite-Specific Anergy in Human Lymphatic Filariasis - Preferential Induction of a Regulatory T-Helper Type-2 Lymphocyte Subset. *Journal of Clinical Investigation* **92**, 1667-1673.

Klein, K.G., Olson, C.L., Donelson, J.E. and Engman, D.M. (1995) Molecular Comparisons of the Mitochondrial and Cytoplasmic HSP70 of *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*. *Journal of Eukaryotic Microbiology* **42**, 473-476.

Knauf, U., Bielka, H. and Gaestel, M. (1992) Over-expression of the Small Heat-shock Protein, Hsp25, Inhibits Growth of Ehrlich Ascites Tumor Cells. *FEBS Letters* **309**, 297-302.

Ko, R.C. and Fan, L. (1996) Heat Shock Response of *Trichinella spiralis* and *T. pseudospiralis*. *Parasitology* **112**, 89-95.

Koltai, H., Spiegel, Y. and Blaxter, M.L. (1997) Regulated Use of an Alternative Spliced Leader Exon in the Plant Parasitic Nematode *Meloidogyne javanica*. *Molecular and Biochemical Parasitology* **86**, 107-110.

Kostrouchova, M., Krause, M., Kostrouch, Z. and Rall, J.E. (1998) CHR3: a *Caenorhabditis elegans* Orphan Nuclear Hormone Receptor Required for Proper Epidermal Development and Molting. *Development* **125**, 1617-1626.

Koyasu, S., Nishida, E., Kadowaki, T., Matsuzaki, F., Iida, K., Harada, F., Kasuga, M., Sakai, H. and Yahara, I. (1986) Two Mammalian Heat Shock Proteins, HSP90 and HSP100, are Actin-binding Proteins. *Proceedings of the National Academy of Sciences USA* **83**, 8054-8058.

Krishna, P., Reddy, R.K., Sacco, M., Frappier, R.H. and Felsheim, R.F. (1997) Analysis of the Native Forms of the 90 kDa Heat Shock Protein (Hsp90) in Plant Cytosolic Extracts. *Plant Molecular Biology* **33**, 457-466.

Krobitsch, S., Brandau, S., Hoyer, C., Schmetz, C., Hubel, A. and Clos, J. (1998) *Leishmania donovani* Heat Shock Protein 100. *Journal of Biological Chemistry* **273**, 6488-6494.

Kroeger, P.E. and Morimoto, R.I. (1994) Selection of New HSF1 and HSF2 DNA-binding Sites Reveals Differences in Trimer Cooperativity. *Molecular and Cellular Biology* **14**, 7592-7603.

Krone, P.H., Sass, J.B. and Lele, Z. (1997) Heat Shock Protein Gene Expression During Embryonic Development of the Zebrafish. *Cellular and Molecular Life Sciences* **53**, 122-129.

Kumari, S., Lillibridge, C.D., Bakeer, M., Lowrie, R.C., Jayaraman, K. and Philipp, M.T. (1994) *Brugia malayi*: The Diagnostic Potential of Recombinant Excretory/Secretory Antigens. *Experimental Parasitology* **79**, 489-505.

Kwa, M.S.G., Veenstra, J.G., van Dijk, M. and Roos, M.H. (1995) β-tubulin Genes from the Parasitic Nematode *Haemonchus contortus* Modulate Drug Resistance in *Caenorhabditis elegans*. *Journal of Molecular Biology* **246**, 500-510.

Laemmli, U.K. (1970) Cleavage of structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **227**, 680-685.

Lai, W.S., Thompson, M.J. and Blackshear, P.J. (1998) Characteristics of the Intron Involvement in the Mitogen-induced Expression of *Zfp-36*. *Journal of Biological Chemistry* **273**, 506-517.

Lammie, P.J., Hitch, W.L., Allen, E.M.W., Hightower, W. and Eberhard, M.L. (1991) Maternal Filarial Infection as Risk Factor for Infection in Children. *Lancet* **337**, 1005-1006.

Landry, J., Chretien, P., Lampert, H., Hickey, E. and Weber, L.A. (1989) Heat Shock Resistance Conferred by Expression of the Human Hsp27 Gene in Rodent Cells. *Journal of Cellular Biology* **109**, 7-15.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988) The Leucine Zipper: A Hypothetical Structure Common to a New Class of DNA Binding Proteins. *Science* **240**, 1759-1764.

Latchman, D.S. and Isenberg, D.A. (1994) The Role of HSP90 in SLE. *Autoimmunity* **19**, 211-218.

Laursen, J.R., di Liu, H., Wu, X. and Yoshino, T.P. (1997) Heat-shock Response in a Molluscan Cell Line: Characterization of the Response and Cloning of an Inducible HSP70 cDNA. *Journal of Invertebrate Pathology* **70**, 226-233.

Lee, M., Hirokawa, M., Kitabayashi, A., Miura, A.B., Itoh, H. and Tashima, Y. (1995) Induction of 73-kDa and 90-kDa Heat-shock Protein Expression in HL-60 Cells During Differentiation. *Tohoku Journal of Experimental Medicine* **176**, 191-202.

Lees-Miller, S.P. and Anderson, C.W. (1989) 2 Human 90-KDa Heat Shock Proteins are Phosphorylated *in vivo* at Conserved Serines That are Phosphorylated *in vitro* by Casein Kinase II. *Journal of Biological Chemistry* **264**, 2431-2437.

Lele, Z. and Krone, P.H. (1997) Expression of Genes Encoding the Collagen-binding Heat Shock Protein (Hsp47) and Type II Collagen in Developing Zebrafish Embryos. *Mechanisms of Development* **61**, 89-98.

Leroux, M.R. and Candido, E.P.M. (1997) Subunit Characterization of the *Caenorhabditis elegans* Chaperonin Containing TCP-1 and Expression Pattern of the Gene Encoding CCT-1. *Biochemical and Biophysical Research Communications* **241**, 687-692.

Leung, P.S.C. and Gershwin, M.E. (1991) The Immunobiology of Heat-Shock Proteins. *Journal of Investigational Allergology and Clinical Immunology* **1**, 23-30.

Levy-Holtzman, R., Clos, J. and Schechter, I. (1995) Differences in DNA Sequence Recognition by the Heat-shock Factors of *Drosophila melanogaster* and the Parasitic Helminth *Schistosoma mansoni*. *Biochimica et Biophysica Acta* **1264**, 134-140.

Levy-Holtzman, R. and Schechter, I. (1994) Schistosome Extracts with Heat Shock Factor Activity Revealed by the Gel Shift Assay. *Parasitology* **108**, 35-42.

Levy-Holtzman, R. and Schechter, I. (1995) Activity of the Promoter of the Hsp70 Gene of the Parasitic Helminth *Schistosoma mansoni* in the Mammalian CHO Cell-line. *Biochimica et Biophysica Acta* **1263**, 96-98.

Liang, P. and MacRae, T.H. (1997) Molecular Chaperones and the Cytoskeleton. *Journal of Cell Science* **110**, 1431-1440.

Lillibridge, C.D., Rudin, W. and Philipp, M.T. (1996) *Dirofilaria immitis*: Ultrastructural Localization, Molecular Characterization, and Analysis of the Expression of p27, a Small Heat Shock Protein Homolog of Nematodes. *Experimental Parasitology* **83**, 30-45.

Linder, B., Jin, Z.J., Freedman, J.H. and Rubin, C.S. (1996) Molecular Characterization of a Novel Developmentally Regulated Small Embryonic Chaperone from *Caenorhabditis elegans*. *Journal of Biological Chemistry* **271**, 30158-30166.

Lindquist, S. (1986) The Heat Shock Response. *Annual Review of Biochemistry* **55**, 1151-1191.

Lindquist, S. (1991) Genetic Analysis of Heat Shock Protein Function in Yeast. In: Marcesca, B. and Lindquist, S. (Eds.) *Heat Shock*, 1st edn. pp. 123-131. Berlin: Springer-Verlag

Lindquist, S. and Craig, E.A. (1988) The Heat Shock Proteins. *Annual Review of Genetics* **22**, 631-677.

Lis, J.T., Xiao, H. and Perisic, O. (1990) Modular Units of Heat Shock Regulatory Regions: Structure and Function. In: Morimoto, R., Tissieres, A. and Georgopoulos, C. (Eds.) *Stress Proteins in Biology and Medicine*, pp. 411-428. Cold Spring Harbor, New York: Cold Spring Harbor Press

Lisowska, K., Krawczyk, Z., Widlak, W., Wolniczek, P. and Wisniewski, J. (1994) Cloning, Nucleotide-sequence and Expression of Rat Heat Inducible HSP70 Gene. *Biochimica et Biophysica Acta* **1219**, 64-72.

Lithgow, G.J. and Kirkwood, T.B.L. (1996) Mechanisms and Evolution of Aging. *Science* **273**, 80

Liu, A., Y-C., Bian, H., Huan, E. and Lee, Y.K. (1994) Transient Cold Shock Induces the Heat Shock Response Upon Recovery at 37°C in Human Cells. *Journal of Biological Chemistry* **269**, 14768-14775.

Liu, R.Y., Kim, D., Yang, S. and Li, G.C. (1993) Dual Control of Heat Shock Response: Involvement of a Constitutive Heat Shock Element-binding Factor. *Proceedings of the National Academy of Sciences USA* **90**, 3078-3082.

Long, M., de Souza, S.J. and Gilbert, W. (1995) Evolution of the Intron-exon Structure of Eukaryotic Genes. *Current Opinion in Genetics and Development* **5**, 774-778.

Loones, M., Rallu, M., Mezger, V. and Morange, M. (1997) HSP Gene Expression and HSF2 in Mouse Development. *Cellular and Molecular Life Sciences* **53**, 179-190.

Louvion, J., Warth, R. and Picard, D. (1996) Two Eukaryote-specific Regions of Hsp82 are Dispensible for its Viability and Signal Transduction Functions in Yeast. *Proceedings of the National Academy of Sciences USA* **93**, 13937-13942.

Lum, L.S., Sultzman, L.A., Kaufman, R.J., Linzer, D.I. and Wu, B.J. (1990) A Cloned Human CCAAT-box-binding Factor Stimulates Transcription from the Human Hsp70 Promoter. *Molecular and Cellular Biology* **10**, 6709-6717.

Luneberg, E., GlennCalvo, E., Hartmann, M., Bar, W. and Frosch, M. (1998) The Central, Surface-exposed Region of the Flagellar Hook Protein FlgE of *Campylobacter jejuni* Shows Hypervariability Among Strains. *Journal of Bacteriology* **180**, 3711-3714.

Luparello, C., Noel, A. and PucciMinafra, I. (1997) Intratumoral Heterogeneity for Hsp90 Beta mRNA Levels in a Breast Cancer Cell Line. *DNA and Cell Biology* **16**, 1231-1236.

Lymphatic Filariasis: Reasons for Hope (1998). Division of Control of Tropical Diseases, World Health Organization. Dzenowagis, J. (Ed.) in collaboration with Ottesen E., Karam M., Behbehani K.

Lyons, R.E. and Johnson, A.M. (1995) Heat-shock Proteins of *Toxoplasma gondii. Parasite Immunology* **17**, 353-359.

Maheswaran, S., Englert, C., Zheng, G., Lee, S.B., Wong, J., Harkin, D.P., Bean, J., Ezzell, R., Garvin, A.J., McCluskey, R.T., DeCaprio, J.A. and Haber, D.A. (1998) Inhibition of Cellular Proliferation by the Wilms Tumor Suppressor WT1 Requires Association with the Inducible Chaperone Hsp70. *Genes and Development* **12**, 1108-1120.

Malone, E.A. and Thomas, J.H. (1996) *Daf-21*, A Gene Required in Several Chemosensory Transduction Pathways, Encodes HSP-90. *The Worms Breeder's Gazette* **14**, 52

Marrs, K.A., Casey, E.S., Capitant, S.A., Bouchard, R.A., Dietrich, P.S., Mettler, I.J. and Sinibaldi, R.M. (1993) Characterization of Two Maize HSP90 Heat Shock Protein Genes: Expression During Heat Shock, Embryogenesis, and Pollen Development. *Developmental Genetics* **14**, 27-41.

Martin, S.A.M., Thompson, F.J. and Devaney, E. (1995) The Construction of Spliced Leader cDNA Libraries from the Filarial Nematode *Brugia pahangi. Molecular and Biochemical Parasitology* **70**, 241-245.

Martin, S.A.M., Hunter, S., Thompson, F.J. and Devaney, E. (1996) Stage Specific Gene Expression in the Post-infective L3 of the Filarial Nematode, *Brugia pahangi. Molecular and Biochemical Parasitology* **79**, 109-112.

Martinez, E., Bartolome, B. and de la Cruz, F. (1988) pACYC184-derived Cloning Vectors Containing the Multiple Cloning Site and LacZ Alpha Reporter Gene of pUC8/9 and pUC18/19 Plasmids. *Gene* **68**, 159-162.

Martinus, R.D., Ryan, M.T., Naylor, D.J., Herd, S.M., Hoogenraad, N.J. and Hoj, P.B. (1995) Role of Chaperones in the Biogenesis and Maintenance of the Mitochondrion. *FASEB Journal* **9**, 371-378.

Maruyama, T., Umezawa, A., Kusakari, S., Kikuchi, H., Nozaki, M. and Hata, J. (1998) Heat Shock Induces Differentiation of Human Embryonic Carcinoma Cells in Trophectoderm Lineages. *Experimental Cell Research* **224**, 123-127.

Matsumoto, K., Wassarman, K.M. and Wolffe, A.P. (1998) Nuclear History of a Pre-mRNA Determines the Translational Activity of Cytoplasmic mRNA. *EMBO Journal* **17**, 2107-2121.

Maxwell, C.A., Curtis, C.F., Haji, H., Kisumku, S., Thalib, A.I. and Yahya, S.A. (1990) Control of Bancroftian Filariasis by Integrating Therapy with Vector Control Using Polystyrene Beads in Wet Pit Latrines. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 709-714.

Mazzarella, R.A. and Green, M. (1987) ERp99, An Abundant, Conserved Glycoprotein of the Endoplasmic Reticulum, Is Homologous to the 90-kDa Heat Shock Protein (Hsp90) and the 94-kDa Glucose Regulated Protein (GRP94). *Journal of Biological Chemistry* **262**, 8875-8883.

McCauliffe, D.P., Yang, Y.S., Wilson, J., Southeimer, R.D. and Capra, J.D. (1992) The 5'-flanking Region of the Human Calreticulin Gene Shares Homology with the Human GRP78, GRP94 and Protein Disulfide Isomerase Promoters. *Journal of Biological Chemistry* **267**, 2557-2562.

McKnight, S.L. and Kingsbury, R. (1982) Transcriptional Control Signals of a Eukaryotic Protein-coding Gene. *Science* **217**, 316-324.

McMillan, D.R., Xiao, X., Shao, L., Graves, K. and Benjamin, I.J. (1998) Targeted Disruption of Heat Shock Transcription Factor 1 Abolishes Thermotolerance and Protection Against Heat-inducible Apoptosis. *Journal of Biological Chemistry* **273**, 7523-7528.

Mehlen, P., Mehlen, A., Godet, J. and Arrigo, A.P. (1997) Hsp27 as a Switch Between Differentiation and Apoptosis in Murine Embryonic Stem Cells. *Journal of Biological Chemistry* **272**, 31657-31665.

Meng, X., Jerome, V., Devin, J., Baulieu, E. and Catelli, M. (1993) Cloning of Chicken Hsp90β: The Only Vertebrate Hsp90 Insensitive to Heat Shock. *Biochemical and Biophysical Research Communications* **190**, 630-636.

Meng, X., Baulieu, E. and Catelli, M. (1995) Isolation of Chicken *Hsp90β* Gene promoter. *Biochemical and Biophysical Research Communications* **206**, 644-651.

Meng, X., Devin, J., Sullivan, W.P., Toft, D.O., Baulieu, E. and Catelli, M. (1996) Mutational Analysis of Hsp90α Dimerization and Subcellular Localization: Dimer Disruption Does Not Impede "*in vivo*" Interaction with Estrogen Receptor. *Journal of Cell Science* **109**, 1677-1687.

Merck, K.B., Groenen, P.J.T.A., Voorter, C.E.M., de Haard-Hoekman, W.A., Horwitz, J., Bloemendal, H. and de Jong, W.A. (1993) Structural and Functional Similarities of Bovine α-crystallin and Mouse Small Heat-shock Protein. *Journal of Biological Chemistry* **268**, 1046-1052.

Meshinchi, S., Grippo, J.F., Sanchez, E.R., Bresnick, E.H. and Pratt, W.B. (1988) Evidence That the Endogenous Heat-stable Glucocorticoid Receptor Stabilizing Factor is a Metal Component of the Untransformed Receptor Complex. *Journal of Biological Chemistry* **263**, 16809-16817.

Mestril, R., Schiller, P., Amin, J., Klapper, H., Ananthan, J. and Voellmy, R. (1986) Heat Shock and Ecdysterone Activation of the *Drosophila melanogaster* Hsp23 Gene; a Sequence Element Implied in Developmental Regulation. *EMBO Journal* **5**, 1667-1673.

Michael, E., Meyrowitsch, D.W. and Simonsen, P.E. (1996) Cost and Cost Effectiveness of Mass Diethylcarbamazine Chemotherapy for the Control of Bancroftian Filariasis: Comparison of Four Strategies in Tanzania. *Tropical Medicine and International Health* **1**, 414-426.

Michael, E. and Bundy, D.A.P. (1997) Global Mapping of Lymphatic Filariasis. *Parasitology Today* **13**, 472-476.

Mimnaugh, E.G., Worland, P.J., Whitesell, L. and Neckers, L.M. (1995) Possible Role for Serine/Threonine Phosphorylation in the Regulation of the Heteroprotein Complex Between the Hsp90 Stress Protein and the $pp90^{v\text{-}src}$ Tyrosine Kinase. *Journal of Biological Chemistry* **270**, 28654-28659.

Minami, Y., Kimura, Y., Kawasaki, H., Suzuki, K. and Yahara, I. (1994) The Carboxyl-terminal Region of Mammalian HSP90 is Required for its Dimerization and Function *in vivo*. *Molecular and Cellular Biology* **14**, 1459-1464.

Minchiotti, G., Gargano, S. and Maresca, B. (1991) The Intron-containing *Hsp82* Gene of the Dimorphic Pathogenic Fungus *Histoplasma capsulatum* is Properly Spliced in Severe Heat Shock Conditions. *Molecular and Cellular Biology* **11**, 5624-5630.

Minota, S., Koyasu, S., Yahara, I. and Winfield, J. (1988) Autoantibodies to the Heat-shock Protein Hsp90 in Systemic Lupus Erythromatosus. *Journal of Clinical Investigation* **81**, 106-109.

Mitsuda, N., Roses, A.D. and Vitek, M.P. (1997) Transcriptional Regulation of the Mouse Presenilin-1 Gene. *Journal of Biological Chemistry* **272**, 23489-23497.

Mobius, J., Groos, S., Meinhardt, A. and Seitz, J. (1997) Differential Distribution of the Mitochondrial Heat Shock Protein 60 in Rat Gastrointestinal Tract. *Cell and Tissue Research* **287**, 343-350.

Moore, S.K., Kozak, C., Robinson, E.A., Ullrich, S.J. and Appella, E. (1987) Cloning and Nucleotide Sequence of the Murine *Hsp84* cDNA and Chromosome Assignment of Related Sequences. *Gene* **56**, 29-40.

Morgan, W.D., Williams, G.T., Morimoto, R.I., Greene, J., Kingston, R.E. and Tijan, R. (1987) Two Transcriptional Activators, CCAAT-box-binding Transcription Factor and Heat Shock Transcription Factor, Interact with a Human Hsp70 Gene Promoter. *Molecular and Cellular Biology* **73**, 1129-1138.

Morimoto, R.I., Sarge, K.D. and Abravaya, K. (1992) Transcriptional Regulation of Heat Shock Genes. *Journal of Biological Chemistry* **267**, 21987-21990.

Mottram, J.C., Murphy, W.J. and Agabian, N. (1989) A Transcriptional Analysis of the *Trypansooma brucei Hsp83* Gene Cluster. *Molecular and Biochemical Parasitology* **37**, 115-128.

Muhich, M.L. and Boothroyd, J.C. (1989) Synthesis of Trypansome Hsp70 mRNA is Resistant to Disruption of *trans*-splicing by Heat Shock. *Journal of Biological Chemistry* **264**, 7107-7110.

Mullis, K.B. and Faloona, F.A. (1987) Specific Synthesis of DNA *in vitro* via a Polymerase-catalyzed Chain-reaction. *Methods in Enzymology* **155**, 335-350.

Nadeau, K., Das, A. and Walsh, C.T. (1993) Hsp90 Chaperonins Possess ATPase Activity and Bind Heat Shock Transcription Factors and Peptidyl Prolyl Isomerases. *Journal of Biological Chemistry* **268**, 1479-1487.

Nagai, N., Nakai, A. and Nagata, K. (1995) Quercetin Suppresses Heat Shock Response by Down Regulation of HSF1. *Biochemical and Biophysical Research Communications* **208**, 1099-1105.

Nagata, K. (1996) Hsp47: A Collagen-specific Molecular Chaperone. *Trends in Biochemical Sciences* **21**, 23-26.

Nakai, A. and Morimoto, R.I. (1993) Characterization of a Novel Chicken Heat Shock Transcription Factor, Heat Shock Factor 3, Suggests a New Regulatory Pathway. *Molecular and Cellular Biology* **13**, 1983-1997.

Nathan, D.F., Vos, M.H. and Lindquist, S. (1997) *In vivo* Functions of the *Saccharomyces cerevisiae* Hsp90 Chaperone. *Proceedings of the National Academy of Sciences USA* **94**, 12949-12956.

Nathan, D.F. and Lindquist, S. (1995) Mutational Analysis of Hsp90 Function: Interactions with a Steroid Receptor and a Protein Kinase. *Molecular and Cellular Biology* **15**, 3917-3925.

Nemoto, T., Ohara-Nemoto, Y., Ota, M., Takagi, T. and Yokoyama, K. (1995) Mechanism of Dimer Formation of the 90-kDa Heat-shock Protein. *European Journal of Biochemistry* **233**, 1-8.

Nemoto, T., Sato, N., Iwanari, H., Yamashita, H. and Takagi, T. (1997) Domain Structures and Immunogenic Regions of the 90-kDa Heat-shock Protein (HSP90). *Journal of Biological Chemistry* **272**, 26179-26187.

Nemoto, T. and Sato, N. (1998) Oligomeric Forms of the 90-kDa Heat Shock Protein. *Biochemical Journal* **330**, 989-995.

Nene, V., Dunne, D.W., Johnson, K.S., Taylor, D.W. and Cordingley, J.S. (1986) Sequence and Expression of a Major Egg Antigen from *Schistosoma mansoni*. Homologies to Heat Shock Proteins and Alpha-crystallins. *Molecular and Biochemical Parasitology* **21**, 179-188.

Neumann, S., Ziv, E., Lantner, F. and Schechter, I. (1992) Cloning and Sequencing of an *Hsp70* Gene of *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* **56**, 357-360.

Neumann, S., Ziv, E., Lantner, F. and Schechter, I. (1993) Regulation of HSP70 Gene Expression During the Life Cycle of the Parasitic Helminth *Schistosoma mansoni*. *European Journal of Biochemistry* **212**, 589-596.

Nicolas, L. (1997) New Tools for Diagnosis and Monitoring of Bancroftian Filariasis Parasitism: The Polynesian Experience. *Parasitology Today* **13**, 370-375.

Nilsen, T.W. (1993) *Trans*-splicing of Nematode, Premessenger RNA. *Annual Review of Microbiology* **47**, 413-440.

Ning, Y. and Sanchez, E.R. (1996) *In vivo* Evidence for the Generation of a Glucocorticoid Receptor-heat Shock Protein-90 Complex Incapable of Binding Hormone by the Calmodulin Antagonist Phenoxybenzamine. *Molecular Endocrinology* **10**, 14-23.

Oesterreich, S., Hickey, E., Weber, L.A. and Fuqua, S.A.W. (1996) Basal Regulatory Promoter Elements of the HSP27 Gene in Human Breast-cancer Cells. *Biochemical and Biophysical Research Communications* **222**, 155-163.

Ohno, K., Fukushima, M., Fujiwara, M. and Narumiya, S. (1988) Induction of 68,000-dalton Heat Shock Proteins by Cyclopentenone Prostaglandins. *Journal of Biological Chemistry* **263**, 19764-19770.

Ohsako, S., Bunick, D. and Hayashi, Y. (1995) Immunocytochemical Observation of the 90-kD Heat Shock Protein (HSP90) - High Expression in Primordial and Pre-meiotic Germ Cells of Male and Female Gonads. *Journal of Histochemistry and Cytochemistry* **43**, 67-76.

Okkema, P.G., Harrison, S.W., Plunger, V., Aryana, A. and Fire, A. (1993) Sequence Requirements for Myosin Gene-expression and Regulation in *Caenorhabditis elegans*. *Genetics* **135**, 385-404.

Ong, R.K.C. and Doyle, R.L. (1998) Tropical Pulmonary Eosinophilia. *Chest* **113**, 1673-1679.

Ottesen, E.A., Duke, B.O.L., Karam, M. and Behbehani, K. (1997) Strategies and Tools for the Control/Elimination of Lymphatic Filariasis. *Bulletin of the World Health Organization* **75**, 491-503.

Ottesen, E.A. (1994) The Human Filariases - New Understandings, New Therapeutic Strategies. *Current Opinion in Infectious Diseases* **7**, 550-558.

Ottesen, E.A. and Nutman, T.B. (1992) Tropical Pulmonary Eosinophilia. *Annual Review of Medicine* **43**, 417-424.

Owens-Grillo, J.K., Hoffmann, T., Hutchison, K.A., Yem, A.W., Deibel, M.R., Handschumacher, R.E. and Pratt, W.B. (1995) The Cyclosporin A-binding Immunophilin CyP-40 and the FK506-binding Immunophilin Hsp56 Bind to a Common Site on Hsp90 and Exist in Independent Cytosolic Heterocomplexes with the Untransformed Glucocorticoid Receptor. *Journal of Biological Chemistry* **270**, 20479-20484.

Page, D.M. (1996) TreeView : Tree Drawing Software for Apple Machintosh and Microsoft Windows. Division of Environmental and Evolutionary Biology, University of Glasgow.

Palmer, G., Louvion, J., Tibbetts, R.S., Engman, D.M. and Picard, D. (1995) *Trypanosoma cruzi* Heat-shock Protein 90 can Functionally Complement Yeast. *Molecular and Biochemical Parasitology* **70**, 199-202.

Patterson, N.A. and Kapoor, M. (1995) Developmentally Regulated Expression of Heat Shock Genes in *Leptosphaeria maculans*. *Canadian Journal of Microbiology* **41**, 499-507.

Pauli, D., Tonka, C.H. and Ayme-Southgate, A. (1988) An Unusual Split *Drosophila* Heat Shock Gene Expressed During Embryogenesis. *Journal of Molecular Biology* **200**, 47-53.

Perisic, O., Xiao, H. and Lis, J.T. (1989) Stable Binding of *Drosophila* Heat Shock Factor to Head-to-head and Tail-to-tail Repeats of a Conserved 5bp Recognition Unit. *Cell* **59**, 797-806.

Picard, D., Khursheed, B., Garabedian, M.J., Fortin, M.G., Lindquist, S. and Yamamoto, K.R. (1990) Reduced Levels of Hsp90 Compromise Steroid Receptor Action *in vivo*. *Nature* **348**, 166-168.

Pleass, R.J. and Bianco, A.E. (1996) The Effect of γ-radiation and Heat Shock on Protein Synthesis and Antioxidant Enzymes in the Gastrointestinal Parasite, *Heligmosomoides polygyrus*. *International Journal for Parasitology* **26**, 353-361.

Polla, B.S. (1991) Heat Shock Proteins in Host-parasite Interactions. *Immunology Today* **12**, A38-41.

Powell-Coffman, J.A., Bradfield, C.A. and Wood, W.B. (1998) *Caenorhabditis elegans* Orthologs of the Aryl Hydrocarbon Receptor and its Heterodimerization Partner the Aryl Hydrocarbon. *Proceedings of the National Academy of Sciences USA* **95**, 2844-2849.

Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A. and Baumeister, K. (1987) A System for Rapid DNA Sequencing with Fluorescent Chain-terminating Dideoxynucleotides. *Science* **238**, 336-341.

Prodromou, C., Roe, S.M., Piper, P.W. and Pearl, L. (1997) A Molecular Clamp in the Crystal Structure of the N-terminal Domain of the Yeast Hsp90 Chaperone. *Nature Structural Biology* **4**, 477-482.

Rallu, M., Loones, M., Lallemand, Y., Morimoto, R.I., Morange, M. and Mezger, V. (1997) Function and Regulation of Heat Shock Factor 2 During Mouse Embryogenesis. *Proceedings of the National Academy of Sciences USA* **94**, 2392-2397.

Rao, M.N., Chernokalskaya, E. and Schoenberg, D.R. (1996) Regulated Nuclear Polyadenylation of *Xenopus* Albumin Pre-mRNA. *Nucleic Acids Research* **24**, 4078-4083.

Rebbe, N.F., Hickman, W.S., Ley, T.J., Stafford, D.W. and Hickman, S. (1989) Nucleotide Sequence and Regulation of a Human 90-kDa Heat Shock Protein Gene. *Journal of Biological Chemistry* **264**, 15006-15011.

Renoir, J.M., Radanyi, C., Faber, L.E. and Baulieu, E. (1990) The Non-DNA-binding Heterooligomeric Form of Mammalian Steroid Hormone Receptors Contains a Hsp90-bound 59-kilodalton Protein. *Journal of Biological Chemistry* **265**, 10740-10745.

Rey-Ladino, J.A., Joshi, P.B., Singh, B., Gupta, R. and Reiner, N.E. (1997) *Leishmania major*: Molecular Cloning, Sequencing, and Expression of the *Heat Shock Protein 60* Gene Reveals Unique Carboxyl Terminal Peptide Sequences. *Experimental Parasitology* **85**, 249-263.

Rico, A.I., DelReal, G., Soto, M., Quijada, L., Martinez, A.C., Alonso, C. and Requena, J.M. (1998) Characterization of the Immunostimulatory Properties of *Leishmania infantum* HSP70 by Fusion to the *Escherichia coli* Maltose-binding Protein in Normal and nu/nu BALB/c Mice. *Infection and Immunity* **66**, 347-352.

Riddihough, G. and Pelham, H.R.B. (1986) Activation of the *Drosophila Hsp27* Promoter by Heat Shock and by Ecdysone Involves Independent and Remote Regulatory Sequences. *EMBO Journal* **5**, 1653-1658.

Riddihough, G. and Pelham, H.R.B. (1987) An Ecdysone Response Element in the *Drosophila Hsp27* Promoter. *EMBO Journal* **6**, 3729-3734.

Robbins, P.F., Eigamil, M., Li, Y.F., Fitzgerald, E.B., Kawakami, Y. and Rosenberg, S.A. (1997) The Intronic Region of an Incompletely Spliced Gp100 Gene Transcript Encodes an Epitope Recognized by Melanoma-reactive Tumor-infiltrating Lymphocytes. *Journal of Immunology* **159**, 303-308.

Rohrer, J. and Conley, M.E. (1998) Transcriptional Regulatory Elements Within the First Intron of Bruton's Tyrosine Kinase. *Blood* **91**, 214-221.

Roseman, A.M., Chen, S., White, H., Braig, K. and Saibil, H.R. (1996) The Chaperonin ATPase Cycle: Mechanisms of Allosteric Switching and Movements of Substrate-binding Domains in GroEL. *Cell* **87**, 241-251.

Rossi, A., Elia, G. and Santoro, M.G. (1997) Inhibition of Nuclear Factor κB by Prostaglandins A_1: An Effect Associated with Heat Shock Transcription Factor Activation. *Proceedings of the National Academy of Sciences USA* **94**, 746-750.

Rothstein, N. and Rajan, T.V. (1991) Characterization of an *Hsp70* gene from the Human Filarial Parasite, *Brugia malayi* (Nematoda). *Molecular and Biochemical Parasitology* **49**, 229-238.

Russnak, R.H., Jones, D. and Candido, E.P.M. (1983) Cloning and Analysis of cDNA Sequences Coding for Two 16 Kilodalton Heat Shock Proteins (Hsps) in *Caenorhabditis elegans*: Homology with the Small Hsps of *Drosophila*. *Nucleic Acids Research* **11**, 3187-3205.

Russnak, R.H. and Candido, E.P.M. (1985) Locus Encoding a Family of Small Heat Shock Genes in *Caenorhabditis elegans*: Two Genes Duplicated to Form a 3.8-kilobase Inverted Repeat. *Molecular and Cellular Biology* **5**, 1268-1278.

Rutherford, S.L. and Zuker, C.S. (1994) Protein Folding and the Regulation of Signaling Pathways. *Cell* **79**, 1129-1132.

Sabbah, M., Radanyi, C., Redeuilh, G. and Baulieu, E. (1996) The 90 kDa Heat-shock Protein (Hsp90) Modulates the Binding of the Oestrogen Receptor to its Cognate DNA. *Biochemical Journal* **314**, 205-213.

Salotra, P., Chauhan, D., Ralhan, R. and Bhatnagar, R. (1995) Tumour Necrosis Factor-alpha Induces Preferential Expression of Stress Proteins in Virulent Promastigotes of *Leishmania donovani*. *Immunology Letters* **44**, 1-5.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Cold Spring Harbor Molecular Cloning: A Laboratory Manual*, 2nd edn.

Sanchez, E.R., Housley, P.R. and Pratt, W.B. (1986) The Molybdate-stabilized Glucocorticoid Binding Complex of L-cells Contains a 98-100 Kdalton Steroid Binding Phosphoprotein and a 90 Kdalton Nonsteroid-binding Phosphoprotein That is Part of the Murine Heat-shock Complex. *Journal of Steroid Biochemistry* **24**, 9-18.

Sanchez, E.R., Meshinchi, S., Tienrungroj, W., Schlesinger, M.J., Toft, D.O. and Pratt, W.B. (1987) Relationship of the 90-kDa Murine Heat Shock Protein to the Untransformed and Transformed States of the L Cell Glucocorticoid Receptor. *Journal of Biological Chemistry* **262**, 6986-6991.

Sanger, F. and Coulson, A.R. (1975) A Rapid Method for Determining Sequences in DNA by Priming Synthesis with DNA Polymerase. *Journal of Molecular Biology* **94**, 441-448.

Santacruz, H., Vriz, S. and Angelier, N. (1997) Molecular Characterization of a Heat Shock Cognate cDNA of Zebrafish, Hsc70, and Developmental Expression of the Corresponding Transcripts. *Developmental Genetics* **21**, 223-233.

Santoro, M.G., Garaci, E. and Amici, C. (1989) Prostaglandins with Antiproliferative Activity Induce the Synthesis of a Heat Shock Protein in Human Cells. *Proceedings of the National Academy of Sciences USA* **86**, 8407-8411.

Sarge, K.D., Zimarino, V., Holm, K., Wu, C. and Morimoto, R.I. (1991) Cloning and Characterization of Two Mouse Heat Shock Factors with Distinct Inducible and Constitutive DNA-binding Activity. *Genes and Development* **5**, 1902-1911.

Sarge, K.D. and Cullen, K.E. (1997) Regulation of Hsp Expression During Rodent Spermatogenesis. *Cellular and Molecular Life Sciences* **53**, 191-197.

Sass, J.B., Weinberg, E.S. and Krone, P.H. (1996) Specific Localization of Zebrafish HSP90-alpha Messenger-RNA to MyoD-expressing Cells Suggests a Role for HSP90-alpha During Normal Muscle Development. *Mechanisms of Development* **54**, 195-204.

Sass, J.B. and Krone, P.H. (1997) *HSP90α* Gene Expression May Be a Conserved Feature of Vertebrate Somitogenesis. *Experimental Cell Research* **233**, 391-394.

Satoh, M., Hirayoshi, K., Yokota, S.I., Hosokawa, N. and Nagata, K. (1996) Intracellular Interaction of Collagen-specific Stress Protein HSP47 with Newly Synthesized Procollagen. *Journal of Cell Biology* **133**, 469-483.

Scharf, K., Rose, S., Zott, W., Schoff, F. and Nover, L. (1990) Three Tomato Genes Code for Heat Stress Transcription Factors with a Region of Remarkable Homology to the DNA-binding Domain of the Yeast HSF. *EMBO Journal* **9**, 4495-4501.

Scheibel, T., Weikl, T. and Buchner, J. (1998) Two Chaperone Sites in Hsp90 Differing in Substrate Specificity and ATP Dependence. *Proceedings of the National Academy of Sciences USA* **95**, 1495-1499.

Schelling, D. and Jones, G. (1996) Analysis of Induction of Hsc70, Hsp82 and Juvenile Hormone Esterase Genes by Heat Shock in *Trichoplusia ni*. *Journal of Insect Physiology* **42**, 295-301.

Schirmer, E.C., Glover, J.R., Singer, M.A. and Lindquist, S. (1996) HSP100/Clp Proteins: A Common Mechanism Explains Diverse Functions. *Trends in Biochemical Sciences* **21**, 289-296.

Schlesinger, M.J., Kelley, P.M., Aliperti, C. and Malfer, C. (1982) Properties of Three Major Chicken Heat-shock Proteins and Their Antibodies. In: Schlesinger, M.J., Ashburner, M. and Tissieres, A. (Eds.) *Heat Shock From Bacteria to Man*, pp. 243-250. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

Schlesinger, M.J. (1994) How the Cell Copes with Stress and the Function of Heat Shock Proteins. *Pediatric Research* **36**, 1-6.

Schmitz, G., Schmidt, M. and Feierabend, J. (1996) Characterization of a Plastid-specific HSP90 Homologue: Identification of a cDNA Sequence, Phylogenetic Descendence and Analysis of its mRNA and Protein Expression. *Plant Molecular Biology* **30**, 479-492.

Schmitz, K.A., Hale, T.J., Rajan, T.V. and Yates, J.A. (1996) Localization of Paramyosin, Myosin and a Heat Shock Protein 70 in Larval and Adult *Brugia malayi*. *Journal of Parasitology* **82**, 367-370.

Schneider, C., Sepp-Lorenzino, L., Nimmesgern, E., Ouerfelli, O., Danishefsky, S., Rosen, N. and Hartl, F.U. (1996) Pharmacologic Shifting of a Balance Between Protein Refolding and Degradation Mediated by Hsp90. *Proceedings of the National Academy of Sciences USA* **93**, 14536-14541.

Schoder, H., Langer, T., Hartl, F.U. and Bukau, B. (1993) DnaK, DnaJ and GrpE Form a Cellular Chaperone Machinery Capable of Repairing Heat-induced Protein Damage. *EMBO J.* **12**, 4137-4144.

Schroder, G., Beck, M., Eichel, J., Vetter, H. and Schroder, J. (1993) HSP90 Homologue from Madagascar periwinkle (*Catharanthus roseus*): cDNA Sequence, Regulation of Protein Expression and Location in the Endoplasmic Reticulum. *Plant Molecular Biology* **23**, 583-594.

Segnitz, B. and Gehring, U. (1997) The Function of Steroid Hormone Receptors is Inhibited by the Hsp90-specific Compound Geldanamycin. *Journal of Biological Chemistry* **272**, 18694-18701.

Selkirk, M.E., Denham, D.A., Partono, F. and Maizels, R.M. (1989) Heat Shock Cognate 70 is a Prominent Immunogen in Brugian Filariasis. *Journal of Immunology* **143**, 299-308.

Serra, E., Zemzoumi, K., Trolet, J., Capron, A. and Dissous, C. (1996) Functional Analysis of the *Schistosoma mansoni* 28 kDa Glutathione S-transferase Gene Promoter: Involvement of SMNF-Y Transcription Factor in Multimeric Complexes. *Molecular and Biochemical Parasitology* **83**, 69-80.

Shapira, M. and Pedraza, G. (1990) Sequence Analysis and Transcriptional Activation of Heat Shock Protein 83 of *Leishmania mexicana amazonensis*. *Molecular and Biochemical Parasitology* **42**, 247-256.

Shi, Y., Kroeger, P.E. and Morimoto, R.I. (1995) The Carboxyl-terminal Transactivation Domain of Heat Shock Factor 1 is Negatively Regulated and Stress Responsive. *Molecular and Cellular Biology* **15**, 4309-4318.

Simon, M.C. (1995) Gotta have GATA. *Nature Genetics* **11**, 9-11.

Simonsen, P.E., Meyrowitsch, D.W., Makunde, W.H. and Magnussen, P. (1995) Selective Diethylcarbamazine Chemotherapy for Control of Bancroftian Filariasis in 2 Communities of Tanzania - Compared Efficacy of a Standard-dose Treatment and 2 Semiannual Single-dose Treatments. *American Journal of Tropical Medicine and Hygiene* **53**, 267-272.

Sistonen, L., Sarge, K.D., Phillips, B., Abravaya, K. and Morimoto, R.I. (1992) Activation of Heat Shock Factor 2 During Hemin-induced Differentiation of Human Erythroleukemia Cells. *Molecular and Cellular Biology* **12**, 4104-4111.

Sistonen, L., Sarge, K.D. and Morimoto, R.I. (1994) Human Heat Shock Transcription Factors 1 and 2 are Differentially Activated and Can Synergistically Induce Hsp70 Gene Transcription. *Molecular and Cellular Biology* **14**, 2087-2099.

Sjottem, E., Andersen, C. and Johansen, T. (1997) Structural and Functional Analyses of DNA Bending Induced by Sp1 Family of Transcription Factors. *Journal of Molecular Biology* **267**, 490-504.

Smith, D.F. (1993) Dynamics of Heat Shock Protein 90-progesterone Receptor Binding and Disactivation Loop Model for Steroid Receptor Complexes. *Molecular Endocrinology* **7**, 1418-1429.

Snutch, T.P., Heschl, M.F.P. and Baillie, D.L. (1988) The *Caenorhabditis elegans Hsp70* Gene Family: a Molecular Genetic Characterization. *Gene* **64**, 241-255.

Snutch, T.P. and Baillie, D.L. (1984) A High Degree of DNA strain Polymorphism Associated with the Major Heat Shock Gene in *Caenorhabditis elegans*. *Molecular Genes and Genetics* **195**, 329-335.

Sorger, P.K., Lewis, M.J. and Pelham, H.R.B. (1987) Heat Shock Factor is Regulated Differently in Yeast and HeLa Cells. *Nature* **329**, 81-84.

Sorger, P.K. (1991) Heat Shock Factor and the Heat Shock Response. *Cell* **65**, 363-366.

Sorger, P.K. and Pelham, H.R.B. (1987) The Glucose-regulated Protein Grp94 is Related to Heat Shock Protein Hsp90. *Journal of Molecular Biology* **194**, 341-344.

Southern, E.M. (1975) Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis. *Journal of Molecular Biology* **98**, 503-517.

Spandidos, D.A. and Riggio, M. (1986) Promoter and Enhancer Like Activity at the 5'-end of Normal and T24 Ha-*ras*1 Genes. *FEBS Letters* **203**, 169-173.

Speith, J., Denison, K., Kirtland, S., Cane, J. and Blumenthal, T. (1985) The *C. elegans* Vitellogenin Genes: Short Sequence Repeats in the Promoter Regions and Homology to the Vertebrate Genes. *Nucleic Acids Research* **13**, 5283-5295.

Srivastava, P.K., Menoret, A., Basu, S., Binder, R.J. and McQuade, K.L. (1998) Heat Shock Proteins Come of Age: Primitive Functions Acquire New Roles in an Adaptive World. *Immunity* **8**, 657-665.

Stancato, L.F., Chow, Y., Hutchison, K.A., Perdew, G.H., Jove, R. and Pratt, W.B. (1993) Raf Exists in a Native Heterocomplexes with Hsp90 and p50 That Can be Reconstituted in a Cell-free System. *Journal of Biological Chemistry* **268**, 21711-21716.

Steel, C., Guinea, A., McCarthy, J.S. and Ottesen, E.A. (1994) Long-Term Effect of Prenatal Exposure to Maternal Microfilaria on Immune Responsiveness to Filarial Parasite Antigens. *Lancet* **343**, 890-893.

Steel, C., Guinea, A. and Ottesen, E.A. (1996) Evidence for Protective Immunity to Bancroftian Filariasis in the Cook Islands. *Journal of Infectious Diseases* **174**, 598-605.

Stege, G.J.J., Brunsting, J.F., Kampinga, H.H. and Konings, A.W.T. (1995) Thermotolerance and Nuclear Protein Aggregation: Protection Against Initial Damage or Better Recovery? *Journal of Cellular Physiology* **164**, 579-586.

Stephanou, A., Amin, V., Isenberg, D.A., Akira, S., Kishimoto, T. and Latchman, D.S. (1997) Interleukin 6 Activates Heat-shock Protein 90β Gene Expression. *Biochemical Journal* **321**, 103-106.

Stewart, M.J., Dipple, K.M., Stewart, T.R. and Crabb, D.W. (1996) The Role of Nuclear Factor NF-Y/CP1 in the Transcriptional Regulation of the Human Aldehyde Dehydrogenase 2-encoding Gene. *Gene* **173**, 155-161.

Streit, J.A., Donelson, J.E., Agey, M.W. and Wilson, M.E. (1996) Developmental-changes in the Expression of *Leishmania-chagasi* GP63 and Heat-shock Proteins in a Human Macrophage Cell-line. *Infection and Immunity* **64**, 1810-1818.

Stringham, E.G., Dixon, D.K., Jones, D. and Candido, E.P.M. (1992) Temporal and Spatial Expression Patterns of the Small Heat Shock (*hsp16*) Genes in Transgenic *Caenorhabditis elegans*. *Molecular Biology of the Cell* **3**, 221-233.

Stuber, D., Matile, H. and Garotta, G. (1990) System for High-level Production in *Escherichia coli* and Rapid Purification of Recombinant Proteins: Application to Epitope Mapping, Preparation of Antibodies, and Structure-function Analysis. In: Lefkovits, I. and Pernis, B. (Eds.) *Immunological Methods*, pp. 121-152. New York: Academic Press

Su, X. and Wellems, T.E. (1998) Sequence, Transcript Characterization and Polymorphism of a *Plasmodium falciparum* Gene Belonging to the Heat-shock Protein (HSP) 90 Family. *Gene* **151**, 225-230.

Sullivan, W.P. and Toft, D.O. (1993) Mutational Analysis of Hsp90 Binding to the Progesterone Receptor. *Journal of Biological Chemistry* **268**, 20373-20379.

Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J., Qui, L., Dear, S., Coulson, A., Craxton, M., Durbin, R., Berks, M., Metzstein, M., Hawkins, T., Ainscough, R. and Waterston, R. (1992) The *C. elegans* Genome Sequencing Project: A Beginning. *Nature* **356**, 37-41.

Swoboda, R.K., Bertram, G., Budge, S., Gooday, G.W., Gow, N.A.R. and Brown, A.J.P. (1995) Structure and Regulation of the *HSP90* Gene from the Pathogenic Fungus *Candida albicans*. *Infection and Immunity* **63**, 4506-4514.

Tanaka, Y., Kobayashi, K., Kita, M., Masuda, H., Kinoshita, S., Nagata, K. and Imanishi, J. (1996) Expression of 47kDa Heat Shock Protein (HSP47) During Development of Mouse Cornea. *Experimental Eye Research* **63**, 383-393.

Tardieux, I., Baines, I., Mossakowska, M. and Ward, G.E. (1998) Actin-binding Proteins of Invasive Malaria Parasites and the Regulation of Action Polymerization by a Complex of 32/34-kDa Proteins Associated with Heat Shock Protein 70kDa. *Molecular and Biochemical Parasitology* **93**, 295-308.

Taylor, D.W., Cordingley, J.S. and Butterworth, A.E. (1983) Immunoprecipitation of Surface Antigen Precursors from *Schistosoma mansoni* Messenger RNA *in vitro* Translation Products. *Molecular and Biochemical Parasitology* **10**, 305-318.

Thomas, J.G. and Baneyx, F. (1996) Protein Folding in the Cytoplasm of *Escherichia coli*: Requirements for the DnaK-DnaJ-GrpE and GroEL-GroES Molecular Chaperone Machines. *Molecular Microbiology* **21**, 1185-1196.

Thompson, F.J., Martin, S.A.M. and Devaney, E. (1996) *Brugia pahangi*: Characterisation of a Small Heat Shock Protein cDNA Clone. *Experimental Parasitology* **83**, 259-266.

Tissieres, A., Mitchell, H.K. and Tracy, U. (1974) Protein Synthesis in Salivary Glands of *Drosophila melanogaster*. Relation to Chromosome Puffs. *Journal of Molecular Biology* **84**, 389-398.

Tranbarger, T.J. and Misra, S. (1995) The Molecular Characterization of a Set of cDNAs Differentially Expressed During Douglas-fir Germination and Early Seedling Development. *Physiologia Plantarum* **95**, 456-464.

Tuohimaa, P., Pekki, A., Blauer, M., Joensuu, T., Vilja, P. and Ylikomi, T. (1993) Nuclear Progesterone Receptor is Mainly Heat Shock Protein 90-free *in vivo*. *Proceedings of the National Academy of Sciences USA* **90**, 5848-5852.

Tweedie, S., Grigg, M.E., Ingram, L. and Selkirk, M.E. (1993) The Expression of a Small Heat Shock Protein Homologue is Developmentally Regulated in *Nippostrongylus brasiliensis*. *Molecular and Biochemical Parasitology* **61**, 149-154.

Udono, H. and Srivastava, P.K. (1993) Heat Shock Protein 70-associated Peptides Elicit Specific Cancer Immunity. *Journal of Experimental Medicine* **178**, 1391-1396.

Uma, S., Hartson, S.D., Chen, J. and Matts, R.L. (1997) Hsp90 is Obligatory for the Heme-regulated eIF-2α Kinase to Acquire and Maintain an Activable Conformation. *Journal of Biological Chemistry* **272**, 11648-11656.

Vamvakopoulus, N.O. (1993) Tissue-specific Expression of Heat Shock Proteins 70 and 90: Potential Implications for Differential Sensitivity of Tissues to Glucocorticoids. *Molecular and Cellular Endocrinology* **98**, 49-54.

Van Breusegem, F., Dekeyser, R., Garcia, A.B., Claes, B., Gielen, J., Van Montagu, M. and Caplan, A.B. (1994) Heat-inducible Rice *Hsp82* and *Hsp70* are Not Always Co-regulated. *Planta* **193**, 57-66.

van Noort, J.M., van Sechel, A.C., Bajramovic, J.J., El Ouagmiri, M., Polman, C.H., Lassmann, H. and Ravid, R. (1995) The Small Heat-shock Protein αB-crystallin as Candidate Autoantigen in Multiple Sclerosis. *Nature* **375**, 798-801.

Vidal, V., Qui, N., Redfield, B., Carlino, A., Brot, N. and Weissbach, H. (1996) ATP Hydrolysis is Not Required for the Dissociation of a Substance P.BiP Complex. *Archives of Biochemistry and Biophysics* **330**, 314-318.

Voellmy, R., Goldschmidt-Clermont, M., Southgate, R., Tissieres, A., Levis, R. and Gehring, W. (1981) A DNA Segment from Chromosomal Site 67B in *D. melanogaster* Contains Four Closely Linked Heat-shock Genes. *Cell* **23**, 261-270.

Wales, A. and Kusel, J.R. (1992) Biochemistry of Irradiated Parasite Vaccines: Suggested Models for Their Mode of Action. *Parasitology* **8**, 358-363.

Walsh, D., Li, Z., Wu, Y. and Nagata, K. (1997) Heat Shock and the Role of the HSPs During Neural Plate Induction in Early Mammalian CNS and Brain Development. *Cellular and Molecular Life Sciences* **53**, 198-211.

Wang, J., Shen, L., Najafi, H., Kolberg, J., Matschinsky, F.M., Urdea, M. and German, M. (1997) Regulation of Insulin PreRNA Splicing by Glucose. *Proceedings of the National Academy of Sciences USA* **94**, 4360-4365.

Warbrick, E.V., Barker, G.C. and Howells, R.E. (1993) The Effect of Invertebrate Hormones and Potential Hormone Inhibitors on the Third Stage Moult of the Filarial Nematode, *Dirofilaria immitis*, *in vitro*. *Parasitology* **107**, 459-463.

Wearsch, P.A. and Nicchitta, C.V. (1996) Purification and Partial Molecular Characterization of GRP94, an ER Resident Chaperone. *Protein Expression and Purification* **7**, 114-121.

Weil, G.J., Lammie, P.J. and Weiss, N. (1997) The ICT Filariasis Test: A Rapid-format Antigen Test for Diagnosis of Bancroftian Filariasis. *Parasitology Today* **13**, 401-404.

Weiss, L.M., Ma, Y.F., Takvorian, P.M., Tanowitz, H.B. and Wittner, M. (1998) Bradyzoite Development in *Toxoplasma gondii* and the HSP70 stress response. *Infection and Immunity* **66**, 3295-3302.

Westwood, J.T., Clos, J. and Wu, C. (1991) Stress-induced Oligomerization and Chromosomal Relocalization of Heat-shock Factor. *Nature* **353**, 822-827.

Whitelaw, M.L., Gottlicher, M., Gustafsson, J. and Poellinger, L. (1993) Definition of a Novel Ligand Binding Domain of a Nuclear bHLH Receptor: Co-localization of Ligand and Hsp90 Binding Activities Within the Regulable Inactivation Domain of the Dioxin Receptor. *EMBO Journal* **12**, 4169-4179.

Whitesell, L., Mimnaugh, E.G., De Costa, B., Myers, C.E. and Neckers, L.M. (1994) Inhibition of Heat Shock Protein HSP90-pp60$^{v\text{-}src}$ Heteroprotein Complex Formation by Benzoquinone Ansamycins: Essential Role for the Stress Proteins in Oncogenic Transformation. *Proceedings of the National Academy of Sciences USA* **91**, 8324-8328.

Wiech, H., Buchner, J., Zimmermann, R. and Jakob, U. (1992) Hsp90 Chaperones Protein Folding *in vitro*. *Nature* **358**, 169-170.

Wiederrecht, G., Shuey, D.J., Kibbe, W.A. and Parker, C.S. (1987) The *Saccharomyces* and *Drosophila* Heat Shock Transcription Factors are Identical in Size and DNA Binding Properties. *Cell* **48**, 507-515.

Williams, N.E. and Nelsen, E.M. (1997) HSP70 and HSP90 Homologs are Associated with Tubulin in Hetero-oligomeric Complexes, Cilia and the Cortex of *Tetrahymena*. *Journal of Cell Science* **110**, 1665-1672.

Wright, K.L., Vilen, B.J., Itoh-Lindstrom, Y., Moore, T.L., Li, G., Crisciteillo, M., Cogswell, P., Clarke, J.B. and Ting, J.P. (1994) CCAAT Box Binding Protein NF-Y Facilitates *in vivo* Recruitment of Upstream DNA Binding Transcription Factors. *EMBO Journal* **13**, 4042-4053.

Wyman, C., Grotkopp, E., Bustamante, C. and Nelson, H.C.M. (1995) Determination of Heat-shock Transcription Factor 2 Stoichiometry at Looped DNA Complexes Using Scanning Force Microscopy. *EMBO Journal* **14**, 117-123.

Xiao, H., Perisic, O. and Lis, J.T. (1991) Cooperative Binding of *Drosophila* Heat Shock Factor to Arrays of a Conserved 5bp Unit. *Cell* **64**, 585-593.

Xiao, H. and Lis, J.T. (1989) Heat Shock and Developmental Regulation of the *Drosophila melanogaster Hsp83* Gene. *Molecular and Cellular Biology* **9**, 1746-1753.

Xu, Y. and Lindquist, S. (1993) Heat-shock Protein Hsp90 Governs the Activity of pp60$^{v\text{-}src}$ Kinase. *Proceedings of the National Academy of Sciences USA* **90**, 7074-7078.

Yabe, N., Takahashi, T. and Komeda, Y. (1994) Analysis of Tissue-specific Expression of *Arabidopsis thaliana* HSP90-family Gene HSP81. *Plant Cell Physiology* **35**, 1207-1219.

Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L. and Sternlicht, H. (1992) TCP1 Complex is a Molecular Chaperone in Tubulin Biogenesis. *Nature* **358**, 245-248.

Yang, J. and DeFranco, D.B. (1996) Assessment of Glucocorticoid Receptor-heat Shock Protein 90 Interactions *in vivo* During Nucleocytoplasmic Trafficking. *Molecular Endocrinology* **10**, 3-13.

Yin, Y., Chen, L. and Beachy, R. (1997) Promoter Elements Required for Phloem-specific Gene Expression from the RTBV in Rice. *The Plant Journal* **12**, 1179-1188.

Yonehara, M., Minami, Y., Kawata, Y., Nagai, J. and Yahara, I. (1996) Heat-induced Chaperone Activity of HSP90. *Journal of Biological Chemistry* **271**, 2641-2645.

Yonezawa, N., Nishida, E., Sakai, H., Koyasu, S., Matsuzaki, F., Iida, K. and Yahara, I. (1988) Purification and Characterization of the 90-kDa Heat-shock Protein from Mammalian Tissues. *European Journal of Biochemistry* **177**, 1-7.

Yost, H.J., Petersen, R.B. and Lindquist, S. (1990) RNA Metabolism: Strategies for Regulation in the Heat Shock Response. *Trends in Genetic Sciences* **6**, 223-227.

Yost, H.J. and Lindquist, S. (1986) RNA Splicing is Interrupted by Heat Shock and is Rescued by Heat Shock Protein Synthesis. *Cell* **45**, 185-193.

Yost, H.J. and Lindquist, S. (1988) Translation of Unspliced Transcripts After Heat Shock. *Science* **242**, 1544-1547.

Yost, H.J. and Lindquist, S. (1991) Heat Shock Proteins Affect RNA Processing During the Heat Shock Response of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **11**, 1062-1068.

Young, D., Lathigra, R., Hendrix, R., Sweetser, D. and Young, R.A. (1998) Stress Proteins are Immune Targets in Leprosy and Tuberculosis. *Proceedings of the National Academy of Sciences USA*

Zarzov, P., Boucherie, H. and Mann, C. (1997) A Yeast Heat Shock Transcriptional Factor (Hsf1) Mutant is Defective in Both Hsc82/Hsp82 Synthesis and Spindle Pole Body Duplication. *Journal of Cell Science* **110**, 1879-1891.

Zemzoumi, K., Dissous, C., Cochu, A., Trolet, J., Capron, A. and McNair, A. (1995) *Schistosoma mansoni*: Interaction of Nuclear Extracts with the CCAAT-binding Site Revealed by the Gel Shift Assay. *Experimental Parasitology* **80**, 149-154.

Zhu, D., Dix, D.J. and Eddy, E.M. (1997) HSP70-2 is Required for CDC2 Kinase Activity in Meiosis I of Mouse Spermatocytes. *Development* **124**, 3007-3014.

Ziemiecki, A., Catelli, M., Joab, I. and Moncharmont, B. (1986) Association of the Heat Shock Protein HSP90 with Steroid Hormone Receptors and Tyrosine Kinase Oncogene Products. *Biochemical and Biophysical Research Communications* **138**, 1298-1307.

Zihai, L. (1997) Priming of T Cells by Heat Shock Protein-peptide Complexes as the Basis of Tumor Vaccines. *Seminars in Immunology* **9**, 315-322.

Zimmerman, J.L., Petri, W. and Meselson, M. (1983) Accumulations of a Specific Subsets of *D. melanogaster* Heat Shock mRNA in Normal Development Without Heat Shock. *Cell* **32**, 1161-1170.

Zuker-Aprison, E. and Blumenthal, T. (1989) Potential Regulatory Elements of Nematode Vitellogenin Genes Revealed by Interspecies Sequence Comparison. *Journal of Molecular Evolution* **28**, 487-496.

Zuo, J., Baler, R., Dahl, G. and Voellmy, R. (1994) Activation of the DNA-binding Ability of Human Heat Shock Transcription Factor 1 May Involve the Transition from an Intramolecular to an Intermolecular Triple-stranded Coiled-coil Structure. *Molecular and Cellular Biology* **14**, 7557-7568.

Zuo, J., Rungger, D. and Voellmy, R. (1995) Multiple Layers of Regulation of Human Heat Shock Transcription Factor 1. *Molecular and Cellular Biology* **15**, 4319-4330.

Appendix I: Distance matrix for HSP90 sequences

	1	2	3	4	5	6	7	8	9	10	11	12	..
1	0.00	49.27	49.44	49.79	52.02	51.60	48.12	49.44	49.07	47.76	48.02	48.29	..
2		0.00	26.30	25.69	51.09	51.50	23.32	23.40	23.36	26.17	25.42	25.42	
3			0.00	14.62	51.22	50.35	23.09	23.32	23.47	24.02	24.25	24.45	
4				0.00	51.22	49.85	27.03	26.48	27.25	26.93	26.73	26.73	
5					0.00	2.31	50.64	50.33	50.50	47.57	49.17	48.63	
6						0.00	51.40	50.84	51.01	48.04	49.39	48.85	
7							0.00	3.81	4.40	14.38	15.28	15.45	
8								0.00	0.96	15.50	15.18	14.85	
9									0.00	16.16	15.84	15.50	
10										0.00	5.61	5.46	
11											0.00	1.12	
12												0.00	
13													
14													
15													
16													
17													
18													
19													
20													
21													
22													
23													
24													
25													
26													
27													
28													
29													
30													

	13	14	15	16	17	18	19	20	21	22	23	24	..
1	50.43	54.34	50.36	110.24	45.67	42.41	53.96	41.40	49.10	41.82	50.01	45.06	..
2	24.13	50.19	14.56	112.34	46.95	35.89	49.39	36.59	26.41	36.36	48.98	41.42	
3	24.05	49.22	27.08	110.74	48.10	40.43	47.36	41.25	26.23	40.01	49.47	45.06	
4	27.87	49.57	27.76	112.22	47.38	39.20	48.90	41.71	28.17	40.95	49.44	44.02	
5	51.88	34.09	51.78	108.32	51.85	42.91	28.54	44.89	49.71	45.94	17.66	49.07	
6	52.69	34.60	51.82	106.89	51.89	43.85	28.97	45.07	50.22	45.07	17.66	48.74	
7	5.42	47.66	23.20	106.54	45.20	34.22	47.98	35.83	17.01	34.91	49.79	40.60	
8	2.08	48.10	24.44	104.23	45.62	34.04	48.53	36.26	16.38	34.94	48.88	40.61	
9	1.66	47.47	24.40	105.38	45.79	34.42	48.71	35.96	17.05	34.65	49.32	40.53	
10	17.00	48.02	25.26	108.32	46.67	37.40	46.25	35.93	6.81	35.30	50.43	41.01	
11	16.53	49.37	24.72	105.96	47.55	36.76	46.53	36.86	2.39	35.76	49.62	41.26	
12	16.36	49.10	24.91	105.96	47.82	36.52	46.53	36.62	2.11	35.76	49.34	41.26	
13	0.00	48.81	25.57	106.54	47.60	35.56	49.55	37.35	17.93	35.79	50.95	41.52	
14		0.00	50.42	111.23	52.99	42.65	35.29	44.37	50.74	45.33	32.71	48.82	
15			0.00	111.47	48.25	39.47	48.67	40.76	25.70	40.76	50.56	44.28	
16				0.00	105.72	105.96	109.34	106.89	107.72	106.89	105.04	109.88	
17					0.00	38.13	52.08	36.49	48.88	36.32	51.64	39.88	
18						0.00	42.33	12.87	37.94	14.43	42.14	17.28	
19							0.00	46.21	47.34	44.30	32.79	47.51	
20								0.00	38.51	7.41	44.80	11.64	
21									0.00	37.40	50.43	42.25	
22										0.00	43.59	8.99	
23											0.00	48.00	
24												0.00	
25													
26													
27													
28													
29													
30													

Appendix I : Distance matrix for HSP90 sequences

	25	26	27	28	29	30
1	46.39	49.34	42.58	46.26	19.02	52.93
2	39.25	23.36	39.32	42.04	50.46	28.83
3	46.26	23.28	42.51	45.46	47.58	29.82
4	43.68	26.64	42.99	46.99	49.62	32.08
5	47.73	49.96	43.85	52.65	52.62	56.09
6	47.03	50.74	43.76	51.78	51.92	55.80
7	41.99	3.66	41.38	42.70	48.04	19.41
8	42.92	0.27	41.31	42.46	48.29	18.99
9	42.67	0.96	41.24	42.38	49.10	18.78
10	43.17	15.11	42.35	42.62	48.12	21.48
11	42.67	14.99	41.94	41.00	48.93	20.26
12	42.92	14.65	41.69	41.48	48.93	20.44
13	43.68	2.08	42.94	43.36	49.91	19.65
14	51.14	47.74	42.31	49.12	57.64	51.19
15	41.34	24.21	42.02	43.69	50.39	29.97
16	106.65	104.23	104.71	106.54	109.39	110.37
17	47.35	45.53	43.27	32.36	45.59	48.69
18	36.50	33.75	11.93	36.06	43.33	40.86
19	49.21	48.43	42.95	52.08	55.83	52.62
20	36.33	35.96	14.71	33.34	42.31	41.10
21	43.68	16.19	43.18	42.45	50.29	21.92
22	34.69	34.65	17.95	34.69	42.22	39.40
23	46.20	48.78	42.15	49.12	50.87	52.76
24	36.57	40.29	21.69	37.22	46.84	46.17
25	0.00	42.42	38.93	40.91	48.74	44.88
26		0.00	41.00	42.14	48.02	18.75
27			0.00	38.65	45.06	43.10
28				0.00	46.72	45.45
29					0.00	53.54
30						0.00

Listed on the right are the species from which protein sequences were used to create a cladogram. Additionally, two different HSP90 sequences from the same species have been used in some cases. Only complete amino acid sequences have been utilized. Not all species possess more than one type of *hsp90* and the full length sequence is not available for all genes. The accession numbers are included for reference and those in parenthesis represent protein translations which were acquired from the DNA sequence.

Note, the common names for some of the species are :

Onchorhynchus tshawytscha : Chinook salmon, *Sus scrofa* : pig, *Oryza sativa* : rice, *Zea mays* : maize, *Cricetulus griseus* : Chinese hamster, *Gallus gallus* : chicken.

Key for column and row indices:

		Accession number
1 *Trypanosoma brucei*		A44983
2 *Anopheles albimanus*		(L47285)
3 *Brugia pahangi*		(AJ005784)
4 *Caenorhabditis elegans*		(Z75530)
5 *Saccharomyces cerevisiae*	HSP82	P02829
6 *Saccharomyces cerevisiae*	HSC82	P15108
7 *Gallus gallus*	HSP90α	P11501
8 *Homo sapiens*	HSP90α	P07900
9 *Mus musculus*	HSP90α	P07901
10 *Gallus gallus*	HSP90β	Q04619
11 *Homo sapiens*	HSP90β	P08238
12 *Mus musculus*	HSP90β	P11499
13 *Cricetulus griseus*		L33676
14 *Schizosaccharomyces pombe*		L35550
15 *Drosophila melanogaster*		P02828
16 *Escherichia coli*	HtpG	P10413
17 *Theileria parva*		P24724
18 *Arabidopsis thaliana*	HSP81-1	P27323
19 *Histoplasma capsulatum*		P33125
20 *Oryza sativa*		P33126
21 *Rattus norvegicus*		P34058
22 *Lycopersicon esculentum*		P36181
23 *Candida albicans*		P46598
24 *Arabidopsis thaliana*	HSP81-3	P51818
25 *Dictyostelium discoideum*		P54651
26 *Sus scrofa*		(U94395)
27 *Zea mays*		Q08277
28 *Plasmodium falciparum*		S49155
29 *Leishmania donovani*		S57415
30 *Onchorhynchus tshawytscha*		(U89945)

Appendix II :

A 545bp region (-781 → -237 in ***Figure 5.1***), containing the HSE2 fragment was analysed using the TFSEARCH analysis tool. The score, in percentage, indicates the significance of the match between a region of sequence and a known transcription factor binding element. The accession numbers denote the entries in the TRANSFAC, transcription factor database. The analysis identified, for example, binding sites for HSF, NF-Y, Sp-1 and the TATA box binding protein.

Appendix II : A TFSEARCH analysis of sequence upstream of *B. pahangi hsp90*

```
** TFSEARCH  ver.1.3 **         (c)1995 Yutaka Akiyama (Kyoto Univ.)
 This simple routine searches highly correlated sequence fragments
 versus TFMATRIX transcription factor binding site profile database
 by E.Wingender, R.Knueppel, P.Dietze, H.Karas (GBF-Braunschweig).
  <Warning> Scoring scheme is so straightforward in this version.
            score = 100.0 * ('weighted sum' - min) / (max - min)
            The score does not properly reflect statistical significance!
 Database:  TRANSFAC MATRIX TABLE, Rel.3.3 06-01-1998
 Query:     hse B. pahangi (545 bases)
 Taxonomy:  ALL
 Threshold: 85.0 point

   1 CGTGGATATC GGTCGTATTG AAGCATTTGT GTGCGAAAGC AAGGATCATC entry        score
                                    ---->              M00028 HSF    91.1
                               ------->                M00100 CdxA   91.0
      <-----                                           M00142 NIT2   88.8
     ---------->                                       M00076 GATA-2 88.5
                                  <-------             M00050 E2F    86.2
                                           ---------- M00230 Skn-1  86.1
       <----------                                     M00076 GATA-2 85.4

  51 TTACAATTTA CAATTCTGCC TACTGCATTA TTTTTTTTCC AGCTTTTACA entry        score
                                                     - M00028 HSF    100.0
                               <-------              M00101 CdxA   97.9
                                                     - M00029 HSF    96.0
                  <----                              M00029 HSF    95.4
                  <----                              M00028 HSF    94.3
                 ------>                             M00101 CdxA   92.1
                                  <---------         M00022 Hb     91.1
                                        <------- M00216 TATA   90.4
                                <----------          M00022 Hb     89.3
                                           --- M00120 dl     89.2
                               ------------>          M00130 HFH-2  88.9
                               ------------>          M00131 HNF-3b 87.9
          ------->                                    M00101 CdxA   87.9
          ------->                                    M00100 CdxA   87.2
                                     <----            M00029 HSF    86.9
                                                  <- M00101 CdxA   86.4
     ->                                              M00230 Skn-1  86.1
                                     <----            M00028 HSF    85.9
                                <-------              M00101 CdxA   85.7
                               -------->              M00253 cap    85.6
                          ----------------->          M00019 Dfd    85.2

 101 GAAAAACATA TTTAATGTTA GCATTAACTG GGACAATTCA GGAATCGAGA entry        score
     --->                                            M00028 HSF    100.0
     --->                                            M00029 HSF    96.0
               ------>                               M00101 CdxA   92.1
        ------>                                      M00148 SRY    90.9
     --                                              M00216 TATA   90.4
     ------->                                        M00120 dl     89.2
                          ---------->                M00183 c-Myb  88.7
```

```
                          ----------------->          M00019 Dfd    87.7
                                <------               M00101 CdxA   87.1
 -----                                                M00101 CdxA   86.4
                                                ---->  M00029 HSF    86.3
                                          <--------    M00241 Nkx-2. 85.3
                                                <----  M00147 HSF2   85.3
          <-------                                    M00101 CdxA   85.0

151 CTTCGGGATT TGATGGATTG GATTGGGATC ATGCACTGTC CAGCTGAACA entry        score
                                              <----------  M00175 AP-4   89.6
                                              <----------  M00176 AP-4   89.2
                <---------                             M00096 Pbx-1  88.2
                             ------------>             M00087 Ik-2   87.7
                   ---------->                         M00106 CDP CR 87.7
     <----                                            M00028 HSF    86.5
                                                 ---->  M00029 HSF    86.3
                                           <--------    M00253 cap    85.7
                                                  <-- M00109 C/EBPb 85.5
 -----                                                M00147 HSF2   85.3
                                                  --- M00190 C/EBP  85.2
          <-----------------                          M00124 Pbx1b  85.0

201 TTGCATGAAC CAAATATGGT TCCTGCTTCT GCCATCAAAC TTCTCTCGGA entry        score
                                  <----               M00028 HSF    95.3
                                           <-----     M00028 HSF    95.3
                                  <----               M00029 HSF    93.7
                                           <-----     M00029 HSF    93.7
                          <-----                      M00029 HSF    90.9
                <-------------                        M00131 HNF-3b 90.2
                          --------->                  M00009 Ttk 69 90.0
   ------------>                                      M00072 CP2    89.6
                                        <----------------  M00090 Abd-B  88.5
                                               --------->  M00253 cap    86.7
       ---->                                          M00029 HSF    86.3
     -------------                                    M00109 C/EBPb 85.5
           ------->                                   M00148 SRY    85.5
     ------------>                                    M00190 C/EBP  85.2
                 ------>                            M00101 CdxA   85.0

251 CACCTCAGAA TGGAACCTCT CCCGAGAGGA AATCACTCCA AACAACACAT entry        score
                                                 ------->  M00148 SRY    96.4
           ----->                                   M00029 HSF    95.4
           ----->                                   M00028 HSF    94.3
                                           <-----     M00048 ADR1   93.8
                 ---->                              M00029 HSF    90.9
                                          ------->    M00253 cap    90.6
      <-----                                        M00271 AML-1a 88.7
                                     ----->          M00029 HSF    86.9
         -------->                                  M00253 cap    86.1
                                     ----->          M00028 HSF    85.9
                              ------------->         M00074 c-Ets- 85.4
```

Appendix II : A TFSEARCH analysis of sequence upstream of *B. pahangi hsp90*

```
301 ACTTCTTCCG GAACATCGTA CAATGCCCAA CCCCTCGAAG AACCTTCCAG entry        score
                                        ----->          M00029 HSF    100.0
                                        <----------     M00147 HSF2    95.5
                                                 --     M00029 HSF     95.4
                                  <------               M00048 ADR1    95.4
     <----                                              M00028 HSF     95.3
                                                 --     M00028 HSF     94.3
     <----                                              M00029 HSF     93.7
                                        ----------      M00169 HSF     93. 3
                                        ---------->     M00147 HSF2    91.7
            ----->                                      M00029 HSF     90.9
                                        <----------     M00146 HSF1    89.9
      <----------                                       M00032 c-Ets-  89.2
                                        ----->          M00028 HSF     88.5
                                        ----------      M00165 HSF     88.0
                                        ---------->     M00146 HSF1    87.7
                               --------->               M00226 P       86.9
                                    ---->               M00028 HSF     86.5
    <--------------                                     M00074 c-Ets-  86.2
       ------------->                                   M00074 c-Ets-  86.2
                                                 --     M00146 HSF1    85.1

351 AATGTCCCAC TCTTCCCAAA TGTTCATATC CCAGAAGTCC CTCGTAGAAA entry        score
                                                 ---->  M00028 HSF    100.0
                                                 ---->  M00029 HSF     96.0
    -->                                                 M00029 HSF     95.4
                                       ---->            M00028 HSF     95.3
              <---------                                M00141 Lyf-1   94.8
    -->                                                 M00028 HSF     94.3
                                       ---->            M00029 HSF     93.7
                                                 -----  M00169 HSF     93.5
    -->                                                 M00169 HSF     93.3
                                                 <----  M00147 HSF2    92.9
                                                 -----  M00147 HSF2    90.4
                                                 -----  M00165 HSF     89.9
          <--------------                               M00087 Ik-2    89.9
        --------->                                      M00253 cap     88.5
    -->                                                 M00165 HSF     88.0
                                 ------>                M00142 NIT2    87.5
                            <----                       M00029 HSF     86.3
                              <----------               M00076 GATA-2  86.2
                                                <--     M00074 c-Ets-  86.2
                            <-----------                M00203 GATA-X  85.8
                              --------->                M00253 cap     85.5
                                                 <-     M00032 c-Ets-  85.3
    ------->                                            M00146 HSF1    85.1
                                                 -----  M00146 HSF1    85.1
                                                 <----  M00146 HSF1    85.1

401 CTTCCAGAAC ATTCTATTGT TCAAATACTT TCATCGTCCA TTCATTGGTT entry        score
        ---->                                           M00029 HSF    100.0
        <----------                                     M00147 HSF2    98.1
        <----------                                     M00146 HSF1    96.4
                 <----------                            M00042 Sox-5   96.1
        ---------->                                     M00147 HSF2    95.5
              <----                                     M00029 HSF     95.4
              <----                                     M00028 HSF     94.3
```

```
    --------->                                          M00169 HSF     93.5
    <----------------                                   M00169 HSF     93.3
         ---------->                                    M00146 HSF1    93.1
    -----                                               M00147 HSF2    92.9
              <-------------                            M00160 SRY     92.2
    ---->                                               M00147 HSF2    90.4
    --------->                                          M00165 HSF     89.9
         ---->                                          M00028 HSF     88.5
    <----------------                                   M00165 HSF     88.0
         <---------------                               M00168 HSF     87.8
                                                <----------  M00185 NF-Y   87.7
                                                 ------->    M00253 cap    87.5
                                                 <--------   M00254 CCAAT  86.4
                    <-----                              M00029 HSF     86.3
                                                 <--------   M00096 Pbx-1  86.3
                                  ------->                  M00253 cap    86.2
    ----------                                          M00074 c-Ets-  86.2
    --------                                            M00032 c-Ets-  85.3
    ---->                                               M00146 HSF1    85.1
    ----->                                              M00146 HSF1    85.1

451 GTCATTGCTG CAAGCACCGC CCACCGATGG TTTTAGAACA TTCCTAGGAC entry        score
                                           ---->        M00029 HSF    100.0
                                           <----------  M00147 HSF2    97.4
                                           ---------->  M00147 HSF2    92.3
                                           <----------  M00146 HSF1    92.0
                                           ---------->  M00146 HSF1    91.7
                                                     <  M00253 cap     91.1
     ------->                                           M00253 cap     88.6
                                           ---->        M00028 HSF     88.5
                          ---------->                   M00075 GATA-1  87.8
    -                                                   M00185 NF-Y    87.7
              <---------------                          M00255 GC box  87.4
                                        -------->       M00253 cap     87.1
    ---                                                 M00254 CCAAT   86.4
                                  <----------------     M00169 HSF     86.3
                                        <-----          M00029 HSF     86.3
                <--------------                         M00196 Sp1     85.9

501 GGAATGATAT AAAAGGGTTT ACGGAAACCT TGGGACACTA GTGAT   entry        score
          <-------                                      M00101 CdxA    92.9
    -------                                             M00253 cap     91.1
                                        <--------       M00253 cap     89.5
    <-------------                                      M00230 Skn-1   89.3
               ---------->                              M00021 Kr      89.1
      <-----                                            M00142 NIT2    88.8
        ---------->                                     M00216 TATA    88.5
     ------>                                            M00101 CdxA    87.9
          <-------                                      M00100 CdxA    87.2
         ---------------->                              M00252 TATA    87.2
                              ---->                     M00029 HSF     86.9
                         -------->                      M00040 CRE-BP  86.7
                         <--------                      M00040 CRE-BP  86.7
                         <---------                     M00223 STATx   86.5
    ---->                                               M00029 HSF     86.3
                              ---->                     M00028 HSF     85.9
```