STUDIES ON STAGE SPECIFIC GENES IN *BRUGIA PAHANGI*

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The research reported in this thesis
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ABSTRACT

Lymphatic filarial worms are mosquito-borne parasites, with a complex life cycle culminating in the mammalian host, where the adult nematodes can live stably in the lymphatics for several years. The L3, or infective stage larvae, is the transmission stage, passing from the arthropod vector to the definitive host and so is the first stage of the parasite to be encountered by the host immune system. It has been shown that the L3 is responsible for eliciting a stage-specific protective immune response and so any antigens which are up-regulated or distinct to this life cycle stage are worthy of further study. This project set out to identify L3 genes of *Brugia pahangi* using two different approaches. The first involved the immunoscreening of an adult cDNA expression library, using a rabbit anti-serum raised against live L3. The serum was first pre-adsorbed against adult antigens in an attempt to make the screen more specific for L3 antigens. However, the degree of cross-reactivity between the different life cycle stages and the immunodominance of a small subset of antigens rendered this method unsuitable for identifying L3-specific genes. The second approach was to differentially screen an L3 cDNA library. The library was made by SL-PCR in an attempt to overcome the problems associated with the limited amount of parasite material available. The library was made from L3 which were removed from the jird at three days post-infection, and was differentially screened using PCR derived probes from mosquito-derived L3, 3d pi L3 and adult worms. Plaques which were positive only on the mosquito-derived L3 filters were chosen for further investigation. This method produced four positive clones which were characterized by sequence analysis and Northern and Southern blotting. The four genes were all found to code for structural proteins, and although highly expressed at the L3 stage were also present in the L4. However, all four cDNAs were only found at a very low level, if at all, in the adult parasite. In order to further examine the expression pattern of these genes, semi-quantitative RT-PCR was carried out, using mosquito and mammalian stages of the parasite. In general, the results confirmed the Northern expression pattern in the post-infective stages, with signals obtained at the L2 stage for three of the genes, and at the pre-transmission L3 stage for all four of the genes. These studies demonstrate the feasibility of adopting a differential screening approach for the identification of stage-specific transcripts from filarial worms.

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LIST OF ABBREVIATIONS

µci	microcurie
µg	microgram
µl	microlitre
µm	micrometre
1D	one-dimensional (electrophoresis)
2D	two-dimensional (electrophoresis)
AP	alkaline phosphatase
APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair (DNA)
BSA	bovine serum albumin
c.p.m.	counts per minute
cDNA	complementary DNA
ddH_2O	double-distilled deionized water
DEC	diethylcarbamazine citrate
DNA	deoxyribonucleic acid
DOC	(sodium) deoxycholate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ES	excretory/secretory material
EtBr	ethidium bromide
FCS	foetal calf serum
gp	glycoprotein
HBSS	Hanks balanced salt solution
HRP	horseradish peroxidase
i.p.	intra-peritoneal
IFAT	immunofluorescent antibody test
Ig	immunoglobulin
IL	interleukin
kb	kilobase (DNA)
kDa	kilodalton
l	litre
L3	third stage larvae
L4	fourth stage larvae
M	molar concentration
McAb	monoclonal antibody
mf	microfilariae
ml	millilitre
mm	millimetre

mM	millimolar
Mr	relative molecular mass
mRNA	messenger RNA
NEPHGE	non-equilibrium pH gradient electrophoresis
NP-40	nonidet P-40
OD	optical density
p.i.	post-infective/infection
PC	phosphorylcholine
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
r.p.m.	revolutions per minute
RNA	ribonucleic acid
RT	reverse transcription
s.c.	sub-cutaneous
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SL	spliced leader
TCA	trichloroacetic acid
TEMED	N,N,N',N',-Tetramethylethylenediamine
TLCK	N-α-p tosyl-L-lysine chloromethylketone
TPCK	L-1-tosylamido-2-phenylethyl chloromethylketone
TPE	tropical pulmonary eosinophilia
Tris	Tris(hydroxymethyl)methylamine
Tween 20	polyoxyethylenesorbitan monolaurate
w/v	weight per volume
WHO	World Health Organisation

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Chapter 1

Introduction

1.1 The Parasite

1.1.1 Filarial Nematodes

The filarial nematodes are blood and tissue dwelling parasites, transmitted by an arthropod vector, that cause a variety of diseases in man and other animals. The most important species of filarial worms, in terms of human disease, are *Onchocerca volvulus*, which causes river-blindness and severe skin disease and *Wuchereria bancrofti* and *Brugia malayi,* which both cause lymphatic filariasis. These parasites are widespread in the tropical regions of the world with approximately 118 million people infected with lymphatic filariasis, 105 million by *Wuchereria bancrofti* and 13 million by *Brugia malayi* (Ottesen and Ramachandran, 1995). Although direct mortality from these diseases is low they do cause much morbidity and disfigurement.

Filarial nematodes tend to be remarkably host specific and *O. volvulus* and *W. bancrofti* only infect man in nature. Chimpanzees can be artificially infected with *O. volvulus*, but this is an expensive system and has made experimental studies difficult. However, *Brugia* parasites have been found in a wide range of vertebrate hosts. *Brugia malayi* and the less common *Brugia timori* are confirmed human parasites, but the sub-periodic form of *B. malayi* is zoonotic and can be used to infect laboratory hosts. The use of *B. malayi* and the closely related species *B. pahangi*, which naturally infects cats and monkeys, in experimental studies has significantly increased our understanding of the human disease. The discovery (Ash and Riley, 1970) that the sub-periodic strain of *B. malayi* and *B. pahangi* would develop to patency in the Mongolian jird (*Meriones unguiculatus*), proved to be a major development in filariasis research. The ability of the parasite to develop within the peritoneal cavity provided a useful model for obtaining all the mammalian stages of the life

cycle in sufficient quantities for molecular and immunological analysis (McCall *et al.*, 1973).

The geographical distribution of the filarial parasites is limited mainly by the distribution of their vectors and also by the number of arthropods which will serve as vectors. Onchocerciasis is only transmitted by black flies of the genus *Simulium*, and its distribution is limited by the breeding requirement of the vector for rapidly flowing water. The disease is found mainly in Africa with smaller foci in Central and Southern America, endemic areas being located around water courses. Bancroftian filariasis can be transmitted by up to forty-eight different species of mosquito and is therefore the most common and widespread species of filaria, with a distribution ranging throughout tropical and subtropical countries. Brugian filariasis is mainly limited to Asia with *Mansonia* and *Anopheles* mosquitoes as the principal vectors.

1.1.2 Life Cycle

Like all nematodes, lymphatic filarial parasites have five developmental stages in their life cycle. Adults, or fifth-stage worms, have a fine, white threadlike appearance. They reside in the lymphatics of the vertebrate host and may live for several years. The female *B. malayi* measure 48.0 x 0.15 mm, while the males are smaller, measuring 22.0 x 0.1 mm. Once the worms become sexually mature they mate and the females produce large numbers of first-stage larvae or microfilarae (mf). For *Brugia* species these measure approximately 210 x 6 μm and are sheathed. The body nuclei extend almost to the tip of the tail with two terminal nuclei being distinctly separate from the others. This fact, together with the presence or absence of a sheath are used to differentiate between mf of different species by examination of stained blood films. The mf travel from the lymphatics to the bloodstream, where they can then be ingested by the mosquito vector during a blood-meal (Denham and McGreevy, 1977).

Mf have a circadian periodicity and are found in the peripheral circulation at certain times of the day. The mosquito vectors also feed according to a circadian rhythm and it has been found that the number of mf in the peripheral blood is highest when the activity of the local mosquito is also at its highest level. The natural vectors of Brugian filariasis include the mosquitoes of the *Mansonia*, *Anopheles* and *Aedes* genera. In those regions where *Mansonia* mosquitoes are the principal vectors, the mf are subperiodic and in these cases the disease has a rural distribution (Denham and McGreevy, 1977). The *Mansonia* larvae need a certain variety of plant, *Pistia stratiotes*, to which they attach by means of their siphon in order to obtain oxygen. These plants grow in ponds, open swamps and ditches, generally low regions, where cats and monkeys act as reservoirs of infection. When the disease is transmitted by *Anopheline* mosquitoes, which prefer to breed in the rice fields surrounding villages and towns, the mf have a nocturnal periodicity and the disease tends to be urban. Throughout most of its range *Wuchereria bancrofti* has a diurnal periodicity, with mf peaking in the blood between 10pm and 2am. The relationship between peak feeding time for the mosquito and peak peripheral microfilaraemia appears to have evolved in order to increase the efficiency of transmission between arthropod vector and vertebrate host.

Once the mf have been taken up by the mosquito, they are carried with the blood meal through the foregut to the midgut, where they ex-sheath and can then penetrate the stomach wall to the haemocoel. Since the blood meal coagulates with varying speed between and within species of mosquito, most of the mf have left the midgut within the first three hours to avoid being trapped here (Ewert, 1965). After migration to the thorax, they invade the muscle cells and here moult twice to the third-stage larvae or L3. This process is temperature dependent and takes between six and twelve days and during this period the infective stage larvae have grown to around 1.5 x 0.03 mm.

L3 then leave the flight muscles and move to the body cavities of the mosquito with particular preference for the mouthparts. When the mosquito bites a

vertebrate host the infective larvae escape, while the mosquito probes the skin and the labium is bent, and travel to the tip of the proboscis and so reach the surface of the skin. Once the mosquito has withdrawn the L3 enter the puncture wound left by the mosquito and then move via the bloodstream into the lymphatics (McGreevy *et al.*, 1974). The larvae are protected against dessication on the surface of the skin by a small drop of haemolymph left by the mosquito, which gives them time to find and penetrate the puncture wound. The successful transmission of infective stage larvae therefore depends upon the relative humidity, as rapid evaporation of the haemolymph droplet makes it much less likely for the L3 to survive and enter the vertebrate host (Ewert, 1967; Ewert and Ho, 1967; McGreevy *et al.*, 1974). This fact, together with the distribution of appropriate vectors, limits filariasis to the more humid areas of the tropics. Once the infective larvae are within the local lymphatic system, usually within a few hours of infection, they migrate with the flow of lymph to the nearest lymph node. In the cat model the next moult to the L4 stage occurs between nine and twelve days post-infection and significant growth only starts after this moult. The L4 then travel against the flow of lymph and return to the afferent lymphatics where they remain (Schacher, 1962). The final moult is less synchronous, with males moulting around day 19 and females around day 24. This fifth-stage larvae, or young adult, continues to grow until sexually mature, when mating occurs and mf production commences, usually between days fifty to sixty post-infection.

1.2 The Disease

1.2.1 Immunology and clinical symptoms

Filariasis is a spectral disease with a wide range of clinical manifestations. It is generally considered that the outcome of infection is related to the type of immune response elicited in the host. Individuals in an endemic area can be broadly divided into five main categories:

1. Endemic normals

This category consists of individuals who show no clinical or parasitological signs of infection. However, when taken as a group, they show significantly greater immune responsiveness to parasite antigens, especially T cell responses (Ottesen *et al.*, 1977), than those who are mf positive. This classification will contain people with sub-threshold and pre-patent infections, as well as those who show effective immunity, as it is difficult to accurately distinguish between those who are truly uninfected, and those who may have occult filariasis, with an undetectable level of circulating mf (Ottesen, 1984). It has been suggested that a proportion of these individuals may have an immunological resistance to infection or a naturally acquired protective immunity. When more sensitive techniques such as detection of circulating antigen (Day *et al.,* 1991a), or PCR-based detection methods are applied to this group, only a very low percentage of the population can truly be described as free from infection. In some areas of the world where transmission rates are very high, such as in parts of Papua New Guinea (PNG), no endemic normals can be detected at all (Day, 1991).

2. Asymptomatic microfilaraemics

This group comprises the largest percentage of infected individuals and again display no overt clinical symptoms of disease, despite having mf circulating in the bloodstream, sometimes in very large numbers, and a low grade eosinophilia. More recent studies have found that these patients have 'silent' renal abnormalities, in the form of microscopic haematuria and proteinuria (Dreyer, 1992). This group is the least immunologically reactive with reduced cellular and humoral responses (Ottesen *et al.*, 1977; Ottesen *et al.*, 1982). However, immune responses in this group to other antigens are normal, the hypo-responsiveness appearing to be specific to filarial antigens. Treatment with diethylcarbamazine (DEC) results in the partial restoration of responses

(Piessens *et al.,* 1981). Asymptomatic microfilaraemics do not necessarily go on to develop pathology and there have been cases reported where the circulating mf levels remain high for decades with no further disease progression. It is believed that the immunological hypo-responsiveness of this group is caused by the parasites themselves, which specifically down-regulate immune responses. Recent data have shown that not all immune responses are suppressed in microfilaraemic individuals; while Th1 responses, as assessed by proliferative capacity, are impaired, Th2 responses are up-regulated (Maizels and Lawrence, 1991).

3. Filarial fevers

Some microfilaraemic patients go on to develop intermittant episodes of lymphatic inflammation. These attacks occur between two and six times a year, lasting for around five days and are characterised by swollen, painful lymph nodes usually accompanied by fever. Occasionally the inflammation spreads to include the lymphatic tracks. Mf are not usually detectable in this group, who have an increased immune responsiveness to filarial antigens compared to group 2. It is presumed that the increased recognition of the parasite, or perhaps less effective immunosuppression, initiates the onset of fevers and local lymphatic inflammatory reactions, while also being responsible for clearing mf from the bloodstream.

4. Lymphatic pathology

While it is not known what triggers some asymptomatic microfilaraemics to develop filarial fevers, the onset of lymphatic pathology is clearly linked to increased lymphatic inflammation. Repeated attacks of inflammation lead to dilation, thickening and eventually fibrosis of the affected lymphatic vessels. Eventually woody induration of the tissues may occur with thickening and verrucous changes of the skin, leading to elephantiasis. Brugian filariasis most commonly affects the limbs and the reduced circulation often leads to

secondary fungal or bacterial infections, which in themselves are responsible for much of the gross pathology of elephantiasis (reviewed by Ottesen and Ramachandran, 1995). Lymphatic pathology also occurs as a result of reactions to developing, adult or dying worms in the lymphatics. The death of an adult worm can lead to severe localised inflammation, an abscess may form around the worm, it may be absorbed or sometimes becomes calcified and can be seen by X-Ray. However, studies in SCID mice have shown that lymphatic pathology can also be caused by the ability of the worm itself to dilate the lymphatic channel and cause local damage, in the absence of an effective immune response (Nelson *et al.,* 1991). Immune responses to filarial antigens, both cellular and humoral, are greater than those found in group 2 (Ottesen *et al.*, 1982), and this increased responsiveness, which is probably responsible for the clearance of mf, also increases local inflammatory responses which cause lymphatic pathology. Studies have shown a direct correlation between the severity of lymphatic damage and the level of immune responses to parasite antigens (Ottesen, 1980; Klei *et al.*, 1981).

5. TPE (Tropical Pulmonary Eosinophilia)

This last group is the most distinct but least common and does not seem to have any lymphatic involvement. Individuals in this group are immunologically hyper-responsive, especially to mf antigens and have very high IgE and eosinophil levels, but rarely have circulating mf in the bloodstream. Clinical symptoms include nocturnal coughing, chest pain, difficulty in breathing and fever, with pulmonary nodules and /or lesions detectable by X-Ray. The respiratory symptoms probably result from allergic responses to large numbers of mf in the lungs. This group responds very well to treatment with DEC but if left untreated chronic pulmonary fibrosis may occur and eventually respiratory failure.

1.2.2 Acquired immunity

The clinical manifestations of lymphatic filariasis are diverse and the pattern of disease progression is poorly understood. Most studies have been cross-sectional and so little is known of the natural history of the disease. Until long term longitudinal studies are carried out, the way in which the different symptoms are related to each other, and the importance of factors such as pre-natal exposure and genetic background in inducing alternative immunological responses, remain unclear. Recent work has found that children of infected mothers were more likely to be infected than those of amicrofilaraemic mothers (Lammie *et al.,* 1991). Since there was no paternal effect, this is probably due to neonatal tolerance, which has been found to increase susceptibility in animal models (Haque and Capron, 1982; Schrater *et al.*, 1983). It may be that neonatal tolerance limits the pathology-inducing immune responses and so reduces the incidence of clinical disease (Maizels and Lawrence, 1991). A recent study on the Cook Islands presented evidence that maternal infection status exerted an effect on T cell responsiveness which lasted up to two decades (Steel *et al.,* 1994).

One of the main features of filarial parasites is their ability to form a long and stable relationship with their host. In order to do this they must somehow be able to evade the hosts immune system or induce immune unresponsiveness in the host. However, immune unresponsiveness to one life cycle stage does not rule out strong immune responses to another, and so the idea that a state of concomitant immunity exists in filarial infections is gaining acceptance. A study in Papua New Guinea by Day *et al* (1991c), found that adult worm burden increased with time in people under 20 years old, but remained static in those over this age. It was hypothesised that resistance to new infection was directed towards the infective stage larvae, in order to control parasite burden. Concomitant immunity has been found in other helminth infections (Smithers

and Terry, 1976), and in experimental filarial infections in cats (Denham *et al.*, 1983).

1.2.3 Diagnosis

There are two main methods of diagnosis, parasitological and serodiagnosis. However, there are problems associated with both methods.

1. Parasitological diagnosis is traditionally made by staining blood films for the presence of mf, but those patients with a low level of circulating mf may not be detected by this method. Alternatives to the stained blood film have been developed and are proving more sensitive than the conventional stained smear.

The counting chamber (Denham *et al.*, 1971) is a cheap and relatively easy method, using only a finger-prick volume of blood, which makes it more attractive to patients. Also the blood can be placed in 3% acetic acid which lyses the blood cells and preserves the mf, especially important after night surveys. However, the main drawback is that differential diagnosis between species is not possible.

The stained smear technique is particularly useful where more than one species of filariae co-exist as it can be used for distinguishing between species; the presence or absence of a mf sheath and the number and position of nuclei in the mf are both used for this purpose. Problems with this method include inaccuracy in estimating levels of circulating mf, due to the loss of mf from the slide, and the fact that it is very time consuming.

A more sensitive method has been developed using a membrane filter, and is especially useful in detecting those people with very low densities of mf, often found after DEC treatment. 1-10 ml of venous blood is passed through a 5μm filter and this is then stained with Giemsa, the sensitivity increasing with the volume of blood filtered. Southgate (1974) found the Nuclepore filter technique to be almost twice as sensitive as more traditional methods, but

disadvantages include expense, the requirement for skilled personnel and the reluctance of local populations to undergo venepuncture (Denham and McGreevy, 1977). All these direct methods of detection are problematic where mf have a diurnal periodicity and peak in the peripheral circulation during the night. Another consideration is occult infection, where mf may not be present in the circulation, as is the case with chronic pathology, single sex infections and during the pre-patent stage.

2. Serodiagnosis would appear to be a more effective method as it is not dependant upon the time of sampling and could theoretically also provide information about the immune status of the individual. However, despite continued efforts there remains no antibody detection test that meets these requirements (Ambroise-Thomas and Kien Truong, 1974). The main problems with ELISA or IFAT tests lie in cross-reactivity between antibody responses to other helminth infections, since polyparasitism is very common in endemic areas of filariasis.

There has been recent development of tests using a circulating antigen bearing the phosphorylcholine (PC) epitope and the levels of this antigen have been shown to decrease following prolonged treatment with DEC (Weil *et al.*, 1988; Day *et al.,* 1991b). In *B. malayi* infected jirds the levels of PC-antigen correspond to the total worm mass and the adult female worm burden (Wenger *et al.*, 1988), and it is now generally considered that levels of circulating PC-antigen give an indirect measure of worm burden (Day *et al.,* 1991b). The PC bearing antigen was identified by immunoblot as a 200 kDa protein (Lal *et al.,* 1987). For *W. bancrofti*, circulating antigen detection assays are available, and are now even commercially produced. They rely on the use of a McAb to the repeated PC epitope which is bound to an ELISA plate. Serum is added and bound antigen detected with the same McAb conjugated to a marker/enzyme. However, for diagnosis of Brugian filariasis, the detection of a circulating antigen has not yet proved successful. In this case the measurement of filarial specific IgG_4 has proved to be a good measure of

active Brugian filarial infections (Kwan-Lim *et al.*, 1990). A concerted effort has been made to develop an immunodiagnostic test for *O. volvulus* infections which would avoid the requirement for taking skin snips. Using a cocktail of recombinant antigens, to overcome the problems associated with heterogeneity in responsiveness, a serodiagnostic test in ELISA format has been developed (Bradley *et al.*, 1993). This test only requires finger prick blood samples and is much more sensitive than the skin snip method.

More recently several groups have developed diagnostic assays based on the polymerase chain reaction (PCR); these tests, which detect parasite DNA in peripheral blood, are extremely sensitive and specific (Nutman *et al.*, 1994).

1.2.4 Treatment and Control

Chemotherapy for filariasis has traditionally meant a 12 day treatment regime using relatively high levels of DEC (diethylcarbamazine), which acts as a microfilaricide, for patients with detectable levels of circulating mf or those displaying clinical symptoms. Side effects resulting from this treatment are often severe, including vomiting, painful lymph nodes, joints and headaches, but tend to decline after the first few days. These effects are due to the large numbers of mf being destroyed over a short period of time, with a massive release of mf antigen. Brugian filariasis patients appear to suffer much more severe side effects than those with Bancroftian filariasis. As a result of these side effects and the length of the course of treatment, compliance with this regime has often been poor, and since adult worms are not always killed by the standard 12 day schedule of DEC, resurgence of microfilaraemia can occur.

More recent treatment strategies involve treating a whole population with single yearly doses of DEC or adding the drug to common cooking/table salt to ensure its daily consumption over a period of 9-12 months. The side effects are very much reduced and at this low dosage level it can also be used in pregnancy. These approaches are simple, cheaper and much more acceptable to the local people. This mass treatment strategy reduces transmission levels

by lowering mf density and prevalence within a population and has been shown to be effective in greatly reducing the incidence of lymphatic filariasis (Ottesen and Ramachandran, 1995).

However, using DEC fortified salt or administering single annual doses of DEC is not recommended in areas where onchocerciasis or loiasis co-exists with lymphatic filariasis, because the side effects are too severe in these diseases, due to the localisation of mf in certain parts of the hosts' body. It is anticipated that ivermectin will become available for treatment of lymphatic filariasis patients and could be used in conjunction with DEC to give a more effective chemotherapy tool.

Other strategies which have been found to be useful in the treatment of filariasis include oral antibiotic therapy for adenolymphangitis episodes and scrupulous local hygiene for elephantiasis patients, together with antifungal and topical antibiotic administration. These measures can limit the progression of the disease and in some cases cause regression of the debilitating limb abnormalities (reviewed by Ottesen and Ramachandran, 1995).

Vector control is an alternative approach in controlling filariasis, requiring local community participation, and ideally should be used in conjunction with the mass treatment strategy. There are two main approaches in reducing transmission. One involves eliminating, or more realistically, reducing the numbers of mosquitoes and the other method is to reduce the biting activity of the mosquitoes. Breeding sites are a good target for control, and biocides have been developed using toxin producing *Bacillus sphaericus* or *B. thuringiensis* which can be sprayed over a known or likely breeding site. In the urban situation, polystyrene beads have proved effective when placed over the surface of water sources (Maxwell *et al.,* 1990). Efforts have been made to reduce host-vector contact by using insecticide impregnated bednets or by indoor spraying of long-lasting, residually active pyrethroids. Encouraging local participation through education and easily accessible information is equally important in an endemic area, since these relatively simple measures can make a significant difference towards the control of filariasis.

1.3 The Importance of the L3

1.3.1 Immune responses elicited by the L3

The L3 stage of the parasite is the infective form to humans. In most endemic areas there is always a small proportion of the population, with no clinical or parasitological evidence of past or current infection, despite life-long exposure to the infective form. This observation suggests that protective immunity may occur naturally. Work in this area has been hindered by the difficulty in distinguishing truly immune members of the population from those who may be infected but are resistant to reinfection. From experimental work on animal models Denham *et al.*, (1983), found that cats repeatedly infected with *B. pahangi* larvae develop some resistance to challenge infection, and can mount an immune response to all of the mammalian stages of the parasite, but only after continued antigenic stimulation over long periods.

Immunisation with radiation attenuated 3rd stage larvae is known to induce strong protective responses against challenge infection with normal larvae in a range of natural hosts of *Brugia* species (Denham, 1980). However, the mechanisms of the development of this protective immunity are poorly understood. The irradiation stunts the larvae and they fail to develop normally into adult worms, suggesting that the L3 or early L4 stage of the parasite is more immunogenic than the later stages.

The gerbil is the only fully permissive rodent host for *Brugia* and can be immunised with radiation attenuated L3 (Yates and Higashi, 1985) but not by exposure to normal L3 (Chusattayanond and Denham, 1986). The BALB/c mouse is not fully susceptible to *Brugia* infections, usually clearing the parasites before they develop into the adult stage. However, it is useful as a model for the early stages of infection and a strong protective immunity to challenge infection has been shown in BALB/c mice immunised with irradiated L3 (Bancroft and Devaney, 1993).

Other workers have reported that the surface molecules of filarial parasites change as the worm differentiates and grows (Philipp *et al.,* 1980, Maizels *et al.,* 1983), and so it would seem likely that stage-specific antigens are responsible for the development of protective immunity. An epidemiological study in PNG (Day *et al.,* 1991c) looked at patterns of age-specific immunity to *W. bancrofti* infection within an endemic area. Using immunofluorescence, the study showed that subjects acquired an antibody response to the surface of mosquito derived L3 which was age-dependant. Measurement of circulating antigen levels suggested that those individuals, who were less than 20 years of age, had no antibodies to the L3 surface, and were still acquiring infection. However, those over 20 years of age, did have antibodies to the L3 surface and were no longer acquiring infection. It would appear that prolonged exposure to the L3 was necessary to induce the immune response. Attempts to isolate the L3 surface antigen were not successful so it remains unclear which L3 antigens are responsible for inducing protective immunity.

1.3.2 L3 specific components/antigens

Since the body of evidence suggests that it is the L3 stage of the parasite which is responsible for inducing an immune response which may be protective, there have been numerous studies looking for antigens which are specific to the L3. The L3 itself is not an easy stage to work on in terms of availability of material, and there are also problems associated with the cross-reactivity of antigens between different life cycle stages. However, if it was possible to identify and isolate L3 specific components, then these antigens would be interesting candidates for vaccine development.

Carlow *et al.,* (1987), used a monoclonal antibody to detect a *B. malayi* L3-surface antigen. The epitope was present in late L2 stage parasites and in the infective form but was rapidly shed, with complete loss of the surface epitope within four to six days *in vitro* and within two to three days *in vivo.* Several groups have used surface and metabolic labelling techniques to examine the

L2 and L3 stages of the parasite within the vector. In *D. immitis*, Ibrahim *et al.*, (1992) showed that synthesis of L3 antigens begins during the late L2 stage, and once the parasite leaves the mosquito the synthesis of these stage specific antigens is very rapidly reduced. Bianco *et al.*, (1990) studied *O. lienalis* and *O. volvulus* larvae within the black fly and again found that once the larvae were removed from their insect host the synthesis of the infective stage antigens was terminated. Lal and Ottesen (1988) identified three L3 specific antigens from *B malayi*, using 2 D gels to compare the protein profiles of adult, L3 and mf extracts. They reported that most of the proteins were present in all of the life cycle stages examined, but three proteins, with molecular weights of 72kDa, 30kDa and 22kDa were only present in the L3 preparation. Monoclonal antibodies were raised to each of these polypeptides and further characterisation carried out. By Western blotting the three antigens appeared to share epitopes with molecules from other stages of the parasite. The 72kDa antigen was localised to the surface of the L3 and was recognised by *W. bancrofti* infected human sera, indicating that it may be cross-reactive with other filarial infections. Freedman *et al.*, (1989) studied sera from two groups of subjects living in the Cook Islands, a *W. bancrofti* endemic area. One study group was made up of endemic normals, those individuals who had no clinical evidence of disease, past or present, while the other group was made up from subjects who were asymptomatic microfilaraemics. Western blotting was carried out using *B. malayi* parasite extracts and sera from the two study groups. There were no differences in antigen recognition between the adult and mf extracts, but all of the endemic normals recognised a 43kDa antigen in the L3 stage whilst only 8% of the asymptomatic microfilaraemics reacted with this antigen. Raghavan *et al.*, (1994) raised an antiserum to the 43 kDa antigen and used it to screen a *W. bancrofti* genomic expression library. A clone was identified which showed significant homology to chitinases from other species, and the expression of this gene was predominantly in L3 and mf life cycle stages.

1.3.3 Cloning L3 genes

As previously discussed the limited availability of L3 material has made the cloning of L3 genes very difficult in the past. However, more recently PCR techniques have been used in the construction of L3 libraries in an attempt to overcome this particular problem. Martin *et al.,* (1996), constructed a *B. pahangi* 3 day post-infection (pi) L3 cDNA library by reverse transcribing total RNA with the oligo dT primer. The resulting first strand cDNA was amplified, using the conserved nematode spliced leader (SL1) and oligo dT as primers, and then cloned into λZap. The library was differentially screened using PCR products, from mosquito-derived L3 and 3day pi L3, as probes. Two clones were isolated that appeared, by Northern blot analysis, to be larval specific. One of these showed homology to the enzyme cytidine deaminase, whilst the other had no known homologues. Yenbutr and Scott (1995), also used a similar RT-PCR based method to construct a late vector-stage (L3) *B. malayi* cDNA library. They cloned a serine proteinase inhibitor gene from this library, which was much more highly expressed (10 to16-fold) in the L3 stage than in adults or mf. A cuticular collagen gene was also cloned from this library (Scott *et al.,* 1995). By using semi-quantitative RT- PCR, it was demonstrated that the collagen gene was expressed at 2-3 times greater levels in the L3, L4 and mf stages than in the adult parasite.

Work is currently underway by Blaxter *et al.,* (1996) using a tag sequencing approach to survey genes expressed in the L3 stage of *B. malayi.* The filarial genome network, funded by the WHO, involves six laboratories throughout the world, using various different libraries to generate expressed sequence tags. The sequence information is fed into the network and is available to everyone. By comparison of the *Brugia* sequences with those in the *Caenorhabditis elegans* genome sequencing project, many new *Brugia* genes are being discovered.

1.4 Aims of the project

The aim of this project was to identify genes that are up-regulated in the L3 stage of *B. pahangi*. As described previously, this stage of the parasite is thought to be responsible for eliciting a protective immune response in the host. Consequently, any genes which are L3 specific or more highly expressed in the infective stage, would be worthy of further study to determine their immunological properties.

Chapter 2

Materials and Methods

2.1 Parasite Life Cycle

The *Brugia pahangi* life cycle was maintained by cyclical passage through the ref^{m} strain of *Aedes aegypti*, the mosquito vector (as described by Macdonald and Sheppard, 1965) and *Meriones unguiculatus*, the Mongolian jird, as the vertebrate host.

2.1.1 Maintenance of mosquitoes

Mosquitoes were kept in an insectary maintained at a temperature of 28°C and a relative humidity of 75-80 %. Adults were kept in wooden and netting cages and fed on sugar cubes and water through a cotton wool pad. Larvae and pupae were kept in plastic trays filled with water and fed on yeast tablets. The pupae were removed from the trays each day and placed into small pots of water inside the cages where they emerged as adults. Stock mosquitoes, having previously been starved of sugar for 24 hours, were fed on anaesthetised guinea pigs. Eggs were collected on moist filter paper and then dried until required. Hatching was achieved by rehydrating the filter paper in a plastic tray containing water and yeast.

2.1.2 Maintenance of parasites

Jirds were infected with 250 L3 (infective stage larvae) into the peritoneal cavity. After 3 months the jirds were sacrificed using CO_2 anaesthesia and exsanguinated by cardiac puncture. Adults and microfilarae (mf) were obtained by washing out the peritoneal cavity with Hanks Balanced Salt Solution (HBSS) at 37°C and pH 7.2 - 7.4. Adult worms were then stored in liquid nitrogen until required. The microfilarae were washed again in HBSS and then resuspended in rabbit blood at a density of 350-450/20µl of blood. Adult mosquitoes were fed through a 12 day old chicken skin membrane following the technique described by Wade (1976). A water jacket surrounds

the blood to maintain the temperature at 37°C and the blood/mf suspension was stirred by a metal paddle to ensure an even distribution of mf.

2.1.3 Recovery of infective larvae

After 8-10 days the progress of infection was assessed by dissecting a small number of adult female mosquitoes. When the parasites have migrated out of the thorax and into the head and abdomen the L3 were considered to be mature and therefore infective. At this point, normally 9 days post-infection, the L3 were recovered using the mass harvesting technique developed by Ash (1974). The mosquitoes were removed from their cages and temporarily stunned by placing them at -20°C for 90 seconds. Then they were crushed on a glass plate and washed into a Baermann funnel lined with several layers of gauze, containing HBSS. After 45 minutes at room temperature the L3 had emerged and were then washed at least twice in fresh HBSS using a fine drawn out glass pipette. The L3 were then used to infect jirds or frozen in liquid nitrogen for RNA or antigen preparation.

2.1.4 Recovery of post-infective larvae

Infective third stage larvae of *B. pahangi* were harvested from mosquitoes as described above. 750 L3 were injected into the peritoneal cavity of each jird to be infected. After the required period of time, usually 3 or 15 days post-infection, the jirds were sacrificed as before and the peritoneal cavity washed out thoroughly with several changes of sterile HBSS. Larvae were counted out into fresh HBSS and then stored in liquid nitrogen until required.

2.2 Immunochemical Techniques

2.2.1 Preparation of parasite antigens

Parasite antigen extracts were prepared from L3, L4 or adult parasites and used for 1 or 2 D gel electrophoresis.

2.2.1.1 DOC extracts

2000 L3, 500 L4 or 200 adult parasites were homogenised on ice in 10mM Tris-HCl pH 8.3, containing the following protease inhibitors: 20mM PMSF, 2mM TPCK and 2mM TLCK. Sodium deoxycholate was added to a final concentration of 1%. After 30 minutes with occasional mixing, the homogenate was centrifuged at 13 000 x g for 10 minutes and the soluble supernatant retained. The protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay kit. Aliquots were stored in liquid nitrogen until required.

2.2.1.2 IEF lysis buffer extracts

Reagents:

Lysis Buffer

Urea	5.5g
NP-40 10%	2mls
Ampholines pH 3.5-10 (LKB Pharmacia)	500µl
DTT	0.08g

Made up to 10 mls with dd H_2O and stored in 500µl aliquots at -70°C.

Procedure:

Approximately 250 adult worms were homogenised in 800µl of the lysis buffer and then left for 10 minutes at room temperature to extract. The mixture was transferred to an Eppendorf tube and the homogeniser washed out with a further 100µl of lysis buffer. Insoluble material was pelleted by centrifugation at 13 000 x g and the supernatant stored at -70°C.

2.2.2 1D SDS-PAGE

Reagents:

12.5% Separating gel

30% acrylamide (Scotlab)	12.5mls
1.5M Tris-HCl, pH 8.8	11.2mls
dd H_2O	6.2mls

After degassing 300µl 10% SDS, 100µl 10% APS and 20µl TEMED were added.

5% Stacking gel

30% acrylamide	1.67mls
0.5M Tris-HCl, pH 6.8	1.25mls
dd H_2O	7.03mls
10% SDS	100µl
10% APS	50µl
TEMED	10µl

Sample Cocktail

0.5M Tris-HCl, pH 6.8	1.88ml
10% SDS	6.0ml
Glycerol	3.0ml
dd H_2O	2.12ml

0.65ml of the Sample Cocktail was mixed with 0.1ml 1.5M DTT, 0.1% Bromophenol Blue and stored in aliquots at -20°C.

Running buffer

Tris base	3.03g
Glycine	14.42g
SDS	1.0g

Made up to 1 litre with dd H_2O and pH to 8.3.

Coomassie Blue Stain

Coomassie brilliant blue	1g
Methanol	450mls
dd H_2O	450mls
Glacial acetic acid	100mls

Destain solution

Glacial acetic acid	350mls
Methanol	1000mls
dd H_2O	3650mls

Procedure:

The separating gel was poured and overlaid with H_2O saturated butanol then left to polymerise. The Hoefer vertical electrophoresis system was used, with 3 different sizes: minigels (10.1 x 8.2cm), longer length minigels (10.1 x 10.6cm) or standard size (17.9 x 16cm) slab gels made according to requirements. The overlay was removed and the stacking gel poured with a suitable comb in place. Samples were prepared by mixing with an equal volume of sample cocktail and then boiling for 3-4 minutes. Molecular weight markers (Bio-Rad) were also prepared in this way and included on all gels (range Mr14 000 to 200 000). Electrophoresis was carried out at 20mA per gel for minigels or 12mA (overnight), 30-40mA (during the day) for larger gels. Gels were then immunoblotted, dried and exposed to film, or the proteins visualised by Coomassie Blue staining.

2.2.3 2D NEPHGE (O'Farrell *et al.*, 1977)

Reagents:

Rod gels

Urea	2.75g
30% acrylamide solution (Scotlab)	0.67ml
10% NP-40	1.0ml
dd H_2O	1.0ml
Ampholines, pH 3.5-10	0.25ml

After the urea was dissolved, using rapid stirring and gentle warming, 10µl 10% APS and 7µl TEMED were added and the gels poured immediately.

Anode buffer

20mM H_3PO_4

Cathode buffer

50mM NaOH

Overlay buffer

200µl lysis buffer mixed with 40µl dd H_2O

Equilibration buffer

1M Tris-HCl, pH 6.8	16.6mls
10% SDS	20mls
DTT	0.76g
Glycerol	12.6mls

Procedure:

1. Rod gels were poured into Bio-Rad glass tubes (170mm x 1.5mm) using a long metal needle, taking care to exclude any air bubbles. The gels were overlaid with dd H_2O and left to polymerise for approximately 45 minutes.
2. Adult IEF lysis buffer extract (45µl) was spiked with ^{125}I labelled adult extract (5µl), L4 extract (6.25µl) or L3 extract (7.44µl), to give approximately 135,000 cpm per gel. The two extracts were mixed together and left for 5 minutes before loading on to the gel. Gels were always run in duplicate. 10-20 µl of overlay buffer was loaded on top of the sample and the rest of the tube filled with anode buffer. Electrophoresis was carried out in a Hoefer tube gel unit at 400V for 4.5 hours.
3. A fine 9cm needle attached to a 10ml syringe filled with dd H_2O was used to extrude the gels into individual 5cm petri dishes. Water was removed from each dish, replaced with 5mls equilibration buffer and left for 30 minutes.
4. The gels were then loaded directly onto SDS-PAGE slab gels for the second dimension, or frozen at -70°C until required.
5. Gels were stained to visualise the proteins using Coomassie Blue Stain, then destained until the background of the gel appeared clear. The gels were dried and autoradiographed at -70°C.

2.2.4 Immunoblotting (Towbin *et al.*, 1979)

Reagents:

Transfer buffer

Tris base	6.05g
Glycine	28.2g
Methanol	400mls
dd H_2O	1600mls

T.B.S. (Tris buffered saline)

NaCl	43.75g
Tris base	12.1g

Adjusted to pH 7.4 with conc. HCl and made up to 5 litres with dd H_2O.

T.B.S./Tween 20

0.05% Tween 20 in T.B.S.

Ponceau S Stain

Dissolve 0.2g Ponceau S in 100mls 3% T.C.A. (Trichloroacetic acid) solution.

Procedure:

Equivalent amounts of adult and L3 DOC extracts (determined by analysing an aliquot of each on a minigel) were run out on a 12.5% acrylamide minigel (as described previously in 2.2.2). The separated proteins were then transferred onto nitrocellulose paper using the Hoefer miniblotting system at a constant current of 200mA for 1 hour. The blot was stained with Ponceau S and cut into strips before non-specific antibody binding sites were blocked with T.B.S./Tween 20/5% B.S.A. Individual strips were probed with control or test rabbit serum at dilutions ranging between 1/100 to 1/600. Goat anti-rabbit alkaline phosphatase conjugate (ICN) was used at a dilution of 1/6000, and then the blot was developed using BCIP/NBT substrate (Dynatech). Washing was carried out between each incubation with T.B.S./Tween 20, for 3 x 15 minutes.

2.2.5 Radiolabelling of parasite proteins with ^{125}I

This method radiolabels proteins that are present in the parasite cuticle. Iodogen catalyses the conjugation of ^{125}I to tyrosine residues in the protein.

Reagents:

Iodogen 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (Pierce Chemicals)

HBSS saturated with L-tyrosine

HBSS containing 10 mM KI

P.B.S. (Phosphate buffered saline)

NaCl	10g
KCl	0.25g
Na_2HPO_4	1.44g
KH_2PO_4	0.25g

Adjust to pH 7.2 with conc. HCl and make up to 1 litre with dd H_2O.

Procedure:

1. Iodogen was dissolved in chloroform at 1 mg/ml and then used to coat 1.5ml screw-top Eppendorf tubes, 200μg Iodogen was used for adult parasites and 400μg for L3 and L4 stages. The $ChCl_3$ was evaporated under a gentle stream of N_2.
2. Adult and L4 parasites were harvested from jirds and were washed twice in HBSS at 37°C. L3 were harvested from mosquitoes as previously described, and washed in HBSS before being incubated at room temperature for 2 hours, to enhance the labelling of this life cycle stage (Devaney and Jecock, 1991). The worms were transferred to 200μl P.B.S. pH 7.2 and added to the Iodogen coated tubes. As it is difficult to get the larval stages into a small volume of buffer, they were taken up in approximately 500μl and then allowed to settle to the bottom of the tube before the excess liquid was removed.
3. Iodination was carried out at room temperature using 200μCi of Na ^{125}I (Amersham) for adults and L4 and 500μCi for L3 parasites. The tubes were left for 15 minutes with gentle agitation.

4. 10µl of HBSS saturated with L-tyrosine was added to each tube to stop the reaction.

5. The worms were then transferred to 15ml centrifuge tubes containing 10ml HBSS/KI and were washed twice in this solution and a further three times in HBSS alone.

6. DOC extracts of the labelled parasites were then made as previously described in 2.2.1.1 and TCA precipitable counts measured in 2.5µl duplicate samples.

2.2.6 Deglycosylation of proteins

This was performed using the N-Glycosidase F (PNGase F) enzyme (Boehringer Mannheim). The rate of deglycosylation is increased by boiling the protein with SDS before incubation with the enzyme. A non-ionic detergent (NP-40) is then used to complex out the SDS and so prevents inactivation of the enzyme.

Reagents:

SDS/2ME

10% SDS	10µl
2ME	1.6µl
dd H_2O	88.4µl

N-glycanase enzyme

Stock is at 200 U/ml. Diluted down in 0.55M sodium phosphate buffer pH 8.6, to give 2 dilutions: a) 10 U/ml and b) 1 U/ml.

Procedure:

1. 67,500 TCA-precipitable counts per lane of ^{125}I - labelled parasite extracts were used, from 3 different life cycle stages, L3, L4 and adult.

2. Three aliquots (2 dilutions of the enzyme and 1 control) of each life cycle stage were taken and made up to 5µl with sodium phosphate buffer to give approximately 135,000 cpm (gels were run in duplicate).

3. 5µl of SDS/2ME was added to each tube and then the samples were boiled for 3 minutes, cooled and spun down.

4. The following reagents were then added to each sample:

    10.8µl 0.55M sodium phosphate pH 8.6

    5µl 7.5% NP-40

5. 3µl of each dilution of the enzyme were added to the test samples, and 3µl of sodium phosphate buffer to the control tube, in place of the enzyme. The samples were incubated at 37°C for 24 hrs.

6. The samples were mixed with an equal volume of sample cocktail, boiled for 3 minutes and then analysed on 12.5% SDS-PAGE gels.

7. Gels were stained with Coomassie Blue, destained and dried before being autoradiographed at -70°C for 10 days.

2.3 Immunoscreening

2.3.1 Antibodies

2.3.1.1 Anti-L3 antibody

This rabbit antiserum was raised against the L3 stage of *B. pahangi* by two intraperitoneal injections of 500 live L3 (Devaney and Jecock, 1991).

2.3.1.2 Preadsorbing anti-L3 serum against adult antigens

100µl adult DOC extract (2.9µg/µl) was added to 20 mls T.B.S./Tween20/1% B.S.A. Four 132mm Hybond-C nitrocellulose filters (Amersham), were incubated in this mixture at room temperature for 1-2 hours on a rocker, to bind adult antigens in the DOC extract to the filter. The filters were then washed thoroughly in T.B.S./Tween20 and blocked in T.B.S./Tween20/ 5% B.S.A. for one hour. After being washed once more and semi-dried on blotting paper, the filters were placed in a 1:50 dilution of the anti-L3 serum, one at a time, for 30 minutes each. Antibodies in the serum, directed against adult antigens, should bind to those on the filter and so reduce the recognition of adult antigens in the immunoscreen.

2.3.1.3 Antibody raised against p27

50μg per lane of adult DOC extract was run out on a 12.5% large gel, as described in 2.2.2, with low range molecular weight markers run alongside. Two gels were run simultaneously at 30 mA overnight to give 18 lanes of antigen. In order to locate the p27 band more easily, a trial gel was run using samples that were spiked with ^{125}I labelled adult DOC extract (2.2.5). Previous work (Devaney, 1988) had shown that p27 was one of only three proteins to be labelled in this way, so that following autoradiography p27 could be more accurately identified. Once the two gels had been run, they were briefly stained in Coomassie and then quickly destained before being rinsed in dd H_2O. Using a light box, and the trial gel as a guide, the p27 band was cut out from all 18 lanes of the gels and put into a sterile 7ml bijou.

The pieces of gel were frozen in liquid nitrogen and ground to a powder using a pestle and mortar. The powder was transferred to another sterile bijou and 300μl sterile H_2O, and 700μl Freunds Incomplete Adjuvant were added. The mixture was emulsified on ice using a mechanical homogeniser (IKA-ULTRA TURRAX) for approximately 10 minutes. The emulsion was taken up into a 2.5ml syringe and injected into a rabbit using three different subcutaneous sites. The process was repeated twice, at two to three week intervals. Serum was taken at various time points and compared by Western blot with pre-bleed serum.

2.3.1.4 Attempts to purify p27

Since the previous attempts to isolate the p27 molecule using 1D gel analysis were not very successful, an alternative method was tried. It was known from earlier experiments (Devaney and Jecock, 1991), that p27 was an extremely basic protein, so it was decided to try and separate it out further from total worm extract using 2D NEPHGE analysis.

Procedure:

1. Adult lysis buffer extract was prepared as in section 2.2.1.2 and was spiked with ^{125}I labelled adult, L4 or L3 extract, prepared as described in 2.2.5, to give

approximately 135,000 cpm per gel. Duplicate gels were run for each life cycle stage.

2. Gels were prepared and electrophoresed as in 2.2.3, stained with Coomassie Blue, destained and then dried.

3. Autoradiography was performed over 2 weeks, and then the labelled spots were lined up with those on the gel. The L4 labelled extract appeared to give the best results, and so 10 more gels were made using radiolabelled extract from the L4 together with unlabelled adult antigen.

Since the protein yield was so low, it was decided to try and purify enough protein for N-terminal amino acid sequencing, rather than to raise another antiserum. This method requires the protein to be blotted onto a PVDF (Polyvinylidene difluoride) membrane, so 1D gels were performed, using the spots from several 2D gels, to try and resolve the protein into a single band which could then be immunoblotted.

4. Spots were cut out from the dried gels using a scalpel and the blotting paper removed. Then the spots were mashed up in liquid nitrogen until a powder was achieved. Sample Cocktail was added until loading consistency was reached.

5. A 12.5% 1D slab gel was prepared, using wide spacers with an extra long stacking gel, and a five well comb to allow more room for loading the sample. The gel was run as in 2.2.2, stained, destained and exposed to film.

2.3.2 The adult cDNA library

A cDNA expression library (average insert size 1.3 kb) was constructed from mRNA isolated from mixed-sex adult *B. pahangi* and cloned into the vector Uni-Zap-XR (Stratagene). The library was prepared by Dr. J. Cox-Singh. (Cox-Singh *et al.*, 1994).

2.3.2.1 Preparation of phage plating cells

Growth Media:

L-Broth

Bactotryptone (Difco)	10g
Yeast extract (Difco)	5g
NaCl	10g

Dissolve in 1l dd H_2O, aliquot into 100ml bottles and autoclave to sterilise.

L-Agar

As for L-Broth but with 15g Agar (Difco), aliquot in 2x 500mls, autoclave and then allow to cool to 45°C (antibiotics added at this stage if required), pour with sterile technique on to plates and allow to set. Store at 4°C for short periods.

Top Agarose

0.7% agarose in L-Broth, autoclave and store at room temperature.

Ampicillin

10mg/ml stock solution, dissolve in sterile dd H_2O, cover bottle with foil and store at -20°C.

Tetracyclin

5mg/ml stock solution, dissolve in ethanol, cover bottle with foil and store at -20°C.

Reagents:

IPTG (isopropyl β-D-thiogalactopyranoside)

100mM stock solution, dissolve in sterile dd H_2O and store at -20°C.

SM buffer

NaCl	5.8g
$MgSO_4.7H_2O$	2g
Tris base	6.05g
2% gelatin	5mls

pH to 7.5 with conc. HCl, make up to 1l with dd H_2O, sterilise by autoclaving and store at room temperature.

Procedure:

1. XL1-Blue MRF cells, from a glycerol stock, were streaked out on an agar plate containing 10μg/ml tetracyclin. The cells were incubated overnight at 37°C.
2. A single colony was picked from the plate and used to inoculate 10mls L-Broth, containing 0.2M maltose, 10mM $MgSO_4$ and 10μg/ml tetracyclin. The cells were incubated overnight in a shaker at 37°C.
3. 1ml of this overnight culture was added to 50mls prewarmed growth medium, as used in step 2. The cells were returned to the 37°C shaker until an O.D. of 0.5 (2.5×10^8 cells/ml) was achieved (approximately 2 hours).
4. The culture was cooled on ice and then centrifuged at 3000 RPM for 10 minutes at 4°C to pellet the cells.
5. The pellet was resuspended in 15 mls ice cold, sterile 10mM $MgSO_4$ and then stored at 4°C, ready for infection with phage.

2.3.2.2 Titration and plating of library

Serial dilutions of phage were made in SM buffer using dilutions of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. 10μl of each of these dilutions were added to 400μl of freshly prepared plating cells and incubated at 37°C for 15 minutes. 5mls of sterile top agarose for each 140mm plate was warmed to 47°C. The cells were added to the top agarose and swirled to mix, then quickly poured onto the previously warmed plate. After 10 minutes at room temperature, to allow the agarose to

set, the plates were placed in a 37°C incubator and left for 4 hours, or until plaques of a reasonable size could be seen. The appropriate dilution was chosen to give confluent plaques and the process repeated. Approximately 40,000 plaques were initially screened in this way.

2.3.2.3 Induction of expression

Hybond-C nitrocellulose filters (Amersham) were soaked in 10mM IPTG (Sigma) and then briefly dried. They were then placed on top of the plaques, with orientation marks made by using a needle to pierce through the filter and the agar below. Care was taken to avoid air bubbles by placing the middle of the filter down first and then rolling down the edges. The plates were returned to the 37°C incubator for another 3 hours, to induce expression.

2.3.2.4 Antibody probing

1. The filters were removed from the plates and washed briefly in T.B.S./T20 (see 2.2.4), while the plates were stored at 4°C.
2. Non-specific antibody binding sites were blocked by incubating the filters in T.B.S./T20 5% B.S.A. with 5% horse serum added, since this has been found (D. Swan, personal communication) to effectively block the *E. coli* binding sites, routinely overnight at 4°C.
3. Filters were washed again before incubation in the appropriate serum. The serum was used at a dilution of 1/5000 in T.B.S./T20 1% B.S.A. with 0.005% sodium azide added to act as a preservative since the serum can be used several times. 5% horse serum was also included to try to reduce the number of false positives. The filters were incubated in the serum for 1 hour at room temperature.
4. Following washing, the filters were placed in the second antibody solution, goat anti-rabbit alkaline phosphatase conjugate (ICN), diluted in T.B.S./T20 1% B.S.A. to 1/10000, for 1 hour at room temperature.
5. The filters were washed again and then developed using BCIP/NBT substrate (Dynatech).

6. Positive plaques developed first, usually within 10 minutes, and were darker than the background levels. After lining up the positive on the filter with the correct plaque, a sterile pipette tip was used to core out the plaque and it was then placed in 500µl of SM buffer with 1% chloroform and stored at 4°C.

2.3.2.5 Secondary screening

Serial dilutions were again used to find the concentration of plaques which would enable a single clone to be picked, which was distinct and separate from those around it. A 1/1000 dilution in SM buffer was chosen and 5µl of this was incubated with 400µl of plating cells at 37°C for 15 minutes before being plated out as before. The screening process was repeated as in 2.3.2.4 and single clones were picked into SM buffer and stored at 4°C.

2.4 Differential Screening

2.4.1 The 3d p.i. L3 cDNA library

The cDNA library in λ uni-ZAP (Stratagene), was made from RNA isolated from mammalian derived L3, harvested at three days post-infection of the jird. As only small numbers of L3 were available, the library was constructed by RT-PCR using the conserved nematode spliced leader (SL1) and oligo dT as primers (Martin *et al.,* 1995).

2.4.1.1 Preparation of phage plating cells

Plating cells were prepared as described in 2.3.2.1.

2.4.1.2 Titration and plating of the library

Serial dilutions of the library were made in SM buffer (see 2.3.2.2), and these were plated out to determine which dilution gave individually distinguishable plaques (approximately 2000 per plate), when grown overnight at 37°C. A dilution of 10^{-2} was chosen, and 30µl of this was incubated at 37°C for 15 minutes along with 400µl of freshly prepared plating cells, before being plated

out on plates containing 10μg/ml tetracyclin. The plates were left overnight at 37°C.

2.4.1.3 Duplicate filter lifts for differential hybridisation

Reagents:

Denaturing solution

1.5M NaCl, 0.5M NaOH, make up to 1l and store at room temperature.

Neutralisation solution

1M Tris base, 1.5M NaCl, adjust to pH 7.4 with conc. HCl, make up to 1l and store at room temperature.

Procedure:

1. The plates were placed at 4°C for 1 hour, to harden the agarose, before filter lifts were taken. To identify L3 specific clones, three filter lifts were taken, so that each set could be differentially screened against adult, p.i. L3 and mosquito derived L3 cDNA probes. Hybond-N filters (Amersham) were used, with the first lift left on the plate for 30 seconds, the second for 1 minute, and the third for 90 seconds. Orientation marks were made with a needle and the filters clearly labelled.
2. Filters were then denatured for 5 minutes by placing them, DNA side up, on 3MM paper that had been soaked in denaturing solution.
3. Neutralisation was carried out in the same manner, but the process was repeated for 2 x 5 minutes.
4. DNA was fixed onto the filters by first baking for 1 hour in an 80°C oven, and then UV crosslinked in a Strata-linker.

2.4.2 Production of stage specific cDNA probes

RT-PCR reactions (see 2.6.1 and 2.6.3) were performed using total RNA from mosquito derived L3, 3 day p.i. L3 and from adult worms as described in 2.5.7. This cDNA was then used as the template for random priming with ^{32}P-dCTP (Amersham). The High Prime method (Boehringer) was used to label

20ng of DNA using 50μCi of 3000mCi/ml ^{32}P-dCTP. Unincorporated nucleotides were removed through a Sephadex G-50 column (Pharmacia). See also 2.5.2.

2.4.3 Hybridisation techniques

Reagents:

SSC (20x stock)

3M NaCl, 0.3M sodium citrate, store at room temperature.

Denhardts' solution (50x stock)

1% Ficoll (type 400), 1% PVP (polyvinyl pyrollidone), 1% BSA, make up to 100mls. Aliquot and store at -20°C.

Prehybridisation solution

5mls 20x SSC, 1ml 50x Denhardts' soln., 1ml 10% SDS, make up to 20mls, and use freshly prepared.

Salmon sperm DNA

10 mg/ml stock in dd H_2O, denature by boiling for 10 mins and cool on ice. Aliquot and store at -20°C.

Procedure:

Southern blots, Northern blots and filter lifts to screen the library were all treated in a similar manner. Detection of bound nucleic acids on the filters was achieved by hybridisation to random primed DNA probes (2.5.2 and 2.4.2). Filters were pre-incubated for ≥4 hours at 65°C in a hybridisation tube (Hybaid) containing 20 mls of prehybridisation solution, with single stranded salmon sperm DNA added to give a final concentration of 100μg/ml. The DNA probe (1 x 10^7 cpm/ml) was denatured by boiling for 10 minutes, and cooled on ice before being added to the prehybridisation solution in the tube. The filters were hybridised overnight at 65°C.

The filters were washed six times at 65°C, twice for 15 minutes in 2x SSC, followed by another two 15 minute washes in 1x SSC/0.1% SDS. The last two washes of 15 minutes each were performed at a concentration of either 0.5x SSC/0.1% SDS (for library screening and Northern blots), or at 0.2x SSC/0.1% SDS (for Southern and PCR blots).

After washing the filters were air dried, covered in cling film and autoradiographed at -70°C.

2.4.4 Selecting L3 specific clones and secondary screening

After the films were developed, they were cut up so that the filters from the same agar plate probed with the three different life cycle stages, could be aligned and compared. Plaques were selected which were strongly positive on the L3 film but which were not present in the adult stage. Since these clones were detected in the 3d p.i. L3 library, they must also be present in that life cycle stage. However, only those clones that appeared very weakly positive in this stage, as compared to the vector derived L3, were picked. Plaques were picked into SM buffer with 1% chloroform and stored at 4°C. To secondary screen these clones, serial dilutions of each was made in SM buffer, then plated out and the appropriate dilution chosen as described in 2.3.2.5. The screening process was repeated and single clones were selected.

2.5 General Molecular Methods

2.5.1 Agarose gel electrophoresis

Reagents:

TAE buffer (10x stock)

48.8g Tris base, 11.4mls glacial acetic acid, 40mls 0.5M EDTA pH 8, make up to 1l with dd H_2O and store at room temperature.

DNA gel loading buffer

30% glycerol, 5% SDS, 0.1% Bromophenol blue, 10mM Tris, 1mM EDTA pH 8, store at room temperature.

Ethidium bromide

10mg/ml stock in dd H_2O, working concentration 0.5μg/ml. Store protected from light at room temperature.

DNA size markers

Lamda (λ) DNA digested with *Hin*d III or *Pst* I. Both used at 1μg/10μl. Store at room temperature.

Procedure:

Gels containing 0.8%-2% agarose w/v in 1x TAE were used to separate and analyse DNA molecules. 30, 100 or 300ml of agar solution was boiled, cooled and then 0.5μg/ml ethidium bromide added to the gel mixture just before pouring to allow visualisation of the DNA. Gels were made in Gibco/BRL horizontal gel tanks with the appropriate comb in place. After setting, sufficient 1x TAE buffer was poured into the tank to cover the gel. Gel loading buffer (1/10 volume) was added to each sample before loading. Molecular weight markers were run alongside the DNA of interest (usually λ *Hin*d III or λ *Pst* I). The gels were subject to electrophoresis at 20 V until the DNA entered the gel, then at 40-60 V for 2-6 hours depending on the size of gel and nature of the samples. Gels were then viewed on a shortwave UV transilluminator and photographed using Polaroid high speed, black and white film, type 667.

2.5.1.1 Purification of DNA from agarose gels

Once the gel had been run far enough to achieve sufficient separation between the DNA bands, the band of interest was cut out of the gel using a sterile scalpel. The gel slice was then subjected to two rounds of freeze/thawing and then spun through a 0.22 μm cellulose acetate filter ("SpinX"; Costar Ltd.) at 14 000 x g for 15 minutes at room temperature. Then an equal volume of a 50/50 phenol/chloroform mixture was added, and after vortexing, spun at 14 000 x g for 5 minutes at room temperature. The clean DNA in the top layer was then carefully removed to a fresh tube and a 1/10 volume of 3M NaOAc

pH 5.2 and 2 volumes of 100% EtOH were then added to precipitate the DNA (-20°C overnight or -70°C , 30 minutes). The pellet was collected by centrifugation, washed with 70% EtOH and then resuspended in 30µl ddH_2O. An aliquot was run out on a gel to check the yield and purity and then the DNA was stored at -20°C.

2.5.2. Production of cDNA probes

10 - 20 ng DNA in an 11µl volume (made up with sterile dd H_2O), was boiled for 10 minutes to denature the double stranded DNA. This was then cooled on ice before adding 4µl of High Prime (Boehringer Mannheim) and 5µl of ^{32}P-dCTP (50µCi of 3000mCi/ml), and then incubating at 37°C for 30 minutes. Labelled DNA was separated from unincorporated nucleotides by passage through a Sephadex G-50 column (Pharmacia). The probe, approximately $1x10^7$ cpm/ml, was boiled for 10 minutes and cooled on ice before being added to the hybridisation tube, already containing 20mls of prehybridisation solution.

2.5.3 *In vivo* excision of pBluescript plasmid

The Bluescript plasmids, containing cDNA inserts were removed from the λ vector by *in vivo* excision.

Procedure:

1. Overnight cultures of XL1-Blue MRF and SOLR™ strain plating cells were grown in 10mls L-Broth containing 200µl 10% maltose, 100µl 1M $MgSO_4$ and 10µl tetracyclin 5mg/ml.
2. The cells were centrifuged at 3000 RPM for 10 minutes at 4°C, and the pellets resuspended in 10mls 10mM $MgSO_4$.
3. 1.5ml Eppendorf tubes were labelled, one for each clone, and 200µl of XL1-Blue plating cells, 200µl phage (containing approximately 10^5 PFU) and 1µl helper phage (Stratagene R408, 10^{10} PFU/ml) were put into each tube. A control with plating cells only was also prepared. The samples were incubated at 37°C for 1 hour.

4. The contents of each Eppendorf tube were then transferred to 15ml centrifuge tubes, each containing 3mls L-Broth, and placed in an orbital incubator at 37°C for 2-3 hours.

5. The samples were heated to 70°C for 20 minutes to lyse the cells and then centrifuged at 4000 RPM for 10 minutes. 1ml of the supernatant (phagemid) was removed to a clean tube and stored at 4°C.

6. 50µl of this phagemid was added to 200µl of SOLR™ plating cells and incubated at 37°C for 15 minutes, before being plated out on 90mm ampicillin plates. Two dilutions, normally 10µl and 100µl, of the transformed SOLR™ cells were used for each clone. The plates were left overnight at 37°C.

7. Once the colonies had grown to a reasonable size, one dilution was chosen for each clone and an overnight culture for each was grown up in 10mls L-Broth containing 100 µg/ml ampicillin. These cultures were used to prepare plasmid DNA.

2.5.4 Preparation of plasmid DNA (using Promega Wizard Minipreps)

1. 3mls of each overnight culture were taken and divided between two 1.5ml Eppendorf tubes. The cells were pelleted by centrifugation at 13 000 x g for 10 minutes. The supernatant was removed and the cells for each clone pooled and resuspended in 200µl Cell Resuspension Solution. (50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/ml RNase A).

2. 200µl Cell Lysis Solution (0.2M NaOH, 1% SDS) was added and the tube inverted several times until the solution became clear.

3. 200µl Neutralisation Solution (1.32M Potassium acetate, pH 4.8) was then added and again mixed by inversion, before centrifugation at 13 000 x g for 5 minutes to clear the supernatant which was then transferred to a fresh tube.

4. 1ml of DNA Purification Resin was added to the supernatant and mixed by inverting the tube. This was put through Minicolumns using 2.5ml syringe barrels and a vacuum manifold. The columns were washed by adding 2mls Column Wash Solution (55% ethanol), and the Resin then dried by continuing to draw a vacuum for an extra 3 minutes.

5. 60µl dd H_2O were added to each Minicolumn and left for 1 minute to allow plasmid to be released from the Resin into the H_2O. DNA was eluted by centrifugation at 13 000 x g for 30 seconds and stored at -20°C.

2.5.5 Enzymatic digestion of DNA

Digestion with restriction endonucleases (Promega and Gibco) was carried out with the recommended buffers at 37°C, using 2-5 units of enzyme per µg DNA. After 3-5 hours the reaction was stopped by the addition of DNA gel loading buffer, prior to agarose gel electrophoresis.

2.5.6 Genomic DNA isolation

Reagents:

TENS buffer

50mM Tris pH 8

100mM EDTA

100mM NaCl

1% SDS

Proteinase K (Sigma)

Working concentration 10mg/ml

TE buffer

10 mM Tris pH 7.4

1mM EDTA

Procedure:

1. 100 adult worms, taken straight from liquid nitrogen, were added to 700µl TENS buffer and the worms chopped up using small stainless steel scissors. 35µl of Proteinase K at 10mg/ml was added to the tube and this was incubated overnight at 55°C.

2. The tube was then filled with phenol, buffered to pH 8 (Gibco), and placed on a vertical rotator for 1 hour. The phases were separated by centrifugation at

13 000 x g for 10 minutes and the top layer and interface removed to a fresh tube.

3. The tube was filled with a 1:1 phenol:chloroform mixture, rotated for 10 minutes and separated by centrifugation as before. The aqueous layer and interface were again placed in a fresh tube.

4. This was then filled with a 24:1 chloroform:isoamyl alcohol mixture and the tube rotated for 5 minutes and then centrifuged for 5 minutes to separate the layers. This time only the top layer was removed to a new tube which was topped up with isopropanol (approximately 500µl). The tube was inverted several times until a stringy precipitate formed.

5. The high molecular weight DNA was removed from the tube by spooling onto a small glass rod. It was then dipped into 70% ethanol, followed by 100% ethanol.

6. The spooled DNA was allowed to air dry and then carefully dropped into a tube containing 500µl TE buffer, by breaking off the tip of the glass rod with a diamond pencil.

7. The DNA was left in the TE buffer, rotating overnight at room temperature, and the glass rod removed the following day using alcohol-flamed forceps.

8. The purity of the DNA was determined by using a 1/100 dilution of the DNA and taking OD readings at wavelengths of A_{260} and A_{280}. The ratio of DNA (A_{260}) to protein (A_{280}) should be between 1.8 and 2.0. The concentration of the DNA, again using a 1/100 dilution, was calculated by the formula A_{260} x 50 x 100 = µg/ml of DNA.

2.5.6.1 Restriction digestion of genomic DNA

Approximately 10µg of high-molecular-weight DNA was digested with 50 units of each restriction enzyme to be used. Typically these were *Eco*R I, *Pst* I, *Bam*H I, *Hin*d III and *Bgl* II. The samples were incubated overnight at 37°C. After ethanol precipitation and a 70% ethanol wash, the pellets were resuspended in 40µl of dd H_2O. DNA loading buffer was added to each sample and then the digests, along with DNA markers, were subjected to agarose gel electrophoresis on a 0.8% gel run overnight at 28 V. The gel was

stained for 30 minutes in dd H_2O containing 0.5µg/ml ethidium bromide, viewed on a shortwave UV transilluminator and photographed using Polaroid high speed, black and white film, type 667. Gels were then depurinated in 0.25M HCl for 30 minutes, to enhance transfer of larger DNA fragments, then denatured and neutralised, both for 30 minutes, before blotting.

2.5.6.2 Southern blotting

The method of Southern (1975) was used to transfer nucleic acids from agarose gels to Hybond-N membranes (Amersham). A glass plate slightly larger than the gel was placed on a support in a dish filled with 20x SSC. 3 layers of 3MM paper were placed over the plate, large enough to dip into the SSC at either side. The gel was placed on top of the damp paper, with care taken to avoid air bubbles. A piece of Nylon membrane was cut to the same size as the gel and placed on top of the gel, followed by two more sheets of 3MM paper. Cling film was used to surround the gel and ensure that the buffer was only drawn up through the gel. A wad of tissue (about 10 cm thick) was placed over the 3MM paper and a weight put on top of this. Blotting was performed overnight.

After removal of the weight, tissues and 3MM paper, orientation marks were made on the membrane and then it was rinsed in 2x SSC. The transferred nucleic acids were fixed to the membrane by baking in a 80°C oven for 1 hour, and then UV cross-linked in a Strata linker.

2.5.7 RNA isolation

To minimise the risk of RNA degradation, all plastics and glassware for RNA work were autoclaved prior to use, dd H_2O was treated with the RNase inhibitor, diethyl pyrocarbonate (DEPC), and 2 pairs of gloves worn at all times. Gel equipment and flasks were rinsed in DEPC water before use.

Reagents:

DEPC H_2O

0.1% DEPC in dd H_2O, leave at room temperature for 1 hour then autoclave, store at room temperature.

Proteinase K

Stock solution 10mg/ml.

RNA lysis buffer

1M Tris-HCl pH 8	1ml
1M NaCl	2ml
10% SDS	2ml
0.5M EDTA pH 8	4ml
DEPC H_2O	1ml

Store at room temperature.

Procedure:

Approximately 2000 L3 or p.i. L3, 750 L4 or 50 adult worms were used to extract RNA, using the hot TRIzol™ method (Gibco/BRL). The parasites were initially digested at 68°C in 200µl RNA lysis buffer, containing 12.5µl Proteinase K, for 10 minutes (adult worms may require 15 minutes). 1ml TRIzol (already warmed to 68°C) was added and after a further 10 minute incubation at 68°C the tube was removed to room temperature and allowed to cool for 3 minutes. 200µl of chloroform was added and the mixture shaken vigorously before centrifugation at 12 000 x g for 15 minutes at 4°C. The aqueous phase was removed and RNA precipitated by adding an equal volume of isopropanol. After 15 minutes at room temperature (or overnight at -70°C) the RNA was recovered by centrifugation at 12 000 x g for 15 minutes at 4°C. The pellet was washed in 80% ethanol and stored as an ethanol precipitate at -70°C until required.

2.5.7.1 RNA isolation from mosquito thoraces

Mosquito thoraces were dissected onto dry ice at 24 hours, 3 days, 6 days and 8 days post-infection with *B. pahangi*. Approximately 10 thoraces were taken from each time point.

Reagents:

Lysis buffer mix (for 5 samples)

RNA lysis buffer	1ml
Proteinase K	62.5µl
β-Mercaptoethanol	20µl

Procedure:

1. The lysis buffer mix was warmed to 68°C, and the mosquito thoraces were transferred to sterile Eppendorfs, still on dry ice.
2. The Eppendorfs containing the mosquito thoraces were placed at 68°C and 220µl of the pre-warmed lysis buffer mix was added to each tube.
3. The tubes were left at 68°C for 30 minutes with occasional vortexing.
4. Then 1ml of TRIzol, pre-warmed to 68°C, was added to each sample and again left for 30 minutes with occasional vortexing.
5. The tubes were removed from the water bath and allowed to cool slightly on the bench.
6. 200µl chloroform was added to each sample, the tube vortexed, allowed to settle slightly, then spun at 12 000 x g for 15 minutes at 4°C.
7. The aqueous phase was removed and the RNA precipitated and recovered as previously described in 2.5.7.
8. Each RNA pellet was resuspended in 50µl DEPC H_2O and then stored as an ethanol precipitate at -70°C.

2.5.7.2 Formaldehyde RNA gel (Maniatis *et al.*, 1982)

Reagents:

10x MOPS (3-[N-Morpholino]propane-sulphonic acid) **buffer**

3M sodium acetate pH 7	8.3mls
0.5M EDTA pH 8	10mls
2M MOPS	50mls

Make up to 500mls with DEPC H_2O, autoclave then store at room temperature covered in foil.

Formaldehyde gel

Agarose	1.2g
10x MOPS	10mls
DEPC H_2O	73mls

Boil to dissolve the agarose, cool to 55°C then add 17mls formaldehyde before pouring the gel.

Procedure:

Approximately 2μg of RNA was resuspended in 50% formamide, 10% DNA gel loading buffer and 10% ethidium bromide (1μl of 500ng/ml) was added to the RNA sample to allow visualisation under UV light. The samples were denatured at 65°C for 10 minutes before electrophoresis on the 1.2% denaturing formaldehyde gel, with 1x MOPS as the buffer. RNA markers were run alongside the samples, and photographs taken before blotting.

2.5.7.3 Northern blotting

RNA gels were blotted overnight and fixed as described in 2.5.6.2.

2.5.8 Sequencing

2.5.8.1 Manual sequencing

Double stranded sequencing was performed using the Sequenase sequencing kit, version 2.0 (Amersham), with the T3 or T7 primer.

Reagents:

10X TBE buffer (stock)

Tris base	121g
Boric acid	51g
EDTA	3.7g

Make up to 1l with dd H_2O, pH 8.2-8.4.

6% Sequencing gel

Easigel (Scotlab)	100ml
10% APS	1ml
TEMED	40µl

Procedure:

1. 10µl plasmid DNA (approximately 5µg) was added to 8µl dd H_2O and then denatured by adding 2µl 2M NaOH, and incubated at 37°C for 15 minutes.
2. The single stranded DNA was then precipitated by adding 8µl of 5M ammonium acetate and 100µl of 100% ethanol. This was left for 1 hour at -70°C and then spun down at 13 000 x g for 15 minutes to pellet the DNA. The ethanol was aspirated and the pellet resuspended in 7µl dd H_2O.
3. Then 2µl of Sequenase Reaction Buffer and 1µl of primer were added to give a total volume of 10µl. This was annealed by heating to 65°C for 2 minutes, and then slowly cooled to <35°C. The reaction tube was then kept on ice until the next step.
4. 2.5µl of each Termination Mixture (A, C, G and T), was put into 4 separate, labelled tubes and then placed at 43°C for 10 minutes.
5. The Labelling Mix was diluted 1:5, in dd H_2O, and then 1µl Sequenase Enzyme was added to 7µl Sequenase Dilution Buffer.

6. For the labelling reaction, the following reagents were added to the 10µl ice cold annealed DNA mixture:

1µl DTT

2µl diluted Labelling Mix

0.5µl ^{35}S

2µl diluted Sequenase Enzyme

The reaction was mixed and left at room temperature for 2-5 minutes.

7. Termination reactions:

3.5µl of labelling reaction was added to each of the four termination tubes, mixed and put at 37°C for 5 minutes.

8. The reactions were stopped by adding 4µl of Stop Solution to each tube which were then put on ice.

9. Before loading, samples were heated to 75°C for 2 minutes.

10. 6% sequencing gels were poured, using the Gibco S2 sequencing rig, to give a 0.4mm thick, 30 x 40 cm gel, with 1X TBE buffer, pH 8.2-8.4, and 3µl of each sample loaded. Gels were run at 60W for 2-3 hours when the samples can be loaded again and the gel run for another 1-2 hours.

11. Gels were fixed, rolled onto blotting paper, covered with clingfilm and dried down for approximately 2 hours, at 80°C.

12. The gels were then exposed to film, for at least 24 hours, then the sequence read from the autoradiograph.

2.5.8.2 Automatic sequencing

Double stranded sequencing was performed using the 'SequiTherm™ Long-Read' Cycle Sequencing Kit (Cambio) and fluorescently labelled (IRD41) T3 and T7 primers (Hybaid) on an automated sequencer (Licor).

Procedure:

1. The reaction mix was prepared as follows;

Double stranded DNA template	5µl (~2.5µg)
IRD41 labelled primer	2µl (2pmol)
10x Sequencing Buffer	2.5µl
SequiTherm™ DNA Polymerase	1µl (5U/µl)
dd H_2O	6.5µl
(to give total volume of 17µl)	

2. 4 x 0.5 ml PCR tubes were labelled A, C, G and T for each sample to be sequenced. 2µl of the appropriate SequiTherm™ LongRead Termination mix was added to each tube, taking care to protect from the light.
3. Then 4µl of the previously prepared reaction mix was added to each tube and a drop of mineral oil placed on top to prevent evaporation.
4. The tubes were then placed in a thermocycler (Perkin Elmer), for the following cycles;

a. 95°C for 5 minutes
b. 95°C for 30 seconds
c. 60°C for 30 seconds
d. 70°C for 1 minute

Steps b-d were repeated for 30 cycles

e. 4°C soak

5. Once the cycling was completed, 4µl of SequiTherm™ Stop Solution was added to each reaction mix, underneath the mineral oil.
6. Samples were denatured at 95°C for 3 minutes before being loaded onto the gel.

2.5.9 Data base searches

Partial nucleotide sequences were used to search the EMBL data base for homology and translated amino acid sequences to search the SWISS-PROT

data base, using both the FASTA and BLAST programs of GCG (Genetics Computer Group).

2.6 Polymerase Chain Reaction

2.6.1 Reverse transcription

To make first strand cDNA from RNA to use as a template in PCR reactions.

Procedure:

1. DNase treatment of RNA - to remove any contaminating genomic DNA

1-2μg total RNA	~7μl (in DEPC H_2O)
DNase buffer (Gibco)	1μl
DNase 1 enzyme (Gibco)	0.5μl

Placed on ice for 10 minutes.

2. The above reaction was stopped by adding 1μl 20mM EDTA to the tube, then placed at 65°C for 10 minutes.

3. Then, while the mixture was still hot, 1μl oligo dT primer (200ng/μl) was added, briefly spun down and kept on ice. Any DNA contamination should have been removed, leaving only RNA annealed to the oligo dT primer.

4. RT reaction mix

RNase IN (Promega)	0.5μl
dNTP's 10 mM each (Pharmacia)	2μl
RT buffer 5x (Promega)	4μl
RT enzyme (100 u) (Promega)	1.5μl
filter sterilised dd H_2O	1μl

9μl of this reaction mix was added to each sample and placed at 42°C for 1 hour, then at 56°C for 30 minutes. Then the resulting first strand DNA was stored at -20°C until required.

2.6.2 Preparation of primers for PCR

Primers for use in PCR reactions were designed from the known sequence to give gene specific primers (see Chapter 5), or the nematode spliced leader 1 sequence and oligo dT were used where appropriate.

Procedure:

1. The lyophilised oligonucleotide was rehydrated in 2 mls of ammonia solution by passing the solution through 2 ml syringes attached to each end of the oligo cartridge. This process was done slowly and gradually by pushing 0.5 mls at a time over the oligo, taking around 30 minutes to complete the rehydration.
2. The solution was then transferred to two screw top Eppendorf tubes and left at 55°C overnight.
3. The next day the primers were dried down under vacuum at 55°C in a DNA concentrator, for 6-7 hours.
4. The resulting pellet was resuspended in 200µl of filter sterilised dd H_2O and left for at least 10 minutes with gentle tapping until dissolved.
5. The OD reading was taken using a 1/100 dilution at a wavelength of A_{260} , and the concentration of the primer calculated according to the following formula;

$$A_{260} \times 30 \times 100 = \mu g / ml$$

6. For PCR reactions the primer needs to be at a concentration of 1mg/ml so an aliquot of the primer was diluted down to this working concentration, while the rest was stored in the more concentrated form at -20°C until required.

2.6.3 PCR reactions

A variety of PCR reactions were carried out to amplify cDNA between two primer sites using the Taq DNA polymerase enzyme (Perkin Elmer). To avoid contamination gloves were worn at all times and sterilised PCR tubes, filter tips and filter sterilised dd H_2O were used.

Procedure:

1. A mix of the required reagents was made and used for all samples, including the controls, so that any contamination could be detected. Perkin Elmer reagents were used for all PCR reactions. PCR reaction mix for 2 reactions (100µl);

DNA	2µl
dNTP's (1.25mM each)	16µl (diluted to 200µM each in sterile dd H_2O)
10x PCR buffer	10µl
Primer 1	1µl
Primer 2	1µl
Taq enzyme (5 U/µl)	0.5µl
dd H_2O	69.5µl

2. 48µl of this mix, initially omitting the primers and DNA, were put into each PCR tube. The primers and cDNA template were then added separately, because these were often different for each reaction.

3. A small drop of mineral oil was placed into each tube, to help prevent evaporation, and then the tube was vortexed, briefly spun, and placed in the PCR machine using the relevant cycle programme.

Standard PCR cycle:

95°C	5 minutes	Initial denaturation temperature
94°C	1 minute	⎫
55°C	1 minute	⎬ Repeated for 30 cycles
72°C	3 minutes	⎭
72°C	10 minutes	Final extension temperature
4°C	10 minutes	Final soak temperature

When using the oligo dT primer the annealing temperature was reduced to 50°C. The number of cycles was also altered according to the circumstances, (for details see Chapter 5).

2.6.4 Semi-quantitative analysis of PCR products

RNA from mosquito thoraces or from different life cycle stages of the parasite was reverse transcribed, using the oligo dT primer, and then the resulting first strand cDNA used in PCR reactions with gene specific primers. The resulting PCR products (10μl) were run out on 2% agarose gels and then denatured and neutralised before being blotted onto nylon membranes (see Southern blotting 2.5.6.2). The blots were then probed with each of the five *Brugia* cDNA clones and autoradiographed. Once the autoradiographs were developed, they were lined up with the nylon membrane, and the hybridising DNA bands from each life cycle stage cut out from the blot using a scalpel. Each piece of nylon membrane was put into 3mls scintillation fluid and then the β-emissions calculated by liquid scintillation counting. A corner of the filter, containing no DNA, was also counted, to act as a control for the background level of radioactivity present on the filter. Graphs were drawn using the ratios of counts for each gene in each life cycle stage, compared to the constitutively expressed ribosomal protein, and in this way a semi-quantitative analysis of the expression pattern of the four genes could be made.

Chapter 3

Cloning L3 Antigens by Immunoscreening

3.1 Introduction

The aim of this section of the work was to attempt to clone L3 antigens using the only library available at the time, an adult cDNA expression library. In order to do this a rabbit antiserum raised against live L3 (Devaney and Jecock, 1991) was used to immunoscreen the library. This anti-L3 serum was known to recognise both adult and L3 antigens by Western blot. A variety of studies have shown that there is a high degree of cross-reactivity between different life cycle stages of filarial parasites. For example, Maizels *et al.,* (1983), demonstrated significant cross reactivity between the ^{125}I labelled cuticular antigens of the L3 and adult stages of *B. pahangi.* Antiserum raised against one life cycle stage was able to immunoprecipitate iodinated antigens of other life cycle stages. Furthermore, Philipp *et al.,* (1986), demonstrated that antibodies which immunoprecipitated the major cuticular glycoprotein of the *Brugia* cuticle (gp29) were present in infected cat serum, prior to the development of the adult parasite. In addition, many of the somatic antigens of both the L3 and the adult worm will be constitutively expressed throughout the life cycle.

Previous studies on the cuticular antigens of *B. pahangi* (Devaney and Jecock, 1991), demonstrated a reciprocal relationship between the apparent abundance of major cuticular antigens of the post-infective L3 and the adult parasite; the L3 appeared to express a protein of approximately 27 kDa and very little gp29, while the adult parasite expresses abundant gp29 and very little of the p27 protein. The antiserum used in this present study was known to immunoprecipitate a number of L3 cuticular antigens which were also present in the adult parasite and so it was anticipated that some of these molecules may have been identified in the screening of the adult library.

3.2 Results

3.2.1 Recognition of adult and L3 antigens by the anti-L3 serum

Prior to being used to immunoscreen the library, the anti-L3 serum was first analysed by immunoblotting, using both L3 and adult DOC extracts to confirm that it recognised antigens present in both life cycle stages. **Figure 3.1** (lanes 2 and 4) shows a Western blot probed with the antibody at a 1:10,000 dilution, demonstrating that the antiserum recognised a range of antigens in both life cycle stages. Antibody dilutions were calculated after performing an end point ELISA, in order to compare the relative strengths of the anti-L3 and anti-adult serums. The anti-L3 serum was found to give a signal that was five times stronger than the anti-adult serum on ELISA, and so dilutions of the antibodies were made (anti-L3 1:10,000, anti-adult 1:2,000) in order to take account of this difference. Lanes 6 and 8 demonstrate the high level of cross reactivity between adult and L3 antigens. In an attempt to restrict the recognition of non stage-specific antigens, the rabbit anti-L3 serum was preadsorbed against an adult DOC extract (2.3.1.2). **Figure 3.1** (lanes 1 and 3) shows a Western blot of adult and L3 DOC extracted antigen probed with the preadsorbed antibody at a 1:10,000 dilution. It can be seen that following preadsorbtion, there was a lower level of reactivity overall, but the same major bands were still recognised in both life cycle stages. Although the anti-L3 serum recognised a range of components in both L3 and adult stages, no obvious L3-specific components were recognised. However, in the absence of a better antibody, it was decided to go ahead and screen the adult cDNA library with this serum.

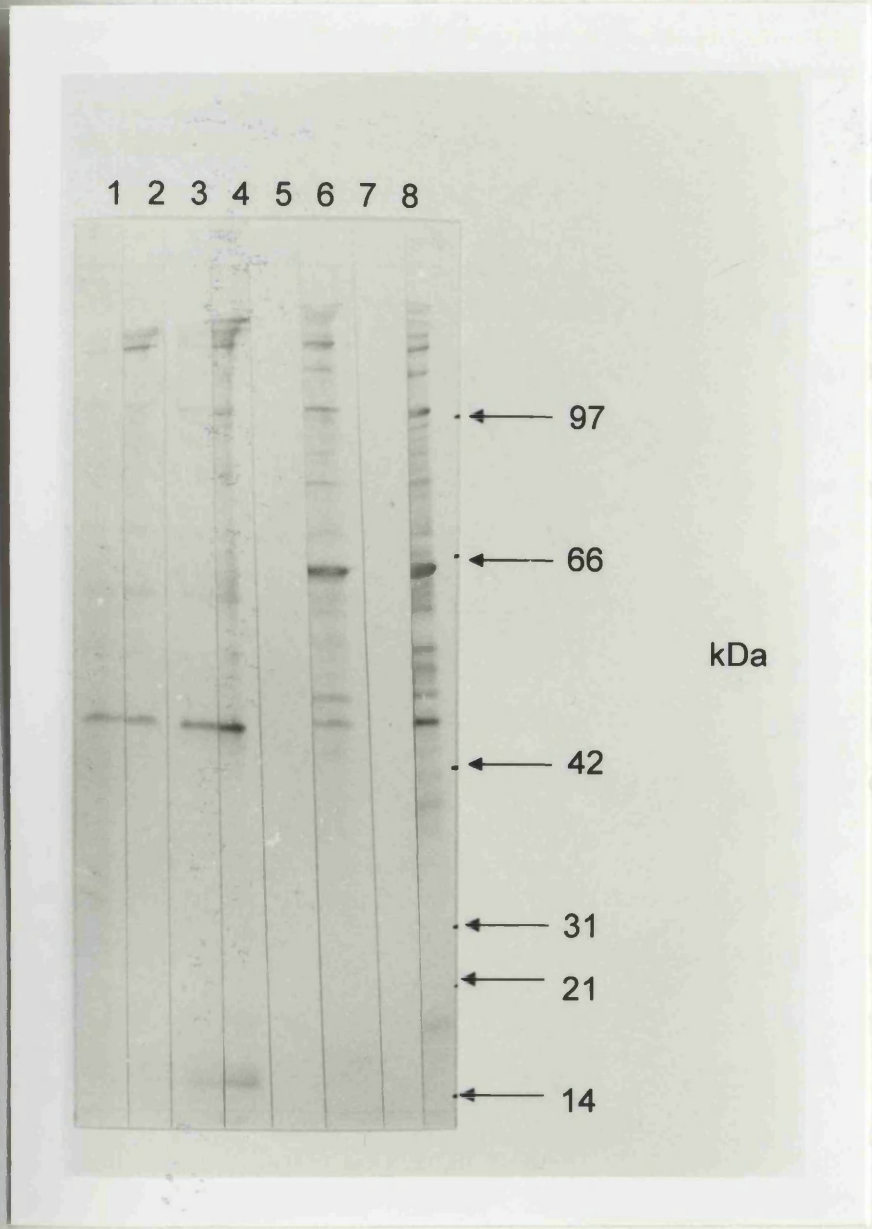
Figure 3.1

Western blot analysis of L3 and adult antigen with the rabbit anti-L3 serum

L3 and adult DOC extracts were run out on a 12.5% SDS PAGE longer length minigel, and immunoblotted onto nitrocellulose paper (see 2.2.4). The strips were incubated in either pre-adsorbed or non pre-adsorbed anti-L3 serum, at a dilution of 1:10,000. Control lanes were incubated with an anti-adult or control rabbit serum at a dilution of 1:2,000, followed by goat anti-rabbit alkaline phosphatase conjugate at a dilution of 1:6,000. BCIP/NBT substrate was used to develop the blot.

Lane 1	L3 extract	pre-adsorbed anti-L3 serum
Lane 2	L3 extract	anti-L3 serum
Lane 3	Adult extract	pre-adsorbed anti-L3 serum
Lane 4	Adult extract	anti-L3 serum
Lane 5	L3 extract	control rabbit serum
Lane 6	L3 extract	anti-adult serum
Lane 7	Adult extract	control rabbit serum
Lane 8	Adult extract	anti-adult serum

Figure 3.1



3.2.2 Immunoscreening

40,000 plaques were initially screened (see 2.3.2), using the pre-adsorbed anti-L3 serum at a dilution of 1:5,000, followed by goat anti-rabbit alkaline phosphatase conjugate at a dilution of 1:10,000. The filters were developed using BCIP/NBT substrate. 22 positive plaques were identified from this first screen. **Figure 3.2** shows an example of a filter probed with rabbit anti-L3 serum; positive plaques are darker and more distinct than background levels. These plaques were picked and taken through a further round of screening. After secondary screening, phage rescue and plasmid DNA preparation, seven of the clones had inserts large enough to warrant sequence analysis. **Figure 3.3** shows the inserts digested with *Kpn I* and *Eco*R *I* with sizes ranging from 300 bp to 1.8 Kbp. These clones were designated SH2, SH3, SH4, SH6, SH7, SH8 and SH12. Manual sequencing of each clone was performed and approximately 200-400 bp of 5' sequence obtained. **Table 3.1** gives the results obtained after searching the gene database (EMBL) using the FAST A program. The three largest clones, SH6, SH8 and SH12, showed homology to the gene encoding the highly immunogenic ladder protein (gp15/400) of *Brugia* (Tweedie *et al.,* 1993). **Figure 3.4** shows the sequence of clone SH12 compared to the gp15/400 sequence. There is a high level of nucleotide homology (96.7% identity) since SH12 appears to contain the 5' end of the ladder gene. Clone SH6 however, contains the 3' end of the gene, and shows a much higher level of homology to the corresponding region of the *Loa loa* gp15/400 (Ajuh *et al.,* 1995), as shown in **Figure 3.5**. The 3' end of the *Brugia* ladder gene has not previously been sequenced. Clone SH2 was homologous to heavy chain myosin mRNA from *C. elegans* (Karn *et al.,* 1983), clone SH3 to a 60S acidic ribosomal protein gene P1, from *D. melanogaster,* (Wigboldus, 1987), while clones SH4 and SH7 had no significant homology, probably because the insert size was very small. These clones were not analysed further.

Figure 3.2

Primary screening of adult cDNA library

An example of a filter from the primary screening of the adult cDNA library, probed with the anti-L3 serum (1:5,000), followed by goat anti-rabbit alkaline phosphatase conjugate (1:10,000) and developed with BCIP/NBT substrate. The arrowheads show the position of positive plaques.

Figure 3.2

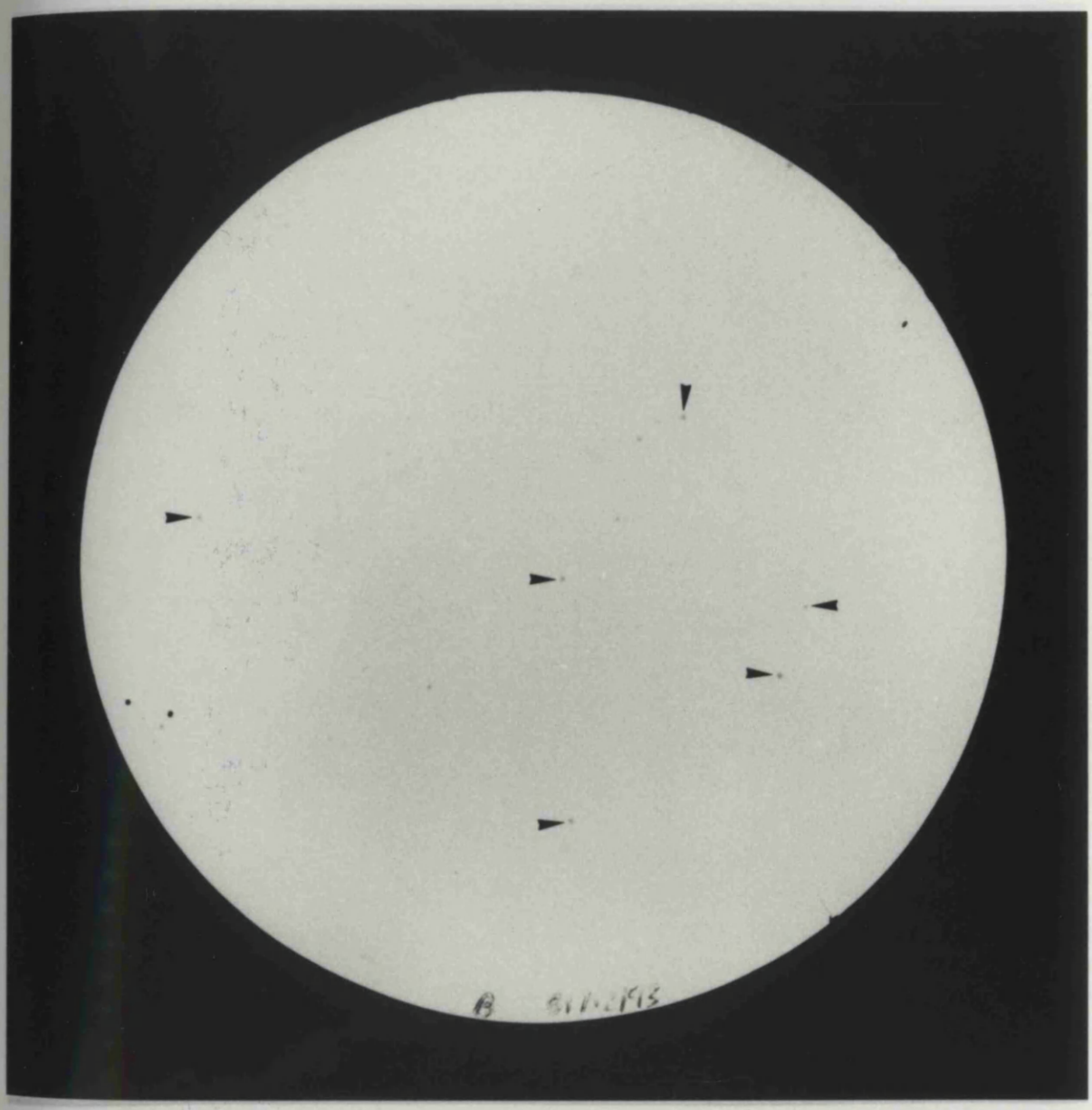


Figure 3.3

Restriction digests of positive clones identified using the anti-L3 serum

1.5% agarose gel showing the size of inserts following restriction digest using *Eco*R I and *Kpn* I.

Lane 1	clone SH4	450 bp
Lane 2	clone SH6	1.8 Kbp
Lane 3	clone SH7	450 bp
Lane 4	clone SH8	1.1 Kbp
Lane 5	clone SH12	1.3 Kbp
Lane 6	clone SH3	300 bp
Lane 7	pBluescript	no insert control
Lane 8	λ*Pst* I markers	

Figure 3.3

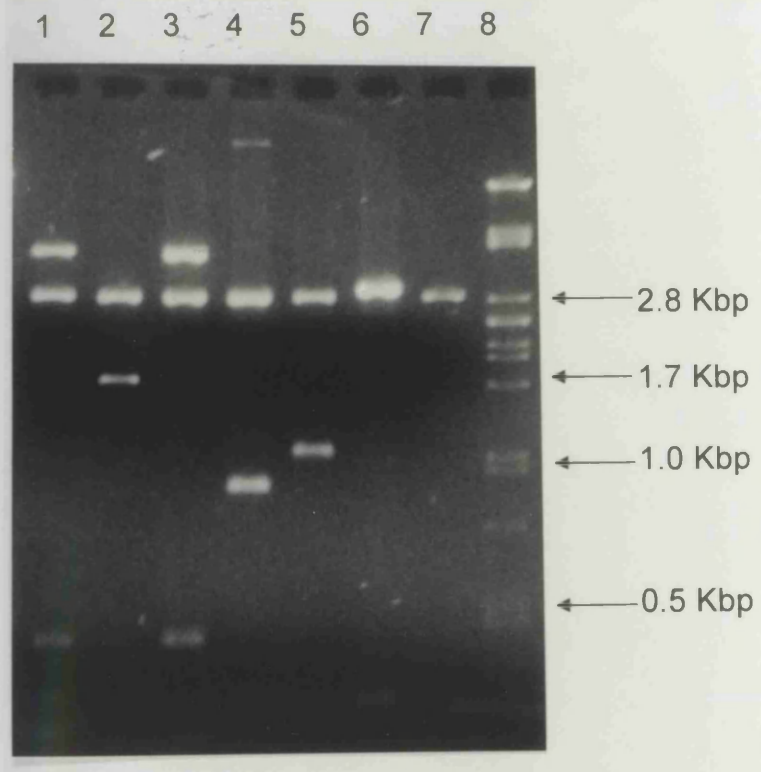


Table 3.1

Sequence homologies of clones picked from the adult cDNA library with the rabbit anti-L3 serum

Table 3.1

Sequence homologies of clones picked from the adult cDNA library with the rabbit anti-L3 serum

Clone	Size (Kbp)	Homology	Species	% Identity
SH 2	0.4	Myosin heavy chain B	*C.elegans*	69.4%
SH 3	0.3	60S acidic ribosomal protein P1	*D. melanogaster*	68%
SH 4	0.45	No significant homology	-	-
SH 6	1.8	gp15/400 ladder protein	*Loa loa*	80.4%
SH 7	0.45	No significant homology	-	-
SH 8	1.1	gp15/400 ladder protein	*B. malayi*	-
SH 12	1.3	gp15/400 ladder protein	*B. malayi*	96.7%

Figure 3.4

Nucleotide sequence of clone SH12 compared to that of the gp15 ladder protein gene from *B. malayi*

Figure 3.4

Nucleotide sequence of clone SH12 compared to gp15/400, the ladder protein gene from *B. malayi*

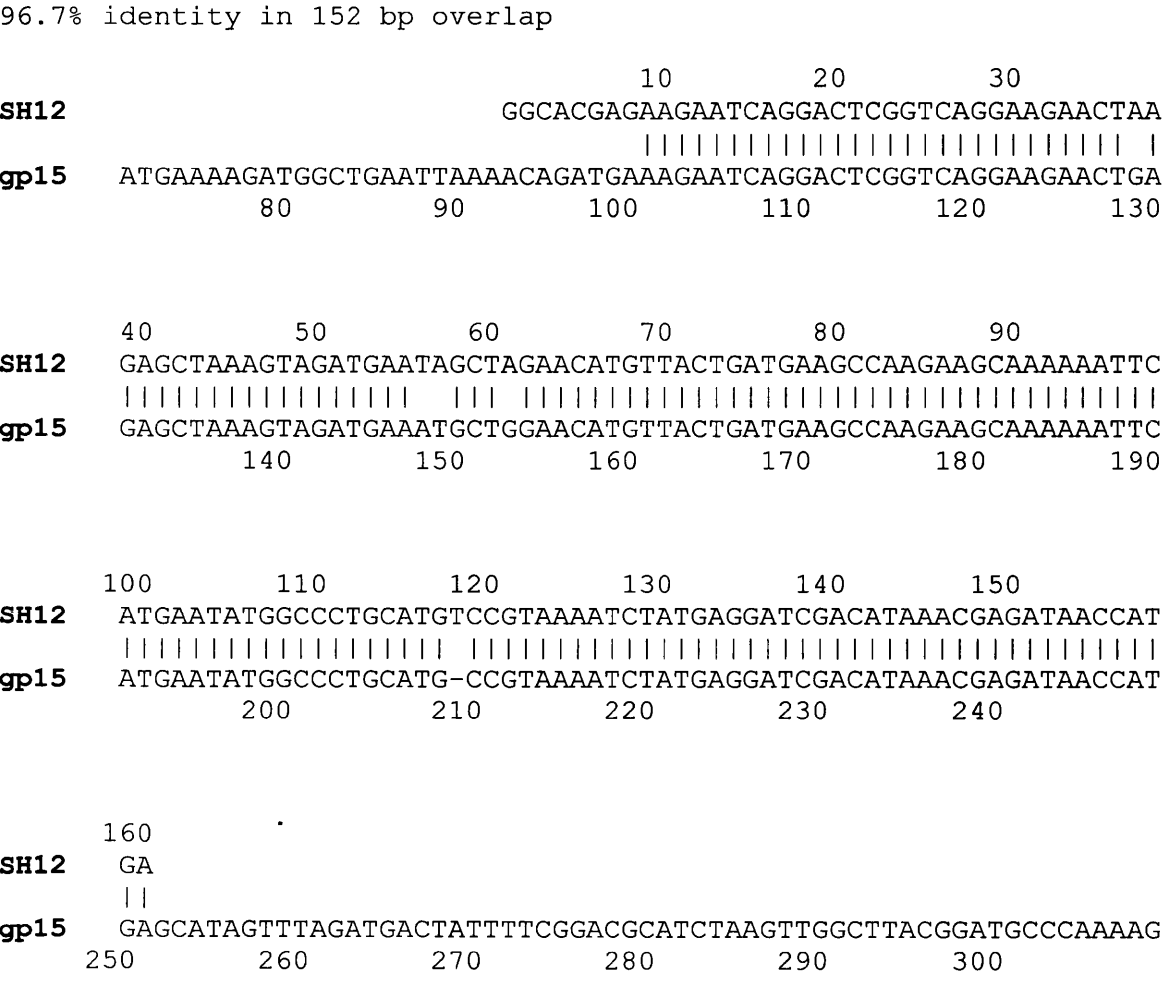
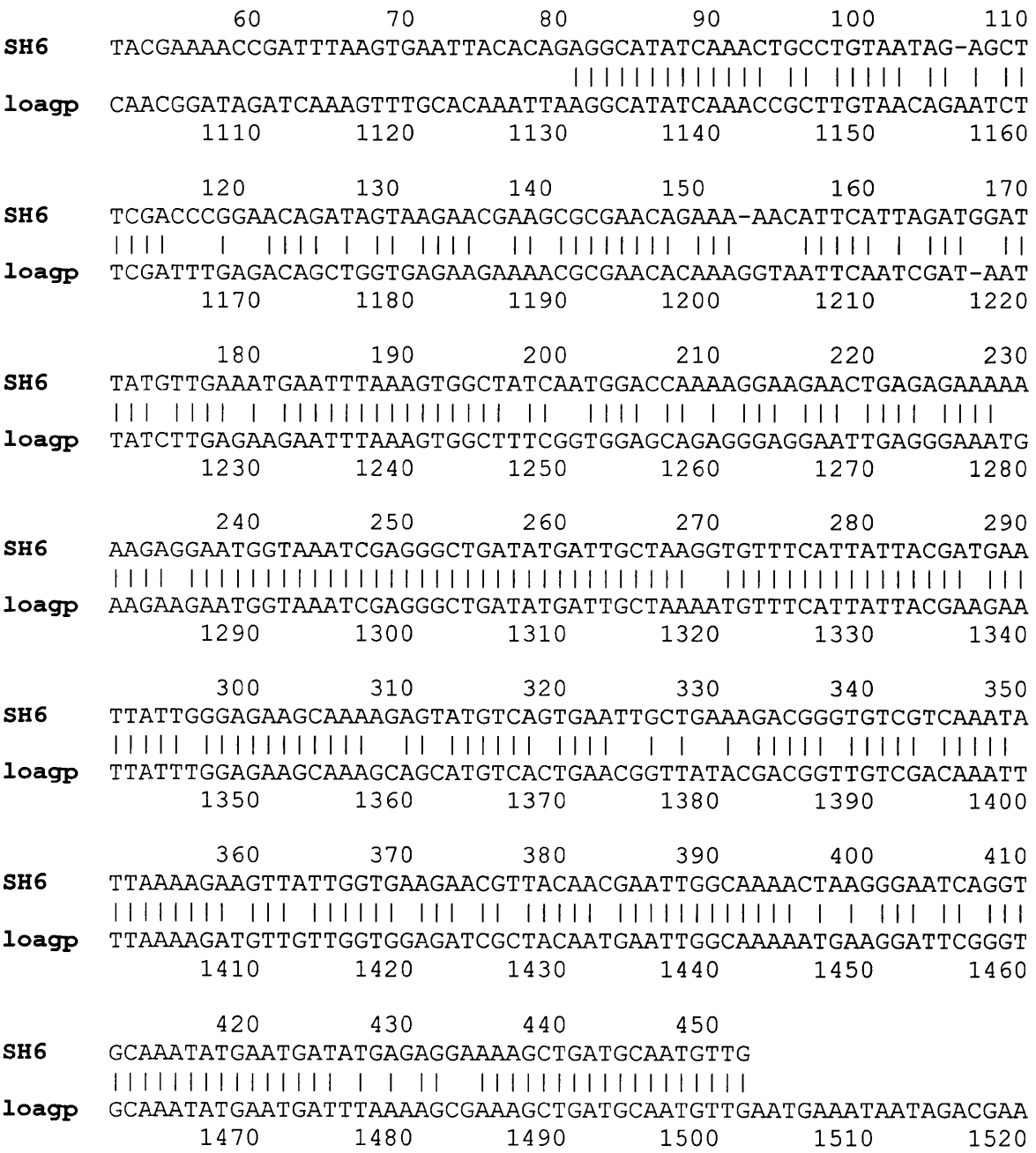


Figure 3.5

Nucleotide sequence of clone SH6 compared to that of the *Loa loa* gp15 ladder protein gene

Figure 3.5

Nucleotide sequence of clone SH6 compared to the gp15/400 ladder protein gene from *Loa loa*



3.2.3 Attempts to raise a monospecific antiserum to p27

Since each of the genes cloned and described above coded for structural or housekeeping proteins, which were unlikely to be stage specific, an alternative approach was employed. The previously identified p27 molecule was known to be present in the adult, but is more highly expressed in the larval stages (Devaney and Jecock, 1991) and is the major ^{125}I labelled component of the cuticle of the L4 (unpublished observations). In the adult parasite, the major ^{125}I labelled component is gp29, a functional molecule, so it was of interest to attempt to further characterise and clone p27. In order to confirm the previous results with p27; L3, L4 (obtained at 12 days post-infection) and adult parasites were labelled with ^{125}I, (by Dr. E. Devaney) extracted in DOC and the soluble extracts analysed on SDS-polyacrylamide gels. **Figure 3.6** (lane 1) shows that in the adult parasite p27 is not labelled to a high specific activity, whereas in the L4 (lane 2) it is more heavily labelled. It can also be seen, from **Figure 3.7**, that the adult gp29 is glycosylated, as treatment of the adult extract with N-Glycosidase F reduces the size of the molecule by approximately 4 kDa (lane 1 compared with lanes 2 and 3). In contrast, p27 does not appear to contain N-linked carbohydrates, as treatment of either the L4 (lanes 4-6) or adult extract with N-Glycosidase did not result in a reduction in molecular weight. It would appear that in the L3 extract (lanes 7-9) there is a protein of approximately 27 kDa which seems to be glycosylated. However, it is not clear whether this is in fact p27, as the L3 extract has not labelled sufficiently to give a clear band.

In order to purify p27 the approach adopted was to run protein gels to separate out the p27 band and to use this to raise a monospecific antiserum, with which the adult library could then be immunoscreened, since p27 is present in both L3 and adult stages. For this purpose 1D SDS polyacrylamide gels were run using an adult DOC extract, as insufficient L3 were available. Gels were run using 50μg of protein per well. The position of p27 was monitored using ^{125}I labelled adult extract followed by autoradiography and the corresponding protein band cut out of the gel after staining with Coomassie Blue. A

Figure 3.6

1D SDS PAGE analysis of ^{125}I labelled L3, L4 and adult extracts

67,500 TCA precipitable cpm of each life cycle stage extract were run out on a 12.5% polyacrylamide gel, which was first stained in Coomassie Blue, destained and then dried down before autoradiography. Lane 1; adult extract, Lane 2; L4 extract, Lane 3; L3 extract. The arrowhead shows the position of p27 which appears to be more heavily labelled in the L4 extract.

Figure 3.6

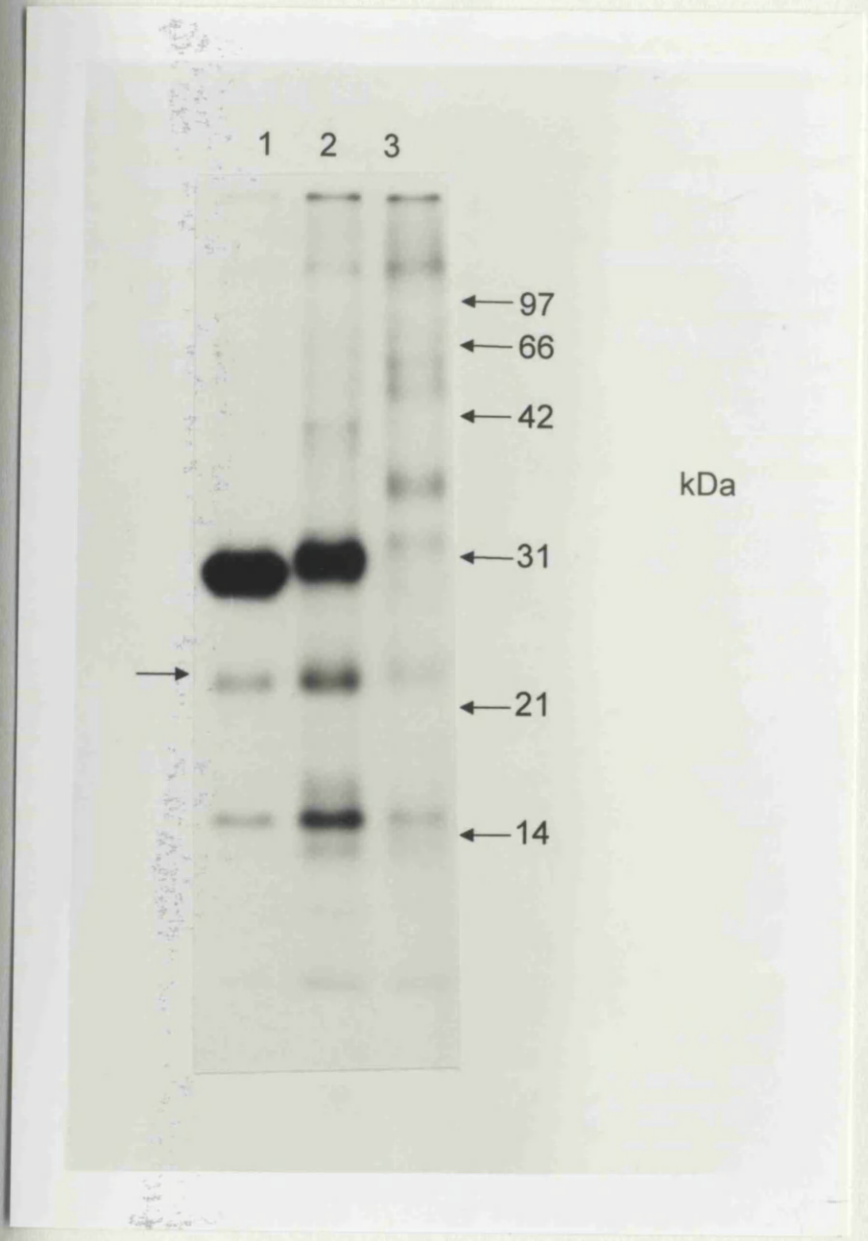


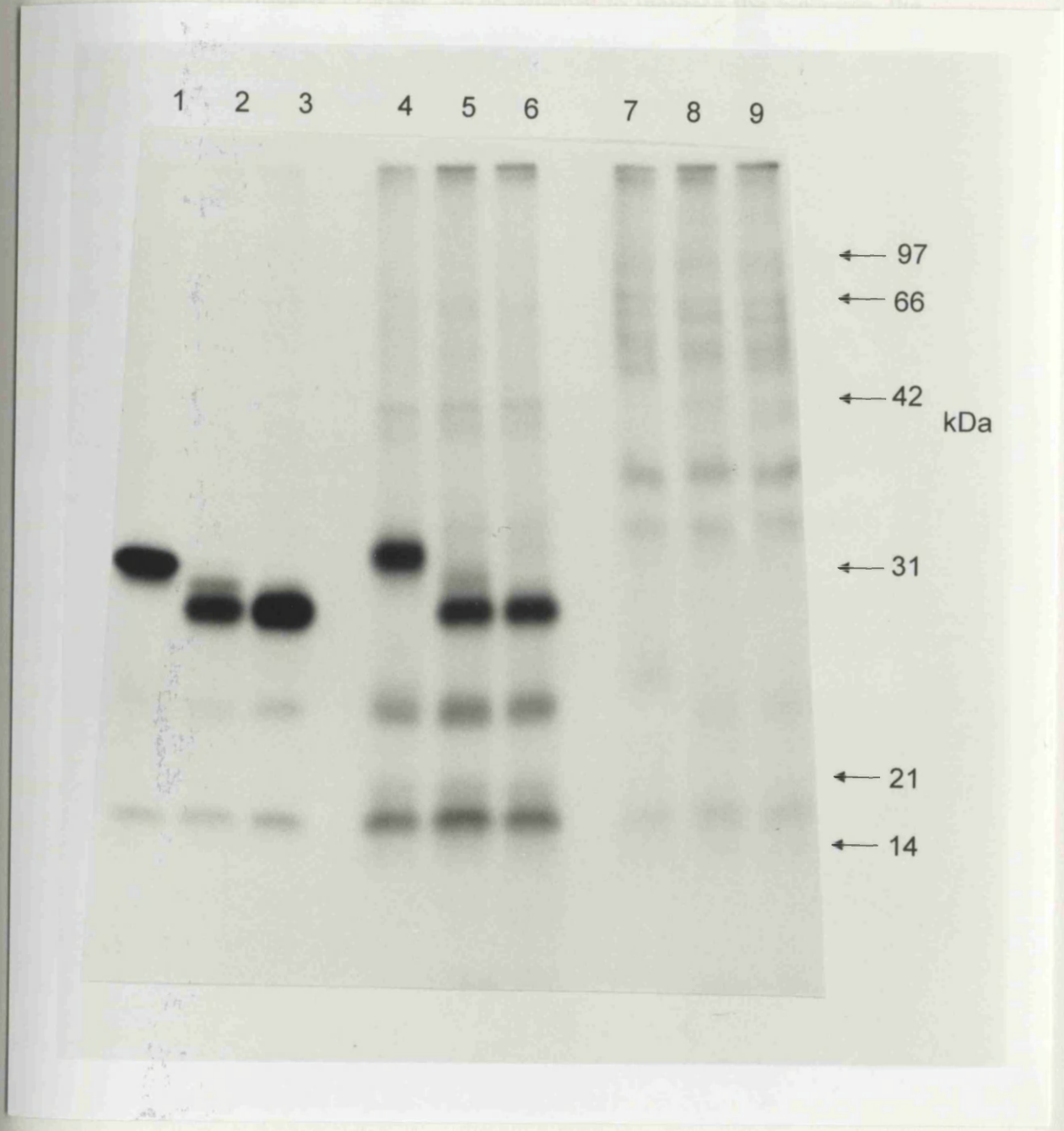
Figure 3.7

Autoradiograph of deglycosylation experiment

A deglycosylation experiment was performed with the N-Glycosidase F enzyme (see 2.2.6), using the ^{125}I labelled extracts from the three different life cycle stages. 7.4µl of L3 extract; 6.2µl of L4 extract and 5.0µl of adult extract were used to give 67,500 cpm per lane. The reactions were run out on 12.5% 1D SDS PAGE gels and then dried and autoradiographed.

Lane 1	Adult extract	no enzyme added
Lane 2	Adult extract	1 U/ml enzyme
Lane 3	Adult extract	10 U/ml enzyme
Lane 4	L4 extract	no enzyme added
Lane 5	L4 extract	1 U/ml enzyme
Lane 6	L4 extract	10 U/ml enzyme
Lane 7	L3 extract	no enzyme added
Lane 8	L3 extract	1 U/ml enzyme
Lane 9	L3 extract	10 U/ml enzyme

Figure 3.7



representative gel is shown in **Figure 3.8**. The bands corresponding to p27, from 18 lanes of antigen, were excised from gels, pulverised and mixed with a 2:1 ratio of Freund's Incomplete Adjuvant, and then used to immunize a rabbit. The rabbit was boosted and test bled after each boost and the serum analysed by Western blot. **Figure 3.9** (lane 2) shows a Western blot of adult DOC extract probed with rabbit serum after the first immunisation and boost. There was a weak antibody response to a protein which resolved in the correct Mr range (approximately 27 kDa). In an attempt to increase the response, the rabbit was boosted again, with additional gel purified antigen and two weeks later another bleed taken. When this serum was analysed by Western blot, the dominant molecule recognised was approximately 37 kDa and there was no increase in reactivity with p27, as shown in **Figure 3.10**.

Despite the fact that the antiserum recognised additional proteins it was decided to go ahead and use it to immunoscreen the adult library. 20,000 plaques were screened using the p27 antiserum at a dilution of 1:5,000, and three positive plaques were identified, designated R1, R2, and R3. After secondary screening, phage rescue and plasmid DNA preparation, only one clone, R2, appeared to have an insert (approximately 300 bp). **Figure 3.11** shows the restriction digest of the three clones, using *Eco*R I and *XHo* I. The insert was sequenced but, after searching the database, no significant homology was found. Since the anti-p27 serum appeared to preferentially recognise a molecule of approximately 37 kDa, a Western blot was performed to compare the reactivity of this serum with L3 and adult extracts. **Figure 3.12** (lane 1) shows that the 37 kDa protein is recognised only very weakly in the L3 extract but is very strongly recognised in the adult extract (lane 3). As the aim of this study was to identify L3 specific components, this part of the work was not taken any further.

Figure 3.8

1D SDS gel of adult antigen demonstrating the position of p27

1D gel (12.5%) of adult ^{125}I labelled DOC extract, Lane 1; 25μg, Lane 2; 50μg, Lane 3; 75μg per well. The arrow shows the position of the 27 kDa protein (upper band of the doublet), used to identify the band on subsequent non-radioactive gels.

Figure 3.8

Figure 3.9

Western blot analysis of adult antigen with the anti-p27 serum (1st test bleed)

12.5% longer length minigel to separate 4 lanes of adult DOC extract, immunoblotted and then probed with pre-bleed and first test bleed serum at different dilutions. Goat anti-rabbit alkaline phosphatase conjugate at 1:6,000 dilution, BCIP/NBT substrate. The arrow shows the weak recognition of a band at approximately 27 kDa.

Lane 1	pre-bleed serum 1/100 dilution
Lane 2	1st test bleed serum 1/100 dilution
Lane 3	pre-bleed serum 1/200 dilution
Lane 4	1st test bleed serum 1/200 dilution
Lane 5	pre-bleed serum 1/400 dilution
Lane 6	1st test bleed serum 1/400 dilution
Lane 7	pre-bleed serum 1/600 dilution
Lane 8	1st test bleed serum 1/600 dilution

Figure 3.9

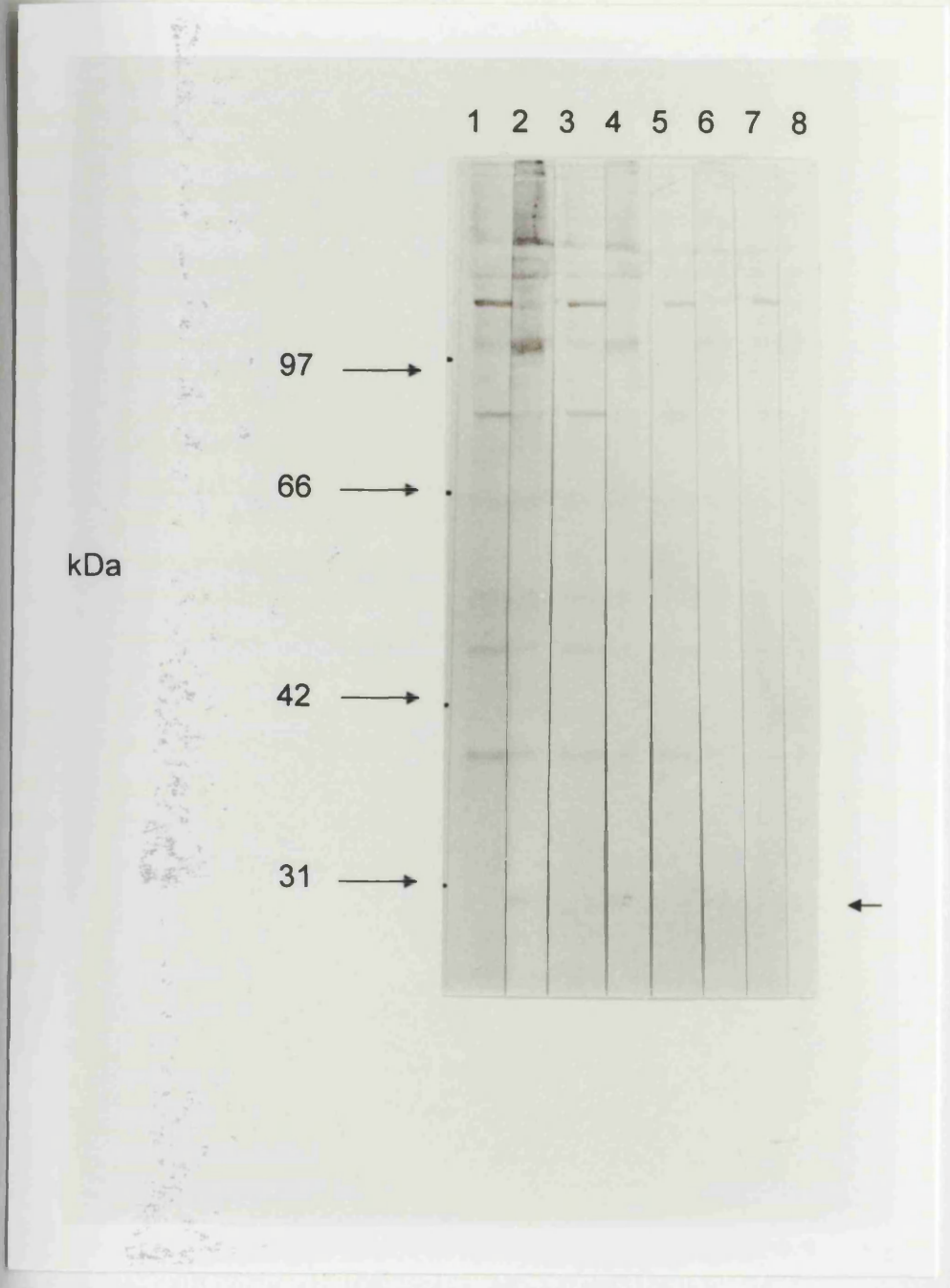


Figure 3.10

Western blot analysis of adult antigen with the anti-p27 serum (3rd test bleed)

Western blot as before, using pre-bleed serum, first bleed serum and third bleed serum in a range of dilutions, with arrow **(a)** showing strong recognition of the 37 kDa protein, and arrow **(b)** the much weaker response to the 27 kDa band.

Lane 1	3rd bleed serum	1/600
Lane 2	1st bleed serum	1/600
Lane 3	pre-bleed serum	1/600
Lane 4	3rd bleed serum	1/400
Lane 5	1st bleed serum	1/400
Lane 6	pre-bleed serum	1/400
Lane 7	3rd bleed serum	1/200
Lane 8	1st bleed serum	1/200
Lane 9	pre-bleed serum	1/200
Lane 10	3rd bleed serum	1/100
Lane 11	1st bleed serum	1/100
Lane 12	pre-bleed serum	1/100

Figure 3.10

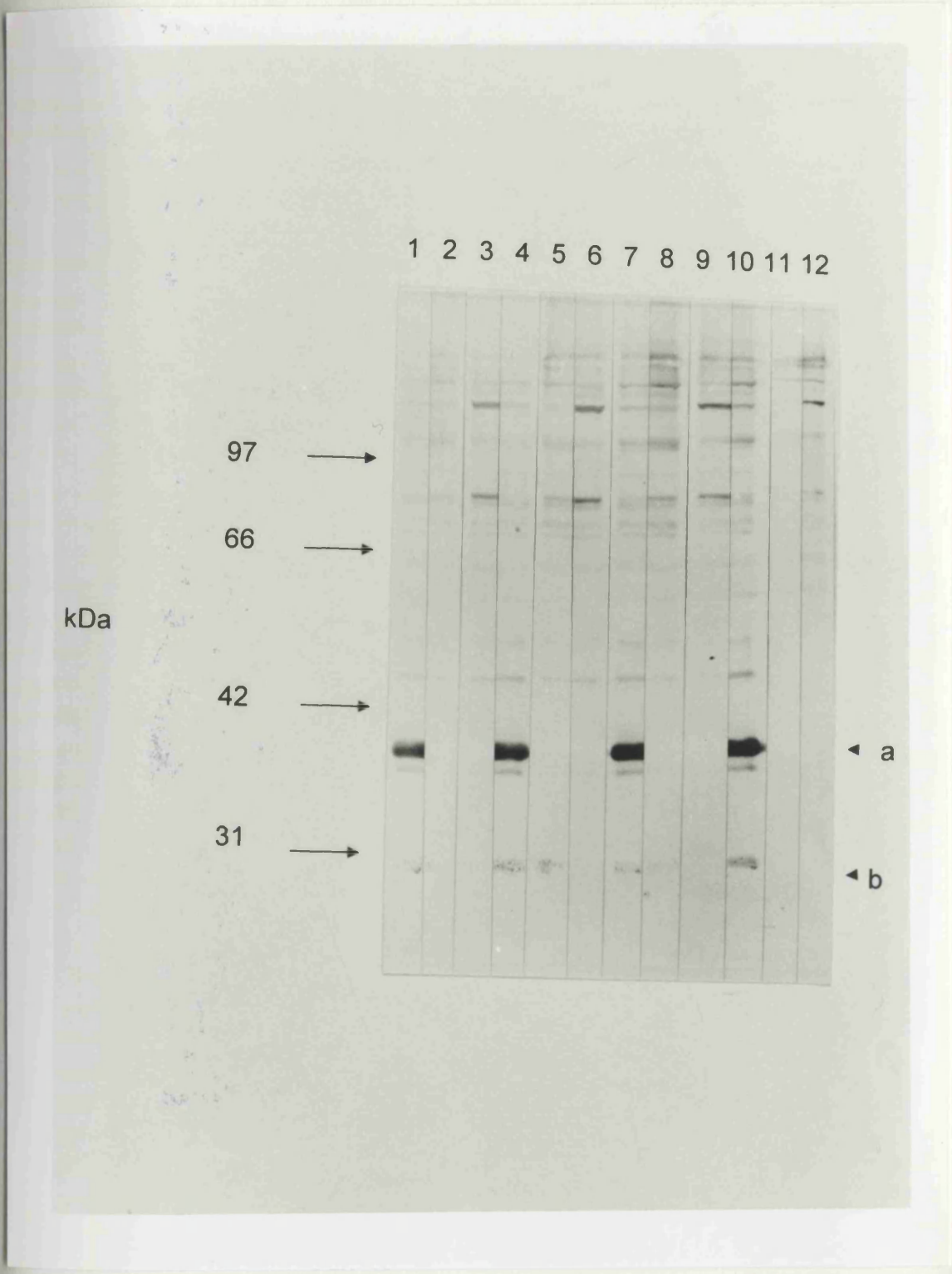


Figure 3.11

Restriction digests of positive clones identified using the anti-p27 serum

1% agarose gel showing insert size (restriction digests using *EcoR* I and *XHo* I) of the three positive clones picked from the adult library using the anti-p27 serum. Lane 1; clone R1, Lane 2; clone R2 (300 bp), Lane 3; clone R3.

Figure 3.11

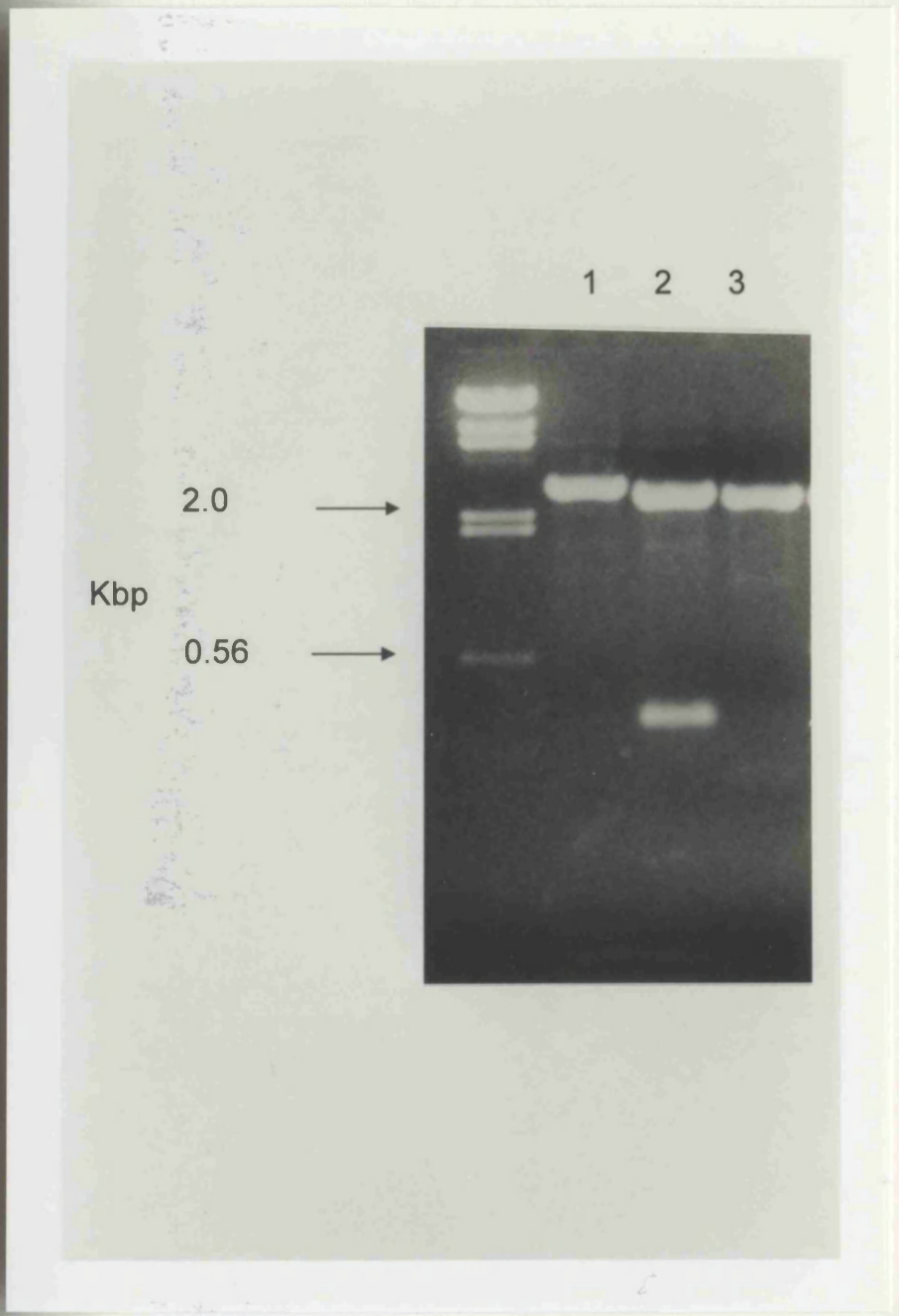


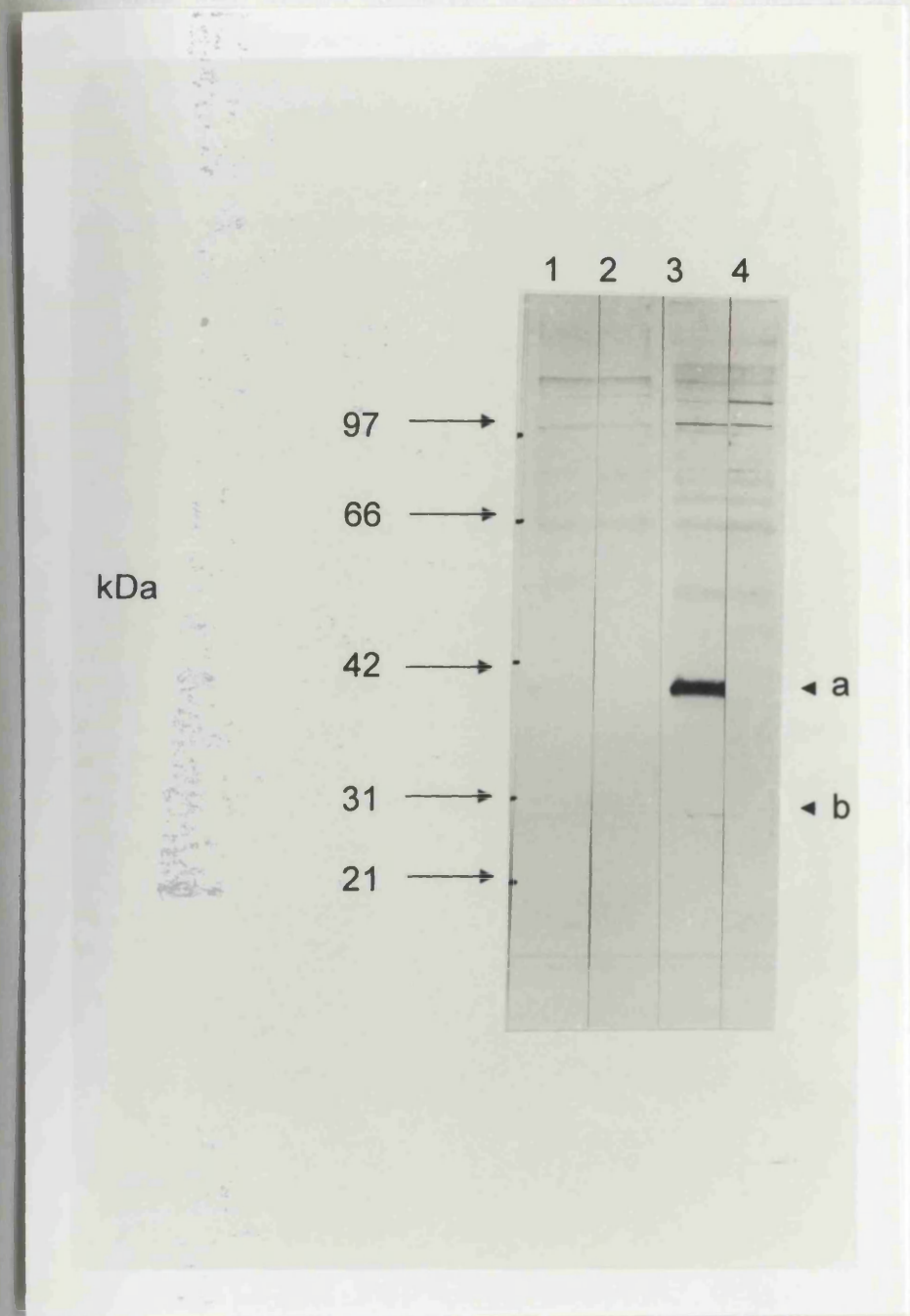
Figure 3.12

Western blot using L3 and adult extracts probed with the anti-p27 serum

L3 and adult DOC extracts were separated on a 12.5% longer length minigel, immunoblotted and probed with anti-p27 serum at a dilution of 1:100, goat anti-rabbit alkaline phosphatase conjugate at 1:6,000 and developed with BCIP/NBT substrate. The 37 kDa antigen (**a**) appears to be much more highly expressed by the adult parasite, arrow (**b**) shows the position of the 27 kDa band.

Lane 1	L3 extract	anti-p27 serum	1/100
Lane 2	L3 extract	pre-bleed serum	1/100
Lane 3	Adult extract	anti-p27 serum	1/100
Lane 4	Adult extract	pre-bleed serum	1/100

Figure 3.12



3.2.4 Attempts to purify p27 for further characterisation

As eluting p27 from 1D gels had not proved successful, it was decided to attempt to purify the protein further. It was known from previous work that the p27 molecule was extremely basic and this was a useful characteristic for attempts to isolate the protein by 2D NEPHGE analysis (2.2.3). If this was successful then another antiserum could be raised or alternatively, the protein could be purified for N-terminal amino acid sequencing. **Figure 3.13** shows a 2D gel of adult IEF lysis buffer extract, spiked with an aliquot of ^{35}S labelled DOC extract of adult parasites (from Dr. E. Devaney), to show the full range of proteins synthesised by the parasite. The arrowhead denotes the position of p27 demonstrating the basic nature of this component. In an attempt to purify sufficient p27 for further analysis, 2D gels were run using adult antigen (because of availability). To localise the p27 protein, the adult extract was spiked with ^{125}I labelled L4, as p27 is best labelled in this life cycle stage. The p27 protein could be easily identified, as shown in **Figure 3.14**, and the corresponding spots were excised from 12 gels. At the same time the spots corresponding to gp29 and the 14 kDa ^{125}I labelled antigen were also excised. To determine whether sufficient protein could ever be obtained by this method, the gp29 and the 14 kDa protein spots were used as test samples. The spots corresponding to both proteins were rehydrated and analysed on a 1D SDS gel. While gp29 could be seen by Coomassie Blue staining using this method, p14, which more closely mirrored p27, in terms of the size of the spots, could not be visualised. It was therefore decided that this method would not be successful, as to obtain N-terminal sequence data the band of interest must be visible by Coomassie Blue staining.

Figure 3.13

2D NEPHGE analysis of ^{35}S labelled parasite proteins

Autoradiograph of 2D NEPHGE analysis using adult IEF lysis buffer extract, spiked with ^{35}S labelled adult DOC extract, showing the full range of proteins synthesised by the parasite. The position of the basic molecule, p27 is denoted by the arrowhead.

Figure 3.13

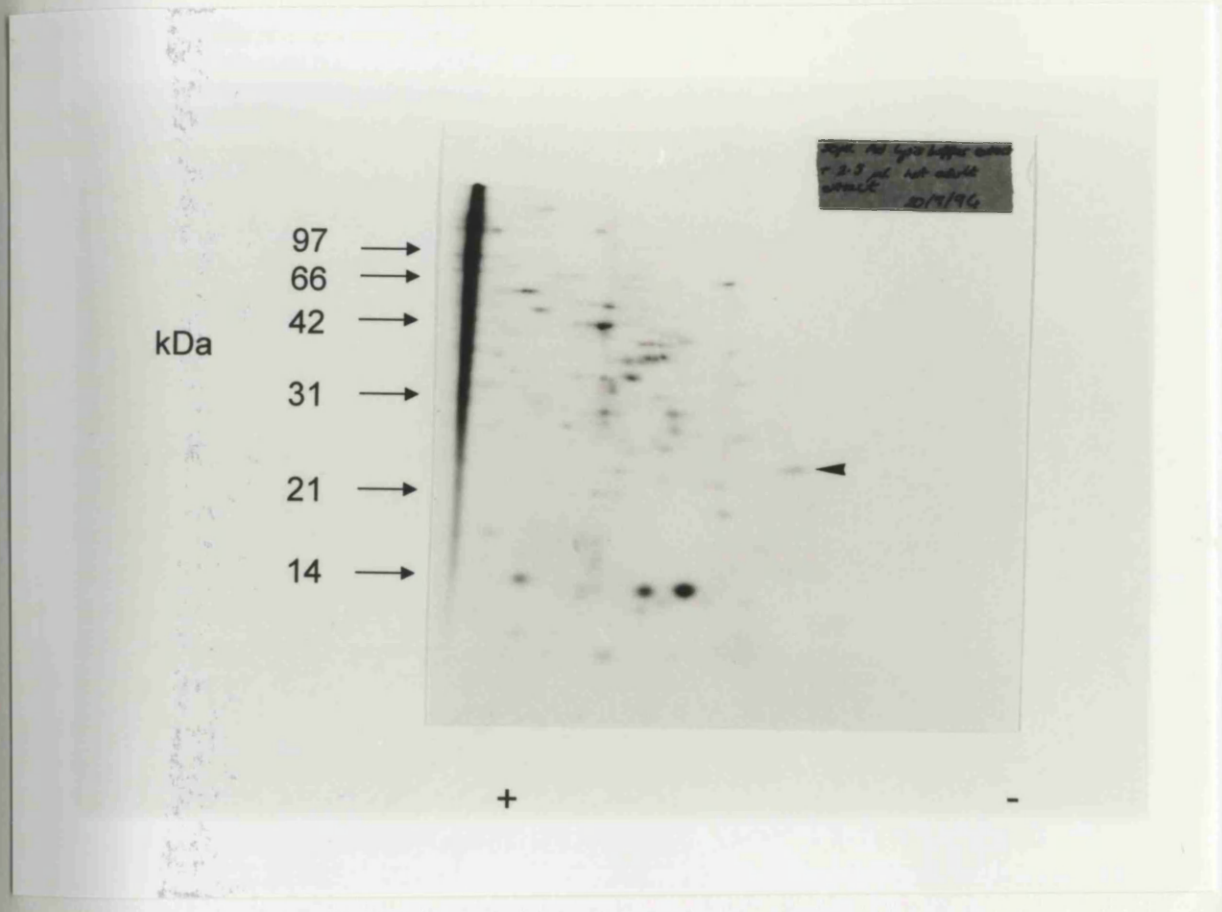
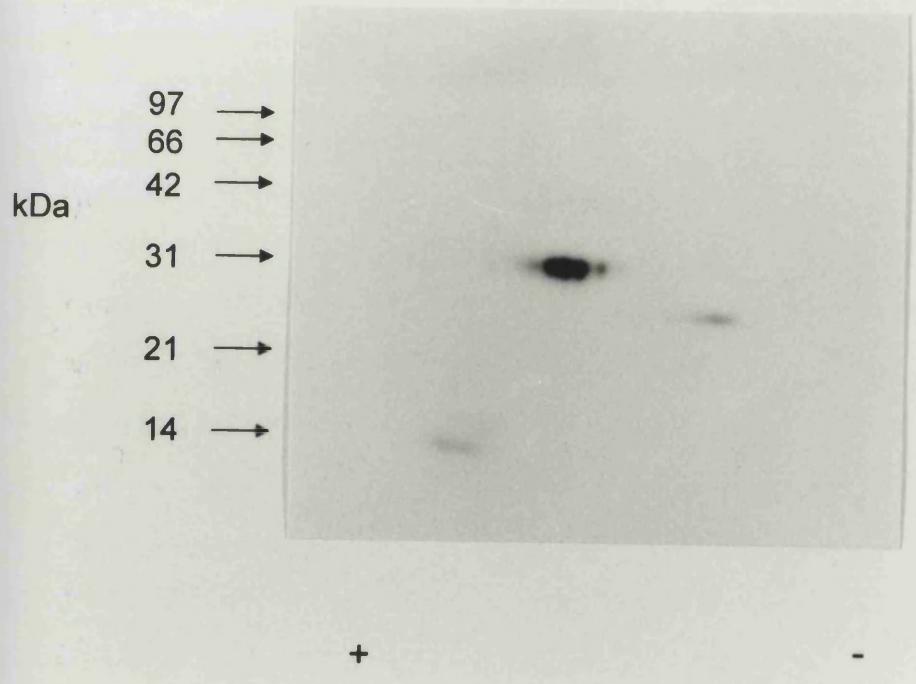


Figure 3.14

2D NEPHGE analysis of ^{125}I labelled parasite proteins

Autoradiograph of 2D NEPHGE analysis using 45µl adult IEF lysis buffer extract, spiked with 6.25µl ^{125}I labelled L4 extract to give 135,000 cpm per gel, showing the position of gp29, p27 and the 14 kDa protein.

Figure 3.14



3.3 Discussion

The rationale behind the experiments described in this chapter was to use an existing adult cDNA library to clone genes which are upregulated in the L3 but which are expressed throughout the life cycle. This was attempted by screening the library with an anti-L3 serum which was known to recognise a variety of L3 antigens, eg. ^{125}I labelled cuticular antigens which, although present in the adult, are more highly expressed in the larval stages. Previous work had shown that the anti-L3 serum immunoprecipitated several cuticular proteins and it was hoped that some of these may have been detected in the immunoscreen.

The initial immunoscreening identified a number of genes which were mostly housekeeping protein genes, such as the ribosomal protein and myosin clones. Three of the clones identified showed homology to the ladder protein, which was not unexpected, since previous work in this and other laboratories has shown it to be a highly immunogenic molecule. The ladder protein has been cloned from a range of nematodes, including *D. immitis, L. loa, Ascaris, D. viviparus* and *C. elegans*. Tweedie *et al.,* (1993), found that in *Brugia* the polyprotein antigen is synthesised as a very large 400 kDa precursor which is then proteolytically cleaved into multiple repeating units of approximately 15 kDa. It is not yet known if these repeats are all identical. The protein appears to be encoded by a single gene with mature transcripts of around 10 kb. The tandemly repeating 15 kDa units are glycosylated and each repeat contains one consensus site for N-linked glycosylation. Kennedy *et al.,* (1995) found that the expressed gp15/400 protein antigen of *B. malayi* acts as a lipid binding protein and is involved in fatty acid transport. The ladder protein can be localised throughout the worm and is also present in excretory/secretory (ES) products (McReynolds, 1993). Ladder proteins are known to be allergenic, stimulating IgE responses in a proportion of patients suffering from elephantiasis (Paxton *et al.,* 1993), and may therefore contribute to the pathology of many nematodiases.

A further gene identified in this screen was shown to have homology to heavy chain myosin from the free living nematode, *C. elegans* (Karn *et al.,* 1983). In this species there are four different myosin heavy chains and the *Brugia* myosin cDNA clone has the highest homology to MHC-B, the protein encoded by the *unc-54* gene, which is found exclusively in the body wall muscle, along with MHC-A. These two heavy chain myosins co-assemble into body wall thick filament where they function in muscle contraction. The myosin molecule is a hexameric protein consisting of two heavy chain units, two alkali light chain units and two regulatory light chain units. The heavy chain may be split into two globular sub-fragments and one rod-shaped fragment. The rod-like tail sequence is highly repetitive and gives a pattern characteristic for alpha-helical coiled coils. The amino acid sequence of this region is less conserved than that of the globular head, which shows a high degree of similarity with the globular head sequences of other muscle and non-muscle heavy chains. The MHC gene has also been cloned and characterised from *B. malayi* (Werner and Rajan, 1992a) and *O. volvulus* (Werner and Rajan, 1992b), and both genes code for a protein of 1957 amino acids with 75% homology to the MHC-B protein from *C. elegans.* In *B. malayi* the Bmmyo-1 gene extends over 11 kb and has 14 introns, which are larger than those found in the *unc-54* gene. The immunogenicity of myosins and paramyosins in helminth infections has been shown by several groups (Dissanayake *et al.,* 1992, Grossman *et al.,* 1990, Ben-Wen Li *et al.,* 1991), and this may explain why the *B. pahangi* myosin clone was detected by immunoscreening.

A further cDNA identified in this screen was demonstrated to code for a ribosomal protein with 68% homology to 60S acidic ribosomal protein P1, from *D. melanogaster* (Wigboldus, 1987), and since the same gene was identified again later, it is discussed in Chapter 4.

In an attempt to clone a more specific L3 cDNA, 1D SDS polyacrylamide gels were used to isolate p27, and then an antiserum was raised against the protein resolving at 27 kDa (p27). Although the resulting serum did recognise a band of the correct size by Western blot, the signal was weak, and it also recognised

very strongly a band of approximately 37 kDa. This serum was used to immunoscreen the adult cDNA library and the clone identified by this method was sequenced but no significant homology was found. However, by Western blot analysis it was shown that the dominant molecule recognised by this serum was more highly expressed by the adult parasite, and so it seemed that this approach had not been successful in identifying L3 specific antigens. As great care was taken in excising the bands from the correct part of the gel, it remains unclear why the antiserum should recognise a molecule of 37 kDa. At the present time there is no evidence to suggest that p27 and the 37 kDa contaminant are related.

It was then decided to try and purify the p27 molecule further, using 2D NEPHGE analysis since it was known to be a very basic molecule and so should be distinct and easily identifiable. Although several gels were pooled together and as much antigen as possible was used on each gel, this method failed to provide enough purified protein for either N-terminal amino acid sequencing or to try and raise a more specific antibody.

Another option may have been to differentially immunoscreen the adult library with anti-L3 and anti-adult serum, but this approach would probably be difficult, due to the cross-reactivity between filarial antigens from different life cycle stages. Alternatively, the adult library could have been screened by differential hybridisation using PCR generated cDNA probes from the adult and L3 life cycle stages. However, during this time an L3 library became available and it was decided to concentrate on that instead.

Chapter 4

Differential Screening

4.1 Introduction

Although several *Brugia* cDNAs were isolated using the approach outlined in the previous chapter, none of these genes was considered to be L3 specific. In the meantime an L3 library had become available, so the rationale behind the work in this chapter was to attempt to differentially screen the post-infective L3 library, using PCR generated cDNA probes from different life cycle stages. Probes were made using mosquito-derived L3, 3 day post-infection L3 and adult worms and used to differentially screen the library which was prepared from 3 day post-infection L3. By selecting plaques which hybridise preferentially to the mosquito-derived L3 probe, and not to the adult probe, it should be possible to identify cDNAs highly expressed in the mosquito-derived L3. Since the library was made using 3 day post-infective L3, any clones picked will also be present at this time, but may be expressed at a much lower level.

The 3 day post-infection L3 library

The library was made in the laboratory by Dr Sam Martin, using L3 which were removed from the jird at 3 days post-infection. In order to overcome the problem of limited availability of material, the library was made by RT-PCR, using the conserved nematode spliced leader (SL1) and oligo dT as primers. SL1 is a 22-nt sequence spliced onto the 5' end of a subset of mRNAs which was first identified in the free living nematode *C. elegans* (Krause and Hirsh, 1987). Since then the spliced leader sequence has been found in a number of parasitic nematodes, including *Brugia* (Takacs *et al.*, 1988), *Ascaris* (Nilsen, 1989), *Haemonchus* (Bektesh *et al.*, 1988) and *Onchocerca* (Zeng *et al.*, 1990). The exact percentage of transcripts which contain the SL1 sequence is not known but is thought to be between 70% and 90% in *Ascaris*. RNA was

first reverse transcribed using oligo dT as a primer, to ensure only polyadenylated RNA was primed for transcription. Then the resulting first strand cDNA was amplified by PCR using SL1 and oligo dT as primers, to try to optimise the amplification of full length cDNAs. The resulting PCR products were cloned into λ uni-ZAP (Stratagene) to create a stage specific library.

It was not possible to immunoscreen the library because recombinant proteins are not expressed, due to the presence of a stop codon in the SL sequence, when reading through from the β-galactosidase fusion protein coding sequence. In order to overcome this problem, 2 extra nucleotides would need to be added between the *Eco*R I site and the SL sequence in the primer, to push the recombinant protein into the same reading frame as β-galactosidase. Therefore, a differential hybridisation approach was adopted to select clones more highly expressed in the mosquito-derived L3 compared to the post-infective L3 or the adult.

4.2 Results

4.2.1 Differential Hybridisation (1)

The 3 day post-infection cDNA library was plated out on four 132mm tetracyclin agar plates to give approximately 2000 plaques per plate (30μl of 10^{-2} dilution of the library per plate). The plates were left overnight at 37°C to give plaques of a reasonable size. Three lifts were taken from each plate using Hybond-N filters (Amersham) and separate orientation marks made for each. The filters were denatured, neutralised, fixed and cross-linked onto the membrane before hybridisation. The cDNA probes were made by using RT-PCR on total RNA from mosquito derived L3, 3 day post-infection L3 and adult worms. The resulting cDNA was used as the template for random priming (see 2.4.2), and each probe was labelled to approximately equal specific activity. Filters were prehybridised for at least 4 hours at 65°C before the labelled probes were added and then left to hybridise overnight, again at 65°C. The following morning the filters were washed down to 0.5 X SSC /

0.1% SDS, and then autoradiographed overnight at -70°C. **Figure 4.1** shows an example of the hybridised filters. Plaques which were positive on the mosquito-derived L3 filters, absent on the adult filters and either absent or very faint on the 3 day post-infective L3 filters, were initially picked. After a second round of screening four positive plaques, SJ1, SJ2, SJ3 and SJ5 were identified. Phage rescue and plasmid DNA preparation were performed on these four clones and then they were digested using *Eco*R I and *Xho* I restriction enzymes. **Figure 4.2** shows a representative agarose gel, and demonstrates that all four clones had inserts; 350 bp inserts in SJ1 and SJ3 and 450 bp inserts in SJ2 and SJ5. The four clones were manually sequenced (see 2.5.8.1) on both strands using the T3 and T7 primers, and it was found that the cDNA inserts of the same size were in fact identical. SJ1 and SJ3 showed homology to nematode muscle tropomyosin mRNA (Frenkel *et al.*, 1989) and SJ2 and SJ5 both appeared to encode a protein very rich in glutamic acid.

4.2.2 Differential Hybridisation (2)

It was decided to rescreen the library in an attempt to identify additional L3 clones so the screening procedure was repeated, using another four plates with three filter lifts taken from each one exactly as described before. This time, after a second round of screening, 10 plaques were picked which hybridised more strongly to the mosquito derived L3 cDNA than to either the 3 day post-infection L3 or to the adult cDNA. These clones were called A,B,C,D,E,F,G,H,J,K. Phage rescue and plasmid DNA preparation were carried out and then the 10 clones were cross hybridised against the previously identified SJ1 and SJ5.

4.2.3 Colony Hybridisation

Thirteen squares were drawn onto two nylon membranes and these were placed on top of an agar plate. Each clone was streaked out onto the relevant square, and a negative control was included on each membrane. The colonies were left to grow overnight at 37°C. The membranes were then denatured, neutralised, fixed and crosslinked as before, but, in order to remove any excess

Figure 4.1

Differentially hybridised filters

An example of duplicate filter lifts of approximately 2000λ plaques from the 3 day post-infection L3 cDNA library. The black arrowheads show differential hybridisation to mosquito derived L3 **(A)** and the position of the same plaques, probed with 3 day post-infection L3 cDNA on the duplicate filter, are shown by open arrowheads **(B)**. The filters were washed to 0.5x SSC 0.1% SDS at 65°C.

Figure 4.1

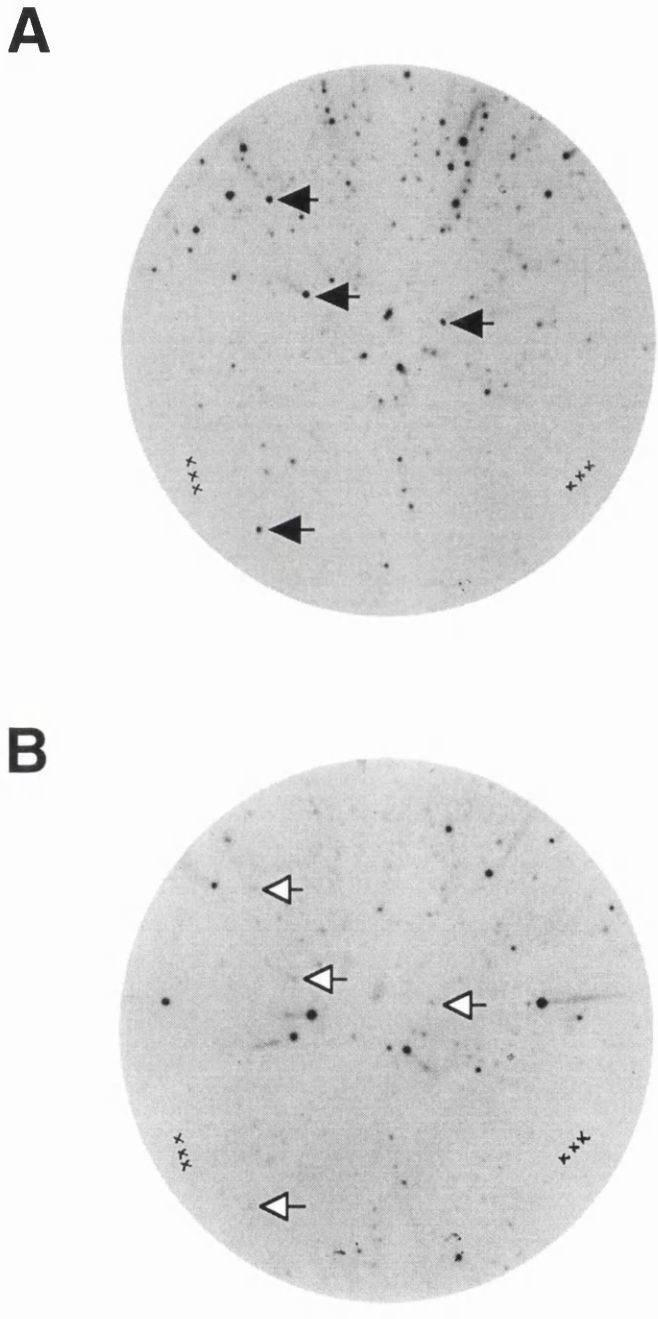


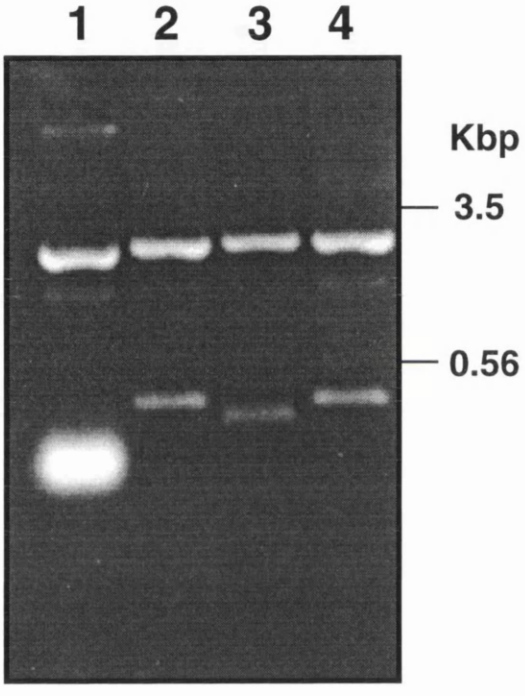
Figure 4.2

Restriction digests of mosquito-derived L3 clones (1)

1% agarose gel, stained with ethidium bromide, showing the size of inserts following restriction digest using *Eco*R I and *Xho* I.

Lane 1	pBluescript	no insert control
Lane 2	Clone SJ5	450bp
Lane 3	Clone SJ3	350bp
Lane 4	Clone SJ2	450bp

Figure 4.2



bacteria, the filters were washed in 5 X SSC / 5% SDS at 65°C for 5-10 minutes. The filters were then rinsed and prehybridised as usual before the labelled clones, SJ1 and SJ5, (see 2.5.2) were added to the hybridisation bottles, and left overnight. The filters were washed to 0.2 X SSC / 0.1% SDS, and exposed to film overnight. **Figure 4.3** (panel A) shows that clone G hybridised to the labelled SJ1 DNA and clones B, H, J and K hybridised to the labeled SJ5 DNA (panel B). So, after colony hybridisation there were five new clones identified, A, C, D, E, and F. Plasmid DNA preparation was carried out on these five clones. **Figure 4.4** shows the restriction digest obtained using *Eco*R I and *Xho* I enzymes. It was decided to sequence A, C and F since the inserts in clones D and E were very small. After manual sequencing (see 2.5.8.1) and searching the database, clone A had no obvious homologues, clone C was homologous to a cuticular collagen precursor gene (*col-12*) from *C. elegans* (Park and Kramer, 1990) and clone F was homologous to a cuticular collagen gene (*col-1*) from *C. elegans* (Kramer *et al.*, 1982).

4.2.4 Constitutive Clone

Since it was important to verify the expression pattern of these genes by Northern blotting, a constitutively expressed clone was needed for comparison. In order to identify such a clone the screening process was repeated in exactly the same way as described previously, except that this time plaques which appeared to be equally expressed in all three life cycle stages were selected. After secondary screening, 6 clones, EE1, EE2, EE3, EE4, EE5 and EE6, were picked which appeared to hybridise with the same intensity to filters probed with each of the different life cycle stages. The inserts were removed from the λ vector by *in vivo* excision and then plasmid DNA preparation was carried out. **Figure 4.5** shows the restriction digest using *Eco*R I and *Xho* I. Four of the clones appeared to have inserts large enough to warrant sequence analysis. Double stranded sequencing was carried out by cycle sequencing using SequiTherm™ DNA polymerase (Cambio) and T3 and T7 fluorescently labelled primers (Hybaid) on an automatic sequencer (Licor).

Figure 4.3

Colony hybridisation

Colony hybridisation of the 10 positive mosquito-derived L3 clones. Clones A-K were streaked in a grid and filter lifts taken:-

(A) Shows the filter hybridised to clone SJ1

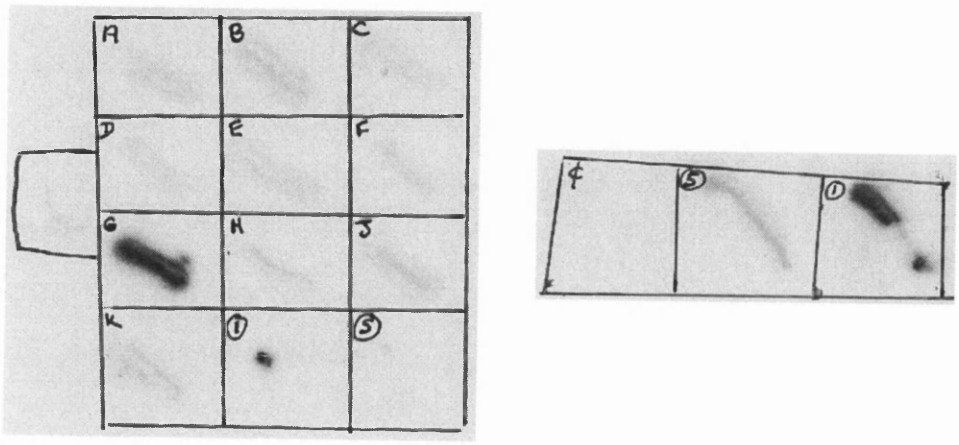
(B) Shows the filter hybridised to clone SJ5

Filters were washed to 0.2x SSC 0.1% SDS at 65°C.

Squares (1) and (5) show clones SJ1 and SJ5 respectively, hybridised to the corresponding radiolabelled probes. The inserts show filter lifts taken from a separate plate to act as a control.

Figure 4.3

A



B

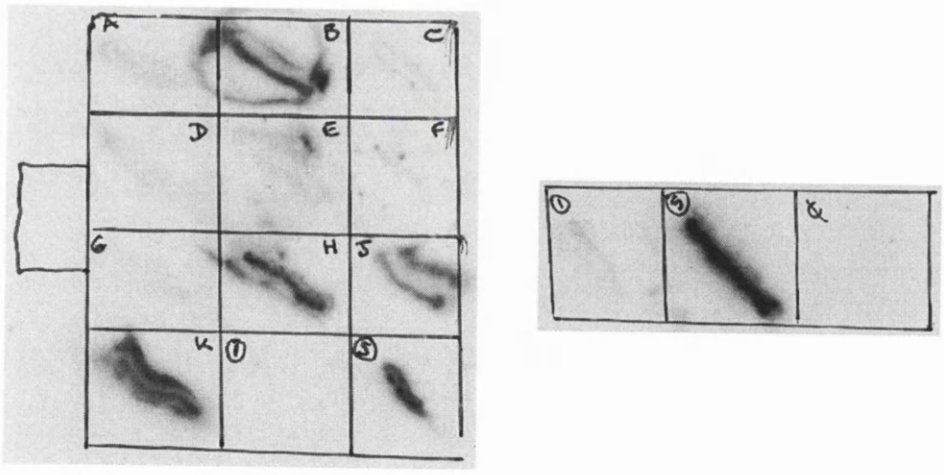


Figure 4.4

Restriction digests of mosquito-derived L3 clones (2)

1% agarose gel, stained with ethidium bromide, showing the size of inserts following restriction digest with *Eco*R I and *Xho* I.

Lane 1	Clone A	700bp
Lane 2	Clone C	250bp
Lane 3	Clone D	-
Lane 4	Clone E	-
Lane 5	Clone F	450bp
Lane 6	pBluescript	no insert control
Lane 7	λ*Hind* III markers	

Figure 4.4

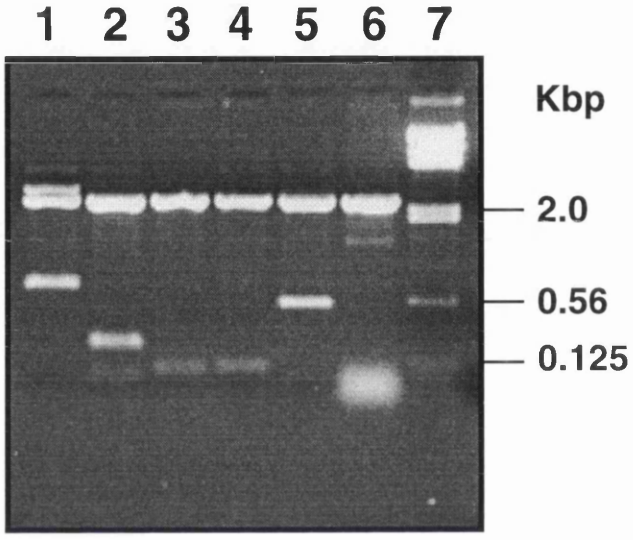


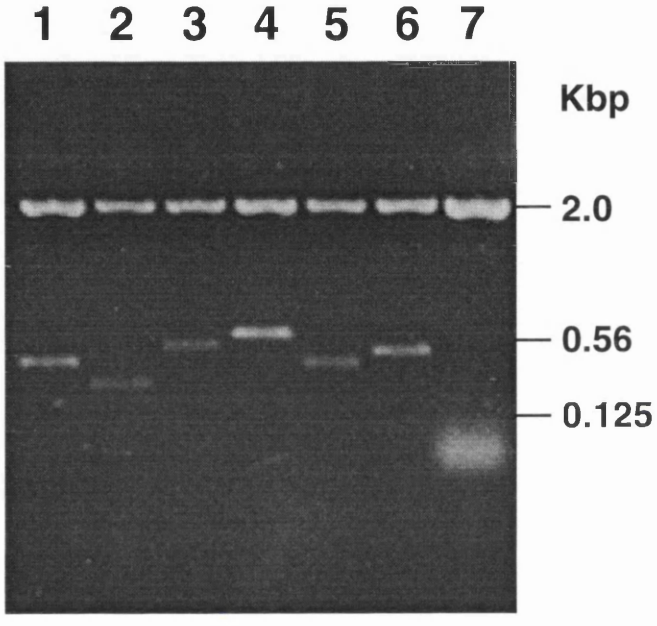
Figure 4.5

Restriction digests of equally expressed clones

1% agarose gel, stained with ethidium bromide, showing the size of inserts following restriction digest using *Eco*R I and *Xho* I.

Lane 1	Clone EE1	350bp
Lane 2	Clone EE2	250bp
Lane 3	Clone EE3	450bp
Lane 4	Clone EE4	500bp
Lane 5	Clone EE5	300bp
Lane 6	Clone EE6	400bp
Lane 7	pBluescript	no insert control

Figure 4.5



The only clone which showed significant homology to sequences in the database was EE4. This gene coded for a ribosomal protein with homology to a 60S acidic ribosomal protein P1 from *D. melanogaster* (Wigboldus, 1987). This clone was the same as the previously identified SH3 clone, selected by immunoscreening the adult cDNA library with anti-L3 serum.

4.2.4.1 Characterisation of EE4, the ribosomal protein cDNA

To confirm that the ribosomal clone was indeed equally expressed throughout the life cycle, a Northern blot was performed using RNA from mosquito-derived L3, 3 day post-infection L3, 15 day post-infection L4, adult worms and 37°C mf. **Figure 4.6** shows a representative ethidium stained gel and the corresponding Northern blot probed with the labelled EE4 clone. It can be seen that the ribosomal protein is expressed in all the life cycle stages tested, hybridising to a 0.56 kb transcript with approximately equal intensity in all the life cycle stages.

In order to further characterise the ribosomal protein gene, a Southern blot of *B. pahangi* genomic DNA, digested with various enzymes was carried out. **Figure 4.7** shows that the labelled EE4 clone hybridises strongly to a single band in each digest, with weaker second bands also being recognised, indicating the presence of other members of the gene family.

The nucleotide and amino acid sequence of the ribosomal protein clone is shown in **Figure 4.8**. The full length clone is 560 bases long and contains an open reading frame. The 5' spliced leader sequence is shown underlined. There is a 3' polyadenylated tail containing 29 adenine bases and, in this particular clone, the polyadenylation signal is the consensus AATAAA. There are 57 bases between the SL sequence and the ATG initiation codon (shown in bold). **Figure 4.9** shows the EE4 sequence compared to the previously identified SH3 clone, confirming that they are the same. The predicted amino acid sequence of the *Brugia* ribosomal protein (EE4) was compared with other ribosomal proteins present in the database. **Figure 4.10** shows a comparison

Figure 4.6

Northern blot analysis of clone EE4 (ribosomal protein)

RNA was isolated from different life cycle stages of *B. pahangi*, as described previously and then ~2μg per lane was analysed on a 1.2% denaturing formaldehyde agarose gel. The gel was blotted and probed with the labelled EE4 cDNA, and washed to 0.5x SSC 0.1% SDS at 65°C.

Panel **A** shows the hybridisation pattern obtained with the labelled EE4 cDNA, while panel **B** shows the ethidium stained gel before blotting.

Lane 1	Mf RNA
Lane 2	Mosquito-derived L3 RNA
Lane 3	3 day pi L3 RNA
Lane 4	15 day pi L4 RNA
Lane 5	Adult RNA

Figure 4.6

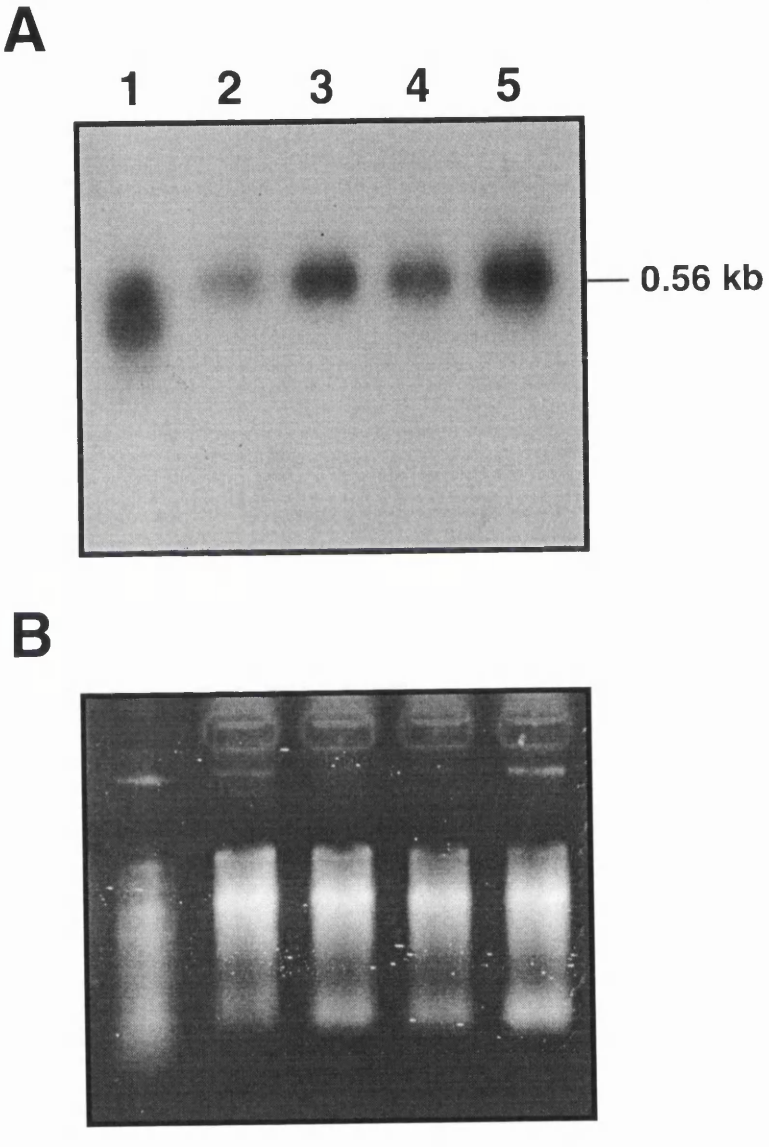


Figure 4.7

Southern blot analysis of clone EE4 (ribosomal protein)

Adult *B. pahangi* genomic DNA (10μg) was digested with the following enzymes;

Lane 1 *Hind* III
Lane 2 *Bgl* II
Lane 3 *Pst* I
Lane 4 *Eco*R I

The blot was hybridised to the labelled EE4 cDNA and washed to 0.2x SSC 0.1% SDS at 65°C.

Figure 4.7

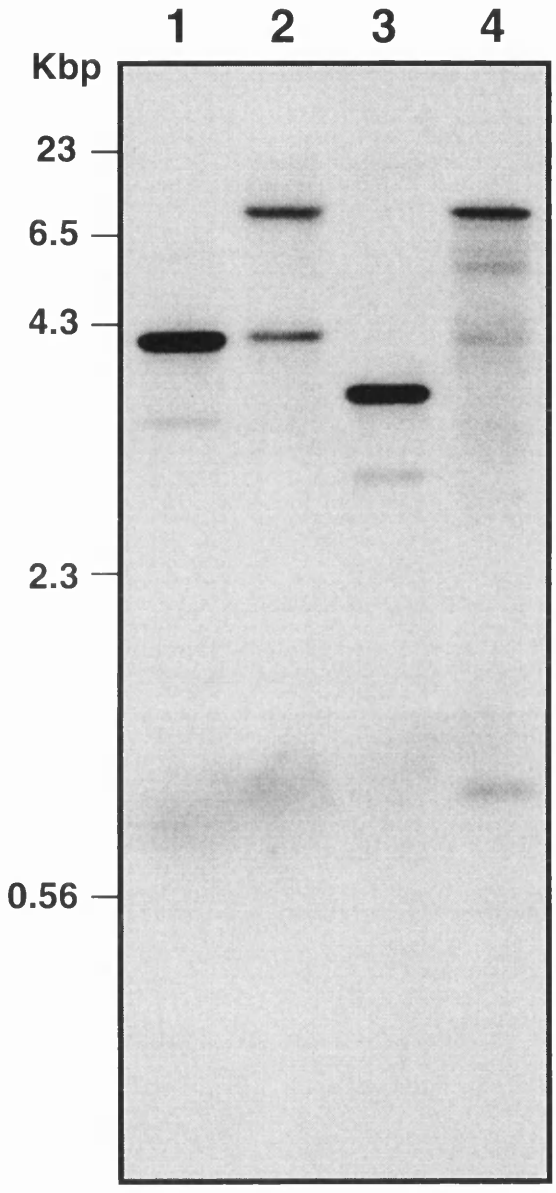


Figure 4.8

Nucleotide and amino acid sequence of clone EE4

The sequence was analysed using the GCG program. The SL1 sequence is shown underlined The initiation codon ATG is shown in bold and the asterisk indicates the termination codon.

Figure 4.8

Nucleotide and amino acid sequence of clone EE4

```
 1                                                                   60
GGTTTAATTACCCAAGTTTGAGACATAGTGCATTGTTCTCAAATAGAGCTAAGGCTTGAT

61                                                                  120
AGCGACGTGCACTTACTAGAGAGGAGAAATGGCAAACCAAGAATTAGCATGCGTCTATGC
                           M  A  N  Q  E  L  A  C  V  Y  A

121                                                                 180
AGCACTTATTTTGCAAGATGATGATGTTGCAATCACTGGTGATAAAATTTCAACCATCTT
 A  L  I  L  Q  D  D  D  V  A  I  T  G  D  K  I  S  T  I  L

181                                                                 240
GAAAGCTGCACATGTTGATGTGGAACCATTCTGGCCAGGATTATTTGCTAAGGCCTTGGA
 K  A  A  H  V  D  V  E  P  F  W  P  G  L  F  A  K  A  L  E

241                                                                 300
AGGAGTTGATGTGAAGTCGTTGATAACTAACATTAGTTCAAGTGTTGGTAGTGGTGGTGG
 G  V  D  V  K  S  L  I  T  N  I  S  S  S  V  G  S  G  G  G

301                                                                 360
TGGTGCAGCAGCAGGAGTAGCGGCACCTTCCGCAACAGCAGCAGCGGCAGCACCTGCAGC
 G  A  A  A  G  V  A  A  P  S  A  T  A  A  A  A  A  P  A  A

361                                                                 420
AGCTGCAGAAGAGAAAGAGGACAAGAAGAAGGAAGAGCCAAAAGAGGAATCAGATGATGA
 A  A  E  E  K  E  D  K  K  K  E  E  P  K  E  E  S  D  D  D

421                                                                 480
TATGGGTTTTGGTCTTTTCGACTAAAATTCGCATTATGTTGTTACTATTTTGAATCACAA
 M  G  F  G  L  F  D  *

481                                                                 540
ATTTGTTTATTTCTCTGCTTTTGGCTTTGGTAATAAAATTATACACAAGCAAAAAAAAAA

541                560
AAAAAAAAAAAAAAAAAAA
```

Figure 4.9

Nucleotide sequence of clone SH3 compared to clone EE4

The sequence of clone SH3, picked by immunoscreening, was compared to that of clone EE4 picked as a constitutive gene in the differential screen. Gaps were introduced to optimise the line up.

Figure 4.9

Nucleotide sequence of clone SH3 compared to clone EE4

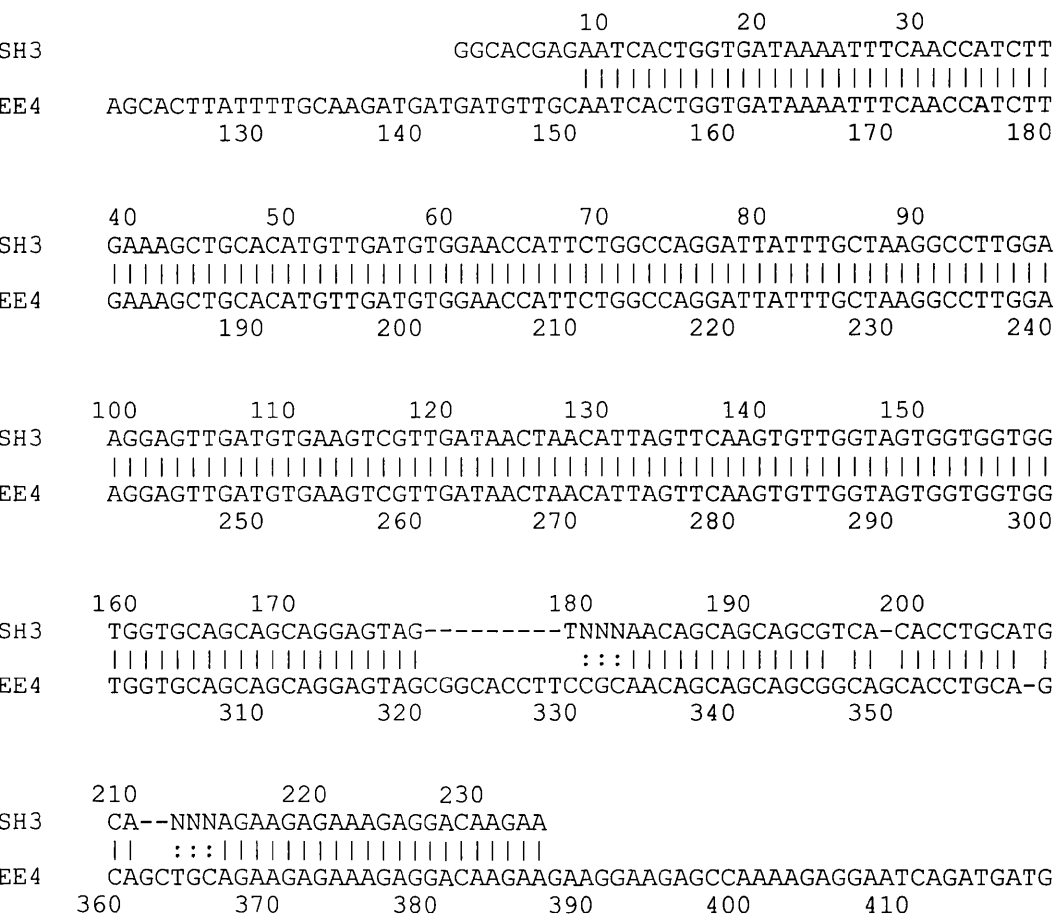


Figure 4.10

Amino acid pile up of 60S ribosomal proteins

The amino acid sequence alignment was produced using the pile-up program of GCG. Gaps were introduced to optimise the alignment.

EE4 - *B. pahangi* 60S ribosomal protein P1 (X91066)

ribod - *D. melanogaster* 60S ribosomal protein P1 (P08570)

riboa - *A. salina* 60S ribosomal protein P1 (P02402)

ribog - *G. gallus* 60S ribosomal protein P1 (P18660)

Figure 4.10

Amino acid pile up of 60S ribosomal proteins

```
         1                                                    50
  EE4   .MANQELACV YAALILQDDD VAITGDKIST ILKAAHVDVE PFWPGLFAKA
ribod   MSTKAELASV YASLILVDDD VAVTGEKINT ILKAANVEVE PYWPGLFAKA
riboa   .ASKDELACV YAALILLDDD VDITTEKVNT ILRAAGVSVE PYWPGLFTKA
ribog   MASVSELACI YSALILHDDE VTVTEDKINA LIKAAGVNVE PFWPGLFAKA

        51                                                  100
  EE4   LEGVDVKSLI TNISSSVGSG GGGAAAGVAA PSATAAAAAP AAAAEEKEDK
ribod   LEAINVKDLI TNIGSGVG.. .....AAPAG GAAPAAAAAA PAAESKKEEK
riboa   LEGLDLKSMI TNVGSGVG.. .....AAPAA GGAAAATEAP AAKEEKKEEK
ribog   LANIDIGSLI CNVGAGGG.. .APAAAAPAG GAAPAGGGAA PAEEKKEEEK

        101                                                 150
  EE4   KKEEPKEESD DDMGFGLFD*
ribod   KKEEESDQSD DDMGFGLFD. .......... .......... ..........
riboa   K..EESEEED EDMGFGLFD. .......... .......... ..........
ribog   K..EESEESD DDMGFGLFD. .......... .......... ..........
```

with the amino acid sequences of 60s ribosomal proteins from *D. melanogaster*, *G. gallus*, and *Artemia salina,* demonstrating the similarity of these sequences.

4.2.5 Attempts to find the full length cDNA for clone SJ1

Clones SJ1 and SJ3, picked in the first differential screen, were shown to have homology to nematode muscle tropomyosin. Since it was clear, from comparing the *Brugia* tropomyosin sequence to other tropomyosin sequences in the database, that the clones did not contain the full length cDNA, two approaches were adopted to try and isolate the complete gene.

4.2.5.1 Screening the adult cDNA library

It seemed likely that tropomyosin would also be present in the adult parasite, albeit at a low level of expression, so the first approach adopted was to screen the adult cDNA library using the labelled SJ1 clone as a probe in the hope of isolating a larger insert. This conventionally made library was more likely to contain full length cDNAs compared to the 3 day post-infection L3 library because PCR tends to preferentially amplify smaller cDNAs and the primers used, especially oligo dT, do not always anneal in the correct position. Altogether 14 plates, with approximately 10,000 plaques per plate, were screened but no positive plaques were identified, suggesting that tropomyosin is not represented in the adult cDNA library.

4.2.5.2 PCR using SJ1 specific primers

The other method adopted was to use PCR (see 2.6.3) to try and amplify tropomyosin from various life cycle stages and libraries. Two internal primers were made (2.6.2) to the SJ1 sequence, one from the 5' end, Tr1 (5' CTAAAGCAAAAATGGATGCGATCAAG 3') and one from the 3' end, Tr3 (5' TCCAAATTAGTATTTGCAACAGC 3'). A primer was also designed from the 3' end of the full length *T. colubriformis* tropomyosin sequence from the database, Tc3 (5' GGTTCTTGAGAGGCCGATAACT 3'). In order to determine whether the tropomyosin gene was represented in

the 3d pi L3 cDNA library, the adult cDNA or the *Brugia* genomic DNA libraries, PCR amplification was carried out using gene specific primers (Tr1 and Tr3), on aliquots of each library. It can be seen from **Figure 4.11** that no PCR product was obtained when using either the adult cDNA library (lane 7) or the *Brugia* genomic library (lane 8), although there was a band in the 3 day pi L3 library (lane 9), of equivalent size to that of the SJ1 cDNA.

The data described above suggested that the tropomyosin cDNA may not be expressed in the adult parasite. In order to further investigate the pattern of expression of this mRNA, PCR was carried out using reverse transcribed RNA from a variety of different life cycle stages. A product of the correct predicted size was obtained in all of the stages used, **Figure 4.11**: lane 2; L3 cDNA, lane 3; 3 day pi L3 cDNA, lane 4; L4 cDNA, lane 5; adult cDNA, lane 6; genomic DNA. A product was obtained using cDNA from adult parasites, despite the fact that no product could be obtained by PCR on either the adult cDNA or the genomic DNA libraries. It would appear that tropomyosin is found in the adult parasite, at a low level of expression, but is not present in the adult cDNA library, confirming the results obtained by screening the adult cDNA library. The smear in the genomic DNA (lane 6) could be due to the amplification of gene copies with different sized introns. The *T. colubriformis* primer (Tc3) did not yield any PCR product (data not shown), probably because the homology between the two species is not sufficient.

In additional experiments on the adult cDNA library, using Tr1 and the T7 primer, several discrete bands were obtained. This gel was blotted and the blot probed with labelled tropomyosin cDNA. However, no hybridisation was obtained.

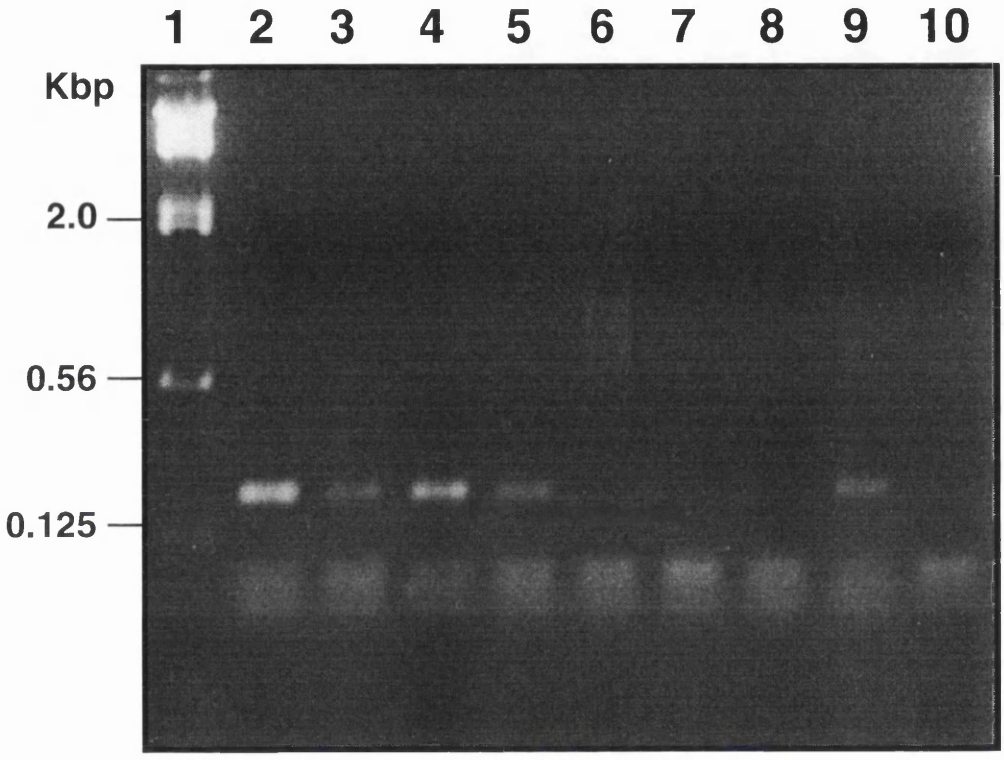
Figure 4.11

PCR products using tropomyosin specific primers

1.5% agarose gel, stained with ethidium bromide, showing the PCR products obtained using Tr1 and Tr3 primers designed from the SJ1 (tropomyosin) sequence, on a range of DNA templates.

Lane 1	λ*Hin*d III markers
Lane 2	L3 cDNA
Lane 3	3 day pi L3 cDNA
Lane 4	15 day pi L4 cDNA
Lane 5	Adult cDNA
Lane 6	Genomic DNA
Lane 7	Adult cDNA library
Lane 8	Genomic library
Lane 9	3 day pi L3 cDNA library
Lane 10	No DNA template control

Figure 4.11



The results obtained from several PCR reactions, using various combinations of the gene specific tropomyosin primers with the *T. colubriformis*, oligo dT and T7 primer, to try and obtain the full length cDNA, are shown below.

DNA template	Primer combination	PCR product obtained
L3 cDNA	Tr1 and Tr3	225 bp tropomyosin product
	Tr1 and oligo dT	Product same size as Tr1/Tr3 reaction
	Tr1 and Tc3	No product obtained
3dL3 cDNA	Tr1 and Tr3	225 bp tropomyosin product
d15 cDNA	Tr1 and Tr3	225 bp tropomyosin product
	Tr1 and oligo dT	Product same size as Tr1/Tr3 reaction
	Tr1 and Tc3	No product obtained
adult cDNA	Tr1 and Tr3	225 bp tropomyosin product
genomic DNA	Tr1 and Tr3	Smear - no distinct bands
adult cDNA library	Tr1 and Tr3	No product obtained
	Tr1 and T7	Several bands - but none recognised by tropomyosin probe
genomic DNA library	Tr1 and Tr3	No product obtained
3dL3 cDNA library	Tr1 and Tr3	225 bp tropomyosin product
	Tr1 and oligo dT	Product same size as Tr1/Tr3 reaction

4.2.6 Characterisation of the four clones up-regulated in the mosquito-derived L3 stage

Basic characterisation of the clones was carried out by Southern blotting as previously described in the Materials and Methods chapter (see 2.5.6, 2.5.6.1, 2.5.6.2, and 2.4.3). Northern blots were also performed to confirm the expression pattern of the four genes. RNA was isolated from mosquito derived L3, 3 day post-infection L3, 15 day post-infection L4 and adult parasites as described in 2.5.7. Since the RNA yield is so low, due to the numbers of parasites available, it is not practical to take OD readings and so an aliquot of RNA was first run out on a 1.2% denaturing formalehyde gel to check the concentration of each preparation by eye. An estimated amount of RNA from each life cycle stage was run out on a gel (see 2.5.7.2) to try and achieve equal loading in each lane. Northern blots were then carried out as previously described in 2.5.7.3 and 2.4.3.

4.2.6.1 Characterisation of clone SJ1, the tropomyosin like cDNA

Clone SJ1 was isolated on the basis of differential hybridisation to the mosquito derived L3 cDNA probe. In order to confirm the expression pattern of this mRNA, Northern blots were carried out with different life cycle stages. **Figure 4.12,** panel A, shows a Northern blot probed with the SJ1 clone, while panel B shows the same blot re-probed with the ribosomal protein gene EE4. The tropomyosin cDNA hybridised to a single transcript of 2.3 kb, confirming that only a small fragment of the cDNA had been cloned. It can be seen that there is a very strong signal in the L3 (Lane 1), a much weaker signal in the 3 day post-infection L3 (Lane 2) and the day 15, L4 (Lane 3), and no apparent signal in the adult (Lane 4). When the blot was re-probed with EE4, a signal of approximately equal strength was obtained from each of the lanes. By comparison of the intensity of signal obtained with the tropomyosin probe to that obtained with EE4 it would appear that the SJ1 clone is much more highly expressed in the L3 stage than in the adult.

Figure 4.12

Northern blot analysis of clone SJ1 (tropomyosin)

B. pahangi RNA was isolated from different life cycle stages, as described previously, and then ~2μg per lane was analysed on a 1.2% denaturing formaldehyde agarose gel. The gel was blotted and probed with the labelled SJ1 cDNA. The blot was washed to 0.5x SSC 0.1% SDS at 65°C.

Panel **A** shows the hybridisation pattern obtained with the SJ1 probe, while panel **B** shows the same blot re-probed with the constitutively expressed labelled EE4 clone.

Lane 1	Mosquito-derived L3 RNA
Lane 2	3 day pi L3 RNA
Lane 3	15 day pi L4 RNA
Lane 4	Adult RNA

Figure 4.12

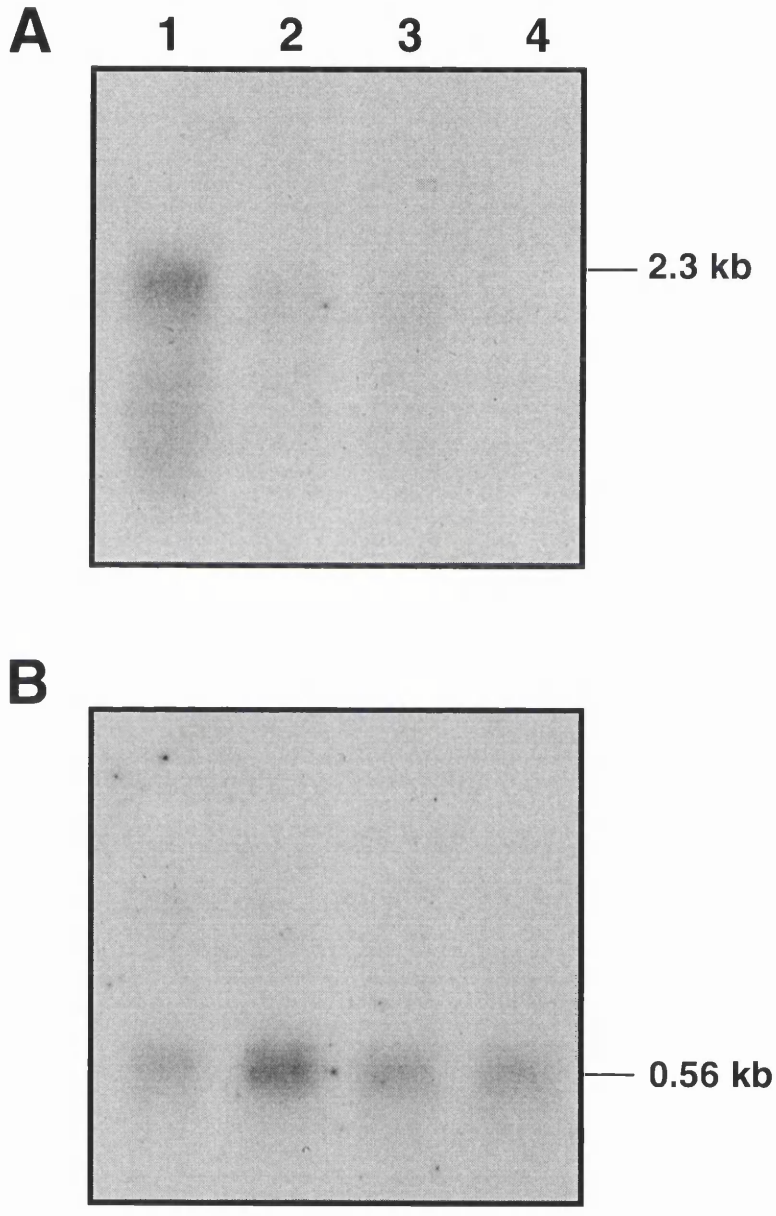


Figure 4.13 shows a Southern blot probed with clone SJ1, and although there is only one dominant band in each restriction digest, there are other weaker bands which would suggest that the SJ1 gene belongs to a small multigene family.

Clone SJ1 had an 89.2% identity in a 74 amino acid overlap with nematode muscle tropomyosin mRNA from *T. colubriformis* (Frenkel *et al.,* 1989). The nucleotide and amino acid sequence of clone SJ1 is shown in **Figure 4.14**. The clone is 303 bases long, with the SL sequence (shown underlined), and the ATG initiation codon (shown in bold), both present. The 5'-untranslated region is 57 bases long. At the 3' end of the cDNA there is a run of A's, but no polyadenylation signal. Presumably the oligo dT primer has annealed to this A rich stretch of sequence. It is unlikely that the full-length cDNA has been cloned, as from the Northern blots, the transcript size is 2.3 kb. A pile up of tropomyosins from various species, including *T. colubriformis* is shown in **Figure 4.15**, demonstrating the high degree of homology between the two nematode species but less so with the vertebrate tropomyosins.

4.2.6.2 Characterisation of clone SJ5 (coding for a glutamic acid rich protein)

The other cDNA isolated in the original screen showed homology to glutamic acid rich proteins. This clone was characterised by Northern and Southern blotting as described for the tropomyosin cDNA. **Figure 4.16** shows a Northern blot probed with the labelled SJ5 clone, and the same blot reprobed with the EE4 clone to demonstrate the equal transfer of RNA to each lane. A transcript of 1.8 kb is present in the larval stages analysed. A very strong signal is obtained from RNA isolated from L3 (Lane 1), with weaker signals in the 3 day post-infection L3 (Lane 2) and day 15, L4 (Lane 3). No signal is obtained from adult RNA (Lane 4). Using EE4, a signal is apparent in all life cycle stages, including the adult. This experiment confirms that it is possible to select for stage specific gene expression using a differential hybridisation approach.

Figure 4.13

Southern blot analysis of clone SJ1 (tropomyosin)

Adult *B. pahangi* genomic DNA (10μg) was digested with the following enzymes;

Lane 1 *Eco*R I
Lane 2 *Pst* I
Lane 3 *Bgl* II
Lane 4 *Hin*d III

The blot was hybridised to the labelled SJ1 cDNA and washed to 0.2x SSC 0.1% SDS at 65°C.

Figure 4.13

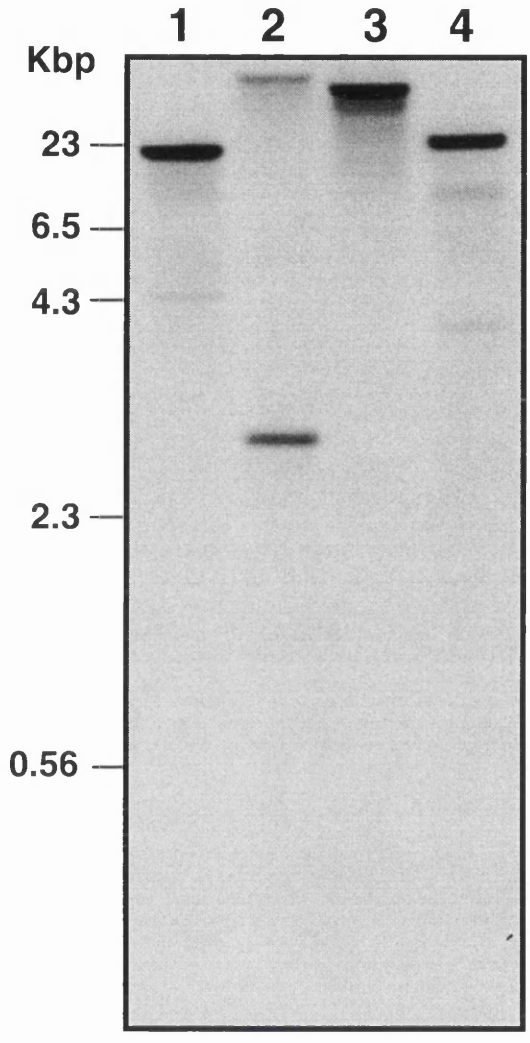


Figure 4.14

Nucleotide and amino acid sequence of clone SJ1 (tropomyosin)

The sequence was analysed using the GCG program. The SL1 sequence is shown underlined and the initiation codon ATG shown in bold.

Figure 4.14

Nucleotide and amino acid sequence of clone SJ1 (tropomyosin)

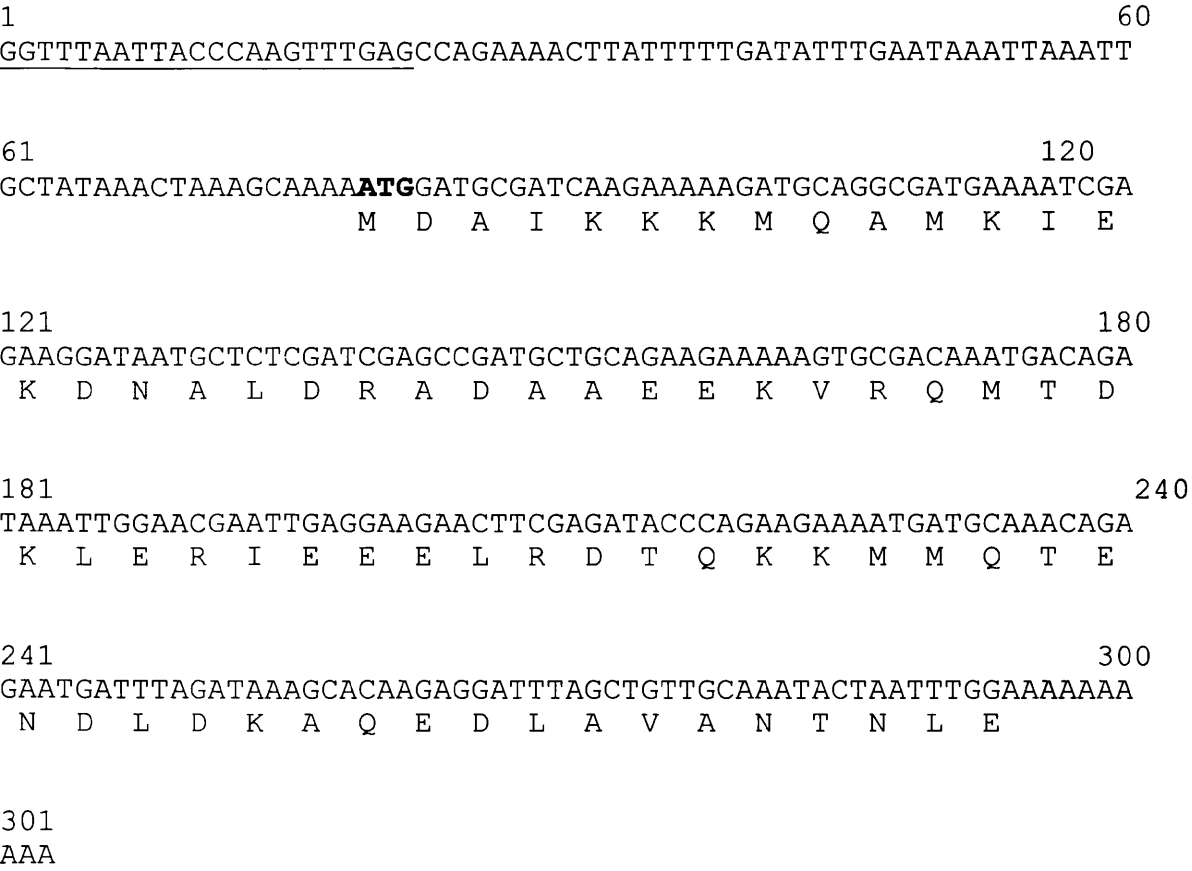


Figure 4.15

Amino acid pile up of tropomyosins

Amino acid sequence alignment was produced using the pile up program from GCG.

tropfr - frog tropomyosin (P13105)

tropze - zebra fish tropomyosin (P13104)

tropco - *T. colubriformis* tropomyosin (P15846)

tropbp - *B. pahangi* tropomyosin (clone SJ1)

tropdr - *D. melanogaster* tropomyosin (P09491)

Figure 4.15

Amino acid pile up of tropomyosins

```
        1                                                   50
tropfr  MDAIKKKMQM LKLDKENALD RAEQAEADKK GAEDKSKQLE DELVAMQKKM
tropze  MDAIKKKMQM LKLDKENALD RAEQAETDKK AAEERSKQLE DDLVALQKKL
tropco  MDAIKKKMQA MKIEKDNALD RADAAEEKVR QITEKLERVE EELRDTQKKM
tropbp  MDAIKKKMQA MKIEKDNALD RADAAEEKVR QMTDKLERIE EELRDTQKKM
tropdr  MDAIKKKMQA MKLEKDNAID KADTCENQAK DANSRADKLN EEVRDLEKKF

        51                                                  100
tropfr  KGTEDELDKY SEALKDAQEK LELAEKKATD AEADVASLNR RIQLVEEELD
tropze  KATEDELDKY SEALKDAQEK LELAEKKATD AEGDVASLNR RIQLVEEELD
tropco  MQTENDLDKA QEDLAAATSQ LEEKEKKVQE AEAEVAALNR RMTLLEEELE
tropbp  MQTENDLDKA QEDLAVANTN LEKK..... .......... ..........
tropdr  VQVEIDLVTA KEQLEKANTE LEEKEKLLTA TESEVATQNR KVQQIEEDLE
```

Figure 4.16

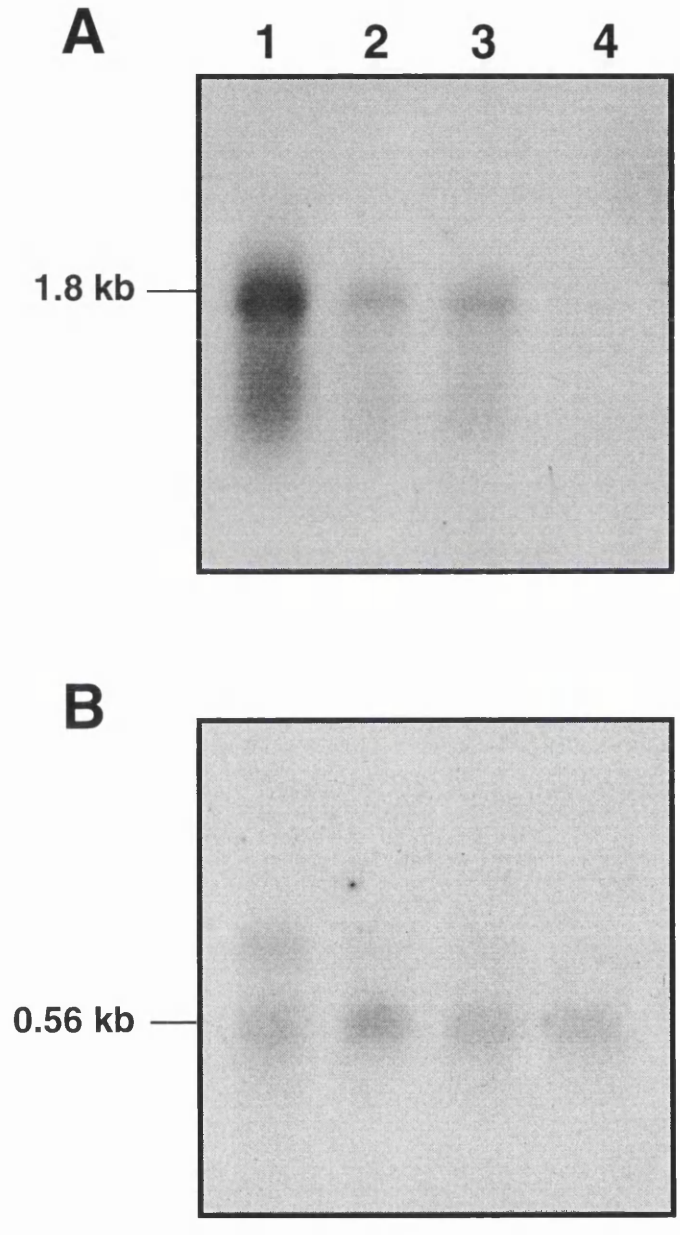
Northern blot analysis of clone SJ5 (glutamic acid-rich protein)

B. pahangi RNA was isolated from different life cycle stages, as described previously, and then ~2μg per lane was analysed on a 1.2% denaturing formaldehyde agarose gel. The gel was blotted and probed with the labelled SJ5 cDNA. The blot was washed to 0.5x SSC 0.1% SDS at 65°C.

Panel **A** shows the hybridisation pattern obtained with the SJ5 probe, while panel **B** shows the same blot re-probed with the constitutively expressed EE4 clone.

Lane 1	Mosquito-derived L3 RNA
Lane 2	3 day pi L3 RNA
Lane 3	15 day pi L4 RNA
Lane 4	Adult RNA

Figure 4.16



A Southern blot probed with clone SJ5 is shown in **Figure 4.17**. It is likely that the SJ5 clone is a single copy gene as there is one major band present in each of the lanes. However, in the *Pst* I restriction digest there are two bands present, and this is due to the presence of an internal *Pst* I restriction site in the DNA fragment used as a probe.

The nucleotide and amino acid sequence of clone SJ5 are shown in **Figure 4.18**. The clone is 404 bases long and has the SL sequence (shown underlined) and the ATG initiation codon (shown in bold). There are 40 bases between the SL sequence and the ATG codon. As with the tropomyosin cDNA, the 3' end contains a run of A's. However, this is unlikely to represent the poly A tail, as there is no termination codon and the transcript size predicted from the Northern blot is 1.8 kb. There are many GAA repeats present which code for glutamic acid (E) and in fact 33% of the predicted partial protein sequence comprises glutamic acid. **Figure 4.19 (A)** shows the SJ5 clone compared to a glutamic acid rich protein (GARP) from *Plasmodium falciparum* (Triglia *et al.*, 1988). Other homologues pulled out from the data base include a number of troponins and neurofilament proteins from various species and **Figure 4.19 (B)** also shows the sequence of chicken troponin (Smillie *et al.*, 1988) compared to clone SJ5. Whether any of these are true homologues remains unclear since the presence of so many GAA repeats could be misleading when searching the database.

4.2.6.3 Characterisation of clone C (coding for a cuticular collagen precursor)

Two collagen genes were isolated in the second differential screen; the first, clone C, showed homology to a cuticular collagen precursor from *C. elegans*. The expression pattern of this collagen was investigated by Northern blot. **Figure 4.20** shows a Northern blot probed with the labelled clone C and the corresponding ethidium stained gel. It can be seen that there is a strong signal from the L3 RNA (Lane 1) and from day 15 L4 RNA (Lane 3), with a weaker signal in the 2 day post-infection L3 (Lane 2) (RNA from this time point was used due to a lack of material at 3 days post-infection). No signal was present

Figure 4.17

Southern blot analysis of clone SJ5 (glutamic acid-rich protein)

Adult *B. pahangi* genomic DNA (10μg) was digested with the following enzymes;

Lane 1 *Hin*d III
Lane 2 *Bgl* II
Lane 3 *Pst* I
Lane 4 *Eco*R I

The blot was hybridised to the labelled SJ5 cDNA and washed to 0.2x SSC 0.1% SDS at 65°C.

Figure 4.17

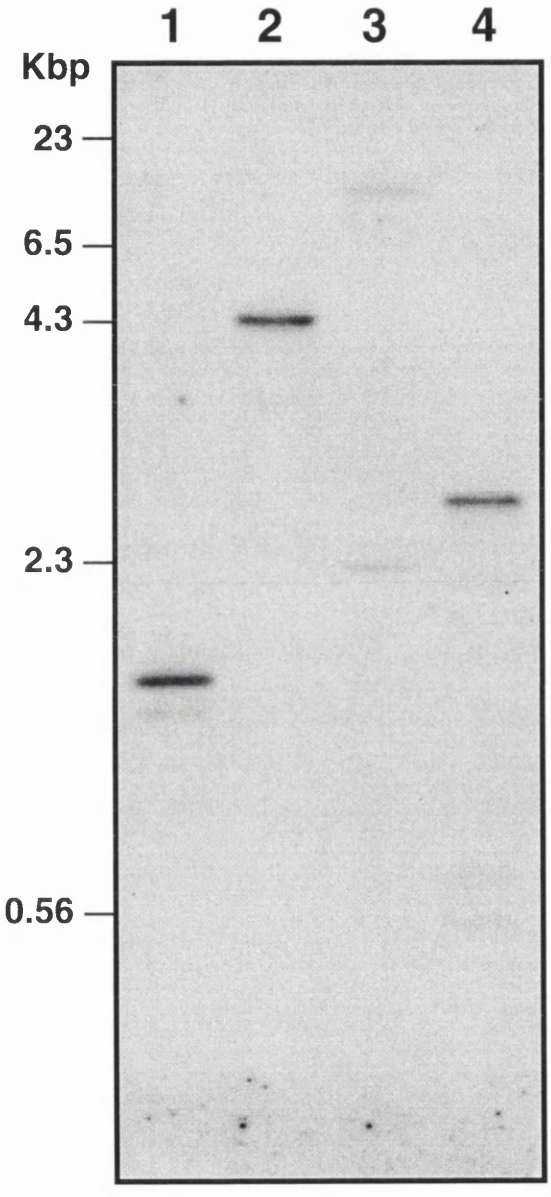


Figure 4.18

Nucleotide and amino acid sequence of clone SJ5 (glutamic acid rich protein)

The sequence was analysed using the GCG program. The SL1 sequence is shown underlined and the initiation codon ATG is shown in bold.

Figure 4.18

Nucleotide and amino acid sequence of clone SJ5 (glutamic acid rich protein)

```
1                                                                    60
GGTTTAATTACCCAAGTTTGAGCTTTTGTTCAATCCATCAAAATAAGATTGCATAGAGAG

61                                                                  120
AATGGCAGACGAAGAAGAAGAGATTACTGAGGAGGAGGAAGATGAAGAAGTAGAAGAAGA
 M  A  D  E  E  E  E  E  I  T  E  E  E  E  D  E  E  V  E  E  E

121                                                                 180
AGAAGAAGAAGCAGCTGAAGCTGAAGCACCAGCAGCTCCAGCATCTGCAGATGATGAAGC
 E  E  E  A  A  E  A  E  A  P  A  A  P  A  S  A  D  D  E  A

181                                                                 240
ACCTGCTGAAAGAGCAGAAGCTACACCTCAATTAAGACGTGCACCACCTCAGGAAAAAGA
 P  A  E  R  A  E  A  T  P  Q  L  R  R  A  P  P  Q  E  K  D

241                                                                 300
TGAAACTCCGGCTGAACAAACTGAAGCTGAAAAGGCTATGCTAGCTGCTAAAAAAAGGCA
 E  T  P  A  E  Q  T  E  A  E  K  A  M  L  A  A  K  K  R  H

301                                                                 360
TCAGGAAGAAGAAGCAGCTAAAATTCAAGATTACGAAGAAAGACGACGAATCGAGAAAGA
 Q  E  E  E  A  A  K  I  Q  D  Y  E  E  R  R  R  I  E  K  E

361                                                 404
ACAGATCGAAGAAGAGTTGCGAACTTTAAAAAAAAAAAAAAAAA
 Q  I  E  E  E  L  R  T  L
```

Figure 4.19

Amino acid sequence of clone SJ5 compared to two of its homologues

The sequences were aligned using the GCG program. **A** shows the *B. pahangi* E rich protein compared to the *P. falciparum* GARP protein (P13816). **B** shows the homolgy between Clone SJ5 and chicken troponin (P12618).

Figure 4.19

Amino acid sequence of clone SJ5 compared to (A) *P. falciparum* glutamic acid-rich protein and (B) *G. gallus* troponin

A

```
P13816; Glutamic acid-rich protein
27.6% identity in 105 aa overlap


                                        10        20        30
sj5                                    MADEEEEITEEEEDEEVEEEEEEEAAEAEAPAA
                                         :|| | :|||||:|| |||||||::|:|: ::
garp_p LQKQKHVDKEEDKKEESKEVQEESKEVQEDEEEVEEDEEEEEEEEEEEEEEEEEEEEE
      540       550       560       570       580       590

             40        50        60        70        80        90
sj5    PASADDEAPAERAEATPQLRRAPPQEKDETPAEQTEAEKAMLAAKKRHQEEEAAKIQDYE
        :::::|: :| :|::::   : ::|::::::|::::|::  :::: :::|:::: :: |
garp_p EEEEEEEDEDEEDEDDAEEDEDDAEEDEDDAEEDDDEEDDDEEDDDEDEDEDEEDEEEEE
      600       610       620       630       640       650

           100
sj5    ERRRIEKEQIEEELRT
       |    ::::|: :||:
garp_p EEEEESEKKIKRNLRKNAKI
      660       670



B
P12618; Chicken troponin
33.3% identity in 45 aa overlap


             30        40        50        60        70        80
sj5    EEAAEAEAPAAPASADDEAPAERAEATPQLRRAPPQEKDETPAEQTEAEKAMLAAKKRHQ
                                       ||| ::|:: ::||:::: :|:||::| ::
trt1_c IELQALIDSHFEARRKEEEELVALKERIEKRRAERAEQQRIRAEKEKERQARLAEEKARR
          90       100       110       120       130       140

             90       100
sj5    EEEAAKIQDYEERRRIEKEQIEEELRT
       |||:|| :: :: :: ::
trt1_c EEEDAKRKAEDDLKKKKALSSMGASYSSYLAKADQKRGKKQTARETKKKVLAERRKPLNI
         150       160       170       180       190       200
```

Figure 4.20

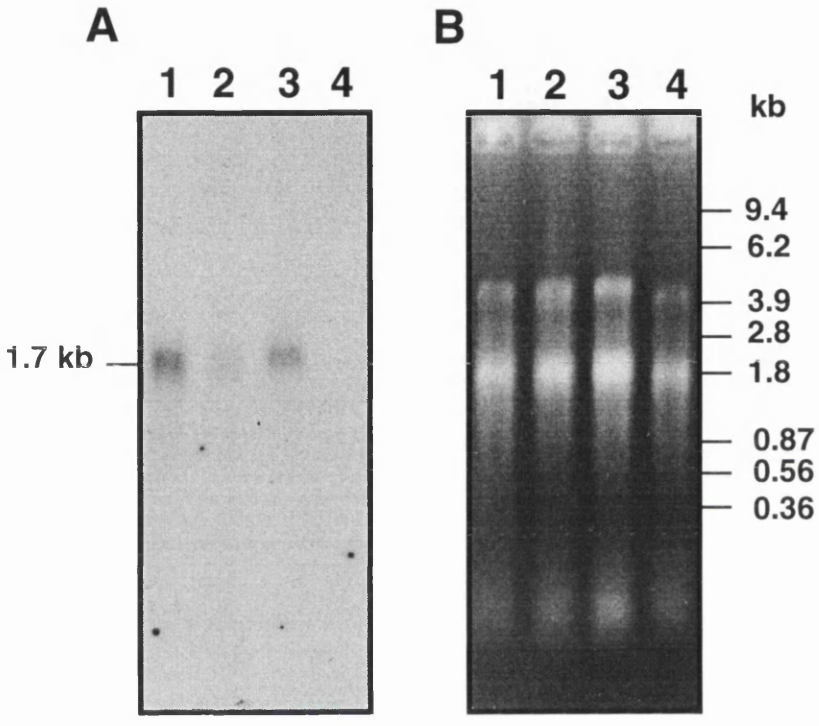
Northern blot analysis of clone C (cuticular collagen precursor)

B. pahangi RNA was isolated from different life cycle stages, as described previously, and then ~2μg per lane was analysed on a 1.2% denaturing formaldehyde agarose gel. The gel was blotted and probed with the labelled clone C cDNA. The blot was washed to 0.5x SSC 0.1% SDS at 65°C.

Panel **A** shows the hybridisation pattern obtained with the clone C probe, while panel **B** shows the ethidium stained gel before blotting.

Lane 1	Mosquito-derived L3 RNA
Lane 2	2 day pi L3 RNA
Lane 3	15 day pi L4 RNA
Lane 4	Adult RNA

Figure 4.20



in RNA isolated from the adult stage (Lane 4). The ethidium stained gel shows that there was less RNA present in the adult lane, but a signal would have been expected if the collagen precursor gene was expressed at the same level as in the L3 life cycle stage. Therefore, the Northern blot seems to indicate that this gene, as with the other genes characterised, is only expressed at a very low level in the adult parasite, if at all.

Figure 4.21 shows a Southern blot probed with clone C. There appears to be one dominant band in each restriction digest, with other weaker bands also present. This would indicate that the collagen presursor belongs to a multigene family.

Clone C had 49.4% identity in a 83 amino acid overlap to cuticle collagen 12 precursor and also to cuticle collagen 13 precursor from *C. elegans* (Park and Kramer, 1990), since these two transcripts are identical when mature. A map of the nucleotide and amino acid sequence of clone C is shown in **Figure 4.22**. The clone is only 294 nucleotides long, but the SL sequence (shown underlined) and the ATG initiation codon (shown in bold) are present. There is no termination codon, although there is a string of A's at the 3' end. From the pile up shown in **Figure 4.23** it can be seen that the *Brugia* clone is not full length. Only the first 81 amino acids are represented, whereas the *C. elegans* transcript is 316 amino acids long. The apparent poly A tail is probably due to spurious priming by the oligo dT primer. The pile up shows that had a longer transcript been obtained, the characteristic collagen $(Gly\text{-}X\text{-}Y)_n$ repeats would have been found. In addition, the transcript size from the Northern blot is in the region of 1.7 kb. However, it can be seen that there is quite a high level of homology between the *C. elegans* collagens 12 and 13 and the *Brugia* clone C, even though the collagen $(Gly\text{-}X\text{-}Y)_n$ repeats are not present, which would confer an even higher level of homology.

4.2.6.4 Characterisation of clone F (coding for a cuticular collagen)

The final clone analysed was clone F which showed homology to a cuticular collagen from *C. elegans*. As with the other L3 cDNAs, it was characterised by Northern and Southern blotting. **Figure 4.24** shows a Northern blot probed

Figure 4.21

Southern blot analysis of clone C (cuticular collagen precursor)

Adult *B. pahangi* genomic DNA (10μg) was digested with the following enzymes;

Lane 1 *Hin*d III

Lane 2 *Bgl* II

Lane 3 *Pst* I

Lane 4 *Eco*R I

The blot was hybridised to the labelled clone C cDNA and washed to 0.2x SSC 0.1% SDS at 65°C.

Figure 4.21

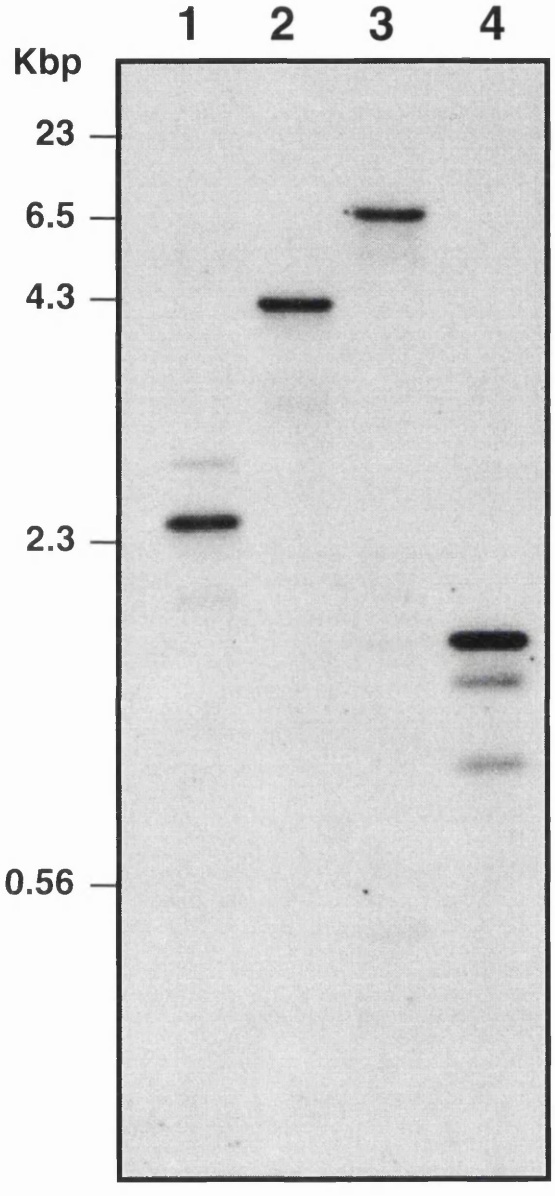


Figure 4.22

Nucleotide and amino acid sequence of clone C (cuticular collagen precursor)

The sequence was analysed using the GCG program. The SL1 sequence is shown underlined and the initiation codon ATG is shown in bold.

Figure 4.22

Nucleotide and amino acid sequence of clone C (cuticular collagen precursor)

```
1                                                                    60
GGTTTAATTACCCAAGTTTGAGGTAGCAAGATGGTTGATTCTGATGATCCGAAACAGCTT
                              M  V  D  S  D  D  P  K  Q  L

61                                                                  120
CTCATTGAGGCAGAAAGCATGAAAAAGCTTGCCTTTTGCGGTGTTGCTGTTTCTACCGTA
L  I  E  A  E  S  M  K  K  L  A  F  C  G  V  A  V  S  T  V

121                                                                 180
GCTACTCTGGTAGCAATTATTTGCGTACCAATGCTCTGCACCTACATGCAAAATGTGCAG
A  T  L  V  A  I  I  C  V  P  M  L  C  T  Y  M  Q  N  V  Q

181                                                                 240
TCTAACTTGCAAGATGAGATTAGCTTCTGCAGGACCCGCGCAATTGGATTACGAGGAGAA
S  N  L  Q  D  E  I  S  F  C  R  T  R  A  I  G  L  R  G  E

241                                                          294
TTCACCAAACTCGAATCATCGCGTTCAGCACTGAAAAAAAAAAAAAAAAAAAAA
F  T  K  L  E  S  S  R  S  A  L
```

Figure 4.23

Amino acid pile up of cuticular collagens

The sequences were aligned using the pile up program from GCG. Gaps have been included to optimise the alignment.

cc12	- *C. elegans* cuticle collagen 12 precursor	(P20630)
cc13	- *C. elegans* cuticle collagen 13 precursor	(P20631)
ccbp	- *B. pahangi* cuticle collagen precursor	(clone C)
cc36	- *C. elegans* cuticle collagen 36	(P34803)
cc34	- *C. elegans* cuticle collagen 34	(P34687)

Figure 4.23

Amino acid pile up of cuticular collagens

```
      1                                                  50
cc12  ...MTEDPKQ IAQETESLRK VAFFGIAVST IATLTAIIAV PMLYNYMQHV
cc13  ...MSEDLKQ IAQETESLRK VAFFGIAVST IATLTAIIAV PMLYNYMQHV
ccbp  .MVDSDDPKQ LLIEAESMKK LAFCGVAVST VATLVAIICV PMLCTYMQNV
cc36  MKIDKEDDQQ .....QQMRR VAFFAVAVST AAVISSIVTL PMIYSYVQSF
cc34  ........MD LETRIKAYRF VAYSAVAFSV VAVISVCVTL PMVYNYVHHV

      51                                                 100
cc12  QSSLQSEVEF CQHRSNGLWD EYKRFQGVSG VEGRIKRDAY HRSLGVSGAS
cc13  QSSLQSEVEF CQHRSNGLWD EYKRFQGVSG VEGRIKRDAY HRSLGVSGAS
ccbp  QSNLQDEISF CRTRAIGLRG EFTKLESSRS ALKKKKKKK. ..........
cc36  QSHLIMETEF CKTRARDMWV EMQVLH..KS GVTRSRRDAG YKEGSGSGGS
cc34  KRTMHNEITF CKGSAKDIWN EVHALKSLPN S.NRTARQAY ..........

      101                                                150
cc12  RKARRQSYGN DAAVGGFGGS SGGSCCSCGS GAAGPAGSPG QDGAPGNDGA
cc13  RKARRQSYGN DAAVGGFGGS SGGSCCSCGS GAAGPAGSPG QDGAPGNDGA
ccbp  .......... .......... .......... .......... ..........
cc36  ......GSGG YGGPTGAGAD IGPTCCPCQQ GPAGPPGPAG DTGPNGNDGH
cc34  .........N DAAVTGGGAQ SGSCESCCLP GPPGPAGTPG KPGRPGKPGA
```

Figure 4.24

Northern blot analysis of clone F (cuticular collagen)

B. pahangi RNA was isolated from different life cycle stages, as described previously, and then ~2μg per lane was analysed on a 1.2% denaturing formaldehyde agarose gel. The gel was blotted and probed with the labelled clone F cDNA. The blot was washed to 0.5x SSC 0.1% SDS at 65°C.

Panel **A** shows the hybridisation pattern obtained with the clone F probe, while panel **B** shows the ethidium stained gel before blotting.

Lane 1	Mosquito-derived L3 RNA
Lane 2	3 day pi L3 RNA
Lane 4	15 day pi L4 RNA
Lane 4	Adult RNA

Figure 4.24

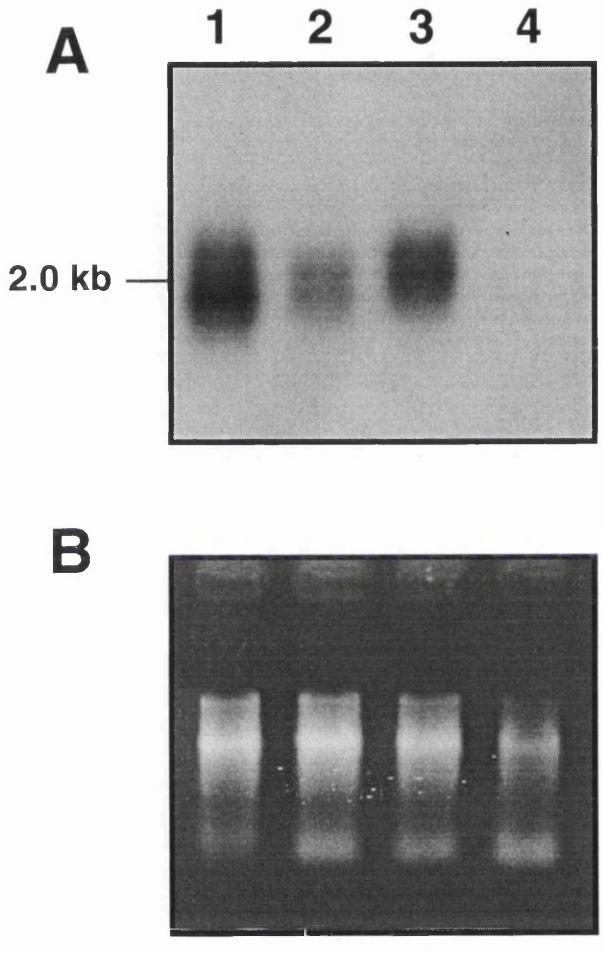


Figure 4.25

Southern blot analysis of clone F (cuticular collagen)

Adult *B. pahangi* genomic DNA (10μg) was digested with the following enzymes;

Lane 1 *Hind* III
Lane 2 *Bgl* II
Lane 3 *Pst* I
Lane 4 *Eco*R I

The blot was hybridised to the labelled clone F cDNA and washed to 0.2x SSC 0.1% SDS at 65°C.

Figure 4.25

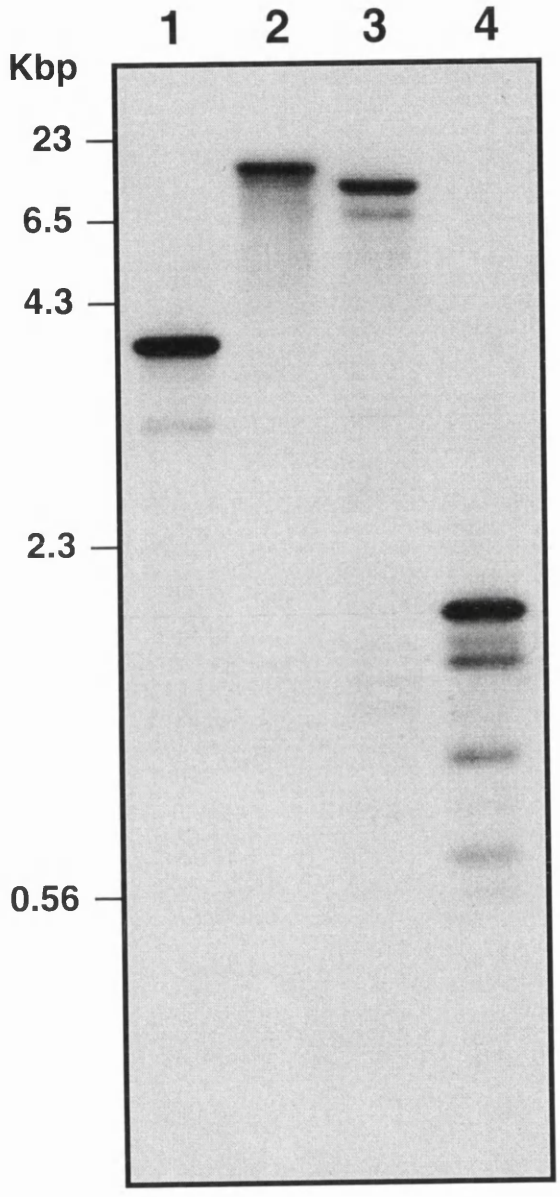


Figure 4.26

Nucleotide and amino acid sequence of Clone F (cuticular collagen)

The sequence was analysed using the GCG program. The SL1 sequence is shown underlined and the initiation codon ATG shown in bold. The apparent termination codon is marked by an asterisk.

Figure 4.26

Nucleotide and amino acid sequence of clone F (cuticular collagen)

```
1                                                                   60
GGTTTAATTACCCAAGTTTGAGAAAAA**ATG**AATTCGAAAATATTTGTTATAATTTGTGGA
                              M  N  S  K  I  F  V  I  I  C  G

61                                                                 120
ATTGTATATTTGGTGAAAGAAGTGGTAATGGAGTTGGATACATCCATGGAATATAATAAT
I  V  Y  L  V  K  E  V  V  M  E  L  D  T  S  M  E  Y  N  N

121                                                                180
ATAGTTAAACAAGTCAATATGCAAAGTGCTATCATCAATAATTTGCAAAGACGTTTGGAT
I  V  K  Q  V  N  M  Q  S  A  I  I  N  N  L  Q  R  R  L  D

181                                                                240
AAATTGCTGGAAAAACAAAGTATGCAAGATGAAATTGTATATCAGAATCAAGGAAAAACC
K  L  L  E  K  Q  S  M  Q  D  E  I  V  Y  Q  N  Q  G  K  T

241                                                                301
AATTGTAAATGCAAAGCAGGACCAAGAGGAGCACCAGGAGCTGCAGGACCACAAGGTGCA
N  C  K  C  K  A  G  P  R  G  A  P  G  A  A  G  P  Q  G  A

301                                                                360
CCAGGACCAGAAGGACCATCAGGACCACAAGGACCACCAGGACCAGTAGGACAGCAAGGA
P  G  P  E  G  P  S  G  P  Q  G  P  P  G  P  V  G  Q  Q  G

361                                                                420
CCACCAGTGATACCGGATGATGATGATACATGCTTTGGTACTTAGTGGGGTAAAATTTAA
P  P  V  I  P  D  D  D  D  T  C  F  G  T  *

421            436
AAAAAAAAAAAAAAAA
```

Figure 4.27

Amino acid pile up of cuticle collagens

The sequences were aligned using the pile up program from GCG. Gaps were introduced to optimise the alignment.

pcce1	- *C. elegans* cuticle collagen 1	(P08124)
pchc	- *H. contortus* cuticle collagen 2C	(P16252)
pchs	- *H. sapiens* collagen alpha 2(x1) chain	(P13942)
pcce2	- *C. elegans* cuticle collagen 8	(P18833)
pcmull	- *E. muelleri* short chain collagen C4	(P18503)
sjf	- *B. pahangi* cuticle collagen	(clone F)

Figure 4.27

Amino acid pile up of cuticle collagens

```
        1                                                          50
pcce1   PQGPPGPPGP PGAPGDPGEA GTPGRPGTDA APGSPGPRGP PGPAGEAGAP
pchc    PQGRPGPPGP IGPPGEPGTP GNPGAPGNDA PPGPPGPKGP PGPPGKAGAP
pchs    KXGPPGPPGV VGPQGAAGET GPMGERGHPG PPGPPGEQGL PGTAGKEGTK
pcce2   IKCPPGCPGP RGPSGLVGPA GPAGDQGRHG PPGPTGGQGG PGEQGDAGRP
pcmull  NDGQPGAPGA PGYDGAKGDK GDTGAPGPQG PKGDQGPKGD QGYKGDAGLP
sjf     .......... .MNSKIFVII CGIVYLVKEV VMELDTSMEY NNIVKQVNMQ

        51                                                        100
pcce1   GPAGEPGTPA ISEPL..... ........TP GAPGEPGDSG PPGPPGPPGA
pchc    GAAGQPGANA PSEPL..... ........VP GPPGPPGPTG PEGPPGPNGA
pchs    GDPGPPGAPG KDGPAGLRGF PGERGLPGTA GGPGLKGNEG PSGPPGPAGS
pcce2   GAAGCPGPPG PRGE...... ......PGTE YRPGQAGRAG PPGPRGPPGP
pcmull  GQPGQTGAPG KDGQDGAKGD KGDQGPAGTP GAPGKDGAQG PAGPAGPAGP
sjf     S.AIINNLQR RLDKLLEKQS MQDEIVYQNQ GKTNCKCKAG PRGAPGAAGP

        101                                                       150
pcce1   PGNDGPPGPP GP...KGAPG PDGPPGADGQ SGP......P GPPGPAGTPG
pchc    PGHPGAPGAP GE...KGPRG QDGHPGAPGN AGH......P GQPGQPGPPG
pchs    PGERGAAGSG GPIGRQGRPG PQGPPGAAGE KGV......P GEKGPIGPTG
pcce2   EGNPGGAGED GNQGPVGHPG VPGRPGIPGK SGT......C GEHGGPGEPG
pcmull  AGPVGPTGPQ GPQGPKGDVG PQGPQGAPGS NGAVVYIRWG NNVCPAGETN
sjf     QGAPGPEGPS GPQGPPGPVG QQGPPVIPDD DDTCFGT*........
```

with the labelled clone F, and the corresponding ethidium stained gel. It can be seen that there is a strong signal in the L3 RNA (Lane 1) and day 15 L4 RNA (Lane 3) with a weaker signal in the 3 day post-infection L3 RNA (Lane 2). Only a very faint signal was obtained with adult RNA (Lane 4), although from the ethidium stained gel there is less RNA present in this lane, which may account for the lack of signal, especially if there is a very low level of expression of this particular collagen gene in the adult parasite.

A Southern blot of digested *Brugia* genomic DNA probed with clone F is shown in **Figure 4.25**. Several bands are present in each lane suggesting that clone F belongs to a multigene family, which would be expected for a gene coding for a collagen protein.

Clone F had 57.8% identity in a 45 amino acid overlap to cuticle collagen 1 from *C. elegans* (Kramer *et al.*, 1982). The nucleotide and amino acid sequence obtained from clone F are shown in **Figure 4.26**. The clone is 436 bases long, with the SL sequence shown underlined. The initiation codon ATG (shown in bold) is only 6 nucleotides after the SL sequence, and then there are 78 amino acids before the characteristic collagen $(Gly\text{-}X\text{-}Y)_n$ repeats start. There is a termination codon TAG present and what appears to be a polyadenylated tail, but this is unlikely to be the true end of the cDNA since the Northern blot recognises a transcript of 2.0 kb. As with the other cDNAs analysed, it is more likely to be a product of spurious priming by the oligo dT primer used in the construction of the library. A pile up of the *Brugia* cuticular collagen with some other homologues is shown in **Figure 4.27** and it can be seen that it is the collagen $(Gly\text{-}X\text{-}Y)_n$ repeats which confer the homology found with the *C. elegans* collagen.

4.3 Discussion

The aim of the work carried out in this chapter was to isolate cDNAs highly expressed in vector derived L3. Differential screening resulted in the identification of four cDNAs which hybridised strongly to the mosquito derived L3 probe, weakly to the 3 day post-infection L3 probe and not at all the the adult probe.

As a first step in characterising these cDNAs, it was important to confirm the pattern of expression in the different life cycle stages. In general, Northern blot analysis confirmed the expression pattern predicted from the differential screening method used to isolate these clones. With all four clones, the strongest signal was obtained in the mosquito derived L3 RNA with weaker signals from L3 obtained at 3 days post-infection. The signal obtained with adult RNA was very weak or absent. It was interesting that L4 (15 days post-infection) RNA gave a strong signal, particularly for the collagen genes. This is a growing stage for the worm and so the expression level of genes coding for structural proteins such as collagens would perhaps be expected to be elevated. However, work by Howells and Blainey (1983) found that, after the moult to the L4 stage, the increase in length of the worm occurred without a significant increase in surface area. Immediately after the moult the cuticle is highly folded and intermoult growth is achieved by the expansion of these folds. The same process occurs after the fifth moult to the adult stage and Selkirk *et al.,* (1989) also found that the synthesis of structural cuticular proteins only takes place at an extremely low level in the adult worm. These findings may explain the very low expression level of the four genes in the adult stage of the parasite, but the processes behind the increased expression at the L4 stage remain unclear.

All of the genes isolated in the differential screen as being more highly expressed in the vector-derived L3, had homology to known sequences in the data bases. This contrasts with the genes that were up-regulated in the 3 day pi L3 (Martin *et al.,* 1996), as only one of these clones had homologues in the data bases. This clone, designated S5, was the *Brugia* homologue of the enzyme cytidine deaminase.

The SJ1 clone, isolated in the first differential screen, showed homology to nematode tropomyosin from *T. colubriformis* (Frenkel *et al.,* 1989). Since the full length cDNA was not cloned, attempts were made, by PCR and by screening the adult cDNA library, to isolate the full length clone. Results from these experiments confirmed that the *B. pahangi* tropomyosin was expressed

at such a low level in the adult worm that it was not possible to identify the gene by these methods. This work could have been taken further by using lower annealing temperatures in the PCR reaction.

This finding was unexpected in that other groups, (O'Donnell *et al.,* 1989; Taylor *et al.,* 1996), have found tropomyosin expression in adult nematodes and since the protein is involved with muscle contraction there seems to be no explanation at present for the low levels of expression that were found in *B. pahangi* adults.

Tropomyosin is an actin-binding protein regulating contraction in muscle and non-muscle cells. It consists of a dimer of two identical α-helical chains which wind around each other to give a coil. This molecule stabilises and strengthens the actin filament by binding along its length. Tropomyosin is evolutionarily highly conserved and the diversity of the many muscle and non-muscle isoforms are generated from a limited number of genes by alternative mRNA splicing (McLeod *et al.*, 1985; Dissous and Capron, 1995). Immunisation with tropomyosin induces partial immunity against two species of parasitic nematodes. O'Donnell *et al.,* (1989) reported that a detergent extract of *T. colubriformis* L3 enriched with tropomyosin induced host-protective immune responses in intraperitoneally vaccinated guinea pigs. Immunofluorescence staining of L4 *T. colubriformis* showed that the antigen was associated with the muscle layer. Western blotting, with a monoclonal antibody, indicated that tropomyosin was found in L3, L4 and adult worms.

Frenkel *et al.,* (1989) cloned and characterised the *T. colubriformis* tropomyosin from an L4 library and found, by Northern blotting, that the expression pattern obtained for the 1.5 kb transcript was greatest in L4, then adult and least in L3, although a strong signal was obtained in all three life cycle stages tested.

More recent work by Taylor *et al.,* (1996) found that vaccination with *O. volvulus* recombinant tropomyosin fused to Maltose-Binding Protein (MBP) significantly reduced the recovery of *O. lienalis* mf from the skin of BALB/c mice. A similar vaccination protocol using Mongolian jirds infected with *A. viteae* produced a 46% reduction in the recovery of adult worms compared to

the control groups. The *O. volvulus* tropomyosin was identified by immunoscreening an adult female *O. volvulus* cDNA library with rabbit antiserum raised against the L3 stage of *O. lienalis*. This serum was found to react with a 42 kD native antigen in all life cycle stages. Immunoelectron microscopy indicated that the tropomyosin was localised mainly to muscle tissue in sections of *Onchocerca* species, as expected. However, antibody reactivity was also detected over cuticular regions. ES products from *A. viteae* L3 contain a component which is recognised by antibodies raised against the recombinant *O. volvulus* tropomyosin. It is not yet clear whether the ES product is an exported form of tropomyosin or whether it is an unrelated protein with a cross-reactive epitope (Taylor *et al.*, 1996).

These preliminary studies seem to indicate the tropomyosin may have some potential as a vaccine candidate but problems may arise due to the immunological cross-reactivity found between invertebrate and vertebrate tropomyosins. Also, work on Schistosomiasis has confirmed the structural conservation of these proteins in lower invertebrates and indicated that a convergent evolution of tropomyosin epitopes might exist between schistosomes and their gastropod hosts (Dissous and Capron, 1995).

Tropomyosin is also being studied in relation to tumour suppression. Several findings suggest that the expression of cytoskeletal proteins, such as tropomyosin, may be incompatible with neoplasia. Tropomyosin isoforms are often missing from tumours whether naturally occurring or experimentally induced (Rastinejad *et al.*, 1993).

The second clone (SJ5) picked in the initial differential screen was found to code for a glutamic acid rich protein. Homologues in the data bases include the GARP gene from *P. falciparum* (Triglia *et al.*, 1988) and the muscle associated protein, troponin from chicken (Smillie *et al.*, 1988). Since the full length *Brugia* cDNA has not been cloned, it is not clear if these are true homologues or whether the high percentage of glutamic acid present in the amino acid sequence may be misleading. Northern blotting of the *B. pahangi* E-rich protein gene demonstrated that this gene was highly expressed in the

vector derived L3 stage, less so in the other larval stages tested, with a very low level of expression in the adult parasite. It is interesting that homologues of clone SJ5 include troponin since clone SJ1 is the *Brugia* tropomyosin homologue and these two accessory muscle proteins interact to mediate the Ca^{2+} regulation of skeletal muscle contraction in vertebrates. In nematodes, work on *C. elegans* has produced information about the nematode muscle structure and organisation (Waterston, 1988). The components of the thin filament consist of actin, tropomyosin and troponin. The troponin molecule in mammalian muscle is composed of three subunits, the first, troponin-T binds to tropomyosin, the second, troponin-C binds to Ca^{2+} and the third, troponin-I binds to actin. The subunit composition of troponin in *C. elegans* has not yet been defined. In vertebrates the troponin complex has an elongated shape, the C and I subunits form the globular head region, while T forms a long tail which binds to tropomyosin. Kimura *et al.,* (1987) isolated the three troponin subunits from *Ascaris* body wall, and found that the troponin-tropomyosin system in the obliquely striated nematode muscle, acted in the same manner as in vertebrate striated muscle, where troponin acts as an inhibitor for actin-myosin interaction and the inhibition is removed by Ca^{2+}.

Two *Brugia* collagen genes (clones C and F) were isolated from the 3 day pi cDNA library. Both appeared, from their homologies, to be cuticular collagens.

Collagens have been extensively studied in vertebrates and now work on *C. elegans* has identified a family of up to 50 genes coding for cuticular collagens alone (Cox *et al.,* 1984). In *C. elegans* there are four main subdivisions of cuticular collagens and *Ascaris* and *H. contortus* cuticular collagen genes can be classified in the same way. Members of each family, even if they are from different species, are more similar to each other than to members of another family. The different cuticle collagen gene families probably serve distinct functions and may form different parts of the cuticle (Cox, 1992).

Nematode cuticle collagens possess a unique structure which is different from nematode basement membrane collagens which in fact have more features in

common with vertebrate collagens. This is due to the fact that the nematode cuticle is a very specialised structure, forming a barrier between the animal and its environment. It consists of an extracellular matrix made up predominantly from extensively crosslinked small collagen like proteins. The cuticle acts as an exoskeleton, determining the shape of the worm and striking a balance between rigidity and flexibility to allow the worm movement. There are four moults in the nematode life cycle and each new cuticle is secreted by the underlying hypodermal cells. The cuticles of different life cycle stages vary and the collagen genes expressed at each moult may also vary as has been found in *C. elegans* (Johnstone, 1994). The majority of cuticular proteins are synthesised for a short period of time immediately prior to and during the deposition of each cuticle.

The collagens are extracellular structural proteins with a characteristic triple-helical rod like structure formed by the association of three polypeptide chains. Every third amino acid must be glycine in order for the proper formation to occur and the repeating $(Gly\text{-}X\text{-}Y)_n$ sequence where X and Y can be any amino acid, but are most commonly proline or hydroxyproline, gives the characteristic collagen amino acid sequence. In contrast to vertebrate and basement membrane nematode collagens, the cuticle collagens are small proteins with molecular weights of between 30-40 kD, which form multimers in the cuticle. Three polypeptide chains are joined covalently by non-reducible tyrosine cross-links with interchain disulphide bonds stabilizing the collagen molecules within the cuticle. It has also been found that cuticle collagens contain only one or two short introns which are usually located outside the $(Gly\text{-}X\text{-}Y)_n$ coding regions and are generally dispersed throughout the genome, which is not the case for vertebrate collagens (Cox, 1992).

The expression of cuticular collagen genes is regulated at several levels. In *C. elegans* peaks of cuticle collagen synthesis coincide with the lethargus periods preceeding each ecdysis (Cox *et al.*, 1981). Selkirk *et al.*, (1989), found that in *B. malayi* the relative rate of cuticle protein synthesis varied according to different stages in the life cycle of the parasite. In *C. elegans* individual cuticle collagen genes are differentially regulated during development

(Johnstone, 1994). Multiple genes are expressed at each moult and others only at particular moults (Kramer *et al.*, 1985; Park and Kramer, 1990). This expression pattern correlates with differences in the structure of the cuticle, found at different developmental stages of the worm.

The *B. pahangi* cuticle collagen (Clone F), was found to have homology to *col-1*, a cuticular collagen from *C. elegans*. Cuticular collagen genes from parasitic nematodes which also show homology to *col-1* have been cloned previously. Shamansky *et al.*, (1989) found that a cuticle collagen gene from *H. contortus* showed high levels of conservation to *C. elegans* cuticle collagen genes in the *col-1* family. Northern blot analysis showed the *H. contortus* gene to have a transcript size between 1.0 and 1.2 kb and to be expressed in an L3/L4 mixed stage population but not in adult worms. The *Brugia* cuticle collagen had a larger transcript size, 2 kb and a very low level of expression in the adult life cycle stage, with high levels at the L3 and L4 stage, therefore showing a similar expression pattern. It may be that the *col-1* family of cuticle collagen genes code for collagens required in the larval cuticles but not in the adult worm cuticle.

The *B. pahangi* cuticle collagen precursor (clone C), was found to show homology to *col-12* from *C. elegans*, a member of the *col-6* cuticle collagen family. Kingston *et al.*, (1989) described two cuticle collagen genes from *Ascaris* that showed homology to the *col-6* family of *C. elegans*. By Northern blotting the size of the transcripts obtained were 1.1 kb and 1.4 kb. One of the *Ascaris* genes appeared to be expressed in the adult stage whilst the other gene gave no signal with adult tissue. No other life cycle stages were tested. Scott *et al.*, (1995) cloned a cuticle collagen gene from *B. malayi* (*Bmcol-2*) using late vector stage L3 and a RT-PCR approach with the SL1 primer. The *Bmcol-2* clone was found to make up 7% of the total SL-PCR cDNA clones and showed highest levels of homology to *col-12*, a member of the *col-6* cuticle collagen family. By semi-quantitative RT-PCR the *Bmcol-2* gene was shown to be transcribed at all life cycle stages tested with L3, L4 and mf stages having a 2-3 fold higher transcription level than adults. By Northern blot the cuticle collagen precursor (clone C) had a transcript size of 1.7 kb and

was found to be expressed in L3 and L4 stages but only at an extremely low level, if at all, in adult worms.

An additional collagen gene has also been cloned from *B. malayi* (Caulagi *et al.,* 1991), but this particular gene appears to show more homology to mammalian basement membrane collagens, and is probably not a cuticular collagen.

The ribosomal protein gene cloned was picked on the basis that it was expressed in all life cycle stages. This was confirmed by Northern blotting, with a signal obtained in all the life cycle stages tested, mf, L3, 3d pi L3, d15 and adult worms. In order for the EE4 clone to be a true constitutive gene it must be expressed in all life cycle stages. This is impossible to test for the whole life cycle of *B. pahangi,* but more recent work using RT-PCR on many more life cycle stages appears to confirm these findings. In the *C. elegans* system the best constitutively expressed markers, at the present time, to control for equal loading on Northern blots are the ribosomal genes (Krause, 1995). The EE4 clone codes for an acidic ribosomal protein, which belongs to a small family of genes, and so is likely to be more specific than other ribosomal genes. It was interesting that the EE4 clone was previously identified by immunoscreening the adult cDNA library with anti-L3 serum (clone SH3, Chapter 3).

The 3d pi L3 library was constructed using SL RT-PCR to try and overcome the problems associated with the limited amount of parasite material available. However, with hindsight, it is clear that there are difficulties with this method. It is known that PCR tends to favour smaller size transcripts and so the library contains mainly small genes/gene fragments, and does not give a true representation of the messages at that particular life cycle stage. Another finding was that there tends to be over-amplification of a few transcripts, presumably at the expense of others. Martin *et al.,* (1996) found that clone S3 represented approximately 3% of the library, while Scott *et al.,* (1995) also

found that the collagen gene isolated from their *B. malayi* SL-PCR cDNA library made up 7% of all inserts.

Another problem concerning the construction of the library involves the use of oligo dT as a primer in the initial amplification. None of the four genes cloned from the library, as being more highly expressed in the vector derived L3, contained the full length cDNA. It would appear that the oligo dT primer tends to anneal to AT rich areas, cutting off the 3' end of the gene and giving a false polyadenylated tail. This is a particular problem as the nematode genome is AT rich. Attempts were made to obtain the full length cDNA for clone SJ1 (tropomyosin), by using the T7 primer or a primer designed to the 3' end of the *T. colubriformis* tropomyosin gene in conjunction with a primer for the 5' end of the *Brugia* tropomyosin. These experiments were not successful in producing any PCR product that was recognised by the *Brugia* tropomyosin probe. PCR reactions with the 5' *Brugia* tropomyosin primer in conjunction with oligo dT also failed to produce a product that was any larger than the original fragment using cDNA as a template or the 3d pi L3 cDNA library. This would appear to suggest that the oligo dT primer was annealing to the same AT rich area of the cDNA. It is also possible that the library does not contain the full length tropomyosin transcript, since the oligo dT may have cut off the 3' end in the initial amplification step. In order to overcome this problem, an adaptor oligo dT primer could be used. This involves designing a 5' extension to the oligo dT, usually containing restriction enzyme sites. This adaptor primer is used in the reverse transcription reaction where RNA is converted to single stranded cDNA. Then the first strand cDNA is amplified using the SL1 primer and the 5' extension only, of the adaptor oligo dT primer. Using this method it should be possible to amplify only full length cDNAs (Ghosh *et al.,* 1995). It is also possible to use a higher annealing temperature than 50°C in the amplification step, if only the adaptor portion of the primer is used, rather than oligo dT, and this should increase the specificity of the reaction, with less chance of spurious priming.

However, despite these drawbacks it was possible, by differential screening, to identify four genes which were highly expressed in the mosquito derived L3,

and their expression pattern was confirmed by Northern blotting. All of the genes appear to code for structural proteins. One explanation for this may be that at this particular stage in the life cycle, the parasite has completed the moult to the L3 stage, and is then developmentally arrested until it enters the mammalian host. This may result in other messages only being expressed at a very low level, until the changed environment within the mammalian host triggers the expression of other genes, related to avoiding the hosts' immune system and adapting to the new environment. The explanation for the seemingly high prevalence of structural protein genes in the vector derived L3 remains unclear, but could simply be due to the fact that there is a low level of expression of other genes at this stage of the life cycle.

Chapter 5

Semi-quantitative RT-PCR

5.1 Introduction

Although the four clones isolated by differential hybridisation are not L3 specific, they are certainly very highly expressed in this life cycle stage. In order to further investigate their expression pattern, especially in those parasite stages found in the mosquito, a semi-quantitative RT-PCR approach was adopted. This method is more sensitive than Northern blotting and since the L1 and L2 stages occur in the mosquito, only very small amounts of material are available, which would not be sufficient for Northern blots. Total RNA was isolated from mosquito thoraces, harvested at varying time points post-infection, and reverse transcription was performed using oligo dT as a primer. This first strand cDNA was then used in PCR reactions with, in the first instance, a primer corresponding to the conserved nematode SL1 sequence together with oligo dT, in order to determine whether it was possible to amplify only worm cDNA, since the first strand cDNA would consist mainly of mosquito cDNAs. Once it was confirmed that there was *B. pahangi* cDNA present after reverse transcription, gene specific PCR primers, designed according to the sequence of each of the four genes, were used to examine the expression pattern of each of the genes. In order to have a base line of expression against which to compare the four clones, RT-PCR of the constitutively expressed ribosomal gene was performed in tandem with each of the four genes. The PCR products were run out on agarose gels, blotted and then probed with the relevant labelled clone. Bands were cut out of the filter and the level of radioactivity counted for each life cycle stage. The counts were then compared to the ribosomal values and expressed as a ratio to show the relative pattern of expression of each of the genes at the different life cycle stages.

5.2 Results

5.2.1 Isolating RNA from mosquito thoraces

Mosquitoes infected with *B. pahangi* were harvested at 24 hours, 3 days, 6 days and 8 days post-infection. Uninfected mosquitoes were also taken to use as a control. 10 mosquitoes were taken from each time point and then the thoraces were dissected onto dry ice. Total RNA was isolated from the whole thorax as described in 2.5.7.1 and checked on a formaldehyde gel (2.5.7.2), before being stored at -70°C as an ethanol precipitate.

5.2.2 Reverse Transcription

First strand cDNA was generated from 7µl of the RNA in DEPC H_2O for each of the life cycle stages used (see 2.6.1), using reverse transcriptase primed with oligo dT. An uninfected mosquito sample was also included as a control. Contaminating genomic DNA was first removed by DNase treatment, and the resulting first strand cDNA stored at -20°C.

5.2.3 PCR using SL1 and oligo dT primers

The first step was to attempt to amplify only nematode cDNAs by using the spliced leader primer together with oligo dT. PCR reactions were carried out as in 2.6.3 using an annealing temperature of 50°C and 30 cycles. **Figure 5.1** shows an example of the smears produced in a typical SL1-PCR reaction. 10µl of PCR product were run out on a 1% agarose gel and then photographed. Mammalian stages were included to act as positive controls. However, it can be seen from lane 6 that cDNA in the uninfected mosquito sample has also been amplified. One possible explanation for the amplification of cDNA in the uninfected mosquito is that the oligo dT primer has annealed to both ends of an A rich cDNA resulting in spurious amplification in the PCR reaction. This gel was blotted and probed with clone F (cuticular collagen). From the

Figure 5.1

PCR products obtained from different life cycle stages using SL1 and oligo dT primers

1% agarose gel, stained with ethidium bromide, showing the PCR products obtained for each life cycle stage tested using SL1 and oligo dT as primers. The amplifications were carried out for 30 cycles with an annealing temperature of 50°C. 10μl of each product was run out per lane and then photographed.

Lane 1 24 hour post-infection mosquito thoraces cDNA

Lane 2 3 days post-infection mosquito thoraces cDNA

Lane 3 6 days post-infection mosquito thoraces cDNA

Lane 4 8 days post-infection mosquito thoraces cDNA

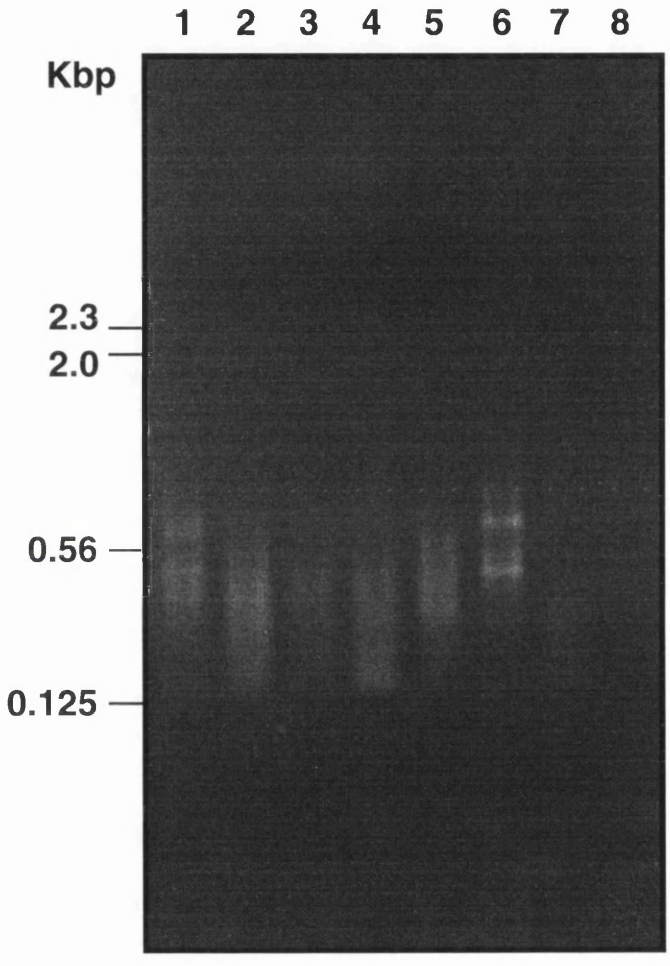
Lane 5 day 15 (L4) post-infection of the jird *B. pahangi* cDNA

Lane 6 uninfected mosquito thoraces cDNA

Lane 7 mosquito derived L3 *B. pahangi* cDNA

Lane 8 no cDNA template control

Figure 5.1



resulting autoradiograph, **Figure 5.2**, it can be seen that the PCR products derived from uninfected mosquito RNA are not recognised by the probe. This was found to be the case when the other four clones were used to probe similar reactions.

This initial part of the work demonstrated that it was possible to selectively amplify *B. pahangi* cDNA from the much larger population of mosquito messages present, and then to detect specific *Brugia* genes by hybridisation.

5.2.4 Gene specific primers

Primers were designed to each of the four clones and to the ribosomal gene, using the available sequence, as shown below;

Clone SJ1 (tropomyosin)

5' (Tr1) 5'CTA AAG CAA AAA TGG ATG CGA TCA AG 3'

3' (Tr3) 5'TCC AAA TTA GTA TTT GCA ACA GC 3'

These primers should give a product of 225 bp.

Clone SJ5 (E rich protein)

5' (Er1) 5'GAT TGC ATA GAG AGA ATG GC 3'

3' (Er3) 5'GCT TCA GTT TGT TCA GCC GG 3'

These primers should give a product of 211 bp.

Clone C (cuticular collagen precursor)

5' (Pc1) 5'CGA AAC AGC TTC TCA TTG AGG C 3'

3' (Pc3) 5'CCA ATT GCG CGG GTC CTG C 3'

These primers should give a product of 179 bp.

Figure 5.2

Autoradiograph showing the SL1/oligo dT PCR products hybridised to clone F (cuticular collagen)

PCR products obtained using the SL1 and oligo dT primers on a range of cDNA templates were first run out on an agarose gel (see **Figure 5.1**) then blotted onto nylon membrane and hybridised to the labelled clone F (cuticular collagen). The blot was washed to 0.2X SSC/0.1% SDS at 65°C.

Lane 1 24 hour post-infection mosquito thoraces cDNA

Lane 2 3 days post-infection mosquito thoraces cDNA

Lane 3 6 days post-infection mosquito thoraces cDNA

Lane 4 8 days post-infection mosquito thoraces cDNA

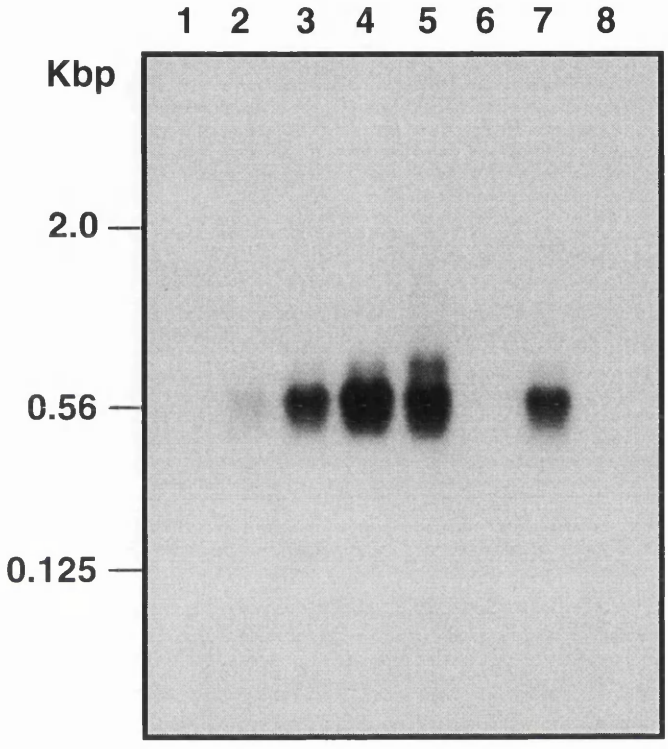
Lane 5 day 15 (L4) post-infection of the jird *B. pahangi* cDNA

Lane 6 uninfected mosquito thoraces cDNA

Lane 7 mosquito derived L3 *B. pahangi* cDNA

Lane 8 no cDNA template control

Figure 5.2



Clone F (cuticular collagen)

5' (Cc1) 5'GGA GTT GGA TAC ATC CAT GG 3'

3' (Cc3) 5'GCA TGT ATC ATC ATC ATC CGG 3'

These primers should give a product of 303 bp.

Clone EE4 (ribosomal protein)

5' (R1) 5'GCA TTG TTC TCA AAT AGA GC 3'

3' (R3) 5'CCC ATA TCA TCA TCT GAT TCC 3'

These primers should give a product of 397 bp.

The primers were taken off the column as described in 2.6.2 and the concentrations calculated. Each primer was then diluted down to a working concentration of 1mg/ml.

5.2.5 PCR titration

Before any semi-quantitative analysis could be undertaken, it was necessary to titrate the number of cycles used in the PCR amplification to find the optimum cycle number for each gene. This needs to be done using hybridisation to visualise the bands since at these levels they are often not visible by ethidium bromide staining. The L3 life cycle stage was chosen for this purpose as there was only limited amounts of first strand cDNA available from the mosquito stages. The cycle numbers chosen were: 15, 20, 25, 30, and 35. This should provide a wide enough range to choose the optimum amplification before a plateau is reached.

The PCR reactions were carried out as before, using the five sets of gene specific primers on L3 first strand cDNA. Reactions were removed from the

PCR machine after the relevant number of cycles, and then run out on five separate gels. The gels were blotted and probed with each of the five genes, and then exposed to film. After autoradiography the filters were lined up with each autoradiograph and the bands cut out as described in 2.6.4. Counts were measured and then graphs drawn to determine the optimum level of amplification. **Figure 5.3** shows an example of the graph obtained for clone C (cuticular collagen precursor), with the level of amplification peaking at 25 cycles. It was decided to choose 23 cycles overall, since the graphs are all rising steeply at this point, before the amplification levels off as one or more of the reagents in the reaction are exhausted. At this level products are not visible by ethidium bromide staining, so that cDNA and primer concentrations are optimised too.

5.2.6 Semi-quantitative RT-PCR

PCR reactions were carried out using the same master mix of dNTP's, buffer, enzyme and water. The mix was divided into five and the gene specific primers added to the relevant tube. The mix was then aliquoted into nine separate PCR tubes, and the first strand cDNA added last. The cDNA samples used for each gene were;

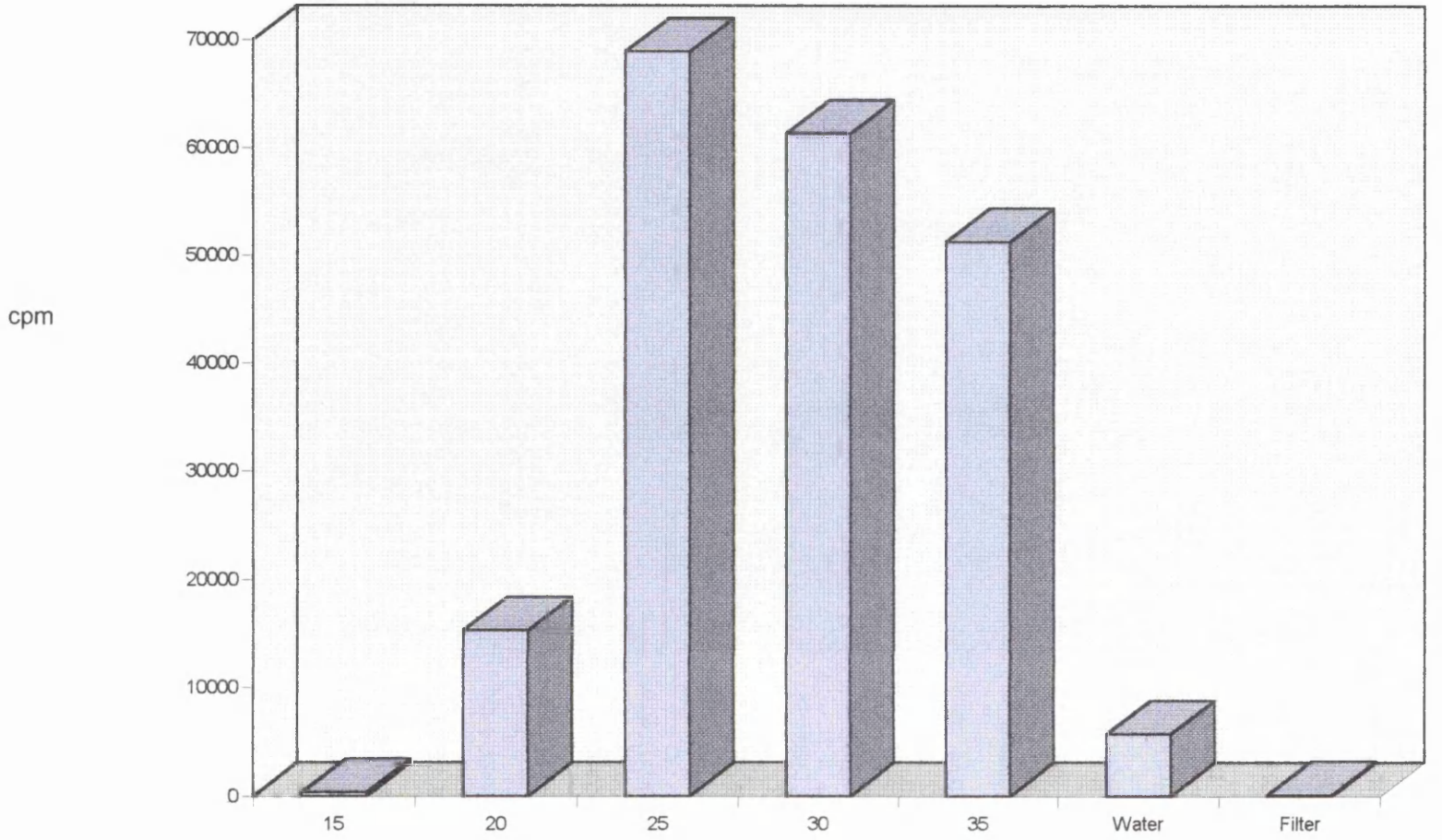
1. Uninfected mosquito - a negative control.
2. Mosquito thoraces - 24 hour post-infection (L1).
3. Mosquito thoraces - 3 days post-infection (L2).
4. Mosquito thoraces - 8 days post-infection (L3).
5. Infective stage parasites, isolated from the mosquito, 9/10 days post-infection in our system (L3).
6. Parasites isolated 3 days post-infection of the jird (L3).
7. Parasites isolated 15 days post-infection of the jird (L4).
8. Adult worms, harvested at approximately 3 months post-infection

Figure 5.3

Titration to determine optimum number of PCR cycles using clone C (cuticular collagen precursor).

PCR reactions were carried out on L3 first strand cDNA using clone C gene specific primers. The reactions were amplified for 15, 20, 25, 30 and 35 cycles and then run out on an agarose gel. The gel was blotted and then probed with the labelled clone C cDNA. After autoradiography, the corresponding bands were cut out from the filter and scintillation counted. The graph shows the cpm obtained from the cut out filter at different numbers of cycles. Also included was a corner of the filter and the reaction which contained no DNA template (amplified for 35 cycles) to act as background controls.

Figure 5.3
Titration to determine optimum number of PCR cycles using clone C (cuticular collagen precursor)



of the jird.

9. No cDNA template - to act as a control for contamination.

PCR reactions were carried out for 23 cycles with an annealing temperature of 55°C. 10µl of each sample were run out on 5 separate 2% agarose gels, then denatured and neutralised before being blotted onto nylon membrane overnight. The DNA was fixed onto the membrane by baking at 80°C for 1 hour and then UV crosslinking. Prehybridisation was carried out at 65°C for at least 4 hours and then the labelled probe was added and left overnight at 65°C to hybridise. The blots were washed down to 0.2 X SSC/0.1% SDS, and exposed to film for 2 hours. **Figure 5.4** shows a panel with representative autoradiographs using the ribosomal, tropomyosin and E rich genes. After the blots were lined up with the relevant autoradiograph, squares were cut out of the filter which corresponded to the bands on the film. A corner of the filter was also cut out to act as a background control. Each square was placed in a scintillation tube with 3 mls of scintillation fluid and the levels of radioactivity counted. The control value was taken away from each reading and then the relative abundance of each gene expressed as a ratio compared to the ribosomal gene values. This method does not give actual levels of expression, but demonstrates the expression pattern of each of the four genes tested in relation to the constitutively expressed ribosomal gene. This relative method of measurement takes into account that there may be varying amounts of RNA and therefore cDNA in each sample. The values obtained by scintillation counting are shown in **Table 5.1**. The control value was then taken away from each sample, and each life cycle stage for the four genes was divided by the appropriate ribosomal value to give a ratio as shown in **Table 5.2**. The values for the L1 stage, 24 hours post-infection of the mosquito, are all very low which may reflect the low biomass of parasite material at this stage. For the other life cycle stages there is always at least one cDNA sample which is well amplified, suggesting that there was sufficient parasite material present.

Figure 5.4

Panel comparing the hybridisation patterns in different life cycle stages by PCR using gene specific primers

Autoradiographs showing the hybridisation patterns obtained for:-

A Ribosomal gene

B Tropomyosin gene

C E rich protein gene

PCR reactions were carried out using gene specific primers for 23 cycles with an annealing temperature of 55°C. 10µl of each product was run out on a 2% agarose gel, then denatured and neutralised before being blotted onto nylon membrane. Hybridisation was carried out overnight at 65°C and the blots were washed to 0.2 X SSC/0.1% SDS.

Lane 1 no DNA template control

Lane 2 adult *B. pahangi* cDNA

Lane 3 15 day (L4) *B. pahangi* cDNA

Lane 4 3 day (L3) *B. pahangi* cDNA

Lane 5 mosquito derived L3 *B. pahangi* cDNA

Lane 6 8 days post-infection mosquito thoraces cDNA

Lane 7 3 days post-infection mosquito thoraces cDNA

Lane 8 24 hours post-infection mosquito thoraces cDNA

Lane 9 uninfected mosquito thoraces cDNA

Figure 5.4

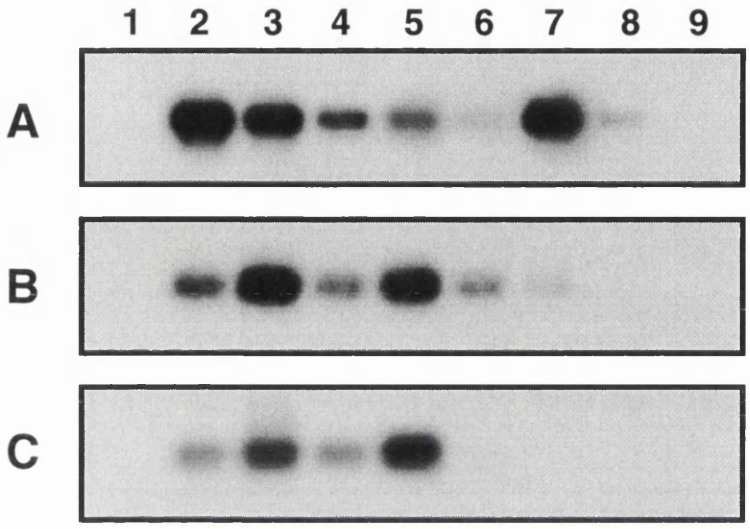


Table 5.1

Values (given as cpm) obtained for each of the five genes, at the different life cycle stages, by scintillation counting.

PCR products using different life cycle stages as the template and gene specific primers, were run out on agarose gels. The gels were blotted and probed with the corresponding labelled cDNA. After autoradiography pieces of the filters corresponding to the PCR products obtained were cut out and scintillation counted, for each of the five clones.

Table 5.1

Values (given as cpm) obtained for each of the 5 genes, at different life cycle stages, by scintillation counting.

Life cycle stage→	Uninfected Mosquito Control	24 Hrs L1	3 Day L2	8 Day L3	Mosquito derived L3	Day 3 pi L3	Day 15 pi L4	Adult	No DNA Control	Filter Control
Clone EE4 Ribosomal	76	349	5165	359	1246	1090	4441	8239	78	91
Clone SJ1 Tropomyosin	73	63	250	637	2679	828	4517	929	68	72
Clone SJ5 E Rich Protein	118	104	85	398	9236	2470	6728	2345	110	100
Clone F Cuticular Collagen	96	52	597	616	2133	1525	3701	1992	95	52
Clone C Cuticular Collagen Precursor	69	75	114	1068	3264	1383	4011	702	63	73

Table 5.2

Ratios of values obtained for each of the four clones, compared to the constitutively expressed ribosomal protein gene.

The control value for each of the five clones was subtracted from each life cycle stage value, to ensure only real signals were included. Then each remaining value at each life cycle stage was divided by the corresponding ribosomal value in order to give a ratio.

Table 5.2

Ratios of values obtained for each of the four genes, compared to the constitutively expressed ribosomal protein gene.

Life cycle stage→	Uninfected Mosquito Control	24 Hrs L1	3 Day L2	8 Day L3	Mosquito derived L3	Day 3 pi L3	Day 15 pi L4	Adult
Clone SJ1 Tropomyosin/ Ribosomal			0.04	2.00	2.24	0.75	1.0	0.1
Clone SJ5 E Rich Protein/ Ribosomal				1.02	7.8	2.3	1.5	0.3
Clone F Cuticular Collagen/ Ribosomal			0.1	1.85	1.74	1.4	0.83	0.23
Clone C Cuticular Collagen Precursor/ Ribosomal		0.04	0.01	3.6	2.7	1.3	0.9	0.08

Figure 5.5 shows the graph obtained for the tropomyosin gene (clone SJ1). There is a very low level of expression in L2 parasites at 3 days post-infection of the mosquito. However, once the parasite has moulted to the L3 stage, the expression of the tropomyosin gene appears to increase, as demonstrated in the 8 day parasites and in the mosquito derived L3. The tropomyosin gene was picked in the differential screen and, as predicted from that process, the relative level of expression appears to decrease in the mammalian derived parasites, with lower levels of expression in 3 day pi L3 compared to mosquito derived L3 and very low levels in the adult. It is interesting that the expression pattern found by RT-PCR, at least in the mammalian stages echoes that found by Northern blotting. **Figure 5.6** shows the ratio of the cuticular collagen (clone F) expression compared to that of the ribosomal gene. Again a signal is detected as early as 3 days post-infection of the mosquito, which would perhaps be expected since the collagens will be involved in the moulting process, and the worm undergoes two moults whilst inside the mosquito. The expression of this gene appears to decline as the worm develops and matures to the adult stage. **Figure 5.7** shows the graph of the cuticular collagen precursor gene (clone C) compared to the ribosomal gene values. It was possible to detect a signal at the L1 stage with this gene, with an extremely low expression level at the L2 stage, and again a large increase of expression at 8 days post-infection of the mosquito, once the L3 moult has been completed. The expression pattern of this gene in the mammalian stages of the life cycle again confirms the Northern blot profile. The graph shown in **Figure 5.8** shows the ratio of the E rich protein gene (clone SJ5) compared to the ribosomal gene. This particular gene appears to be the most L3 specific with a huge increase of expression at this stage. Expression of this gene was only detected in mosquito stages at 8 days post-infection and not in earlier stages. Levels of expression in the later mammalian derived parasites are also low.

One feature of these results is the low level of ribosomal expression at the 8 days post-infection of the mosquito time point, which results in seemingly very high levels of expression for three of the genes. This could be due to a

Figure 5.5

Graph showing the ratio of the tropomyosin gene (SJ1) compared to that of the constitutively expressed ribosomal protein gene

Figure 5.5
Graph showing the ratio of the tropomyosin gene (SJ1) compared to that of the constitutively expressed ribosomal protein gene

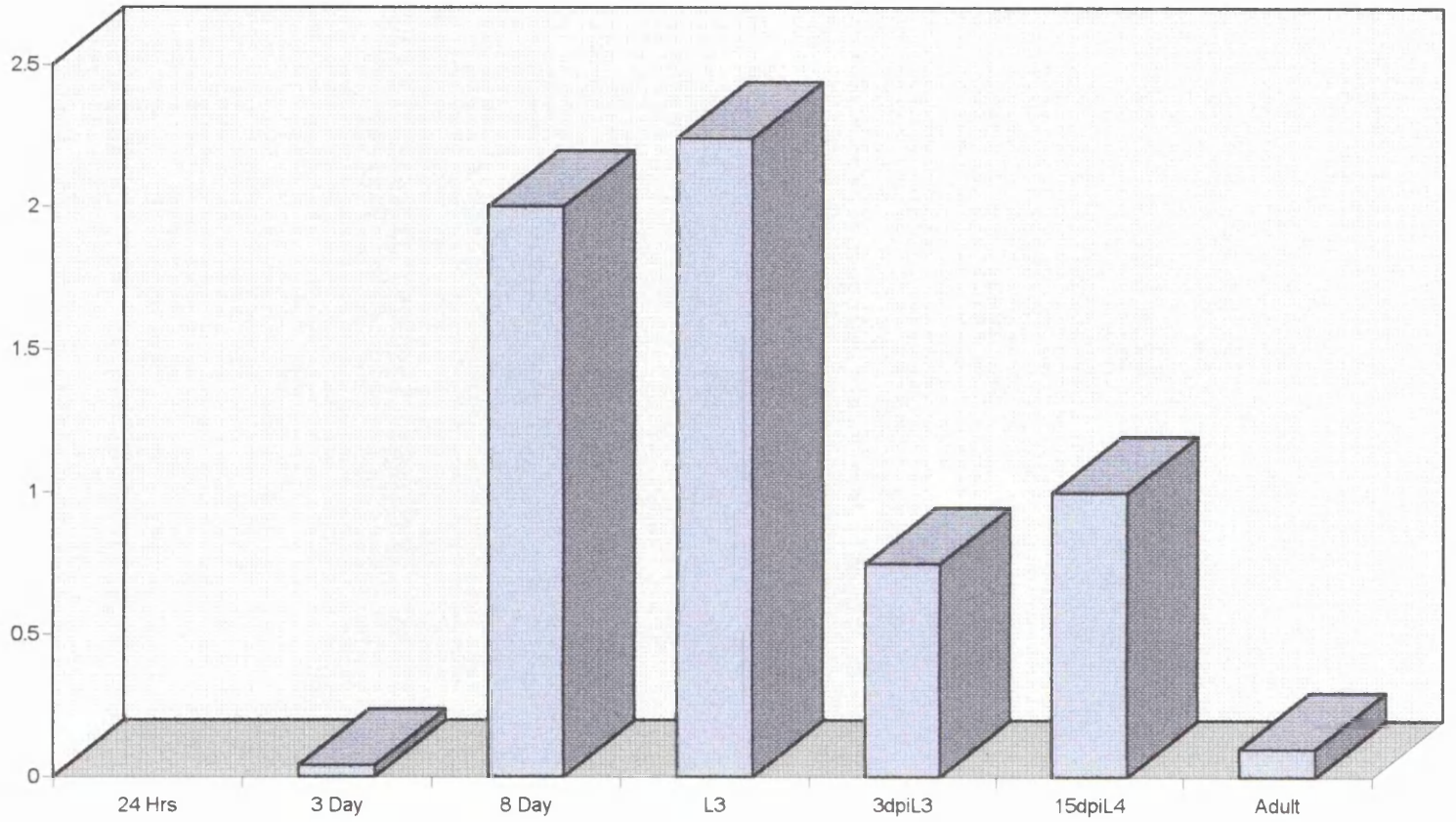


Figure 5.6

Graph showing the ratio of the cuticular collagen gene (Clone F) compared to that of the constitutively expressed ribosomal protein gene

Figure 5.6
Graph showing the ratio of the cuticular collagen gene (Clone F) compared to that of the constitutively expressed ribosomal protein gene

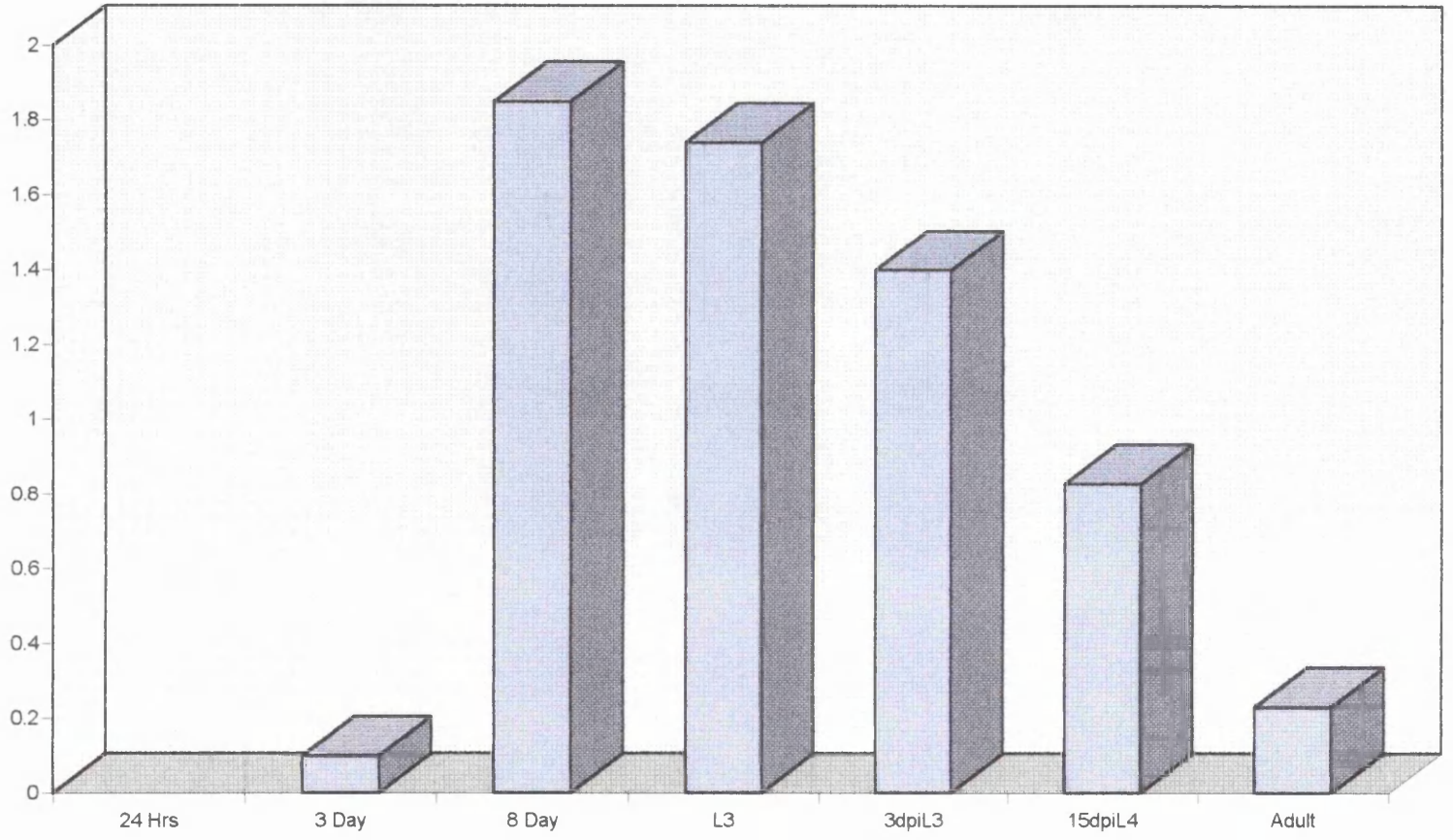


Figure 5.7

Graph showing the ratio of the cuticular collagen precursor gene (Clone C) compared to that of the constitutively expressed ribosomal protein gene

Figure 5.7
Graph showing the ratio of the cuticular collagen precursor gene (Clone C) compared to that of the constitutively expressed ribosomal protein gene

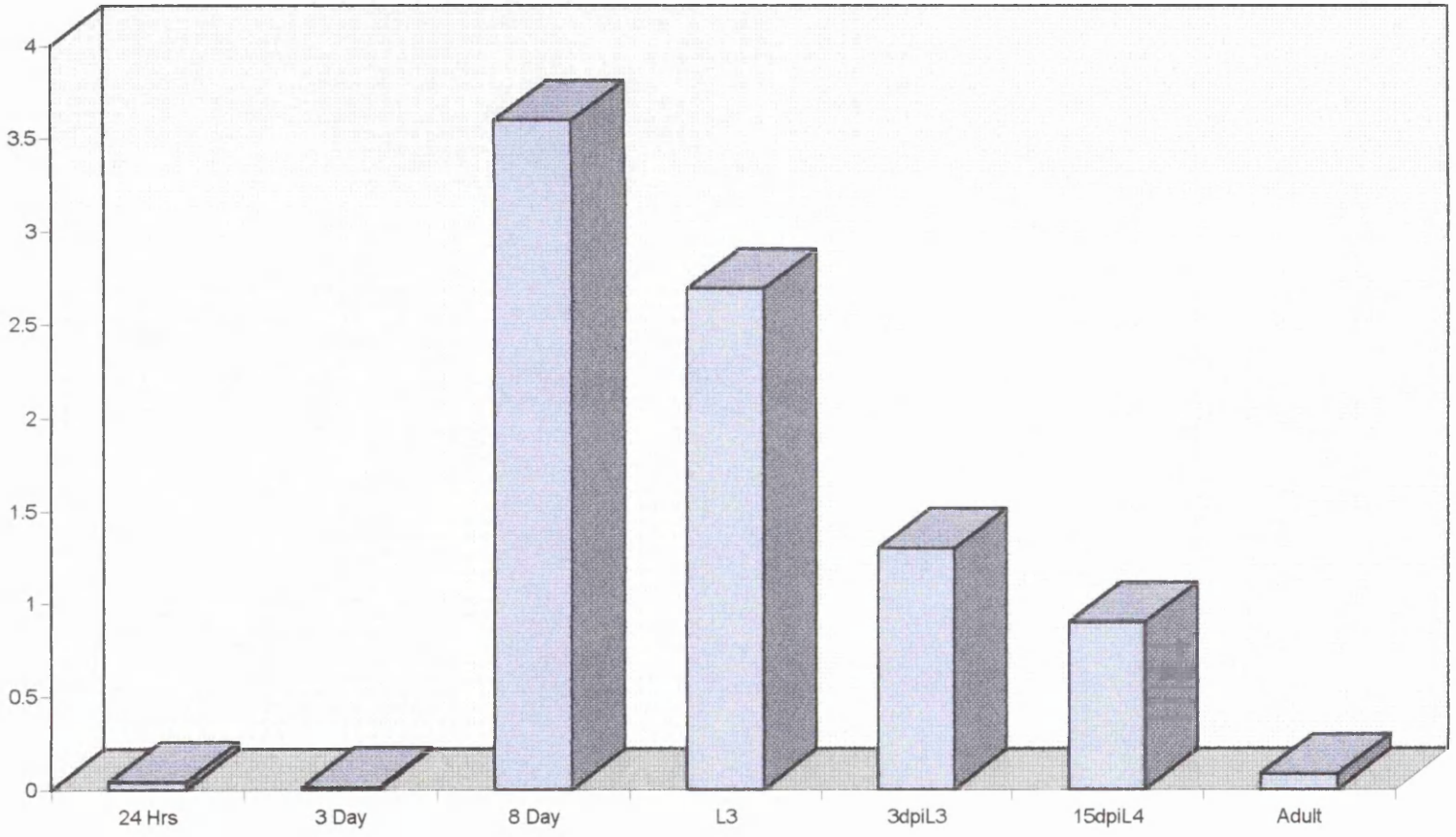
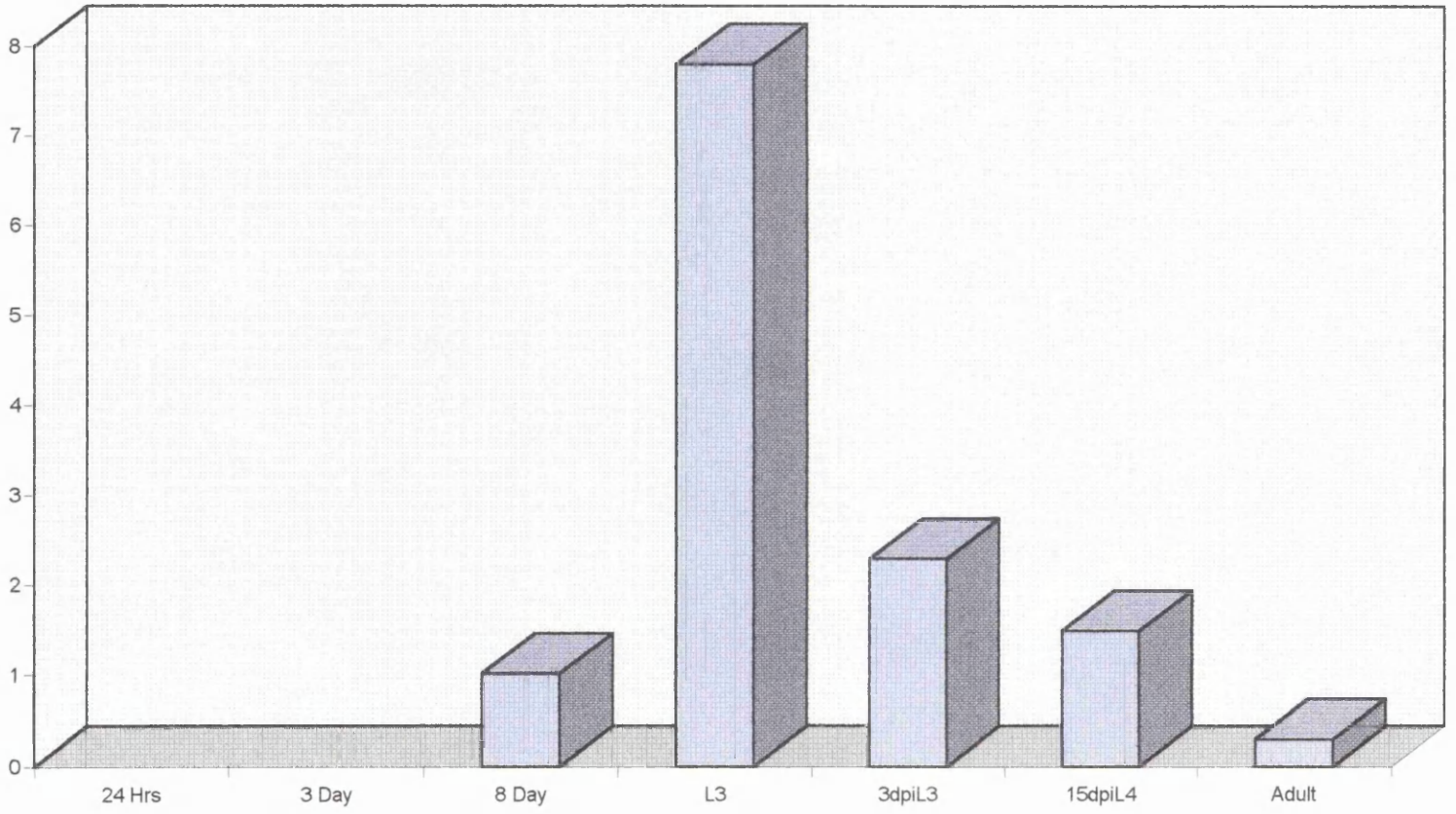


Figure 5.8

Graph showing the ratio of the E rich protein gene (SJ5) compared to that of the constitutively expressed ribosomal protein gene

Figure 5.8
Graph showing the ratio of the E rich protein gene (SJ5) compared to that of the constitutively expressed ribosomal protein gene



low concentration of cDNA in this particular sample, although the results obtained for the cuticular collagen precursor for this life cycle stage would seem to suggest otherwise. It could also be that although the ribosomal gene is expressed throughout the life cycle, the level of expression may fluctuate, in relation to the expression of other genes which may be turned on and off according to the developmental stage of the worm.

5.3 Discussion

Each of the four clones studied in this chapter was highly expressed in the mosquito derived L3 stage of the parasite. In order to take a closer look at the expression pattern in the earlier mosquito stages of the life cycle an RT-PCR approach was adopted, since Northern blotting was not possible due to the lack of parasite material. By using the constitutively expressed ribosomal protein gene in tandem with each of the four clones, it was possible to use semi-quantitative RT-PCR, to measure the fluctuations in the relative abundance of each transcript at different life cycle stages.

The timing of the expression of these genes was of interest since some genes in the mosquito stages are expressed in readiness for the transfer to the mammalian host. Ibrahim *et al.,* (1992), used surface and metabolic radiolabelling to look at the L2 and L3 stages of *B. malayi* during vector-stage development. They found that as the larvae develop within the mosquito, they produce an increasingly complex repertoire of surface components and that L2's appear to initiate the synthesis and expression of many of the L3 components. Carlow *et al.,* (1987), used a monoclonal antibody to a *B. malayi* L3 surface epitope, to show that the antibody also recognised the L2 stage. The epitope is rapidly lost after the parasite is removed from the vector. It is interesting that many workers have found that L3 surface components are lost fairly rapidly after they enter the mammalian host. In *D. immitis* all of the infective stage L3 antigens are synthesised during late L2-early L3 development and once the parasite leaves the mosquito the synthesis of these antigens is very rapidly reduced to undetectable levels (Ibrahim *et al.,* 1992).

Bianco *et al.,* (1990), labelled developing *Onchocerca lienalis* and *Onchocerca volvulus* larvae within black flies and found that the synthesis of the major labelled components was terminated once the larvae were removed from the black flies. These findings seem to suggest that the environmental changes that occur during and after the transmission event act as triggers for the regulation of protein synthesis within the parasite.

The semi-quantitative approach employed in this part of the work demonstrated that it was possible to detect *B. pahangi* single copy genes from RNA isolated from mosquitoes, even though there was a vast excess of mosquito RNA present. Other systems have used similar methods to detect parasites in a pool of mosquitoes (Chanteau *et al.,* 1994), or in blood samples (McReynolds *et al.,* 1986), but have used highly repeated DNA sequences rather than single copy genes.

The patterns of expression obtained by semi-quantitative PCR for the four clones analysed mirrors those results obtained by Northern blotting in Chapter 4. The results do not give actual expression levels, but rather show the relative abundance of each of the four genes compared to the constitutively expressed ribosomal protein gene (clone EE4), at each of the life cycle stages tested. The tropomyosin gene (clone SJ1), is first expressed in the L2 stage and the levels increase until the infective stage L3 is fully developed. Once within the mammalian host, the level of expression decreases, except for an upturn in expression at day15, and then drops to very low levels in the adult parasite. The *B. pahangi* E-rich protein gene (clone SJ5), appears to show the greatest increase of expression at the L3 life cycle stage. There is no detectable expression at the L2 stage, a low level at early L3, with a huge increase in the fully developed infective L3. Once within the mammalian host the expression gradually declines through the larval stages until only a very low level of expression is found in adult parasites. The two *B. pahangi* cuticle collagen genes (clones C and F), show a similar pattern of expression, they are detectable at the L1 and L2 stages, albeit at very low levels. Once the moult to the L3 has been achieved the expression levels greatly increase and then

gradually decline as the parasite develops to the adult stage. Work by Johnstone and Barry (1996), using a similar semi-quantitative RT-PCR method on the free living nematode *C. elegans*, found that collagen expression increased during lethargis, in the period immediately preceeding each moult. Different collagen genes had different temporal expression patterns, with some being expressed at each moult, whilst others are only expressed at specific moults. It would be of interest to look at the expression levels of the *Brugia* collagen genes in more detail, using those time points within the life cycle that correspond to the pre-moult period, to see if a similar pattern of expression is found in filarial worms. However, this would be more difficult in the *Brugia* system, since the development of the parasite is not synchronous, whereas in *C. elegans* it is possible to artificially synchronize the cultures.

By using this semi-quantitative method it was possible to detect *Brugia* specific genes from a pool of much more numerous mosquito messages. However, there are many problems associated with this method, which require adaptation before it can be successfully used to look at expression patterns of various genes within the *B. pahangi* life cycle.

The first issue is the problem of finding a truly constitutive gene, which is expressed at a reasonably constant level throughout the life cycle. It is particularly difficult to establish this fact in the filarial system, due to the limited amount of parasite material available. The ribosomal gene used in this study has certainly been found to be expressed at all of the life cycle stages so far tested, but whether the levels of expression are constant is difficult to determine. If the levels of expression of the constitutively expressed gene vary considerably between life cycle stages, then this could skew the results obtained, so that an apparently huge increase in abundance of a particular mRNA may not be quite as dramatic as it seems.

The oligo dT primer was used in the synthesis of first strand cDNA to ensure that only polyadenylated RNA is primed for reverse transcription. However, it was found that this particular primer often anneals to AT rich areas and this spurious priming can result in the presence of more than one band being

recognised by the probe. Joshua *et al.,* (1995), also reported problems associated with the oligo dT primer when they used a differential display technique to identify stage specifically expressed genes in the parasitic nematode *Angiostrongylus cantonensis*. 50% of their stage specific cDNA's were primed from both ends by oligo dT. As was the case with the four clones isolated in this study, many of their cDNA's did not have a polyadenylation signal, because the oligo dT had primed in an internal portion of the mRNA. Work is currently underway to compare the use of random ninemers (I. Johnstone, personal communication) with oligo dT in the RT reaction, in the hope that this may provide a better method of first strand cDNA synthesis.

Although the RNA used in the semi-quantitative analysis was DNAse-treated to remove any genomic DNA, it would have been better to use primers which were designed over an intron, which would allow cDNA-derived PCR products to be distinguished from possible genomic DNA (Krause, 1995). The resulting genomic band can then serve as a control for equal loading. Since each RT reaction can vary in efficiency and it is hard to quantify the starting amount of RNA, it is impossible to have exactly equal amounts of cDNA going into each PCR amplification. By using primers that span an intron it would give some idea of the relative amounts of starting material. This would probably have involved more work than the present study allowed for, but is currently being carried out in the laboratory.

Another issue in the battle for a truly semi-quantitative RT-PCR method is the question of using only one tube to amplify both the gene of interest and the constitutive gene together. This avoids any problems of uneven pipetting which may occur. The work presented here used the same master mix for these reactions so that everything except the cDNA was controlled for. Again, this particular problem is presently being addressed for future work.

Although there are many problems associated with using this semi-quantitative RT-PCR approach in our system, the work presented in this chapter does show that it is possible to identify *Brugia* transcripts from infected mosquito RNA. By comparing the levels of signals obtained for each of the genes, at the

chosen points within the life cycle, with that of the constitutively expressed ribosomal protein gene, an expression pattern for each of the four genes was determined. Despite it's limitations, this has proved to be a useful technique for examining expression patterns when only very small amounts of parasite material are available.

Chapter 6

Discussion

Epidemiological studies on lymphatic filariasis, (Vanamail *et al.,* 1989; Bundy *et al.,* 1991), have shown that the rate of gain of infection peaks in the 16-20 year age group. The reduced rate of gain in adults is thought to be a consequence of the development of an age-acquired immune response, by individuals with a history of infection. The most likely target of this immune response is the infective L3 stage as the parasite makes the transition from the arthropod vector to the mammalian host. A strong immunity to re-infection can also be produced in experimental models, by immunising with irradiated L3 (Oothuman *et al.,* 1979). These studies suggest an important role for L3 antigens in the development of a host-protective immune response, and so this project set out to identify genes that were up-regulated in the L3 stage of the parasite.

The initial approach was to immunoscreen an adult cDNA library with an antiserum raised against the L3. It was hoped that genes isolated by this method would be highly expressed in the infective form although still present in the adult. Since there is such a high level of cross-reactivity between the L3 and the adult, the serum was pre-adsorbed against adult antigens, in an attempt to make the immunoscreen more specific for L3 genes. A similar approach was adopted by Abdel-Wahab *et al.,* (1996), who differentially immunoscreened an adult *O. volvulus* cDNA library with sera raised in cattle against the related species *O. lienalis*. They isolated a clone (OvB20), which was recognised by antibodies from cattle immunised with irradiated L3 but not by antibodies from animals infected with non-irradiated larvae. The OvB20 gene is expressed in developing stages from embryo to the L4 larval stage but not in adult worms, and the recombinant OvB20 protein has been shown to induce partial protection in a rodent model of onchocerciasis (Taylor *et al.,* 1995). However, in the present study, this approach was not successful in that the genes identified were not L3 specific. The ladder protein was cloned on

more than one occasion from the *B. pahangi* adult library, confirming the immunogenicity of this protein. The other genes isolated by this method include myosin, which although not L3 specific, has been found to have immunogenic properties (Dissanayake *et al.,* 1992). The ribosomal protein gene was also identified in the immunoscreen; this gene was later isolated in a screen for genes which were equally expressed in both adult and L3 stages, suggesting that the immunoscreening method was unsuitable for identifying genes upregulated in the L3. It is now clear that many antigens are only expressed for a short length of time within the life cycle (Bianco *et al.,* 1990), especially between the mf and early L4 stages, which is a period of rapid change for the parasite. The adult worms however, have a relatively stable existence, often living for several years within the lymphatics. These observations suggest that it would be necessary to use an L3 library in order to isolate potentially interesting antigens, which may play a role in the establishment of infection, or be responsible for initiating an immune response in the host. However, at the time that this work was carried out, there was no conventionally prepared L3 library available for immunoscreening.

Once an L3 library was prepared in the laboratory, a differential screening method was used to isolate genes which were more highly expressed in the mosquito derived L3, compared to the post-infective L3. This SL-PCR cDNA library was made from parasites harvested at 3 days post-infection of the jird (Martin *et al.,* 1995), and was screened using PCR derived probes from adult parasites, mosquito-derived L3 and 3 days pi L3. Plaques which hybridised selectively to the mosquito-derived L3 probe were picked and four cDNA clones isolated in this way. After sequencing and searching the data bases, it was discovered that all the clones had homology to structural proteins of other nematodes. Other groups have also found a high prevalence of structural, ribosomal and housekeeping genes in their PCR-based libraries (Blaxter *et al.,* 1996). The reasons for this are unknown, although PCR libraries do tend to contain more small transcripts than a conventionally made library, and this could account for the high number of ribosomal genes that were cloned. For example, in the late vector-stage L3 SL-PCR library analysed by Blaxter *et al.,*

(1996), 24% of cDNAs coded for ribosomal proteins, compared to 10% from a conventionally made cDNA library from the same life cycle stage. The cuticular collagen precursor gene that was isolated in this study has also been identified from L3 libraries by other groups involved in the *B. malayi* tag sequencing project, and it appears to be an an abundant transcript at the L3 stage (Blaxter *et al.,* 1996).

The expression pattern of each of the four genes was confirmed by Northern blotting and semi-quantitative RT-PCR. All of the genes were found to be most highly expressed at the L3 stage, with a very low level of expression in the adult worm. From work on *C. elegans* (Cox *et al.,* 1981; Johnstone, 1994), it is known that individual cuticular collagen genes are differentially regulated during the development of the worm, and the expression patterns of the two cuticular collagen genes from *B. pahangi* fit this pattern, with high levels of expression in the larval stages but not in the adult. However, the tropomyosin and the E Rich protein, both almost certainly structural proteins involved in muscle contraction, present more of a puzzle. It would seem reasonable to expect that these types of structural protein, which are required throughout the life of the worm, would have a more constant expression pattern. It may be that the vector derived L3 is in an arrested state before it enters the mammalian host, and so the expression of many genes is reduced. This could result in an apparent increase in expression of the structural proteins, simply because other genes are switched off, or down-regulated, at this time point. The expression pattern of each of the four cDNAs was also studied during larval development in the mosquito. Although parasite transcripts must represent a minimal proportion of mRNA within the mosquito, it was interesting to note that all four mRNAs could be detected.

The immunological relevance of the four genes cloned is unknown, although tropomyosin has been shown to induce protective immunity when used to immunise experimental animals against *T. colubriformis* (O'Donnell *et al.,* 1989). A tropomyosin cDNA has also been cloned from *O. volvulus* (MOv14) and is known to induce a significant level of protection, in immunized jirds, against the filarial nematode *A. viteae*. (Taylor *et al.,* 1996). Vaccination with

the recombinant fusion protein of MOv14 also reduced the recovery of *O. lienalis* mf from the skin of BALB/c mice. Attempts are presently underway to express the E Rich protein (clone SJ5), in order to raise an antibody, which would allow further investigation of this protein. Other studies from this laboratory have demonstrated that infection with the L3 of *B. pahangi* induces an immediate burst of IL-4 production (Osborne *et al.,* 1996), yet nothing is known of the antigens that drive this response. It would be interesting to test the cloned cDNAs, as recombinant proteins, to see whether any of these induce IL-4 production in the mouse model.

Although the four genes cloned in this study coded for structural proteins, it has proved possible to identify *B. pahangi* transcripts, that are highly expressed in the L3 stage, using a differential screening approach. Now that additional vector-derived L3 libraries are available, through the filarial genome sequencing project, it would be interesting to differentially screen these libraries to determine whether additional cDNAs with important functional roles in the parasite life cycle, could be identified.

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