Studies on the cuticlin homologues of *Brugia* species

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DECLARATION

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

For Caroline Lewis,

with love and thanks.

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SUMMARY.

The aim of this project was to ascertain the existence of the *Brugia* homologues of the *C.elegans* cuticlin genes, and to isolate and characterise them. Preliminary experiments in which Southern blots of cleaved genomic DNA from various parasitic nematodes was hybridised to a ^{32}P-labelled fragent of *cut-1* revealed the presence of at least one hybridising band in each of the digests, implying the presence of at least one *cut-1*-like gene in each of the nematode genomes. The probe hybridised to several bands in the *B. pahangi* digests. During the course of the project various approaches were used in the search for the *Brugia* cuticlin homologues.

The initial approach was to screen an adult *B. pahangi* cDNA library with antisera raised against recombinant proteins corresponding to fragments of *C. elegans* CUT-1 and CUT-2. This resulted in the isolation of four clones, two of which carried the same insert, a truncated version of the other two. The full length clone is 2.2 Kb and comparison with the data base revealed it to be the *B. pahangi* homologue of a *C. elegans* dead-box ATP-binding RNA helicase gene (f01f1.7), identified during the course of the *C. elegans* Gerome Sequencing Project. The specific recognition of this unrelated cDNA is attributed to a combination of the following two factors: the rabbits in which the anti-cuticlin antisera were raised having a previous nematode infection resulting in the pesence of antibodies to nematode RNA helicases in the 'pre-immune' rabbit sera; and the high representation of RNA helicase cDNA species in the adult library, due to the the role of these enzymes in translation.

PCR using primers based on the sequence of the *Ascaris cut-1* gene (*ascut-1*) was carried out on *B.pahangi* genomic DNA, and resulted in the isolation of a 358bp fragment of a *B. pahangi cut-1*-like gene, subsequently named *bpcut-1*, showing a 71% nucleotide homology and a 96% amino acid homolgy with the corresponding region of the *C. elegans cut-1* gene, *cecut-1.* Primers based on the sequence of *bpcut-1* were used to isolate the corresponding cDNA from *B. pahangi* day 7 p.i. cDNA (this time-point in the life-cycle corresponds to the L3-L4 moult), revealing that *bpcut-1* has an intron not present in the *cut-1*-like genes of the other nematodes studied.

Screening a *B. malayi* genomic library with *bpcut-1* resulted in the isolation and subsequent sequencing of *bmcut-1*, a 613bp clone which is the *B. malayi* homologue of *bpcut-1.* There are three introns in the clone, and when they are removed the coding sequence corresponds to that of *bpcut-1*, resulting in a 97.5% homology between the two amino acid sequences.

Screening a *B. pahangi* genomic library with *bmcut-1* resulted in the isolation of 4 clones which cover the same 17kb region of the *B. pahangi* genome. The presence of the *bpcut-1*-specific intron in all four clones allows them to be identified as *bpcut-1*, rather than *bpcut-1*-like. PCR using one of the genomic clones with a 5' primer based on the linker region of EMBL3 (in which the cDNA library was made) and a 3' primer based on the *bpcut-1* sequence, allowed the

direction of transcription of the gene to be determined, and enabled the gene to be positioned within the genomic clone.

A 948 bp clone was isolated from an adult *B. pahangi* cDNA library by screening with *bmcut-1*. Sequencing and analysis revealed the clone to represent a mis-transcription of the *bpcut-1* gene; the resultant cDNA is apparently the result of transcription of the non-coding strand of the *bpcut-1* RNA.

The pattern of *bpcut-1* mRNA abundance throughout the mammalian stages of the life-cycle of the parasite strongly implies that it encodes a component of the nematode cuticle: peaks of abundance occur before the L3-L4 and the L4-adult moults, when the components of the cuticle are being maximally synthesised. The mRNA abundance profile also shows that the *bpcut-1* transcript can be detected during the intermoult period, but at a very much lower abundance. There seems to be little or no *bpcut-1* transcript detected in the adult mRNA, implying that the protein encoded by *bpcut-1* is incorporated into the insoluble component of the cuticle at the time of cuticle synthesis, and that it is not renewed throughout the adulthood of the parasite.

Localisation with an antiserum raised against recombinant ASCUT-1 showed a cuticular pattern on all life-cycle stages tested. Immunogold labelling showed that there was no labelling of the external surface of the cuticle. Interestingly, there was also strong recognition of an epitope on the surface of mature mf, implying the presence of an embryonic *cut-1*-like gene which was not detected by RT-PCR using *bpcut-1*-specific primers.

LIST OF ABBREVIATIONS

µci	microcurie
µg	microgram
µl	microlitre
µm	micrometre
APS	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phospahate
bp	base pair (DNA)
BSA	bovine serum albumin
c.p.m.	counts per minute
cDNA	complementary DNA
ddH_20	double-distilled deionized water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOC	(sodium) deoxycholate
EDTA	ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FITC	fluorescein isothiocyanate
g	gram
HRP	horseradish peroxidase
i.p.	intraperitoneal
IFAT	immunofluorescent antibody test
Ig	immunoglobulin
kb	kilobase (DNA)
KDa	kilodalton
l	litre
λ	lamda
L3	third stage (infective) larva
L4	fourth stage larva
M	molar concentration
mf	microfilariae
ml	millilitre
mm	millimetre
mM	millimolar
Mr	relative molecular mass
mRNA	messenger RNA
OD	optical density

p.i.	post-infective/infection
PCR	polymerase chain reaction
r.p.m	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
s.c.	sub-cutaneous
SL	spliced leader
TEMED	N,N,N',N',- Tetramethylethyldiamine
Tween 20	poly oxyethylenesorbitan monolaurate
w/v	weight per volume
WHO	World Health Organization

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION.

This project is concerned with the cloning of homologues of the *C. elegans* cuticlin genes from the filarial nematodes of the genus *Brugia*. The introduction attempts to discuss the parasite, the use of *C. elegans* as a model for parasitic nematodes and the importance of the nematode cuticle in the life-cycle of free-living and parasitic nematodes. Moreover it aims to discuss how a knowledge of the cuticlin genes and the proteins which they encode could contribute considerably to what is already known about this complicated and dynamic structure, which in turn could contribute to the development of novel methods of control for the diseases caused by parasitic nematodes. And finally it aims to show that a detailed knowledge of the nematode cuticle and how it is constructed could provide a model for the genetic and molecular control of a complex developmental process, which again could be applied to disease control.

1.1. THE PARASITE.

The lymphatic filariae (superfamily Filarioidae) are arthropod-borne parasitic nematodes. Their importance to man lies in the fact that three lymphatic filariae, namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, are the causative organisms of lymphatic filariasis, a serious and debilitating human disease. The species which was mostly used in the following studies, *Brugia pahangi*, is a close relative of *B malayi*. The natural host for *B. pahangi* is the cat.

1.1.2. The life-cycle.

In common with all nematodes, the filariae have five developmental stages in their life-cycle, marked by a moult, or shedding of the old larval cuticle. See Figure 1.1. a diagramatic representation of the *B. pahangi* life-cycle. The adult worms dwell in the afferent lymphatics of the mammalian host, where they can live for many years. After mating, the viviparous females release microfilariae (L1)directly into the lymphatics, from whence they migrate to the blood-stream. When in the peripheral circulation of the mammalian host, they can be ingested by a blood-feeding mosquito, and move to the mid-gut in the blood-meal. Here they exsheath, and penetrate the stomach wall into the haemocoel. The larvae then migrate to muscle cells in the thorax and undergo the L1 - L3 moults. The L3 stage is infective, and the larvae distribute themselves throughout the body cavities of the mosquito, showing a preference for the mouth parts (Denham &,McGreevy. 1977). The L3 larvae enter the vertebrate host via the puncture wound made when the mosquito takes a blood meal (Ewart & El Bihary, 1971). Studies on cats and monkeys (Ewart & El Binary, 1971; Ewart and Ho, 1967) have shown that the larvae penetrate the local lymphatic within a few hours of infection, move to the periodontal lymphatic sinus of the nearest lymph node (where they moult to the L4), and later migrate to the afferent

lymphatic, where they moult to the adult worm and remain for the rest of their lives. Adult females measure 40mm x 0.25mm, while the males are around 25mm long and 0.1mm wide. Timings of the larval migrations of the lymphatic filariae within their mammalian hosts vary from species to species, whilst development in the arthropod vector is temperature dependent.

1.1.3 Back-ground on the parasites and their vectors.

Lymphatic filariasis in humans has a prevalence of 118 million (Ottesen & Ramachandran, 1995), and is caused by three filarial parasites: *Wuchereria bancrofti* (which accounts for around 90% of infections) and *Brugia malayi* and *Brugia timori*, which together account for the remaining 10% of infections (WHO, 1984). The disease distribution is sub-tropical and tropical, defined largely by the survival capacity of the mosquito vector. Most infections are in Asia and Africa, with some in the Americas and in the Western Pacific. Two thirds of the people infected are in China, India and Indonesia.

The differences between the *Wuchereria* and *Brugia* genera and between the *Brugia* spp. are generally categorised on the basis of microfilarial periodicity, natural mammalian host range and vector susceptibility. Morphological differences between them are minor.

The microfilariae are not present continuously in the peripheral circulation of the infected mammalian host, but remain for much of the time in the blood-vessels of the deep tissues of the body, largely in the pulmonary vessels next to the pulmonary arterioles of the lung. The mosquito vectors of the filariae show a circadian rhythm in which they bite at certain times during the day or night. The microfilariae hence demonstrate a circadian periodicity which ensures that they are present at high densities in the peripheral blood only when the mosquito vector with which they are associated is actively feeding (Wharton, 1963). It is thought that this periodicity is effected by the difference in oxygen tensions between arterial and venous blood in the lungs, which drops at night when breathing is shallow and the body is inactive. A lowering of the venous-arterial oxygen tension will cause microfilariae to leave the environs of the lung for the general circulation, whilst raising oxygen tension results in the return of the mf to the arterioles (Hawking et al, 1967).

W. bancrofti is most prevalent in the nocturnal periodic form, and as such is largely transmitted by night-feeding mosquitoes: in urban areas the parasite is transmitted by *Culex quinquefasciatus* which thrives in shallow, dirty water associated with inadequate urban sanitation; in rural, agricultural areas the mosquito vectors are of *Anopholes* and *Aedes* spp., which require respectively clean and brackish water for completion of their life-cycles. Bancroftian filariasis is not a zoonosis; man is the only known natural vertebrate host of *W. bancrofti.* This makes disease control easier because there is no animal population which can act as a reservoir for parasite infection. The disadvantage from a research point of view is that there is no animal model for the disease, which makes experimental studies difficult. However, it should be noted that the silver leaf monkey (*Presbytis* spp.) has been experimentally

infected with *W. bancrofti* (Cross et al, 1979), and subsequent experimental studies (e.g. Morgan et al, 1986) have been carried out.

Brugia malayi is mainly found in South-East Asia. It has two main strains: the nocturnal periodic strain is found in agricultural areas, is transmitted via anopholine and aedine vectors, and, although experimental infections can produce patent infections in cats and monkeys, man is the only known natural vertebrate host (Laing et al, 1961); the nocturnal sub-periodic strain is largely found in the forest, is transmitted by Mansonoid vectors, and is widely believed to be zoonotic, infecting a range of feral monkeys, cats, dogs and other carnivores (Laing et al, 1960). Man and his domestic animals, dwelling in villages surrounded by forest, are infected by mosquitoes moving in and out of the forest. The feral animals serve as a reservoir for the parasite which makes the effective control of sub-periodic *Brugian* filariasis virtually impossible (Mak et al, 1982). In addition to the periodic and sub-periodic forms, there are a number of intermediate variants which have presumably arisen in response to local host/environmental conditions such as mosquito feeding and breeding habits and mammalian host availability.

Brugia timori has a tiny focus in the Indonesian islands of Timor, Flores, Rote and Alor, is nocturnally periodic, found only in man and transmitted by *Anopholes barbirostris.*

It is thought that *Brugia pahangi*, which infects a wide range of wild and domestic animals in South-East Asia, could also be responsible for some human infection. The parasite can be experimentally transmitted to man (Edeson at al, 1960), and Palmieri et al (1985) reported natural human infections from Indonesia. However, the infections were never passaged back into animals, and no natural human infection has been reported from Malaysia, despite numerous searches (Mak, 1987).

In 1947 it was estimated that at least 189 million people world-wide were infected with either *B. malayi* or *W. bancrofti* (Stoll, 1947). Diethylcarbamazine (DEC) which has been the only human microfilaricidal drug available for the last fifty years, was also discovered in this year (Hewitt et al, 1947). It is worth mentioning that other macrofilaricidal drugs have been used over the years in the treatment of lymphatic filariasis, but have all been abandoned due to unacceptable levels of toxicity. Despite the fact that DEC is relatively easy to administer and highly effective against the microfilariae of the lymphatic filariae, most recent estimates suggest that the infection prevalence in 1994 was at least 118 million. This failure to appreciably reduce the disease incidence is thought to be largely due to the rapid and ill-managed urbanisation which has taken place in many countries where lymphatic filariasis is endemic. Poor sanitation results in the creation of conditions which are ideal for maintenance of a *Culex* population, and over-crowding with people and their domestic animals facilitate the spread and maintenance of the disease.

It is hoped that the imminent licencing of Ivermectin as a human microfilaricide will have an impact on the control of lymphatic filariasis.

1.2. THE DISEASE.

1.2.1. The spectrum of infection.

Filariasis is a spectral disease with a wide range of clinical symptoms. The outcome of an individual infection is mediated by the type and intensity of immune response elicited by the parasite in the host (Otteson, 1980; Otteson, 1992). The two extremes are asymptomatic microfilaraemia caused by the parasite-specific down-regulation of the immune response; and amicrofilaraemic chronic disease caused by a strong immune response to the adult worms in the host lymphatics. Individuals in an endemic area can be divided into four main categories A-D, which are briefly discussed below.

A	B	C	D	E
None ? Infected ? Immune	Asymptomatic microfilaraemia	Filarial fevers	Chronic pathology	Tropical pulmonary eosinophilia

FIGURE 1.2. Spectrum of clinical manifestations of lymphatic filariasis in endemic regions. Adapted from Otteson (1984).

A. Endemic normals. Individuals who show no parasitological signs of infection, despite life-long exposure to infection. This could be due to effective immunity or to sub-threshold, pre-patent or single sex infections, resulting in undetectable levels of circulating mf, and known as occult infections (Otteson,1984). It is now accepted that there are very few, if any, truly infection-free individuals in an endemic population, the use of more sensitive diagnosis methods showing many endemic normals to be infected at very low levels (Day, 1991).

B. Asymptomatic microfilaraemics. This group makes up the majority of the infected endemic population. Despite having extremely high levels of mf circulating in their blood, they show no overt disease symptoms, and it is only relatively recently that this group has been identified as suffering from sub-clinical abnormalities of lymphatic and renal function (Dreyer, 1992). This group is immunologically hypo-responsive, specifically to filarial antigens: the parasites down-regulate the immune response to the infection (Otteson et al 1977; Otteson et al, 1982). In this way, asymptomatic amicrofilaraemics serve as a reservoir for continued transmission of infection.

C & D. Disease pathology amicrofilaraemics. Characteristic clinical symptoms are caused by the adult, lymphatic-dwelling worms. The pathology seen in these individuals is though to result from a break-down of tolerance, and the resultant immune response against the adult

worms in the lymphatics brings about severe lymphatic inflammation and intermittent fevers. Several studies have shown a direct correlation between the severity of lymphatic damage and the level of immune responses to parasite antigens (Otteson, 1980; Klei et al, 1981). Repeated acute inflammatory attacks result in dilation, thickening and eventually fibrosis of the lymphatic vessels, with accompanying oedema. This in turn leads to the hardening of tissues and skin surrounding the enlarged affected areas, producing chronic elephantiasis. Resultant reduced circulation encourages secondary fungal and bacterial infections, which are responsible for much of the gross pathology of elephantiasis (reviewed by Otteson and Ramachandran, 1995). Paradoxically, mf are not usually detectable in the blood of clinically symptomatic individuals, presumably due to the decrease in immunosuppression, and subsequent clearing of the parasites.

E. Tropical Pulmonary Eosinophilia (TPE). This is a rare condition, brought about by immunological hyper-responsiveness to parasite antigens. This group rarely have circulating mf in the blood stream. Symptoms are respiratory and are thought to result from allergic responses to large numbers of degenerating mf in the lungs, trapped following antibody opsonisation.

1.3. THE IMMUNOLOGY OF FILARIASIS.

The immunology of filarial infections in the vertebrate host is a complex field. However, this is not the place for a discourse on the current issues of filarial immunology (see Maizels et al, 1995, for a review of recent advances) and the following is a conceptual glance at the subject. The most striking feature of filarial (and helminth in general) infections of humans, is the long and stable nature of the relationship. Filarial adult worms can live for more than five years in the lymphatics of the host (Vanamail et al, 1996), producing untold millions of microfilariae which circulate in the blood and facilitate the extremely efficient transmission of disease throughout the endemic population, in the vast majority of cases without causing the host severe pathology, and never resulting in mortality. This is a good example of a long-evolved, finely-tuned and therefore successful host-parasite relationship, depending on immunological tolerance of the host to the parasite, with an eventual mechanism for the prevention of super-infection (Maizels and Lawrence, 1991). It should be remembered at this point, however, that although the parasites are not responsible for mortality, many individuals suffer greatly from morbidity and disfigurement for a great number of years.

The natural history of a filarial infection, creating an accurate picture of the progression of the disease, depends upon continuous re-infection over many years. Basically, naive infection is accompanied by a parasite-specific T-cell response which does not succeed in curing the infection, allowing the parasites to establish themselves and reach maturity in the lymphatics. The first appearance of microfilariae in the blood is accompanied by a down-regulation of all effective parasite-specific immune responses (Leiva & Lammie, 1989). That the mf are responsible for this down-regulation is shown by the fact that treating microfilaraemics with the

microfilaricidal drug DEC results in a partial restoration of the immune response (Piessens et al, 1981; Lammie et al, 1992; Sartono et al, 1995). Two distinct types of tolerance to filarial infection have been described. The first derives maternally from a microfilaraemic mother (Lammie et al, 1991) and results in long-term (up to twenty years) tolerance in the off-spring, extending to Th1 and Th2 responses (Steel et al, 1994). The mechanism for neo- or pre-natal tolerance is thought to be a deletion of antigen-specific lymphocytes *in utero* by exposure to micrfilariae or filarial antigens during thymic development (Weil et al, 1983), and is known as clonal deletion. The second type is that seen in microfilaraemics and is thought to be the result of T-cell anergy, is probably specific to the Th1 sub-set, and may be due to inadequate antigen presentation (Schwartz et al, 1990; Jenkins et al, 1987).

Resistance to super-infection is achieved by acquired protective immunity to the incoming, infective L3s, independent of tolerance to other life-cycle stages of the parasite. This concomitant immunity as a defence against super-infection has also been described for other helminths (Maizels et al, 1993). It has long been known that repeated infection with irradiated *B. pahangi* L3s results in high levels of protective immunity in cats (Denham et al, 1972; Denham et al, 1983). These results are mirrored in studies of endemic human populations where individuals are exposed continuously to infective mosquitoes. Two large-scale quantitative studies on the rate of gain of infection in age-stratified populations have demonstrated that children and adolescents up to the age of twenty show a gradually increasing worm burden, at which point the rate of gain of infection begins to slow down, resulting in a stable worm load in adults (Day et al, 1991a & 1991c; Bundy et al, 1991).

So far what has been described is a stable host-parasite relationship wherein the parasite is transmitted effectively throughout the host population at a level which does not cause appreciable pathology in the host, and is the status enjoyed by microfilaraemic asymptomatics. This equilibrium depends upon the maintenance of tolerance, which unfortunately is not an infinitely stable state. According to a well-accepted model (Maizels and Lawrence, 1991), with increased years post-infection tolerance breaks, and the host mounts an effective response against both the adult and mf stages of the parasite. In the case of a host having built up an effective degree of protective response against incoming L3s, this will result in an 'immune' endemic normal. However, if the anti-L3 response is not sufficiently protective, the host will be subjected to continuous re-infection leading to the mounting of an aggressive immune response against the maturing parasites and ensuing mf, which results in the pathology of the disease.

1.4. DIAGNOSIS, TREATMENT AND CONTROL.

Traditionally, parasitological diagnosis was by the direct detection of mf in the blood, via stained blood smears. More sensitive variations involve the use of a counting chamber (Denham et al, 1971) and concentrating blood samples using a Nucleopore filter (Southgate, 1974), but these methods do not circumvent the problems of diurnal periodicity where mf are

present in the peripheral circulation in the middle of the night, or of occult infections which do not result in the production of mf.

A recent lymphatic imaging technique, lymphosintography, can detect lymphatic abnormalities in infected but otherwise asymptomatic individuals, allowing the direct assessment of chemotherapeutic measures on adult worms for the first time (Otteson and Ramachandran, 1995).

Serodiagnosis methods employ indirect detection of parasite antigens or parasite-specific antibodies. For *W. bancrofti*, the circulating levels of the phosphorylcholine-containing antigen (PC-antigen), detected by a MAb, have been shown to give an indirect measure of worm burden (Weil et al, 1988; Day et al, 1991b). For *Brugian* filariasis the measurement of filarial-specific IgG4 has been shown to be a good measure of active *Brugian* filarial infections, even in the absence of other clinical or parasitological evidence (Kwan-Lim et al, 1990; Kurniawan et al, 1993).

The use of species-specific DNA probes and PCR techniques for diagnosis (Williams et al, 1996; McCarthy et al, 1996) and for detecting filarial parasites in vectors to assess transmission of infection (Dissanayake and Piessens, 1992; Chanteau et al, 1994) is also gaining wider use.

Successful control of the disease uses a two-pronged approach to prevent the transmission of the parasite: reducing human-vector contact by mosquito eradication programs; and treating the human population to reduce microfilaraemia. In the past, individuals with pathology or detectable levels of microfilariae were dosed with the highly effective microfilaricide DEC over a 12 day period (Otteson, 1985; Partono, 1989). However, the need for repeated treatment, combined with a variety of unpleasant side-effects, meant that compliance with the chemotherapy regime was poor. It is now accepted that a more effective, and cheaper, alternative is to add low-dose DEC to the cooking salt of an endemic population, ensuring its daily consumption over a 9-12 month period, or to administer a semi-annual single dose treatment to the whole population (Meyrowitsch et al, 1996). This ensures that asymptomatic microfilaraemics and individuals with undetectable levels of microfilariae who would normally not seek treatment but continue to act as a reservoir for parasite transmission, are cleared of parasites.

Treatment of individual symptoms have recently been shown to limit the progression of chronic pathology, and can cause regression of debilitating limb abnormalities (Otteson and Ramachandran, 1995). Such measures include oral antibiotic treatment for adenolymphangitis episodes and scrupulous local hygiene, in combination with anti-fungal and topical antibiotic application for elephantiasis patients.

Vaccine development strategies focus on the L3 stage of the parasite, as it provokes a protective immune response in the host, and to this end there are many groups working on the identification of L3-specific genes and antigens. The search for an effective adulticide would also be an asset in the total disease eradication of lymphatic filariasis (although its use would

have to be carefully monitored as much of the lymphatic pathology of filariasis is caused by severe inflammatory responses due to the death of adult worms). However, it must be said that there is no reason why the effective use of mosquito control measures combined with mass-treatment with DEC or a similar microfilaricide, should not lower transmission of the parasite to levels which result in disease eradication in endemic areas.

1.5. *C. elegans* AS A MODEL ORGANISM FOR STUDIES ON PARASITIC NEMATODES.

1.5.1. Suitability as a model.

The free-living soil-dwelling nematode *Caenorhabditis elegans* was proposed, by Sydney Brenner in 1965, as a model to study animal development and behaviour, with an emphasis on the nervous system. The organism was chosen for a range of practical reasons which have been more than justified over the years. The life-cycle is simple and rapid, with a three-day generation time, and the worm populations can be maintained on agar plates, fed on *E. coli,* and can be easily frozen and stored indefinitely with no loss of viability. The animal is small (about 1mm long) and transparent, which has permitted reconstruction of the entire anatomy at the ultrastructural level with serial section electron microscopy. *C. elegans* populations consist normally of hermaphrodites, with males arising at a frequency of about 0.01% (Hodgkin et al, 1979). Consequently, reproduction is usually by self-fertilisation but cross-fertilisation is possible, which makes genetic studies very straightforward: mutants can be generated, propagated and analysed efficiently and relatively easily. Cellular development and position is invariant, permitting a complete account of cell lineages, from zygote to adult (Sulston et al, 1988). The genome is small for a metazoan: 10×10^8 base pairs, about 1/30 the size of the human genome (Waterston, Sulston and Coulson, 1997). This has permitted the construction of an almost complete physical map and the release, to date, of more than half the assembled sequence, obtained largely from gene-rich regions of the genome. In addition, randomly generated partial cDNA sequences known as expressed sequence tags (ESTs) have been cloned and sequenced to give some idea of how *C. elegans* genes are transcribed and processed. ACeDB, the *C. elegans* database, aims to co-ordinate and up-date the data being produced by the *C. elegans* research community, and as such provides an interactive display of genetic and physical maps, with corresponding genomic and cDNA sequence data, and additional information.

In addition to the simplicity of obtaining and analysing genetic mutants of *C. elegans*, allowing the phenotype of a gene to be understood with relative ease, transformation of hermaphrodites by injection of genetic material directly into the gonads, which results in a clonal population of a transgenic worm, can be used in a variety of ways to study the expression of a gene (Fire and Waterston, 1989; Fire et al, 1990; Mello et al, 1992a). Genes

can also be specifically 'knocked-out' by the use of transposons, and the resultant mutant transgenic organisms used to analyse the phenotype of the gene (Plasterk & Groenen, 1992).

1.5.2. Potential as a model for metazoans.

As a model organism for metazoan genes, *C. elegans* has proved to be even more of a success than was hoped: approximately 48% of the genes so far sequenced have significant homologies to genes previously sequenced in other organisms; half of all cloned human disease genes have similarities to *C. elegans* genes, which in some cases is the only homology to be found in all of the available data bases (Waterston et al, 1997). Obtaining the *C. elegans* homologue of a gene from another organism provides the opportunity of sequence comparison which can highlight conserved regions and provide information on intron organisation and even promoter structure of the gene. Additionally, studies of the expression and function of the *C. elegans* homologue could indicate parallel function in other metazoans.

1.5.3. Potential as a model for other nematodes.

The possibilities outlined above are obviously vastly expanded for studying other nematodes. Despite huge adaptive differences within the phylum, which have enabled nematodes to colonise every imaginable ecological niche, the basic nematode features are conserved to a remarkable extent: uniform early embryonic cell lineages; the retention of a basic body plan of two concentric tubes separated by a pseudo-coelomic space containing the gonads and bound by the extra-cellular cuticle (Wood, 1988); four moults resulting in five stage-specific cuticles and a basic layered cuticle pattern (Zuckerman et al, 1973). With the realisation that the 22-nucleotide *trans*-spliced mRNA leader sequence (SL1), originally discovered (outside the trypanosomatids) in *C. elegans* (Krause & Hirsch, 1987) was also present in all the parasitic nematodes so far studied, it would seem that certain regulatory sequences within the nematode phylum are also conserved. This raises the possibility of transforming *C. elegans* with the regulatory sequences from the genes of parasitic nematodes fused to a reporter gene such as β-galactosidase, in order to establish the spatial and temporal expression of the genes *in vivo* (Fire et al, 1990).

1.6. THE NEMATODE CUTICLE.

1.6.1. Basic structure of the cuticle.

The nematode cuticle is a multi-layered, extra-cellular matrix structure which completely surrounds the worm apart for small openings into the pharynx (which is lined with cuticle), anus and vulva. The cuticular components are synthesised by the outermost cellular layer of the worm, which is known as the hypodermis (Kan et al, 1968; Lee, 1970). A cuticle is synthesised five times during the life-cycle of the worm: once in the egg prior to hatching (or, in the case of filarial worms, within the microfilarial sheath) and during the moult at the end of

each larval stage. The new cuticle is synthesised underneath the old one, which is then sloughed off during a process called ecdysis. During cuticle deposition, large Golgi bodies can be seen in the hypodermal cells and vesicles can be observed budding off from the Golgi apparatus and moving to the surface of the hypodermis, presumably carrying cuticular components where the new cuticle is being deposited (Singh & Sulston, 1978; Bonner et al, 1971).

1.6.2. Evolution of the cuticle.

The cuticle is a complex, multi-layered structure, the particular nature of which varies between nematode species and between different life-cycle stages of the same species. These variations obviously reflect the different requirements of the worm surface depending on the environment in which it finds itself. However, the basic structure of the nematode cuticle is surprisingly invariant (reflecting the general evolutionary uniformity of the group), consisting of the epicuticle, external and internal cortical, medial, fibrous and basal layers. In addition, a loosely associated, carbohydrate-rich surface coat external to the epicuticle can be detected in some nematodes (Zuckerman et al, 1979), although the adult stages of the lymphatic filariids have not been shown to possess such a structure (Blaxter et al, 1992).

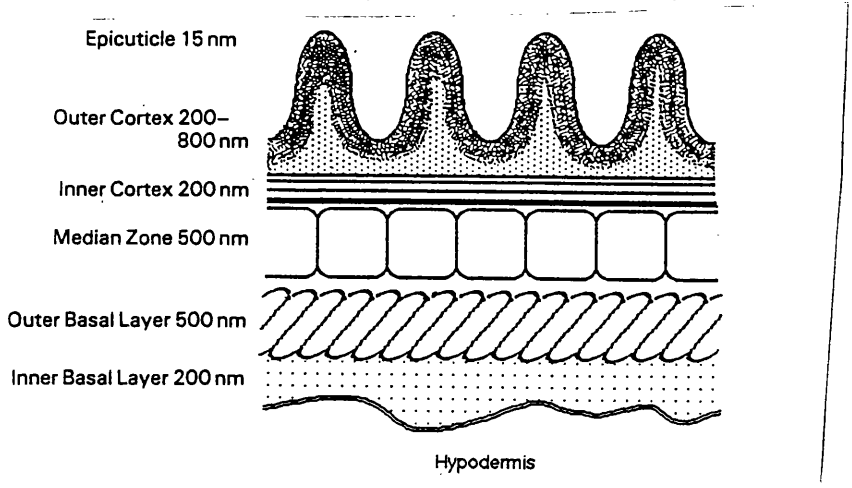

Figure 1.3. Diagrammatic representation of the cuticle of adult *B. malayi* (Selkirk, 1991).

The basal, fibrous and medial layers of the cuticle are composed mainly of di-sulphide cross-linked collagens (Cox et al, 1981a; McBride et al, 1967; Leushner et al, 1979), whilst the cortical layers are made up of non-collagenous, insoluble 'cuticlin' proteins (Cox et al, 1981a; Fujimoto & Kanaya,1973) and the epicuticle contains lipids (Bird &Bird, 1991; Proudfoot et al, 1991). Various antigenic surface/cuticular molecules have also been identified from parasitic and free-living nematodes (Parkhouse et al, 1981; Maizels et al, 1984; Zwelebil et al, 1993). In the case of parasitic nematodes, much interest has been generated by these molecules on the grounds that they could be used to devise rational control strategies for nematodiases.

It would seem likely that the nematodes have evolved from an ancestor which had only one larval stage, and therefore only one cuticle. Inglis (1964) proposed that moulting evolved as a way of increasing the radial strength of the cuticle by changing its morphology. This would allow the hydrostatic pressure of the organism, on which locomotion depends, to increase with growth, whilst maintaining the integrity of the worm morphology. However, this theory does not fit easily with observations that some nematodes produce identical, but thicker, cuticles at each moult (e.g.. *Panagrellus silusiae*) and that others are capable of enormous growth between moults and after the final moult (e.g. the adult *Ascaris* increases in length nearly thirty-fold without moulting). However, there seems very little doubt that it was the gradual acquisition of different larval stages with accompanying changes in cuticular morphology which has allowed the nematodes to colonise such an enormous range of environments. Nematode life-styles range from free-living in the soil and on the ocean floor, to parasitic on vertebrates, insects and other helminths, and cover all intermediate stages of inter-organism dependency; their ability to adapt to diverse ecological niches is largely dependent upon the specific adaptations of the cuticle (see Bird, 1980, for examples).

C. elegans is often portrayed as a 'prototype pre-parasitic nematode species' (Blaxter, 1993), from which the basic nematode architecture is elaborately adapted to aid survival in a range of host organisms. However, there is some reason to believe that *C. elegans* could in fact be a secondarily derived free-living form, having re-evolved a free-living life-style from a parasitic ancestor (Cox et al, 1981c). It is true that members of the Rhabditis genus are insect parasites, and that members of the same genus associate with specific annelid and arthropod vectors to aid dispersal (Chitwood & Chitwood, 1974). As is discussed further below, the cuticle of *C. elegans* changes substantially at each moult with respect to structural protein components, and each life-cycle stage has a distinct set of surface proteins/antigens (Politz et al, 1987; Blaxter, 1993). Also, the lipid nature of the epicuticle undergoes changes within an instar in response to alterations in temperature and pH (Proudfoot et al, 1993). Immunofluorescence antibody experiments have demonstrated that *C. elegans* larvae shed/release antibody-bound surface antigens (Hemmer et al, 1991) which is a well-documented mechanism used by parasitic nematodes to evade the host immune response. These seem extreme adaptations for an organism which undergoes the entirety of its life-cycle in one, relatively stable and inert soil environment.

The size of the collagen gene family in *C. elegans* is huge, estimated at between 50-150. This estimate was made on the basis of the ratio of collagen to actin-hybridising clones in *C. elegans* genomic phage libraries, given that there are four actin genes in the genome (Cox et al, 1984). Most of the collagens are thought to be cuticular proteins. This number contrasts with that of the collagen genes of parasitic nematodes, which does not exceed twenty (Selkirk et al, 1989; Shamansky et al, 1989; Kingston, 1991, Bisoffi & Betschart, 1996). This is despite the fact that the parasitic nematodes have complex life-cycles which involve adaptation to many fluctuating physical parameters, for which a more flexible and adaptive cuticle, provided

by a wider spectrum of collagen proteins, would presumably be useful. To reiterate: why does *C. elegans* require this level of potential adaptive sophistication at both the structural and surface protein levels of its cuticle?

It is of course possible that *C. elegans* is the evolutionary result of a passing flirtation with, and retraction from, parasitism. On the other hand, it could be that the free-living environment is in fact much more challenging to the nematode than is the parasitic life-style, at least with respect to the demands made on the adaptability of the cuticle. Whilst the parasite has to undergo major adaptations when it changes from one host to another, once established in the new host environment the surroundings remain relatively stable. However, the soil-dwelling nematode, especially one which is as ubiquitous as *C. elegans*, has to adapt to continuously changing parameters imposed by fluctuations in the environment and the behaviour and success of other organisms. As such the surface molecules, acting either defensively or, a more likely scenario, as signal receptors for environmental or developmental cues, would be expected to be expressed in a complex manner. Likewise a more immediately responsive cuticle structure would justify the large collagen gene family.

Ultimately, whether the direction of evolution is towards or away from parasitism, the similarities and differences in the cuticles of free-living and parasitic nematodes mean that techniques and information from one group can always be used and extended to further the understanding of the other.

1.6.3. Basic function of the nematode cuticle.

The cuticle forms the exoskeleton of the worm, and as such maintains morphology and, in combination with muscular action and the high internal hydrostatic pressure of the animal, allows motility. In addition, the cuticle is the surface presented to the external environment, and is hence responsible for the protection of the worm: in free-living nematodes such as *C. elegans*, defence is against dehydration and predation by nematophagous fungi and bacteria, the spores and hyphae of which adhere specifically to the nematode surface (Barron, 1977; Wharton et al, 1990); whilst in parasitic nematodes defence is against the immune system of the host. Many parasitic nematodes have extremely complicated life-cycles, involving very different host organisms and free-living stages. Helminth infections of vertebrates are also characterised by the long length of time that the adult parasite inhabits the host. Therefore the cuticle must be adaptive to different environmental conditions, in addition to having properties which will allow evasion of the host immune response by the parasite.

For some nematodes the cuticle also has a physiological function, as a site for nutrient acquisition. It has been shown that the L3 stage of *B. pahangi* has an occluded gut until four days post-infection of the vertebrate host (Collin, 1971) and that nutrients are obtained via a trans-cuticular route (Howells & Chen, 1981). Trans-cuticular up-take of glucose had previously been reported in *Dirofilaria immitis* (Chen & Howells, 1981), *Trichinella spiralis* (Stonor & Hankes, 1958) and *Mermis negrescens* (Rutherford and Webster, 1974).

1.7. DYNAMIC FEATURES OF THE NEMATODE CUTICLE.

The nematode cuticle is an extraordinarily dynamic structure, responding rapidly to the demands of the nematode life-style at a number of levels. It has long been accepted that the characteristics of the nematode cuticle vary with life-cycle stage, and that the formation of an entirely new cuticle at each moult allows this variation (Cox et al, 1981b). Largely through research on parasitic nematodes, it has been shown that the surface-associated molecules and the basic biophysical properties of the epicuticle are also highly variable between moults, responding to the changing environment of the nematode.

1.7.1. The epicuticle .

It has been shown that the lipid nature of the epicuticle of various parasitic nematodes changes when the parasite moves to the mammalian host environment (Proudfoot et al, 1993). This could enable the parasite to cope with the rapid changes in the parameters of the physical environment without loss of integrity of the outer layer of the cuticle; or it could confer increased resistance to host immune defence mechanisms. The mechanisms involved in these rapid changes are as yet unknown. Similar changes occur in the dauer stage of *C. elegans*, which is often likened to the developmentally arrested L3 stage of parasitic nematodes, when exposed to fresh *E. coli,* which acts as a stimulus for the reinitiation of development. This would imply that the mechanism is developmental and/or adaptive, rather than being evasive of an immune response. The same group noted that, following the transition to the vertebrate host, the L3 of *Acanthocheilonema viteae* and *Nippostrongylus brasiliensis* actively take up host lipids. This could be because they are required for metabolic or biosynthetic purposes; they could be essential for the delivery of messages involved in signalling pathways concerned with parasite development; or they could be involved in the masking of surface antigens which may otherwise act as a target for the host immune response.

1.7.2. Surface / cuticular antigens.

There are various examples of surface antigens being involved in maintenance of the cuticle or in defence strategies. Changing the antigens in a life-cycle specific manner allows the parasite to avoid the host immune response; by the time the immune system has managed to raise a response, the surface proteins have been completely changed and the parasite is unrecognisable. This method is obviously useful only in a primary infection, and is utilised by *T. spiralis* (Ortega-Pierres et al, 1984a). Parasites can also produce large amounts of surface antigen which is then released and acts as a 'decoy' to the host immune response (Maizels et al, 1984a). Alternatively, surface antigens can act as an immunosorbent, reacting with host immune factors but undergoing such a high rate of turnover that the nematode remains undamaged, as occurs in the case of *Toxocara canis* (Maizels et al, 1984a).

Other surface antigens have been shown to have recognisably protective functions in the nematode cuticle. The major cuticular antigen of the lymphatic filarid adults is an N-glycosylated glutathione peroxidase (GPX) homologue (Devaney, 1988; Maizels et al, 1989, Cookson et al, 1992). This filarial GPX is made in the hypodermis, secreted across the cuticle, and is active against lipid peroxidases, so probably protects the epicuticle of the tissue-dwelling adult parasites from peroxidative disruption (Tang et al, 1995). This molecule is also a potent antigen in human lymphatic filariasis (Maizels et al, 1983) and is therefore a possible candidate for immunoprophylaxis against the disease. A second cuticular antigen from *B.malayi* has been identified as a secreted superoxide dismutase (Tang et al, 1994) which is thought to be involved in eliminating superoxide generated by the lipoperoxidase. A surface protein from the cutaneous filarid *Onchocerca volvulus* was cloned and characterised as a cystatin proteinase inhibitor, again thought to be involved in cuticle maintenance or in neutralising the effects of proteases released by host immune effector cells (Lustigman et al, 1992).

The surface-expressed proteins of *C. elegans* have also been shown to be stage-specific: an adult-specific polyclonal antiserum, made by adsorbing antiserum raised against the cuticles of all stages of wild-type *C. elegans* onto the cuticles of larval *C. elegans*, showed no reactivity, in immunofluorescence staining experiments, to the surface of larval *C. elegans*, but reacted against the adult worms (Politz et al, 1987). This demonstrated that there is a distinct class of adult-specific surface molecules. The same adult-specific antiserum was then used to identify adult worms in natural populations of wild-type adults which did not stain, and therefore lack the stage-specific surface antigens. That the expression of adult-specific antigens is dependent upon the presence of an adult-type cuticle rather than sexual maturity, was shown by the fact that the antibody will not stain the surface of adult *lin-4* mutants, which are characterised by the fact that the sexually mature worms continue to express a larval cuticle (Ambrose & Horvitz, 1984). Subsequent genetic analysis by linkage testing and using immunofluorescence of live worms as a phenoptypic marker, mapped the gene or genes responsible for the adult-specific expression of surface antigens to a single locus. This naturally-occurring polymorphism in antigen expression could be important for parasitic nematodes as a mode of surviving attack by the host immune response; recognition of one set of surface antigens by the host immune response could result in the selection of a parasitic population bearing a set of 'new' surface antigens, which are not recognised by the host. Indeed, the surface antigens of the infective larvae of *A. lumbricoides* do appear to show heterogeneity (Fraser & Kennedy, 1991).

Subsequently, the adult-specific antiserum was adsorbed onto wild-type adult cuticles and used to screen the adult progeny of worms which had been chemically mutagenised by ethyl methanesulphonate (EMS) (Politz et al, 1990). Worms that did react were therefore mutants arising from a mutation which results in the 'unmasking' of antigens which are normally hidden in the wild-type cuticle, presumably due to a post-translational modification or modifications.

Again, this has relevance to parasitic nematode research; surface antigen diversity can clearly be generated by the unmasking of an epitope already present in the cuticle, and could therefore represent another mechanism for evading a host-immune response. In addition, it could be a mechanism used for adapting to a new environment, or indeed becoming infective, without changing the cuticle completely, i.e. between moults. This would be relevant for parasites such as *Brugia* spp, the L3 stage of which develops in the mosquito to the form which is infective to the vertebrate host, without moulting, or indeed changing the morphology of the cuticle in any way. Other surface antigen mutants have since been identified which also effect internal structures in the worm, presumably due to the loci being involved also in expression of basal membrane extra-cellular matrix proteins (Link et al, 1992). A third type of surface protein mutation results in the mis-timing of surface antigen expression; these mutants express an L1-specific surface antigen at later larval stages (Hemmer et al, 1991).

1.8. The structural proteins.

From cuticle isolation and characterisation studies carried out on *C. elegans, Panagrellus silusiae* and *Ascaris lumbricoides*, it was established that the basal layer, struts, and internal cortical layers of the nematode cuticle are made up of collagens, cross-linked by di-sulphide bonds. This is shown by the fact that they can be solubilised from the cuticle using di-sulphide bond reducing agents such as 2-mercaptoethanol, and hydrolysed by clostridial collagenase. In contrast, the external cortex is made up of a mixture of covalently cross-linked, highly insoluble proteins which are known as cuticlins (Fujimoto & Kanaya, 1973; Leushner et al, 1979; Cox et al, 1981a, Fetterer & Urban, 1988).

From ultrastructural studies on the cuticles of *B. pahangi* and *C. elegans*, it would seem that the structure of the larval cuticle at the L2-L4 stages is fairly invariant, becoming thicker in proportion to increasing body diameter (Cox et al, 1981b; Rogers et al, 1974). The adult cuticle has the additional layer of struts not seen in the larval cuticles. All stages have annulations on the cuticle surface, which are thought to be essential for locomotion; closely-spaced annuli minimise radial distortion whilst giving resistance and elasticity which can be used against the high hydrostatic pressure of the organism and the longitudinal muscles to allow sinusoidal movement (Clark, 1964). In *C. elegans*, the cuticle of the dauer larva differs from the other life-cycle stages (Cassada & Russell, 1975). The dauer larva is a developmental alternative to the normal L3 juvenile, and arises in response to adverse environmental conditions such as over-crowding and lack of food. Instead of L2 larvae moulting to L3, they moult to the dauer, a specialised resistant stage which does not feed and can survive for months, in addition to having certain behaviours which facilitate their transport, by other organisms, out of the unfavourable environment. It is thought that the dauer is the equivalent of the dispersal/infective stage of the parasitic nematode. The cuticle of the dauer larva is extremely tough and resistant; dauers can survive in 1% SDS, which kills all other life-cycle stages of the worm. The cuticle is correspondingly thicker, with a thicker insoluble

external cortical and a striated basal layer not seen in other life-cycle stages. In favourable conditions, the dauer will resume normal development to the L4 and adult stages (although such individuals have been shown to express different surface molecules than those which undergo an uninterrupted development, Blaxter, 1993). Cox et al (1981b) showed that the proportion of insoluble to soluble cuticle proteins is much higher in the dauer cuticles.

As has already been mentioned, the nematode cuticle collagens are encoded by a large gene family, and it now appears that the insoluble cuticle proteins, named the 'cuticlins', are similarly encoded by a gene family. The following is a compressed review of the enormous amount of work that has been done on the nematode collagen gene family, and a review of what is known about the cuticlin genes and the proteins which they encode.

1.8.1 The cuticlins versus the collagens.

There are many apparent similarities between the collagens and cuticlins which make up the extracellular matrices, in particular the cuticle, of the nematodes. This means that the research already done on the nematode collagens, working on *C. elegans* and on other free-living and parasitic nematodes, can often be looked towards to provide guidelines in the much newer field of cuticlin research.

Both the cuticlins and the collagens are structural proteins which comprise the components responsible for the complex infrastructure of the nematode cuticle. It would now appear that both the soluble and insoluble protein components are encoded by gene families which are, at least in *C. elegans*, quite extensive. The genes from both families can be classified according to sequence and structure, and in both cases the similarities within the same class between different nematode species have been shown to be greater than the similarities between members of different classes from the same species. Both gene families are also ubiquitous throughout the genomes of the nematodes so far studied. The expression of both gene families appears to be stage-specific, enabling the nematode cuticle to be optimally adapted to the differing demands which are placed on each different life-cycle stage of the worms. Furthermore, the expression of the genes of both families appear to be regulated at a complex level: the collagen genes are expressed differentially, both quantitatively and qualitatively, within as well as between moults; and the cuticlin genes so far studied appear to show great variation and specificity with respect to spatial expression. As would be expected for the genes encoding cuticle structural proteins, the overall quantitative expression of collagen and cuticlin genes peaks immediately before a moult, but expression apparently continues at a much reduced rate during the intermoult period. Both sets of proteins are synthesised in the hypodermal cells underlying the cuticle, and are transported into the extracellular matrix via unknown mechanisms, the timing of which could well represent another level of developmental control. It would seem, from the extensive amount we know of the collagens and the more limited evidence available thus far from the cuticlins, that the proteins are synthesised and transported from the hypodermis as monomers, which are then polymerised

and cross-linked during the construction of the cuticle. This means that the collagen and cuticlin proteins encoded by the gene family members of each group certainly interact with one another, and most probably with the proteins of the other group. These intimate protein interactions mean that it is difficult to link genotype to phenotype during the process of genetic analysis. However, the extensive analysis of collagen gene mutants carried out using *C. elegans* has created a well-established field of study which will almost certainly be used in the future to analyse the cuticlin genes.

As will be shown, the cuticlin gene family encodes proteins which are not as structurally or functionally homologous as the collagens. The collagens are specifically structural and supportive, whereas it is quite possible that the cuticlin-like proteins have adapted, via a more complex and as yet speculative evolutionary pathway, to fulfil a number of niches in the development of the nematode. However, with respect to their role in the nematode cuticle, the knowledge gained from one gene family will certainly contribute to what is already known about the other.

1.9. The collagens.

Collagens are a group of heterogeneous extra-cellular structural proteins with a characteristic structure. They have been extensively studied in vertebrates, which have at least twenty-five collagen genes which can be grouped into thirteen types based upon structural differences in the molecules (Vuorio & de Crombrugghe, 1990). The molecules undergo extensive post-translational type-specific modification. Each type has a distinctive supramolecular structure, structural function and tissue distribution. The nematode collagens are of two types: the basement membrane collagens, which have many features in common with vertebrate basement membrane collagens (Guo & Kramer, 1989); and the cuticular collagens, which possess a unique structure.

The collagen gene family in *C. elegans* comprises between 50 and 150 genes, and the full-length genomic sequences of more than thirty of these have been determined (Kramer, 1997). Based on computational analyses of 7299 predicted genes, there are 97 *C. elegans* collagen proteins (Wormpep release 11), the vast majority of which are thought to be cuticular collagens. The genes are distributed relatively evenly throughout the genome of the worm (Cox et al, 1985). There are examples of collagen genes situated close to one another with highly conserved sequence similarity, presumably as a result of gene duplication (Park & Kramer, 1990; Levy et al, 1993) whilst there are other pairs showing high levels of sequence similarity which are widely dispersed in the genome. The genes are small (between 1-2kb) and have 1-3 short introns, and their triple helical regions are often interrupted by non-helical amino acid sequence (Kramer et al, 1982). It has also been shown that the cuticular collagens are encoded by a gene family in other nematode species, as indicated by numbers of hybridising bands on genomic Southern blots when collagen-specific probes are used. A *C. elegans* collagen probe identifies about fifteen putative collagen genes in the *Ascaris*

genome (Kingston, 1991); several different *Haemonchus contortus* collagen cDNA clones recognise the same number (about twenty) of bands of varying intensities (Shamansky et al, 1989); and Selkirk et al (1989) used a probe coding for the Gly-X-Y domain of a chicken type-1 collagen to demonstrate hybridisation to seventeen bands in *Brugia pahangi*.

Figure 1.4. shows a generalised representation of the structure of a nematode cuticle collagen translation product (pre-pro-collagen), which is derived from the sequences of 38 *C. elegans* genes.

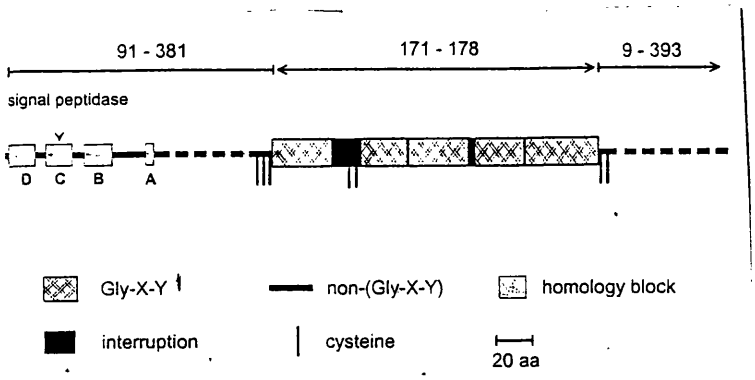

Figure 1.4. (After Kramer, 1997).

Basically, the structure consists of: an amino terminus containing four short sequence motifs (the Homology Blocks A-D) which are conserved in most or all of the collagens, one of which is a fairly typical membrane transport signal sequence (Kingston, 1991); a highly-conserved stretch of the amino acid repeats Gly-X-Y, where X and Y can be any amino acid but are frequently proline and hydroxyproline (Cox, 1992), and a short carboxyl domain which is fairly variable amongst the collagen genes. It is the Gly-X-Y domain which is characteristic of all collagens, and which permits their distinctive triple helical rod-like structure: collagen monomers form a helical structure with the glycine residues running along one side of the helix. Monomers then form triple helical structure with the glycine edge of each helix facing inwards, forming the core of the triple helix. The internal positioning of the glycine is for spatial reasons: the residue is small and therefore does not distort the structure (Johnstone, 1993). Most importantly, there are cysteine residues flanking the Gly-X-Y domain, which are thought to stabilise the definitive triple helical structure of all collagen molecules, as well as allowing di-sulphide bonds between triple helical fibres.

Unlike vertebrate collagens, which are large molecules, the primary translation products of the nematode cuticle collagen genes are small (Mr 26-35 kD), whilst the mature collagens are much larger (53-200kD). Politz et al (1984) showed that low Mr collagenase-sensitive products can be detected on Western blots of *C. elegans* protein from worms undergoing a moult, using antisera raised against high molecular weight adult cuticle proteins. This was interpreted as suggesting the conversion of low-to-high Mr collagen products in the formation of the cuticle. Betschart & Wyss (1990), working on the collagens of *Ascaris suum*, using rotary shadowing electron microscopy, found that under reducing conditions the dimensions

the collagen molecules correspond to what one would expect if three small pre-pro-collagen triple helices were joined end to end by non-reducible covalent cross-links, effectively functioning as a single molecular species. Whilst covalent cross-links in vertebrate collagens are known to occur between modified lysine residues, the non-reducible cross-links in nematode cuticles have long been known to be between tyrosine residues (Fujimoto, 1975; Fetterer & Rhoads, 1990): a single tyrosine residue is found in the carboxyl region of most cuticle collagens, and is presumably involved in cross-linking. In *Ascaris*, it is known that cross-links in the collagens are primarily via iso-tri-tyrosine residues, whereas in the insoluble fraction of the cuticle i.e. the cuticlin, the cross-links identified are di- and tri-tyrosine residues (Fujimoto et al, 1981; Sakura & Fujimoto, 1984; Fetterer et al, 1993). The mature collagen is then made by linking the insoluble procollagens via di-sulphide bonds between the cysteine residues. Additionally, it is thought that further insoluble cross-links occur between mature collagens in the process of cuticle construction.

1.9.1. The collagens of other nematodes.

It has been shown that the isolated and characterised collagen genes from *Ascaris* (ucol-1; Kingston et al, 1989) and *Haemonchus contortus* (Shamansky et al, 1989) encode small collagen precursors which are cross-linked to form the mature cuticle collagens, as has been demonstrated in *C. elegans.* In the case of the free-living nematode *Panagrellus silusiae,* however, collagenase digestion of *in vitro* translated products yielded proteins which correspond in molecular weight to those of the mature cuticular collagen proteins, implying that they are not made up of more than one small collagen precursor (Leushner et al, 1979). Original characterisation of the *Brugia* collagens appeared to show the same mode of collagen synthesis: procollagens identified via collagenase digestion of *in vitro* translation products apparently correlated broadly in molecular weight with those seen by metabolic labelling of worms *in vitro* (Selkirk et al, 1989). However, the subsequent isolation, sequencing and characterisation of collagen genes from *B. malayi* (Scott et al, 1995) and *B. pahangi* (Bisoffi & Betschart, 1996), has revealed the encoded products to correspond to the size predicted by the collagen precursor molecules of *C. elegans*, *Ascaris* and *Haemonchus*, thus showing conclusively that the mechanism of collagen synthesis in *Brugia* is similar to that of the majority of other nematodes so far studied. Interestingly, whilst Southern blot analyses carried out under high-stringency conditions using a probe made from the B. *malayi* collagen gene *bmcol-2* imply the presence of homologues in the parasitic nematodes *B. pahangi*, *Dirofilaria immitis* and *Onchocerca volvulus*, the probe did not hybridise to DNA from *Panagrellus*, showing that the relatedness of the collagen genes is high between parasitic nematodes and considerably lower between parasitic nematodes and *Panagrellus.* Hence it may be predicted that the mechanism of synthesis of the proteins will be similarly conserved between the parasitic nematodes.

The *C. elegans* cuticle collagens are divided into sub-families dependent upon the spacing of the cysteine residues, and the amino acid sequence of the carboxyl domain (Cox, 1990; Kramer, 1994b; Johnstone, 1994). In *C. elegans* there are four sub-families: *col-1, col-6, col-8* and *sqt-1*, named after the first discovered member of each sub-family. The genes encoding cuticular collagens from parasitic nematodes that have so far been cloned and sequenced can also be classified into the same sub-families: the two sequenced *H. contortus* genes are members of the *col-1* sub-family (Shamansky et al, 1989) whilst the two *Ascaris suum* genes (Kingston et al,1989; Kingston & Pettit, 1990), the *B. malayi* gene (Scott et al, 1995) and the three *B. pahangi* genes (Bisoffi & Betschart, 1996; S. Hunter, unpublished M.Sc thesis) all belong to the *col-6* sub-family; and a fully-sequenced gene from the plant parasite *Meloidogyne incognita* belongs in the *col-8* sub-family (Vandereycken et al, 1994). Genes within a sub-family have greater similarity to one another than to genes in other sub-families, even across nematode species. Using an antibody raised against a peptide with the sequence of the carboxyl region of one of the *Haemonchus col-1*-type genes, a sequence which is diagnostic of the *col-1* sub-family, Cox et al (1990) found that the antiserum reacted specifically with collagen proteins extracted from nematodes of eight other species: *Ostertagia ostertagi*, *C. elegans*, *Panagrellus redivivus, Heterorhabditis bacteriophora, Neoaplectana carpocapsae*, *Toxocara canis*, *Dirofilaria immitis* and *Trichinella spiralis*. Interestingly, one of the *H. contortus* collagen genes has an intron in the same position, in Homology Block B, which is conserved amongst half of the 38 *C. elegans* genes so far characterised, implying a common ancestor for the genes.

1.9.2. Stage-specificity and expression of the cuticle collagens.

It has long been known that the collagen composition of the *C. elegans* cuticle changes at each moult, resulting in stage-specific cuticles as a result of non-reiterative moults: Cox et al (1981b) labelled soluble cuticle proteins with ^{35}S methionine and showed by SDS-PAGE that there was a unique banding pattern for each of the stages studied (adult, L1, dauer and L4). Obviously these results could not determine between whether the stage-specific cuticle differences were due to stage-specific post-translational modifications of a small set of cuticular collagens, or to expression of distinct sets of genes. Again working on *C. elegans*, Politz & Edgar (1984) isolated polyA+ RNA populations from worms early and late in the L4-adult moult and during the L2-dauer moult, and carried out *in vitro* translations. Two-dimensional gel analysis of the translation products identified at least 60 distinct collagenase-sensitive products, which hybridised to chick and nematode ^{3}H-proline-labelled collagen peptides, only three of which were found in both moults. The collagens isolated early and late during the same moult were also shown to be different, implying an intra- as well as inter-moult control of expression of the collagen genes.

To establish the expression pattern of collagen genes in *C. elegans*, Cox & Hirsh (1985) constructed a sub-library of recombinant phages containing collagen genes and then

screened it with single-stranded 32P-labelled cDNA prepared from polyA+ RNA isolated from animals undergoing the L2-dauer moult, the dauer-L4 moult, the L4-adult moult, and from eggs. Hybridisation was carried out at a stringency which ensured that there was no cross-reactivity between collagen genes (Cox et al, 1984). The results demonstrated that collagen gene expression varies between life-cycle stages temporally as well as quantitatively, and that more collagen genes appear to be expressed at each consecutive moult, presumably reflecting an increase in complexity of the worm cuticle. In general, a larger proportion of a nematode adult cuticle can be solubilised than that of a larval cuticle (Cox et al, 1981a; Reddigari et al, 1986; Fetterer & Urban, 1988), which could either reflect a greater proportion of collagens being made in the adult cuticle, or alternatively a stage-specific difference in the nature and extent of the cross-links in the cuticles.

Kramer et al (1985) probed northern blots of RNA isolated from worms at different life-cycle stages with gene-specific probes from the *C. elegans* collagen genes *col-1* and *col-2*: whilst the *col-1* transcript was found in all the developmental stages tested, and showed a variation in the abundance between the life-cycle stages, the *col-2* transcript was only present during formation of the dauer larvae cuticle. This study supports the theory that fluctuations in collagen abundance are controlled at the transcriptional level as opposed to, or in addition to, control at the translational and post-translational levels. I

A more recent study of collagen genes (Johnstone & Barry, 1996) appears to show that the patterns of expression are even more complex than was previously imagined. The study addressed the qualitative and quantitative fluctuations in the expression of six genes, which represent the four main categories of *C. elegans* collagen genes, using semi-quantitative RT-PCR. Two separate synchronous worm cultures were sampled to provide RNA/cDNA samples for two distinct developmental time-courses. The first time-course spanned development from early L1 all the way through to adulthood, with samples taken every two hours. The pattern found for each gene, demonstrated by PCR on the cDNA with gene-specific primers, showed that mRNA abundance oscillates, peaking once during every larval stage. However, the periods of abundance do not coincide, different genes being expressed at different times relative to one another within the moulting cycle. The results did not contradict any expression studies already in existence (Park & Kramer, 1994; Cox & Hirsh, 1985; Park & Kramer, 1990; Kramer et al, 1985; Liu et al, 1985; Liu & Ambrose, 1991; Levy & Kramer, 1993): they just showed a more complete picture which contradicts the idea of a qualitatively non-reiterative moulting cycle; each gene clearly shows a tightly controlled temporal expression pattern which is repeated during each of the four larval stages.

The second time course was designed to examine this phenomenon in more detail, and consisted of RNA/cDNA samples taken from a synchronous population every fifteen minutes from early L1 to the L1-L2 moult. The results of RT-PCR using gene-specific primers confirmed the earlier experiments: mRNA abundance of individual collagen genes peaks in consecutive waves throughout the course of a moulting cycle. An interesting observation was

that two of the genes show an additional wave of expression after the L4-adult moult, presumably related to growth of the adult cuticle during worm development. That only two of the genes studied show this expression pattern would imply that a sub-set of the collagens are involved in the post-moult growth of the adult cuticle. They do not belong to the same cuticle collagen gene sub-class. In order to establish whether or not embryonic expression patterns were similar to post-embryonic patterns, the authors went on to examine the expression pattern of the two genes which represented the earliest and latest peaks of expression (as demonstrated by the RT-PCR experiments), by using GFP reporter gene fusions. Again, these results confirmed earlier experiments: the 'early' gene (*dpy-7*; Johnstone et al, 1992) fusion showed the onset of fluorescence, and hence expression, at the 'comma' stage, which is about 4 hours before the embryo secretes the cuticle of the L1 larva; the 'late' gene (*col-12;* Park & Kramer, 1990) showed the onset of fluorescence when the embryo is already elongated, around the time of the L1 cuticle secretion.

It has been demonstrated in *C. elegans* (Cox et al, 1981c), in *Panagrellus silusia* (Leushner & Pasternak, 1975) and in *B. malayi* (Selkirk et al, 1989) that the synthesis of cuticle collagens during development peaks immediately before the larval moults, with a reduced level of transcription occurring during the intermoult period, although there is evidence from *Ascaris* (Kingston et al, 1989) and *C. elegans* (Johnstone & Barry, 1996) that a sub-group of the cuticle collagen genes are expressed at significant levels in adult worms. It is known from genetic analysis of collagen mutants that the products of different collagen genes interact in the formation of the structural components of the cuticle of *C. elegans* (Kusch & Edgar, 1986). However, one of the questions that remains unanswered, despite the extensive amount of work done on the cuticle collagens at both the molecular and genetic level (see reviews by Cox, 1992; Kramer, 1994; Johnstone, 1994) is whether interaction occurs between separate triple helices, or within the collagen monomers that make up the triple helices. It is fairly well accepted that correct trimerisation is achieved via the relationship between the precise lengths of the Gly-X-Y repeats and the specific positioning of the adjacent cysteine residues in relation to the repeats (Cox at al, 1989). Presumably, this means that similar lengths and spacings are required for monomers to trimerise within a triple helix, which therefore implies that collagen monomers making up a single triple helix must be derived either from the same gene or from a very similar gene in the same 'sub-family'. It is possible that the ordered expression of the collagen genes seen in the work of Johnstone & Barry, results from a mechanism which has evolved in order to control and reduce the variety of collagen monomers available for trimerisation in the hypodermal syncitium at any one time; this, in theory, could assist in the formation of appropriate interchain interactions between the products of similar collagen genes. Evidence for this is that there is a correlation between the timing of the waves of expression and the sub-class of collagen gene expression seen in *C. elegans* i.e. genes of the same structural class, which would be expected to trimerise, are expressed at similar times during the intermoult period (Johnstone & Barry, 1996).

As can be seen, the expression of cuticle collagen genes of *C. elegans*, which can largely be extrapolated into other nematodes, is developmentally controlled on a number of different levels. Many different genes are expressed at each moult, and whilst some are expressed at every moult, others are specific to certain life-cycle stages; mRNA levels for individual collagen genes vary between life-cycle stages as well as between different times within the same intermoult period. Furthermore, the expression of different collagen genes results in stage-specific differences in the cuticle architecture, implying that localisation of the collagens is also spatially controlled; it would seem that particular gene products are used to form specific morphological features of the nematode cuticle.

The relatedness of the genes, both at the structural and sequence levels, encoding all the nematode collagens so far characterised means that convergent evolution of the genes is highly unlikely, leaving the probability that all the members of the nematode cuticle collagen gene family evolved from a single ancestral gene, presumably by numerous gene duplications. The ancestral gene therefore must have encoded a component of the primitive nematode cuticle. Due to the ubiquity of the morphology of the L1 cuticle of nematodes, it is thought that it represents this so-called 'primitive' cuticle (Bird, 1971), and that the more elaborate stage-specific morphologies arose through modifications of this primitive, L1-like cuticle (Inglis, 1964). One would imagine that the expansion of the collagen gene family, perhaps in combination with the genes encoding the insoluble structural proteins of the cuticle, allowed the evolution of the moulting cycle, and the subsequent specialisation of the stage-specific cuticles. The fact that the complexity of the cuticle structure increases with each moult could mean that each larval cuticle evolved from the one preceding it, as the evolution of structural component proteins allowed. Clearly this is an over-simplification; there must have been parallel evolution of genes controlling regulatory switches (the heterochronic genes of *C. elegans* are obvious candidates), but the extraordinary expansion of the collagen gene family, combined with the extreme conservation of basic structural features, must mean that the evolution of the collagen gene family played a primary role in the evolution of the nematode cuticle. The structural diversity of the cuticle allowed by the cumulative possibilities afforded by a huge multi-gene family, interaction between primary gene products, and post-translational modification, has been responsible for the diversity of the nematode cuticle. And, as will be discussed later, it is largely the evolution of diversity in the nematode cuticle which has allowed the phylum to radiate into virtually every ecological niche on the planet.

1.9.3. *C. elegans* cuticle collagen genetics.

Apart from the fact that most of what was originally known about the nematode collagens was learnt via the analysis of genetic mutants of *C. elegans*, the most important point to make about the genetics of the cuticle collagen genes of *C. elegans* is that the relationship between genotype and phenotype is complex and can often be confusing, not to say misleading. This is generally accepted to be the case for all genes encoding structural proteins (Kusch & Edgar,

1986), where the products encoded for by the genes commonly interact and form multi-protein complexes which play a significant part in the maintenance of the gross morphology of the organism. What follows is not intended to be a comprehensive review of nematode cuticular collagen genetics, which is a relatively complex field in which there remain many more questions than answers, but an overview designed to show how genetics can potentially provide information on the assembly and functioning of the extracellular matrix, specifically with respect to the cuticle.

The relationship between defects in cuticle function and gross changes in the overall morphology of the of the worm is well established in *C. elegans* research (Brenner, 1974; Cox et al, 1980; Kusch & Edgar, 1986). The use of a self-fertilising hermaphroditic species has permitted the use of classical genetic techniques to investigate the genes responsible for the cuticle defects; most of the resultant phenotypes render males incapable of mating. The genes are characterised according to the resultant mutant phenotype: bli - blisters on cuticle; dpy - dumpy, short and fat; lon - long and thin; lrol - twisted in a left-handed helix; rrol - twisted in a right-handed helix. Additionally, mutations that cause dominant Rol and recessive Dpy phenotypes are known as Sqt (squat). There are currently over forty identified genes, mutations of which result in cuticle defects: 27 *dpy*, 6 *bli*, 6 *rol*, 3 *lon* and 3 *sqt* genes. As has been previously mentioned, seven of these have been cloned and shown to encode cuticular collagens; it seems very likely that in addition to those already characterised, many of the other genes will also encode collagen genes. In fact, Thacker & Rose have just demonstrated that another *dpy* gene (*dpy-5*) encodes a cuticle collagen (Worm Breeders Gazette: Oct, 1997). Mutations in the cuticle collagens so far cloned can generate all of the previously noted phenotypes, indicating that the collagens have different functions in the cuticle, and that different mutations in a single collagen can alter its function in different ways.

Stage specificity of phenotype.

Interestingly, mutations in several of these genes only show phenotypes at particular developmental stages, and in some cases the phenotype of a single allele can differ at different stages (Cox et al, 1980; Park & Kramer, 1994). Presumably, this could either be due to the previously discussed stage-specific expression of the relevant gene, and/or the influence of other gene products, collagenous and perhaps non-collagenous, which are expressed at the same stages. Another strange phenomenon is that *sqt-1* and *rol-6* mutants show strong Rol phenotypes at the L2, L2d and dauer larval stages, despite the fact that *sqt-1* and *rol-6* transcripts cannot be detected after the completion of the L1-L2d moult (Park & Kramer, 1994). This is thought to be due to the fact that the hypodermis forms a template for the physical form of the new cuticle at each moult, and that since the L2d animals are Rol, the hypodermis would already be 'locked' into a helical configuration at the L2-dauer moult. Consequently, the hypodermis would synthesise a dauer cuticle that is wild-type with respect to protein components, but made on a twisted, helical template (Kramer, 1997).

Mutations.

Null mutations which result in the complete absence of a collagen component of the cuticle have highly variable phenotypes. This is presumably the result of functional redundancy between components of the cuticular structure; some collagens are more important than others in the maintenance of normal morphology. It is also possible that in the complete absence of one collagen component, a very similar species could take its place in the structure without severe disturbance of the cuticle, and hence morphology of the worm. This precedent was defined by Greenwald and Horwitz (1980), who suggested that genes identified by dominant mutations and exhibiting a wild-type null phenotype are members of a gene family in which other member genes can replace the deleted gene product. For example, null mutations in *sqt-1* and *rol-6* result in a more or less wild-type cuticle (Kramer et al, 1990), whilst null mutations of *dpy-10* and *dpy-13* have very strong phenotypes (Levy et al, 1993; von Mende et al, 1988). Interestingly, although the complete absence of SQT-1 and ROL-6 do not severely affect the cuticle, the presence of abnormal forms of either protein results in severe morphological abnormalities (Kramer, 1994). An interpretation of these observations is that although SQT-1 and ROL-6 are not absolutely required for normal morphology, they interact with other collagen components that are required for normal morphology, and that disturbing these interactions will destroy the integrity of the composite structure within which the collagens interact.

Due to the interactions between the proteins, cuticle collagen gene mutations are often dominant. Furthermore, they are frequently temperature-sensitive, because the mutations result in collagens which destabilise the triple helix formation and/or the higher order interactions between triple helices that occur during the polymerisation of the cuticle. Different mutant alleles of the same gene will also result in widely different phenotypes due to the variable effect that the mutation has on the interactions of the mutant collagen chain with others. *Sqt-1* has several identified mutant alleles which result in Dpy, Rrol, Lrol and long phenotypes. Additionally, animals for two different *sqt-1* alleles often show phenotypes which are impossible to predict from the homozygous phenotype of either mutant allele alone (Kusch & Edgar, 1986).

Glycine substitutions.

Most collagen mis-sense mutations (in human as well as nematode genes) are substitutions of glycine residues in the Gly-X-Y repeat domains. This is because glycine is an unusually small residue, and substitution with a larger residue results in the inhibition of triple-helix formation by interfering with hydrogen bond formation, which in turn leads to abnormal modification and degradation of the mutant collagen chain and other chains associated with it

(Prockop & Kivirikko, 1995). Unexpectedly, glycine substitutions are recessive, despite the fact that similar mutations in vertebrate collagen genes are dominant (Byers, 1990; Prockop, 1992), and usually result in a phenotype which is similar to, but slightly more severe than the null phenotype for the gene, presumably because the mutation results in an extreme reduction in collagen levels in both cases. The fact that the phenotype is more disruptive than the null phenotype implies that the mutant collagen chains interfere with the function of other molecules involved in cuticle synthesis, assembly or structure. The recessive nature of the mutations could be explained if mutant collagen peptide chains are not secreted from the hypodermis, either due to dismantling via the action of accessory proteins, or directly via instability due to the mutation (Johnstone, 1994). This would mean that the heterozygote would continue to make some chains which could be secreted and incorporated into the cuticle, albeit at a much lower rate than occurs in the wild-type animal. The homozygous mutant, however, would not produce any peptides viable for secretion, hence the similarity to the null mutation. Unfortunately, the two theories contradict one another; how can a mutant collagen gene that is not secreted result in a phenotype that is more severe than the null phenotype? One possibility is that a similar but non-identical collagen is substituted in the glycine replacement mutant and not in the null phenotype mutant, which results in the disruption of the triple helix and hence the cuticle morphology of the animal.

Other mutations.

Dominant mutant alleles in *sqt-1, rol-6* and *dpy-10* have been identified and sequenced (Kramer & Johnson, 1993). They all show a substitution of cysteine for argenine in the highly conserved HBA. It is unknown whether the severity of the phenotype can be attributed to the loss of the arginine or the gain of the cysteine. Gaining a cysteine could result in the inappropriate and disruptive formation of di-sulphide bonds between collagen peptides or between triple helices. Engel & Prockop (1991) proposed that correct interchain di-sulphide bond formation is essential for the aligning of the Gly-X-Y repeats which is essential for triple helix formation. On the other hand, Yang & Kramer (1994) have showed that argenine (or lysine) is absolutely required in HBA for normal collagen function.

The importance of cysteine residues is seen again in several mutant alleles of *sqt-1* and *rol-6*, where either one or both of the conserved carboxyl domain cysteine residues is substituted with serine or tyrosine, resulting in an Lrol phenotype (Yang & Kramer, 1994). Western blot analysis of cuticle extracts from *sqt-1* Lrol mutant animals shows that non-reducible cross-link formation is severely reduced in these animals (Kramer, 1997). Interestingly, a tyrosine residue immediately precedes the first carboxyl residue in SQT-1 and ROL-6, so it could be that substituting the cysteine results in the inability of the neighbouring tyrosine to form cross-links during the polymerisation of the cuticle. Alternatively, the di-sulphide bonds between the cysteine residues may be required to enable the cross-linking to occur between the tyrosine residues.

Interactions between cuticle collagen genes.

Because the collagens do not function individually, but as components of a single complex structure, genetic interactions such as intergenic enhancement and suppression of phenotype and cryptic dominance can be observed.

An example of suppression is the interaction between *rol-6* and *sqt-1* mutations: Lrol (glycine substitution) mutations of *sqt*-1 suppress *rol-6* Rrol alleles (Kramer & Johnson, 1993); whilst *rol-6* Lrol mutations can suppress *sqt-1* Rrol phenotypes (Yang & Kramer, 1994). In the case of collagen genes, the suppression of phenotype of one mutation by another can be explained by the fact that the two encoded products normally interact with each other, and the alterations in the two mutant collagen chains are compensatory. In fact, ROL-6 and SQT-1 almost certainly do interact, as is shown by the fact that ROL-6 requires SQT-1 to function, since *rol-6* phenotypes are suppressed in a *sqt-1* null back-ground.

An example of enhancement is the conversion of a *dpy-10* Dpy phenotype mutant animal to the more severe DpyLrol phenotype by the addition of a single copy of any non-null *sqt-1* mutation (Kusch & Edgar, 1986). The fact that enhancement does not occur with the null mutation demonstrates that the effect is due to the presence of the mutant SQT-1 rather than the absence of normal SQT-1.

'Cryptic dominance' is a term invented by Kusch and Edgar (1986) to describe the phenomenon of an apparently recessive allele behaving as a dominant in certain genetic back-grounds. For example several normally recessive *sqt-1* alleles are dominant in mutants of *dpy-7, dpy-2* and *dpy-10*. The obvious explanation for this is that the products of these *dpy* genes are so closely associated with the those of the sqt genes in the architecture of the cuticle that, even in the presence of wild-type products, the presence of the abnormal collagen chains will not permit the construction of a morphologically normal cuticle. However, the exact mechanism of interaction is unknown. Another unpredictable interaction of the collagen genes is intergenic non-complementation: this is when animals heterozygous for two recessive mutations nonetheless have a mutant phenotype, presumably due to a highly disruptive interaction between the two abnormal collagen chains.

It is possible that classical genetics of the type described in the characterisation of the collagen genes could also be used to define the cuticlin components of the nematode cuticle. From the work already done on relating the genotype and phenotype of cuticle protein components genes, it should be possible to predict whether a gene encodes a protein which is implicated in the formation of the cuticle. As has been discussed, the behaviour of the gene with respect to interaction and variability of dominant and recessive expression is characteristic when it encodes a structural protein which is involved in the formation of the cuticle. So far no mutant strains of *C. elegans* have been identified which represent a mutation in a gene encoding a cuticlin component of the cuticle.

1.10. The cuticlin genes.

Although the major protein component of the nematode cuticle is collagen-like, there is an insoluble residue which remains after treatment with SDS and the reducing agent β-mercaptoethanol. This was originally defined by Fujimoto and Kanaya (1973), working on the cuticles of adult *Ascaris lumbricoides*. Analyses of the residue showed it to be different from the cuticle collagens: the amino acid composition was similar to but distinct from that of the collagens which had been isolated at that time; wide-angle X-ray diffraction pattern diagrams, which show an easily recognisable pattern for collagens, were completely different for the insoluble residue; and the residue was insusceptible to treatment with bacterial collagenase. Additionally, the residue did not contain an appreciable amount of carbohydrate, and was insoluble in all the protein solvents tested (including SDS, formic acid, 8M urea and 0.1M NaOH). The residue was named 'cuticlin'. Studies on the cuticles of *C. elegans* identified a cuticlin residue (Cox et al, 1981a) and went on to show that the amount of insoluble protein present in the worm cuticle was stage-specific (Cox et al, 1981b). By radio-labelling the *E. coli* on which the worms are fed, the cuticle proteins subsequently synthesised by the developing worms can be obtained by cuticle isolation procedures and characterised by SDS-polyacrylamide gel electrophoresis. Using this method it was demonstrated that 25% of the adult cuticle proteins are cuticlins whilst the remaining 75% are soluble collagen-like components. In the dauer larvae cuticles the percentages are inverted; 75% cuticlin and 25% soluble proteins. Microscopy of solubilised cuticles at the two life-cycle stages confirms this data; BME-treated adult cuticles show that the basal layer, struts, and most of the internal cortical layer have been removed, leaving only the external cortical layer and some of the internal cortical layer; the gross morphology of dauer cuticles, however, is only mildly affected by the same treatment. The dauer larvae can remain viable for very long periods under unfavourable environmental conditions, and have been shown to be highly resistant to fixatives, detergents and anaesthetics (Cassada & Russell, 1975). When conditions improve, the dauer larvae moult to the L4 stage and progress normally through the rest of the life cycle. The cuticle is obviously largely responsible for the resistance of the dauer larvae, and the dauer cuticles of various nematodes have been shown to be both structurally and physiologically different from the cuticles of the equivalent 'normal' stage larvae (Bird, 1980). Dauer larvae of *C. elegans*, for example, have a cuticle thickness to body diameter ratio which is more than double that of other life-cycle stages, and have a distinctive striated zone present in the basal zone of the cuticle, made up of two sets of interwoven laminae, one of which is oriented longitudinally and the other circumferentially, and both of which are insoluble and unaffected by treatment with BME (Cox et al, 1981b), so are presumably made up of 'cuticlin'. The correlation between an increase in the proportion of cuticlin in the worm cuticle and an increase in resistance and decrease in permeability would imply that the purpose of the insoluble cuticlin proteins is to increase the resistance and impermeability of the cuticle by

forming strong, inflexible structures which could well provide the framework of the nematode cuticle onto which the soluble proteins are deposited.

The insolubility and strength of the cuticlin proteins is provided by extensive, non-reducible, covalent bonds which cross-link the proteins. Fujimoto (1975) identified these cross-links as di- and tri-tyrosine bonds formed between tyrosine side chains in the cuticlin of *Ascaris*. In *Ascaris*, the tyrosine cross-links between collagens are iso-tri-tyrosine residues, whereas those found in the insoluble cuticlins tend to be di-and tri-tyrosine residues (Fetterer et al, 1993). Tyrosine cross-links have also been identified in the cuticlin residue of *C. elegans* (Kramer, 1997) and in that of *Haemonchus contortus* (Fetterer & Rhodes, 1990). Significantly, insolubilisation and hardening of extracellular layers or matrices via di-tyrosine, tri-tyrosine and iso-di-tyrosine formation has been shown to occur in several other diverse biological systems since it was first discovered in resilin, an insect wing ligament protein (Anderson, 1964): the basement membranes of *Drosophila* (Nelson et al, 1994); the cell wall of *Chlamydomonas* (Waffenschmidt et al, 1993); the highly resistant ascospore cell wall of *Saccheromyces cerevisiae* (Briza et al, 1994); and a component of the fertilisation envelope of sea urchin embryos (Foerder & Shapiro, 1977; Nomura et al, 1990).

That cuticlin-like proteins are present throughout the nematode phylum was demonstrated by Betschart et al (1990). An antiserum raised against a purified, collagen-free cuticlin preparation from adult *Ascaris suum* was shown, by immuno-electron microscopy, to cross-react with the cuticles of adult *Acanthocheilonema viteae*, *Brugia pahangi* and *Onchocerca volvulus*. The localisation of the antiserum was to the external cortical layer of the cuticle in all four parasitic nematode cuticles.

Further biochemical and molecular studies of cuticlin were made virtually impossible due to the insolubility of the residue; it was not even possible to ascertain whether or not the term 'cuticlin' applied to a single protein or a number of proteins with similar properties. The genes encoding two cuticlin-like proteins were eventually isolated from *C. elegans*; they were subsequently cloned, sequenced, fully characterised and named *cut-1* and *cut-2* (Sebastiano et al, 1991; Lassandro et al, 1994). The genes were discovered serendipitously; the probe used to screen an adult *C. elegans* genomic library was a 138-bp cDNA fragment coding for a region conserved amongst three different vitelline membrane genes of *Drosophila melanogaster* (Gigliotti et al, 1988).

1.10.1. *cut-2.*

The *cut-2* gene encodes a novel, secreted protein of 231 amino acids, with a putative cleavable signal peptide of 16 amino acids. The CUT-2 protein is characterised by a central region containing 13 repetitions of a short hydrophobic motif (often degenerate with substitutions and deletions): AAP(A/V/I). A similar pattern of the repeated motif has been seen in various proteins which are involved in the formation of extracellular insoluble protective layers: various larval cuticle proteins of *Locusta migratoria* (Hojrup et al, 1986); a chorion

protein of the medfly *Ceratitis capitata* (Konsolaki et al, 1990); two of the components of the *Drosophila* vitelline membrane (Scherer et al, 1988; Popodi et al, 1988); and CUT-1, the predicted protein encoded by *cut-1*. Significantly, 70% of the 56 repeated motifs compared contain a tyrosine residue.

An antiserum was raised in rabbits against a recombinant fragment of CUT-2, and was shown to recognise epitopes in the cortical layer of the *C. elegans* cuticle at all stages of the life-cycle (Ristoratore et al, 1994). In addition, the antiserum recognises epitopes in the fibrous layer beneath the alae of the dauer larvae, in the cuticle-secreting hypodermis, and in the cuticle lining the pharynx. Reactions using the same antiserum on the insect parasite *Heterorhabditis spp.* demonstrates high levels of cross-reactivity between the two species: the localisation of the recognised epitopes is exactly the same in both nematodes (Favre et al, 1995). *cut-2* mRNA can be detected by northern analysis in the RNA of all the life-cycle stages of *C. elegans*. To establish the transcription pattern of the gene, RNA was harvested from a sample of a highly synchronised population of worms every two hours between hatching and a few hours after the L1-L2 moult. The RNA was reverse transcribed and the resultant cDNAs amplified with *cut-2* primers. The results show clearly that *cut-2* transcription is discontinuous, occurring immediately before the L1-L2 moult, strongly implying that the *cut-2* gene encodes a component of the *C. elegans* cuticle (Lassandro et al, 1994).

As has already been mentioned, it has long been hypothesised that oxidation of tyrosine to form di-, tri- and iso-tri-tyrosine is one of the cross-linking mechanisms responsible for the insolubilisation of insect and nematode cuticles (Anderson, 1964; Fujimoto et al, 1981; Fetterer & Rhoads, 1991). Prior to the finding of di-tyrosine in natural sources (Anderson, 1963), it had previously been enzymatically synthesised from free tyrosine in an *in vitro* system using horseradish peroxidase as the enzyme and H_20_2 as the oxidative substrate: the tyrosine residues are oxidised to form a covalent bridge between the phenolic rings of the residues (Gross & Sizer, 1959). Aeschbach et al (1976) subsequently used the same HRP/H_20_2 system to cross-link various globular and structural proteins with the formation of di-tyrosine in the products. The presence of regularly repeating tyrosine residues in CUT-2 prompted the hypothesis that the protein is rendered insoluble in the worm cuticle via the formation of covalent tyrosine-derived cross-links (Lassandro et al, 1994). To test this theory, recombinant, soluble, monomeric CUT-2, produced in *E. coli*, was labelled with ^{14}C alanine and incubated in the HRP/H_20_2 enzyme system. SDS-PAGE analysis demonstrated that within minutes of initiating the reaction, a large fraction of the protein is polymerised into high-molecular weight complexes which cannot enter the gel. After fifteen minutes of incubation, centrifugation revealed that 90% of the TCA precipitable radioactivity is present in an insoluble pellet. Fluorimetric analysis was used to demonstrate that between 50-100% of the tyrosine present in the recombinant CUT-2 is oxidised to di-tyrosine in this cross-linking reaction. Interestingly, the enzymatic formation of di-tyrosine from free tyrosine is much less efficient, reaching only 15-20% after 24 hours (Anderson, 1964). Lysozyme, BSA and recombinant

CUT-1 were also tested in the same enzyme system: none of the three proteins showed appreciable polymerisation after 24 hours incubation. These results strongly imply that recombinant CUT-2 polymerises into an insoluble residue via di-tyrosine cross-links. The fact that recombinant CUT-1, which in fact has an equivalent number of tyrosine residues to recombinant CUT-2, does not cross-link via di-tyrosine, combined with the observation that CUT-2 is a more efficient substrate for the enzymatic formation of di-tyrosine than free tyrosine, indicates that the structure of the protein somehow aligns the tyrosine residues such that they are optimally available for cross-linking. The regular spacing of the tyrosine residues as part of the repeating central motif of CUT-2 could contribute to this structure.

Further studies on recombinant CUT-2 (R-CUT-2) revealed that the monomeric protein has a tendency to aggregate into large complexes: a time course of the production of the protein in bacterial cells reveals that ten minutes after induction the protein is detectable in the soluble fraction of the extract. After 60 minutes more than 95% of the R-CUT-2 is in the insoluble fraction of the extract. However, the aggregated protein is not yet covalently cross-linked, as demonstrated by the fact that it runs as a monomer in reducing SDS-PAGE analysis (Parise & Bazzicalupo, 1997). Furthermore, the addition of substances (to the HRP/H_20_2 cross-linking assay) which reduce hydrophobic interactions, reduces the rate of R-CUT-2 cross-linking, and even when the reaction is slowed to the point at which most of the R-CUT-2 is monomeric (by reducing the HRP concentration), no dimer or trimer intermediates can be detected. It would therefore seem that the formation of non-covalent, hydrophobic bonds between monomers results in the formation of a CUT-2 aggregate which is the only competent substrate for the cross-linking of R-CUT-2. Although the mechanisms of aggregation are unknown, there are six cysteine residues in the N-terminal region of CUT-2 which are likely to be involved in di-sulphide bridges, and there are glutamine and lysine residues which could be cross-linked via trans-glutamination. Lustigman et al (1995) have shown that transglutaminase-catalysed reactions are important in the formation of the larval cuticle of the filarial nematode *Onchocerca volvulus*.

A second recombinant CUT-2 was made in pMalP, wherein the same recombinant fragment is fused to the maltose binding protein (MBP). This recombinant is completely soluble, does not form aggregates (presumably due to interference by the MBP, which is very large), and is a very poor substrate for cross-linking by HRP. Interestingly, while the R-CUT-2 will not form cross-links with free tyrosine, the reaction between MBP-CUT-2 and free tyrosine results in the formation of di-tyrosine bridges between the protein and the tyrosine residues. Hence the aggregation of CUT-2 not only results in the alignment of tyrosine residues in such a way that cross-linking between monomers is facilitated, but that tyrosine residues are not available for cross-linking with any other residue. It is possible to imagine a scenario whereby, in the nematode, CUT-2 is secreted by the hypodermal cells of a newly-formed cuticle and rapidly forms ordered complexes with itself and possibly with other cuticle protein components via non-covalent, hydrophobic interactions. It could be that such an aggregate is inserted into

the structure of the cuticle in this form, and is hardened and insolubilised instantly by the developmentally regulated release of a suitable enzyme and/or oxidative substrate. This would explain why low molecular weight dimers and trimers of CUT-2 cannot be isolated from a cross-linking reaction; the protein is incorporated into a macromolecular aggregate as soon as it is secreted.

The above scheme is not entirely unprecedented; it is suggested by the actual events which occur in the hardening of the sea urchin fertilisation membrane, which has long been studied as a model for the production, assembly and modification of a complex extracellular matrix. In this system, the egg responds within minutes to fertilisation, by producing a un-crosslinked soft fertilisation membrane (SFM) which is made up of a number of different polypeptides stabilised by divalent-cation-dependent interactions (Kay et al, 1982) thought to be di-sulphide bonds (Lallier, 1970). As appears to be the case for CUT-2, correct assembly of the component proteins into the SFM seems to be critical to the subsequent cross-linking process (Kay et al, 1982). Within 5-10 minutes the SFM has been converted into the hard fertilisation membrane (HFM) which is a rigid, impermeable extracellular coat which serves the dual purpose of protecting the early embryo and preventing the entry of additional sperm (Shapiro et al, 1981). The hardening process is achieved by the formation of intermolecular cross-links via the oxidation of protein-bound tyrosyl residues, catalysed by an enzyme known as ovoperoxidase (Foerder & Shapiro, 1977; Hall, 1978). Ovoperoxidase is synthesised in and released from cortical granules within the egg a few minutes after fertilisation (Foerder & Shapiro, 1977) and is inserted into the assembling fertilisation membrane by the action of an intermediate protein called proteoliaisin (PLN) which contains two distinct cation-dependent binding domains: one for interacting with ovoperoxidase and one for interacting with the vitelline layer of the egg-shell (Somerset & Shapiro, 1991). The H_20_2 which functions as the oxidative substrate is produced by the egg in a burst after fertilisation (Foerder et al, 1978). In addition to the major tyrosine-derived cross-linking amino acids, di- and tri-tyrosine, Nomura et al (1990) identified a novel trivalent compound which they called pulcherosine (it was isolated from the sea urchin *Hemicentrotus pulcherrimus).* Interestingly, pulcherosine is an isomer of iso-tri-tyrosine, which is found in *Ascaris* cuticle collagen (Fujimoto et al, 1981).

No clear homologues of *cut-2* have so far been identified in *C. elegans* or in any parasitic nematode. However, Bisoffi et al (1996) isolated a cDNA clone (named *ascut-1*) from *Ascaris suum* which encodes a non-collagenous protein that has four repeats of a 51-aa peptide motif, each of which includes one AAPA/V/P motif, and three tyrosine residues, one of which is closely associated with the APAA motif. The cDNA was identified via recognition by a mAb which was the result of the fusion of spleen cells of mice immunised with cuticlin from *A. suum.* The mAb was shown to react strongly with cuticlin extracts and not at all with collagen extracts in immuno-blotting experiments, and localised to the epicuticular layer of the *Ascaris* cuticle. An antiserum raised against rASCUT-1 was used to detect epitopes in the cortical layers of the cuticle, confirming the protein as a non-collagenous protein component of the

Ascaris cuticle, i.e. a cuticlin. The anti-ASCUT-1 antiserum also recognised a protein of 97kDa in total extracts of *B. pahangi*, implying the presence of a filarial homologue of the novel cuticlin gene.

It is not possible to say for sure that the AAPA/V/P motif with accompanying tyrosine residue has been conserved throughout the evolution of the insects and nematodes in which it has so far been identified: the motif is very short and is made up of common amino acids. The fact that it occurs in proteins which are all structural extracellular components could be due to convergent evolution, rather than retention of the motif. The presence of a proline residue is known to result in a bend in a polypeptide, whilst alanine residues are very small and would not therefore interfere with a putative hinge-type mechanism which could be utilised to align the tyrosine residues on adjacent protein monomers. It is also possible that the tyrosine residues are not functionally connected to the AAPA/P/V motif, although this is unlikely as proteins which have equivalent numbers of tyrosine residues uninvolved with the motif do not appear to cross-link via tyrosyl residues, as determined by the HRP/H_20_2 assay. However, the fact that the motif is evidently involved in the aligning of structural extracellular proteins for tyrosine cross-linking makes it extremely interesting.

When CUT-2 was first characterised and shown to be an extraordinarily efficient substrate for the enzymatic cross-linking of tyrosyl residues, it was thought that an assay could be developed to try and identify the enzyme/s responsible for cross-linking the nematode cuticle, using R-CUT-2 as the substrate and substituting enzyme candidates for the HRP. Thus far, however, the cross-linking enzymes from *C. elegans* and other nematodes have not yet been identified, despite the investigation of several candidate enzymes (Tang et al, 1995; Cox-Singh et al, 1994; Fetterer & Hill, 1994).

1.10.2. *cut-1*.

cut-1, the first cuticlin gene to be isolated from *C. elegans* (Sebastiano et al, 1991), was identified in an adult genomic library by hybridisation to the same *Drosophila* vitelline membrane protein probe used to isolate *cut-2* (Gigliotti et al, 1988). However, obtaining the *cut-1* cDNA clone proved to be somewhat more difficult: screening seven cDNA libraries from different life-cycle stages of *C. elegans* with a genomic *cut-1* fragment containing the region of homology to the *Drosophila* sequence did not result in a single positive clone. The cDNA was eventually found by making and screening a cDNA library using poly(A+) RNA from L2 larvae undergoing the transformation to the dauer stage larva. The cDNA encodes a secreted protein of 423 amino acids, beginning with a putative cleavable signal peptide of 18 residues (von Heijne, 1986). The gene maps to LGII of the *C. elegans* genome.

The dauer-specific expression of *cut-1* was confirmed by northern and western blot analysis (Sebastiano et al, 1991). RNA was prepared, at various time points, from L2 larvae undergoing transformation to the dauer larval stage, following the protocol of Golden & Riddle (1984) to obtain a synchronised population of dauers. Probing northern blots of the RNA with

a labelled *cut-1* genomic fragment revealed the presence of a band corresponding to the size of the *cut-1* cDNA, increasing in strength during the time period in which the worms were moulting to the dauer stage. No signal was detected either in RNA from the L2 population, from the dauer larvae resulting from the cultures, or from L4 larvae deriving from the cultures.

Two CUT-1 fusion proteins were made in *E. coli*, one containing sequence coding for 131 amino acids from the penultimate exon, and one containing sequence coding for the last 79 amino acids of the *cut-1* cDNA; the two fusions overlap by 13 amino acids. The resultant recombinant proteins were used to raise antisera in rabbits. The antisera were purified by affinity purification: cuticlin residue was prepared from isolated *C. elegans* cuticles (Cox et al, 1981a & b) by the solubilisation of all other cuticle proteins using SDS and BME (Fujimoto & Kanaya, 1973). This residue was used to adsorb the CUT-1-specific antibodies present in the antisera, and it was demonstrated that the antibodies eluted from the treated cuticlin recognised the blotted recombinant proteins, and not *E. coli* extracts. These reagents were used to localise the CUT-1 protein in the worm, and to establish when the protein was synthesised. A time-course of SDS/BME-treated protein extracts of *C. elegans* was western blotted and probed with purified anti-CUT-1 antibodies; a protein of 40kDa was detected in extracts from worms entering the dauer developmental pathway, but in no other stages, confirming the results of the Northern analysis. Immunolocalisation experiments showed that the anti-CUT-1 antisera only recognised epitopes in the cuticle of the dauer larvae and only in the fraction of the cuticle not solubilised by SDS and mercaptoethanol, signifying that CUT-1 is a dauer specific component of the cuticlin residue of *C. elegans*. The fact that it cannot be detected in the soluble extract of dauer cuticles demonstrates that it is synthesised as a soluble protein of 40kDa and then assembled into the cuticle via an unknown insolubilisation mechanism. Immunofluorescence microscopy with goat anti-rabbit secondary antibodies on whole dauer preparations was used to localise the protein in the worm: it appears to form two ribbons 1-2mm wide which run laterally down the length of the worm, in the fibrous layer of the cuticle immediately under the alae (Ristoratore et al, 1994). This localisation suggests that the function of CUT-1 may be related to the strengthening of the seam between the two halves of the cuticle. Subsequent analysis involving transformation of *C. elegans* using a β-galactosidase reporter gene fused to the promoter sequences of *cut-1* shows clearly that the protein is synthesised exclusively in the seam cells (as opposed to the hypodermal syncitium) in the advanced stages of the dauer transformation process (Hilliard & Bazzicalupo, personal communication).

Whilst homologues of *cut-2* have not been isolated either from *C. elegans* or from any other nematodes studied, at the start of this project *cut-1*-like genes had been cloned from the plant parasitic nematode *Meloidogyne artiella* and from the intestinal nematode *Ascaris lumbricoides*. Additionally, anti-CECUT-1 antibodies have recently been shown to recognise cross-reacting epitopes in the cuticle of the insect parasitic nematode *Heterorhabditis spp.*(Favre et al, 1995). Much of the following data was obtained and published throughout the

duration of this project, and information and reagents from the study of one parasite system were used freely to advance what was known in the other systems. The *Brugia* project benefited enormously from this exchange.

1.10.2.2. *Ascaris cut-1.*

Ascaris is an intestinal nematode that infects mammals (*A. suum* is a parasite of pigs, whilst *A. lumbricoides* infects humans). The infective stage of the parasite is the L2 larva, which is contained within an egg-shell. The infective eggs are ingested by the host, wherein they hatch, penetrate the gut and migrate to the liver and lung; they then return to the intestine where they develop to adulthood. Eggs are shed into the external environment, where they embryonate and undergo the L1-L2 moult to become infective. Moulting and ecdysis are associated with the migration from site to site within the host, and the pathogenesis of Ascariasis is caused largely by the effects of these migrating larvae. As in other nematodes, the nature of the cuticle changes at each moult; in *A. suum* there is a particularly marked increase in the thickness of the median and basal layers as the parasite grows to adulthood, correlated with a progressive increase in collagen-like proteins in these layers (Fetterer & Urban, 1988). Much work on the nematode cuticle has been carried out on *Ascaris*. This is partly because the parasite is large (the adult female is 200-350 mm long) and the cuticle can be separated from adult and larval stage worms by the combination of mechanical disruption and SDS extraction, without disrupting the structure of the cuticle and with very little contamination by other tissues (Cox et al, 1981a; Fetterer & Urban, 1988). Additionally, *Ascaris* can be cultured *in vitro* (reviewed in Douvres & Urban, 1987) and highly developmentally-synchronised populations can be generated via the mechanical hatching of eggs at room temperature (Urban et al, 1981). As has previously been mentioned, cuticlin was originally defined in *Ascaris*, as the insoluble residue remaining after treatment of the cuticle with SDS and BME (Fujimoto & Kanaya, 1973).

Three *cut-1*-like regions were identified by screening an *Ascaris lumbricoides* genomic library with a *C. elegans cut-1* DNA fragment containing the second and third exons of the gene (Timinouni & Bazzicalupo, 1997). Two of the hybridising regions were demonstrated to map to the same region of the *Ascaris* genome, and their sequences show opposite orientations. At present it is not known whether these clones represent genes or pseudogenes; they have not been fully characterised and it has not been demonstrated that they are transcribed. The third clone maps to a region not less than 12-15kb from the other two.

The cDNA of the third clone has been identified and the gene named *ascut-1* (Timinouni & Bazzicalupo, 1997). The RACE procedure of Frohman et al (1988) was used to obtain the cDNA clone, using RNA from adult *Ascaris lumbricoides*. At this stage it was not known whether or not the gene was transcribed in the adult worm; northern analysis of adult RNA probed with a labelled fragment of *ascut-1* did not result in a specific signal. RACE resulted in

the isolation and cloning of three 'mature' cDNA species; all three were *trans*-spliced via the SL1 spliced leader. The procedure to obtain the cDNAs was arduous; several rounds of amplifying PCR using gene-specific secondary primers internal to the first were required to obtain a signal that could only be visualised by blotting and hybridisation to the *ascut-1* genomic probe, implying that the *ascut-1* mRNA is extremely rare in the adult mRNA pool. However, a portion of the longest cDNA clone was used to make a recombinant fusion protein in *E. coli* against which an antiserum was raised in rabbits (Paiva-Nunes, personal communication). The antiserum was shown to strongly recognise recombinant ASCUT-1. . Anti-R-ASCUT-1 was then used to localise the protein in the worm and identify when the protein was synthesised. *Ascaris* eggs were incubated and synchronously hatched and RNA, DNA and protein were made from fifty worm sub-populations every day for 28 days. Additionally, samples were taken at representative larval stages of the life-cycle.

RT-PCR carried out on cDNA reverse transcribed from the extracted mRNA demonstrated that the gene is transcribed only at the L1-L2 moult, which occurs within the egg. Cloning and sequencing carried out on this, the 'true' isolated *ascut-1* cDNA, revealed that it is not trans-spliced and that it is shorter than that which was obtained by RACE; the last part of the RACE-derived species was in fact shown to be intron sequence. Thus the cDNAs obtained by RACE presumably represent extremely rare mis-priming events, whereby the transcription machinery of the cell wrongly recognises a promoter, or alternatively fails to recognise a termination signal at the end of the transcription of another gene. In this case these meaningless and deviant transcripts were amplified to the point of recognition, but do not represent any biologically meaningful mRNA species (Bazzicalupo, personal communication).

Western blotting of the proteins extracted from the *Ascaris* time-course, hybridised with the anti-R-ASCUT-1 antibody confirmed the RT-PCR results: a signal was seen around the time of the L1-L2 moult and was not seen before or after this time; the antiserum clearly does not recognise the protein once it has been assembled into the cuticle, as is the case for CECUT-1. Interestingly, two bands are recognised on the blot, one much larger than the other. The smaller band is about the same size as the ASCUT-1 monomer whilst the larger band could represent an aggregate of the protein. When the protein is first recognised by the antibody the monomer signal is much stronger than that of the dimer, whereas by the time the signal is lost (several days later) antibody recognition is largely of the 'aggregated' protein. This implies a scenario whereby CUT-1, like CUT-2, forms some sort of non-covalently bonded aggregate before it is cross-linked insolubly into the cuticle.

A characterisation of the secondary structure and 3-dimensional nature of R-ASCUT-1 was carried out using Fourier transform infrared, circular dichroism and fluorescence spectroscopy methods (D'Auria et al, submitted for publication). The addition of DTT (which is known to destroy di-sulphide bonds between cysteine residues) to the protein solution results in a major change in the organisation of the protein, with an overall loss in 3-D structure, implying that at least some of the cysteine residues present in the protein are forming intra-molecular di-

sulphide bonds which play an important part in the tertiary structure of R-ASCUT-1. The combined data shows that the tertiary structure of the protein demonstrates the formation of β-sheet-like structures as a consequence of protein intermolecular interactions (aggregation) between secondary β-structures, which are disrupted by the addition of SDS. Phenylalanine residues are also likely to be involved in aggregation via hydrophobic molecular interactions, as shown by the fact that the addition of disaggregating SDS also results in an increase in the dichroic activity of the phenylalanine. The protein structure is extremely stable to heat. Overall, these results are what would be expected for a cuticlin protein: a strong tendency to aggregate via a number of hydrophobic intermolecular bonds, forming a highly stable multimolecular substrate for the cross-linking of the protein into the insoluble structure of the cuticle.

The anti-R-ASCUT-1 antiserum was used in immunolocalisation experiments on cross-sections of *Ascaris* at various different life-cycle stages: the antibody recognised epitopes in the cuticle of the L2 larva (as would be predicted from the transcription data) as well as in the vitelline membrane of the egg-shell and in the matrix of the egg, surrounding the developing embryo. As will be discussed later, it is thought that these non-cuticular localisations are the result of cross-reaction with extracellular matrix proteins encoded by genes showing some homology to the cuticular *cut-1*-like genes.

1.10.2.3. *Meloidogyne artiella cut-1.*

A *cut-1*-like gene has also been isolated and characterised from the plant parasitic nematode *Meloidogyne artiella* (De Luca et al, 1994). These parasites are commonly known as the root-knot nematodes, and make up the most widely distributed group of plant parasitic nematodes, affecting the quality and quantity of a wide number of harvests (Sasser & Freckman, 1987). Adult females live within plant roots, and deposit gelatinous egg sacs, containing around 500 eggs at different stages of development, on the outer surface of the root. The J1-J2 moult occurs within the egg prior to hatching. The J2 larva is the infective stage, which migrates into the soil and invades plant roots, where it completes the life-cycle, undergoing three further moults. Adult males are free-living in the soil, and reproduction can be either via cross-fertilisation or parthenogenesis (Agrios, 1978).

The M. *artiella cut-1* gene (*macut-1*) was isolated from a genomic library as a result of screening with a labelled fragment of *cecut-1*. The library was also screened with a *cecut-2* probe, but no positive clone was identified, although the authors report that experiments demonstrate that a *cut-2* homologue is present in the M. *artiella* genome (De Giorgi et al, 1996). The transcription pattern of the gene was obtained by RT-PCR analysis, using cDNA from RNA derived from the egg, the free-living L2 and adult male stages, and the adult female. Products were amplified from the cDNA from eggs, J2 larvae and adult males; the *macut-1* mRNA does not appear to be present in the adult female (Di Giorgi et al, 1997). No data are available on the localisation of MACUT-1 in the cuticle of M. *artiella*. Reddigari et al (1986) observed differences in the cuticle structure of *Meloidogyne spp*: in males and in the J2 larvae

the cuticle is thinner but more complex, consisting of three layers; whereas in the adult female the cuticle is considerably thicker but has a more basic, laminate structure. Hence it would appear that in *M. artiella* the transcription of *cut-1* correlates with the production of a more complex cuticular structure required for the free-living life-cycle stages of the nematode.

The authors (Di Giorgi et al, 1997) also report that RT-PCR results in the additional amplification of an un-spliced *cut-1* message in the J2-derived mRNA population, which is not present in the egg or adult male mRNAs. They interpret this as signifying that there is a reduction in the rate of ma*cut-1* transcription after the J1-J2 moult, but that transcription continues in the intermoult period, and that the retention of the unprocessed transcript in the J2 serves to prevent cessation of ma*cut-1* gene-expression until the formation of the adult worms. The possibility of the larger transcript representing a functional alternative splicing event is ruled out by the fact that there is no polyadenylation signal within the introns. The authors go on to propose that repression of splicing of the primary transcript of ma*cut-1* may be responsible for the regulation of splicing, and hence expression, of the gene. Considering the fact that the transcripts were identified via amplification-based methods from small amounts of original material, I would suggest that it was more likely that the un-spliced transcript was the result of a rare transcriptional error which was enhanced and amplified during the subsequent experimental procedures. An example of such an occurrence has already been described in the case of *ascut-1*.

1.10.2.4. The *cut-1* gene family.

In addition to the *cut-1*-like genes isolated from *C. elegans*, *Ascaris suum* and *Meloidogyne artiella*, nineteen hypothetical proteins deduced from sequences generated by the *C. elegans* Genome Sequencing Project (Wilson et al, 1994) have been identified, which show significant homology to the CUT-1-like proteins characterised thus far. They were discovered by using the sequence of the conserved region of the *cut-1* genes in the GENEFINDER program. Sequence CEF22B5.3 (Accession No. Z50044) from the Sequencing Project encodes a putative protein which is most similar to the *C. elegans* and parasitic nematode CUT-1-like proteins; it has been named CUT-3 for the purpose of ease of comparison. A line-up of some of these proteins is shown in Appendix 1. The alignment of the proteins was initially done using the 1.6 version of Clustal W (Thompson et al, 1994), and was altered manually to enhance the efficacy of the alignment. Figure 1.5. is a more focused line-up of the homologous regions of the four protein sequences which are most like one another: CECUT-1 and CECUT-3 from *C. elegans*, ASCUT-1 and MACUT-1 (Timinouni & Bazzicalupo, 1997). A generalised account of the structure of these CUT-1-like proteins follows:

The proteins start with a putative cleavable signal peptide, as predicted by PSORT, a specialised expert system for the prediction of protein localisation sites in cells (Nakai & Kaneheisa, 1992). The region is a short stretch of hydrophobic amino acid residues (usually between 15 and 22), the presence of which indicates that the proteins are translocated from

```
CUT-1        MTWKPIICLAALVLSASA              cleavage
CUT-3        MARYSLGLGLCLLVASVSA             cleavage
ASCUT-1      MCRAVSFLALFGLAAA                cleavage
MACUT-1      MRKLLFAIGVFVALNAIFTVRA          cleavage

CUT-1        IPVDNNVEGEPEVECGPNSITVNFNTRNPFEGHVYVKGLYDQAGCRSDEGGRQVAGIELPFDSCNTARTRSLNPKGVFVSTTVVISFHPQFVTKVDRAY
CUT-3        IPVDNNVEGEPEVECGPTSITVNFNTRNAFEGHVYVKGLFDQQECRNDEGGRQVAGIELPFDTCNVARTRSLNPKGVFVTTTVVVSFHPQFVTKVDRAY
ASCUT-1      IPVDNGVEGEPEIECGPTSITVNFNTRNPFEGHVYVKGLYDQEGCRSDEGGRQVAGISLPFDSCNVARTRSLNPRGIFVTTTVVISFHPLFITKVDRAY
MACUT-1      IPVDNGVEGEPEIECGPTSITVNFNTRNPFEGHVYVKGLFDQAGCRSDEHGRQVAGIELPFDSCNVARTDA-EPKGVFVSTTVVISFHPQFVTKVDRAY
consensus    IPVDN-VEGEPE-ECGPTSITVNFNTRN-FEGHVYVKGL-DQ-GCR-DEGGRQVAGIELPFDSCNVARTRSLNPKGVFVSTTVVISFHPQFVTKVDRAY

CUT-1        RIQCFYMESDKTVSTQIEVSDLTTAFQTQVVPMPVCKYEILDGGPSGQPIQFATIGQQVYHKWTCDSETTDTFCAVVHSCTVDDGNGDTVQILNEEGC
CUT-3        RVQCFYMEADKTVSTQIEVSDLTTAFQTQVVPMPICKYEILNGGPTGEPVQFATIGQQVYHKWTCDSETVDTFCAVVHSCTVDDGNGDTVQILDENGC
ASCUT-1      RVQCFYMEADKTVSTQIEVSEITTAFQTQIVPMPVCRYEILDGGPTGQPIQFATIGQQVYHKWTCDSETVDTFCAVVHSCFVDDASGDTIQIHNEEGC
MACUT-1      RVQCFYMEADKTVSAQLEVSEITTQFQTQVVPMPVCKYEILEGAALGQPIQFATIAQQVYTSGTCDSETIDTFCAVVHSCVVDDGNGDTVQILNEGSC
consensus    RVQCFYMEADKTVSTQIEVS--TTAFQTQVVPMPVCKYEIL-GGP-GQPIQFATIGQQVYHKWTCDSETVDTFCAVVHSC VDDGNGDTVQILNE-GC

CUT-1        ALDKFLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQISITIKDPGSECARPTCSEPQGFGAVK
CUT-3        ALDKFLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQISITVKEPNEECARPTCSEPQGFGAVK
ASCUT-1      ALDKYLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQITITIKEPNSECPRPTCSEPQGFGAVR--
MACUT-1      ALDKFLLNNLEYPTDLTAGQEAHVYKYADRSQLFYQCQISITIKEPHSECARPKCAEPRAFNAVK-
consensus    ALDKFLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQISITIKEPNSECARPTCSEPQGFGAVK
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Figure 1.5.

Multiple line-up of CUT-1-like proteins of nematodes. ASCUT-1 is the putative protein coded for by *ascut-1* (Bazzicalupo & Timinouni, 1997). CECUT-1 is the first cuticlin protein described in *C. elegans* (Sebastiano et al,1991). CECUT-3 is a cuticlin-like protein of *C. elegans* coded for by CEF22B5.3, accession no. z50044, sequenced by the *C. elegans* sequencing project. MACUT-1 is a cuticlin homologue of *M. artiella* (de Giorgi et al, 1996).

their site of synthesis: most secreted proteins have such a sequence (Kreil, 1981). This is to be expected: the nematode cuticular proteins are known to be synthesised in the hypodermis and transported to the extracellular matrix of the cuticle (Bird & Bird, 1991).

Then follows the defining region of the CUT-1 proteins, the CUT-1 'box', consisting of 262 conserved amino acid residues. The homology varies amongst all the proteins between less than 20% and more than 90%. However, between the four most conserved proteins the homology is extremely high, between 84% and 90%. In addition, most of the substitutions are conservative, i.e. the replacement residues have similar properties. This region is also characterised by the presence of 12 cysteine residues, the positions of which are highly conserved throughout the proteins. This would imply that they are involved in maintaining a highly conserved secondary structure which is retained by all of the proteins. In fact, as was previously mentioned, the integrity of R-ASCUT-1 is destroyed by the addition of DTT, which disrupts di-sulphide bonds between cysteine residues by reducing them (D'Auria et al, 1997). In this region it can also be seen that there are insertions of short stretches of gene-specific amino acid residues (for example see CUT-6), but even in these cases the position of the cysteine residues are remarkably conserved.

Immediately carboxyterminal to the *cut-1* box, there is what appears to be a gene-specific domain. In this region MACUT-1 and CECUT-1 carry regions of 36 and 44 amino acids, respectively, which are rich in alanine and proline and which are reminiscent of the conserved, repetitive motif present in the central region of CUT-2 (Lassandro et al, 1994). This is the only region of homology between CECUT-2 and the CUT-1-like proteins, and the reason that both *cecut-1* and *cecut-2* were recognised by the same *Drosophila* vitelline membrane protein probe. Only two of the CUT-1-like proteins (MACUT-1 and CECUT-1) appear to contain it. Interestingly, the AAPA motif results in a distinctive hydrophobic peak, as predicted by hydrophobicity plots of the predicted proteins,.

Homology between the proteins then resumes with a short, highly charged, basic region which is rich in arginine and lysine residues. The nature and spacing of these residues is reminiscent of that which is found in HBA of the *C. elegans* collagen genes (Yang & Kramer, 1994). This spacing of the conserved residues in HBA suggests that they could form the cleavage site for a subtilisin-like endoproteinase, and indeed it has been shown that HBA mutants produce a collagen which is larger than that produced by wild-type worms by the amount expected if cleavage normally occurs at HBA (Kramer, 1997). It is thought that cuticle collagens are synthesised as procollagens and that they are subsequently endoproteolytically processed at HBA during their maturation. Many vertebrate collagens are also proteolytically processed during their maturation (e.g. Smith et al 1992), though not via the action of a subtilisin-like protease, which appears to be nematode-specific. It could be that the CUT-1-like proteins also undergo post-translational modification and maturation via a similar proteolytic cleavage mechanism, which would appear to be unique to nematode cuticle proteins.

The terminal residues of the proteins show some homology to one another and, interestingly, the genes encoding CECUT-1, CECUT-3, MACUT-1 and ASCUT-1 all have an intron at the same position in the middle of this region.

At the carboxyterminal end of all of the CUT-1-like proteins there is an hydrophobic stretch of 17-21 residues. The amino acid sequences of this stretch are not conserved between the proteins but all result in a pronounced hydrophobic peak in hydropathy profiles, implying that the region represents a functional trans-membrane domain. The proteins all end with a short charged tail which shows no homology within the group.

It would seem that the CUT-1 proteins of *C. elegans* are encoded by a gene family. That the sequences obtained from the Genome Sequencing Project are real, rather than pseudogenes, is shown by the fact that all the sequences have open reading frames which run uninterrupted through exons. Additionally, it would seem that the family has representatives in the parasitic nematodes so far studied, at least three in the *Ascaris* genome and one in the *Meloidogyne artiella* genome. This scenario is immediately reminiscent of the cuticular collagen gene family of *C. elegans* and the corresponding homologues found in parasitic nematode genomes. The *cut-1*-like genes encode proteins which have highly conserved and highly divergent domains; it is possible to see that they could have evolved, like the collagens, to fulfil the same general structural function in different larval cuticles or in different localisations of the worm cuticle, or indeed in other extracellular matrices.

This theory is supported by retrospective analysis of the transcription and immunolocalisation data already available on the *cut-1*-like genes. In *C. elegans* it is known that *cut-1* is transcribed and synthesised in the seam cells exclusively during the transformation to the dauer larval stage, and that anti-CECUT-1 antisera shows a very specific localisation pattern in the dauer larvae, forming a lateral ribbon running beneath the alae for the entire length of the worm. There is enough unambiguous data available to enable a definitive statement: *cecut-1* is transcribed and translated in the hypodermal seam cells specifically and only during the formation of the dauer cuticle, and CECUT-1 is subsequently insolubilised and utilised to construct a ribbon of protein which, considering its position and character, probably serves to strengthen the seam which joins the two segments of the *C. elegans* dauer cuticle. However, anti-CECUT-1 antibodies recognise epitopes in the cuticles of all life-cycle stages of *C. elegans*, and of other parasitic nematodes, demonstrating a cross-reactivity that is due to the fact that the antisera was raised against a conserved region of CECUT-1. Consequently the antibodies are almost certainly recognising epitopes encoded by the conserved domains of a number of different *cut-1*-like genes, each of which presumably serves a different function in the extracellular protein component of the worms. Localisation experiments using an antiserum raised against R-ASCUT-1 showed recognition of epitopes in the egg-shell and in the proteinaceous matrix of the egg, in addition to a cuticular recognition. Transcription data shows that *ascut-1* is transcribed for the first and only time in the life-cycle of *Ascaris* prior to the L1-L2 moult, accounting for the cuticular localization of the antiserum,

but not for the localisation in the egg. The antiserum was raised against R-ASCUT-1, which includes the highly conserved *cut-1* box. Therefore, a reasonable explanation is that the *Ascaris* genome, like that of *C. elegans*, contains a *cut-1* gene family, members of which encode not only cuticular proteins, but other extracellular protein components. Supporting this theory is the recent identification of one of the *C. elegans cut-1*-like genes as *mig-4. Mig-4* mutants show disruption of attachment of the gonad primordium to its substrate within embryos and early L1 larvae. Also, the pharynx is mis-aligned and the M mesoblast is unattached. *Mig-4* apparently encodes a 500 amino acid predicted protein which, in addition to the *cut-1* box, contains Willebrand type A domains, which are found in many extracellular proteins known to be involved in cell adhesion and migration. The authors suggest that a likely role for the protein is as an adapter molecule binding a larger molecule to the matrix itself, or to a cell surface receptor (Proenca et al, 1997).

Interestingly, it is not possible to find EST's of most of the *cut-1*-like genes of *C. elegans*, including *cecut-1* itself. This is presumably because they are highly specialised proteins which are expressed only for a very short space of time in the life history of the worm. Hence their cDNAs are not well represented in the total cDNA population and have consequently not been detected by the random sequencing strategy of the EST program. Information from ACeDB has revealed the presence of a cDNA relating to *cecut-3* in an embryonic *C. elegans* library.

The isolation of the dauer-specific *cecut-1* was the result of screening with a probe which did not contain the *cut-1* box: the very short region of homology between the two genes which is present in the probe sequence probably represents the only link between the *cut-1* and *cut-2* gene families. Screening the same *C. elegans* library with the conserved region of the gene would probably result in the isolation of numerous other *cut-1*-like genes. In fact the genes isolated from parasitic nematodes are more homologous to each other and to *cecut-3* than they are to *cecut-1*.

The *cut-1*-like genes so far isolated and characterised are obviously cuticlin components, in as far as they are transcribed prior to a moult, the proteins that they encode appear to be cross-linked into the insoluble residue of the cuticle, and antibodies raised against them have been shown to recognise epitopes in the cuticle. It could be that, like the cuticle collagen gene family of *C. elegans*, the *cut-1* gene family encode a number of cuticlin proteins that are expressed at different moults for incorporation into the stage-specific structure of the nematode cuticle.

The structure of the proteins, in addition to what is known of their synthesis and localisation, led to, and complies with, the following model: the CUT-1 proteins are synthesised and inserted into the membrane of the hypodermal cell; initially they are anchored in the cell membrane at both ends; at the N-terminal end by the hydrophobic cleavable signal peptide, and at the C-terminal end by the putative, highly hydrophobic trans-membrane domain. The protein is then cleaved at the cleavable signal peptide and at the putative proteolytic cleavage site and released into the extracellular matrix, where it undergoes

aggregation via non-covalent bonds with other molecules, either of the same or similar cuticlin proteins, and is eventually inserted into the layered structure of the cuticle and cross-linked to form the insoluble scaffolding of the cuticle. However, it must be said at this point that the *Ascaris* R-CUT-1 protein, as detected in western blots of protein extract from *Ascaris* eggs, is too long to comply with this model, and in fact incorporates the putative cleavage signal site and the subsequent C-terminal sequences (P. Bazzicalupo, personal communication).

To extrapolate this model even further, it is possible to imagine that the ancestral *cut-1* gene may have originally encoded an integral membrane protein and that the process of evolution first cleaved it from the cell surface to become an extracellular protein and then caused its radial divergence into a number of specialised extracellular matrix and protein function niches. There is already evidence that anti-CUT-1-like antibodies recognise epitopes in the cuticle and in the vitelline membrane, where the protein function is almost certainly to strengthen and support the extracellular matrix; and also in the extracellular proteinaceous matrix of the nematode egg, where the protein and its function is unknown. And the *mig-4* gene apparently encodes a protein which is extracellular but non-structural, perhaps with an adapter function. Furthermore, anti-CUT-2 antisera and an antiserum raised against the purified cuticlin residue of *C. elegans*, (but not anti-CUT-1 antisera) recognise epitopes on the lining of the pharynx (Favre et al, 1995), which is made up of thickened basement membrane, defined as thin sheets of extracellular matrix material closely associated with cell membranes (Kramer, 1997). The basal membrane and the cuticle are the two nematode extracellular matrices. The basement membrane covering the pharynx is known to be twice as thick as that found on other tissues (Albertson & Thompson, 1976), presumably due to the fact that the pharynx is an active organ, so it would be reasonable to assume that the CUT-2-like protein localised in the pharynx has a role in the strengthening and reinforcement of the pharyngeal basal membrane.

This raises the question of the evolutionary relationship of *cut-1* to *cut-2*. Does *cut-2* represent a further evolutionary specialisation step from a branch of the *cut-1* family, in that it seems to be only present in insoluble extracellular matrices, and shows homology to at least one other *C. elegans cut-1*-like protein (CECUT-1 itself)? Or has it evolved separately, or convergently, as could well be implied by the previously discussed conservation of the defining motif throughout the various proteins which make up insoluble extracellular protective layers of insects and nematodes? Certainly, if the CUT-1 and CUT-2 type proteins have a common ancestry, the gene encoding the original protein was present an extraordinarily long time ago, before nematodes diverged from arthropods. Somewhat surprisingly, the evolutionary relationship of the nematodes to other metazoans is somewhat contentious (Conway Morris, 1993) but the current thinking, based on molecular data, is that the nematodes form an "outgroup" to most metazoan forms, including vertebrates and arthropods (Sidow & Thomas, 1994). This means that at a certain point in evolution the metazoans diverged and that the nematodes represent one branch and the vertebrates and arthropods

are representatives of another; or, more simply still, this means that arthropods and vertebrates are more closely related to one another than either of them are to nematodes. The early divergence of the nematodes during evolution means that they share many unique features, and also that any features shared with other metazoan groups (unless of course achieved by convergent evolution) must resemble features present in the common, ancestral metazoan (Fitch & Thomas, 1997). The further definition of the cuticlin gene family, and clarification of the various functions of the encoded proteins, would probably reveal whether or not the genes have a common ancestor and, if they do, what were the features of the ancestral gene and protein that have allowed it to evolve and diverge so successfully throughout the metazoans. And conversely, what advantage did the divergence of the gene and its product give the organisms that carried it, allowing them to evolve so comprehensively in the intervening millions of years?

In *C. elegans*, the *cut-1*-like genes identified by the GSP do not appear to be evenly distributed throughout the genome; at least half of the genes are clustered on LGII. This would point to duplication, probably via unequal crossing-over mechanisms, of large chunks of chromosome, occurring more than once in the evolutionary process.

1.11. Immunoreactivity of cuticlin.

Unlike the collagen fraction of the parasitic nematode cuticle, the cuticlin residue does not appear to be particularly immunoreactive. Selkirk et al (1989) showed that although the cuticular collagens of *B. malayi* and *B. pahangi* appear to be inaccessible to the immune system in intact worms, immunoprecipitation analyses show that the serum of lymphatic filariasis patients contains antibodies to cuticle collagens. This is presumably due to the fact that the host is exposed to the collagens during moulting as well as a result of the attrition of the parasites due to immune responses against other targets. Individuals infected with other filarial worms (*Wuchereria, Mansonella, Onchocerca* and *Loa*) also have antibodies which cross-react with the cuticular collagens of *B. malayi*, whilst serum from individuals with non-filarial nematode infections (Hookworm and Toxocariasis) does not appear to contain cross-reacting antibodies. Archer et al (1985), injected rats with cuticlin residue and whole cuticle preparations of *Ascaris suum* and found that whilst the whole cuticle provoked eosinophilia and mast cell hyperplasia, a response to the cuticlin residue was virtually undetectable (and probably due to contamination of the cuticlin residue with collagen). Immunoblotting experiments with the 2-ME-soluble cuticular proteins of *Onchocerca volvulus* probed with a sera from hyper-immune individuals showed recognition of several strongly reactive antigens, whilst the cuticlin residue was unrecognised by the sera (Sakwe et al, 1997). This is relatively unsurprising; there is no evidence that cuticlins locate to the nematode surface, but are components of the internal layers of the cuticle structure. In addition, the insoluble nature of the residue means that it is probably an immunologically inert structure, with few epitopes available for the purposes of host recogniton.

Two anti-cuticlin antisera were made in rabbits, raised against the whole cuticlin residue from *C. elegans* dauer larvae and *Ascaris suum* adults. The residues were exhaustively purified to ensure that there was no contamination with collagenous material. Both the resultant antisera strongly recognise recombinant CECUT-1. Furthermore, specific anti-CUT-1 antibodies can be affinity purified from both antisera, by adsorbing the sera onto nitrocellulose strips carrying blotted R-CECUT1, and then eluting the recognised antibody off the nitrocellulose. These experiments showed that there is a CUT-1-like cuticular component present in both nematodes which is antigenic even when assembled into the insoluble residue of the cuticle.

The antiserum raised against the *Ascaris* cuticlin residue was used in immunolocalisation experiments. In the dauer larvae of *C. elegans*, the antiserum recognises the same epitopes that are recognised by the anti-R-CECUT-1 antiserum, i.e. forming a ribbon running laterally down the worm, immediately under the alae. The same antiserum recognises epitopes under the alae, in addition to those in various other localisation's including the cortical layers of the cuticle. Hence it would appear that the major cross-reacting epitope present in the cuticlin is CUT-1-like (P. Bazzicalupo, personal communication).

1.12. Why study the cuticlins?

It is my opinion that the study of the genes which we refer to as cuticlin-like, and belonging to a gene family, could be useful and important on a number of levels. Firstly, the study of multigene families, which appear to be ubiquitous components of metazoan genomes, can yield much information on the mechanisms of gene evolution, by their radiating progress not only throughout the development and life-cycle of a single species, but across species as they adapt to newly colonised niches. Knowledge of the differential expression and localisation of the proteins encoded by the individual members of a gene family could also lead to the identification of the factors involved in controlling gene expression, and elucidation of complex developmental pathways.

Secondly, the cuticlins are a vital component of the nematode extracellular matrix (ECM), a multipurpose structure which is produced by all metazoans. Many of the same molecules found in mammalian ECM (collagens, proteoglycans, laminins) have been identified in *C. elegans*, with a remarkable level of conservation. Whether or not the cuticlins are similarly conserved throughout the metazoans, their study will certainly contribute to what is known about the assembly of complex extracellular structures. And this in turn could be used as a model to address basic questions of gene regulation and cell differentiation. As in many biological systems, the absence of a component from a ubiquitous system (in this case the ECM) can be just as interesting and informative as its presence, both in evolutionary and functional studies.

Thirdly, the fact that the nematode cuticlins, like the collagens, are stage-specific, mean that they could in theory be used to identify and characterise additional genes in the regulatory

network that controls larval development throughout the life-cycle of the nematodes. In fact, it is already known that genes identified as being responsible for the regulation of stage transition during *C. elegans* development (the heterochronic genes) are also responsible for controlling the normal cuticle switches during development. This is not a simplistic process, and depends upon the strictly adhered-to progression through a number of well-defined post-embryonic cell lineages, including the hypodermal cell lineages. It would certainly be inappropriate to enter into a discussion on the heterochronic genes here (for the latest review of the field see Ambrose et al, 1997). However, it is of interest to mention that mutants in the heterochronic gene *lin-4* cause certain post-embryonic lineages to be reiterated, and the animals possess a juvenile cuticle as sexually mature adults. Meanwhile, mutants of a second heterochronic gene, *lin-14*, have been shown to 'skip' certain lineages, and the animals possess an adult cuticle at the L4 larval stage (Sulston & Horwitz, 1984).

And last but not least, because the functioning of the cuticle is vital to the morphogenesis and survival of all nematodes, and cuticlin clearly plays an indispensable role in the maintenance of the integrity of the cuticle, it is possible that studies on the cuticlins could lead to the discovery of novel targets for the therapeutic control of parasitic nematodes.

As has been consistently referred to throughout this introduction, *C. elegans* is acknowledged to be an excellent model system for genetic, biochemical and molecular studies of genes from throughout the metazoan kingdom: and the studies done in *C. elegans* have very often set the pace, both technically and conceptually, for parasitic nematode research in particular. However, there is still room for the contribution of parallel studies on parasitic nematodes to *C. elegans* research, particularly with respect to evolutionary studies which can be extremely instructive as to the importance and functionality of a gene and its encoded product.

The field of cuticlin research is new and, it seems to me, promising. This project represents preliminary work on the topic, and it is hoped that it will help, along with the *C. elegans*, *Ascaris* and *Meloidogyne* cuticlin studies, to carry the study of the cuticlins to a point where it is clear that it would be very worthwhile, interesting and challenging to continue to learn more about this group of proteins.

CHAPTER TWO: 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 wood and netting cages and fed on sugar 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 picked from the trays every day and added to 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 artificially heated and stirred bovine blood through a synthetic membrane. Eggs were subsequently 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 three months the jirds were sacrificed using CO_2 anaesthesia and exsanguinated by cardiac puncture. Adults and microfilariae (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 mf were washed again in HBSS and then resuspended in rabbit blood at a density of 350-450/20µl of blood. Adult mosquitoes were fed via the artificial feeder mentioned above at 37°c.

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 are considered to be mature and therefore infective. At this point the infective L3 are recovered using the mass harvesting technique developed by Ash (1974). The mosquitoes are removed from their cages and temporarily stunned by placing them at -20°c for 90 seconds. They are then 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 L3s have emerged and are then washed at least twice in fresh HBSS using a fine drawn-out pipette. The infective larvae 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 L3s were injected into the peritoneal cavity of each jird to be infected. After the required period of time, dependent upon the time-point post-infection required, 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 IMMUNOCHEMISTRY.

2.2.1 SDS-PAGE (Laemmli, 1977).

Reagents:

12.5% Separating Gel.

30% acrylamide (Scotlab)	12.5ml
1.5M Tris-HCl, pH 8.8	11.2ml
dd H20	6.2 ml

After degassing 300µl 10% SDS, 100µl 10%w/v APS and 20µl TEMED were added to polymerise the gel.

5% stacking gel.

30% acrylamide	1.67ml
0.5M Tris-HCl, pH 6.8	1.25ml
ddH_2O	7.03ml
10% SDS	100ml
10% APS	50µl
TEMED	10µl

Sample Cocktail.

0.5M Tris-HCl, pH 6.8	1.88ml
10% SDS	6.0ml
Glycerol	3.0ml
ddH_20	2.12ml

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

Running Buffer.

Tris base	3.03g
Glycine	14.4g
SDS	1.0g

Make up to 1 litre with ddH_20 and pH to 8.3.

Coomassie Blue Stain.

Coomassie Brilliant Blue	1g	
Methanol		450mls
ddH_20		450mls
Glacial Acetic Acid	100mls	

Destain Solution:

Glacial Acetic Acid	350mls	
Methanol		1l
ddH_20		3.65l

Procedure:

The separating gel was poured and overlaid with H_20-saturated butanol and left to polymerise. The Hoefer vertical electrophoresis system was used. The overlay was removed and the stacking gel was poured with a suitable comb in place. Samples were prepared by mixing with an equal volume of sample cocktail and than boiling for 3-4 minutes. Molecular weight markers (Bio-Rad) were also prepared in this way, and included on all gels (range Mr 14000 to 200000). Electrophoresis was carried out at 30-40mA. Gels were then immunoblotted, dried and exposed to film, or the proteins visualised by Coomassie Blue staining.

2.2.2 Immunoblotting.

Reagents:

Transfer buffer.

Tris base	6.05g
Glycine	28.2g
Methanol	400mls
ddH_20	1.6l

TBS (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 ddH_20.

TBS/Tween 20.

0.05%v/v Tween 20 in TBS.

Ponceau S Stain.

Dissolve 0.2g Ponceau S in 100ml 3% v/v TCA (trichloroacetic acid) solution.

Procedure:

Brugia pahangi proteins or purified recombinant proteins were run out on a 12.5% acrylamide gel, as previously described. The separated proteins were then transferred onto nitrocellulose paper using the Hoefer blotting system at 200mA for one hour. The blot was then stained with Ponceau S to visualise the transferred protein. Blotted proteins were then incubated with TBS/Tween 20/5% BSA to block non-specific antibody binding sites. Individual strips were then probed with control or test rabbit serum at varying dilutions. Goat anti-rabbit alkaline phosphatase conjugate (ICN) was used as the second antibody, at a dilution of 1/6000. The blots were developed using BCIP/NBT substrate (Dynatech). Washing was carried out between each incubation with TBS/Tween 20, for 3 x 15 minutes. Hybridisation of the blots was carried out using the MINIBLOT®16 system (Immunetics) which is designed for use with very small volumes of antiserum.

2.3. IMMUNOLOCALISATION STUDIES USING ANTI-rASCUT-1 ANTISERUM.

2.3.1 The recombinant proteins and the raising of the antiserum.

There are two fusion proteins consisting of the same recombinant ASCUT-1 fragment, UNI (see Chapter six for the fragment sequence), inserted into two different vectors. They are: pmalUNI TB1, which is the recombinant protein fused to the MBP in the vector pmalcRI (TB1 refers to the *E. coli* host strain) and pt7.7UNIBL21$^-$, which is the vector pt7.7 with the *Ascaris* UNI insert. The pt7.7UNI recombinant protein was electroeluted from an acrylamide gel and injected into a rabbit to raise the anti-rASCUT-1 antibodies. This work was done by Claudia Paiva-Nunes at IIGB, Naples.

2.3.2 Expression of the recombinant proteins.

1) Overnight cultures of the appropriate *E. coli* host carrying the two recombinant fusion proteins were grown up overnight in L-Broth containing 50mg/ml ampicillin.
2) The next day, 250ml of pre-heated LB + amp was inoculated with 500µl of the overnight cultures. The cultures were grown, with shaking at 37°c, for 2.5 hours or until the OD was about 1.0.

3) IPTG was added to 0.5mM final concentration, and the culture grown as above for at least another two hours.

4) The cells were collected by centrifugation at 8000 RPM for 15 minutes and the supernatant discarded.

5) The cells were resuspend in 12.5mls of: 1% Triton, 50mM Tris-HCl pH6.8 and 1mM PMSF (phenyl methyl-sulfonyl fluoride, a protease inhibitor).

6) They were then frozen in dry-ice ethanol, and thawed quickly at 37°c, followed by sonication at maximum power for 6 cycles of 30 seconds on and 30 seconds off.

For pt7.7UNI :

7) Another 15mls of the Triton/Tris/PMSF mixture was added to the broken cells, incubated at 37°c for 20 minutes and centrifuged at 10,000 RPM for 10 minutes.

8) The supernatant was discarded, and the pellet resuspended in 9mls 2M urea, 50mM Tris pH 6.8. and 1mM PMSF. The cells were incubate at 37°c for 20 minutes, and centrifuged at 12,000 RPM for 10 minutes.

9) The supernatant was discarded and the pellet resuspend in 4mls of loading buffer. The resultant protein was run on an acrylamide gel to check the size of the band which should be around 26kDa.

For pMalUNI:

After sonication, the broken cells were centrifuged for 10 minutes at 10,000 RPM. The pellet was resuspended in 4mls of 3M urea, in which the protein dissolves easily. The cells were centrifuged for 10 minutes at 10,000 RPM and the supernatant retained, combined with loading buffer and run on an acrylamide gel. The fused protein is a prominent band at 80kDa.

2.3.3 Affinity purification of anti-rASCUT-1 antiserum.

The antiserum used in the immunolocalisation experiments was pT7.7UNI, which was affinity purified using pMalUNI as follows:

1) A 40ml culture of pMalUNI was grown as above and the protein extracted and run on an acrylamide gel.

2) The gel was blotted and lightly stained with Ponceau S to identify the correct band.

3) The band was then cut out of the nitrocellulose, washed in TBS/Tween 20 until the Ponceau was rinsed off, and blocked in 3% BSA for 30 minutes.

4) After rinsing briefly, the nitrocellulose strip was added to a tube containing 1ml of the anti-rASCUT-1 antiserum (IgG fraction, purified by ammonium sulphate precipitation to remove proteases present in the whole serum). The strip was incubated, rotating, at 4°c overnight.

5) The next day the strip was removed from the antiserum and rinsed three times for ten minutes in TBS/Tween20.

6) The antibodies were eluted off the nitrocellulose as follows: the nitrocellulose strip was added to 500µl of glycine pH2.8, and neutralized immediately with 140µl of Tris pH8.5 to give a pH of 7.5. Add 64µl of 10% BSA. This is Eluate One.
7) The strip can be washed well, re-blocked in 3% BSA and re-used to affinity-purify specific IgG from the same sample of antiserum as described above, but in our experience the resultant antiserum was not as potent as the first eluate.

2.4 IMMUNOFLUORESCENCE MICROSCOPY.

In order to identify the structures to which the anti-*cut-1* antiserum bound in adult *B. pahangi*, cryostat sections were labelled with the affinity-purified antiserum. As the preliminary experiments indicated that the uterine contents of the adult female were differentially stained by the antiserum, further experiments were carried out to investigate antibody binding to mf at different developmental stages.

2.4.1.Preparing *Brugia pahangi* adults and mf for IFAT.

1) To obtain microfilariae at different developmental stages, adult female worms were cut into four equal parts and each part was laid on a separate glass slide. Mf were then extruded from the adult worm using a fine dissection needle. The slides were allowed to air dry and were then fixed by immersion in ice-cold acetone for thee minutes.
2) Adult worms were removed from the peritoneum of the jird, and were then embedded in TissueTek within gelatine capsules. The capsules were frozen by immersion in liquid nitrogen, and then sectioned (5µm) using a cryostat, and the sections placed on multispot slides.
   All slides were stored at -80°c until they were used.

2.4.2 Immunofluorescence antibody testing (IFAT)

1) The slides from the -80°c freezer were allowed to come to room temperature and dry.
2) The slides were then blocked in 5% BSA/PBS at room temperature for 30 minutes and washed 3 x 5 minutes in PBS (in a Coplin jar).
3) They were then incubated in affinity-purified anti-rASCUT-1 at a 1/10 dilution (in PBS/1% BSA) for an hour at room temperature, and washed as above 3 x 5 minutes in PBS.
4) The secondary antibody (goat anti-rabbit FITC, Sigma) was diluted 1/80 in 1% BSA/PBS and added to the slides. They were then incubated in the dark for 60 minutes at room temperature, after which they were washed as above.
5) The sections were counter-stained for 2 minutes in Evans Blue and washed again before being mounted in Dabco/glycerol (which prolongs the flourescence) and viewed either on a LEITZ microscope or a Zeiss Axial Plan microscope.

2.4.3.Immunogold Staining.

Fixation and embedding of adult worms for immunoantibody staining was carried out by Dr. Lawrence Tetley at the E.M unit, IBLS, University of Glasgow. The procedure was as follows.

1) Worms were fixed in freshly prepared 2% (w/v) paraformaldehyde, 0.1% gluteraldehyde in PBS, pH7.4, at 4°C for 30 minutes.
2) Pellets were washed twice for 5 minutes in cold PBS and cold-processed through an increasing series of alcohol concentrations: 50% ethanol, 4°C for 10 minutes; then 70%, 95% and 100% ethanol steps at -20°C for 20 minutes each, with agitation at 10 minutes.
3) The samples were then treated with a 50:50 mixture of LR White Medium resin and absolute ethanol at -20°C for 30 minutes, then with a 75:25 resin/ethanol mixture at -20°C for 30 minutes, then with 100% resin for 8 hours (during which time the specimens were brought up to 20°C) and subsequently allowed to infiltrate fully overnight with mild agitation.
4) Worms were then transferred to gelatine capsules in fresh LR White resin and polymerised at 20°c under 350nm indirect UV light for two days.
5) Ultrathin sections were collected onto 300 mesh nickel grids.
6) On-grid immunostaining was performed as follows: Blocking and conditioning steps using 0.2M glycine and 1% BSA in PBS for 30 minutes each were followed by incubation for 30 minutes in affinity-purified anti-CUT-1 antibody diluted 1/10 in PBS/1% BSA; and then a 30 minute incubation in goat anti-rabbit IgG, conjugated to 10nm of gold (Aurion), diluted 20 fold in the same buffer.
7) Sections were briefly counter-stained in 0.5% uranyl acetate and viewed with a Zeiss 902 electron microscope.

2.5 IMMUNOSCREENING.

2.5.1 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).

2.5.2 Preparation of plating cells.

Growth media.

L-Broth: 10g bacto-tryptone (Difco), 5g yeast extract (Difco), 10g NaCl, made up to 1 litre with distilled H_2O and adjusted to pH7.0 with NaOH.

L-agar: As L-broth with the addition of Bacto-agar (Difco) to 1.5% for growth plates.

L-agarose: 0.7% agarose in L-broth, for top agarose.

All growth media were sterilised by autoclaving at 120°c for 15 minutes at 15psi.

Antibiotics: When required either ampicillin, at a final concentration of 100μg/ml, or tetracycline, at a final concentration of 10μg/ml, were added to broth or agar. Ampicillin was dissolved in dd$H_2$0 to make a stock solution of 10mg/ml which was stored, foil-covered, at -20°c. Tetracycline was made as a 5mg/ml stock in ethanol, and stored, foil-covered, at -20°c.

Reagents:

IPTG (Isopropyl B-D-thiogalactopyranoside).

100mM stock solution, dissolved in sterile ddH_2O and stored, foil-covered, at -20°c.

Reduced strength TNT.

10mM Tris-Cl (pH 8)

150mM NaCl

0.025% Tween 20

SM buffer.

NaCl	5.8g
$MgSO_4.7H_2O$	2.0g
Tris base	6.05g
2% gelatine	5mls

pH to 7.5 with conc. HCl, make up to 1l with ddH_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 10mg/ml tetracycline. 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 10mg/ml tetracycline. The cells were incubated overnight, with shaking.
3) 1ml of this overnight culture was added to 50mls of pre-warmed growth medium, as used in step 2. The cells were returned to the 37°c shaker until an OD of 0.5 (2.5 x 10^8 cells/ml) was achieved (approximately 2 hours).
4) The culture was then cooled on ice and centrifuged at 3000RPM for 10mins at 4°c to pellet the cells.

Titration and plating of the library.

Serial dilutions of phage were made in SM buffer using dilutions 10^{-3} - 10^{-6}. 10ml 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. The plates were allowed to solidify at room temperature before inverting them and incubating them for four hours at 37°c, or until plaques could be seen on the plates. The appropriate dilution was chosen and the process repeated to screen a representative number of plaques. The number of plaques screened in each case is specified in the relevant results chapter.

2.5.3 Induction of expression.

Hybond-C nitrocellulose filters (Amersham) were soaked in 10mM IPTG (Sigma) and then briefly dried. They were then laid on top of the plated 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 forming between the plate and the filter by placing the middle of the filter down first and then rolling down the edges. The plates were returned to the 37°c incubator overnight, to induce expression.

2.5.4 Antibody hybridisation.

1) The filters were removed from the plates and washed in reduced strength TNT. The plates were stored at 4°c.
2) Non-specific antibody binding sites were blocked by incubating the filters in 5% Marvel (commercially available powdered skimmed milk), dissolved in reduced strength TNT.
3) Filters were washed again before incubation in the appropriate serum, the details of which are discussed in Chapter Three. The antisera were diluted in 5% Marvel, in order to reduce the number of false positives. The filters were incubated on a shaker at room temperature for an hour.
4) The filters were well washed to wash away any unbound primary antibody. They were then placed in the secondary antibody solution: goat anti-rabbit alkaline phosphatase conjugate (ICN), diluted in TNT/5% Marvel to 1/10000, for one hour at room temperature.
5) The filters were washed again and then developed using BCIP/NBT substrate (Dynatech).
6) Positive plaques will develop first, usually within ten minutes, and be darker than the background levels. After lining up the positive on the filter with the corresponding plaque, a sterile pipette tip was used to core out the plaque, and it was placed in 500µl of sterile SM and 10µl of chloroform.

Secondary screening.

Serial dilutions were again used to find the appropriate concentration of plaques such that a single positive plaque could be picked without contamination by those surrounding it. 5ml of

the correct dilution was incubated with 400ml of plating cells before being plated out as previously described. The screening process was repeated as before, single plaques were picked into SM buffer and stored at 4°c.

2.5.5 pBluescript transformants.

Phage DNA from the clones which had been identified as positive in the screening process was isolated by the plate-lysate method (Sambrook et al, 1989). The pBluescript phagemids, carrying the cDNA of interest, were removed from the phage molecule by *in vivo* excision.

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 tetracycline 5mg/ml.
2) The cells were centrifuged at 3000 RPM for 10 minutes at 4°c, and the pellets resuspended in 10mls 10mM $MgSO_4$ in SM buffer.
3) 1.5ml Eppendorf tubes were labelled, one for each clone. 200µl of XL1-Blue plating cells, 200µl λ (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 one 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 4000RPM for 10 minutes. 1ml of the supernatant (containing the pBluescript phagemid) was transferred 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 and grown overnight at 37°c.
7) Once the colonies had grown to a reasonable size, a single colony was picked and used to inoculate an overnight culture of L-broth containing ampicillin. These cultures were used to prepare plasmid DNA for analysis of the clones.

2.6 SCREENING THE GENOMIC LIBRARY.

The *B. pahangi* genomic library was made by Jorge Hirzmann (given by Prof. Gird Hobom) at the University of Geissen. It was constructed in EMBL3 using size-fractionated (9-23kb) partial *Mbo*I digests of high molecular weight DNA from adult, mixed-sex *B. pahangi*.

Reagents:

NZY Broth: 5g NaCl, 2g $MgSO_47H_2O$, 5g yeast extract, 10g NZ Amine (casein hydrolysate). Adjust to pH7.5 with NaOH.

Top Agar (per 100ml): NZY broth, 0.7% (w/v) agarose.

NZY Plates (per litre): NZY broth, 15g of Difco agar.

Denaturing solution: 1.5M NaCl, 0.5M NaOH, make up to 1l with ddH_2O and store at room temperature.

Neutralising solution: 1M Tris base, 1.5M NaCl, adjust to pH 7.4 with conc. HCl, make up to 1l with ddH_2O and store at room temperature.

Reagents for hybridisation:

20 X SSC: 3M NaCl, 0.3M sodium citrate (pH 7.0)

Church and Gilbert Hybridisation Solution (200ml working solution):

100mls phosphate hybridisation stock*, 70mls 20% SDS, 0.4mls 0.5M EDTA, 29.6mls ddH_2O.

*** Phosphate Stock (0.5M Na_2HPO_4 pH 7.2):**

71g Na_2HPO_4 (anhydrous), 4mls 85% orthophosphoric acid, make up to 1l with ddH_20. pH should be 7.2.

Procedure:

Primary screen:

1) The XL1-Blue MRA strain was used as plating cells. Preparation was as previously described for MRF plating cells, except that ampicillin is the appropriate antibiotic. The library was titrated to determine the concentration, using serial dilutions of the library.
2) 600µl of plating cells (OD600 = 0.5) were combined with 50000 pfu of the library and incubated for 15 minutes at 37°c to allow the phage to attach to the cells. This mixture was combined with 6.5mls of top agar, plated onto large (150mm) NZY plates and grown overnight at 37°c.
3) Next morning the plates were chilled to 4°c to prevent the warm top agar sticking to the nitrocellulose filters.
4) Filter lifts were taken from each plate, using Hybond-N filters (Amersham). Duplicate lifts were taken. Orientation marks were made using a needle, and the filters clearly labelled. For the first filter lift the plaques were left to transfer for two minutes, whilst the duplicate filters were left to transfer for four minutes.
5) Filters were denatured by lying them, plaque side up, on 3MM paper soaked with denaturing solution for 2 minutes.
6) Neutralisation was carried out in the same way, but for five minutes.
7) Filters were briefly rinsed (for no more than 30 seconds) in a solution of 0.2M Tris-HCl pH 7.5 and 2 x SSC.
8) After air-drying at room temperature, the filters were UV cross-linked using the Strata-linker.

Hybridisation procedure:

In addition to the filters used in the library-screening process, southern and northern blots were all hybridised using the same technique and reagents. Detection of bound nucleic acids on the filters was achieved by hybridisation to random-primed DNA probes. The reactions were carried out using temperature-controlled hybridisation ovens and hybridisation tubes; the system is manufactured by Hybaid. 15mls of Church and Gilbert Hybridisation Solution was added to the hybridisation tube and allowed to heat to the hybridisation temperature. The blots were wetted in 2 x SSC and added to the tube at the same time as the labelled probe. Hybridisations were generally carried out overnight at 65°c, unless otherwise specified in the results.

The filters were washed six times at 65°c: twice for fifteen minutes in 2x SSC, 1%SDS; two further fifteen minute washes in 1x SSC, 1% SDS; and the last two fifteen minute washes were carried out at a concentration of either 0.5x SSC, 0.1% SDS (for library screening and Northern blots) or 0.2x SSC, 0.1% SDS (for Southern and PCR blots). After washing, the filters were air-dried briefly, bagged in plastic, and autoradiographed at -70°c.

Secondary screen.

1) The filters were oriented with regard to the autoradiograph by lining up the needle 'dots', and the strongest putative positives, which were also repeated on both of the duplicate filters, were marked.
2) Squares of about 0.5cm^2 were cut out of the stock plates where the putative clones lined up with the film spot. The agar was added to 1ml of SM buffer and 20µl of chloroform, vortexed, and left at room temperature for four hours to allow the phage to elute from the top agar.
3) At this stage the putative clones were checked for the presence of a cuticlin insert by doing a confirmatory PCR reaction: reactions were done in 20µl volumes using 1µl of the eluted phage in SM, and two *cut-1*-specific primers. The control was a non-hybridising lamba clone. The putative clones which resulted in amplification products of the right size were selected to carry through to a secondary screen. This process of selection was also carried out between the secondary and tertiary screens.
4) Each putative clone was titrated as previously described, and plated such that the plaques were well-separated on the agar plates.
5) Filter lifts were carried out as before, and the filters hybridised. Duplicate lifts were not taken.
6) A large number of the plaques should hybridise at this stage (depending, obviously, on how densely the primary plaques were plated).

Tertiary screen.

The tertiary screen was carried out exactly as the secondary screen. It should result in a clonal population of the putative clones; every plated plaque should hybridise strongly to the probe. The cored putative positives from the tertiary screen were stored in SM and chloroform at 4°c, and high-titre phage stocks were made as described later.

2.7 GENERAL MOLECULAR METHODS.

2.7.1 AGAROSE GEL ELECTROPHORESIS.

Reagents.

10 X TBE Buffer: 109g Tris-HCl, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre with ddH_2O (pH8.3).

DNA gel loading buffer: 30%v/v glycerol , 5%w/vSDS , 0.1%w/v Bromophenol blue, 10mM Tris, 1mM EDTA pH8. Store at room temperature.

Ethidium bromide: 10mg/ml stock in H_2O, working concentration 0.5mg/ml. Store protected from light at room temperature.

DNA size markers: Lamda DNA (Promega) digested with *Hind* III or *Pst* I. Both used at 1mg/10ml. Store at room temperature.

Procedure:

Gels containing 0.8-2% agarose w/v in 1 x TBE were used to separate and analyse DNA molecules. The agarose solution was boiled and cooled before the addition of EtBr to allow visualisation of the DNA. The mixture was then cast in Gibco/BRL horizontal gel tanks with the appropriate comb in place. After setting, the gel tank was filled with 1 x TBE so that the gel was completely covered. Gel loading buffer (1/10 volume) was added to each sample before loading. Molecular weight markers were run alongside the DNA of interest. The gels were subjected to electrophoresis at 50V until the DNA entered the gel, then at a variable voltage for an appropriate amount of time depending on the size of gel and nature of the samples. Gels were then viewed on a short wave UV trans-illuminator and photographed using Polaroid high speed, black and white film, type 667.

2.7.2 PURIFICATION OF DNA FROM AGAROSE GELS.

Once the gel had been electrophoresed for long enough to achieve sufficient separation between DNA bands, the band or bands of interest were cut out of the gel using a clean scalpel. The DNA was then extracted using the QIAEX© II system (QIAGEN Ltd.). The procedure used followed precisely the QUIEX II Agarose Gel Extraction protocol in the QUIEX© II Handbook (1995).

2.7.3 PRODUCTION OF DNA PROBES.

2.7.3.1 Double stranded DNA probes.

10-20ng of DNA in an 11µl volume (made up with sterile ddH_2O), was boiled for 10 minutes to denature the double stranded DNA. The DNA was then cooled on ice before the addition of 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, at least 1 x 10^7 cpm/ml, was boiled for ten minutes to separate the double strands of the probe DNA, and cooled on ice before being added to the hybridisation tube, already containing 20mls of prehybridisation solution.

2.7.3.2 Oligonucleotide probes.

100-200ng of single-stranded oligonucleotide was combined with 1µl 10x kinase buffer, 2µl (100µCi) of γ-AT^{32}P and made up to 10µl with ddH_2O. 5 units of polynucleotide kinase (PNK), Boehringer, was then added and the reaction mixed well by pipetting. After 20 minutes incubation at 37°c a second 5 units of PNK were added, mixed and incubated as before. The probes were then diluted to 1ml by the addition of STE buffer (100mM NaCl; 20mM Tris-Cl pH 7.5; 10mM EDTA) and purified of unincorporated nucleotides by passing them through NucTrap® Probe Purification Columns (Stratagene), using a Push Column Beta Shield Device (Stratagene). The probes were then added directly to the hybridisation tubes, already containing 20mls of pre-heated hybridisation solution.

2.7.4 PLASMID DNA ISOLATION.

Plasmid isolation was carried out by the alkaline lysis method of Birnboim and Doly (1979), using the Promega Wizard® Plus Miniprep DNA Purification System kit. For all purposes other than sequencing, the procedure used was as specified in the Wizard Miniprep handbook, purifying the plasmid DNA by binding it to a purification resin and passing it through a Wizard® Minicolumn from which the DNA was subsequently eluted. For sequencing the protocol was followed until after the neutralisation step and subsequent centrifugation and removal of the supernatant containing the plasmid DNA. At this point the plasmid solution was extracted once with phenol/chloroform and twice with chloroform. The aqueous phase was 0.3M in sodium acetate (pH5.2) and the DNA was precipitated by the addition of two volumes of ethanol.

2.7.5 BACTERIOPHAGE LAMBDA DNA ISOLATION.

A high titre phage lysate stock was made by the liquid culture method. A single phage plaque was picked from an agar plate of well-separated λ plaques and the phage were eluted from the agar in 100µl of SM buffer. A fresh culture of an appropriate strain of *E.coli* was grown in 5mls of LB medium supplemented with 50µl of 20% maltose and 50µl of 1M $MgSO_4$. 500µl of the overnight culture was added to a tube containing 10-20µl of the agar plug eluate and incubated for 20 minutes at 37°c. 500µl of the infected culture was added to a 250ml flask containing 100mls of pre-warmed LB medium supplemented with 1ml of 1M $MgSO_4$. The

culture was incubated at 37°c with vigorous shaking until lysis occurred (around 6 hours), at which point 500µl of chloroform and 4g NaCl were added, and shaking was continued for a further 10-15 minutes. The lysate was centrifuged at 8000xg for 10 minutes to remove the cellular debris. The supernatant was transferred to a sterile tube and stored at 4°c. DNA was obtained from the phage stock using the Promega Wizard® Lamda DNA Purification System kit, following the protocol outlined in the handbook accompanying the kit. Proteinase K (to a final concentration of 0.5mg/ml) was added after the phage resuspension step, in order to prevent degradation of the DNA by the nuclease mixture used to remove the lambda phage coat.

2.7.6 ENZYMATIC CLEAVAGE OF DNA.

Restriction-endonuclease cleavage of DNA was carried out using Promega and Gibco enzymes. The reactions were carried out using the buffers and temperatures recommended by the manufacturers at a concentration of 2-5 units of enzyme per µg of DNA. After 3-5 hours the reactions were stopped by the addition of DNA gel loading buffer, prior to agarose gel electrophoresis.

2.7.7 GENOMIC DNA ISOLATION.

Reagents:

TEN buffer: 50mM Tris pH8, 100mM EDTA, 100mM NaCl, 1% SDS

TE buffer: 10mM Tris pH 7.4, 1 mM EDTA

Procedure:

1) 100 adult worms were added to 700µl TEN buffer and chopped finely using small, sterile stainless steel scissors. 35µl of Proteinase K (10mg/ml stock) was added to the tube, which was incubated overnight at 55°c.
2) Next morning, the DNA was RNase treated (0.26µg) and incubated at 37°c for 1-2 hours.
3) The tube was then filled with pH8 buffered phenol (Gibco), and placed on a vertical rotator for one hour. The phases were separated by centrifugation at 13000xg for 10 minutes and the top layer and interface removed to a fresh tube.
4) 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 transferred to a fresh tube.
5) The tube was then filled with a 24:1 chloroform/isoamyl alcohol mixture and the tube rotated for five minutes and subsequently centrifuged for 5 minutes to separate the layers. This time only the top layer was removed to a fresh tube which was topped up with isopropanol. The tube was inverted several times until a stringy precipitate formed.

6) The high molecular weight DNA was removed from the tube by spooling onto a small glass rod. It was then dipped into 70% followed by 100% ethanol.
7) The spooled DNA was allowed to air dry briefly, and then dropped into a tube containing 500µl TE buffer, by breaking off the tip of the glass rod with a diamond pencil.
8) 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.
9) The purity and quantity of the DNA was determined by using a 1/100 dilution of the solution 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 was calculated using the formula A_{260} x 50 x 100 = mg/ml of DNA.

2.7.8 RESTRICTION ENZYME CLEAVAGE OF GENOMIC DNA.

Between 10-20µg of total genomic DNA was digested with 50 units of each restriction enzyme to be used. Typically these were *Eco*RI, *Pst*I, *Bam*HI, *Hind*III and *Bgl*II. The samples were incubated overnight at the temperature recommended by the enzyme manufacturer. After ethanol precipitation and a 70% ethanol wash, the pellets were left for several hours at 37°c to resuspend in 40µl of ddH_20. After the addition of DNA gel loading buffer to each sample, the digested DNA was subjected, alongside DNA markers, to agarose gel electrophoresis on a 0.8% gel run overnight at 28V.

The gel was stained for thirty minutes in ddH_20 containing 0.5mg/ml ethidium bromide, viewed via a UV trans-illuminator and photographed as previously described. 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.

2.7.9 SOUTHERN BLOTTING.

The method of Southern (1975) was used to transfer nucleic acids from agarose gels to nylon Hybond-N membranes (Amersham UK), overnight via capillary action, as instructed by the manufacturer. The transferred DNA was fixed to the membrane by UV cross-linking in Strata linker.

2.7.10 RNA ISOLATION.

In order to minimise the risk of RNA degradation, all chemicals and receptacles for RNA work were made RNase-free: glassware was baked at 300°c for a minimum of 4 hours and plasticware and reagents (except those containing Tris) were treated with 0.1% DEPC overnight and then autoclaved at 120°c for 15 minutes at 15psi. Tris-containing solutions were made by adding new ingredients to DEPC-treated distilled water which had been autoclaved to break down the DEPC. Two pairs of gloves were worn at all times when doing RNA work.

Reagents:

DEPC H20: 0.1% DEPC in ddH_2O, leave at room temperature for an hour, then autoclave. Store at room temperature.

RNA lysis buffer: 0.1M Tris-HCl pH8, 0.2M NaCl, 2% SDS, 0.2M EDTA pH8. 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 of pre-heated RNA lysis buffer, containing 12.5µl Proteinase K (10mg/ml), for at least 15 minutes. Adult worms invariably required longer. 1ml TRIzol®, pre-heated to 68°c, was added and after a further ten minutes incubation at 68°c the tube was transferred to room temperature and allowed to cool for three minutes. 200µl of chloroform was added and the mixture shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes, before centrifugation at 12000xg for 15 minutes at 4°c. The aqueous phase was removed and the 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 12000xg for 15 minutes at 4°c. The pellet was washed in 80% ethanol and stored as an ethanol precipitate at -70°c until required.

2.7.11 RNA purification.

An essentially pure fraction of mature mRNA was obtained from *B.pahangi* adult RNA via the removal of unprocessed RNA, contaminating DNA and proteins. This was carried out using the PolyATtrack® mRNA Isolation System (Promega), which utilises MagneSphere® Streptavidin Paramagnetic Particles and a Magnetic Separation Stand. The procedure used followed exactly the protocol in the PolyATtrack® mRNA Isolation System III handbook.

Formaldehyde RNA gels (Sambrook et al, 1989).

Reagents:

10 x MOPS buffer 41.8g MOPS (3-{N-Morpholino}propane-sulphonic acid), 4.1g sodium acetate, 1.89g Na_2EDTA made up to 1 litre with DEPC-treated H_2O (pH7.2).

Formaldehyde gel: 1.2g Agarose, 10mls 10x MOPS, 73mls DEPC H_20

Boil to dissolve the agarose, cool to 55°c then add 17mls formaldehyde before mixing well and pouring the gel.

Procedure:

RNA samples were resuspended in 50% formamide, 10% DNA gel loading buffer and 10% ethidium bromide (10mg/ml stock). The samples were then denatured at 65°c for 10 minutes

and put on ice for a further 10 minutes before electrophoresis on the 1.2% denaturing formaldehyde gel, with 1 x MOPS as the running buffer. 1µg RNA markers (Boehringer Mannheim) were prepared as the other RNA samples and run alongside the samples, and the gels were photographed before blotting.

Northern blotting

RNA gels were blotted overnight and fixed as previously described.

2.7.12 *IN VITRO* MANIPULATION OF DNA.

2.7.12.1 Cloning PCR products.

Two different vectors were used in the course of the project to clone PCR-generated fragments; each vector is part of a kit which was developed for the rapid, direct cloning of fragments generated by PCR. The systems take advantage of the fact that non-proof-reading DNA polymerases such as Taq often add single A overhangs to the 3' ends of amplified products (Marchuk et al, 1990; Holton et al, 1990). The PCR cloning vectors, pre-cut and adapted to have complementary T overhangs, are therefore ready for direct ligation to the PCR product. Both vectors are derived from pUC plasmid vectors. The two vectors used were pTAg (R & D Systems) and pCR™II (Invitrogen). The ligation and transformation procedures were carried out as specified in the handbooks supplied by the manufacturer of each kit. However, MAX Efficiency DH5α™ competent cells (Gibco BRL) were used in all transformations.

2.7.12.2 Sub-cloning.

Restriction enzyme cleavage of DNA was carried out as recommended by the manufacturers. Sub-cloning of DNA into plasmid vectors, and introduction of recombinant plasmids into *E. coli,* were performed essentially as described by Sambrook et al (1989).

Reagents.

S.O.C medium: Prepare 1M KCl and 1M NaCl stock solutions. Prepare and filter sterilise a 2M Mg^{++} stock (1M $MgCl_2.6H_2O$ + 1M $MgSO_4.7H_2O$, filter-sterilized) and a 2M glucose stock. To 97ml dd H2O add 2g bactotryptone, 0.5g yeast extract, 1ml 1M NaCl, and 0.25ml 1M KCl. Stir to dissolve, autoclave, and cool to room temperature. Make medium 20mM in Mg^{++} with the addition of 1ml 2M Mg^{++} stock. Add 1ml 2M glucose to make the medium 20mM. Filter the complete medium through a 0.2mm filter unit. The pH should be 7.0 +/- 0.1.

Preparing the plasmid.

10µg of plasmid was cut with the appropriate restriction enzyme. In the last 30 minutes of the plasmid digest, 2.5µg of CIP (calf intestinal phosphatase) was added to the reaction. This

results in the dephosphorylation of the cut ends of the plasmid which reduces religation of the plasmid and hence increases the efficiency of the ligation of the DNA to be sub-cloned into the plasmid. The cut plasmid was then extracted once in phenol-chloroform, and once in chloroform alone, before being precipitated in ethanol and 0.3M sodium acetate. The pellet was air-dried, resuspended in 100µl of TE and then analysed on an agarose gel to check that the majority of the plasmid was cut, and to estimate the concentration.

Ligations.

It was observed throughout the course of the project that ligation efficiency varied considerably with the ratio of plasmid to DNA fragment. Small fragments are easier to ligate and therefore ligation efficiency will be higher and the ratio of plasmid molecules to DNA fragment molecules can be smaller. Ratios were calculated stoichiometrically, and several ratios of plasmid:DNA fragment were used in different ligation reactions, to optimise the chances of ligation. A control ligation of cut plasmid alone was also set up in order to establish the frequency of self-ligation in the reactions.

1) The following reagents were combined in a 1.5ml tube:

ligase buffer (5x)	2µl
DTT (100mM)	0.5µl
ATP (10mM)	0.5µl

Cut plasmid, DNA fragment and ddH_2O were added to make a volume of 9.5µl. The tube was then mixed and spun briefly to collect the contents, and 0.5µl (2-3 U) of High Concentration T4 DNA Ligase (Gibco BRL) was added.

2) The reactions were incubated overnight at 16°c.

Transformations.

The use of pUC-based plasmid vectors means that blue-white screening of transformants can be used to select successfully transformed bacterial colonies. The basis of this colour selection is that the plasmid vector contains a LacZα peptide sequence, which when functionally produced complements the N-terminal truncated LacZ peptide synthesised in the competent cells. The resulting enzyme, ß-galactosidase, cleaves X-Gal to result in blue colonies. IPTG derepresses the expression of the LacZα gene in cells containing the plasmid. When an insert is successfully ligated into the cut plasmid the LacZα peptide sequence is interrupted. This interferes with the function of the peptide and the resultant colonies are white.

1) 1µl of the ligation reaction was mixed with 20µl of competent cells which had been thawed on ice. The reaction was mixed gently without pipeting the mixture.

2) The tube was incubated on ice for 30 minutes.
3) The cells were heat shocked by placing the tube in a 42°c water bath for exactly 45 seconds.
4) The tube was transferred to ice for another 2 minutes.
5) 90µl of complete SOC medium was added and incubated for 1 hour, with shaking, at 37°c.
6) Two plates were spread for each reaction; one with 50µl and one with 10µl. The plates were LB plates with ampicillin to 50µg/ml; IPTG to 0.5mM; and X-Gal to 80µg/ml.
7) The plates were inverted and grown overnight at 37°c. White colonies were picked, grown up in overnight cultures, and digested with the appropriate restriction endonuclease to check for an insert of the correct size.

2.8 SEQUENCING.

2.8.1 Manual sequencing.

The sequencing of the putative positive clones resulting from the original antibody screen was done manually. Double-stranded sequencing was performed by the dideoxy chain-termination method first described by Sanger et al (1977) using the Sequenase™Sequencing Kit, version 2.0 (Amersham). The polymerase was Sequenase™, a modified T7 DNA polymerase (Tabor & Richardson, 1987). Sequencing reactions were carried out using the conditions recommended in the Sequenase™ Version 2.0 manual supplied by the manufacturers (United States Biochemical Corporation).

Polyacrylamide gels:

Products of DNA sequencing reactions were separated on denaturing acrylamide wedge gels: 6% acrylamide (acrylamide:bisacrylamide, 19:1), 7M urea, in 1x TBE. Polymerisation was initiated by the addition of 1ml of 10% ammonium persulphate and 20µl TEMED to 150ml of 6% acrylamide/urea. Gels were allowed to polymerise for at least two hours before use and were then pre-run at 60W for 30-45 minutes in order to pre-heat them to 45-50°c before loading. Samples were denatured by boiling for two minutes in the buffer provided by the manufacturer, and then loaded onto the gel with the aid of a sharks-tooth comb. Gels were run for 2-10 hours, soaked in 10% acetic acid/10% methanol for 30-60 minutes, and then dried onto Whatmann 3MM paper under vacuum. Autoradiography was carried out with intensifying screens at -70°c for 48 hours, and the sequence was read from the autoradiograph.

2.8.2 Automatic sequencing.

The sequencing carried out in the latter half of the project was performed automatically, using the SequiTherm™ Long-Read Cycle Sequencing Kit (Cambio) and fluorescently-labelled (IRD41) T3 and T7 primers (Hybaid) on an automated sequencer (Licor). The reactions were carried out as stipulated in the SequiTherm™ Long-Read Cycle Sequencing

Kit manual. The reactions were processed in a thermocycler (Perkin Elmer), using the following cycle program:

1) 95°c for 5 minutes
2) 95°c for 30 seconds
3) 60°c for 30 seconds
4) 70°c for 1 minute

Steps 2-4 were repeated for 30 cycles.

5) 4°c soak.

Once the cycling was completed, 4µl of SequiTherm™ Stop Solution was added to each reaction mix. Samples were denatured at 95°c for 3 minutes and then put onto ice prior to loading.

2.9 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.

2.10 POLYMERASE CHAIN REACTION.

Reverse Transcription.

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

Procedure:

1) 1-2µg of RNA were first treated with DNase to remove any contaminating DNA: the RNA was resuspended in 7µl DEPC H_2O and 1µl of DNase buffer (Gibco) and 0.5µl Dnase 1 enzyme (Gibco) added. The reaction tube was placed on ice for 10 minutes, after which the reaction was stopped by adding 1µl 20mM EDTA and heating to 65°c.
2) While the mixture was still hot, 1µl oligo (dT) primer (200ng/µl) was added, briefly spun down and put on ice. Any contaminating DNA should have been removed, leaving only RNA annealed to the oligo (dT) primer.
3) RT reaction mix:

RnaseIN (Promega)	0.5µl
dNTPs 10mM each (Pharmacia)	2.0µl
RT buffer 5x (Promega)	4.0µl
RT enzyme 100u (Promega)	1.5µl
ddH_2O	1.0µl

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. The resultant first strand DNA was stored at -20°c until required. The cDNA was generally diluted 1/20 in ddH_2O for use in PCR reactions.

2.10.1 Preparation of primers for PCR.

Oligonucleotide primers for use in PCR reactions were designed from the available sequences to give gene-specific primers. Throughout the course of the project they were variously made in-house, by Cruachem Ltd or by Pharmacia Biotech. They were quantified by taking an OD reading of a 1/100 dilution at a wavelength of A_{260}, and the concentration of the primer calculated according to the following formula:
A_{260} x 30 x 100 = mg/ml. The primers were diluted to a working concentration of 1mg/ml and stored in aliquots at -20°c.

2.10.2 PCR reactions.

A variety of PCR reactions were carried out to amplify cDNA between two primer sites using the AmpliTaq™ DNA polymerase enzyme (Perkin Elmer). In order to avoid contamination of the reactions, gloves were worn at all times and sterilised PCR tubes, filter tips and filter-sterilised ddH_2O were used.

Procedure:

1) A mix of the required reagents was made and used for all samples, including the controls, allowing contamination to be easily detected. Perkin Elmer PCR reagents were used for all PCR reagents.

2) PCR reaction mix for 2 reactions (100µl): n.b. smaller reaction volumes were also used for various purposes: optimising PCR conditions, testing reagents, establishing the correct number of cycles required for new primer sets, checking for the presence of inserts in new transformants etc., but for the semi-quantitative RT-PCR experiments described in the results chapter dealing with expression, the larger reaction volume was used. This meant that experiments could be repeated using the same reactions, which was useful when the semi-quantitative PCR protocol was being developed.

DNA	2µl (<1mg)
dNTPs	(1.25mM each)
10 x PCR buffer	10µl
primers 5' & 3'	1µl (0.2mM) each
AmpliTaq DNA polymerase	0.5µl (5U/µl)
ddH_2O	69.5µl

3) 48µl of this mix was added to each tube. The primers and cDNA template were usually added subsequently because they were often different for each reaction. Controls were included in every round of PCR, and were as follows: a reaction with no DNA template; a reaction with no primers; a single-primer reaction; and, when using a new primer aliquot, a

reaction using the primer as the template and a set of primers constitutive for DNA. from all life-cycle stages of *Brugia*

4) A small drop of mineral oil was placed into each tube, to prevent evaporation, the tube was vortexed, briefly spun, and placed in the thermocycler (Perkin Elmer). The following is the standard amplification program, consisting of a variable number of cycles incorporating melting, annealing and extending steps:

95°c	5 minutes	initial denaturation temperature
94°c	1 minute	melting
55°c	1 minute	annealing
72°c	3 minutes	extending
72°c	10 minutes	final extension temperature
4°c	10 minutes	final soak temperature

The annealing temperature was variable with the Tm of the primers used in the reaction (for example, when using the oligo (dT) primer the annealing temperature was lowered to 50°c; the extension time varied with the length of the fragment to be amplified; and the number of amplification cycles was optimised, as discussed in Chapter Six, to ensure that the reaction kinetics were still in the log phase for the semi-quantitative PCR experiments.

2.10.3 Semi-quantitative analysis of PCR products.

RNA from different life-cycle stages of the parasite was reverse transcribed, using the oligo (dT) primer, and the resultant first strand cDNA was used in PCR reactions with *bpcut-1*-specific primers and with primers based on the DNA sequence of a constitutively-expressed *Brugia* ribosomal protein. The resulting PCR products (10µl) were run out on 2% agarose gels and then denatured and neutralised before being blotted onto nylon membranes. The blots were then hybridised to labelled fragments of the *Brugia* cuticlin gene and the *Brugia* ribosomal protein gene and autoradiographed. Once developed, the autoradiographs were lined up with the nylon membrane, and the bands corresponding to each life-cycle stage cut out from the blot using a scalpel. Each piece of nylon membrane was added to 3mls of scintillation fluid and then the ß-emissions calculated by liquid scintillation counting. A corner of the filter 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 life-cycle stage compared to the constitutively expressed ribosomal protein. In this way a semi-quantitative analysis of the expression pattern of the *Brugia* cuticlin gene could be made.

2.11 RAPID AMPLIFICATION OF cDNA ENDS (RACE).

The attempts to clone the 3' and 5' ends of the *bpcut-1* cDNA were carried out according to the method of Frohman et al (1988).

2.11.1 3' RACE.

First strand cDNA synthesis.

First strand cDNA synthesis was catalysed by SuperScript RnaseH reverse transcriptase. The advantage of using this enzyme is that it has been engineered to eliminate the Rnase H activity (found in other reverse transcriptases) that degrades mRNA during the first strand reaction, hence resulting in higher yields of cDNA, a higher proportion of which is full-length.

1) 1µg of RNA, in 13µl of ddH_20, was added to a 0.5ml microcentrifuge tube.
2) 1µl of a 10mM stock of oligo (dT)-containing adapter primer was added to the tube. The adapter sequence contains several endonuclease sites which are incorporated into the cDNA which is amplified using the oligo (dT) primer. The primer was annealed at 65°c for ten minutes and then chilled on ice for two minutes before extending the primer by the addition of :

10x RT synthesis buffer	2µl
10mM dNTP mix	1µl
RNaseIN (Promega)	0.5µl
ddH_2O	2µl

Mix gently and equilibrate the mixture to 42°c for 2 minutes.

3)1µl of SuperScript RT was added. The tube was incubated at 42°c for 30 minutes.

4) After a brief centrifugation, the tube was placed on ice and 2U of Rnase H were added (to degrade any remaining RNA template). The reaction was incubated for 10 minutes at 55°c. The cDNA was stored at -20°c.

Primary amplification of the target DNA.

In this step a *cut-1*-specific 5' primer was used in conjunction with the adapter primer. Use of the adapter primer means that only full-length cDNA should be amplified, and also facilitates post-amplification cloning into vectors which have the same endonuclease restriction sites in the polylinker. Taq DNA polymerase (Gibco BRL) was used in the amplification reactions.

1) To a fresh 0.5ml microcentrifuge tube the following were added:

10x synthesis buffer	5µl
sterilized ddH_20	39.5µl
10mM dNTP mix (Pharmacia)	1µl
cut-1 5' primer	1µl (of a 10mM solution)
adapter primer (10mM)	1µl
Taq DNA polymerase	0.5µl (5U/ml)

And finally 2µl of the first-strand cDNA, diluted 1/100, was added. The reaction was mixed gently and collected by brief centrifuation.

2) Incubate the reaction at 95°c before performing 40 cycles of PCR:

95°c	40 seconds
50°c	2 minutes
72°c	3 minutes

After the cycling reaction, incubate the reaction for 15 minutes at 72°c.n.b. PCR protocol details are included earlier in the chapter.

3) Following the amplification, extract with 50µl of chloroform and transfer the aqueous layer to a fresh tube.

4) 15µl of the amplification product was analysed using agarose gel electrophoresis (1% gel) and ethidium bromide staining. In our experience, this first round of amplification either did not result in a visible product, or resulted in a smear, rather than a discreet band. Consequently two further steps were required: the agarose gels were blotted and probed to see if any *cut-1*-like products were present in the first-round reaction products, and a second round of amplification was carried out to increase the specificity of the reaction.

Hybridisation.

The blots were probed with an end-labelled oligo corresponding to the *cut-1* sequence interior to the 5' primer used in the amplification reaction. Oligos are synthesised without a phosphate group at their 5' termini so are easily labelled by transfer of the γ-^{32}P from γ-^{32}P ATP using the enzyme bacteriophage T4 polynucleotide kinase. Using this method should result in labelling the oligo to a very high specific activity. The protocol for making the probe has already been out-lined. The hybridisation was carried out overnight at 53°c in Church and Gilbert hybridisation solution. The washes were carried out in horizontal dishes without rotation at 42°c. The blots were washed 3x for 15 minutes in 6x SSC, 0.1% SDS before autoradiography.

Secondary Amplification.

If there was a hybridisation signal on the probed blot, a secondary amplification was carried out, using the primary amplification product, diluted 1/200, as the template. The primers used were the adapter primer and a 5' *cut-1* specific primer internal to that which was used in the primary amplification. In this way the specificity of the amplification is increased enormously. Again 40 cycles of amplification were used, followed by 15 minutes extension at 72°c. The reaction product was analysed as for the primary amplification, blotted and hybridised using the same protocol. This should result in hybridisation to a single band or, in the case of a gene family of the type that the *cut-1* genes appear to represent, several bands. In theory, the bands can then be purified from the gel, cloned and sequenced to establish the structure of the 3' end of an incomplete cDNA clone.

2.11.2 5' RACE.

In contrast to 3' RACE, first strand cDNA synthesis is primed using a gene-specific primer, in this case a *cut-1*-specific 3' anti-sense oligonucleotide (2A3Med). This permits cDNA conversion of specific mRNA, or related families of mRNAs. Following cDNA synthesis, the first strand product is purified from unincorporated dNTPs and excess primer. Terminal deoxynucleotidyl transferase (TdT) is used to attach homopolymeric tails to the 3' ends of the cDNA, and the tailed cDNA is then amplified using a nested 3' gene-specific primer (Br3/2) and an 'anchor primer' (complementary to the homopolymer tail). This allows the amplification of the cDNA sequence between the second gene-specific primer and the 5' end of the clone.

First strand cDNA synthesis.

1) 2.5 pM (10-25ng) of 2A3Med and 1$\mu\gamma$ of RNA were added to a sterile tube and made up to 15µl with sterile DEPC-treated H_20. The reaction was heated at 70°c for 10-15 minutes to denature the RNA, chilled on ice for 1 minute and collected with a brief spin.
2) The following were added to the tube:

10x reaction buffer	2.5µl
25mM $MgCl_2$	3µl
10mM Ultrapure dNTP mix (Pharmacia)	1µl
0.1M DTT (Gibco BRL)	2.5µl

   The reaction was mixed, spun and incubated for 2 minutes at 42°c.
3) 1µl (8U) SuperScript II Reverse Transcriptase was added and the reaction incubated for 30 minutes at 42°c.
4) The tube was placed at 65-70°c for 5 minutes to denature the reverse transcriptase.
5) The reaction was spun down then transferred to 55°c. 2U of Rnase H were added and incubated for 10 minutes then chilled on ice.

Purification of first strand cDNA.

Because of the large amounts of 2A3Med relative to cDNA product, the excess primer has to be removed using a stringent purification procedure; if not, the residual primer will be tailed by TdT and will compete for anchor primer during PCR amplification. The GlassMAX™ DNA Isolation Spin Cartridge (Life Technologies) system was used: this method, first described by Vogelstein and Gillespie, involves the entrapment of cDNA >200bp on a silica-based membrane in the presence of sodium iodide. All the smaller cDNA species are washed through, and the purified cDNA is eluted off the membrane. The protocol used was that described in Protocol 2 of the '5' RACE System for Rapid Amplification of cDNA Ends' Instruction Manual (Gibco BRC).

Homopolymeric tailing of cDNA.

The purified first strand cDNA, which should consist only of *cut-1*-like and related cDNA species, was then given a poly(C) tail using Terminal Deoxynucleotidyl Transferase (TdT).

1) Purified cDNA was made up to a final volume of 16µl with DEPC-treated water and denatured at 70°c. The mixture was chilled on ice and spun briefly to collect the reaction.

2) The following were added to the reaction:

10x synthesis buffer	1µl
25mM $MgCl_2$	0.5µl
2mM dCTP (Gibco)	2.5µl
TdT (10U/ml, Gibco)	1µl

n.b. In order to evaluate the specificity of the amplification reaction from the oligo (dC) tail, a control reaction omitting the TdT was also set up. The reactions were incubated at 37°c for 10 minutes.

3) The TdT was inactivated at 65°c for 10 minutes, and then put on ice.

Amplification of the (dC)-tailed cDNA.

This was carried out using Br3/2 and the 'anchor primer' provided in the Life Technologies 5' RACE System kit. The primer is complementary to the poly(C) tail at the 3' end, with an adapter region at the 5' end.

1) The following were added to a 0.5ml PCR tube:

10x reaction buffer	4.5µl
25mM $MgCl_2$	3µl
10mM Ultrapure dNTP mix	1µl
primer Br3/2 (10mM)	2µl
anchor primer (10mM)	2µl
dC-tailed cDNA	5µl
sterilized ddH_2O	28.5µl

The reaction was overlaid with mineral oil and denatured for 5 minutes at 95°c, then maintained at 80°c while 2U of Taq DNA polymerase (Perkin Elmer) were added to the reaction mixture.

2) 40 cycles of PCR amplification were carried out, with an annealing temperature of 55°c, and the reaction was incubated for 10 minutes at 72°c following the last cycle of PCR.

3) 15µl of the reaction was run on a 1% agarose gel and visualised by ethidium bromide staining. Bands should represent the 5' ends of *cut-1*-like, or closely-related cDNA species.

CHAPTER THREE: SCREENING A *B. pahangi* cDNA LIBRARY WITH ANTI-CUTICLIN ANTISERA.

3.1 INTRODUCTION

When this project was initiated, the *C.elegans cut-1* & *cut- 2* genes had been isolated and characterised and the *Meloidogyne artiella cut-1*-like gene had been isolated from a genomic library, using a *C. elegans cut-1*-derived recombinant fragment as the probe, all of which has been discussed at length in the Introduction. It was now a priority to obtain and characterise the cuticlin homologues of parasitic nematodes of veterinary and medical importance, so as to establish their role in the architecture of the nematode cuticle.

From what was known at the time about the cuticlin genes and the proteins that they encoded , *cut-2* appeared initially to be a stronger candidate for playing an important role in the basic structure of the nematode cuticle. This gene is known to be transcribed in all life-cycle stages of *C. elegans*, discontinuously and immediately before a moult. Antisera raised against a recombinant fragment of CUT-2 localized to the cortical layer of the cuticle (Favre et al, 1995). Hence it is reasonable to assume that the protein is incorporated into the cuticle when it is being synthesised. The structure of CUT-2, with the regular arrangement of tyrosine residues permitting di-, tri- and iso-tyrosine cross-links, implies that it plays a part in the insolubilisation of the nematode cuticle (Lassandro et al, 1994). In short, its ubiquity throughout the life-cycle of the worm combined with its ability to form insoluble cross-links with great efficiency, would suggest a possible role for CUT-2 in forming the frame-work or skeleton of the nematode cuticle, onto or around which other cuticular proteins are added.

Previous attempts to screen an *Ascaris lumbricoides* genomic library with a recombinant DNA fragment corresponding to the final 161 amino acids of *cut-2* had been unsuccessful (Bazzicalupo, personal communication). However, the screening protocol had resulted in a number of false positives, probably due to the fact that the *cut-2* probe had a very high GC content which consequently made it non-specifically 'sticky' to the *Ascaris* DNA, at the relatively permissive conditions required for cross-species hybridisation.

In the initial attempt to obtain a *Brugia* cuticlin gene homologue it was decided to immunoscreen a *B. pahangi* cDNA library with antisera raised against recombinant fusion proteins of CUT-1 and CUT-2. There were several reasons for choosing this option over the screening of a genomic library with labelled fragments of the cuticlin genes. Firstly, at that stage the extent of the DNA sequence homology between the nematode *cut-1* genes was not fully realised, and it was thought that lowering the stringency of screening conditions to a point where heterologous sequences could be detected would result in the hybridisation of an overwhelming number of false positives, which had been the case in the screening of the *Ascaris* library with *cut-2* . Under these circumstances it was felt that screening with antisera would be more specific and reliable. Secondly, the reagents and library were already available and known to be functional: the antisera localized to the cuticle of *C. elegans* and had been

shown, by western blot, to react with *B. pahangi* proteins; the cDNA library had been successfully screened using antisera on a number of occasions. Hence the likelihood of isolating a positive clone seemed relatively high. Finally, directly obtaining a cDNA clone can make the rapid characterisation of a gene more straightforward; only the coding regions of the gene are present and the mRNA is representative of the peptide product of the gene. Fusion proteins can then be made from the predicted amino acid sequence and antisera can be raised for use in localisation experiments. Such experiments are extremely important, in that they yield substantial information about the real biological function of a protein within the organism. In the case of the filarial nematodes, for example, it was important to establish whether a *Brugia* homologue of either of the cuticlin genes encoded a protein which appeared to localise to the cuticle.

3.2 OPTIMISING THE CONDITIONS USED IN THE ANTIBODY SCREENING OF THE cDNA LIBRARY.

3.2.1 Antisera used in library screening.

Figure 3.1 shows the amino acid sequences of *C. elegans* CUT-1 and CUT-2, with the sequences used to raise the antisera used in this chapter marked.

- **Anti-CUT-1**

NA5 - raised against fusion 914.1, expressed in pEx34 vector.

F3 - raised against fusion 912.1 Expressed in pEx34.

The two fusion proteins over-lap by 12 amino acids, and the antisera against both were raised in rabbits. The antisera were purified by adsorbing them extensiveley onto a lysate of the *E. coli* strain carrying pEx34 with no insert.

- **Anti-CUT-2** - a recombinant was made from the last 161 amino acids of CUT-2 fused to the maltose binding protein, and cloned into the pMalP plasmid. Antisera against the construct were raised in rabbits, and were purified by adsorption against the cold osmotic shock fraction of TB1, which is the bacterial strain carrying pMalP with the maltose binding protein but without a CUT-2 insert.
- The library was screened with a 'cocktail' of the above antisera, diluted in 5% Marvel.

3.2.2 The library.

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

3.2.3 Experimental conditions for screening.

The experimental procedures used in the library screening are described fully in the Materials and Methods section. Outlined below are the optimisations that were carried out to maximise the chances of finding a positive clone with the reagents used.

cut-1 recombinant phage stock (carrying the insert which was used to raise NA5) was plated out and the plaques induced, blotted onto nitrocellulose filters and probed with the same 'cocktail' of antibodies, under the same conditions that were subsequently used in the screening of the library. These plaques were considered to be the 'positive control', for the experimental conditions, which were adjusted such that the control plaques reacted strongly with the antisera. It must be said that the positive control was representative of only one of the fusion proteins used to raise the three antisera which made up the 'cocktail' used to screen the library. However, previous experiments had shown that all three antisera reacted with plaques carrying the recombinant proteins against which they were raised, under the same experimental conditions.

To show that the positive plaques from the first round of the screening react specifically with the anti-cuticlin antisera, controls were carried out using pre-immune rabbit sera (data not shown).

A number of separate experiments were carried out to ascertain the conditions under which the protein from the plated library phage recombinant plaques could be optimally transferred onto the nitrocellulose filters used in the screening. Within this experimental system it was found that growing the phage for three hours prior to overlaying the plates with damp nitrocellulose filters soaked in 10mM IPTG, then leaving the filters on the plates overnight at 37°c and processing them the next day, appeared to yield the best results.

Control experiments were carried out using a number of different high protein substances to ascertain which would block non-specific antibody binding sites most effectively. Substances tested were: BSA (at 3% and 1%), Marvel (5%) and a commercially available skimmed milk (80%). All dilutions were done in reduced strength TNT. It was concluded that 1% BSA and Marvel were equally effective in their blocking function, although filters blocked with BSA were somewhat 'cleaner' than those blocked with Marvel. Obviously, this did not effect the efficacy of the specific antisera binding, so it was decided to use Marvel in all subsequent experiments.

To ascertain whether or not the secondary antibody was binding non-specifically to epitopes, an experiment was carried out whereby filters were treated exactly as usual apart from the fact that no antisera were added at the primary incubation. After incubation in the secondary antibody at various different dilutions, the filters were washed and developed as usual. At a 1/1500 dilution of the secondary antibody, the filters were completely clear on development. This demonstrates that, within this system, there was no spurious binding of the secondary antibody.

Before screening the library with antibodies, about 30,000 plaques were screened by hybridisation using a labelled *cut-1* recombinant fragment, 914.1 (the recombinant used to raise NA5). Hybridisation was carried out at 58°c and the filters were washed down to 0.5 X SSC and 0.01% SDS. Several hybridising plaques were picked and taken through to a secondary screen, when all the plaques appeared to hybridise to the probe. These were

clearly false positive results, because isolated plaques had certainly not been picked from the first screen. In fact, when these false positives were induced and the resultant proteins screened with the anti-cuticlin antisera 'cocktail', none of them reacted. There is the possibility that the inserts which reacted were true cuticlin homologues which were not inserted 'in frame' into the vector; consequently they could not be expressed correctly so would not be recognised by the antisera. However this is relatively unlikely, as the vector used is designed to maximise the chances of an insert being 'in frame'. The fact that none of the clones picked expressed a protein which was recognised by the antisera confirmed their diagnosis as false positives. This result confirmed the conclusions drawn from the screening of the *Ascaris* library with a recombinant *cut-2* DNA probe: that the conditions which must be used to permit heterologous hybridisation at the DNA level are not stringent enough to eliminate the non-specific hybridisation of heterologous sequences to the probe. Thus it was decided to continue with the antibody screening.

3.3 LIBRARY SCREENING.

Approximately 100,000 plaques were screened, over several experiments. Fifteen putative positive plaques were picked throughout the screening process. They were picked on the grounds that they stood out clearly against a pale background of staining on the nitrocellulose filter. There did appear to be differential antibody recognition of the putative positives, in that some were definitely more darkly stained than others. It was assumed that this was due to reaction of plaques to different components of the antisera 'cocktail'. Positive plaques were picked and the phage eluted in SM buffer. A clonal phage population of each putative positive was obtained by consecutive screenings, always using the same primary antibody 'cocktail'.

Plate lysates were made of the fifteen putative positives, plus the XL1 Blue bacterial strain carrying untransformed λZapp. *In vivo* excision of the pBluescript phagemids, carrying the putative cuticlin inserts, was then carried out using the f1 helper phage. These phagemids were then used to transform bacterial cells and the resultant transformant strains were used in all further analyses.

3.4 Analysis of the putative positive clones isolated from the library screen.

3.4.1 Western blotting of recombinant proteins.

The fifteen clones which were positive in successive rounds of antibody screening were induced with IPTG and analysed by western blotting with the same cocktail of antiserum used in the original screening. Untransformed cultures were induced in parallel. None of the expressed proteins showed reactivity with the antisera. In retrospect, this could be due to the fact that the excised plasmid was not expressing the recombinant protein as effectively as in the lambda phage, and that this was the explanation for the lack of recognition by the antisera.

At this stage it was not known whether the clones isolated were all recognised by a single component of the anti-cuticlin antiserum 'cocktail', or whether they had reacted with different

components of the cocktail. Additionally, it was thought that the negative results of the western blot may be due to the fact that the antisera cocktail had been re-used. To identify whether the clones picked encoded a CUT-1 or a CUT-2- like protein, and to analyse the recombinant proteins with fresh aliquots of antisera, it was decided to screen them separately, by western blotting, with specific anti-CUT-1 and anti-CUT-2 antisera.

The antisera used in the first round of western analysis were as follows:

- The anti-CUT-1 NA5 antisera, purified by adsorption onto a lysate of the bacterial strain carrying the induced, untransformed pEx34 plasmid, as previously mentioned.
- The anti-CUT-2 antisera, purified by adsorption onto the cold osmotic shock (periplasmic) fraction of an induced lysate of TB1, the bacterial strain carrying untransformed, induced pMalP vector. The recombinant CUT-2 is known to be secreted into the periplasm of the bacteria, so it is the *E. coli* periplasmic proteins which are used to adsorb the non-specific antibody present in the anti-CUT-2 antiserum.

As is shown in Figure 3.2A & C, western blotting of the recombinant proteins with purified anti-CUT-1 and anti-CUT-2 revealed a single band of the same size in all the clones, which was not present in cultures of untransformed λ. However, the size of the band was much smaller than that predicted by the size of either of the *C. elegans* cuticlin proteins.

The recognition of a single component in all induced clones by both anti-CUT-1 and anti-CUT-2 antisera has a number of possible explanations, the most likely being: the antisera are recognising a component of the plasmid/bacterial strain carrying the insert used to immunise the rabbit; or the antisera are recognising a single immunodominant shared epitope highly represented in the cDNA library.

Thus it was decided to further purify the antisera by adsorbing them onto excised, untransformed, induced pBluescript phagemid. These results, shown in Figure 3.2 B & D were again very discouraging, despite the blots being 'cleaner' with respect to the first experiment, due to the further purification of the antisera. Both antisera recognized a component in induced and uninduced cultures, including that of untransfomed λ. In summary, any reactivity seen is not specific for the peptide product of any of the cloned inserts, or indeed for expressed vector proteins.

3.4.2 DNA analysis of the putative positives.

3.4.2.1 Which of the clones have inserts, and what is their size?

Despite the disappointing results of the antibody screen, it seemed worthwhile to proceed with a preliminary analysis of the isolated clones. It seemed prudent to ascertain the presence of an insert in each of the clones before proceeding further. To this end, the insert of each clone was amplified using 25mer T3 and T7 primers, the sequences of which are present in the pBluescript polylinker, flanking the insert. A high annealing temperature of 65°c was used in the PCR amplification reactions. In this way it was shown that nine of the fifteen clones originally picked had inserts, ranging in size between 0.75 and 2.5 Kb. Table 3.1 shows the

sizes of the inserts of each clone, established by amplification of DNA using T3 and T7 primers.

CLONE	INSERT SIZE (BP)
2	2400
4/6	1800
3/12	1261
8	550
9	900
13	1800
14	1640

3.4.2.2 Do the clones have a cuticlin identity by DNA hybridisation?

To determine whether or not any of the cloned fragments had a cuticlin identity, amplification products from the above PCR reactions were gel-purified and digested with *Tac*1 (a two-base cutter which cuts frequently and therefore was likely to cut all the clones), run on two identical 2% gels and blotted. Each of the gels was then probed with either recombinant *cut-1* or *cut-2*, labelled with ^{32}P. Hybridisation conditions were moderate (65°c, washed down to 0.5 X SSC, 0.1% SDS) and the blots were exposed for three days. Despite these permissive conditions, there was no hybridisation to any of the clones with either of the recombinant cuticlin probes.

3.4.2.3 Restriction enzyme analysis of the cloned inserts.

The nine clones were cut with twelve different restriction enzymes. These data were used to determine whether any of the clones were apparently identical or similar to one another, and to establish whether the clones have restriction sites which can be used for sub-cloning to generate fragments which can be easily sequenced. Single restriction enzyme analysis strongly suggested that cloned inserts 3 and 12 were the same, and that clones 4 and 6 were the same. This is shown in Figure 3.3.

Further restriction analysis, including double digests of the clones, enabled the determination of the size of the restriction fragments and where, in relation to one another, they are positioned in the clone. This information was used to construct restriction maps of the clones, and revealed that cloned inserts 4 and 6 were identical, and were in fact a truncated version of clone 3/12. Figure 3.4 is a restriction map of the combined clones.

3.4.2.4 Sub-cloning and sequencing the pBluescript inserts.

Sub-cloning of the inserts facilitate not only their sequencing, but also any future work on the expression of the clones, for the purposes of further characterisation and the raising of antisera.

Clone ELBP3

This clone represents a gene which was isolated four times during the screening process: it is clone 3/12 (see above) and incorporates 4/6. The sub-cloning strategy used for ELBP3 is shown in Figure 3.4.

The full 1261bp of ELBP3 was sequenced and the appropriate coding frame established. The clone has a polyA tail, showing that it has a complete 3' end. A BLAST search of the *C. elegans* data base ACeDB resulted in the identification of a region of high homology to ELBP3 in the randomly generated cDNA clone CEcm12g11, which physically maps to linkage group III of the *C. elegans* genome. At the time, nothing was known about the nature of the *C. elegans* homologue, so it did not yield any information about a potential role for the product of ELBP3 in *Brugia*, or a possible explanation of why it may have been recognised by antisera raised against cuticlin genes.

Subsequently, however, the region of CEcm12g11 shown to be homologous to ELBP3 was sequenced by the *C. elegans* genome sequencing project and identified as an RNA helicase, known as f01f1.7. Figure 3.6 is a comparison of the predicted amino acid sequences of ELBP3 and f01f1.7. The coding sequence of f01f1.7 initiates 330bp before the start of the ELBP3 clone. This knowledge, combined with the fact that there is no methionine residue preceded by a stop codon at or near the 5' end of the clone, demonstrates that ELBP3 almost certainly does not represent the full-length cDNA of the *Brugia* RNA helicase homologue. The predicted amino acids of the two clones show a 62% homology over 185 residues. In this conserved region of the two predicted peptides, differences in the sequence are generally due to the substitution of one amino acid by another which shows very similar properties in terms of charge, hydrophilicity and acidic or basic nature. For example valine and isoleucine, argenine and lysine, and aspartic and glutamic acid, often substitute for each other in the two proteins. Thus the peptides coded for by the two sequences will show greater functional similarity then that which is indicated simply by their amino acid sequence identity. Further analysis of f01f1.7 identifies it as a DEAD-box RNA helicase, carrying the distinctive amino acid motif ILDEADRML, which is the signature of the ATP-dependent helicase sub-family. It also has an ATP-GTP binding site. Interestingly, the stretch of sequence containing the DEAD box motif is not present in ELBP3. The homology runs for about 185 amino acids, at which point there is a stretch of about 160 amino acids (containing the DEAD box motif and the ATP-GTP binding sites) which is deleted from ELBP3, followed by a stretch of homology running for a further 20 amino acid residues. After this point, which is half way through the sequence of ELBP3, there is no further homology to f01f1.7. However, a BLAST search of peptide data bases using only the latter part of the clone showed character homologies with RNA helicases

from *Arabidopsis thaliana, Dictyostelium discoidem*, *Saccharomyces cerevisiae* and a second *C. elegans* gene. The yeast clone is specified as being a pre-mRNA splicing factor RNA helicase, whereas the second *C. elegans* clone does not appear to be an ATP-dependent DEAD box helicase.

ELBP13 was fully sequenced (see Figure 3.5 for the restriction map and sub-cloning strategy used for clone 13) and the sequence compared with various data bases. The clone showed no meaningful homology at the DNA or amino acid level to any sequence in the data bases.

The remainder of the clones were characterised less comprehensively using restriction analysis and incomplete sequence data. Simple maps of the clones are shown in Figure 3.5 to show that they appear to be different from each other. Clones 8 and 9 were not sub-cloned, but were sequenced from their 3' and 5' ends. None of the clones showed any homology to either of the *C. elegans* cuticlin genes. When compared to sequence data bases, the sequences did not show homology to any other gene present in the data bases.

3.5 Raising antisera against ELBP3.

The identity of CEcm12g11 as the RNA helicase gene f01f1.7 was not known when ELBP3 was being characterised. Consequently there was no way of predicting the nature or function of the protein encoded by ELBP3. It was thought that localisation of the protein in the worm would help to determine whether or not it appeared to be a component of the cuticle. Therefore it was decided to synthesise a peptide coded for by part of the ELBP3 predicted amino acid sequence, and to raise antisera against the peptide by immunisation of a rabbit. Experiments using the resulting antiserum on *Brugia* worms should result in recognition by the antiserum to the region of the worm where the protein localises (provided, of course, that the epitope/s recognised by the antiserum are not lost due to post-translational protein processing, or masking by tertiary structural modification).

A peptide of twenty amino acids coded by the DNA sequence of ELBP3 was commercially synthesised (Genosys) and used in the raising of the antiserum. The peptide was conjugated to KLH and used to immunise a rabbit, as described in Materials and Methods. The sequence was chosen on the grounds that it covered a region showing high homology to CEcm12g11, and that the computer protein prediction was for a hydrophilic, and therefore antigenic peptide, suited for the production of an antiserum. Figure 3.8 shows the amino acid sequence of ELBP3 with the sequence of the peptide marked on it.

Immunofluorescence antibody testing was carried out using the antisera on sections of adult *Brugia pahangi.* As a negative control, the experiments were also carried out in parallel using pre-immunisation rabbit sera.

Unfortunately, the reactivity on the worms treated with the anti-ELBP3 antisera appeared to be exactly the same as the reactivity with the sera from the non-immune rabbit, despite the fact that the experiment was carried out on several different occasions, using worm sections fixed at different times. It was concluded from these localisation experiments that the protein product of ELPBP3 is not a component of the *Brugia* cuticle, nor does it appear to localise

internally in the adult worm. However, as can be seen in 3.7, when western blots of *Brugia* adult and mf protein extracts were reacted with the anti-ELBP3 antiserum and the pre-immunisation control rabbit serum, the same bands were recognised, implying that the antiserum is not recognising a *Brugia* protein at all. The most likely explanation for these results is that the rabbit did not recognise the peptide, and therefore the immunisation was not successful.

3.6 DISCUSSION.

This chapter summarises initial attempts to obtain a *Brugia* cuticlin homologue using specific anti-cuticlin antibodies to screen an adult *B. pahangi* cDNA library. Although a number of putative positive clones were isolated by this method, none of these shared a homology with either of the cuticlin sequences. In retrospect, it could be argued that it was an error to continue with the characterisation of these clones, having established (by DNA hybridisation and antisera recognition) that they did not have a cuticlin identity. However, in the early stages of sub-cloning and sequencing, it could be seen that four independently-selected clones corresponded to the same DNA sequence: two identical clones representing a truncated version of a longer identical pair. In the case of the longer clone, it was selected twice from separate screening experiments, while the shorter clones originated from different plates within the same experiment. Hence it is possible to say that the antisera independently recognised an epitope present in all four clones, which would strongly imply that recognition was specific to the recombinant polypeptide expressed by the plaque, and not co-incidental.

Once the clone had been sequenced and the homology to the *C. elegans* randomly generated cDNA had been established, the clone seemed even more interesting. The extent of the homologies, at the DNA and amino acid levels, between the homologues from different nematodes, is significant in itself, if only because it demonstrates evolutionary retention of a gene throughout a phylum, which could signify its fundamental importance to the *Nematoda.*

For these reasons it was decided to continue with the characterisation of ELBP3. It was hoped to at least ascertain, by further sequencing, why the anti-cuticlin antisera recognised the clone. There was clearly no homology to *cut-1* or *cut-2* at the DNA level, but it was thought that with a more comprehensive knowledge of the amino acid content of the clone, it might be possible to detect a motif or sequence that is suggestive of a tertiary configuration, or interaction, which may have been recognised by the anti-cuticlin antisera. Such elucidation was not forthcoming, and the subsequent raising of antisera and experiments did not contribute anything further to our understanding of the nature of ELBP3.

The knowledge that the clone was in fact the *Brugia* homologue of an RNA helicase was interesting, but the issue of why the antisera recognised it with such apparent specificity remained unresolved. However, there does appear to be one meaningful explanation for the recognition of the *Brugia* helicase homologue by the anti-cuticlin antisera.

In 1983, Strome and Wood, working on the development of *C. elegans*, published a paper on the generation of asymmetry and segregation of germ-line granules in *C. elegans* embryos

(Strome and Wood, 1983). In the paper they report the use of an antibody raised against these germ-line granules (known as P-granules) as a marker for the mechanisms of cytoplasmic determination of an asymmetrical cell lineage. P-granules are cytoplasmic granules which segregate in early development to germ-line cells, up to gametogenesis when they are excluded from the sperm and become dispersed in the cytoplasm of mature oocytes. Later still they have a cytoplasmic localisation in the embryo (Strome and Wood, 1982). It is not known how, or even if, these cytoplasmic granules play a determinative role in germ-line development in *C. elegans*, but they serve as a marker for the mechanisms involved. The identity of the components of the P-granules in *C. elegans* remained completely unknown, although genes required for P-granule assembly in *Drosophila* had previously been identified; all of them code for novel proteins apart from one, named *vasa*, which codes for an ATP-dependent RNA helicase (Hay et al, 1988).

There may be a significance of these discoveries to ELBP3: Strome et al noted that sera from non-immune rabbits stain P-granules in the germ line at all embryonic stages in *C. elegans,* as well as reacting with non-gonadal tissues in later-stage larvae and adults. It was assumed from this that the antisera contained anti-P-granule antibodies, produced in response to a previous, naturally occurring nematode infection, and that P-granules were in fact very immunogenic. It is possible that it is the helicase component of the P-granules which is immunogenic, and recognised by the non-immune antisera. Hence there is a rational explanation, and a possible precedent, for the specific recognition of ELBP3: i.e. one or more of the 'anti-cuticlin' antisera contained antibodies against a germ-line specific helicase of the type which is present in nematode P-granules, raised in the experimental rabbit against antigen from a previous naturally occurring nematode infection.

Basically, the role of ATP-dependent RNA helicases is to bind and unwind mRNA, and to bind them to ribosomes in preparation for translation (Ray et al, 1985). In *Drosophila*, the *vasa* product has been demonstrated to be maternally required in the formation of polar-granule and germ cells (Mahowald, 1968). So, in *Drosophila*, it is possible that RNA helicases have a role in the translation of maternal RNA in the germ-line development of the embryo. The *C. elegans* homologue of *vasa* has since been cloned and identified as an ATP-dependent germ-line RNA helicase named *glh-1* (Roussel et al, 1993). The gene for a second helicase component of the P-granules, *glh-2*, was identified, cloned (Gruidl et al, 1996) and shown to have high homology to *glh-1*. They are both DEAD-box helicases containing zinc fingers of the type known to bind RNA, and glycine-rich N-terminal repeats which are a characteristic of likely RNA-binding proteins (Hay et al, 1988).

Experiments on *C. elegans* demonstrate that anti-GLH-1 and anti-GLH-2 monoclonal antibodies stain the same granules recognised by anti-P-granule monoclonal antibodies (Gruidl et el, 1996), supporting the theory that the helicase component of the P-granule is the immunogenic component identified by Strome (Strome & Wood, 1982), and hence that recognition of ELBP3 could have been by antibodies raised against a previous nematode

infection. Interestingly, f01f1.7 has both the DEAD-box motif and an ATP-binding site, although otherwise it does not show a particularly high homology to *glh-1* or *glh-2*, which map very close to one another on chromosome I of the *C. elegans* genome. The *C. elegans* sequencing project has identified a third potential RNA helicase which maps to the same region, nowhere near the location of f01f1.7. ELBP3, despite its high homology to f01f1.7, shows a deletion of about 160 amino acids over the region containing both the DEAD-box motif and the ATP-binding sequence. Hence there is no conclusive evidence to imply either that f01f1.7 codes for a helicase component of the P-granules, or indeed that ELBP3 would be recognised by antibodies raised against such a protein. However, it could be that immunogenicity is a feature of RNA helicases in general, not just those of the ATP-dependent DEAD-box sub-family. The role of RNA helicases in translation means that they must be abundant in the early embryonic development of nematodes, when maternally-derived proteins are being maximally synthesised; a nematode infection of the types common in rabbits would almost certainly result in the animal being exposed to significant amounts of embryonic antigen, as they are shed in enormous numbers to facilitate effective transmission of the parasite. Thus the component of the 'anti-cuticlin' antisera which recognised ELBP3 would not necessarily have to be raised against a DEAD-box RNA helicase present only in germ-line cells, it could well have a more ubiquitous distribution and function in the parasitic nematode cells. Perhaps more significantly, the ubiquity of the helicases might mean that they are highly represented in the adult cDNA library, enhancing the probability of recognition by the antisera during a screening of the library. The presence of several helicase sequences in the Brugia Genome Sequencing Project, which consists of randomly sequenced ESTs, is evidence for this theory.

It is most unfortunate that the antisera raised against the peptide coded for by ELBP3 did not reliably recognise a band on a western blot of Brugia proteins; if it were not for this fact, the identical staining of *Brugia* with non-immune and anti-ELBP antisera could readily be explained by the presence, in the non-immune sera, of antibodies recognising the same epitopes, raised against a helicase component of the P-granules from a previous nematode infection. However, the likely explanation of these results is that the rabbit failed to make antibody against the immunogen and therefore the 'immune' anti-serum did not contain specific antibodies. The fact that the pre-immune anti-serum also recognised similar bands on western blots of *Brugia* protein extracts, and similar structures on fixed worm sections, probably relates to the fact that the 'immune' antiserum was used at high concentrations, in an attempt to gain a positive signal.

To address the function of GLH-1 and GLH-2 in the *C. elegans* germ-line, antisense RNA made against *glh-1* and *glh-2* was micro-injected into the gonads of wild-type worms (Gruidl et al, 1996). Antisense inhibition has been used for some years to confirm the cloning and identity of genes in *C. elegans*: injection into wild-type hermaphrodite gonads of constructs carrying DNA which is antisense to the gene of interest, will result in off-spring showing the

same phenotype as the deletion mutant (Fire et al, 1991). Presumably the antisense DNA, once incorporated into the extra-chromosomal array of the f1 generation, will code for an RNA antisense to the mRNA of the gene, hybridising to it and hence preventing its transcription. A more direct method of inhibition analysis was developed for genes which are predicted to be expressed in the early embryo, from maternal mRNAs (Guo et al, 1995): antisense RNA is injected directly into the gonads of wild-type worms, resulting in sterile off-spring if the injected RNA corresponds to a gene whose product is essential for germ-line development. Injecting both *glh-1* and *glh-2* antisense RNA into wild-type worms resulted in sterile off-spring which failed to stain with monoclonal antibodies raised against GLH-1, GLH-2 and four other unknown P-granule epitopes. This could imply that the germ-line helicases are essential for the assembly of the P-granules in *C. elegans.* Injection of antisense RNA produced against the gene-specific 5' and 3' ends of *glh-1* and *glh-2* did not result in sterile off-spring. The authors attribute this result either to inefficient antisense inhibition by the shorter RNAs, or, more interestingly, to the possibility that GLH-1 and GLH-2 may functionally compensate for one another.

Clearly it would be very interesting to obtain f01f1.7, make antisense RNA to the gene, and inject it into *C.elegans* wild-type worms to see if the off-spring were sterile. This would strongly imply a germ-line-specific function for the gene product. If an antibody raised against the gene localized to the P-granules in developing embryos, it would be possible to say that the gene was another member of the *C. elegans* germ-line helicase family, related more distantly to *glh-1*, 2 and 3. And by this inference it could be assumed that, in all possibility, ELBP3 was the *Brugia* homologue of this gene, perhaps coding for a product that fulfilled the same function in the parasitic nematode. The most conclusive evidence for ELBP3 having an RNA helicase, P-granule component identity, would be if injecting antisense RNA of ELBP3 into wild-type *C. elegans* resulted in off-spring sterility.

The conclusions of this chapter are by definition unsatisfactory: the only scientific explanation of why ELBP3 was recognised by the anti-cuticlin antisera is unsupported by experimental evidence, which would take a considerable amount of time to obtain. At the same time the identity of the clone is very interesting and quite possibly worthy of further study. I have attempted to acknowledge the potential of the clone, and to some extent outline the various paths that could be taken to confirm the identity and function of ELBP3, without having done more than the most preliminary characterisation.

The project was concerned with obtaining a *Brugia* cuticlin gene homologue, and the results of this chapter show that, using the reagents available at the time, screening an adult *Brugia* cDNA library with antisera raised against recombinant CUT-1 and CUT-2 was not a suitable method for identifying such a clone.

Figure 3.1

The recombinant proteins used to raise the anti-CUT-1 and anti-CUT-2 antibodies used to screen the *B. pahangi* adult cDNA library.

MTWKPIICLAALVLSASAIPVDNNVEGEPEVECGPNSITVNFNTRNPFEG 50

HVYVKGLYDQAGCRSDEGGRQVAGIELPFDSCNTARTRSLNPKGVFVSTT 100

VVISFHPQFVTKVDRAYRIQCFYMESDKTVSTQIEVSDLTTAFQTQVVPM 150

PVCKYEILDGGPSGQPIQFATIGQQVYHKWTCDSETTDTFCAVVHSCTVD 200

DGNGDTVQILNEEGCALDKFLLNNLEYPTDLMAGQEAHVYKYADRSQLFY 250

QCQISITIKDPGSECARPTCSEPQGFGAVKQAGAGGAHAAAAPQAGVEEV 300

QAAPVGAAPVAAPVAAAAAAPAVPRATLAQLRLLRKKRSFGENEG**ILDVR** 350

VEINTLDIMEGASPSAPEAAALVSEESVRRRATSTGISSTPIGLPSFLGM 400

RTIVATALSATIFYVARPTSHKHZ 450

Fig. 3.1a The amino acid sequence of the *C.elegans* CUT-1 protein (Sebastiano et al, 1991). The anti-serum NA5 was raised against a recombinant protein 914.1, which is underlined. The antiserum F3 was raised against recombinant 912.1, which is double underlined. The two recombinants over-lap for 12 amino acids; this region is marked in bold. The recombinants incorporate the amino acid homology with the predicted *B. pahangi* CUT-1-like protein.

MQKLIVFFTTIAAAQAFLLPSGGGGGCGCAPPPPPPPCGCGAPALPPLQL 50

PRFELPRLSLPSLGGGCGGPAPCAAAPIAAPAGGYATAPAAPVGGYATGP 100

AFGGAAPIGGAYQAAPAFVGAAPVGGAYQSGPAFGGAAPAGGAYQSGPAF 150

GGAAPAGGAYQSGPAFGGAAPAVGGAYQAGQAAVESAPLGGAAPAGGYQA 200

SAPAAVEAAPAAGGYQAAAPAGGAYAGHKKNZ 250

Figure 3.1b The coding sequence of the *C. elegans* CUT-2 gene (Lassandro et al, 1994). The anti-CUT-2 antiserum used was raised against the recombinant protein underlined in the sequence.

Figure 3.2

Western blots of the recombinant proteins encoded by the putative positive clones isolated in the antibody screen of the adult *B. pahangi* cDNA library, probed with anti-cuticlin antisera in order to establish a cuticlin identity for the cloned inserts, and to determine whether they had a CUT-1 or CUT-2 identity.

Expression of the cloned inserts was induced with IPTG, after which 100µl of each culture was spun down, the supernatant discarded and the pellet resuspended in 40µl of loading buffer. The samples were boiled and 10µl of each loaded onto 12.5% acrylamide mini gels. Untransformed λ was used as a control and, in the case of panels **B** & **D**, the protein extract from each uninduced clone was run next to the expressed extract. The gels were blotted onto nitrocellulose and then reacted with either anti-CUT-1 or Anti-CUT-2 antiserum, diluted 1/100 in 5% Marvel. Full experimental details are to be found in the Materials and Methods chapter.

In panels **A** & **C** the blots were reacted with antisera which had been affinity purified by adsorption onto a lysate of the bacterial strain carrying the untransformed vector used to clone the CUT-1 or CUT-2 recombinant protein. Panel **A** was reacted with anti-CUT-1 antiserum and Panel **C** was reacted with anti-CUT-2 antiserum. All the clones showed reaction to a component which was not present in the untransformed λ. As can be seen in panels **A** & **C**, the recognized component (indicated by an arrow) is the same size for both antisera, and is much smaller than predicted by the sizes of *C.elegans* CUT-1 and 2 (26KDa for CUT-2 and 40KDa for CUT-1).

In panels **B** & **D** the anti-CUT-1 and 2 antisera as described above were further purified by adsorption onto excised, untransformed, induced pBluescript phagemid. Panel **B** was reacted with anti-CUT-1 and panel **D** with anti-CUT-2. There was no difference in recognition of the protein extracts between induced and uninduced cultures.

A and C

lane 1	clone 14
lane 2	clone 13
lane 3	clone 12
lane 4	clone 9
lane 5	clone 8
lane 6	clone 6
lane 7	clone 4
lane 8	clone 3
lane 9	untransformed λ

B and D

lanes 1 & 2	uninduced & induced clone 3
lanes 3 & 4	uninduced & induced clone 6
lanes 5 & 6	uninduced & induced clone 13
lanes 7 & 8	uninduced & induced λ
lanes 9 &10	uninduced & induced clone 14
lanes 11 & 12	uninduced & induced clone 2

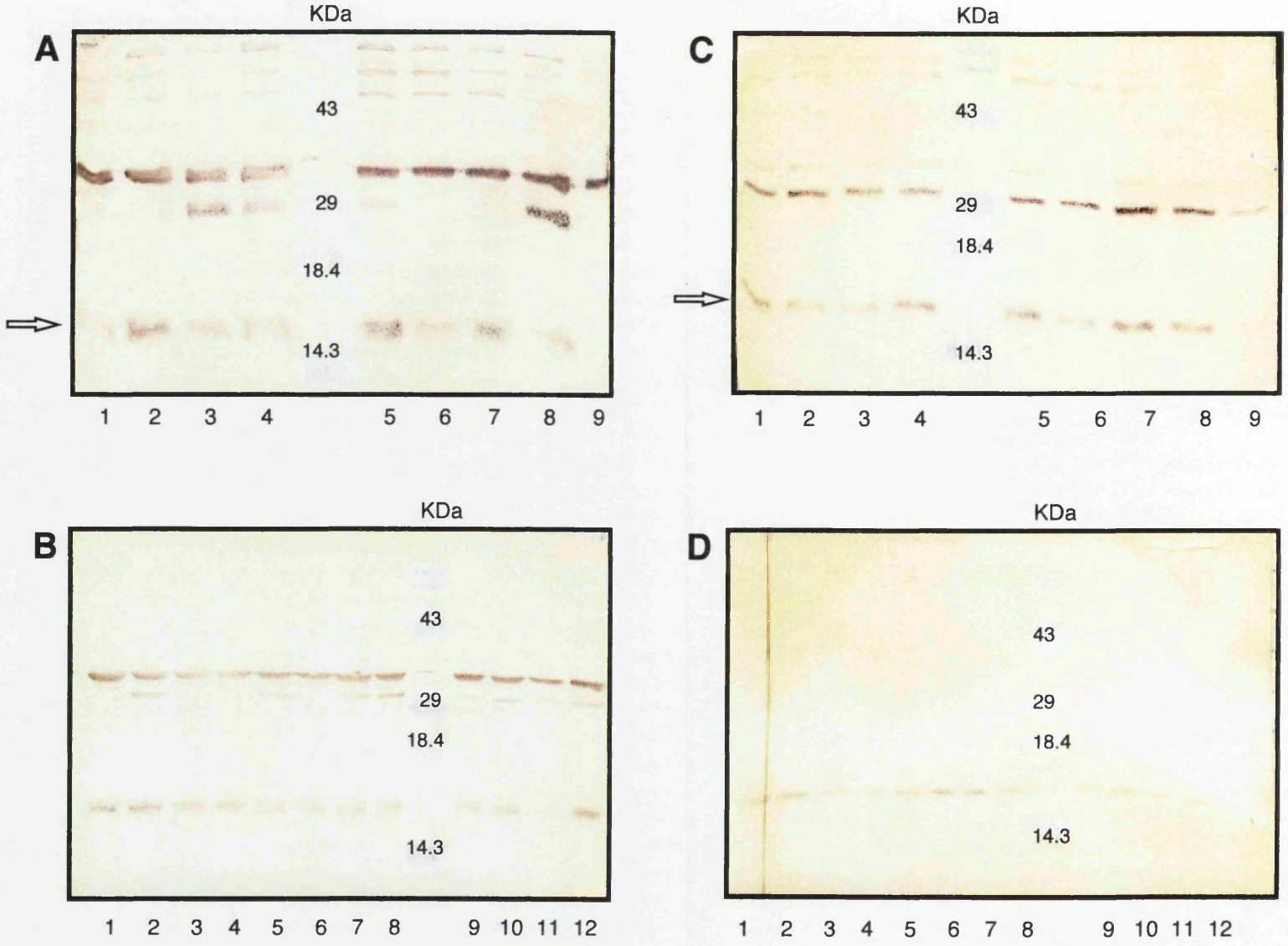

A
KDa
43
29
18.4
14.3
1 2 3 4 5 6 7 8 9
C
KDa
43
29
18.4
14.3
1 2 3 4 5 6 7 8 9
B
KDa
43
29
18.4
14.3
1 2 3 4 5 6 7 8 9 10 11 12
D
KDa
43
29
18.4
14.3
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.2

Figure 3.3

Figure showing, by restriction enzyme analysis of DNA from the clones isolated during the antibody screening of the *B. pahangi* adult cDNA library, that the inserts present in clones 4 & 6 are identical; that the inserts present in clones 3 & 12 are identical, and that insert 3/12 is different from insert 4/6.

In each case 10µg of plasmid DNA was digested with the appropriate enzyme for at least two hours. The digests were cleaned by ethanol precipitation and the products were run on 1.5% agarose gels.

Panel A.

lane	clone	enzyme
1	4	*Sac*I
2	6	*Sac*I
3	4	*Tac*I
4	6	*Tac*I

n.b. the un-marked lanes contain cleaved DNA from clone 5, which appeared to have lost its insert. This figure shows that clones 4 and 6 have the same restriction pattern and therefore carry the same insert.

Panel B.

lane	clone	enzyme
1	3	*Sac*I
2	12	*Sac*I
3	3	*Tac*I
4	12	*Tac*I
5	3	*Xba*I
6	12	*Xba*I
7	3	*Hind*III
8	12	*Hind*III

This figure shows that clones 3 and 12 have the same restriction pattern and therefore carry the same insert.

Panel C shows DNA from clone 4/6 and **Panel D** shows DNA from clone 3/12, digested with the following enzymes:

lane	enzyme
1	*Pvu*II
2	*Eco*RV
3	*Kpn*I
5	*Sal*I
6	*Pst*I
7	undigested plasmid

These figures show that the restriction patterns for clones 3/12 and 4/6 are different, and therefore carry dfferent inserts.

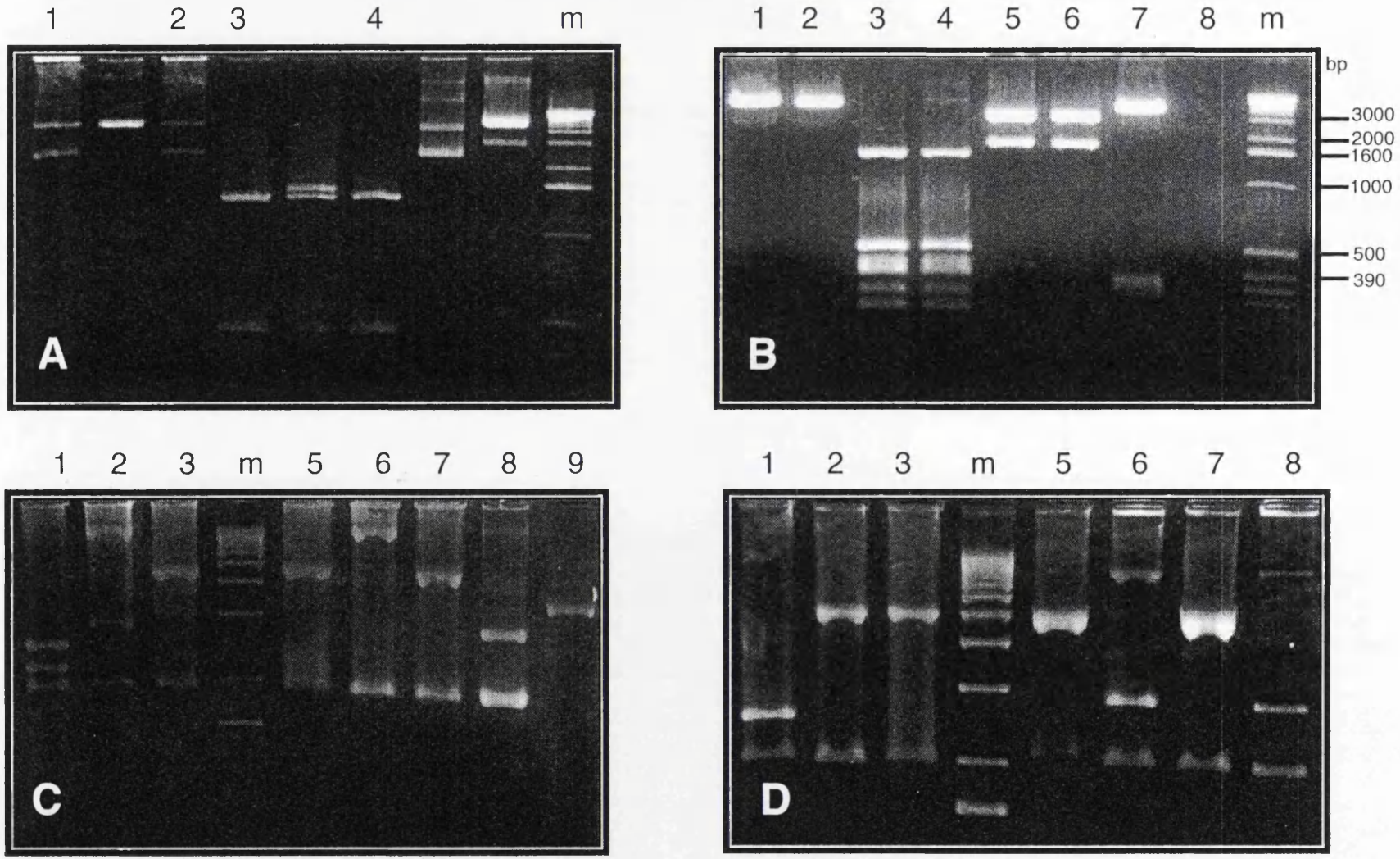

1
2
3
4
m
A
1
2
3
4
5
6
7
8
m
bp
3000
2000
1600
1000
500
390
B
1
2
3
m
5
6
7
8
9
C
1
2
3
m
5
6
7
8
D

Figure 3.3

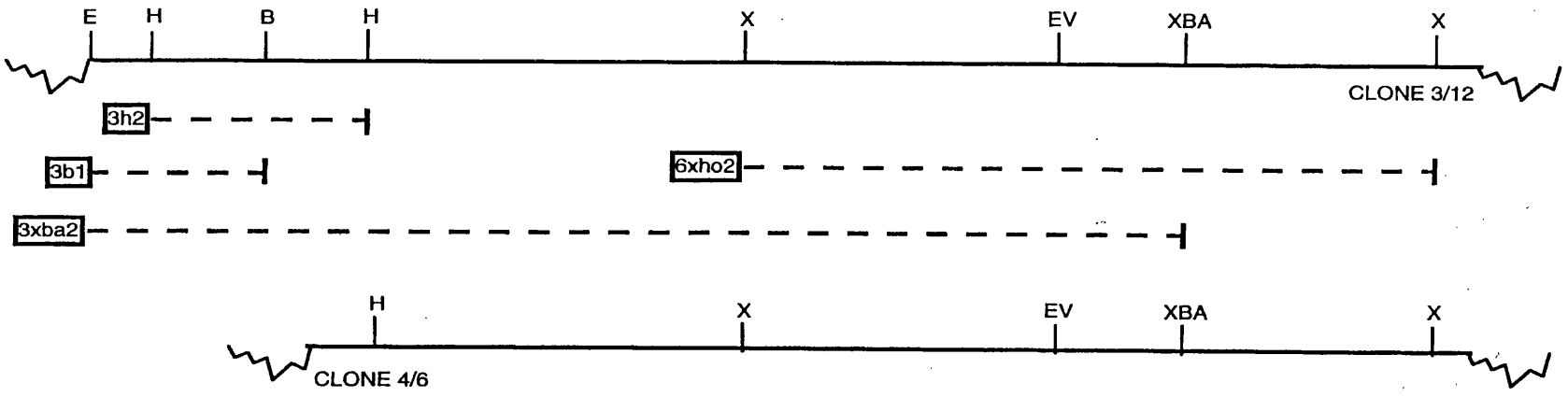
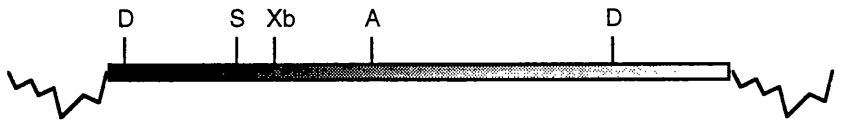


Figure 3.4 Restriction map of ELBP3, which incorporates the inserts from clones 4, 6, 3, & 12. The dotted lines show restriction fragments which were sub-cloned into pUC18, and the remaining sequence re-ligated onto itself.

H	*Hind*III	**X**	*Xho*I	zig-zag line = pBluescript polylinker.
E	*Eco*RI	**XBA**	*Xba*I	
B	*Bam*HI	**EV**	*Eco*RV	—— = 100bp

CLONE 13

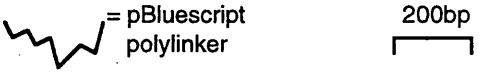


CLONE14

CLONE 2

FIGURE 3.5 Restriction maps of clones 13, 2 and 14, showing that the inserts are all different. Clones 8 and 9 were sequenced from either end of the insert to determine that they were different. Clone 13 - the shaded boxes represent restriction fragments which were sub-cloned into pUC18, and the remaining sequence re-ligated onto itself.

E	*Eco*R1
S	*Sac*I
D	*Dra*I
X	*Xho*I
A	*Acc*
Xb	*Xba*



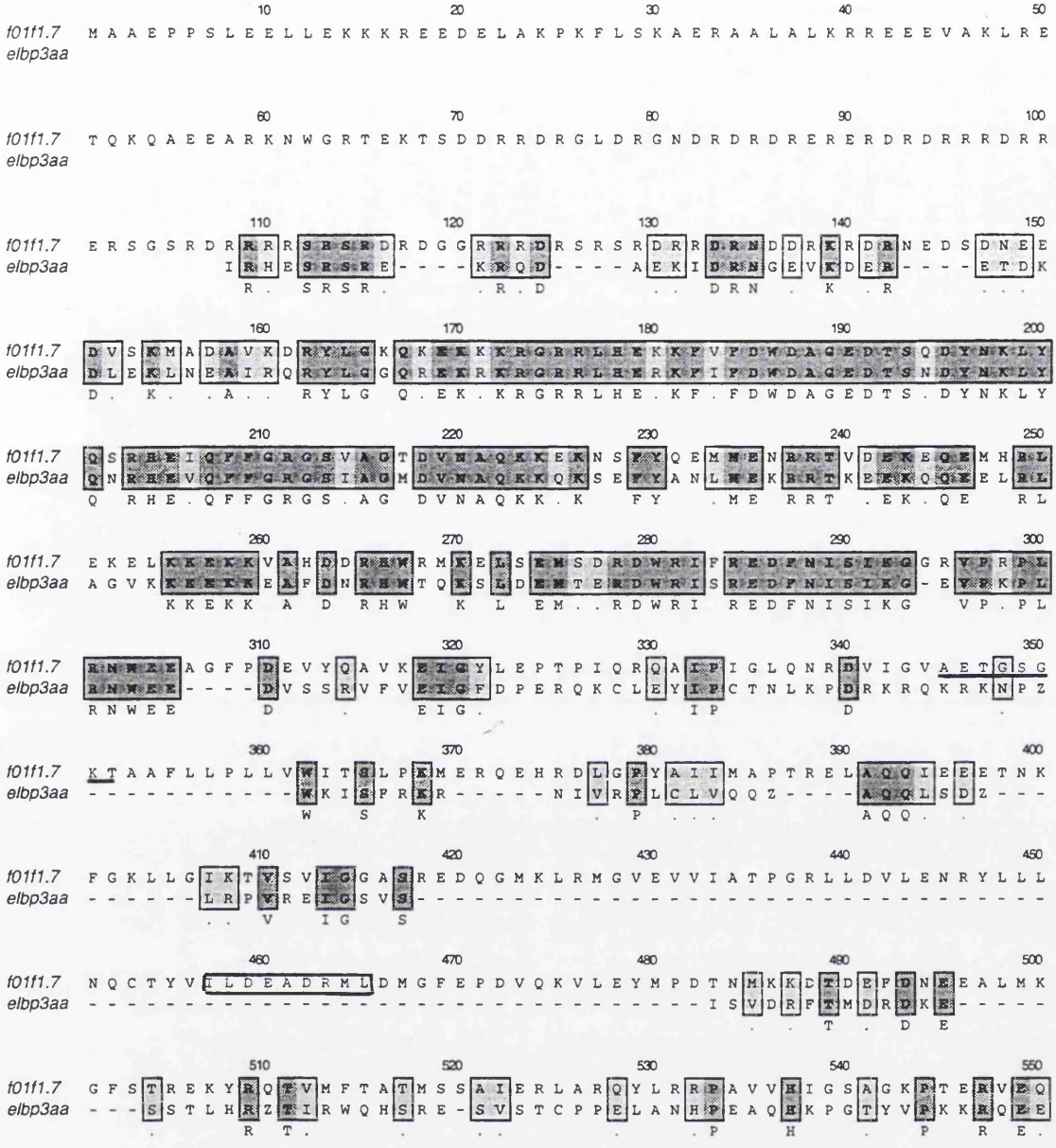


Figure 3.6

ClustalW formatted alignment of the region of amino acid homolgy between f01f1.7, the *C.elegans* DEAD-box ATP-binding RNA helicase, and ELPB3. The program adjusts the sequences to maximise alignment. Darker shading shows complete homology whilst lighter shading represents conservative amino acid substitutions. The ATP/GTP-binding site present in f01f1.7 is underlined at position 350, whilst the defining DEAD-box RNA helicase motif at position 460 is boxed. As can be seen, neither are present in the amino acid sequence of ELBP3.

Figure 3.7

WESTERN BLOT ANALYSIS OF *B. pahangi* ADULT AND MICROFILARIAL ANTIGEN WITH ANTI-ELBP3.

B. pahangi adult and mf protein extracts were run on 12.5% minigels, and immunoblotted onto nitrocellulose paper. The blots were incubated with various dilutions of anti-ELBP3 or pre-immune rabbit sera. The second antibody was goat anti-rabbit alkaline phosphatase conjugate diluted 1/6000. BCIP/NBT substrate was used to develop the blot.

Panel A - *B. pahangi* mf protein extract reacted with:

lane 1	anti-ELBP3	1/100
lane 2	anti-ELBP3	1/200
lane 3	anti-ELBP3	1/400
lane 4	pre-immune serum	1/100
lane 5	pre-immune serum	1/200
lane 6	pre-immune serum	1/400

Panel B - *B. pahangi* adult protein extract reacted with:

lane 1	anti-ELBP3	1/100
lane 2	anti-ELBP3	1/200
lane 3	anti-ELBP3	1/400
lane 4	pre-immune serum	1/100
lane 5	pre-immune serum	1/200
lane 6	pre-immune serum	1/400

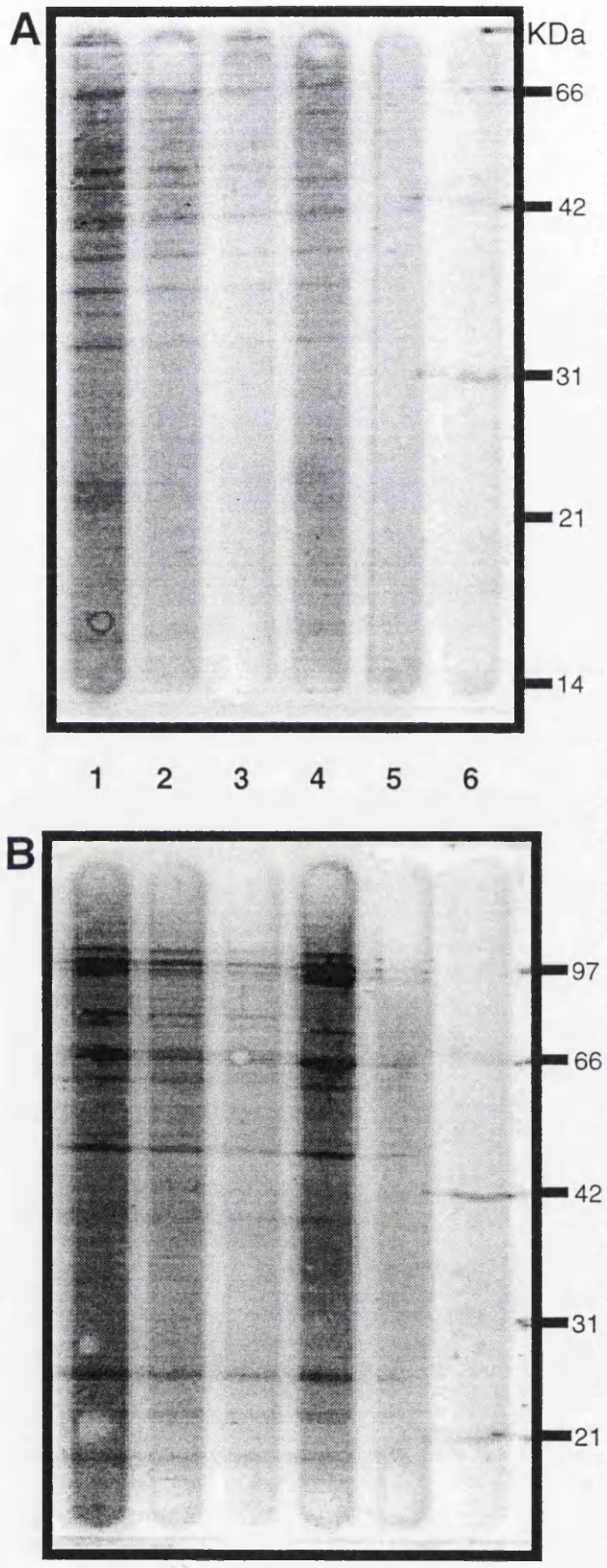


Figure 3.7

IRHESRSREKRQDAEKIDRNGEVKDERETDKDLEKLNEAIRQRYLGGQREKRKRGRRLHE 60

RKFIFDWDAGEDTSNDYNKLYQNRHEVQFFGRGSIAGMDVNAQKKQKSEFYANLMEKRRT 120

KEEKQQEELRLAGVKKKEKKEAFDNRHWTQKSLDEMTERDWRISREDFNISIKGEVPKPL 180

RNWEEDVSSRVFVEIGFDPERQKCLEYIPCTNLKPDRKRQKRKNPZWKISFRKRNIVRPL 240

CLVQQZAQQLSDZLRPVREIGSVSISVDRFTMDRDKESSTLHRZTIRWQHSRESVSTCPP 320

ELANHPEAQHKPGTYVPKKRQEETLFRNZSCSLPIICFSLFFFFFITLLZVMFVLSLFIL 380

FLNFIRLIRIDRZIYVYVRZQYIVIEHVYIDLKNGIIZIRKYHIFFZSZAZZNZFSKKKK 440

KNS 443

FIGURE 3.8

Amino acid sequence of ELPB3, with the peptide used to raise the antiserum anti-ELBP3 marked by double underlining. The peptide corresponds to the region which is most conserved between ELBP3 and the *C. elegans* RNA helicase gene f01f1.7. It also has a predicted hydrophilic character, so should be antigenic, and therefore suitable for raising an antiserum.

CHAPTER FOUR: ISOLATING PART OF A *BRUGIA CUT-1* HOMOLOGUE.

4.1. Introduction.

As has previously been discussed in the Introduction, this project began with the knowledge of two cuticlin genes of *C. elegans*, *cut-1* & *cut-2* , one *cut-1*-like gene of *Meloidogyne artiella*, and one *cut-1*-like gene of *Ascaris lumbricoides*. The extent of the homology between the genes of the three nematodes, one free-living, one parasitic on plants and one an animal parasite, implied that there may also be cuticlin homologues present in the genomes of parasitic nematodes of man and other animals of economic importance.

The initial experiments of the project, out-lined below, were carried out in order to to establish the presence of cuticlin-like genes in the *Brugia* genome, subsequent to screening an adult *Brugia* expression library with antisera raised against recombinant fragments of the *C. elegans cut-1* and *cut-2* genes. The antibody screening is the subject of Chapter Three, and did not result in the discovery of a *Brugia* cuticlin gene. The aim of the later experiments in this chapter was to use newly discovered information on the cloned *C. elegans* and *Ascaris* cuticlin genes to isolate and clone a *Brugia*-specific cuticlin gene fragment.

4.2. Confirming the presence of cuticlin-like genes in the genomes of *Brugia* and other nematodes.

Initially, fragments of recombinant *C. elegans cut-1* and *cut-2* were ^{32}P-labelled and used to hybridise to Southern blots of high molecular weight DNA of *Brugia pahangi.* There was never appreciable hybridisation using a *cut-2* probe, and *cut-1* hybridisation was quite poor, making it difficult to estimate the number and size of the hybridising bands. Subsequent hybridisations were carried out using a labelled fragment of the *Ascaris cut-1* homologue, originating from the region showing high homology to the *C. elegans* and *Meloidogyne* genes. The *Ascaris cut-1* probe hybridises to multiple bands in blotted high molecular weight DNA digests from *Ascaris, C. elegans* and *Brugia pahangi,* as shown in Figure 4.1.The presence of more than one band which apparently hybridise to the probe to varying degrees would imply that all three nematode genomes contain several closely-related but not identical '*cut-1*-like' genes, perhaps constituting a small 'gene family'. The various genes may code for products which are utilized at different times in the life-cycle of the worm, or which are used in different locations in the worm.

It is difficult to explain why the *C. elegans* probe did not hybridise strongly to bands in the *Brugia* genomic DNA: the probe sequence shows high homology to what was subsequently discovered to be the *Brugia cut-1* sequence in the same region of the gene (see Chapter Five). At the time it was assumed that the evolutionary position of *Ascaris* was mid-way between that of *C. elegans* and *Brugia*, in that *C. elegans* is a free-living soil dweller, *Ascaris* is parasitic but spends some of the life-cycle outside the host, and *Brugia* development is entirely parasitic between two hosts, one mammalian and one insect. It would therefore be

reasonable to suppose that the sequence homology of the cuticlin genes of *Ascaris* would be mid-way between *Brugia* and *C. elegans*, and that hybridisation would hence be stronger between *Ascaris* and *Brugia* material. It was with this logic that it was decided to use a labelled *Ascaris cut-1* fragment as the probe in further experiments to find the *Brugia* cuticlin gene homologues.

4.3. The Zoo Blot - establishing the presence of *cut-1*-like genes in the genomes of a variety of parasitic nematodes of medical and veterinary importance.

High molecular weight DNA was made from the adult stages of various parasitic nematodes, quantified by absorbance at OD_{260}, and cleaved with two different restriction enzymes, *Eco*RI and *Hind*III. In addition to DNA from *Brugia pahangi*, material from *Toxocara canis, Haemonchus contortus* and *Dictyocaulus viviparus* was used. As can be seen in Figure 4.2, the *Ascaris cut-1* probe hybridised to at least one band in each of the digests, implying the presence of at least one *cut-1*-like gene in all four genomes. The hybridisation conditions were stringent (65°C, washed down to 0.2 X SSC, 0.1% SDS) and further hybridisation experiments in fact revealed the presence of two hybridising bands in *Brugia* digests (see Figure 4.13), but this 'zoo-blot' confirmed that *cut-1* was represented in all the nematodes so far tested.

4.4. Northern blots.

Having established the presence of more than one *cut-1*-like gene in the *Brugia pahangi* genome (see fig 4.13), it was decided to hybridise northern blots of *Brugia* RNA from different stages in the life-cycle with the *Ascaris cut-1* probe in order to ascertain when the gene/s were transcribed, and the size of the transcripts. RNA was made from adult worms and from post-infective L3, L4 and juvenile adult stages. See the legend for Figure 4.3 for details.10μg of total RNA from each life-cycle stage was analysed.

12μg of *Ascaris* adult RNA was also blotted and hybridised in order to provide a positive control for the hybridisation conditions. At the time it was thought that the cloned *Ascaris cut-1*-like gene was transcribed in the adult worm on the grounds that the 3' end of the gene had been obtained via 3' RACE using adult cDNA as the template. However, subsequent RT-PCR experiments show that the mRNA is hardly represented at all in adult cDNA, and that transcription occurs largely in the embryonated egg of the worm, which is the infective stage of the parasite (M. Timinouni, personal communication).

The probe did not hybridise to a band on any of the northern blots.

Figure 4.3 shows ethidium stained gel pictures to demonstrate the amount and quality of *Brugia* RNA used. The *Ascaris* probe did not hybridise to RNA from any of the life-cycle stages tested.

4.5. RT-PCR to obtain a *Brugia*-specific fragment of *cut-1*.

As has already been discussed, screening the adult cDNA library with anti-cuticlin antisera was not successful, so there was no real evidence to suggest that a *Brugia cut-1* gene product was represented in adult mRNA. At that point a *Brugia pahangi* adult genomic library was not available for screening, and northern analysis had not proved sensitive enough to detect *Brugia cut-1* transcription at any of the life-cycle stages tested. At this stage of the project it was imperative to obtain a fragment of a *Brugia cut-1* homologue for several reasons: firstly to confirm that the gene was actually transcribed; secondly to ascertain the point in the life-cycle at which the gene was transcribed so that efforts to obtain a clone could be concentrated on material from the relevent stage; and thirdly to provide a fragment of *Brugia*-specific *cut-1* DNA which could be used with confidence in hybridisation experiments. *Brugia*-specific *cut-1* sequence would also permit the design of primers that could be used in PCR and related amplification techniques.

To this end it was decided to use reverse transcriptase PCR on first strand cDNA made from RNA from *Brugia* worms taken at days 5, 16 and 28 post-infection.

4.5.1. RT-PCR using primers based on the *C. elegans cut-1* sequence.

Figure 4.4 shows the coding sequence of the *C. elegans cut-1* gene, with the primers used in these experiments marked on the sequence.

Initially, *cut-1*-specific primers cut1 and cut1R were used, together and in combination with an oligo (dT) primer and an SL1 primer. The PCR reactions were done for thirty cycles with an annealing temperature of 55°C. This annealing temperature is permissive, and there was clearly a considerable amount of non-specific priming occurring. However, all PCR products were run on agarose gels, blotted and hybridised with the *Ascaris cut-1* probe: in this way it was hoped to detect any band hybridising to the probe in an oligo (dT)-SL1 smear, as well as to identify whether or not the bands in an amplification were spurious.

Unfortunately, the only reproducible result came from using primers which spanned a region not covered by the sequence of the *Ascaris* probe. The combination of cut1 and the SL1 primer amplified a band of around 200bp when 16 and 28 dpi cDNA were used as the template, but not when mature adult cDNA was used. Figure 4.5a (lanes 2 & 6) and b (lane 1) shows the results of the PCR experiments on first strand cDNA from days 16 and 28 p.i. life-cycle stages. At that stage it was anticipated that the homologue would not be found in adult material and therefore any product obtained using a gene-specific primer, from larval and not adult cDNA, was worthy of further investigation.

The fragments amplified from first strand cDNA from days 16 and 28 p.i., with cut1 and cut1R were the same size and were therefore assumed to have the same identity. The day 28 p.i. fragment was cloned into the TA cloning vector pCRII, and named LS1. At the same time a Southern blot of *Ascaris*, *C. elegans* and *B. pahangi* genomic DNA was hybridised with a labelled fragment of LS1 which had been gel-purified. There was hybridisation to only one

band in both the *Eco* RI digest and the *Hind*III digest of *B. pahangi* DNA, and the size of the bands did not correspond to those seen in the same digests when probed with the *Ascaris cut-1* probe. There was no hybridisation of the LS1*Brugia* PCR fragment to the *Ascaris* or *C. elegans* DNA. Figure 4.6 shows the results of the Southern hybridisation experiment.

A northern blot of *Brugia* RNA from adult worms and days 16 and 28 p.i. larval worms was also hybridised with the labelled fragment. There was no hybridisation.

The discouraging hybridisation results were explained when the cloned fragment was sequenced and the data analysed: the sequence of the fragment showed no homology to cuticlin at either the DNA or amino acid levels. Neither the cuticlin-specific primer nor the SL1 primer were present in the sequence.

4.5.2. RT-PCR using primers designed to flank the stretch of *C. elegans cut-1* sequence showing homology to the *Ascaris cut-1* probe.

New primers were made from the *C. elegans* sequence, flanking the region in the third exon which was known to be highly conserved between *C. elegans*, *Ascaris* and *Meloidogyne*. Therefore, any products amplified using these primers can be reliably screened using the labelled *Ascaris cut-1* recombinant, which is derived from the same region. The primers are marked on Figure 4.4, and are named: ELcut1, ELcut2 and ELcut3.

Amplifications primed with ELcut1 and the oligo (dT) primer resulted in a number of bands on days 16 and 28 p.i. cDNA, but not on adult cDNA. An example is shown in Figure 4.5a lane 5.The results were reproducible and the two products contained two bands in common and two which were unique to the individual cDNA time-points. All the fragments were individually gel-purified, and when the products were blotted and probed with the *Ascaris cut-1* fragment, the largest fragment (800bp), common to the day 16 and 28 p.i. material, hybridised (data not shown). This was taken to imply that the fragment was differentially transcribed at the different life-cycle time points. The hybridisation was very weak, but at the time the extent of the homology between the *Ascaris* and *Brugia cut-1* genes, which in fact results in an extremely powerful hybridisation signal, was not appreciated. Consequently it was decided to determine whether or not the fragment (known as LS2) actually had a cuticlin identity. Whilst attempting to clone and sequence LS2, a number of experiments were carried out with gel-purified, ^{32}P-labelled LS2.

A Southern blot of *Brugia*, *Ascaris* and *C. elegans* DNA was hybridised to labelled LS2. The resultant hybridisation pattern was compared to that obtained using the labelled *Ascaris cut-1* probe. As shown in figure 4.7, some of the hybridising bands corresponded to bands which also hybridised to the *Ascaris cut-1* probe, whilst others were unique to the LS2 hybridisation pattern. The hybridisation was very strong and an optimistic interpretation of the results was that LS2, having a *Brugia*-specific cuticlin identity, was hybridising to additional bands which could represent additional cuticlin-like genes in the worm genomes which show significantly higher homology to the *Brugia cut-1* than to the *Ascaris* homologue.

Probing a northern blot of RNA from adult worms and worms from days 16 and 28 p.i. resulted in hybridisation to all three samples, but without a pattern of clear bands. There appeared to be much more hybridisation to the day 28 p.i. RNA, but this was probably due to unequal loading of the gel (data not shown).

Nested PCR is frequently used to ensure that priming in an initial PCR amplification is not spurious, and to confirm that the amplified product has a sequence which is recognized by gene-specific primers. Using the original PCR product as a template, a second round of amplification, using gene-specific primers internal to those originally used, should result in the amplification of a fragment correspondingly smaller (by the number of base pairs that the second set of primers are internal to the original primers) than the original PCR product, in this case LS2. No 5' primer internal to Elcut1 was available, but Elcut2 & Elcut3 are obviously internal to an oligo (dT) primer, so it was decided to use the primers available to do some 'semi-nested' PCR. Table 4.1. below shows the primers used in the original and 'semi-nested' PCR experiments, and gives the sizes of the resultant amplified bands.

PRIMER ONE	PRIMER TWO	Fragment size predicted from *C. elegans* sequence	*Brugia* fragment size
oligo (dT)	ELCUT1	720bp	800bp
ELCUT1	ELCUT2	480bp	450-500bp
ELCUT1	ELCUT3	360bp	300-350bp

Semi-nested PCR, using Elcut1 and Elcut2, resulted in amplification products which did not show a clear banding pattern on an ethidium-stained gel. Blotting and hybridising the products with labelled LS2, however, gave clear results, again showing that the predominant amplified band hybridising to the LS2 probe was present almost exclusively at the days 16 and 28 p.i. time-points. Additionally, the hybridising band was also reduced in size by approximately the right amount in the semi-nested PCR products, based on the *C. elegans cut-1* sequence. Figure 4.8 shows the results of hybridising labelled LS2 to nested and non-nested PCR products.

Obviously the most important proof of genetic identity is sequence, and the experiments out-lined above were all carried out whilst cloning and sequencing LS2. The fragment was cloned into the TA cloning vector pCRII and sequenced using the the automatic sequencer. When the sequence was compared to the data base, there was no homology to a cuticlin sequence, or indeed to any related gene. The fragment appeared to show highest homology to a ribosomal protein gene.

However, in the process of writing this chapter, the sequence was once again used to scan the database, and the results revealed that LS2 has an 100% homology, at the DNA level, to a recently isolated fragment of *Bm-col-3* (Gregory et al, 1997). Upon comparison with the sequence of the only isolated and characterised*B. pahangi* collagen gene (Bisoffi et al, 1996) the sequence of which is not in the database, it can be seen that LS2 represents a small fragment of this gene. The search also revealed LS2 to show significant homology (82% and 84% respectively) to *C. elegans* and *C. briggsae col-12* precursors. As has been described in the Introduction, the *Caenorhabditis* collagens are grouped into sub-families, and *Bp-col-1,* represented here by LS2, is clearly the *Brugia* homologue of a member of the col-6 sub-family, of which *col-12* is a member. Interestingly, Bisoffi et al isolated *Bpcol-1* by screening a *B. pahangi* adult cDNA library with a polyclonal antisera raised against the insoluble components of the cuticle of adult *B. pahangi* i.e the cuticlin component of the cuticle. The presence of an anti-collagen antibody in the antisera was probably due to contamination of the insoluble residue with collagen.

Gregory et al isolated *Bm-col-3* in the course of a project which was undertaken in order to identify abundant, stage-specific transcripts from infective and post-infective *B. malayi*, in the hope of identifying proteins which are important in the adaptive and developmental processes involved in the transfer of infective larvae between the insect and mammalian host. The *Bm-col-3* transcript is present at both the L3 and L4 stages of the parasite, and its abundance is clearly indicated by the fact that there are eleven ESTs containing the same sequence which have been separately deposited into Genbank by the Filarial Genome Project, representing 0.6% of the total L3 and L4 ESTs deposited.

4.5.3. Using primers based on the *Ascaris cut-1* sequence to obtain a *Brugia cut1* fragment.

It was fortunate that newly produced and tested primers made against the conserved region of the *Ascaris cut-1* sequence became available from the Naples laboratory at this stage (see Figure 4.9). The annealing temperature used in the PCR reactions was optimized to ensure the amplification of clear bands with minimum spurious priming. Initially the primers were used with *Brugia* genomic DNA as the only template, in order to obtain some authentic *Brugia pahangi cut-1* sequence which could then be labelled and used to probe a newly-made adult genomic library.

Four of the *Ascaris* primer pairs reliably resulted in a clear amplified band, the sizes of which were compatible with the *Ascaris* sequence (see the table below). All four amplification products were gel-purified, ligated into the TA vector pCRII and used to transform competent cells. All 27 resultant colonies potentially carrying an insert (white or sky-blue in colour) were confirmed byhybridisation, using labelled *Ascaris cut-1* as the probe. DNA was made from all 9 potentially positive clones, and digested to demonstrate the presence of an insert.

Subsequent sequencing, using the automatic sequencer, resulted in the discovery of a *Brugia pahangi cut-1* fragment clone amplified from genomic DNA using the primers 2ab5ext and 2a3med (see Figure 4.10).The fragment is 358 bp long and has a 74.7% homology with the corresponding *Ascaris* fragment and a 71.3% homology with the corresponding *C. elegans* fragment. Sequencing the fragment on both strands revealed that virtually all differences between the three fragments are at the third base of a codon and that the amino acid homologies across this stretch of the three nematode *cut-1* sequences are consequently extremely high (96% homology with the *C.elegans* and 91% homolgy with the *Ascaris* peptides). Figure 4.11 shows a pile-up of the three amino acid sequences. The *Brugia pahangi cut-1*-like fragment (named 32) contains an intron of 150 base pairs which is not present in the other two nematode genes. When this intron is removed, the amino acid homology with the conserved regions of the *C. elegans* and *Ascaris cut-1*-like genes continues until the end of the clone. The intron splice sites agree to a very high level with those established from *Dirofilaria immitis* by Claude Maina (CWBG). Table 4.2 below shows the predicted sizes of *B. pahangi* fragments resulting from amplication with the various primer combinations used.

5' PRIMER	3' PRIMER	*BRUGIA* FRAGMENT SIZE (BP)
2AB5Ext	2A3Med	358
2AB5Ext	12A3Int	150
2AB5Ext	12A3EXT	260
UNI5Int	12A3Int	125
Br5/3	2A3Med	150 (cDNA)

4.5.4. Using primers based on the *Brugia cut-1* fragment to obtain a corresponding fragment of cDNA.

Brugia pahangi-specific *cut-1* primers were designed on exon sequence from the '32' clone, initially for use in isolating a fragment of cDNA which could then be used in a RACE or RACE-like protocol to obtain a full-length cDNA clone. The sequence and position of the primers are shown in Figure 4.10. The newly-made primers were used in combination with the *Ascaris* primers known to amplify *Brugia* genomic DNA. RT-PCR was carried out using first-strand cDNA from the following time-points in the *Brugia pahangi* life-cycle: days 3, 5, 7, 15, 16, 28 post-infection, and adults. A band of 150bp was consistently amplified using day 7 p.i. cDNA as the template, and the *Ascaris*-derived 3' primer 2a3med (used to amplify 32) with the *Brugia*-derived 5' primer 5/3. This fragment was gel-purified and cloned into the TA cloning vector pCRII. It was subsequently sequenced on both strands. As shown in Figure 4.12, the fragment (referred to as 2133) clearly derives from the gene represented by 32; when the intron is removed, the cDNA and genomic sequences show 100% homology to one another.

4.5.5. PCR using SL1 and oligo (dT) primers to obtain a larger piece of *Brugia cut-1* cDNA.

An approach which can be used to obtain a full-length cDNA, in theory very simply, is to use two primers: the 5' primer designed on the SL1 sequence and the 3' oligo (dT) primer. The oligo (dT) primer is made to complement the polyA tail present at the end of virtually all mature mRNA species in most animal cells. The spliced leader sequence (SL1) is a 22-nt sequence spliced onto the 5' end of a subset of mRNAs, in a process known as *trans*-splicing. In nematodes, *trans*-splicing via the spliced leader sequence was first identified in *C. elegans* (Krause & Hirsh, 1987). The percentage of *Brugia* transcripts *trans*-spliced via SL1 is not known, but in *Ascaris* an estimated 70-90% of mRNAs have the SL1 sequence (Nilsen, 1989; Maroney et al, 1995), and it has been estimated that approximately 70% of *C. elegans* genes are *trans*-spliced, either via the SL1 or SL2 spliced leaders (Zorio et al, 1994).

Carrying out PCR reactions on cDNA from different points in the *Brugia* life-cycle, using the afore-mentioned primers with a permissive annealing temperature and a reasonably long extension time, should result in the amplification of all the full-length cDNAs transcribed at that life-cycle time-point and *trans*-spliced via the spliced leader. If the reaction products are then run on an agarose gel, blotted and hybridised with a labelled fragment of the gene of interest, the probe should hybridise to a discreet band or bands representing the appropriate cDNA, providing of course that it is SL1 *trans*-spliced.

This approach was used with cDNA from many different life-cycle time-points (days 3, 5, 6, 7, 9, 12, 18, 19, 20, 21, and 28 post-infection, and adult worms), without a positive result. Using a diluted sample of the initial reaction product as the template for a second round of amplification with gene-specific primers resulted in an apparently non-specific 'smear' of material, which did not hybridise to a labelled cuticlin gene probe.

4.6. Hybridising a Southern blot of Brugia genomic DNA to labelled *Brugia cut-1.*

Figure 4.13 shows the results of the hybridisation of labelled 32 to *B. pahangi* genomic DNA. The probe hybridises to two bands in each of two restriction enzyme digests, and the hybridisation is of equal intensity to both bands. There is no recognition site for *Eco*RI or *Hind* III (the enzymes used to cleave the genomic DNA) in the sequence of the probe, therefore the two hybridising bands are unlikely to represent two fragments of the same gene. This would imply either that the *B. pahangi cut-1* gene represented by 32 either exists as a double copy in the genome, or that the probe is recognizing a second *cut-1*-like gene with a very high homology to the original, at least in the region covered by the probe.

4.6. Attempts to find the 5' and 3' ends of the *Brugia cut-1* cDNA via PCR using SL1 and oligo (dT) primers.

Using *Brugia* cDNA from a life-cycle stage at which it is known that *cut-1* is transcribed as a template, it is in theory possible to obtain the 3' end of the gene by amplification using the oligo (dT) primer in combination with a *cut-1*-specific 5' primer. If the *Brugia cut-1* gene was SL1 *trans*-spliced, it would be possible to use the SL1 primer and a *cut-1*-specific 3' primer to amplify the 5' end of the cDNA. Primers based on the sequence of a previously characterized *Brugia pahangi* cytidine deaminase gene, known to be SL1 *trans*-spliced (Martin et al, 1996) were used in positive control reactions: the cytidine deaminase-specific 3' and 5' primers, CD1 and CD2, in combination with either the SL1 or oligo (dT) primers gave clear bands of known sizes using *Brugia pahangi* cDNA as the template (see Figure 4.14). The experiment demonstrates that although the oligo (dT) and SL1 primers were fully functional, and that the cDNA was competent as a template, the combination of either primer with a *cut-1* gene-specific primer did not result in amplification of a product.

The SL1 primer, in combination with all the 3' *Ascaris* and *Brugia* primers demonstrated to amplify *Brugia* cDNA, was used in amplification reactions with *Brugia* cDNA from life-cycle time points at which it was known that the *cut-1*-like gene was transcribed (as descibed in Chapter Six). The days chosen were 5, 7, 19, 20 & 21 post-infection. Controls showing the competence of the cDNA, the primers and the PCR reactions were positive. Despite blotting and hybridising reaction products with labelled 32, the 5' end of the *Brugia cut-1* cDNA was never found using this method.

A primer corresponding to a second spliced leader sequence (SL2) was also available in the laboratory. Again, the SL2 sequence was initially identified in *C. elegans* (Huang & Hirsh, 1989), where it has been suggested that *trans*-splicing via SL2 may be used to mature the 5' ends of genes which are internally located within polycistronic transcription units (Spieth et al, 1993). The SL2 primer was also used in a variety of amplification reactions with different 3' *cut-1*-specific primers on *Brugia* cDNA. A product was never amplified.

Similarly, amplification of *Brugia* cDNA from different life-cycle time-points was carried out using the oligo (dT) primer in combination with several *cut-1*-specific 5' primers. The oligo (dT) primer has a very low Tm compared to the gene-specific primers used; consequently the annealing temperature of the PCR reactions had to be low, resulting in the amplification of numerous false positives. These were identified by hybridising the blotted reaction products with labelled 32, to which they did not hybridise.

Table 4.3 gives the sequences of the non-cuticlin-derived primers used in the preceding experiments.

PRIMER	SEQUENCE 5'∅ 3'
SL1 (with EcoRI tail)	GCCGGAATTCGGTTTAATTACCCAAGTTTGAG
SL2 (with EcoRI tail)	GCCGGAATTCGGTTTAACCCAGTTACTCAAG
oligo (dT) with Xho site	GCCGCTCGAG$(T)_{17}$
CD1	GATGCTTTAACAAGTGCTGCTCG
CD2	GTGGTATTAGCTGTGAAAGTT

4.7. DISCUSSION.

The ubiquity of the cuticlins.

The apparent presence of cuticlin-like genes in the genomes of various nematodes of medical and veterinary importance was a welcome result. At that time it was known that all nematodes so far studied had an insoluble component of the cuticle which was referred to as cuticlin, but the nature of the proteins making up the cuticlin were completely unknown. Prior to the cloning of *cut-1* and *cut-2*, Betschart et al (1990), had raised antibodies in mice against a purified (i.e. collagen-free) 2ME-insoluble fraction of the *Ascaris* cuticle, which was therefore assumed to be an anti-cuticlin antiserum. IFAT studies on sections of adult and L3 *A. lumbricoides* localized the antibody to the external cortical layers of the worm cuticle, with no labelling on the epicuticle of the worm. Cross-reactivity was also seen on the external cortical layers of the filarial worms *B. pahangi*, *Acanthocheilenoma viteae* and *Onchocerca volvulus*, demonstrating that similar epitopes are clearly present in the cuticlin proteins of both filarial and ascarid nematodes. Hence it was already known that there were proteins present in the cuticlin residue of parasitic nematodes which were fairly well-conserved. The means by which the *C. elegans* cuticlin genes were discovered (using a *Drosophila* probe, previously described in the Introduction), and the high homology between the *Ascaris*, *Brugia* and *C. elegans* *cut1*-like-genes, shows that the cuticlin components of the cuticle are very well-conserved. It is still not known how many proteins make up the cuticlin residue of the nematode cuticle; it could be that whilst some are conserved within the Nematoda phylum, others are species-specific, adapted to the particular life-cycle requirements of the worm. The

fact that a *cut-1* homologue seems to be present in all nematodes so far tested, both parasitic and free-living, would imply that it codes for a protein which is very important in the basic structure and function of the nematode cuticle.

The cuticlins may be encoded by a gene family

Evidence from this chapter points to the existence of at least two *cut-1*-like genes in *Brugia,* at least one conserved gene in the other parasitic geneomes studied, and multiple *cut-1*-like genes in the C.elegans geneome. As has been covered in the Introduction, this situation has a precedent in the form of the nematode cuticular collagens; they are coded for by a number of genes which are scattered throughout the genomes of the worms. The exact numbers are not known, but in *C. elegans* there are thought to be between 40-150 collagen genes, most of which are cuticular (Cox et al 1984, Cox et al 1985) whilst in *Ascaris* there are at least 15 (Kingston et al, 1989), with a similar number thought to be represented in the *Brugia* genome (Selkirk et al,1989). Much work has been done on the characterisation of the cuticular collagen genes of *C. elegans* and, to a much lesser extent, of several plant and animal parasitic nematodes (discussed in more detail in the Introduction). In general, nematode cuticular collagens are structurally very similar, but differ in the spacing of the cysteine residues via which they trimerise. Individual genes code for small pro-collagen molecules which interact to produce the mature collagen protein (this is in contrast to vertebrate collagens, where the mature collagen is coded for by a single gene). Thus it would be reasonable to assume that the collagen genes have evolved from a common ancestral structural cuticular protein, presumably via a series of gene duplications (Johnston, 1994). In all probability the insoluble proteins of the nematode cuticle i.e. the cuticlins, evolved in a similar way, possibly even from a common ancestral protein or proteins.

It is interesting to note that the cuticle collagen gene *Bp-col-1* is transcribed at days 16 and 28 p.i., as demonstrated by the cloning of LS2. This shows that cuticular components are definitely being synthesised at these stages of *Brugia* development.

The evolution of the cuticlin genes.

It has been shown in *C. elegans* that the cuticle changes with respect to collagen composition between larval stages, presumably to allow specialization of the cuticle, which is advantageous to the nematode at each life-cycle stage (Cox et al, 1981C). This adaptability is equally important for parasitic nematodes which have to survive in hostile host environments and also successfully make the transition between hosts, and in many cases between host and external environments. The evolution of the nematode life-cycle to include four cuticular moults to adulthood has enabled the diversification of the Phylum; each larval stage has the potential to adapt its cuticle to a new environment. It has been suggested that the nematode ancestral life-cycle had only one post-embryonic stage, and evolution of the multiple larval

forms was gradually permitted largely by the diversification of the cuticular structural proteins (Inglis, 1964).

The nematode phylum is classified as shown in Figure 4.15, and it has been proposed that the Rhabditid line gave rise to about 92% of all nematode vertebrate parasites (Anderson, 1992). The *cut-1*-like gene has been shown, by identification and cloning, and by hybridisation during the course of this project, to be generously distributed throughout the Rhabditid line. Hence it is a gene which has retained high levels of homology from the earliest ancestral form, whilst having been exposed to extremely high levels of selection pressure. For these reasons it would be reasonable to assume that the protein/s encoded by the *cut-1*-like gene/s play an indispensable role in the survival of the nematode, and have done so since the evolution of the multi-larval form, perhaps even contributing to its emergence.

B. pahangi cut-1 is not *trans*-spliced.

From the experiments out-lined in this chapter, there is no evidence to suppose that the *cut-1* gene so far isolated from *B. pahangi*, is *trans*-spliced.

Trans-splicing via the SL1 sequence is now accepted to be widely used in the maturation of mRNA signals in nematodes, in addition to the more conventional *cis*-splicing. *Trans*-splicing occurs in the kinetoplastids, where it has been fully described in the trypanosomes, in the nematodes (Donelson et al, 1990), in *Euglena* (Tessier et al, 1991) and in flatworms (Rajkovic et al, 1990; Davis et al, 1994). The spliced leader in trypanosomes (39 nt) is longer than the nematode SL (22nt) and shows considerable sequence variation between species, unlike that of the nematodes. In contrast to eukaryotic *cis*-splicing, whereby intervening RNA structures (introns) are removed from a single primary transcript during message maturation, *trans*-splicing is defined as the ligation of exon sequences originating in independently transcribed RNAs (Krause, 1995). In trypanosomes *trans*-splicing is the only mode of maturing mRNA species; there is no *cis*-splicing. In nematodes, the initial SL donor, a small (109-nt)RNA, is cleaved to produce the 22-nt SL1 leader sequence, which is donated (or *trans*-spliced) to the 5' end of mRNAs. The only requirement for *trans*-splicing is intron-like RNA at the 5' end of the pre-mRNA (quaintly termed an 'outron') which has a 3' splice acceptor lacking an upstream 5' splice donor site which is the substrate for the *trans*-splice (Conrad et al, 1993). It is now thought that more than 70% of *C. elegans* genes are *trans*-spliced (Zorio et al, 1994) and a sequence identical to SL1 has been identified at the 5' ends of of mRNA transcripts from a variety of free-living nematodes, as well as from the parasitic nematodes *Brugia* (Takacs et al, 1988), *Ascaris* (Nilsen, 1989), *Haemonchus* (Bektesh, 1988) and *Onchocerca* (Zeng et al, 1990). Nematodes are also the only animals known to use *cis*- and *trans*-splicing in the maturation of a single mRNA message (Krause, 1987).

The precise function of *trans*-splicing via the SL1 has not yet been established, although work on *C. elegans* shows that *trans*-splicing is required for viability (Ferguson et al, 1996); and analysis of mutants shows that the SL RNA is required for embryogenesis. There is also

no clear indication as to why *trans*-splicing should be an advantage for nematodes: it has been suggested that it could stabilise an mRNA species; enhance its translational efficiency; or be involved in directing intracellular transport or location (Blumenthal & Steward, 1997).

There was no real way of predicting whether the *Brugia cut-1*-like gene would be *trans*-spliced or not. So far, there has been no unifying feature, functional or structural, which enables *trans*-spliced genes to be grouped together, and hence no way of predicting which mRNA species will carry the SL1 sequence. In fact, Park et al (1990), demonstrated that two collagen genes from *C. elegans* were identical in all respects apart from the fact that one was *cis*-spliced and the other was *trans*-spliced, suggesting that *cis*-and *trans*-splicing can be interchanged during evolution. In *C. elegans*, *cut-1* is *trans*-spliced whilst *cut-2* is not; the *Meloidgyne cut-1*-like gene is not *trans*-spliced. The full-length *Ascaris* transcript, which is extremely abundant, and known to be translated in the infective egg stage of the life-cycle, is not *trans*-spliced. However, when a RACE-like protocol is carried out on adult mRNA, three SL1 *trans*-spliced transcripts are obtained: one corresponds to the full-length mRNA transcript; the other two fragments are smaller. In all three cases the SL1 donor sequence has been added at the *cis*-splice sites (Timinouni & Bazzicalupo, 1997). There is no evidence that the shorter transcripts are translated, and they are most probably rare artefacts, detected by hybridisation only after two rounds of RACE amplification. A possible explanation for this phenomenon is that under circumstances in which an mRNA is not transcribed (in this case at an inappropriate point in the life-cycle) and the regulating *cis*-splicing mechanism is therefore not 'activated', the SL1 is added spuriously to the outrons of an mRNA, resulting in redundant and untranslated messages. It is an intriguing (and completely hypothetical) possibility that the 'accidental' translation of one of these mis-spliced messages could result in selection for a new polypeptide, the gene of which could be added to a growing gene family.

The experiments described in this chapter would imply that there was no *cut-1*-like gene in the *Brugia* genome that was SL1 *trans*-spliced (assuming, from hybridisation experiments, that there are more than one present); the SL1 primer was used in tandem with a variety of 3' primers made from the highly conserved region of the *cut-1* gene as well as from a region that appears to be specific to the sequence of a *Brugia pahangi* cDNA clone subsequently cloned (as described in Chapter Five), without ever obtaining a product.

Whilst it was impossible to predict whether or not the *Brugia cut-1*-like gene would be SL1 *trans*-spliced, it was extremely unlikely that it would turn out to be SL2 *trans*-spliced: SL2 has been found in *C. elegans* genes (Zorio et al, 1994) and in the genes of the closely-related soil-dweller*C. briggsae* (Lee et al, 1992). No parasitic nematode messages have thus far shown *trans*-splicing via SL2. All messages that acquire SL2 have so far proved to be immediately down-stream of, and in the same orientation as, an up-stream transcription unit, *trans*-spliced with SL1 (Spieth et al, 1993). They concluded that, as happens in trypanosomes, the juxtaposed genes constitute a polycistronc transcription unit in which a single promoter initiates continuous transcription through two or more genes, resulting in mRNAs which are

subsequently processed (via cleavage, polyadenylation and *trans*-splicing) such that they are functionally monocistronic. They further postulated that the genes in the polycistronic transcription units already discovered contained genes of related function, thus drawing a parallel with the bacterial operons, clusters of co-regulated genes whose products perform related functions (Lewin, 1990). So the discovery of a parasitic nematode gene which was SL2 *trans*-spliced, especially one which possibly belongs to a gene family and codes for a protein which shows a close functional relationship with other similar gene products, would have been quite exciting.

In conclusion, it would be reasonable to assume that maturation of the mRNA of *cut-1* in *Brugia* is accomplished by conventional *cis*-splicing, as is also the case in *Ascaris* and *Meloidogyne*. However, it must be added that the only way of stating this for certain using the data shown here would be to confirm the RACE results shown in Figure 4.14 by attaining a signal using bpcut-1 primers internal to those used.

Discussion of experimental detail.

Use of the oligo (dT) primer.

It is in some ways difficult to explain why amplification using the oligo (dT) primer, in tandem with a *cut-1* specific primer, never amplified a *cut-1*-like fragment from *B. pahangi* DNA. Cleavage and subsequent polyadenylation in the 3' maturation of a primary transcript is virtually ubiquitous in animal cells: the only group of mRNAs which do not have a poly-A tail are the major histones (Wahle et al, 1992). In *C. elegans*, a recent analysis of 1300 cDNA clones revealed only 6% which did not appear to have a poly-A tail (Krause, 1995). So there is very little doubt that the mature *cut-1* transcript has a poly-A tail, and the problem must lie in the practicalities of the experimental detail. As has previously been mentioned, the nature of the oligo (dT) primer means that it must be used at a low annealing temperature which results in the amplification of many non-specific products, due to the oligo (dT) primer 'sitting down' at sequences in the DNA template which do not exactly match the primer sequence. Spurious annealing of the oligo (dT) primer to nematode sequences has been reported by Martin et al (1995) and Joshua et al (1995). It is thought that the *cut-1*-like genes are not transcribed at a very high level at any point in the life-cycle, so in a reaction which is not stringently selecting for a *cut-1*-like sequence, it is easy to imagine that the message may fail to be amplified. A similar problem was noted by Gregory et al (1997), who used the oligo (dT) primer in tandem with the SL1 primer on cDNA from vector-derived L3 and L4 larvae in order to identify full-length cDNA transcripts which were abundantly and differentially expressed around the time of the larval transfer between the insect and mammalian hosts of *B. malayi*. Many of the clones were truncated by mis-priming of the oligo (dT) primer, an example being the *Bm-col-1* clone, of which LS2 represents the *B. pahangi* homologue. This region of the *Brugia* collagen gene must contain a region which is effectively recognised by the oligo (dT) primer. It is, however, more difficult to explain the 5' recognition of a collagen sequence by the cuticlin-specific

primer, the sequence of which has no known homology to any collagen gene sequence. The only explanation is a combination of the already discussed apparent abundance of the transcript with the permissive annealing temperatures required when doing PCR using the oligo (dT) primer.

The false positive clones obtained using oligo (dT) and SL1 primers in tandem with *C. elegans* and *Ascaris cut-1*-specific primers serve to illustrate the pit-falls of working with heterologous reagents, where obtaining any result at all must be carefully balanced against the pursuit and characterisation of false positives.

NORTHERN BLOTTING.

Hybridisation of a *cut-1* probe to a northern blot of total *Brugia* RNA was never seen, despite the use of several different cut-1 recombinant sequences throughout the course of the project. The experiment was carried out a number of times with RNA from various time-points in the *Brugia* life-cycle, at first using the *Ascaris cut-1* fragment as a probe, and later using a *Brugia*-specific probe. Subsequently probing the negative blots with a labelled fragment of a cloned ribosomal protein, which hybridises to a band of known size in all life-cycle stages tested, served as a positive control for the experimental conditions.

In *C. elegans*, the *cut-1* probe hybridises to 10µg of total adult RNA, clearly demonstrating the transcript size of the gene. Using 12µg of *Ascaris* DNA, hybridisation could not be detected with an homologous probe. Also, the *M. artiella cut-1* homologue did not show hybridisation to 50µg of *Melodogyne* adult RNA (Di Giorgi et al, 1996). RT-PCR subsequently revealed that *cut-1*-like transcription occurs in adult male but not in adult female *Meloidogyne* (Di Giorgi et al, 1996), and that in *Ascaris* the levels of transcription are very low in the adult worms (P. Bazzicalupo, personal communication). Experiments carried out during the course of the project (see Chapter Six) show that the *cut-1*-like mRNA is not represented in adult first strand cDNA, and at the stages where it does appear to be transcribed it was very difficult to obtain enough parasite material to provide 10µg of RNA. In conclusion, hybridisation of northern blots is not sensitive enough to detect *cut-1*-like gene transcripts at the low levels at which they are clearly present in the relevant life-cycle stages, taking into consideration the practical difficulties of obtaining sufficient material from representative time-points in the parasitic nematode life-cycle.

CONCLUSION.

The experimental work shown in this chapter eventually resulted in the cloning of cDNA and genomic fragments of a *Brugia pahangi cut-1*-like gene. The sequence information taken from these fragments was then used to move the project forward with more confidence, in the knowledge that the primers and probes which were being used were homologous to the *cut-1* sequence specific to *B. pahangi*.

Figure 4.1.

Southern blots of cleaved nematode genomic DNA probed with *Ascaris cut-1.*

a. Adult *Brugia pahangi* genomic DNA (20μg) was cleaved with the following enzymes:

lane 1 *Hind* III

lane 2 *Eco*RI

b. *Ascaris suum* and *C.elegans* genomic DNA was cleaved with the following enzymes:

lane 1 *A. suum Eco*RI

lane 2 *A. suum Hind* III

lane 3 *C. elegans Eco*RI

lane 4 *C. elegans Hind* III

The blots were hybridised to labelled *Ascaris cut-1* at 65°c, and washed to 0.2 x SSC, 0.1% SDS at 65°c.

n.b. The EcoRI digest shown in 4.1a is incomplete/partial and therefore does not show the same pattern seen in figures 4.2 and 4.13.

Fig. 4.1.

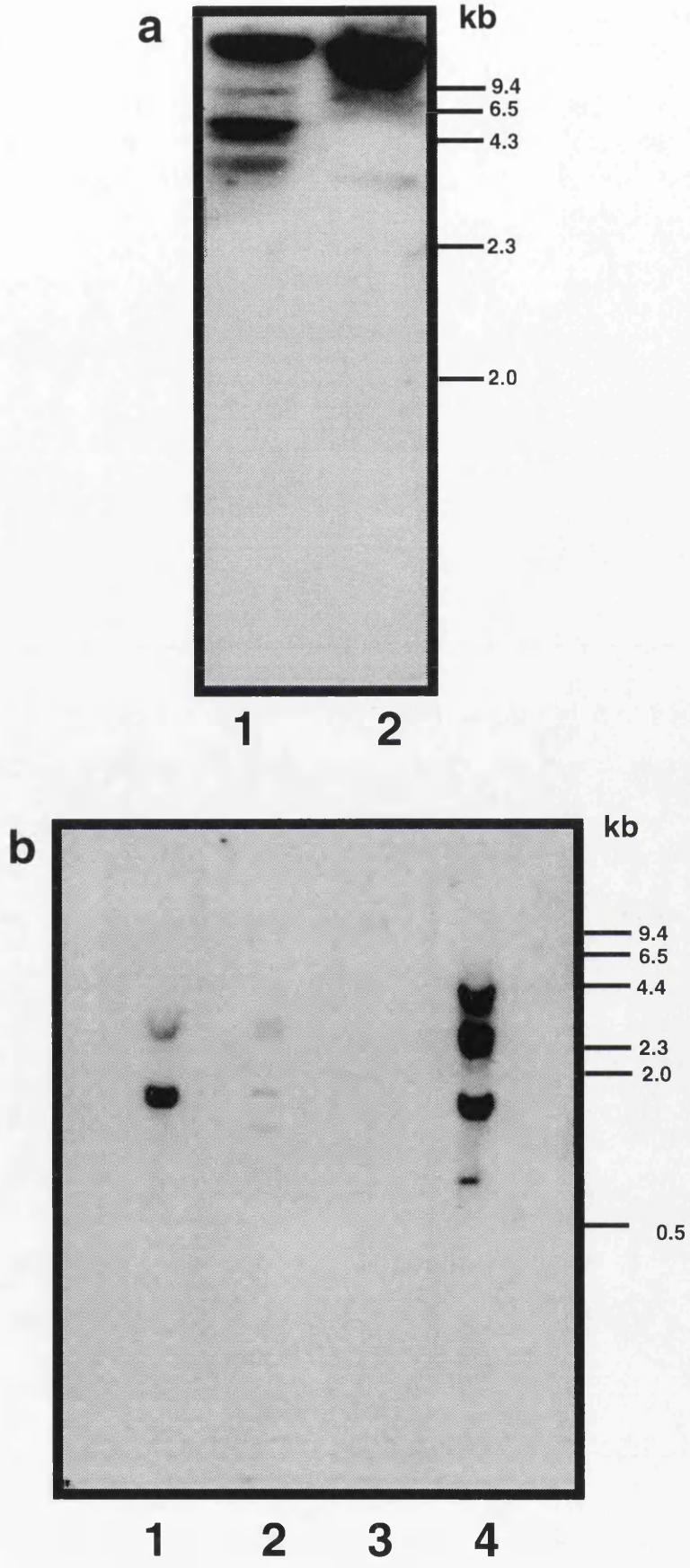


Figure 4.2. 'The Zoo-Blot'

a. Southern blot of cleaved genomic DNA from a variety of parasitic nematodes of medical and veterinary importance, hybridised to *Ascaris cut-1.* Hybridisation was carried out at 65°c and washed down to 0.2 x SSC 0.1% SDS at the same temperature.

b. Ethidium gel which was subsequently blotted and probed (see a). 10-15µg of genomic DNA was cleaved with the following enzymes:

lane 1 *Toxocara canis Eco*RI

lane 2 *Toxocara canis Hind* III

lane 3 *Haemonchus contortus Eco*RI

lane 4 *Haemonchus contortus Hind* III

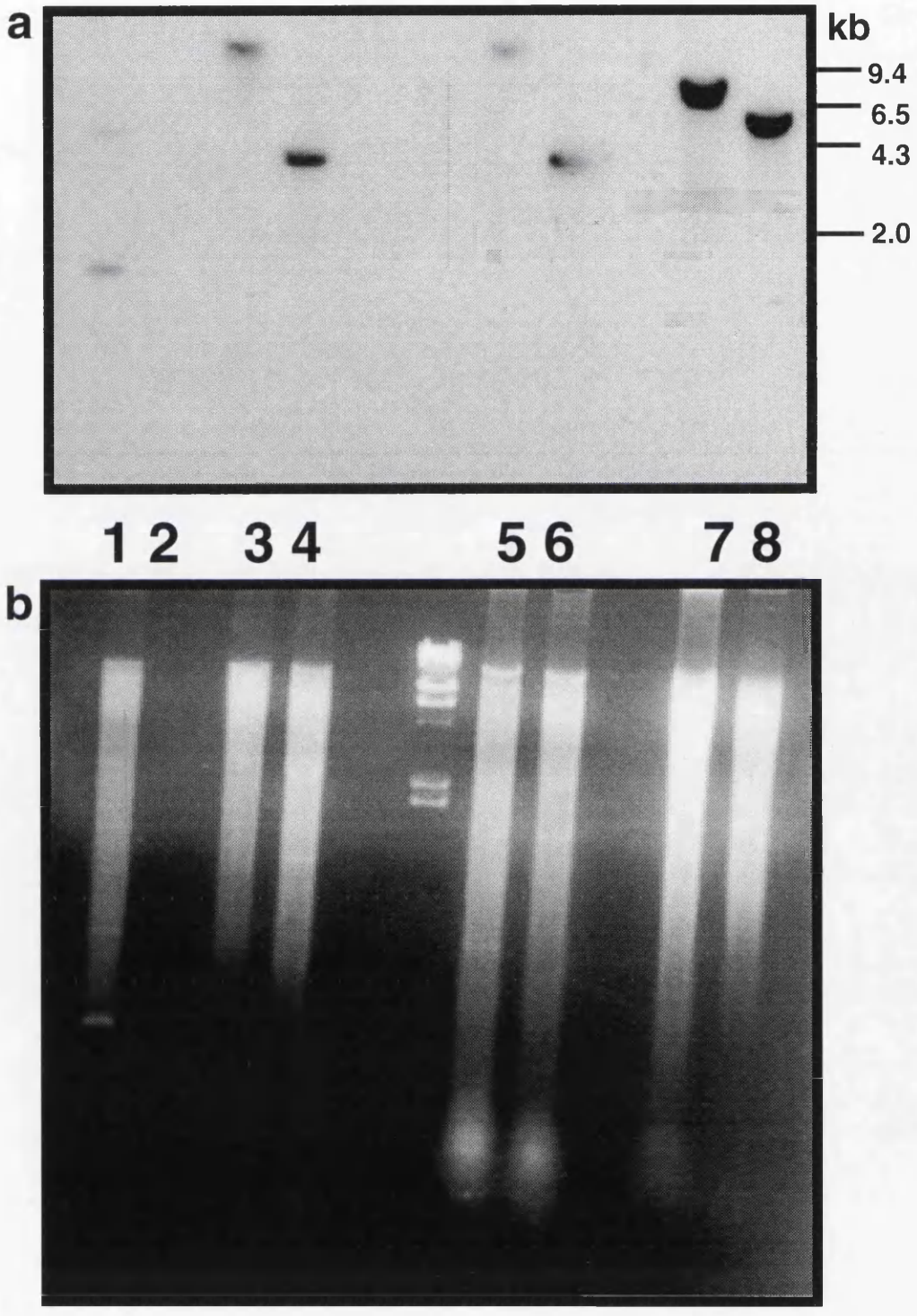
lane 5 *Dictyocaulus viviparus Eco*RI

lane 6 *Dictyocaulus viviparus Hind* III

lane 7 *Brugia pahangi Eco*RI

lane 8 *Brugia pahangi Hind* III

Fig. 4.2.



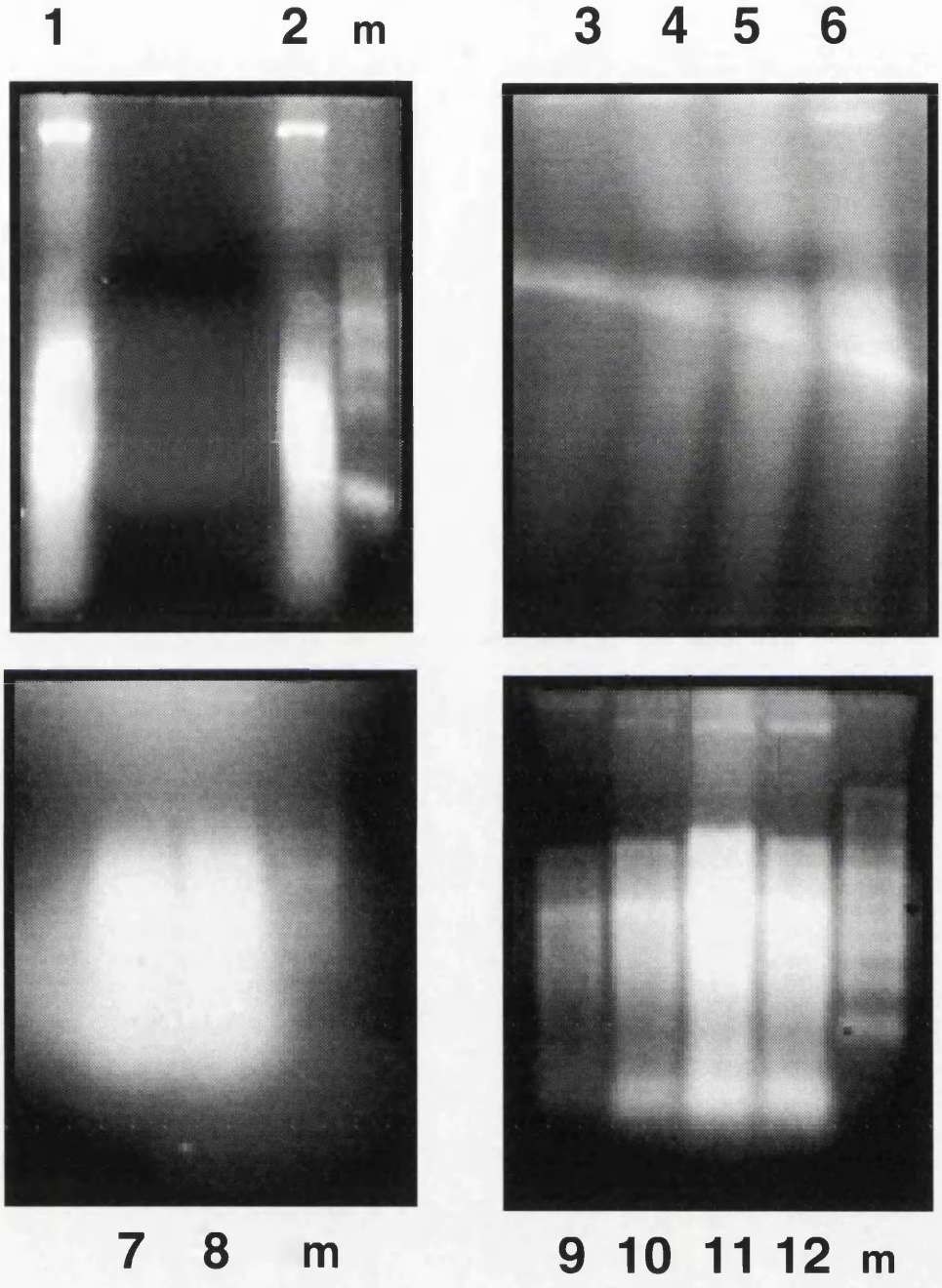


Fig. 4.3.

RNA from various life-cycle stages of *B. pahangi*, run on 1.2% formaldehyde gels.

1	adult male	7	day 7 p.i.
2	day 17 p.i.	8	mixed adult
3	day 7 p.i.	9	day 7 p.i.
4	day 20 p.i.	10	day 19 p.i.
5	day 27 p.i.	11	day 21 p.i.
6	mixed adult	12	day 23 p.i.
m	1µg of RNA markers		

FIGURE 4.4.

Coding sequence of the *C. elegans cut-1* gene (the full genomic sequence has the Accession no. M55997). ⇓ signifies the position of an intron. The primers used in the PCR strategy by which the larval/immature adult-specific fragments LS1 and LS2 were obtained are marked in red, the blue arrows indicating the direction of priming.

Figure 4.5.

EtBr stained gel of PCR products obtained using the *C.elegans* cut-1 primer in combination with the SL1 primer, showing that the amplified product is specific to pre-adult *B. pahangi* cDNA.10μl of the following PCR products were electrophoresed in each lane.

a Using day 16 p.i. L4 first strand cDNA as the reaction template:

lane 1 cut-1 and elcut-3

lane 2 **SL1 and cut-1**

Using genomic DNA as the reaction template:

lane 3 elcut-1 and elcut-2

lane 4 elcut-1 and elcut-3

Using day 28 p.i. L5 first strand cDNA as the reaction template:

lane 5 elcut-1 and oligo(dT) primer

lane 6 **SL1 and cut-1**

b Using day 28 p.i. first strand cDNA as the reaction template:

lane 1 **SL1 and cut-1**

lane 2 cut-1 and cut-1R

lane 3 cut-1 alone (single primer control)

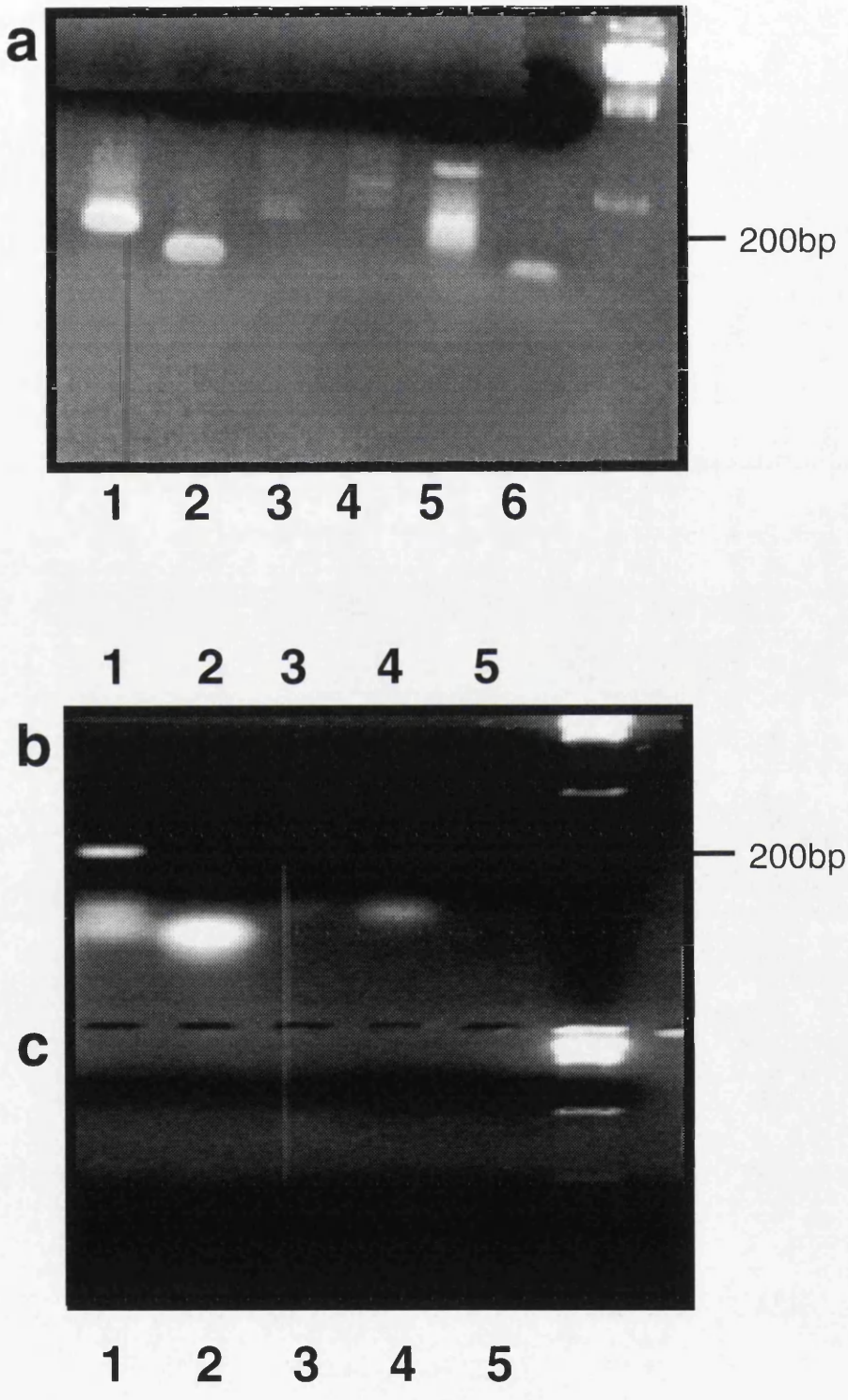
lane 4 SL1 alone (single primer control)

lane 5 no primer control

c Using adult first strand cDNA as the reaction template:

The lane scheme is identical to that described for **b.**

Fig. 4.5.



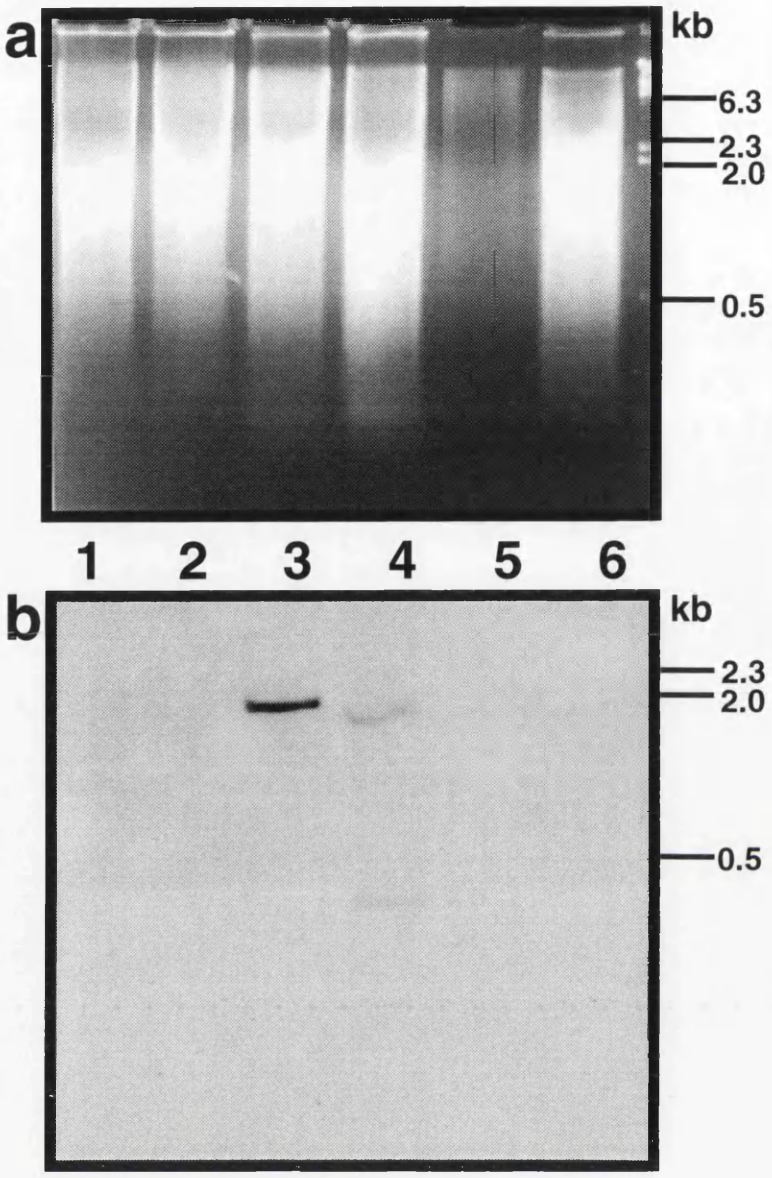


Figure 4.6.

EtBr stained gel of cleaved nematode genomic DNA, and corresponc
Southern blot hybridised to LS1, the fragment amplified from d 28 p.
cDNA using SL1 and cut-1 primers.
20µg of genomic DNA was cleaved for each lane. Hybridisation and
washing were carried out at 65°c.
The blot was washed to 0.2 x SSC, 0.1% SDS.

lane 1 *Ascaris lumbricoides* *Eco*RI
lane 2 *Ascaris lumbricoides* *Hind*III
lane 3 *Brugia pahangi* *Eco*RI
lane 4 *Brugia pahangi* *Hind*III
lane 5 *C. elegans* *Eco*RI
lane 6 *C. elegans* *Hind*III

Figure 4.7.

Southern blots of cleaved genomic DNA from various nematodes hybridised with *Ascaris cut-1* and the pre-adult PCR-derived clone LS2, to compare the hybridisation patterns.

20 µg of DNA was cleaved with Eco*RI* or Hind*III* and run on a 08% agarose gel before blotting. Hybridisation was carried out at 65°c and the blots were washed down to 0.2 x SSC and 0.1% SDS, at the same temperature.

A - Hybridised to ^{32}P-labelled LS2

B - Hybridised to ^{32}P-labelled *Ascut-1.*

lane 1	*C. elegans* DNA cleaved with Eco*RI*
lane 2	*C. elegans* DNA cleaved with Hind*III*
lane 3	*B. pahangi* DNA cleaved with Eco*RI*
lane 4	*B. pahangi* DNA cleaved with Hind*III*
lane 5	*A. suum* DNA cleaved with Eco*RI*
lane 6	*A. suum* DNA cleaved with Hind*III*

Bands which hybridise to both probes are marked with either a filled or empty circle or arrow head.

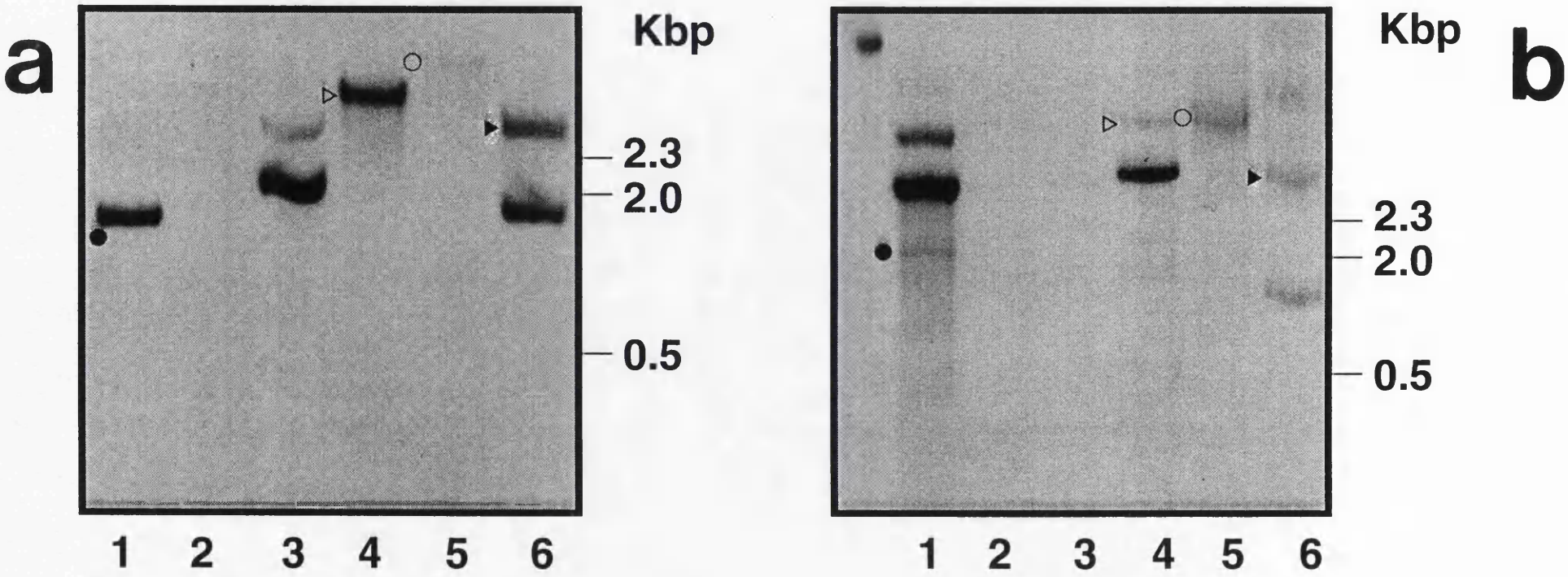


Figure 4.7.

Figure 4.8.

Semi-nested PCR products hybridised to labelled LS2, showing that LS2 is amplified almost exclusively from cDNA made from *B. pahangi* worms taken at days 16 and 28 p.i. of the jird.

PCR reactions were carried out using first strand cDNA from: days 5, 16 and 28 p.i. of the jird, and adult worms, as the template. 10µl of each reaction were then electrophoresed and blotted onto nitrocellulose, and the blots probed with ^{32}P-labelled LS2. Hybridisation and subsequent washing were carried out at 65°c. The blots were washed down to 0.1 x SSC and 0.1% SDS.

A - PCR products from reactions using ELcut1 and the oligo (d)T primer.

B - PCR products from reactions using ELcut1 and ELcut2.

The figure shows that the predominant hybridising band hybridising to the LS2 probe (marked with an arrow) is present almost exclusively at days 16 and 28 p.i. It also shows that the hybridising band is also reduced in size by approximately the right amount in the semi-nested products, as predicted from the *C. elegans cut-1* sequence.

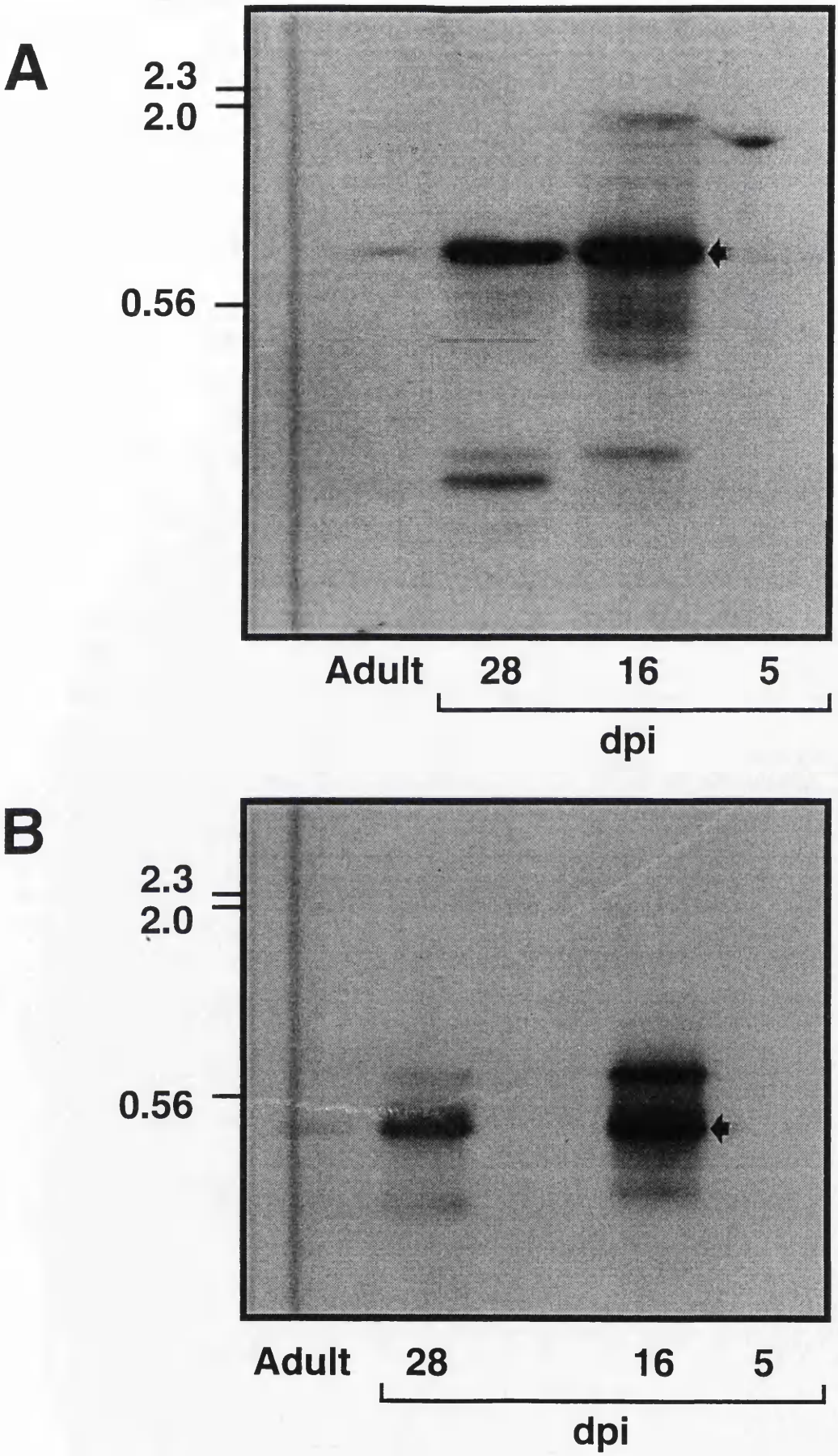


Figure 4.8.

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GGTTTAATTACCCAAGTTTGAGGATTTCTTCGGTGCGCATCCCGACTGTTTTGATGACCC      60

CAATCGCCAGGTCGATGCTCAGTTCATGGCATCTATAAAAGACTAACACTCCTGGGCGGG      120

GCGGATATCTGCTGGAGAATGTGCCGTGCGGTCTCCTTCCTCGCTCTTTTCGGGCTCGCC      180

GCAGCTATTCCTGTCGACAACGGAGTCGAAGGAGAGCCGGAGATTGAATGTGGTCCTACG      240

TCAATCACGGTCAACTTCAACACGAGAAATCCATTCGAGGGTCATGCATACGTGAAGGGA      300

CTCTATGATCAAGAGGGATGCCGCAGCGACGAGGGTGGACGTCAAGTGGCTGGCATCTCG      360

CTGCCTTTTGACTCGTGCAACGTCGCACGAACACGCTCGCTGAATCCTCGTGGTATCTTT      420

GTCACGACGACGGTTGTCATCTCGTTCCATCCGCTCTTCATCACCAAAGTTGACCGTGCC      480

TACCGCGTCCAATGCTTCTACATGGAGGCCGACAAGACTGTCAGTACGCAAATCGAAGTG      540

TCTGAGATTACGACCGCTTTCCAGACGCAAATCGTTCCTATGCCAGTCTGCAGATACGAG      600
                  →                    2AB5EXT
ATTCTCGACGGAGGTCCAACGGGACAGCCGATACAGTTCGCAACCATCGGTCAGCAAGTA      660
    →          UNI5INT
TACCACAAGTGGACTTGCGATTCGGAAACTGTGGATACGTTCTGTGCTGTCGTTCACTCA      720

TGCTTCGTTGATGATGGCAGCGGTGATACGATCCAGATCCTCAACGAGGAGGGTTGTGCG      780
          ←              12A3INT                  ←
CTTGACAAATACCTGCTGAACAACCTCGAGTACCCCACCGATTTGATGGCCGGCCATCAA      840
 2A3MED                                    ←          12A3EXT
CGAGGAGGGTTGTGCGCTTGACAAATACCTGCTGATTCTATCAGTGCCAGATTAGATTT       900

GATGGCCGGCCAAGAAGCGCACGTCTACAAATATGCGGACCGATCACAGCTCTTCTATCA      960

GTGCCAGATTACTATCACCATAAAAGAGCCAAACAGCGAATGCCCCAGACCCACTTGCAG      1020

TGAGCCGCAAGGATTTGGGGCTGTGCGTCCAGGTGGCTCCATAGCGCCGAAAAAGCAGCG      1080

GCGCTGCCAACTCCGCCTGATCAAAAAGAGTGGGGGTGACTATGACAACACCCTTGATGT      1120

ACGCACCGACTTCAGCGCCCTCGACATTAGTGATCGGGATGAGGCGCTTCCAATGGATCT      1180

TCGCCATCGCGCTCGTCATGCGCGCGGCCAACAGGTGATTCTGTCGCCAGCGAATGAAGG      1240

AATATGCATGTCGCCATTTGGATTCTCGATCTTCATGGGCTTAGCTGTCGCACTGGCTGC      1300

AGCTGTGGTCGTCGTTGTCTCGTTCAAACTTCGACCTCAGCAAAAAGCTTGAAGCATCTG      1360

AGGGAGCCAACGCATGTTCACCACTTCCGTGGCCTTCTATCACATATCCTACAATCTGTC      1420

TCATTCACATTCGCACATACCGCTATGCACCTCTGTCTACGTCTGCAAATTCGTATATGA      1480

GCTCGGACTATACGATAGAAAGAACAACTCTTGTATTGCCACTTCCAAAGTGTGTCGGAA      1520

ACCTGGATTTTCTCCCAAATAATTCCGTGCACGTGGAAGATGTCACTAACCTACTATTCA      1580

TCTGTTTAAGCATCATCTATCAATATGTCTTGCTATCTTTCTTTCCGATCAGTGCAATTG      1640

TATGTAATCTCTCGCCCAAATAAGGGTGTCAACGAGTGCAATATTATACATTAACCACAC      1700

CGAATAAAACTTTCTCTCTTAAAAAAAAAAAAAAAAAAA                           1760
```
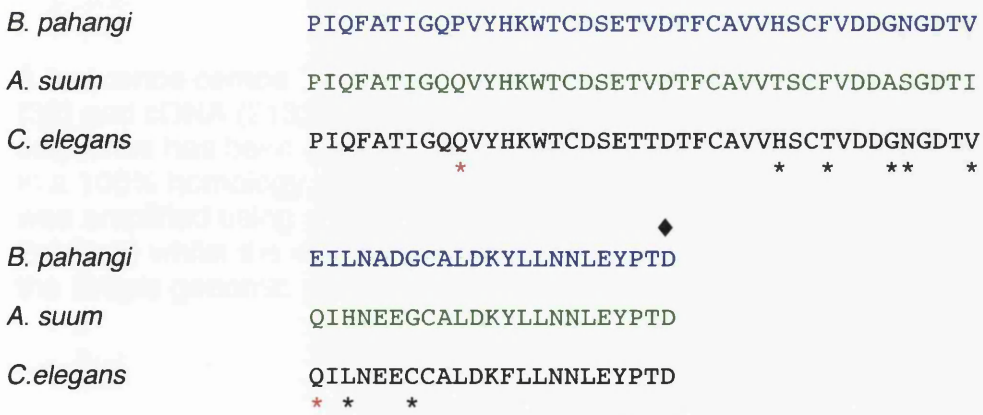

Figure 4.9. The *Ascaris cut-1* coding sequence. Primers are marked in red. The underlined sequence was 32P-labelled for use in hybridisation experiments.

Figure 4.10.

The sequence of the first *Brugia*-specific *cut-1* fragment '32' showing the primers which were subsequently used to isolate the corresponding cDNA fragment from day 7 p.i. first strand cDNA (see figure below for comparison of genomic and cDNA sequences). The intron is marked in red and the primers in blue.

Figure 4.11.



The predicted amino acid translation of '32' compared with the homologous regions of the *C. elegans* and *Ascaris suum* CUT-1 peptides. * marks where the '32' amino acid differs from one of the other two sequences, whilst * marks amino acids which differ from the *Ascaris* and *C. elegans* sequences. The position of the *B. pahangi*-specific intron is indicated by ♦.

Figure 4.12.

A sequence comparison of fragments of *Brugia pahangi cut-1* genomic DNA (32) and cDNA (2133). The red text represents intron sequence. Primer sequence has been deleted. As can be seen, the removal of the intron results in a 100% homology between the two fragments. n.b. the genomic fragment was amplified using primers based on the *Ascaris cut-1* sequence (2a3med and 2ab5ext) whilst the cDNA fragment was obtained using one primer based on the *Brugia* genomic sequence (Br5/3) and the *Ascaris* primer 2a3med.

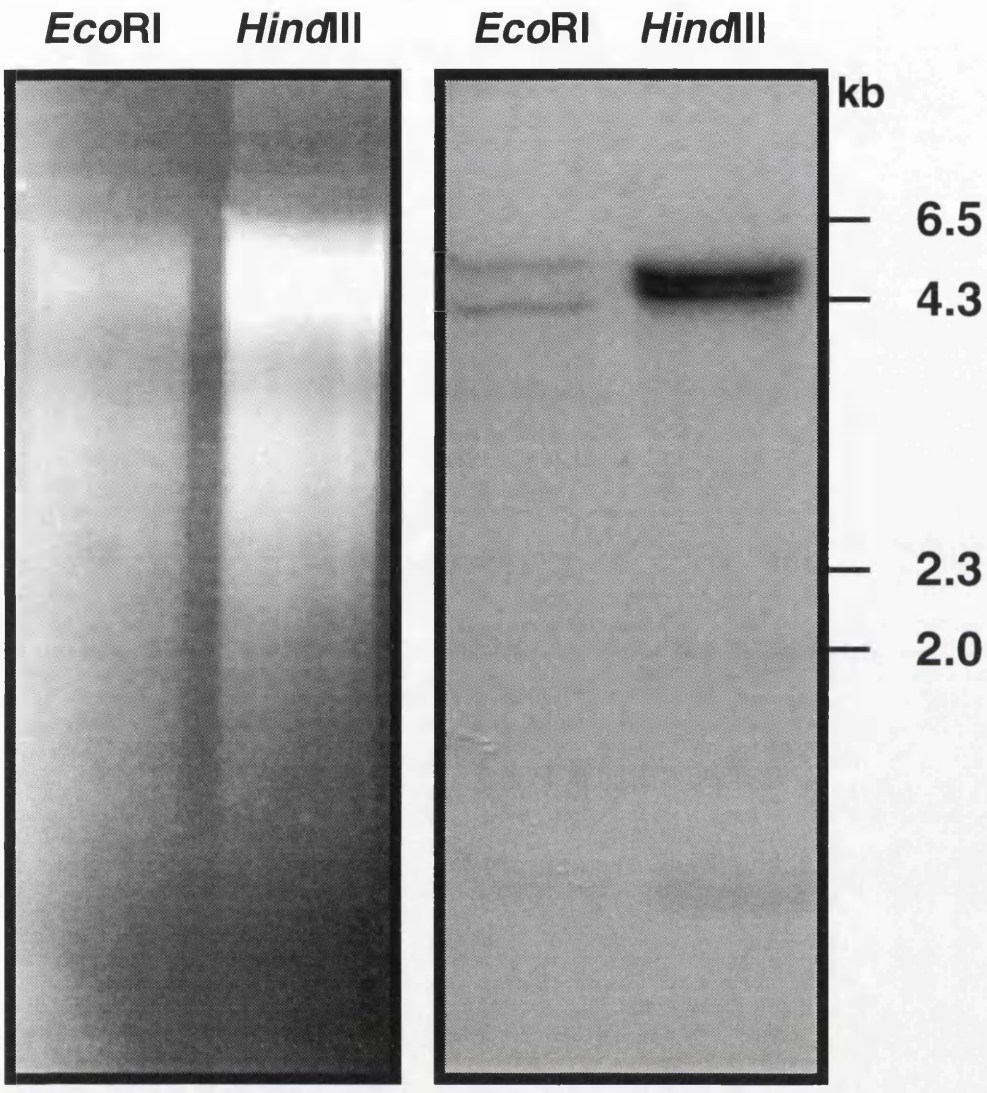


Figure 4.13.

Ethidium gel of cleaved *B. pahangi* genomic DNA, and corresponding Southern blot hybridised to '32', the first *Brugia*-specific cuticlin gene fragment.

20µg of DNA was cut with *Eco*RI and *Hind*III. Hybridisation was carried out at 65°c and the blot washed to 0.2xSSC, 0.1% SDS at the same temperature.

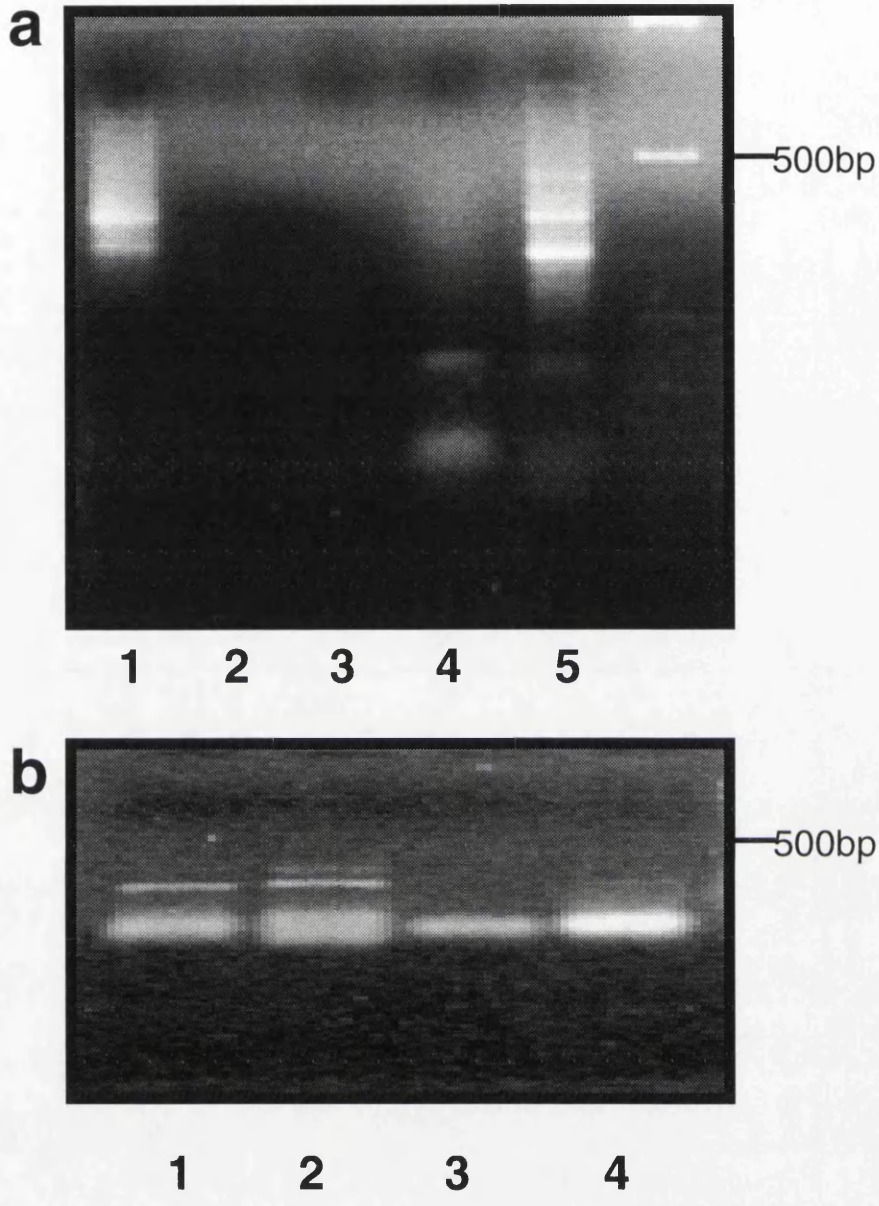


Fig. 4.14. EtBr-stained gels showing electrophoresis of PCR products (10µl per lane).The template for all reactions was *B.pahangi* day 7 p.i.cDNA which was amplified using the following primers:

a. lane 1 oligo(dT) and SL1
lane 2 oligo(dT) and Br5/2
lane 3 SL1 and Br3/2
lane 4 SL2 and Br5/2
lane 5 oligo(dT) and Br5

b. lane 1 SL and CD2
lane 2 oligo(dT)and CD1
lane 3 oligo(dT)primer alone
lane 4 no primer control

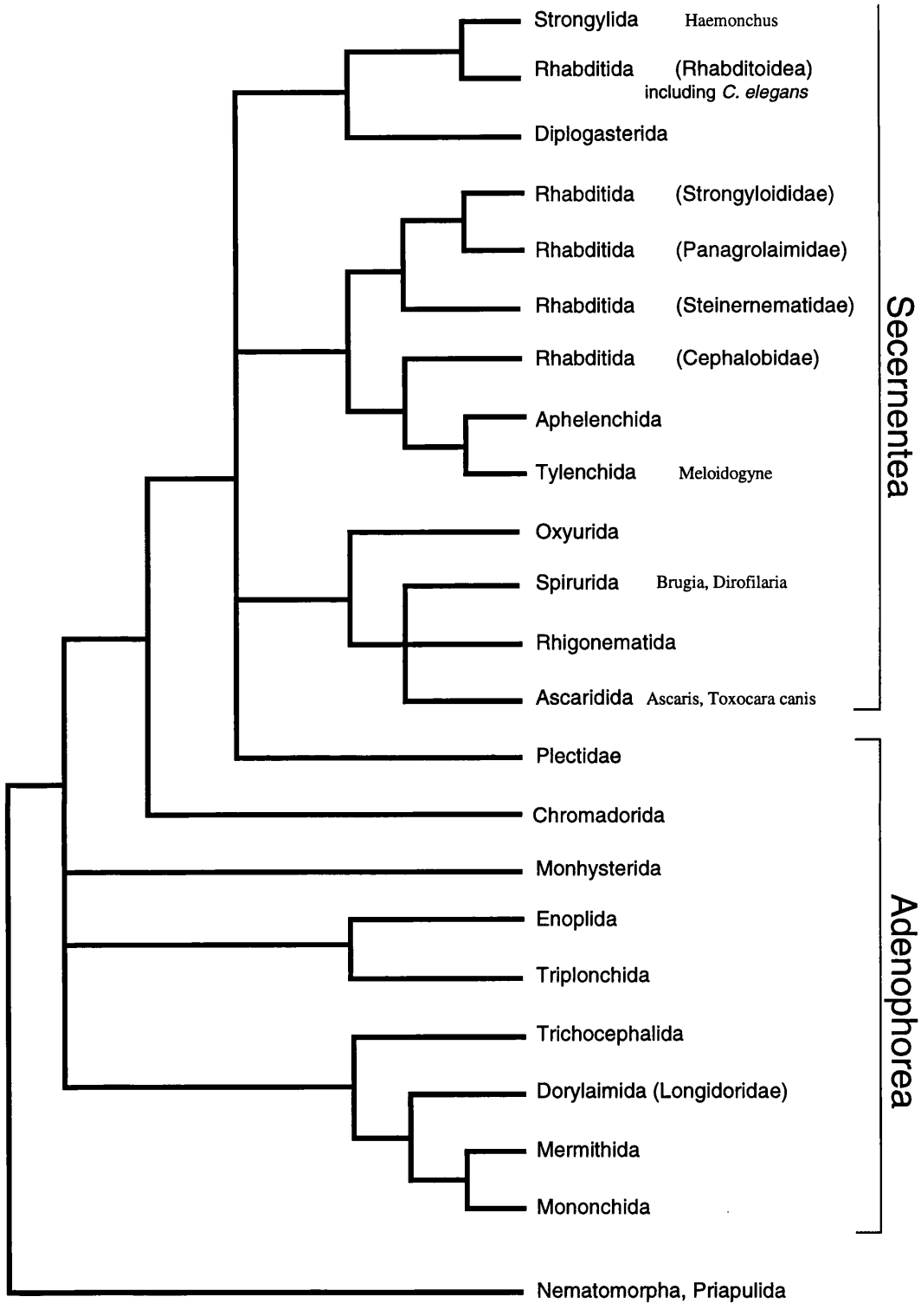


Figure 4.15.

Relationships of the nematodes mentioned in this chapter. The presence of a *cut-1*-like gene has been established throughout the Secernentia. Taxonomy taken from Blaxter et al, in press. Figure courtesy of Mark Blaxter.

CHAPTER FIVE: THE ISOLATION OF GENOMIC AND cDNA *cut-1* CLONES FROM *BRUGIA* DNA LIBRARIES.

5.1 Introduction.

From the experiments outlined in the previous chapter it was known that the *cut-1* genes of *C. elegans* and *A. lumbricoides* have a *B. pahangi* homologue and that it is expressed at 7 days post-infection. Using primers based on the *A. lumbricoides cut-1* sequence, fragments of *Brugia* genomic and cDNA were amplified, cloned and sequenced, to conclusively prove a *Brugia cut-1* identity. It was now decided to use the genomic fragment '32' (known forthwith as *bpcut-1*) to screen *Brugia* genomic DNA and cDNA libraries in an attempt to isolate genomic and cDNA clones of *Brugia cut-1*.

Four libraries were screened, three of the screens resulting in the isolation of *Brugia cut-1* clones. The clones were isolated from a *B. malayi* genomic library, a *B. pahangi* genomic library, and a *B. pahangi* adult cDNA library. In this chapter the screening process is outlined, and analysis of the structure of the clones presented and discussed.

The only genomic *Brugia* library available in the lab at the time was an adult, mixed-sex *B.malayi* library, so the search for a *Brugia cut-1* clone started in *B. malayi,* despite the fact that the initial experiments were carried out using genomic DNA and cDNA from *B. pahangi*. *B. malayi* is a natural parasite of humans whereas *B. pahangi* has a feline natural host, although it is possible to infect humans with both species (Buckley, 1958a; Edeson et al, 1960). It is virtually impossible to distinguish between the species morphologically, and it was only twenty years ago that Denham et al (1975) showed definitively that crossing *B. pahangi* and *B. malayi* did not result in fertile off-spring, and that the two are therefore biologically distinct species. It is estimated that the overall genetic similarity between the two species is 96% (Jorg Hirzmann, personal communication).

5.2 *B. malayi* genomic library.

The library was a gift from Dr. Fran Perler at New England Biolabs, and was made as a genomic expression library. The average insert size is small, 1.3kb. It was constructed using partially digested adult mixed sex *B. malayi* DNA ligated into the vector, λgt11, via cohesive *Eco*R1 adapters. The library represented a sub-set of an original library and was estimated to contain about $3X10^5$ original phage. Full practical details of library screening are given in Materials and Methods. $4X10^5$ plaques were screened in duplicate, to reduce the risk of picking false positives. The filters were probed with ^{32}P-labelled *bpcut-1*. Hybridisation and wash conditions were stringent (65°c, washed down to 0.2XSSC, 0.1% SDS) to prevent non-specific hybridisation to the probe. This high stringency was permitted by the use of the homologous probe, known to be a *Brugia* sequence. At each round of the screening process, putative positive plaques were checked for a cuticlin identity by amplification using *cut-1*-specific primers. Two clones were carried through to a final screen and were found to

represent the same DNA fragment. PCR using primers corresponding to the the λgt11 linker sequence was used to obtain the full-length of the insert, which was then cloned into the TA cloning vector pTag and sequenced on both strands. The sequence of the *bmcut-1* clone is shown in Figure 5.1. The clone covers the highly-conserved region of the *cut-1* gene, and is 613bp long. There are three introns present in the clone, and when they are removed, the coding sequence corresponds to that of *bpcut-1*, and to the *cut-1*-like fragment amplified from day 7 p.i. cDNA.This is shown in Figure 5.2. Two of the intron positions are the same as they are in the *B. pahangi* homologue, while a third appears to be unique, as is shown in Figure 5.3, a pile-up of CUT-1-like proteins from various nematodes. A predicted translation of the coding region gives a peptide sequence which shows 97.5% similarity to the corresponding *B. pahangi* coding sequence over the full length of the clone.

Subsequent to its isolation, the *B. malayi* clone was labelled and used to screen the following libraries.

5.3 Adult *B. pahangi* genomic library

This library was a gift from Prof. Gird Hobom at the University of Geissen. It was constructed in λEMBL3 using size-fractionated (9-23kb) partial *Mbo*I digests of high molecular weight DNA from adult, mixed-sex *B. pahangi.*

$2X10^5$ plaques were screened in duplicate, using labelled *bmcut-1.* Sixteen putative positives were picked in the primary screen and were used as the templates inPCR reactions using primers corresponding to the sequence of *bpcut-1.* The amplification products were run on a gel, blotted and probed with labelled *bmcut-1.* Nine of the sixteen amplification products hybridised strongly to the probe, despite the fact that the hybridising band was barely visible by ethidium staining, as is shown in Figure 5.4. The four phage samples corresponding to the amplified products which hybridised most strongly to the probe were selected to carry through to a secondary screen. All four phage were successfully taken through to a tertiary screen and clonal plaque populations confirmed by hybridisation. The λ clones were named 1a, 4ab, 4ac and 4b, and the inserts are all approximately12Kb long. Preparation of λ clone DNA is described in Materials and Methods.

5.3.1. Restriction enzyme analysis of the genomic clones.

Preliminary restriction enzyme analysis of the cloned DNAs (shown in Figure 5.5) revealed that 4ab and 4ac contain the same insert, and that the inserts in 4b and 1a differ from each other and from 4ab/c.Further analysis facilitated the construction of simple maps of the three separate clones 4a, 4b and 1a. These maps reveal that the three clones correspond to the same region of the *B. pahangi* genome, and that the inserts partly overlap. Fragment 4a was inserted into the vector in the opposite orientation to the other two clones.

The clones share a central *Kpn-Sal*I fragment of about 3.5kb which hybridises to the *cut-1* probe, and is used to orientate the clones in Figure 5.6. The hybridisation pattern of the digest

shows that, at the up-stream end of the clones, 1a and 4a contain an extra 4kb and 6kb of sequence compared to 4b. The clones also have a common *Eco*RI site down stream of the *Kpn-Sal*I fragment, and 4a and 1a have a second *Eco*RI site not present in 4b, as indicated by the fact that the hybridising band in 4b is very large, showing the fragment to be fused to the λ arm. 4b extends down-stream of the other two clones, as shown by a smaller, non-hybridising band in the *Eco*RI digest due to a second site not present in 1a and 4a. The very close proximity of the *Eco*RI and *Hind*III sites on 1a is demonstrated by a double digest where the hybridising band in the *Hind*III and *Eco*RI-*Hind*III digests appear to be the same. The same digest reveals that 4a does not have this HindIII site, as the hybridising band is clearly the λ arm fused to the fragment, so 4a must terminate between the *Hind*IIIand *Eco*RI sites.

5.3.2. PCR using EMBL3 primers.

Primers made against the linker region of EMBL3 were available in the lab.

EMBL3for 5'TCTTGCAGACAAACTGCGCAACTCG3'

EMBL3rev 5'GTTGATTACTGAACACTCTCGTCCGAG3'

The forward and reverse primers were used in combination with 3' and 5' *cut-1*-specific primers to orientate the clone with respect to the direction of transcription of the gene, and to provide further proof that the clones carried a *cut-1*-like sequence. Because the length of DNA to be amplified was unknown and potentially very long, the extension time required in the amplification reaction was considerably prolonged, to seven minutes. DNA from each of the λ clones was used as the template for the amplification reactions. 4b was the only clone to be successfully amplified in this way. It was felt that the increase in the PCR parameters could result in the premature exhaustion of the enzyme activity of the Taq DNA polymerase used routinely in PCR. Consequently, Vent(exo) DNA polymerase was used as a comparison. This DNA polymerase was originally isolated from an organism capable of growth at 98°c and was engineered specifically to give high yields of high-fidelity product over long periods at high temperatures. However, PCR using Vent resulted neither in the amplification of 1a and 4a, nor an increase in the yield of product from 4b. The product size, using the 5' EMBL3 Forward primer and each of two *cut-1*-specific 3' primers from the conserved region of the gene (Br3/2 and Br5/2rev), was about 3.5kb. Using the size of the *C. elegans cut-1* gene as a rough indication of the predicted size of the *B. pahangi* homologue, this PCR fragment should contain all the coding region of the 5' end of the gene. Assuming the map of clone 4b to be correct, the region falls within the 3.5kb Sal*I*-Kpn fragment and, again using the *C. elegans cut-1* gene as a size reference, it is possible that the fragment could also contain the coding sequence of the gene 3' of the primer sequence used in the amplification. However, the

product was not cloned, so the only irrefutable conclusions that could be reached from this line of investigation were that 4b definitely contains sequence homologous to *cut-1,* and that the homologous region appears to be at the amino terminus end of the λ clone; and that transcription is in the *Kpn* - *Sal*I direction, with respect to insertion into the vector. As can be seen from Figure 5.6, clones 1a and 4a extend 5' of clone 4b in the direction which would be amplified by the primers used. Presumably, the failure of the same primer sets to amplify clones 1a and 4a is due to the fact that they are 5kb longer, making the predicted fragment size around 8kb. Extension time in a PCR reaction is roughly calculated as requiring a minute for each kb to be amplified. Even the prolonged extension time of seven minutes could not be expected to result in the successful amplification of a fragment of this length.

5.3.3. Obtaining sequence from the λ clones.

Although restriction enzyme analysis strongly implied that the three clones were representative of the same *cut-1*-like gene, and gene-specific PCR had been carried out successfully on 4b (see above), it was important to obtain sequence data from all three clones to prove definitively that the clones were from the same gene, and that they had a cuticlin identity. To these ends, PCR was carried out on DNA from all three λ clones, using *bpcut-1*-specific primers designed to span the intron known to be unique to the *cut-1*-like gene of *B. pahangi.* The reactions resulted in the amplification of a single product from each clone, the sizes of which were identical. The reactions were repeated, along with two negative controls: one with no DNA template, to ensure that the primers were uncontaminated; and one using an unrelated λ clone, to ensure that the experiment was free of phage contamination. The amplified fragments from 4a, 4b and 1a were subsequently cloned into the TA cloning vector pCRII, and sequenced. The fragments were only sequenced on one strand because the purpose of the experiment was to obtain sequence to show the presence of the 'diagnostic' *B. pahangi* intron in each of the clones. The sequence line-up in Figure 5.7 shows clearly that the clones are identical to each other and to *bpcut-1*, demonstrating that they are derived from the same gene. It is very unlikely that two different genes would have intron sequences which are conserved to the extent seen here, even if they were closely related, so the experiment is conclusive.

To reiterate - at this stage of the project the following clones had been isolated: a *B. pahangi* cDNA clone from day 7 p.i. parasites (2133), the corresponding partial genomic PCR fragment *bpcut-1*, the *B. malayi cut-1* homologue (*bmcut-1*), and four λ clones corresponding to *bpcut-1*. In an attempt to obtain additional cDNA sequence it was decided to screen two *B. pahangi* cDNA libraries.

5.4. *B. pahangi* spliced leader day 3 p.i. cDNA library.

The first library screened was a spliced leader library made from *B. pahangi* L3 worms taken from the jird host at three days post-infection (Martin et al, 1995). The library was made

from 2μg of total RNA. The RNA was reverse transcribed using oligo (dT) as a primer, to ensure that only polyadenylated RNA is primed. The resultant first strand cDNA was then amplified using an SL1 primer containing an *Eco*RI site and oligo (dT) containing an *Xho*I site as primers to optimise the amplification of full-length cDNAs in the PCR reaction. The resultant cDNA PCR products were then digested with *Eco*RI and *Xho*I and directionally cloned into the vector λ Uni-Zap. The library had already been screened successfully to obtain full-length, stage-specific cDNA clones (Devaney et al, 1996). RT-PCR analysis had shown that *bpcut-1* is expressed prior to a moult (outlined fully in Chapter Six), and as early as 3 days post-infection, so it was reasonable to expect a representative clone to be present in a day 3 p.i. cDNA population.

2 $X10^5$ plaques were screened in duplicate using labelled *bpcut-1* as the probe. Several plaques were carried through to a second round of screening, but subsequent investigation, including amplification of eluted phage using *cut-1*-specific primers, revealed that none of the putative positive clones were true positives. In light of what is now known about the structure and expression of the *cut-1* homologue of *B. pahangi*, this result is not surprising: and numerous experiments (recounted in Chapter Four) have demonstrated that there is no reason to suppose the gene to be *trans*-spliced. These facts were obviously unknown at the point at which the library was screened.

5.5. *B. pahangi* conventional adult cDNA library.

This was the library previously used in for antibody screening Chapter Three. It is a *B. pahangi* expression library constructed from mRNA isolated from mixed-sex adult adult worms and cloned into the vector Uni-Zap-XR. 5 X 10^5 plaques were screened in duplicate with labelled *bmcut-1*. Hybridisation conditions were stringent (65°c, washed to 0.1% SDS, 0.1 x SSC). Two hybridising plaques were selected and carried through to a tertiary screen, by which time a clonal population had been achieved. The library was made in λ Uni-Zap, so *in vivo* excision of the pBluescript phagemid was done, allowing further analysis of the inserts to be carried out in a plasmid system. Preliminary restriction enzyme analysis showed the two clones to be the same size, almost 1kb long. The clones were sequenced on both strands and shown to carry the same insert. The clone is 948bp long and is known as ned. The clone commences with a run of lysine residues followed by a sequence of about a hundred base pairs which show no homology to any of the *cut-1*-like genes. Initially this region was thought to be an artefact of the library construction process, when the insertion of two cDNA fragments into the same set of vector arms occurs. This is not uncommon. Homology to the other *cut-1* genes begins approximately twenty codons after the region encoding the proteolytic cleavage sites of the other homologues, and this point in the insert was assumed to be the beginning of the *B. pahangi cut-1* cDNA. As can be seen in Figure 5.3, the predicted amino acid sequence encoded by ned is virtually identical to the corresponding CUT-1-like protein sequences from *C. elegans*, *M. artiella* and *A. lumbricoides.* However, there are two deletions, one encoding

thirty two amino acids and one encoding fifty two amino acids, with respect to the other proteins. At this point it was difficult to know if the cDNA originated from a second *B. pahangi cut-1*-like gene, if it was an alternative splicing of *bpcut-1*, or if it represented an aberrant transcription event unrelated to a functional cDNA species.

5.5.1. Obtaining genomic sequence of ned.

A pair of primers, NF and NR, were designed on the sequence of ned which was not present in *bpcut-1* (32). The primers are shown in Figure 5.9. The purpose of the primers was primarily to carry out RT-PCR on various life-cycle stage cDNAs of *B. pahangi,* in order to establish whether or not the corresponding gene has the same expression pattern as that of *bpcut-1.* Also, it was hoped to establish whether or not the amino acid deletions observed in ned were biologically meaningful, as opposed to being library-construction artefacts, by amplifying a band from adult cDNA which was the same size as that amplified from ned, using the same primers. The results of these experiments are presented and discussed in Chapter Six.

The primers were also used to amplify *B. pahangi* genomic DNA. The primers span a region corresponding to 237bp of ned, and amplify a 700bp fragment from genomic DNA. The PCR product was cloned into the TA cloning vector pTAg, and sequenced on both strands. The genomic DNA from which ned derives (known as ngen) is 756 bp long and contains the whole of the *bpcut-1* (32) sequence, including the intron, leading to the conclusion that 32 and ngen derive from the same gene. Thus it would appear that the gene is 'alternatively' spliced to give two different peptides: the first splice results in a cDNA coding for a product which corresponds exactly to the other CUT-1-like homologues present in *A. lumbricoides* and *C. elegans* and in 2133, the PCR derived cDNA clone from day 7 p.i. *B. pahangi* and corresponding to *bpcut-1* (32); whilst the 'alternative' splicing results in ned, coding for a protein with substantial amino acid deletions in a region of extreme conservation amongst the other CUT-1 proteins. The 'alternative' splicing involves deletions from regions which are coding regions according to the 'common' splice: the position of the first intron in ngen is shared with the *B. malayi* genomic clone in the common splicing, whilst in the alternative splicing, sequence from the coding region surrounding the intron is removed, resulting in the first 55 amino acid deletion; and the intron of *bpcut-1* (32) is contained within a much larger region which is removed in the alternative splice to give the second 32 amino acid deletion seen in ned. Figure 5.8 shows the ngen sequence with the two intron/exon patterns marked in order to compare them.

There are several explanations for the occurrence of ned as the result of screening the adult cDNA library. Firstly, it could represent a second *cut-1*-like gene. This is unlikely as PCR on genomic DNA using primers made on the sequence of ned gave a product identical to *bpcut-1* (32). Secondly, ned could derive from an alternative splicing event, either with or without biological significance. Thirdly, the clone could be an artefact produced during

construction of the cDNA library. And finally, it could be an artefact which arose as a result of mis-transcription in the worm, and was randomly reverse transcribed and hence represented in the library. These alternatives are analysed in more detail in the Discussion.

5.6 3' and 5' RACE on *B. pahangi* cDNA.

RACE (rapid amplification of cDNA ends) was developed in order to obtain full-length cDNA clones of low-abundance mRNA transcripts (Frohman et al, 1988). It uses PCR techniques to amplify cDNA between a known, short internal sequence and its unknown 3' and 5' ends. The technique was used at two different points in the project: it was first attempted in order to preclude the screening of a cDNA library, subsequent to cloning and sequencing 2133, the *bpcut-1* cDNA fragment obtained using RT-PCR on day 7 p.i. cDNA; and it was used a second time in order to obtain the full-length cDNA of ned.

In the first instance both 3' and 5' RACE were used. A brief outline of the principles involved are included here; full experimental details are included in the Materials and Methods chapter. In 3' RACE, first strand cDNA synthesis is carried out using a primer which is based on the oligo (dT) sequence, with the addition of an 'adapter' region, consisting typically of restriction enzyme recognition sites common in the polylinker region of cloning vectors. In this way all amplified cDNAs should encode the 3' end of a gene, and should carry the adapter sequence that can later be used to facilitate cloning of the product.

oligo (dT) - adapter primer

*Mbo*I *Mlu*I *Xho* I

GTCAGATCTACGCGTCGACCTCGAG $(T)_{17}$

*Bgl*II *Sal*I

Adapter primer

*Mbo*I *Mlu*I *Xho* I

GTCAGATCTACGCGTCGACCTCGAG

*Bgl*II *Sal*I

A gene-specific amplification is then carried out, using a 5' primer based on the known sequence of the gene to be amplified, and the 3' adapter primer. The use of the adapter primer increases the specificity of the reaction by ensuring that only those cDNAs already tagged with the adapter-oligo (dT) primer, and therefore full-length, are amplified. Hence the amplification products should be made up only of cDNA species representing the 3' end of the gene of interest. In theory the use of an oligo (dT) primer in combination with a 5' gene-specific primer should amplify the same specific sub-set of cDNAs, but in reality the oligo (dT) primer has a tendency to anneal to sequences internal to the poly-A tail, resulting in truncated

cDNA clones. If the mRNA species is represented at a particularly low level, or the amplification of the target cDNA results in a mixture of products, it is necessary to do a second round of amplification, using a second gene-specific primer internal to the original. This step increases the product specificity and should result in a band that can be visualised on an ethidium gel.

In 5' RACE (otherwise known as 'anchored' PCR), first strand cDNA is synthesised using a gene-specific primer which amplifies towards the 5' end of the gene, so that only specific mRNA, or related mRNAs, are represented in the cDNA population. A homopolymer tail is then added to the 3' ends of the cDNAs. A second gene-specific primer is then used in combination with a primer complementary to the homopolymer tail to amplify the 5' end of the cDNA.

First strand cDNA was synthesised from days 5 and 7 p.i. RNA because it was already known, from RT-PCR experiments, that the mRNA was transcribed at these stages. In addition, preliminary immunofluorescence experiments (Chapter Six) appeared to show that the gene product was present in the cuticle of mature mf, so it was decided to also use adult RNA to make first strand cDNA, on the grounds that it would also contain abundant microfilarial mRNA. Total RNA was used in these experiments. The primers used in the course of the RACE are shown in Figure 5.9.

5.6.1 3' RACE.

Two 5' primers based on the *A. lumbricoides cut-1* sequence (2AB5Ext and 5MRExt) but known to amplify *bpcut-1*, and a 5' primer corresponding to the *bpcut-1* (32) sequence (Br5), were used as the first gene specific primer to amplify all three cDNA samples. A more internal *Brugia* primer, BR5/2, was used in the second round of amplification. To ensure that the first strand cDNA synthesis had worked, amplification reactions were carried out using the cDNA as a template with a pair of internal *Brugia cut-1* primers, and with primers made on the sequence of a ribosomal protein known to be constitutively expressed. The amplification of a product of the expected size indicates, in the case of the ribosomal primers, that first strand synthesis has been successful. In the case of the cuticlin primers it implies the presence of cDNAs with a cuticlin identity in the cDNA population. Despite the fact that these controls were positive for the day 7 p.i. and adult first strand cDNA, further gene-specific amplification did not result in the isolation of the 3' end of a cuticlin cDNA. No product could be visualised after the first round of gene-specific amplification, and the second round resulted in a mixture of products that could be visualised on an ethidium gel as a smear which, when blotted and hybridised with either an end-labelled *cut-1* oligonucleotide or labelled *bpcut-1*, did not show a signal, implying that amplification in the second round had been spurious.

5.6.2 5' RACE.

RNA from adult and day 7 p.i. worms was used to make first strand cDNA. The gene-specific 3' primer 2A3Med was used in the first strand synthesis. It was made on the *A. lumbricoides cut-1* sequence but amplifies *bpcut-1* very well. A *Brugia*-specific primer, Br3/2, was used as the second gene-specific primer. As a control, the gene-specific primers provided in the LIFE TECHNOLOGIE 5' RACE kit were used in parallel with the cuticlin primers. If all the steps of the technique are successful, the control product should be seen as a distinct band of a known size. In this case the adult RNA gave a negative result (first strand synthesis did not appear to be successful) and the final amplification product for the day 7 p.i. and control reactions were visualised as smears on an ethidium gel. When probed with a labelled *cut-1* oligonucleotide internal to those used in the amplifications, the probe hybridised non-specifically to the smear, rather than to specific bands in the product. Further amplifications of the product with cuticlin primer pairs and different 3' cuticlin primers with the anchor primer were carried out in an attempt to obtain a distinct band or banding pattern. These attempts were uniformly unsuccessful.

At this point it seemed evident that obtaining a full-length *Brugia* cuticlin cDNA clone by the use of RACE was not a straightforward procedure, and that screening a cDNA library was therefore the remaining option (the screening process has already been described earlier in this chapter).

5.6.3 The use of RACE in an attempt to obtain the 3' end of the putative embryonic *B. pahangi cut-1*-like gene.

As has already been discussed, the screening of an adult *B. pahangi* cDNA library resulted in the isolation of a *cut-1*-like clone, which was sequenced, and is known as ned. The length of the clone in comparison to the *A. lumbricoides* and *C. elegans* homologues, implied that the clone lacked a significant amount of the 3' region. This sequence is the most interesting with respect to comparison of the cuticlin genes within and between nematode species, because the genes show greatest divergence at the 3' end. On these grounds it was decided to make a second attempt at 3' RACE. Primers were made corresponding to the sequence of ned. To increase the probability of successfully amplifying a cuticlin transcript, mRNA from adult *B. pahangi* was purified from total RNA and contaminating proteins and DNA. This procedure is more fully described in Chapter Six and in Materials and Methods, and yields an essentially pure fraction of polyA+ mRNA, which was used in the first strand cDNA synthesis step. Three primers corresponding to the the *cut-1* sequence were used in the first gene-specific amplification: Br5/2 (corresponding to the 32 sequence), 5mrext (corresponding to the *A. lumbricoides cut-1* sequence), and NF (corresponding to the ned sequence). R1, the 5' ribosomal protein primer, was also used as a positive control. Br5/2 was used as the second gene-specific primer in all cases. As can be seen in Figure 5.10, the first round of amplification resulted in products which could not be visualised on an EtBr-stained gel. A second round of amplification, carried out on two separate occasions, resulted in a confusing

array of bands. However, hybridisation of the blotted products with labelled 2A3Med clarified the results to some extent: the probe hybridised strongly to several bands in the products amplified using Br5 and 2AB5Ext, and did not hybridise to any of the bands in the NF amplification products.

5.7 DISCUSSION.

The screening of *B. pahangi* genomic and adult cDNA libraries resulted in the isolation of four λ clones representing a single *bpcut-1* gene and a cDNA clone (ned) corresponding to the same gene, but spliced differently from the *cut-1*-like genes already isolated from various nematodes. The *B. malayi* homologue was isolated by screening a genomic expression library. The PCR derived genomic clone '32' and day 7 p.i. cDNA clone '2133' are also representative of the same *bpcut-1* gene. The Chapter Six demonstrates that the day 7 p.i. cDNA cannot be detected in adult parasite mRNA, but that anti-CUT-1 antisera recognise a protein present in mature microfilariae. This would imply the existence of a second *cut-1*-like gene which encodes an embryonic CUT-1 protein. When the alternatively-spliced cDNA ned was isolated it was thought that it might represent the embryonic gene, but subsequent experiments and sequence analysis revealed that this was not the case (the nature of ned is discussed further in the latter part of the discussion) and therefore that the second, embryonic *B. pahangi cut-1* gene has not been isolated in the course of this project.

Other *B. pahangi cut-1*-like genes?

The *Brugia* genome is presumed to be the same size as that of *C. elegans*, which is $1X10^8$bp, and contains approximately 12,500 genes (Hodgkin et al, 1995). Taking into consideration the average insert size of the genomic library, it can be calculated that screening 5 $X10^4$ clones will provide a 95% chance of finding a clone representing a single copy gene in the library, assuming that the screening conditions will allow its recognition. The library had been amplified, which in fact makes any subsequent calculations unpredictable, if not meaningless, but the number of plaques screened in this experiment vastly exceeded the number required. In retrospect, the reasoning used to choose the putative positives with which to continue analysis was somewhat flawed: clones were selected on the basis of strength of hybridisation to bands resulting from amplification of the phage with two *Brugia*-specific *cut-1* primers. The four clones which hybridised most strongly were selected, and the others discarded when a cuticlin identity had been irrefutably proven for the four chosen. It now seems obvious that the clones chosen by this method will have all derived from the same gene, having the same specificity for the primers and the same affinity for the probe (this assumes that the input DNA in the PCR reactions i.e the number of plaques, is approximately equal). If clones had been picked which showed a range of hybridisation affinities, it is much more likely that they would have derived from different *cut-1*-like *B. pahangi* genes, with sequences not quite specific to the primers and not completely homologous to the probe. The

analysis carried out on the four genomic clones, however, leaves no doubt that they are all derived from the same gene. Thus it is perfectly possible that a relatively limited re-screening of the *B. pahangi* library would result in the isolation of another *cut-1*-like gene or genes.

Splicing.

From the results outlined in this chapter, it seemed initially that the *B. pahangi cut-1* homologue thus far isolated represents a single gene which is alternatively spliced. In this way a single gene product can give rise to two (or more) separate mRNA species, and ultimately differential expression from the same gene. Alternative splicing has been exhaustively studied in a variety of viruses, and in *Drosophila*, where the pathway of sex determination involves the interaction between a series of genes in which alternative splicing events distinguish between males and females (Saltz et al, 1989). As well as splicing alternative 5' sites to the same 3' site, and the same 5' site to alternative 3' sites, there are examples of internal exons being substituted, added or deleted from primary transcripts (Lewin, 1994).

There are many examples of nematodes using alternative splicing for widely differing purposes. In some cases the alternative splicing is regulatory, whilst in other cases it is the result of a complex and often not fully-understood regulatory pathway. Developmentally regulated alternative splicing of the alpha 2(IV) collagen gene has been reported in *C. elegans* (Sibley et al, 1993) and *A. lumbricoides suum* (Pettit et al, 1994). Dodemont et al (1994), working on the multigene family encoding the cytoplasmic intermediate filament proteins of *C. elegans*, report alternative splicing in three of the genes via the use of different promoters and alternative *cis*-and *trans*-splicing sites, correlated with cell-type as well as developmental expression of the 'original' pre-mRNAs. A recent discovery (Zorio et al, 1997) identifies an alternative splicing in the gene of U2AF, a protein which forms part of the spliceosome of *C. elegans.*, and is essential in the binding of the U2 snRNP to the 3' splice site during splicing of an mRNA.

The cuticlin genes could be regarded as being reasonable candidates for alternative splicing, in that they appear to constitute a small multigene family and probably encode proteins that perform a range of related functions: alternative splicing of a limited number of ancestral genes represents an economical way of achieving functional protein divergence. However, in this case the evidence would imply that ned, the cDNA isolated in the library screening, is not the result of a biologically significant alternative splicing event. Firstly, all the *cut-1*-like genes of the nematodes so far isolated, in addition to those which have been identified by the *C. elegans* genome sequencing project, share an extremely conserved amino acid sequence which is retained almost to identity between all the genes, despite the fact that the sequences flanking the region show little homology. This is a very clear indication that this particular amino acid sequence, more than the nature of the rest of the gene, is what has been stringently selected for during evolution, and therefore it must have an extremely important function. Hence it is somewhat odd that the alternative splice seen in ned results in the deletion of this most conserved region of the gene. Of course this could indicate a biological

significance; if the cuticlins turn out to be a family of structural proteins which, like the collagens, are the result of evolutionary fine tuning wherein different genes encode proteins which are spatially, temporally and stage-specifically regulated, it would make perfect sense that the alteration of an important amino acid sequence could result in a useful novel adaptation of a vital protein which would be selected for and retained. Sequence analysis and comparison do not favour this hypothesis however. As seen in Figure 5.3, the sequence of ned conforms to the homology between the polypeptides apart from three unambiguous deletions which vary in length. However, the original sequence of ned reveals that in all three cases these deletions are also accompanied by a frame-change in the translation of the cDNA sequence i.e. there is no continuous reading frame running throughout the clone. If this 'alternative' splicing encoded a functional protein, it would by definition have to comprise a continuous open reading frame which could be read without interference by the translation apparatus in whichever cell and life-cycle stage the protein contributed to. This is strongly indicative that ned represents a mis-transcription rather then an 'alternative' splice.

A further proof that the clone was the result of a nonsense splice is that the intron boundary sequences in no way match the recently released *C. elegans* intron consensus sequences, which are the result of the full characterisation of 200 genes with over 650 introns. The survey reveals that the nematode introns obey the vertebrate GU-AG rule, but that in *C. elegans* there is an extended, highly conserved 3' splice site consensus (Blumenthal et al, 1997). As previously stated, ned has, at it's 5' end, a string of T residues which were initially assumed to be an artefact from the library-making process. It would now seem that the clone is in fact the result of a mis-splicing which probably initiated with *bpcut-1* being transcribed on the 'wrong' strand i.e. on the template rather than the coding strand, and that the T residues in fact indicate the end of the cDNA, the poly-A tail. The most significant piece of evidence for this is that when the sequence of ngen is reversed and complemented, as shown in Figure 5.11, the intron boundaries conform well to those predicted by the *C. elegans* intron consensus sequences. Additionally, there is an *Xho* I site at the beginning of ned and an *Eco*RI site at the 3'end, which indicates the direction of transcription of the clone: one of the primary advantages of using the UNI-ZAP vector is that it selects for unidirectional cloning; cDNA was made directionally for inclusion in the library by the use of an oligo (dT)-*Xho*I primer in the first strand cDNA synthesis, such that the direction of transcription of an isolated clone can always be known.

Supposing this to be the case, how would such an error arise? The most reasonable explanation is that the mistake was initially made at the promoter recognition level of RNA processing. From the RT-PCR data shown in Chapter Six, it is known that the *B. pahangi cut-1* gene product resulting from the 'classic' splicing, is expressed around the L3-L4 and the L4-adult moults. It cannot be detected, with the primers used, by amplifying adult first strand cDNA, implying that although the protein encoded by the gene may be present in the adult worm, the gene itself is not expressed at this stage of the life-cycle. Much is known about the

transcriptional regulation of genes, but in many cases the precise details of how this complex regulation is accomplished are still under investigation. Very simply, proteins or 'transcription factors' interact with promoters and enhancers on the DNA to attach the transcription complex, consisting of RNA polymerase II (in the case of mRNA synthesis) and various associated proteins, to the correct starting point for initiation of transcription, which then proceeds, the result being a strand of pre-mRNA identical to the coding strand of the DNA. In this case it would be possible to imagine a scenario where a transcription factor or factors recognised a sequence on the *bpcut-1* DNA that was not a functional promoter, in the sense that transcription from that starting point did not result in an mRNA of the right length, sequence or configuration. It is known that differential use of starting points on a pre-mRNA transcript is one method of generating alternatively-spliced mRNAs, so if a pre-mRNA has been inappropriately transcribed using the 'wrong' promoter, this could well result in the type of aberrant alternative splicing that would seem to be represented by ned. Perhaps the transcription was initiated at the functional promoter of an upstream gene to the *cut-1* gene, on the opposite strand, and transcription termination was not fully accomplished, resulting in the transcription and subsequent mis-processing of the *cut-1* sequence.

There are in fact examples of biologically significant alternative gene products generated by transcription and processing of an already characterised gene on the opposite strand (Varkey et al, 1995). The product, which may or may not be translated, could potentially be used in the regulation of the 'original' gene. However, there is not enough evidence available to make a judgement on whether or not ned might have such a regulatory function for *bpcut-1*. However, the fact that ned was isolated from an adult cDNA library, whilst the *bpcut-1* mRNA transcript is not represented in adult mRNA (as the results of Chapter Six demonstrate), imply that the chances of ned autogenously regulating *bpcut-1* are relativley small.

These results beg the question of why the screening of the library resulted in the isolation of a presumably rare aberrant cDNA clone, rather than that of the cDNA relating to a cuticlin gene expressed either in the adult worm or in the microfilariae. This is presumably the result of the conditions under which the screening was carried out: hybridisation and subsequent washing conditions were stringent, and the probe used was labelled *bmcut-1*, the homologue of *bpcut-1*. Assuming that all *cut-1* like homologues carry the highly conserved amino acid sequence seen in those already identified, the third base changes seen between nematode *cut-1* genes already take the DNA sequence homology down to around 70%, even in stretches which code for an amino acid homology of almost 100%. If the amino acid homology is not maintained to this level in other homologues, sequence homology with the *B.malayi* probe would not be good enough to detect a strong signal under stringent hybridisation conditions. In summary, the cDNA corresponding to *bpcut-1* is not represented in the adult library, and presumably the conditions used were not permissive enough to identify other potential *cut-1* clones, which may be present in an adult cDNA library.

Comparing the nematode *cut-1*-like genes.

The cloning of two homologous *cut-1*-like sequences from *B. malayi* and *B. pahangi* allow us to compare genes from near identical species, and allow their addition to the sequence and structural comparison of *cut-1* genes from widely differing nematode species that exists already. The structural and evolutionary relationships, and their significance, between the various *C. elegans cut-1*-like genes and the *A. lumbricoides* and *Meloidogyne* homologues, has already been discussed in the Introduction. The addition of the limited *Brugia* genes sequences allows the emergence of an interesting pattern of intron position in the stretch of sequence encoding the region of highly conserved amino acids: two introns are shared between the *C. elegans* genes that are not present in the parasitic homologues; the four genes from parasitic nematodes have a common intron not present in the *C. elegans* sequence; there is one *Brugia*-specific intron not seen in the *A. lumbricoides* or *Meloidogyne* genes; and finally, the *B. malayi* clone has an intron additional to those shared with *B. pahangi*. There is also an intron which seems to be unique to the *A. lumbricoides* gene, but the sequence is up-stream of the point where there is *Brugia* sequence available, so it is not possible to say whether or not it is present in *B. pahangi*. As is well known, the evolutionary pressure on intron sequences is weaker than on coding sequences, because they do not contain deleterious sequence and are therefore not selected against. Hence introns can be lost or gained or extensively modified without altering the fidelity of the protein encoded by the gene i.e. the changes are selectively neutral, and therefore occur frequently and rapidly (in evolutionary terms!). Intron position in autologous genes can therefore imply certain things about the point at which related organisms diverged, although it may be unwise to draw firm conclusions as to relatedness by these means alone. But the fact that introns are shared exclusively between the *C. elegans* genes and between the parasitic nematode genes would imply that the parasitic nematodes diverged from the soil-dweller simultaneously, or at least at time points very close together. This is surprising because one hypothetical scenario for evolutionary relationships between Nematode orders predicts *Meloidogyne* to be more closely related to *C. elegans* than to either of the vertebrate parasites (Malakhov, 1994), which makes sense in terms of the ecology of the Nematode groups. It is to be expected that the two *Brugia* genes would share a common intron, and the fact that *B. malayi* appears to have a unique intron could imply that *B. pahangi* evolved from *B. malayi*, due to the fact that there are few examples of introns being gained by evolution, which is reductionist in its approach to introns.

Discussion of the RACE results.

Although the 3' RACE carried out on adult polyA+ mRNA did not result in obtaining the 3' end of ned, the results were nonetheless interesting.

In retrospect it is obvious that this experimental procedure could not work using the primer NF to obtain the 3' end of ned: as can be seen in Figure 5.8, a comparison of ned and *bpcut1* show that Br5/2, the gene-specific primer used in the second round of amplification, is deleted

from the sequence of ned; the sequence corresponding to the oligo 2A3Med, which was labelled and used to probe the blotted products, is also deleted from the cDNA of ned, although both sequences are present in ngen, the genomic DNA corresponding to ned. It would seem most likely that ned does not represent a biologically meaningful alternative splicing of *bpcut-1*, but instead arose due to faulty recognition by a promoter, or some other error in transcription. As such the transcript is likely to be represented in the mRNA in minute quantities that would not be detected in a first round of amplification by ethidium staining, and will not be detected by hybridisation to the oligonucleotide used in these experiments. To confirm this theory, it would be interesting to re-do the second round of amplification using a primer known to be present in the sequence of ned, and to probe it with either an internal oligonucleotide or simply a labelled fragment of *bpcut-1*.

It is also interesting to find that the amplifications were apparently positive using the primers which are known to amplify the cDNA which represents the 'classic' splicing, found in the *C. elegans* , *B. malayi* and *A. lumbricoides* *cut-1* genes. Primers from the same region were used in the RT-PCR experiments described in the following chapter, which clearly show that the transcript is not present in adult *Brugia* mRNA at a level which can be detected by hybridisation after thirty rounds of PCR amplification. These results are not contradicted by the RACE result, where hybridisation was only detected after a second round of amplification. There are three possible explanations for this result: the first is that the true transcript is present at a very low level in the adult worm which is not detected by normal RT-PCR; the second is that there is a second aberrant splice which results in a transcript which can be amplified using these primers; and the third option is that the primers are amplifying a third *cut-1*-like gene, which is normally expressed either in the adult worm or the microfilariae at levels which would be detectable using specific primers, but which is amplified fortuitously but only at very low levels, by these primers. From the immunofluorescence results, it is known that CUT-1, or at least a protein which is recognised by anti-CUT-1 antisera, is being expressed at high levels in the mature microfilariae, and that therefore the transcript must be represented at significant levels in adult worm RNA. Meanwhile, it is also known from the RT-PCR results that the 'classic' *bpcut-1* mRNA is not present in mRNA from adult worms. Therefore there must be a second *Brugia* *cut-1*-like gene which codes for the product seen to localise on the surface of the microfilariae. Obviously it would be a piece of serendipity if the 3' RACE product, obtained using mRNA from adult worms, was in fact the cDNA relating to this second *cut-1*-like gene. To establish whether or not this was in fact the case it would be worthwhile cloning and sequencing the hybridising products.

The 3' RACE products resultant from a primary round of amplification with 2AB5Ext and a secondary round with Br5/2, show two strongly hybridising bands which are between 0.5-1kb long, and a larger band of around 1-1.2kb. A hybridising band of the same size is also present in the product resultant from a primary amplification with Br5, and a secondary with BR5/2. The RACE protocol was carried out twice to show reproduceability (see Figure 5.10) and

show that the products of the same reactions hybridised strongly to the cut-1 probe, altough the pattern of hybridising band s was different. Assuming that the length of the *A. lumbricoides* and *C. elegans cut-1* proteins will be similar in the *B. pahangi* homologue, the predicted size of the product of an amplification between Br5/2 and the 3' end of the cDNA is 600-700bp. Therefore the bands hybridising in the experiment could represent the 3' end of a *cut-1*-like cDNA. However, it must be stated that the *bpcut-1* oligo, Br5/2, which was used as a probe in the hybridisation of the RACE products, does not give a signal when used in amplification reactions on adult Brugia cDNA (see Chapter Six for a full description of these experiments). This is not in agreement with the theory that the RACE products could represent the embryonic *cut-1* gene, which would obviously be represented in the adult first strand cDNA. To reiterate what has been stated in the previous paragraph: the only way to confirm whether or not the RACE products represent the 3' end of the *Brugia* embryonic *cut-1*-like gene is to sub-clone and sequence the products. The fact that more than one band hybridises could be because the primers are recognising more than one *cut-1*-like transcript representing different genes in the cDNA population. Alternatively, it could be the result of the oligo (dT) primer recognising and 'sitting down' on a sequence internal to the 3' end of a *cut-1*-like mRNA, resulting in the reverse transcription of a truncated cDNA species. Although the presence of the adapter primer considerably reduces spurious priming in subsequent amplifications, it cannot prevent it during first strand synthesis. Consequently, amplification using gene-specific primers could result in the amplification of truncated versions of the cDNA, in addition to its full 3' length.

An explanation for the fact that the first attempts at 3' and 5' RACE were unsuccessful is that the cDNA was made from total RNA, rather than the purified, mature mRNA used in the later 3' RACE. This could either be because enriching for mature mRNA increases the likelihood of reverse transcribing rare transcripts such that they are represented in the first-strand cDNA pool. An alternative explanation is that purifying the mRNA of all extraneous material allows the reagents and conditions in subsequent reverse transcription and amplification reactions to operate cleanly and therefore optimally, yielding better results.

5.8 SUMMARY OF RESULTS.

- Four genomic clones covering the same region of the *B. pahangi* genome have been isolated, and encode part of a *cut-1*-like gene, corresponding to the *B. pahangi cut-1* fragments isolated by PCR from genomic and day 7 p.i. cDNA.
- A *B. malayi* genomic clone of 613bp has been isolated and sequenced and appears to be the homologue of the *cut-1*-like gene mentioned above.
- A cDNA clone of 1kb was isolated from a *B. pahangi* library and sequenced. The genomic sequence corresponding to the cDNA clone was obtained by PCR. Comparison and analysis of the sequences reveal that the genomic sequence corresponds to the previously isolated *cut-1*-like gene and that the cDNA clone represents a transcription on

the opposite strand of the DNA from which the *bpcut-1* message is transcribed. However, thus far this sequence represents the longest stretch of *Brugia* specific *cut-1*-like gene that has been isolated.

5.9 OUTSTANDING ISSUES.

- Obviously the next step in this project would be to isolate the embryonic *cut-1*-like gene. As has already been discussed, this could be attempted using RACE, or by the further screening of the *B. pahangi* genomic library using more permissive hybridisation conditions.
- The discovery that ned represents an aberrant splicing event means that the project has not succeeded in isolating the full-length cDNA corresponding to *bpcut-1*. Using primers made on the sequence of ned known to be homologous to other *cut-1*-like cDNAs (and therefore more likely to be present in the 'real' *bpcut-1* cDNA) further RACE could be carried out on cDNA from the life-cycle stages of the parasite where transcription of the gene is known to be occur. To maximise the chances of success the mRNA used in the first strand cDNA synthesis could be purified of immature RNA species and other contaminants.

- The λ clones isolated by screening the adult *B. pahangi* genomic library certainly incorporate the entire *bpcut-1* gene. According to the mapping of the clones that has already been done, it is likely that the gene is situated in the central *Sal*I-*Kpn*I fragment of the clone. Sub-cloning and sequencing this fragment would therefore probably yield the entire sequence of *bpcut-1*.

```
1     tgtaattccc tccatccact gtccGTCACA AAAGTGATCG AGCATACCGA
51    GTGCAGTGCT TCTATATGGA AGCTGACAAA ACGGTCAGCA CCCAGATTGA
101   GGTGTCTGAA ATCACAACTG CTTTTCAAAC TCAAATTGTC CCCATGCCTG
151   TTTGTCGATA CGAGgcatgt ctttttgaat tttttgaatt tgcatttatt
201   tatacaattc aaatcaaaca tgaaaaatct atttatcaca tagtagatgg
251   tgacaaatat tttagATTTT GGATGGTGGA CCAACCGGAC AGCCAATTCA
301   ATTTGCTACC ATTGGCCAAC CAGTTTATCA CAAATGGACC TGTGATTCCG
351   AAACCGTTGA TACCTTCTGC GCAGTTGTCC ACTCCTGCTT TGTGGATGAT
401   GGCAACGGTG ATACGGTGGA AATTCTGATG CAGATGGTTG TGCTCTCAAA
451   ATACTTGCTG AAAATTTGGA ATATCCGACA Ggtaagtgag ataatcggta
501   aggcaatcaa caattagcac taatttcttc actttccttt cgaattccgc
551   ggaattccgc ggaatgccag ctgagcgccg gtcgctacca ttaccagttg
601   gtctggtgtg agaatcacga attctggtcc
```

Figure 5.1a Sequence of the *B. malayi cut-1* genomic clone isolated from a *B. malayi* genomic library, showing introns (lower case).

```
      GTCACAAAAGTGATCGAGCATACCGAGTGCAGTGCTTCTATATGGAAGCTGACAAAACGG
    1 ---------+---------+---------+---------+---------+---------+ 60
      CAGTGTTTTCACTAGCTCGTATGGCTCACGTCACGAAGATATACCTTCGACTGTTTTGCC

        H   K   S   D   R   A   Y   R   V   Q   C   F   Y   M   E   A   D   K   T   V

      TCAGCACCCAGATTGAGGTGTCTGAAATCACAACTGCTTTTCAAACTCAAATTGTCCCCA
   61 ---------+---------+---------+---------+---------+---------+ 120
      AGTCGTGGGTCTAACTCCACAGACTTTAGTGTTGACGAAAAGTTTGAGTTTAACAGGGGT

        S   T   Q   I   E   V   S   E   I   T   T   A   F   Q   T   Q   I   V   P   M

      TGCCTGTTTGTCGATACGAGATTTTGGATGGTGGACCAACCGGACAGCCAATTCAATTTG
  121 ---------+---------+---------+---------+---------+---------+ 180
      ACGGACAAACAGCTATGCTCTAAAACCTACCACCTGGTTGGCCTGTCGGTTAAGTTAAAC

        P   V   C   R   Y   E   I   L   D   G   G   P   T   G   Q   P   I   Q   F   A

      CTACCATTGGCCAACCAGTTTATCACAAATGGACCTGTGATTCCGAAACCGTTGATACCT
  181 ---------+---------+---------+---------+---------+---------+ 240
      GATGGTAACCGGTTGGTCAAATAGTGTTTACCTGGACACTAAGGCTTTGGCAACTATGGA

        T   I   G   Q   P   V   Y   H   K   W   T   C   D   S   E   T   V   D   T   F

      TCTGCGCAGTTGTCCACTCCTGCTTTGTGGATGATGGCAACGGTGATACGGTGGAAATTC
  241 ---------+---------+---------+---------+---------+---------+ 300
      AGACGCGTCAACAGGTGAGGACGAAACACCTACTACCGTTGCCACTATGCCACCTTTAAG

        C   A   V   V   H   S   C   F   V   D   D   G   N   G   D   T   V   E   I   L

      TGATGCAGATGGTTGTGCTCTCAAAATACTTGCTGAAAATTTGGAATATCCGACAG
  301 ---------+---------+---------+---------+---------+------     356
      ACTACGTCTACCAACACGAGAGTTTTATGAACGACTTTTAAACCTTATAGGCTGTC
        M   Q   M   V   V   L   S   K   Y   L   L   K   I   W   N   I   R   Q   -
```

Figure 5.1b Coding sequence of the *B. malayi cut-1* clone, with amino acid translation.

```
                                                                    10
B.pahangi                                                           CCAATTCAATTTG>
                                                                    |||||||||||||
B.malayi                                                            CCAATTCAATTTG

                20          30          40          50          60          70
B.pahangi       CTACCATTGGCCAACCAGTTTATCACAAATGGACCTGTGATTCCGAAACCGTTGATACCT>
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B.malayi        CTACCATTGGCCAACCAGTTTATCACAAATGGACCTGTGATTCCGAAACCGTTGATACCT

                80          90          100         110         120         130
B.pahangi       TTTGCGCAGTTGTCCACTCCTGCTTTGTGGATGATGGCAACGGTGATACGGTGGAAATTC>
                | |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B.malayi        TCTGCGCAGTTGTCCACTCCTGCTTTGTGGATGATGGCAACGGTGATACGGTGGAAATTC
```

Figure 5.2a Line-up of the nucleotide coding sequences of *bpcut-1* (32) and the *B. malayi* genomic clone.

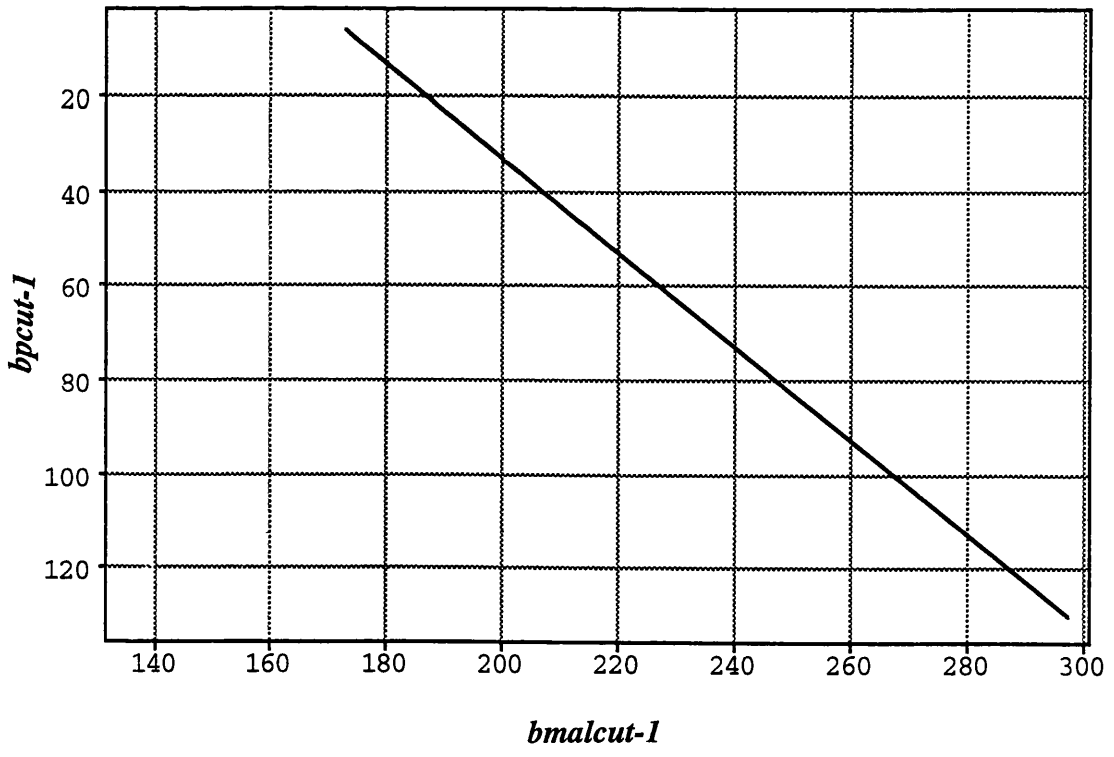


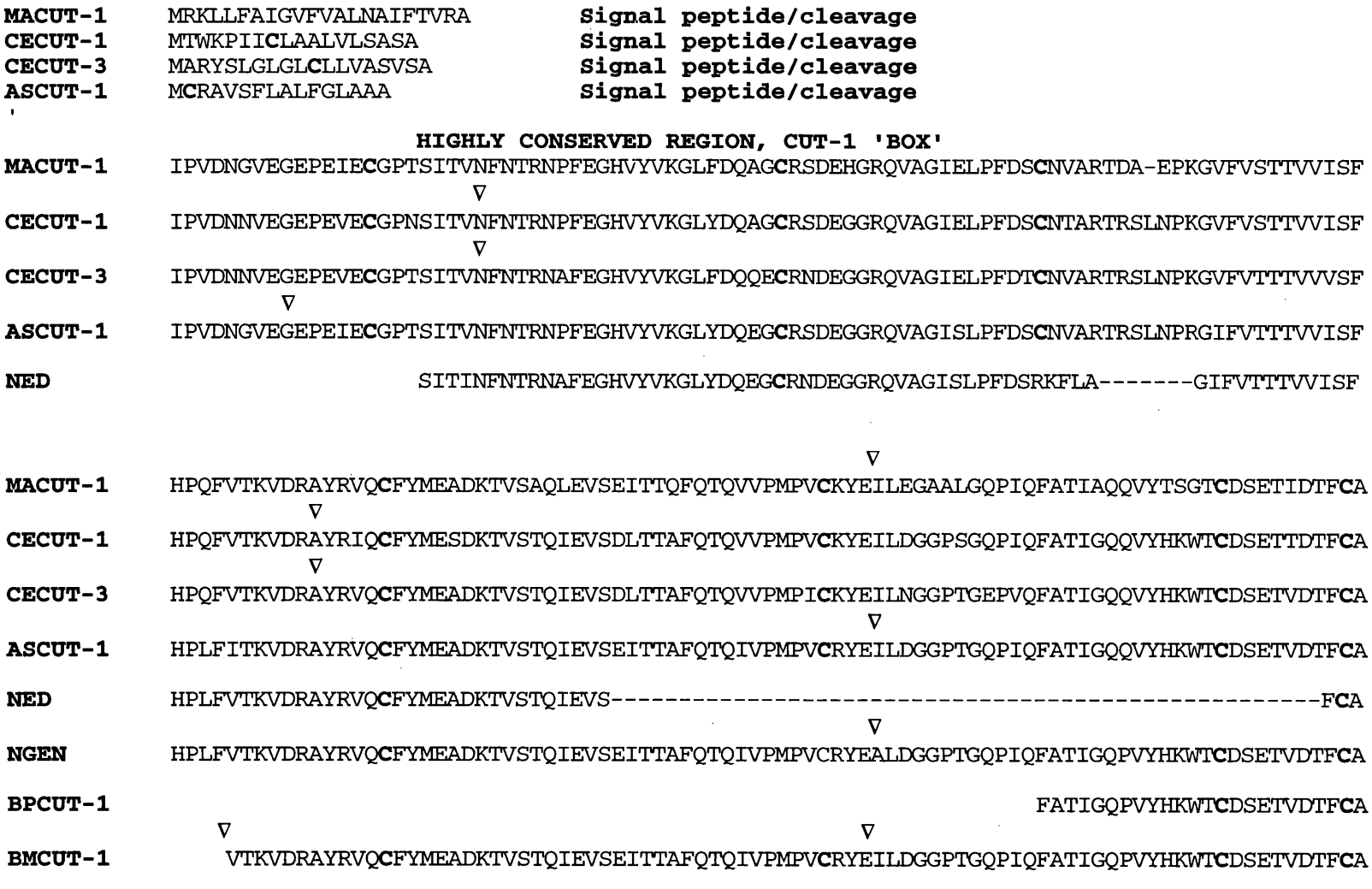
Figure 5.2b Schematic comparison of *bpcut-1* and *bmalcut-1* coding sequences. The figure shows a Matrix Plot (Mac Vector) which plots two nucleotide sequences against eachother to give a straight line if the sequences are identical, and a broken line where the sequences diverge.

Figure 5.3

Multiple alignment of *cut-1*-like proteins, including the clones isolated during this project.

- **MACUT-1** is the cuticlin homologue from *M. artiella* (De Giorgi et al, 1996).
- **CECUT-1** is the first cuticlin studied, from *C. elegans* (Sebastiano et al,1991).
- **CECUT-3** is a cuticlin-like protein of *C. elegans* encoded by CEF22B5.3, accession No. Z50044, sequenced by the *C. elegans* Genome Sequencing Project.
- **ASCUT-1** is the cuticlin homologue from *A. lumbricoides* (Timinouni & Bazzicalupo, 1997).
- **NED** is the predicted peptide encoded by ned, the *B. pahangi cut-1* cDNA isolated from an adult *B. pahangi* cDNA library and described in this chapter.
- **NGEN** is the predicted peptide encoded by the genomic sequence of NED, obtained by amplifying *B. pahangi* genomic DNA with ned-specific primers, as described in this chapter.
- **BPCUT-1** is the predicted peptide encoded by *bpcut-1* (32), the PCR fragment isolated from *B. pahangi* genomic DNA using primers based on the sequence of *ascut-1*, as described in Chapter Four.
- **BMCUT-1** is the predicted protein encoded by *bmcut-1*, the genomic clone isolated from a *B. malayi* genomic expression library, described in this chapter.

- Down-pointing arrows mark introns.
- The amino acid deletion seen in NED, referred to in the text, are marked with a dotted line.


MACUT-1 MRKLLFAIGVFVALNAIFTVRA Signal peptide/cleavage
CECUT-1 MTWKPIICLAALVLSASA Signal peptide/cleavage
CECUT-3 MARYSLGLGLCLLVASVSA Signal peptide/cleavage
ASCUT-1 MCRAVSFLALFGLAAA Signal peptide/cleavage
HIGHLY CONSERVED REGION, CUT-1 'BOX'
MACUT-1 IPVDNGVEGEPEIECGPTSITVNFNTRNPFEGHVYVKGLFDQAGCRSDEHGRQVAGIELPFDSCNVARTDA-EPKGVFVSTTVVISF
CECUT-1 IPVDNNVEGEPEVECGPNSITVNFNTRNPFEGHVYVKGLYDQAGCRSDEGGRQVAGIELPFDSCNTARTRSLNPKGVFVSTTVVISF
CECUT-3 IPVDNNVEGEPEVECGPTSITVNFNTRNAFEGHVYVKGLFDQQECRNDEGGRQVAGIELPFDTCNVARTRSLNPKGVFVTTTVVVSF
ASCUT-1 IPVDNGVEGEPEIECGPTSITVNFNTRNPFEGHVYVKGLYDQEGCRSDEGGRQVAGISLPFDSCNVARTRSLNPRGIFVTTTVVISF
NED SITINFNTRNAFEGHVYVKGLYDQEGCRNDEGGRQVAGISLPFDSRKFLA-------GIFVTTTVVISF
MACUT-1 HPQFVTKVDRAYRVQCFYMEADKTVSAQLEVSEITTQFQTQVVPMPVCKYEILEGAALGQPIQFATIAQQVYTSGTCDSETIDTFCA
CECUT-1 HPQFVTKVDRAYRIQCFYMESDKTVSTQIEVSDLTTAFQTQVVPMPVCKYEILDGGPSGQPIQFATIGQQVYHKWTCDSETTDTFCA
CECUT-3 HPQFVTKVDRAYRVQCFYMEADKTVSTQIEVSDLTTAFQTQVVPMPICKYEILNGGPTGEPVQFATIGQQVYHKWTCDSETVDTFCA
ASCUT-1 HPLFITKVDRAYRVQCFYMEADKTVSTQIEVSEITTAFQTQIVPMPVCRYEILDGGPTGQPIQFATIGQQVYHKWTCDSETVDTFCA
NED HPLFVTKVDRAYRVQCFYMEADKTVSTQIEVS----------FCA
NGEN HPLFVTKVDRAYRVQCFYMEADKTVSTQIEVSEITTAFQTQIVPMPVCRYEALDGGPTGQPIQFATIGQPVYHKWTCDSETVDTFCA
BPCUT-1 FATIGQPVYHKWTCDSETVDTFCA
BMCUT-1 VTKVDRAYRVQCFYMEADKTVSTQIEVSEITTAFQTQIVPMPVCRYEILDGGPTGQPIQFATIGQPVYHKWTCDSETVDTFCA

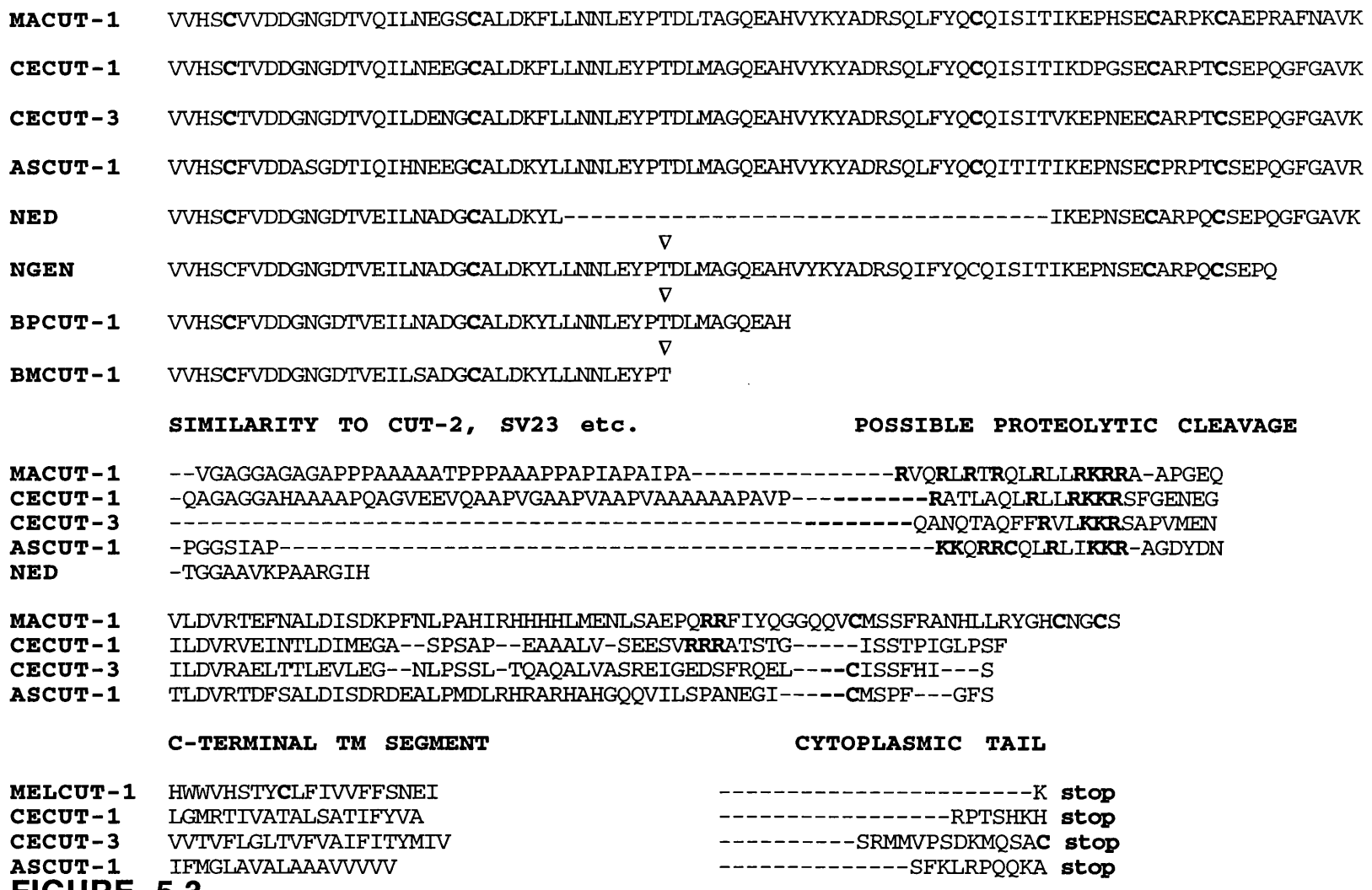

MACUT-1 VVHSCVVDDGNGDTVQILNEGSCALDKFLLNNLEYPTDLTAGQEAHVYKYADRSQLFYQCQISITIKEPHSECARPKCAEPRAFNAVK
CECUT-1 VVHSCTVDDGNGDTVQILNEEGCALDKFLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQISITIKDPGSECARPTCSEPQGFGAVK
CECUT-3 VVHSCTVDDGNGDTVQILDENGCALDKFLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQISITVKEPNEECARPTCSEPQGFGAVK
ASCUT-1 VVHSCFVDDASGDTIQIHNEEGCALDKYLLNNLEYPTDLMAGQEAHVYKYADRSQLFYQCQITITIKEPNSECPRPTCSEPQGFGAVR
NED VVHSCFVDDGNGDTVEILNADGCALDKYL----------IKEPNSECARPQCSEPQGFGAVK
NGEN VVHSCFVDDGNGDTVEILNADGCALDKYLLNNLEYPTDLMAGQEAHVYKYADRSQIFYQCQISITIKEPNSECARPQCSEPQ
BPCUT-1 VVHSCFVDDGNGDTVEILNADGCALDKYLLNNLEYPTDLMAGQEAH
BMCUT-1 VVHSCFVDDGNGDTVEILSADGCALDKYLLNNLEYPT
SIMILARITY TO CUT-2, SV23 etc.
POSSIBLE PROTEOLYTIC CLEAVAGE
MACUT-1 --VGAGGAGAGAPPPAAAAATPPPAAAPPAPIAPAIPA----------RVQRLRTRQLRLLRKRRA-APGEQ
CECUT-1 -QAGAGGAHAAAAPQAGVEEVQAAPVGAAPVAAPVAAAAAAPAVP----------RATLAQLRLLRKKRSFGENEG
CECUT-3 ----------QANQTAQFFRVLKKRSAPVMEN
ASCUT-1 -PGGSIAP----------KKQRRCQLRLIKKR-AGDYDN
NED -TGGAAVKPAARGIH
MACUT-1 VLDVRTEFNALDISDKPFNLPAHIRHHHHLMENLSAEPQRRFIYQGGQQVCMSSFRANHLLRYGHCNGCS
CECUT-1 ILDVRVEINTLDIMEGA--SPSAP--EAAALV-SEESVRRRATSTG-----ISSTPIGLPSF
CECUT-3 ILDVRAELTTLEVLEG--NLPSSL-TQAQALVASREIGEDSFRQEL----CISSFHI---S
ASCUT-1 TLDVRTDFSALDISDRDEALPMDLRHRARHAHGQQVILSPANEGI-----CMSPF---GFS
C-TERMINAL TM SEGMENT
CYTOPLASMIC TAIL
MELCUT-1 HWWVHSTYCLFIVVFFSNEI ----------K stop
CECUT-1 LGMRTIVATALSATIFYVA ----------RPTSHKH stop
CECUT-3 VVTVFLGLTVFVAIFITYMIV ----------SRMMVPSDKMQSAC stop
ASCUT-1 IFMGLAVALAAAVVVVV ----------SFKLRPQQKA stop

FIGURE 5.3

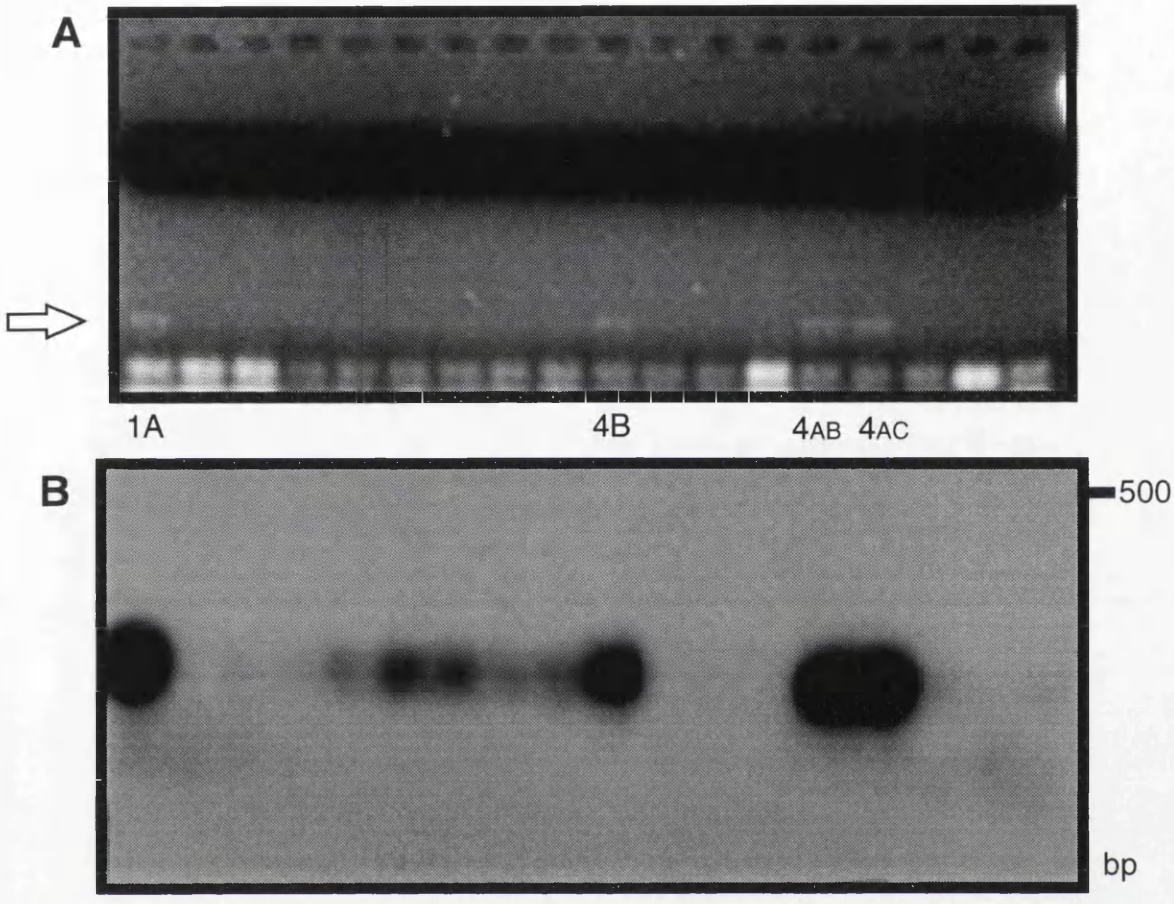


Figure 5.4

Reactions were carried out to confirm that the putative positive clones picked in the first round of screening a *B. pahangi* genomic library had a *cut-1* identity.

Phage DNA from each clone served as the template in reactions using gene-specific primers Br5/2 and Br3/2 (expected fragment size 210 bp). 16 clones were tested and the last two lanes are the negative controls: one reaction carried out with no template and one with no primers.

A. EtBr-stained gel of PCR products. 10µl reactions were run on an agarose gels.
The faint uppermost band represents a *cut-1* like fragment; the lower bands represent excess primer.
The gel was blotted and the blot hybridised to a labelled fragment of *bmcut-1*
Conditions were stringent: hybridisation and washing of the blots were done at 65°c; the blots were washed to 0.1 x SSC, 0.1% SDS.

B. Autoradiograph of the blot after a 4 hour exposure to film. The probe is showing strong hybridisation to at least 9 of the 16 clones, after a very short exposure time. The clones that were chosen to be taken through to a secondary screen are marked. They were chosen because they showed strongest hybridisation to the probe.

Figure 5.5

Restriction enzyme analysis and subsequent Southern blotting and hybridisation to show that λ Clones 4ab and 4ac carry the same insert and that Clones 1a and 4b carry inserts which differ from one another and from Clones 4ab/c.

The upper panels are 0.8% EtBr-stained agarose gels on which the products of various restriction enzyme digests have been electrophoresed. 5µl of λ clone DNA was cleaved with a variety of restriction enzymes to establish a restriction pattern for each clone. In some cases the DNA was cleaved with more than one enzyme. The gels were blotted and hybridised to ^{32}P-labelled *bpcvt-1.* The blots were hybridised and washed at 65°c. They were washed down to 0.1 x SSC and 0.1% SDS.

The lower panels represent the autoradiograph resulting from exposing the blots to film for 6 hours, clearly showing the major hybridising band in each digest.

lane	Restriction enzyme/s
1	*Eco*RI
2	*Hind* III
3	*Eco*RI & *Hind*III
4	*Eco*RI
5	*Kpn*I
6	*Eco*RI& *Kpn*I
7	*Sal*I
8	*Pst*I

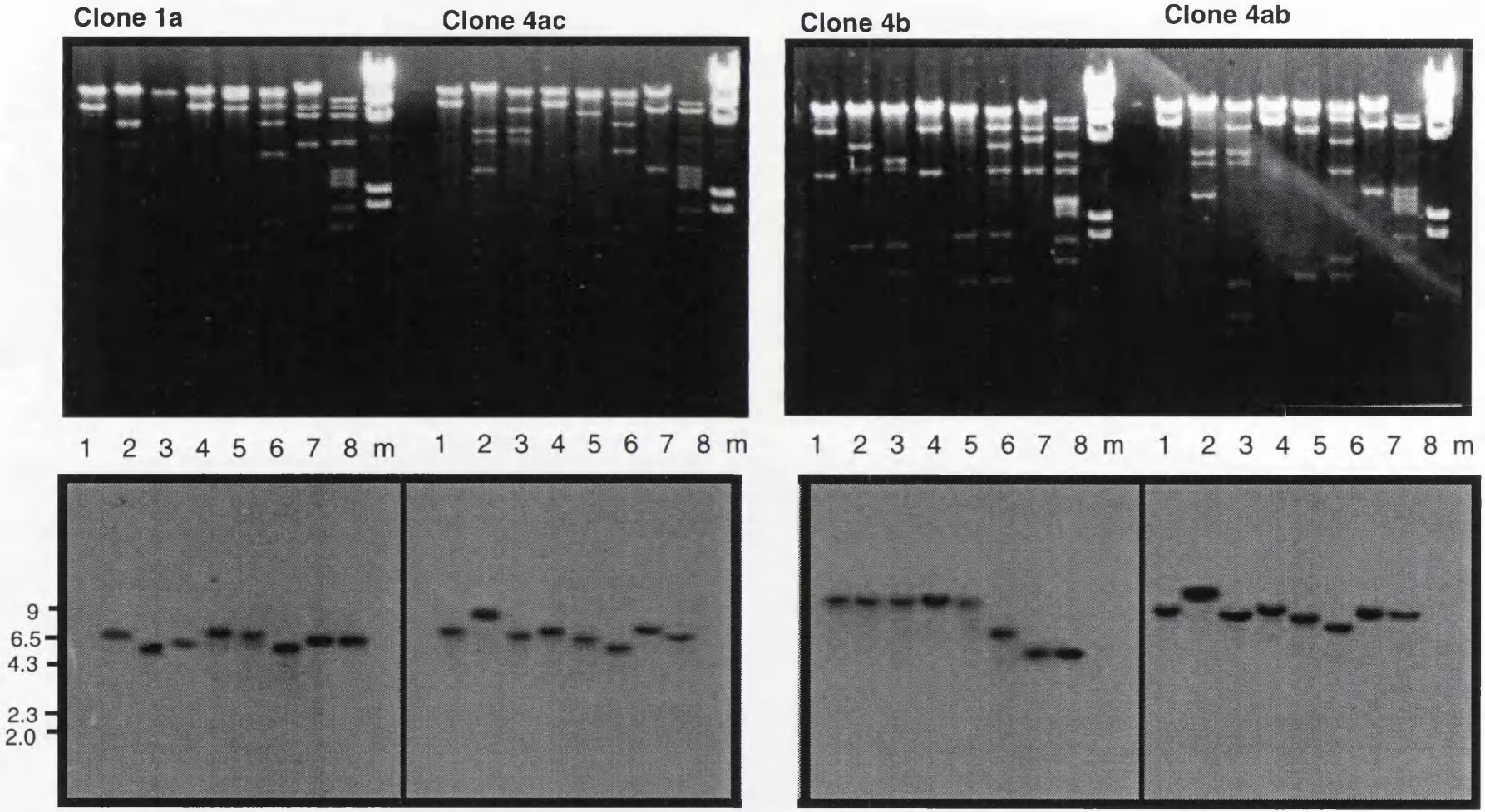

Clone 1a
Clone 4ac
Clone 4b
Clone 4ab
1 2 3 4 5 6 7 8 m 1 2 3 4 5 6 7 8 m
1 2 3 4 5 6 7 8 m 1 2 3 4 5 6 7 8 m
9
6.5
4.3
2.3
2.0

Figure 5.5

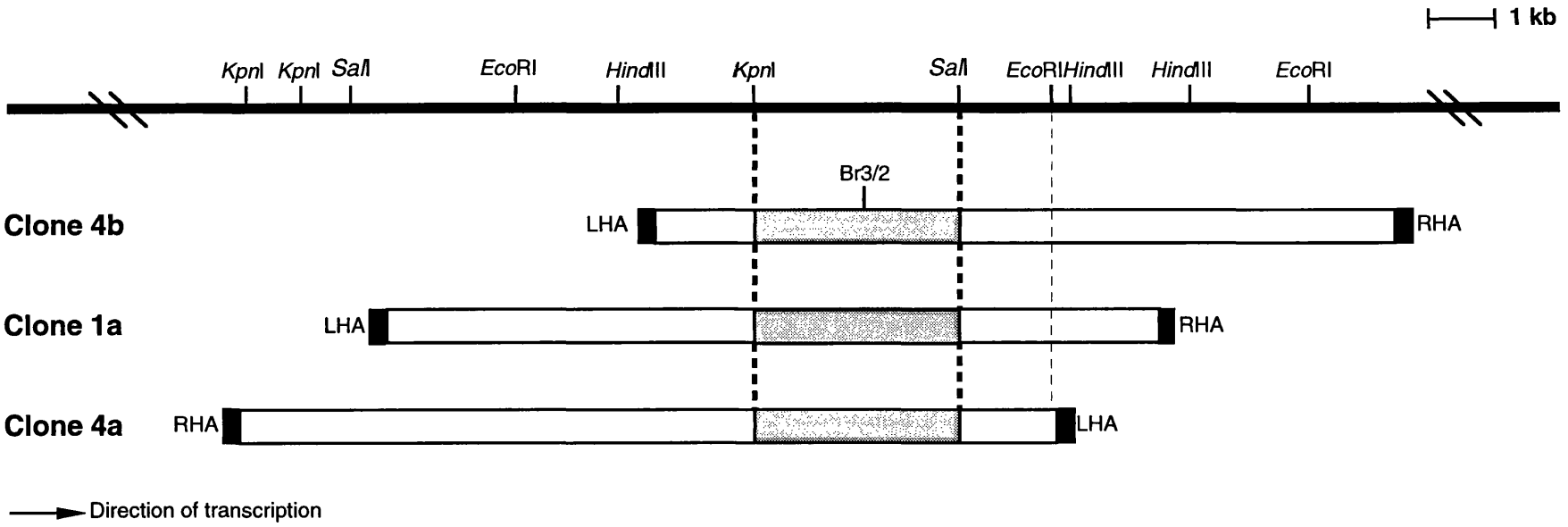


Figure 5.6 Schematic diagram of the region of the *B. pahangi* genome from which the λ clones 4b, 1a & 4a derive.

Shaded box = hybridising region Dotted lines = restriction enzyme sites shared by all three clones.

LHA ▮ = λ left hand arm RHA ▮ = λ right hand arm

n.b. The *Eco*RI and *Hin*dIII recognition sites at 11kb are too close together to distinguish them accurately. Clone 4a terminates between the two sites.

Figure 5.7

Nucleotide pile-up of the sequences obtained by amplification of the λ clones 1a, 4a & 4b with primers 2a3med and Br5/2, which span the *bpcut-1*-specific intron. Also included are the corresponding sequences of *bpcut-1* (32) and ngen, to show that all the sequences have the same intron, and that all the introns have the same sequence, thereby demonstrating that they all derive from the same *B. pahangi cut-1*-like gene.

Figure 5.8

Figure showing the two different splicing patterns seen in ned and in *bpcut-1*. In both cases the sequence of ngen is used, as it represents the longest stretch of *Brugia*-specific *cut-1*-like genomic DNAsequence available.

- Intron sequence is in blue, exon sequence is black.
- Primer sequences are marked in red.

Figure 5.8a

This shows ngen spliced to give the cDNA seen in 2133 (the cDNA corresponding to *bpcut-1* amplified from *B. pahangi* day 7p.i. cDNA using *bpcut-1*-specific primers). As is shown in Figure 5.3, it also encodes a predicted peptide which has an extremely high homolgy to the CUT-1-like proteins of other parasitic nematodes. The primers used in the 3' RACE are marked on the sequence to demonstrate that their sequence is present in the cDNA. 2A3MED, which was end-labelled and used to hybridise to RACE products, is also marked to show its presence.

Figure 5.8b

This shows ngen spliced to give the cDNA seen in ned, the cDNA clone isolated by screening an adult *B. pahangi* cDNA library, as described in this chapter. The corresponding predicted protein results in the deletion of 55 amino acids from the highly conserved region of the CUT-1-like protein. As can be seen in the figure, both primers used in the 3' RACE procedure are present within intron sequence, as is the sequence used to probe the amplification products.

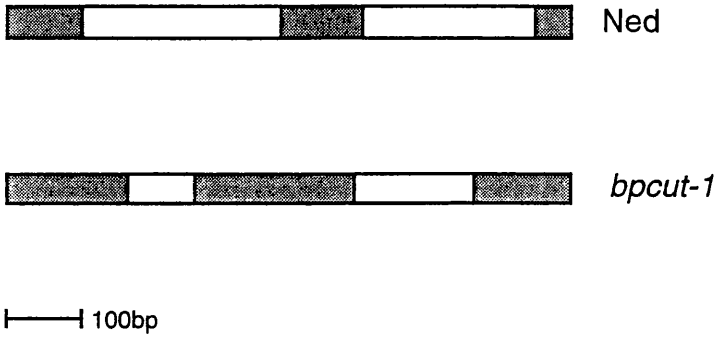


Figure 5.8c Schematic diagram to show the alternative splicing of ngen.

shaded = Exon unshaded = Intron

TTCCATCCACTGTTCGTCACAAAAGTTGATCGAGCATACCGAGTACAGTGCTTCTATATGGAGGCTGATAAAACGGTCAGCACCCAGATTGAGGTGTCTG 100

AGATCACAACTGCTTTTCAAACTCAAATTGTCCCCATGCCTGTTTGTCGATACGAGGCATGTTTTTGAATTTGCATTTATTTCTACAATTCAAATTCAAA 200

BR5→ BR5/2→

TATGAAAGATCTATTTATCACATAGTAAATGGTGACAAATATTTTAGATTTTGGATGGTGGACCAACCGGACAGCCAATTCAATTTGCTACCATTGGCCA 300

ACAAGTTTATCACAAATGGACCTGTGATTCCGAAACCGTTGATACCTTTTGCGCAGTTGTCCACTCCTGCTTTGTGGATGATGGCAACGGTGATACGGTG 400

GAAATTCTGAATGCAGATGGTTGTGCTCTCGACAAATACTTGCTGAACAATTTGGGATATCCAACAGGTAAGTGAGATAAATCGGTAAAGCAATTAACAA 500

TTAGGCACTAATTTCTTCATTTTCCTTTAAAATGTAAACGAAACAGTTTTTGGTTATAAAAAAATGATTTTCAGGATGCTTAAATTAAACTTATCTT 600

←2A3MED

TGTAAATTAACTTAAATTGTTAGATCTTATGGCTGGTCAAGAAGCGCATGTGTATAAATACGCGGATCGATCACAAATTTCTATCAATGCCAGATCAGT 700

ATTACCATTAAAGAACCAAACAGTGAATGTGCTCGACCACAGTGTTCAGAGCCGCAA 800

FIGURE 5.8A

TTCCATCCACTGTTCGTCACAAAAGTTGATCGAGCATACCGAGTACAGTGCTTCTATATGGAGGCTGATAAAACGGTCAGCACCCAGATTGAGGTGTCTG 100

AGATCACAACTGCTTTTCAAACTCAAATTGTCCCCATGCCTGTTTGTCGATACGAGGCATGTTTTTGAATTTGCATTTATTTCTACAATTCAAATTCAAA 200

BR5→ BR5/2→

TATGAAAGATCTATTTATCACATAGTAAATGGTGACAAATATTTTAGATTTTGGATGGTGGACCAACCGGACAGCCAATTCAATTTGCTACCATTGGCCA 300

ACAAGTTTATCACAAATGGACCTGTGATTCCGAAACCGTTGATACCTTTTGCGCAGTTGTCCACTCCTGCTTTGTGGATGATGGCAACGGTGATACGGTG 400

GAAATTCTGAATGCAGATGGTTGTGCTCTCGACAAATACTTGCTGAACAATTTGGGATATCCAACAGGTAAGTGAGATAAATCGGTAAAGCAATTAACAA 500

TTAGGCACTAATTTCTTCATTTTCCTTTAAAATGTAAACGAAACAGTTTTTGGTTATAAAAAAATGATTTTCAGGATGCTTAAATTAAACTTATCTT 600

←2A3MED

TGTAAATTAACTTAAATTGTTAGATCTTATGGCTGGTCAAGAAGCGCATGTGTATAAATACGCGGATCGATCACAAATTTCTATCAATGCCAGATCAGT 700

ATTACCATTAAAGAACCAAACAGTGAATGTGCTCGACCACAGTGTTCAGAGCCGCAA 800

FIGURE 5.8B

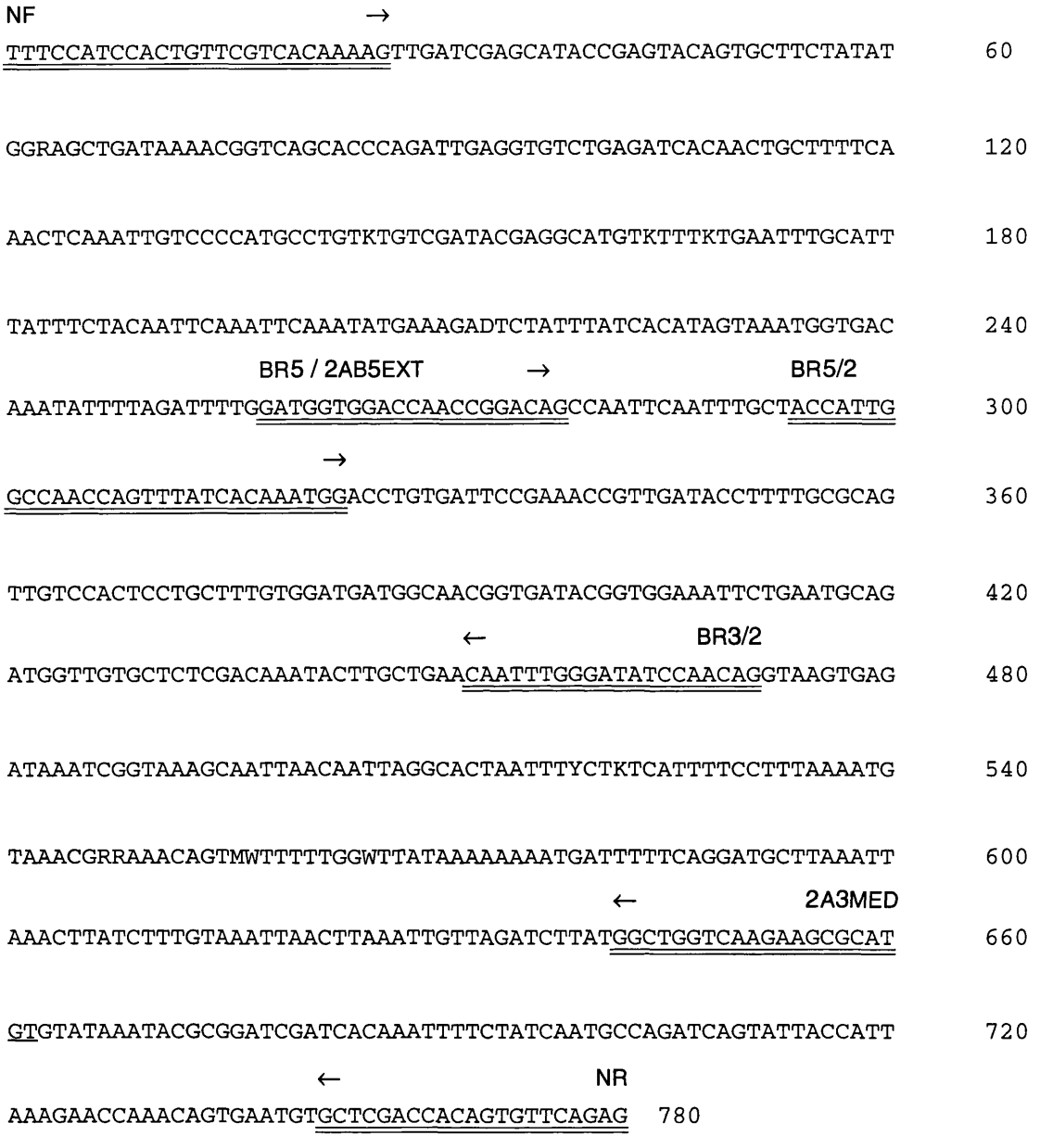


Figure 5.9

The ngen sequence (the longest *Brugia*-specific *cut-1*-like genomic DNA sequence available) showing the primers used in the 3' RACE on cDNA made from polyA mRNA. To note: 5MREXT is not shown because it was made on a region of the *ascut-1* sequence which is 5' of the region spanned by the ned-specific primers NF and NR. Br5 was designed on *Brugia*-specific sequence , whilst 2ab5Ext was designed on the equivalent *ascut-1* sequence.

Figure 5.10

3' RACE was carried out in an attempt to obtain the 3' end of the *bpcut-1* cDNA.. Adult polyA mRNA was used to make first strand cDNA using the oligo (dT)-adapter primer, and gene-specific amplifications were carried out using a variety of 5' primers based on the *cut-1* sequence (Br5, 2ab5Ext and NF), and the adapter primer. The secondary gene-specific amplifications were all carried out with Br5/2, the most internal 5' *brugia*-specific *cut-1* primer, and the adapter primer. Control reactions were carried out using R1, a primer based on the sequence of the constitutive *Brugia* ribosomal protein gene.

A EtBr-stained agarose gel. 10µl of each reaction were run in lanes 1-12 on a 1.8%. The gel was blotted, and the blot hybridised to γ-^{32}Pend-labelled oligonucleotide 2a3Med. Hybridisation was carried out at 50°c. The blot was washed 3 x 15 minutes in 6 x SSC, 0.1% SDS at 42°c.

B Autoradiograph of hybridised blot after 2 hours of exposure to film.

Reactions 1-5 represent the primary gene-specific amplifications, and were carried out using adult cDNA as the template.

	Primers used
lane 1	adapter primer & R1
lane 2	adapter primer & Br5
lane 3	adapter primer & 2ab5Ext
lane 4	adapter primer & NF

Reactions 5-8 represent the secondary gene-specific amplifications, and were carried out using reactions 1-4 (diluted 1/200) as the template.

	Primers used	**Template**
lane 5	adapter primer & R1	reaction 1
lane 6	adapter primer & Br5/2	reaction 2
lane 7	adapter primer & Br5/2	reaction 3
lane 8	adapter primer & Br5/2	reaction 4

Reactions 9-12 are exactly as 5-8, and were carried out to verify the reproducibility of the results.

Lanes 6 & 7, and 10 & 11 show reactions which, using the same primer sets, result in products which hybridise strongly to an oligo based on the sequence of *bpcut-1*.

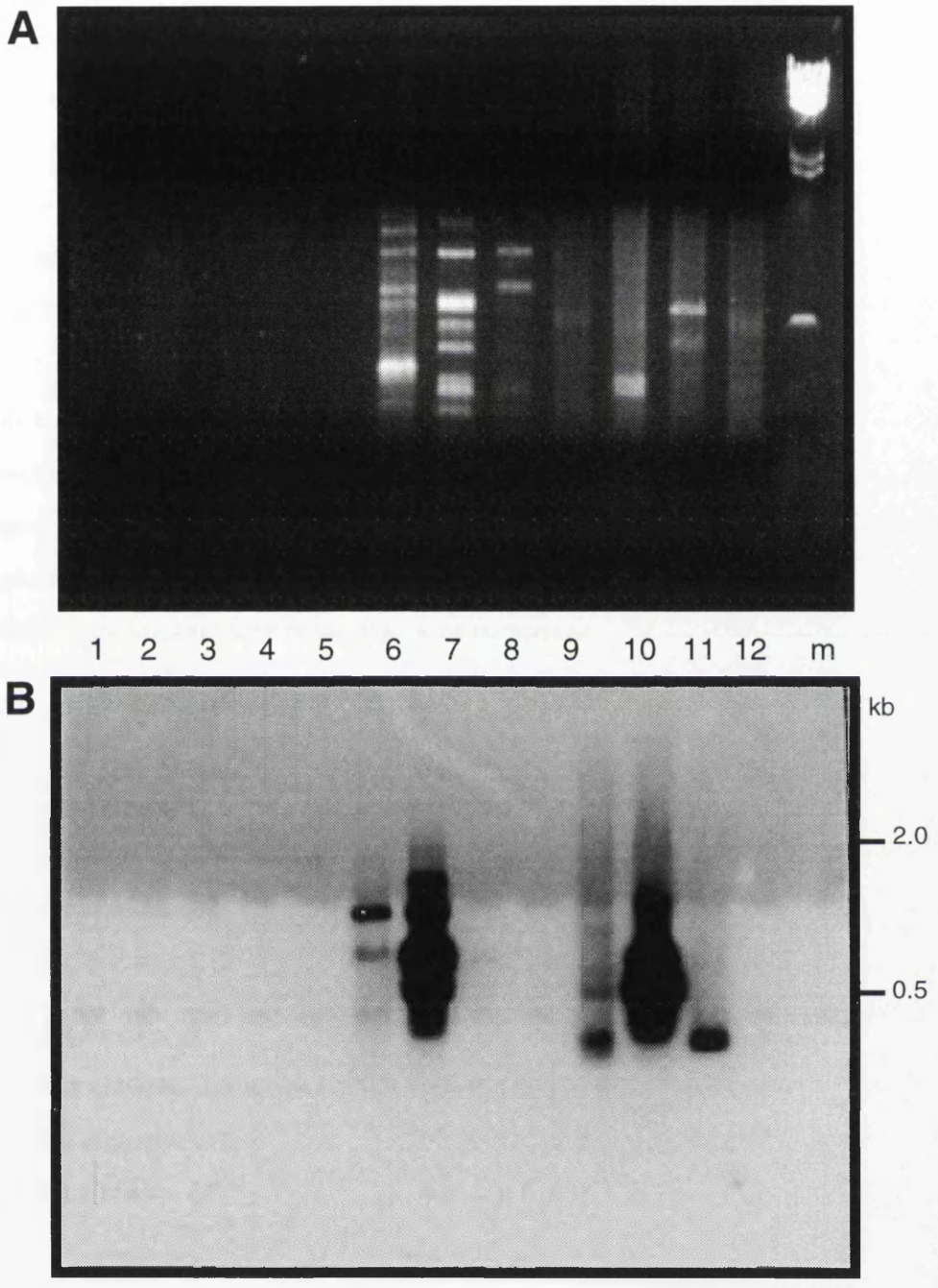


Figure 5.10

3' RACE on cDNA made from *B. pahangi* polyA adult mRNA.

CTCTGAACACTGTGGTCGAGCACATTCACTGTTTGGTTCTTTA**ATGgtaatact**gatctg 60

gcattgatagaaaatttgtgatcgatccgcgtatttatacacatgcgcttcttgaccagc 120

cataagatctaacaatttaagttaatttacaaagataagtttaatttaagcatcctgaaa 180

aatcatttttttataawccaaaaawkactgtttyycgtttacattttaaaggaaaatga 240

magraaattagtgcctaattgttaattgctttaccgatttatctcacttacctgttggat 300

atcccaaat**tgttcagC**AAGTATTTGTCGAGAGCACAACCATCTGCATTCAGAATTTCCA 360

CCGTATCACCGTTGCCATCATCCACAAAGCAGGAGTGGACAACTGCGCAA**AAGgtatcaa** 420

cggtttcggaatcacaggtccatttgtgataaactggttggccaatggtagcaaattgaa 480

ttggctgtccggttggtccaccatccaaaatctaaaatatttgtcaccatttactatgtg 540

ataaatagahtctttcatatttgaatttgaattgtagaaataaatgcaaattcamaaama 600

catgcctcgtatcgacamacaggcatggggacaatttgagtttgaaaagcagttgtg**atc** 660

tcagACACCTCAATCTGGGTGCTGACCGTTTTATCAGCTYCCATATAGAAGCACTGTACT 720

CGGTATGCTCGATCAACTTTTGTGACGAACAGTGGATGGAAA 762

5' SPLICE SITE USAGE

C. elegans 5' splice site consensus	A/C A G | G T R A G T T T
5' splice site at position 54	A T G | G T A A T A C T
5' splice site at position 413	A A G | G T A T C A A C

3' SPLICE SITE USAGE

C. elegans 3' splice site consensus	T T T T C A G | R
3' splice site at position 317	T G T T C A G | C
3' spice site at position 665	A T C T C A G | A

Figure 5.11

This figure shows the sequence of ngen reversed and complemented with respect to the coding sequence of *bpcut-1*, i.e. it represents the non-coding strand of the mRNA. Upper case indicates exon and lower case indicates intron, as seen in the cDNA clone ned. The agreement with the *C. elegans* intron consensus sequences (Blumenthal & Steward, 1997) demonstrates that ned is an aberrant cDNA species resulting from the transcription of the opposite strand to the coding strand of *bpcut-1.*

CHAPTER SIX: THE EXPRESSION PATTERN OF *bpcut-1.*

6.1. INTRODUCTION.

Although sequence data provides information on the structure of a gene, and can be used to predict the nature of the protein which it encodes, it cannot elucidate the biological function or significance of the gene or its product within the organism from which it derives. To characterise a gene it is obviously important to know its expression pattern i.e. when it is transcribed, when and where the message is translated, and where the resultant protein localises on or in the organism. This chapter reports the transcript profile corresponding to the *B. pahangi cut-1*-like gene which has been isolated, and describes the localisation pattern of an anti-CUT-1 polyclonal antiserum on *B. pahangi* worms.

6.2. RT-PCR.

Reverse transcriptase PCR (Kawasaki & Wang, 1989) was devised as a technique for determining gene-specific RNA transcript abundance. The method is very much more sensitive than northern blotting for the detection of transcripts, and a result can be obtained using a tiny initial input of RNA. Thus the technique is particularly useful for working with parasites, when it is often very difficult to obtain enough material from various life-cycle stages to yield sufficient RNA for northern blot analysis.

Total RNA was isolated from worms harvested from the jird at various time points post-infection, and DNase treated to reduce contaminating genomic DNA. The time points used were: days 3, 5, 6, 7, 9, 12, 18, 19, 20, 21 and 28 p.i. and adult worms. It is worth mentioning at this stage that obtaining sufficient worms from the earlier post-infective time-points to make RNA for the first strand cDNA synthesis was often very difficult, and that synchronising populations became problematic in the later time-points, when males begin the L3-L4 moult several days before the female worms. These are representative of the difficulties which are commonly encountered when working within a parasite system.

First strand cDNA was reverse transcribed from each RNA sample using oligo (dT) as a primer. A comparison using oligo (dT) and random hexamers as the primer in the reverse transcription step showed that while the two primers yielded similar amounts of first strand cDNA from the same RNA, reactions using the oligo (dT) primer were more reliable (Joyce Moore, personal communication). Due to the difficulty of obtaining sufficient material from the early time-points post-infection to make enough RNA for reverse transcription, reliability of the reaction was of primary importance.

The first strand cDNA was amplified with a set of primers specific to a constitutively expressed ribosomal protein of *B. pahangi*, to ensure that the first strand cDNA synthesis had worked. The primers were designed to span an intron and could therefore be used to check whether any contaminating genomic DNA remained in the cDNA; the presence of genomic

DNA is indicated by the amplification of a product larger, by the number of bases corresponding to the intron, than the cDNA-derived product (Krause, 1995).

In order to establish the pattern of expression of *bpcut-1*, the first strand cDNA was then used as the template in amplification reactions using *cut-1*-specific primers, 2a3med and Br5/2, designed to span the *bpcut-1* intron. In order to quantify the gene-specific product, a control reaction must be carried out using primers specific for a constitutive gene, R1 and NR3. This provides a base-line of expression with which to compare the expression of *bpcut-1* at each time point. The ribosomal protein primers used to check the first strand cDNA synthesis were used to amplify the 'reference transcript'. The abundance of the *bpcut-1* transcript is expressed as a fraction of the abundance of the ribosomal transcript at the same life-cycle stage, to give a relative abundance. See Materials and Methods for full experimental details. In this way the assay is independent of the total amount of cDNA in each reaction, and hence RNA in each sample.

Table 6.0 Primers used in RT-PCR experiments.

PRIMER	SEQUENCE	AMPLIFIED CDNA BAND
NR3	GAGGAACAAGAAGAAGGAAGAGCC	380bp
R1	GCATTGTTCTCAAATAGAGC	
2A3MED	CATGTGCTTCTTGGCCAGCC	180bp
BR5/2	ACCATTGGCCAACCAGTTTATCACAAATGG	

It is important to choose the optimum number of PCR cycles for each primer set, i.e. to stop the reactions before the reagents are exhausted, when the amplification reactions are still in the log phase, and when the abundance of control product is proportional to the amount of input cDNA. As can be seen in Figure 6.1b., the quantity of product could sometimes barely be visualised by ethidium staining, demonstrating that the reagent concentrations are not limiting.

It could be argued that relative quantification is only possible if both transcripts are simultaneously amplified in a single reaction, thus ensuring that each primer set is operating under identical conditions, i.e. controlling for human error in setting up the reaction mixes. The experimental method used in this study, of using the same 'master-mix' of PCR reagents for the reference transcript and gene-specific reactions, means that the only variable is the amount of cDNA added to each reaction. In theory it should be possible to check that equivalent amounts of cDNA were added to each reaction by using contaminating genomic DNA as an 'internal control', assuming that the probe used will hybridise to both genomic and cDNA amplification products. The genomic DNA product abundance could then be quantified along with the transcript abundance and compared between different primer pairs amplifying

the same time-point DNA: equivalent abundance of genomic DNA transcript between samples would demonstrate equivalent cDNA input. However, if it is felt that the presence of contaminating genomic DNA is interfering with the interaction between the primers and cDNA, it is possible to design primers which hybridise across an intron/exon boundary in the genomic sequence. Theoretically, such primers would amplify cDNA and not contaminating genomic DNA.

Preliminary trials using both primer sets in the same tube were not successful in this project: an increase in the amplification of one transcript resulted in a corresponding decrease in the amplification of the other. The dynamic of the reactions varied between cDNA samples apparently meaninglessly, as shown by the fact that patterns of relative abundance varied from one experiment to the next. The results imply an interaction of some kind either between the primers or between the transcript/s and the primers. The only way to confirm an interaction of this type would be to titrate each set of primers against a steady concentration of the other set, to observe whether increasing the concentration of one set resulted in a decrease in product amplification by the other. The reagents used in the reactions were in excess of what should be required for optimum amplification, and increasing the concentration of potentially limiting reagents did not resolve the problem. As in the amplification of any DNA, certain primer pairs are more efficient than others for reasons which often cannot be predicted or explained. By the same token, Krause (1995) notes that certain primer pairs are incompatible when used in multiplex reactions to assay more than one template simultaneously. Presumably this was the case with the *bpcut-1* and ribosomal gene primer pairs. Consequently it was decided to carry out the *bpcut-1* and control reactions in separate tubes. As can be seen from Figures 6.1 and 6.2, although there are minor variations in the abundance values between the same time points in different experiments, the overall pattern of relative abundance is reproducible.

The PCR reactions were electrophoresed and Southern blotted, and were then hybridised to radio-labelled oligonucleotides internal to those used to amplify the products. In comparison with random-priming large fragments of cloned DNA as probes in the hybridisation step, the use of end-labelled, gene-specific oligonucleotides results in a much higher specific activity for the probe. In our experience, this gave stronger hybridisation signals and 'cleaner' blots, which facilitated accuracy in cutting out the signal from the nitrocellulose blots in the post-autoradiography quantification step of the experiments. Oligonucleotides made from exon sequence from both *bpcut-1* and the reference gene were radiolabelled and used as probes. In this way both genomic DNA and cDNA transcripts could be visualised by autoradiography (in the interests of clarity, the autoradiographs presented in this chapter are the result of short exposure times of the blot to film, and hence the hybridisation to the much less abundant genomic transcript is not visualised). Figure 6.1 c & d, and Figure 6.2 a & b show the autoradiograpic results of two separate experiments carried out as described above.

After autoradiography, bands corresponding to the *bpcut-1* product and the ribosomal reference transcript were cut out of the membrane and counted in scintillant. The relative abundance of the *bpcut-1* transcript was then expressed as a ratio of the signal corresponding to the ribosomal protein gene transcript at every time-point tested. Tables 6.1 and 6.2 give ratio values for the two separate experiments shown in Figures 6.1 and 6.2. The log value of these ratios were plotted against time post-infection to give a graph of the mRNA abundance of *bpcut-1*. Figures 6.1 e and 6.2 c show the graphs resulting from the data generated by the two separate RT-PCR experiments.

Viewing the combined results of the ethidium stained gels, the autoradiography of the resultant Southern blot hybridisations, and the graphs of the relative abundance of the transcript throughout the mammalian stages of the life-cycle of *B.pahangi*, it would appear that *bpcut-1* is expressed maximally around the L3-L4 moult and the L4 -adult moult. In the case of *B. pahangi*, in the cat (and in the jird), the L3-L4 moult is synchronised, occurring for both male and female worms at between 7-9 days p.i of the mammalian host. The final L4-adult moult is much less synchronised: males moult between days 19 and 23 p.i, whilst females moult between days 25-30 p.i. (Schacher, 1962). The autoradiographic results and the graph showing the relative abundance values of the *bpcut-1* transcript, clearly demonstrate that transcript abundance increases enormously around the time of the final moult, appearing to peak at day 19p.i. By day 28 p.i the abundance is extremely low, and no signal was ever obtained using *bpcut-1* primers on adult cDNA. The relative abundance of bpcut-1 is less at the L3-L4 moult.

6.3 IMMUNOLOCALIZATION EXPERIMENTS.

6.3.1 Raising antisera against recombinant *Ascaris* CUT-1

Although the RT-PCR results show a clear transcription pattern for *bpcut-1*, which corresponds to what one would expect for a protein component of the nematode cuticle, it would be very useful to establish where, in the *B. pahangi* worm, the CUT-1 protein is expressed. This was done using antisera raised against the conserved region of the CUT-1 amino acid sequence of *Ascaris*. As has been previously mentioned, *ascut-1* was isolated by Mohammed Timinouni at IIGB, Naples. The recombinant protein was made and expressed by Claudia Paiva-Nunes (IIGB), who also raised the antisera in rabbits, and did the subsequent affinity-purification.

The recombinant ASCUT-1 was designed and produced before the cDNA of the gene had been isolated, and consequently the terminal part of the sequence was subsequently shown to be intron sequence. The sequence covers the region over which the *cut-1*-like genes of *C. elegans, Meloidogyne artiella, Ascaris lumbricoides, B. malayi* and *B. pahangi* are highly conserved, and their amino acid sequences are virtually identical (see Figure 5.7). The fragment was cloned into the pT7 vector, which uses the bacteriophage T7 RNA

polymerase/promotor system to exclusively express the cloned gene, without fusion to a carrier protein. The fragment must be cloned in-frame, into a polycloning site downstream from the bacteriophage T7 promotor. The construct is used to transform *E. coli* and the expressed protein is present in cytoplasmic granules, known as inclusion bodies. The inclusion bodies were isolated from lysed bacterial cells, solubilised, and analysed by SDS-PAGE. The band representing the recombinant protein was electro-eluted out of the gel, at which point it was estimated to be about 95% pure, and used to immunise a rabbit. The resultant antiserum was purified by ammonium sulphate precipitation to yield an IgG fraction of the serum. The antiserum was then affinity purified (see below) and tested for specific reactivity on Western blots of *A. lumbricoides* and *C. elegans* protein extracts. The results showed, in the case of both nematodes, that the antiserum recognised a protein of the size predicted by the *ascut-1* sequence, with no background at all.

6.3.2. Western blot of *B. pahangi* protein extracts with anti-rASCUT-1.

To establish whether or not it was worthwhile affinity-purifiying the anti-rASCUT-1 antiserum for use in the following localisation experiments, a western blot of *B. pahangi* adult and mf protein extracts was reacted with the affinity purified IgG, and with the whole antiserum. As can be seen in Figure 6.3., the whole antiserum reacted with many components of the protein extracts, in addition to a fair amount of back-ground staining. On the basis of this experiment, it was decided to use the affinity-purified IgG fraction of anti-rASCUT-1 in the localisation experiments.

The western blot also demonstrated that the anti-rASCUT-1 antiserum recognized *B. pahangi* proteins, in both the adult and mf extracts. Using the affinity-purified antiserum, the antiserum is definitely recognizing a prominent band at 34KDa, in both mf and adult protein extracts, not seen in the control rabbit serum lanes.

The antiserum is obviously recognizing a protein which is solubilised by treatment with SDS and mercaptoethanol; presumably this represents a pre-cursor to the mature cuticlin protein, which is present before being cross-linked into the insoluble matrix of the cuticle.

6.3.3. Immunofluorescence.

In these experiments, adult worms and L4s were sectioned and fixed onto slides, and microfilariae were extruded from adult females and also fixed onto slides (see Materials and Methods). Slides were blocked with BSA and then incubated with affinity purified anti-rASCUT-1 antisera. Control slides were incubated with non-immune rabbit sera. A goat anti-rabbit-FITC conjugate was used as the secondary antibody. The sections were counter-stained using Evans Blue, and mounted in DAB/glycerol, which inhibits quenching of the fluorescence. The slides were viewed and photographed immediately, using a fluorescence microscope.

In the adult worms there were two separate localisations. The first was in the cuticle. Using this technique it was impossible to determine whether the localisation was to the epicuticle i.e.

the surface of the cuticle, or to an internal layer of the cuticle structure. On some images, the localisation appeared to be to a double layer of the cuticle, as seen in Figures 6.4 and 6.6, whilst in others (for example Figure 6.7), the fluorescence revealed a single layer of localisation. The cuticular localisation was observed on both male and female adult worms, and on fourth stage larvae (L4s). Figure 6.8 C and D show cross-sections through L4 worms taken from the jird at day 15 p.i., clearly demonstrating cuticular localization of the epitope/s recognised by the antiserum.

The second localisation appears to be on the surface of mature microfilariae. The microfilarial fluorescence appears to be much stronger than that seen on the cuticle, as is seen particularly clearly in Figure 6.4. Cross-sections of the mature microfilariae (see Figure 6.9) reveal that there is no internal localisation of the antiserum, the antibody recognition is of epitopes on the surface of the larvae. Localisation experiments on whole microfilariae extruded from the adult female show that the antiserum does not localise to the microfilarial sheath, so the surface labelling is specifically cuticular. Figure 6.8A shows an exsheathed worm, demonstrating antibody recognition of the cuticle. In Figure 6.8B, above the two fluorescing worms, there is a worm which does not show binding of the antiserum; although it cannot be seen whether or not the worm is exsheathed, it is tempting to speculate that it has retained its sheath and that binding of the anti-cuticlin antiserum is therefore being blocked.

In Figure 6.6 (dic image), the cross-section is of the posterior end of an adult female worm: the uteri do not fill the body cavity of the worm and the alimentary tract can still be visualised as a tube-like structure. At this stage it is important to note that the ovaries are situated in the posterior part of the adult female worm, and the female gametes are developed from a syncitium situated at the posterior tip of the ovary (McLaren, 1973). Development, including fertilisation of mature primary oocysts, occurs along the female reproductive tract, culminating in the expulsion of live, mature microfilariae from the anteriorly-positioned vulva. In fact, depending on how posterior the cross-section is, the two structures could well represent the ovaries before they constrict to the oviducts and reopen into the uteri, in which case they would contain unfertilised oocytes rather than zygotic embryos (Delves et al, 1989). Neither the embryonic structure nor the boundaries between embryos can yet be identified. The corresponding FITC-labelled image shows that although the antiserum is localising to the cuticle, it is not recognising an epitope on or in the contents of the gonads.

In contrast, Figure 6.5 is a cross-section of the anterior part of a female worm. The dic image shows the uteri to be filling the entire body cavity of the worm, and clear boundaries can be seen between the embryos developing within, especially in the left-hand uterus. As in Figure 6.4, the FITC image demonstrates that the antiserum is strongly recognising epitopes on the surface of the developing embryos. Also, the orientation of some of the cross-sections allows it to be seen that the embryos at this stage have a curved, 'worm-like' appearance, showing that they have developed beyond the stage where they are just a fertilised ovum within an egg-shell.

Figure 6.10 and 6.7 illustrate a common observation made in the course of the immunofluorescence experiments, namely that whilst epitopes present on the surface of the embryos in one of the two uteri were recognised by the antiserum, the embryos in the other uterus were unrecognised. The dic image of Figure 6.10 shows very clearly that there is a qualitative difference in the embryos present in the left and right hand uteri. Those in the right hand uterus, in addition to being recognised by the antiserum, are bigger and more widely spaced, and are visualised as having a different texture than those in the left-hand uterus.

In an attempt to understand more fully the apparent selective embryonic recognition by the antiserum, adult female worms were cut into four equal lengths, and the intra-uterine contents of each portion extruded and fixed onto a slide. The extruded mf from each segment were fixed onto different spots on a multispot slide. In this way all the embryos from the same worm but corresponding to different longitudinal position in the adult female were represented on the same slide. Immunofluorescence was carried out as above, using the anti-rASCUT-1 antiserum. The results showed that whilst the embryos from the most anterior, and the two posterior sections of the uteri do not appear to have epitopes which are recognized by the antiserum (data not shown), the cuticles of the extruded embryos from the second section are strongly recognized by the antisera. Figure 6.8B shows recognition of extruded mf from the second section. As mentioned previously, the microfilarial sheath does not seem to be recognized by the antisera.

6.3.4. Immunogold localisation experiments.

The immunogold labelling and electron microscopy was kindly carried out by Dr. Lawrence Tetley (IBLS). Ultrathin sections of *B. pahangi* mixed-sex adult worms were collected on platinum grids and reacted with the anti-rASCUT-1 antisera, using a second antibody labelled with colloidal gold. Details of the procedure are out-lined in Materials and Methods.

The results obtained from these experiments enabled us to localise the epitopes recognised by the anti-rASCUT-1 more precisely within the layered structure of the *B. pahangi* cuticle. Figures 6.11 and 6.12 show that the localisation pattern in the adult cuticle of both males and females appears to be extremely discrete, and, using the defining paper of Rogers et al (1974) as a guideline, restricted to the basal layer of the cuticle, which lies between the external cortical and internal fibrous layers of the parasitic nematode cuticle (Lee, 1966). In Figure 6.11, the antiserum also appears to recognise clusters of epitopes in the hypodermis, which underlies the cuticle. It has long been established that the components of the nematode cuticle are synthesised in, and secreted from, the hypodermal cells (Singh and Sulston, 1978), therefore the antiserum could be recognising cuticlin-secreting hypodermal cells.

The results of the immunogold localisation of the antiserum on microfilariae confirm what was observed in the immunofluorescence experiments. As can be seen in Figures 6.13 and 6.14, there is definitely no recognition of epitopes on the microfilarial sheath. Neither is there recognition on the surface of immature microfilariae, as is shown in Figure 6.13. As in the

adult worms, the embryonic localisation is restricted to a single layer in the cuticle of mature microfilariae, and Figure 6.14 demonstrates that the epitopes recognised by the antiserum are in the basal layer of the mf cuticle.

In contrast with the immunofluorescence results, the immunogold labelling of the mf does not appear to be any more pronounced than that of the adult cuticle. This may relate to the different microscopy techniques; the E.M. sections contain a single section of mf, whereas at the light level many more mf are visualised.

6.4. EXPERIMENTS USING NED PRIMERS.

When ned (the cDNA clone isolated in Chapter Five during the screening of a *B. pahangi* cDNA library, and subsequently shown to be a mis-transcription on the non-coding strand of *bpcut-1*)was first isolated and sequenced, it was optimistically thought that it may represent a second *B. pahangi cut-1*-like gene, which had already been shown, by immunolocalisation experiments (fully described in this chapter), to have an embryonic expression pattern. Consequently, the following experiments were carried out using primers which were made on what was thought to be ned-specific, and therefore embryonic *cut-1*-specific sequence.

6.4.1. RT-PCR using ned primers.

These experiments were done in order to establish an embryonic transcript profile using the RT-PCR technique. To this end, first strand cDNA was made from microfilariae, and from adult worms which had been separated according to sex. If the primers represented a gene which was expressed only in the embryonic stage of the life-cycle, it would be expected that they would amplify a message from adult female cDNA, and possibly from microfilarial cDNA, but not from male cDNA or cDNA from any other larval stage. The primers were used to amplify cDNA from every life-cycle stage available in the laboratory, with consistently negative results. The primers appeared to be functional only on the ned clone itself, and on *B. pahangi* genomic DNA. When it was discovered that ned represented a mis-transcription event, and that the NF and NR primers (thought to be ned-specific) were derived from *bpcut-1*, efforts to understand why the primers did not recognise cDNA from any of the life-cycle stages were abandoned. Their main use was to provide, via amplification of the genomic DNA, the longest piece of sequence of *Brugia*-specific *cut-1* available to the project. As was mentioned earlier, the efficacy of primers varies with apparently little explanation. The sequences of the primers are known to be present in the coding sequence of the gene, and amplify ned and genomic DNA, wherein the sequence is no doubt represented more abundantly than in the cDNA from the various life-cycle stages. Therefore their failure to amplify is probably due to the fact that one or more of the PCR components is not optimised for these specific primers. Titrating the primers, the cDNA, and possibly altering the number of amplification cycles and the annealing temperature, would quite possibly yield better results. However, in light of the fact that ned is not biologically significant, there seems little justification for continuing these experiments.

6.5. RT-PCR ON ADULT FEMALE WORM SECTIONS.

Preliminary immunolocalisation results showed that a CUT-1-like epitope is recognised on or in the cuticle of mature microfilariae, but is not present in earlier embryonic stages. The following experiment was done in an attempt to establish when the gene encoding the embryonic CUT-1-like protein was transcribed. The experiment was carried out using the following assumptions: that embryonic development is synchronous in the uteri of adult females; and that development begins with a syncitium at the tip of the ovaries, situated at the posterior end of the worm, and ends with the expulsion of mature microfilariae from the anteriorly-situated vulva (McClaren, 1973). In effect, the longitudinal correlation between distance down the genital tract and embryonic maturity is being exploited in this experiment. Adult *B. pahangi* were isolated from a jird and males and females were separated. Female worms were then stretched out on glass slides and cut lengthwise into four equal pieces. The four pieces from each worm corresponding to the same longitudinal portion were combined, and RNA was then made from each, from which first strand cDNA was synthesised. The second assumption of this experiment was that the aforementioned ned primers were specific to the embryonic *cut-1*-like gene. RT-PCR was carried out on the cDNA samples, initially using the ribosomal protein primers as a control for first strand cDNA synthesis. The primers used to establish the mRNA abundance profile for *bpcut-1* were used as a negative control. Genomic DNA contamination made the results messy and difficult to interpret, but in retrospect, knowing that ned and *bpcut-1* derive from the same moult-specific gene, the fact that neither primer set amplified any of the adult female cDNA samples, whilst the ribosomal protein control primers worked on all four samples, makes perfect sense. In fact, further immunolocalisation experiments also revealed that uterine development is not synchronous between paired uteri at the same point in the female worm, so it is unlikely that this experiment would yield clear-cut results, even using primers that were truly specific to the embryonic *cut-1*-like gene. However, it is possible that such results would vary quantitatively, giving some idea of variation in mRNA abundance.

6.6 NORTHERN BLOT ANALYSIS.

As has been discussed in Chapter Four, probing northern blots of total RNA from various *B. pahangi* life-cycle stages with *bpcut-1* gave consistently negative results. Northern analysis of the cuticlin genes has had variable success amongst the nematodes studied: in *C. elegans* the transcripts of both *cut-1* and *cut-2* were detected using 10 and 15µg respectively of total RNA; in *Meloidogyne artiella*, however, no signal was detected in total RNA isolated from any life-cycle stage of the parasite, despite using 50µg of total RNA.

Having established, by the preceding RT-PCR experiments, that *bpcut-1* is transcribed maximally in day 19 p.i. worms, it was decided to purify polyA+ mRNA from total RNA from day19 p.i. worms. Approximately 20µg of total RNA were used in the purification. The resultant

pellet of purified mRNA was barely visible, and not visible when run on a denaturing gel. This is unsurprising, as polyA+ mRNA constitutes 2-2.5% of total RNA, therefore the amount would not exceed 200ng, even if the purification process was 100% efficient. The gel was blotted and probed with end-labelled oligonucleotide Br3/2, the sequence of which is present in the exon of *bpcut-1*. Despite a long exposure time, a signal could not be detected. As a control for the experimental conditions, the blot was re-probed with a labelled oligonucleotide corresponding to the sequence of the ribosomal protein gene known to be constitutively expressed in *B. pahangi*. No signal was detected, therefore the only conclusions that can be drawn from this experiment are concerned with technical details: either the RNA was lost during the purification process, or the blotting and probing conditions were not appropriate for hybridisation of the probe to the transcript.

On the supposition that there was a second *B. pahangi cut-1*-like gene with an embryonic expression (see immunolocalisation results), it was decided to perform a northern blot using purified, polyA+ mRNA from adult worms (containing a high proportion of embryonic message), in order to establish the existence of a transcript. The amount of total RNA initially purified was estimated by ethidium staining to be approximately 60µg, and the resultant purified fraction could be visualised on an ethidium gel. The gel was blotted and probed three times: the first time with a labelled fragment of the ribosomal protein clone as a positive control for experimental conditions; the second time with end-labelled oligonucleotide 2A3med; and the third time with random-primed *bpcut-1*. The ribosomal protein gene probe hybridised to a single band of the right size, showing that the conditions at all stages of the experiment were suitable for detection of a transcribed mRNA species (data not shown). The 2A3med hybridisation did not result in the detection of a band, despite lowering the stringency of the hybridisation and washing conditions in accordance with using an oligonucleotide probe. This experiment probably did not work because the embryonic *cut-1*-like gene does not contain a sequence similar enough to that of 2A3Med to enable hybridisation of the probe to the mRNA. The third hybridisation, to ^{32}P-labelled *bpcut-1,* did not result in the detection of a band.

6.7. DISCUSSION.

RT-PCR.

Before discussing the relevance, importance and reliabilty of semi-quantitative RT-PCR in the establishment of an mRNA abundance pattern for *bpcut-1,* it is worthwhile considering some aspects of the methodology of the technique, both generally and in its specific application to this project.

Methodology.

The first issue is whether the oligo (dT) primer should ideally be used in the first strand cDNA synthesis step. Theoretically, the advantages are a) that only polyadenylated i.e. mature, processed mRNA is primed for reverse transcription, resulting in a cDNA population

which should ideally consist entirely of mature cDNA species corresponding to the mRNA population and b) as previously mentioned, in our experience the primer is very reliable in the synthesis of first strand cDNA. However, it would appear that the use of the oligo (dT) primer has some major draw-backs. Firstly, it obviously primes from the 3' end of the mature mRNA. Therefore, if the message of your gene is long, or if the gene-specific primers are made to 5' sequence, the probability of the relevant cDNA being available for amplification is considerably reduced. Secondly, it has a tendency to anneal to A-rich regions of the mRNA, resulting in spurious priming and truncated cDNAs (Martin et al, 1995). Additionally, it has been reported that it is common for the oligo (dT) primer to prime from both ends of an mRNA species (Joshua et al, 1995). This means that the resultant cDNA population may not be representative of the mRNA present at a given time-point in the life-cycle. If a gene of interest is transcribed very briefly or at a very low level, this mis-priming could mean that the full-length messenger would not be present (or present in such a small amount) in the cDNA pool, that its transcription would be undetected by subsequent PCR amplifications using gene-specific primers on the cDNA template. RT-PCR is used to establish the expression pattern of a gene, not to obtain a full length cDNA clone, for which other techniques would be much more suitable. Hence the use of random primers in the priming step of the reaction are likely to be more efficient with respect to guaranteeing the presence of a cDNA molecule represented at very low levels in the mRNA.

The second issue is the choice of a constitutive control gene on which to design primers to amplify a base-line product for comparison with the fluctuating gene of interest. It is surprisingly difficult to find a truly constitutive gene, and even more difficult to find a constitutive gene which maintains a constant level of mRNA abundance throughout the life-cycle of the worm, or at least does not fluctuate enough to interfere with its use as a reference transcript. As has already been mentioned, the difficulties of establishing these requirements are enhanced in the filarial system, due to the often extremely limited amounts of parasite material available. The ribosomal protein gene used in these experiments was originally isolated and cloned for use as a comparison control in northern blotting experiments. This was done using a differential screening method, and the clone was picked on the grounds that it appeared to hybridise with equal specificity to cDNA probes made from total RNA from mosquito-derived L3s, 3 day p.i L3s, and adult worms (S. Hunter, personal communication). The clone was subsequently labelled and used to probe a Northern blot of RNA from the three aforementioned life-cycle stages, with the addition of day 15 p.i. L4s and microfilariae. This demonstrated that the probe hybridised to a transcript of the same size, and with equal intensity, in all five life-cycle stages (Martin et al, 1995). Primers were made spanning an intron of the gene and have been used in subsequent RT-PCR experiments on a comprehensive range of mammalian-derived cDNA sample time-points, and the gene appears to be constitutive and relatively invariant in its mRNA abundance. Incidentally, recent comparisons of constitutively expressed genes to use as controls for equal loading of *C.*

elegans RNA for Northern blots, have found the ribosomal protein genes to be the most useful (Krause, 1995).

Although the abundance of the ribosomal protein transcript appears to be constant when visualised by autoradiography, it is impossible to judge whether or not there are minor fluctuations in the transcript profile. This could affect the ratio calculation if the difference between the abundance of the test and control transcripts was too large, and result in a skewing of the results obtained. Krause (1995) noted that quantification of relative amounts of message in different samples was not possible when starting out with a very small RNA input. He attributed this to interference by contaminants which become more overbearing when there is less RNA present, and interfere with the reverse transcription, the PCR reaction, or both. A combination of these factors could result in unreliable ratios and hence expression profiles.

It must be reiterated that the results of these experiments do not provide a real value for the abundance of *bpcut-1* mRNA throughout the life-cycle, but a relative abundance with respect to the constitutive ribosomal reference gene. Comparisons of abundance can only be made within the same experiment due to unavoidable experimental variables between experiments. For example, the oligonucleotide probes used to detect the reference and gene-specific transcripts will vary in their precise specific activity from one labelling to the next; and it is difficult to exactly reproduce the conditions for washing the blots between experiments.

The transcript profile of *bpcut-1*.

The mRNA abundance of *bpcut-1* is as one would expect for the *B. pahangi* homologue of *cut-1* of *C. elegans,* a component of the nematode cuticle, peaking prior to each moult with no detectable signal in the adult worm. In *C. elegans*, RT-PCR using primers specific to *cut-2* (the gene encoding a second cuticlin protein present constitutively in the cuticle) was carried out to establish whether the mRNA is present continuously during development and growth, or whether it occurs just before each moult. RNA for reverse transcription was obtained by harvesting worms every two hours, between hatching and a few hours after the L1-L2 moult, from a highly synchronised worm population. The results showed clearly that the gene is transcribed only between 12-16 hours after hatching, immediately before the L1-L2 moult, which occurred between 13-16 hours post-hatching (Lassandro et al, 1994). In *M. artiella*, RT-PCR reveals that the *cut-1* homologue is transcribed at high levels in the egg, at the L1-L2 moult, and at lower levels in the (infective) J2 larvae and in the adult male, while no signal was detected in the adult female (De Giorgi et al, 1997). Interestingly, the J2 and adult male are the only free-living stages of the plant parasite, and have previously been demonstrated to have a thinner but more complex three-layered cuticular structure than that of the adult females, which are thick and bilayered (Reddigari et al, 1986). The authors interpret this as signifying that a more complex cuticle is required for protection in the external soil environment, and propose that cuticlin may be a candidate for toughening the cuticle against

external attacks. Reddigari et al demonstrated that the *Meloidogyne* J2 cuticle is analogous to the dauer larva cuticle in *C. elegans*, with respect to structure and solubility behaviour when treated with strong detergents. The *cut-1* gene of *C. elegans* has been shown to be expressed only in the dauer larvae (Sebastiano et al, 1991) strengthening the hypothesis that the role of the product encoded by *cut-1* is to contribute to the resistance and durability of the nematode cuticle. In *A. lumbricoides,* the expression pattern of the *cut-1* homologue was determined directly (P. Bazzicalupo, personal communication). Eggs were incubated at room temperature and protein extracted every second day for 28 days. The protein samples were western blotted and probed with an antibody raised against recombinant CUT-1 of *Ascaris.* This will recognise soluble CUT-1 monomers and multimers but not the mature, insoluble, cross-linked, cuticlin product which is incorporated into the cuticle. The results mirror those found in *Meloidogyne*, showing clearly that the protein is made, within the egg, at the time of the L1-L2 moult, when the L2 cuticle is being synthesised. As is the case for the J2 stage in *Meloidogyne*, the L2 stage in *Ascaris* is the infective stage of the parasite, and the stage which must survive outside the vertebrate host, albeit within the egg-shell.

It has long been established, by pulse-chase experiments on the synthesis of cuticular proteins in *C. elegans,* that cuticular components are synthesised discontinuously, peaking in the period prior to and during the deposition of a new cuticle (Cox et al, 1981). It has also been demonstrated, in *C. elegans*, that mRNA abundance of the collagens, which are known to make up the bulk of the nematode cuticle proteins, is temporally regulated and peaks prior to a moult (Cox & Hirsch, 1985). Selkirk et al (1989) showed the same pattern of discontinuous cuticular collagen synthesis in *B. malayi* , using metabolic labelling *in vitro* with ^{3}Hproline. Labelling of adult male and female worms and day 21p.i. worms (the majority undergoing the L4-adult moult) revealed maximum collagen synthesis in the adult female, considerable synthesis in the day 21p.i. worms, and virtually none in the adult males. The interpretation of these results is that the peak of collagen synthesis at day 21 p.i. provides the material for the synthesis of the adult cuticle, whilst the collagen synthesised in the adult female is destined for the microfilarial cuticle, the high levels of production reflecting the fecundity of the organism. The fact that the adult males do not synthesise collagen would imply that the majority of cuticular collagens, and presumably cuticular proteins, are made prior to or during the moult, with no significant further additions to the mass during adulthood. These observations support the work of Howells and Blainey (1983) who, working on *B. pahangi*, demonstrated that the surface area of L4 larvae does not increase throughout the intermoult period, despite the fact that the worms increase in length. This phenomenon is explained by the fact that the newly-formed cuticle is highly folded into deep, closely packed, narrow annulations. As the worm grows in length the number of annuli remain constant, but the distance between them increases and their depth decreases. So the larvae apparently grow, via a 'concertina' effect, without the incorporation of additional material throughout the intermoult period. Interestingly, the same mechanism is apparently not seen in *A.*

lumbricoides, wherein all the layers of the cuticle increase in volume throughout the growth of the adult worm (Watson, 1965). Fetterer (1996), working with an *in vitro* cultivation system for *A. suum* between the L3-L4 stages (Urban et al, 1981) determined the pattern of cuticular protein synthesis by metabolic labelling and subsequent isolation of collagenous proteins and cuticlin. He observed that cuticular collagen synthesis increased in a constant manner throughout intermoult development, and that synthesis of cuticlin was relatively constant during most of the intermoult period, increasing sharply just before the moult. It must be noted, however, that the culture system used results in a highly asynchronous worm population, such that it is not really suitable for the study of moulting and cuticular synthesis. These results can readily be explained by a staggered L3-L4 moult, rather than by continuous intermoult cuticle protein synthesis.

Although the *bpcut-1* RT-PCR results show obvious and repeatable peaks of transcript abundance around the two moults, and intermoult time-points do not result in easily visualised signals by autoradiography, scintillation analysis reveals that a small amount of transcript can be detected during the intermoult period. This is probably due to an asynchrony in the moulting times of the *B. pahangi* population, as observed and documented by Howells and Blainey (1983). They observed morphological changes related to L2-L3 moulting as early as 5 days p.i and as late as 9 days p.i, and the L4-adult moult was even less synchronous (as established by Schacher, 1962) extending from day 19 to day 25p.i, when the study was terminated. However, there is a possibility that the primers could be amplifying, at a lower efficiency, the transcript of a similar but different *cut-1*-like gene encoding a protein which is synthesised in the intermoult period.

Various morphological studies on nematodes (Bonner et al, 1969; Lee, 1970; Kozek, 1971) have shown that the epicuticle and associated cortical layers of the cuticle are those which are first synthesised, prior to ecdysis of the previous cuticle, during the moulting process. And it is these outer layers of the nematode cuticle which have long been known to contain the insoluble non-collagen proteins cross-linked by non-reducible covalent bonds (Cox et al, 1981) that we can now identify more specifically as cuticlin. Consequently, it would be expected that the the protein encoded by *bpcut-1* would be (along with any other *B. pahangi* CUT-1-like proteins) one of the first cuticular proteins synthesised and incorporated into the new cuticle. So, if we assume translation to be closely linked to transcription of an mRNA, we would expect the abundance of the *bpcut-1* transcript to peak early in the moulting process, which is indeed the case in the results shown. The only unexpected value is that of the 3 day p.i relative abundance, which is much higher than was to be expected. This could well be due to a skewing of the *bpcut-1*/ribosomal protein control transcript ratios due to the necessarily tiny amounts of RNA used to reverse transcribe the first strand cDNA; or it could in fact be a true reflection of the early synthesis of cuticlin during the moulting process. The fact that the transcript abundance peaks at the absolute outset of the L4-adult moult would support the idea of a tight regulation between transcription and translation of the *bpcut-1* gene, and its

incorporation into the newly-synthesised adult cuticle. Presumably the greater abundance of the transcript at the later moult is a reflection of the need for the adult cuticle to be thicker and more resistant than the larval cuticles. This would not be because they must adapt to a new habitat, but because while the mammalian larval stages of *Brugia* last for a total of about a month, the adult worms can live stably in the mammalian host for up to five years (Vanamail et al, 1996) presumably at least in part due to the properties of the adult cuticle.

On one hand it is to be expected that no *bpcut-1* transcript would be detected in the adult worm. The cuticle has already been assembled and any further growth can been accounted for by the aforementioned 'concertina' effect. However, the longevity of the adult worms would imply that there might be at least minimal turnover of the cuticular proteins in the adulthood of the worm. Presumably this occurs at such a low rate, if at all, that it cannot be detected by the RT-PCR method described here.

The anti-rASCUT-1 antiserum recognizes the product of more than one *cut-1*-like gene.

There is very little doubt that the anti-rASCUT-1 antiserum used in these experiments recognises the protein products of more than one *cut-1*-like gene. The antiserum was raised against a stretch of amino acids which we now know to be definitive of the CUT-1-like proteins within and between nematode species. It is therefore presumably CUT-1-specific, rather than being specific to the product of a particular *cut-1*-like gene. The RT-PCR results in combination with the localisation data appear to show that the anti-rASCUT-1 antiserum is recognising two CUT-1-like proteins encoded by two different *B. pahangi cut-1*-like genes, only one of which has been isolated in the course of this project. The evidence for this is as follows:

The RT-PCR results strongly imply that *bpcut-1* is a gene which encodes a protein which is a cuticular component: its transcript is present prior to and during a moult, and no transcript can be detected in cDNA from adult worms, therefore strongly suggesting that there is no transcription occurring either in the adult worms of either sex, or in the developing embryos. However, the immunolocalisation results, visualised by two different methods which confirm and complement each other, show conclusively that a CUT-1-like protein is present in the cuticle of the L4s and adult worms of both sexes, and in the cuticle of the mature microfilariae. The presence of the protein in the L4 and adult worms can be attributed to the transcription (and subsequent translation) of the *bpcut-1* mRNA, detected by RT-PCR, around the times of the L3-L4 and L4-adult moults. The presence of a CUT-1-like epitope in the cuticle of the microfilariae must be explained by the existence of a second functional *B. pahangi cut-1*-like gene which codes for an amino acid sequence very homologous to that of the other CUT-1-like polypeptides, but has a cDNA sequence which differs enough from that of *bpcut-1* to be unrecognisable to primers designed on the *bpcut-1* sequence.

Immunolocalization.

The immunogold labelling shows very clearly that the localisation in *B. pahangi* is not to the external cortical layers of the cuticle, but to the more internal median matrix layers. This is in contrast to the localisation of anti-CUT-1 antisera on the cuticles of *C. elegans* dauer larvae and the L_2 larvae of *Ascaris,* where the epitopes appear to be present in the external cortical layers of the cuticles. In some of the immunogold photos there appears to be some localisation of the antibody to the fibre bundles in the internal layers of the cuticle. This may correspond to the localisation of anti-CUT-1 antisera to the fibrous material under the alae of *C. elegans* and *Heterorhabditis* (Favre et al, 1995). It must be remembered that the anti-CUT-1 antisera is likely to recognise several proteins encoded by different cuticlin genes which have evolved to fulfil different functions within the structure of the nematode cuticle.

Embryo-specific localization.

There are several points of interest to be noted in the embryo-specific localisation of the antiserum. From the evidence outlined above, it would appear that the gene is only expressed in the more mature fertilised embryos. Rogers et al (1976) note that the developing zygote does not develop a cuticle until relatively late in intrauterine development, when the embryos pack the uterine lumen completely, and differentiation has proceeded to a point where the zygotes can be visualised in ultrastructural studies as having a c-shaped, worm-like form. It would therefore seem likely that expression of the embryonic *cut-1*-like gene coincides with the synthesis of this first embryonic cuticle. The microfilariae of *Brugia* are shed into the blood-stream of the vertebrate host where they are taken up by the insect host, within which they continue their development. Hence the microfilariae are complex larval nematodes, fully evolved to life in, and transmission from, the vertebrate host (Laurence& Simpson, 1974). The cuticle is a correspondingly complex structure, consisting of a trilaminate external epicuticle, which is arranged into annulae, and an inner cuticle of a more homogeneous nature (Araujo et al, 1994). As such, it would be expected that the microfilarial cuticle would contain a representative of the CUT-1 protein family, the genes encoding which have been detected in all nematode cuticles so far studied.

Anti-rASCUT-1 does not recognize epitopes on the sheath.

Interestingly, there is no recognition by the antibody of epitopes on the microfilarial sheath. The sheath is derived from the egg-shell of *Brugia*, which is present from fertilisation, and which stretches as the embryo develops to eventually form a loose sheath around the mature microfilariae (Rogers et al, 1976). Ultrastructural studies have shown the egg shell and microfilarial sheath to be identical in structure, proving that the second is derived from the first (Zaman, 1987). The sheath is known to play an important role in the protection of the parasite against host-cell damage (Srivastava, 1985). Immunolocalisation studies using the anti-rASCUT-1 antiserum on *A. lumbricoides* have demonstrated that epitopes recognized by the antiserum are present in the egg-shell as well as in the matrix surrounding the embryo within the egg-shell and on the surface of the infective L2 larvae (Paolo Bazzicalupo, personal communication). SHP2 is one of the main constituents of the microfilarial sheath, and is

thought to constitute the bulk of its insoluble component (Zahner et al, 1995). The authors comment that the SHP2 is reminiscent of a *C. elegans* cuticlin, presumably because it has a highly hydrophobic central region featuring several repeats of a simple motif, which is conserved among the filarial parasites. The protein may be encoded by a member of the putative filarial cuticlin gene family, and in fact the repeated motif is similar to that seen in CUT-2 of *C. elegans*, but this work demonstrates that it is not a CUT-1-like protein, and that, in contrast to *Ascaris*, there is no CUT-1-like protein present in the egg-shell.

Anti-rASCUT-1 differentially recognizes epitopes on intrauterine embryos.

The observation that paired uteri can contain embryo populations with different antigenic properties, demonstrated here by the differential recognition of epitopes by the anti-rASCUT-1 antiserum, is surprising. As has already been discussed, expression of the CUT-1 protein depends on the age or developmental stage of the developing embryo. Delves et al (1989) state clearly that progressive embryonic development along the two genital tracts of the female reproductive system of *B. pahangi* is identical, implying that a cross-section of the adult female worm at any point along its length will show embryos which are at the same stage of development. If this were the case, the embryos in both uteri should initiate the presentation of CUT-1 epitopes simultaneously, which does not appear to be the case. There are several explanations for this phenomenon. The first is technical: perhaps the cross-sections showing this phenomenon were cut at a slant, such that the sheared cross-section incorporates different distances down the length of the worm, by coincidence encompassing the developmental moment when the CUT-1 epitopes begin to be expressed on the embryonic surface. This is quite unlikely, especially since the images show the cross-sections to be round, implying a clean transverse cut. A second explanation is that the microfilariae which are not recognised by the antiserum are dead. There are several reports of intrauterine embryonic death in filarial nematodes (Terry et al, 1961; Schacher et al, 1967; and Vincent et al, 1975). Rogers et al (1976), reported the initiation of embryonic death in the central uterine lumen of *B. pahangi* just before the cuticle appeared, presumably due to overcrowding and/or lack of nutrients. The death rate increased throughout the development of the worms, until the mature embryos were no longer a compacted mass, which is the case in Figure 6.10 However, in this case you would expect to see reactivity with the antisera on the live microfilariae, in contrast to a lack of reactivity on the surface of the dead embryos, which does not appear to be the case. The final explanation is that development in paired uteri is not completely synchronous, and perhaps becomes less so with increasing time of development.

Anti-rASCUT-1 recognition of epitopes on extruded mf.

There is also more than one potential explanation for the initially baffling results of the whole, fixed microfilariae immunofluorescence experiments. If the expression of the CUT-1-like protein is correlated with microfilarial maturity, why is there no reaction of the antiserum on the surface of mf in section one, where the embryos are most mature? The most likely explanation is that when the CUT-1-like protein is first synthesised it is present as a monomer

or multimer on the microfilarial surface, in a form that renders the relevant epitope accessible to the antiserum, a situation which is seen in the microfilariae from section two. As the cuticle matures the protein might adopt a tertiary structure which obscures the epitope recognised by the antisera, for example via cross-linking. The same effect could be achieved by the protein being incorporated into the increasingly complex and insoluble developing cuticle. Hence the explanation for the lack of reactivity of the fully mature microfilariae in section one could be that the epitope is present, but not accessible to the antiserum. There is a precedent for this phenomenon: immunofluorescence localisation experiments carried out using antisera against recombinant CUT-1 of *C. elegans* on the dauer larvae of *C. elegans* were initially unsuccessful, despite the fact that it was known that the gene was expressed at this life-cycle stage (Sebastiano et al,1991). However, when the larval cuticles were broken open by freezing the worms between two glass slides and subsequently prying the slides apart, prior to fixing them, the reaction with the antiserum was strongly positive. The antigen is clearly not easily accessible to antibodies once the protein has been incorporated into the cuticle.

Another possibility is that as the microfilarial sheath matures it becomes impermeable, so the antiserum can not enter and react with the epitopes of cuticle. This seems unlikely, as one proposed function of the sheath is as a 'placenta', conveying nutrients from the uterine wall to the developing embryo (Rogers et al, 1976). Also, it was observed that even when the sheath was obviously ripped, and therefore fully permeable, microfilariae from section one did not react with the antibody. A third explanation relies on the observation that almost-mature microfilariae burrow into the uterine wall (Rogers et al, 1976), presumably to maximise nutrient up-take, before emerging and being expelled as fully-mature microfilariae from the vulva. It is possible that in section one the mature and functional microfilariae were not available for reaction with the antisera, having been retained in the uterine wall of the female worm. The microfilariae that remain in the lumen of the uterus may be dysfunctional and incapable of burrowing into the uterine wall, and as such may be incapable of expressing the CUT-1 epitope.

Immunogold labelling.

Overall, the immunogold experiments enhanced and clarified the results obtained using the immunofluorescence technique. The apparent double layer of reactivity sometimes seen in the immunofluorescence experiments was not seen with immunogold labelling, and is therefore probably a relic of either the sectioning or fixing of the worms, caused by the cuticle curling over slightly at the edge of the cross-section. The immunofluorescence labelling revealed the embryonic reactivity with the antiserum to be much stronger than that of the cuticle, an observation which was not confirmed by the immunogold labelling. This again is likely to reflect differences in technique.

CONCLUSIONS.

1) The mRNA abundance of *bpcut-1*, the *B.pahangi cut-1*-like gene isolated during the course of this project, peaks around the times of the moults in the vertebrate host, suggesting that the gene encodes a component of the L4 and adult male and female cuticles.

2) An anti-CUT-1 antiserum raised against a recombinant CUT-1 protein of *Ascaris*, known to be highly conserved amongst all the nematodes so far studied, shows a dual localisation pattern in *B. pahangi*: an internal cuticular localisation, presumably to an epitope defined by *bpcut-1*; and an additional localisation to the surface of mature, intrauterine microfilariae.

FIGURE 6.1.

Showing the process by which a semi-quantitative RT-PCR was carried out. As is discussed in the text, PCR reactions were carried out on first strand cDNA from *B. pahangi* parasites obtained at different time-points post-infection of the jird, using primers specific to either the *bpcut-1* gene or to a constitutive *B. pahangi* ribosomal protein gene. PCR reactions were carried out for 23 cycles with an annealing temperature of 55°C.

a & **b** - 10µl of each product was run on a 2% agarose gel. Each lane represents a different time point post-infection. C is a control reaction, using the primers with no DNA template.

a - reactions carried out with ribosomal protein constitutive gene primers.
b - reactions carried out with 2a3med & BR5/2, *cut-1*-specific primers.

The gels were then blotted and hybridised to labelled fragments of the DNA corresponding to the primers used in the PCR reactions.

C - Autoradiograph of blotted PCR reactions done using primers designed on the sequence of the constitutive ribosomal protein gene (R1 and NR3), hybridised to a ^{32}P-labelled fragment of the gene. Each lane represents a different post-infective time point at which first strand cDNA was made and used as the reaction template.

d - Autoradiograph of blotted PCR reactions done using primers designed on the sequence of *cut-1* (2a3med and Br5/2), hybridised to a ^{32}P labelled fragment of the *bpcut-1* gene. Each lane represents a different post-infective time-point at which first strand cDNA was made and used as the reaction template.

After autoradiography, hybridisation to each product was quantified by scintillation counting. For each life-cycle stage, the value of the cut-1 hybridisation was divided by the corresponding constitutive gene product in order to give ratio values.

e - A graph of log values of the ratios calculated as above against days post-infection, giving a semi-quantitative transcription pattern for the *bpcut-1* gene.

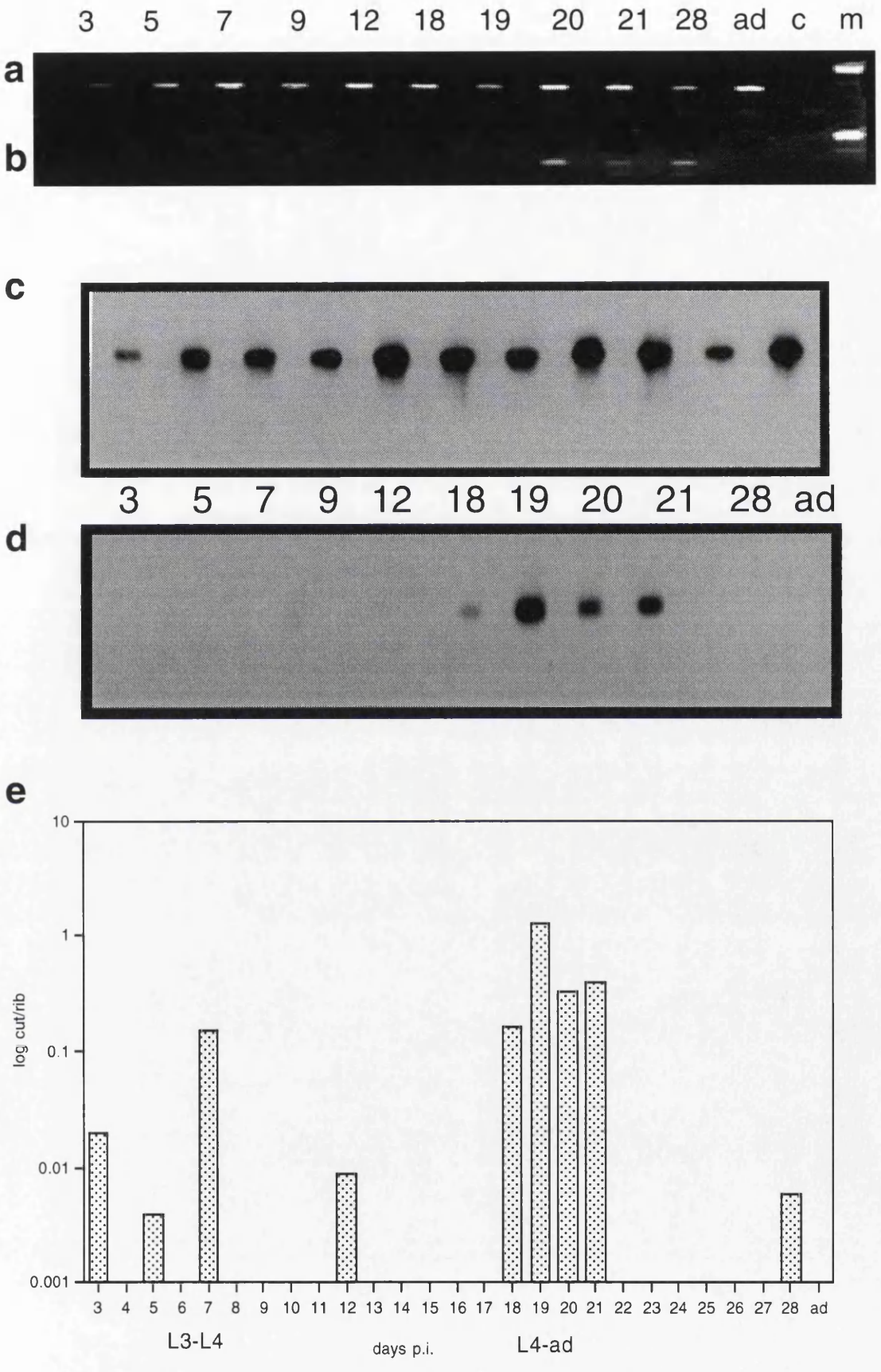

3 5 7 9 12 18 19 20 21 28 ad c m
a
b
c
3 5 7 9 12 18 19 20 21 28 ad
d
e
log cut/nib
10
1
0.1
0.01
0.001
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 ad
L3-L4
days p.i.
L4-ad

Figure 6.1

Figure 6.2.

Figure 6.2 presents the results of a second semi-quantitative RT-PCR experiment, to show the reproducibility of the method.

a - Autoradiograph of blotted PCR reactions done using primers designed on the sequence of the constitutive ribosomal protein gene (R1 and NR3), hybridised to a ^{32}P-labelled fragment of the gene. Each lane represents a different post-infective time point at which first strand cDNA was made and used as the reaction template.

b - Autoradiograph of blotted PCR reactions done using primers designed on the sequence of *cut-1* (2a3med and Br5/2), hybridised to a ^{32}P labelled fragment of the *bpcut-1* gene. Each lane represents a different post-infective time-point at which first strand cDNA was made and used as the reaction template.

After autoradiography, hybridisation to each product was quantified by scintillation counting. For each life-cycle stage, the value of the cut-1 hybridisation was divided by the corresponding constitutive gene product in order to give ratio values.

c - A graph of log values of the ratios calculated as above against days post-infection, giving a semi-quantitative transcription pattern for the *bpcut-1* gene.

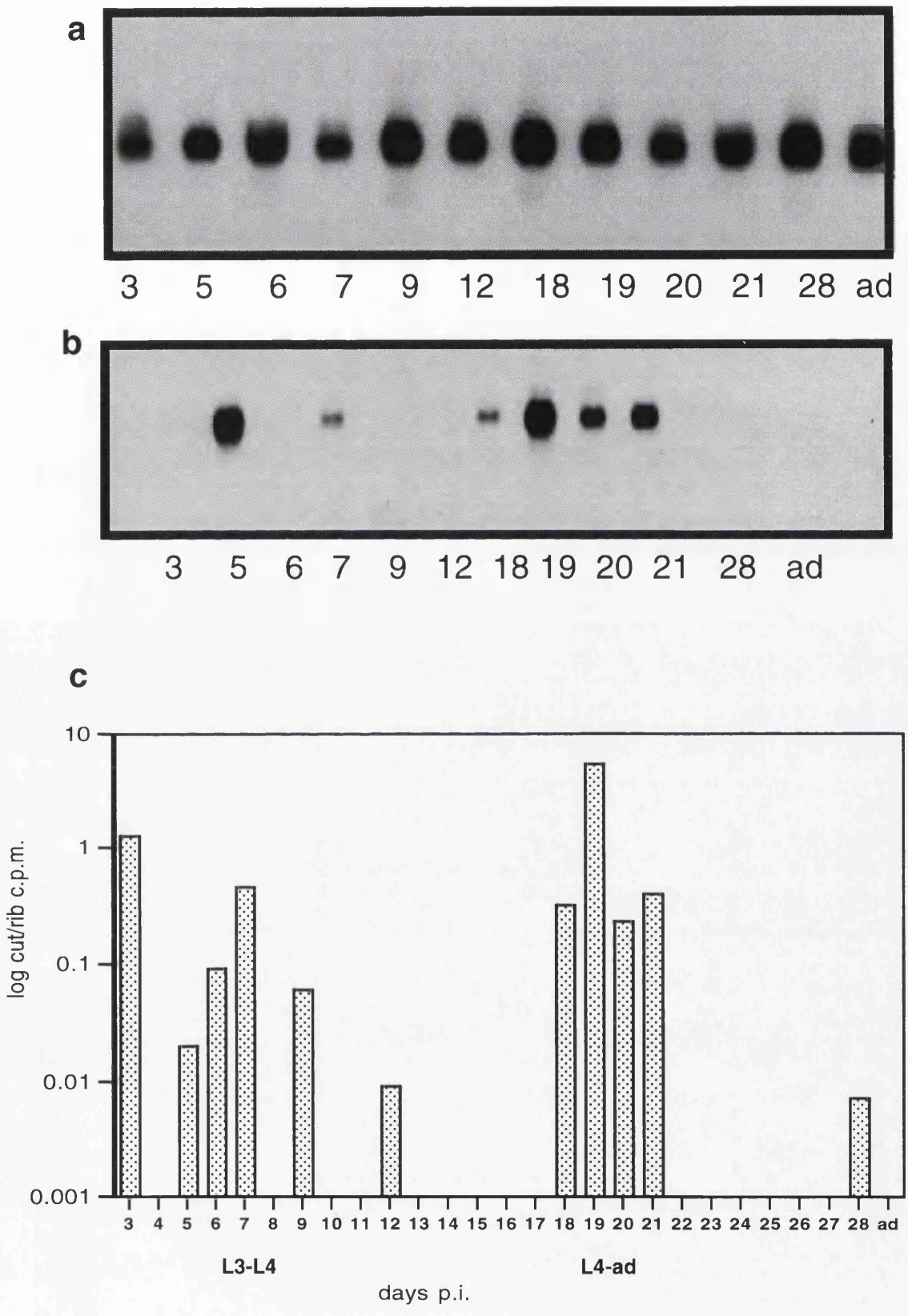

a
3 5 6 7 9 12 18 19 20 21 28 ad
b
3 5 6 7 9 12 18 19 20 21 28 ad
c
log cut/rib c.p.m.
10
1
0.1
0.01
0.001
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 ad
L3-L4
L4-ad
days p.i.

Figure 6.2.

DAYS POST-INFECTION cDNA	CUTICLIN/RIBOSOMAL PROTEIN CPM
3	0.02
5	0.004
7	0.15
9	less than control
12	0.009
18	0.16
19	1.25
20	0.33
21	0.39
28	0.006
adult	less than control

TABLE 6.1. Ratios of scintillation counts (cpm) obtained from Experiment 6.1.

DAYS POST-INFECTION cDNA	CUTICLIN/RIBOSOMAL PROTEIN CPM
3	1.3
5	0.02
6	0.09
7	0.46
9	0.06
12	0.009
18	0.32
19	5.3
20	0.23
21	0.4
28	0.007
adult	less than control

TABLE 6.2. Ratios of scintillation counts (cpm) obtained from Experiment 6.2.

PCR products using different life-cycle stages as the template and *cut-1* or ribosomal protein gene specific primers, were electrophoresed on agarose gels. The gels were blotted and hybridised with the corresponding gene-specific labelled DNA fragment. After autoradiography, pieces of the filters corresponding to the regions showing hybridisation of the probe were cut out and scintillation counted. A control region was cut from the filter and subtracted from each value. For each life-cycle stage, the value for the *cut-1* product was divided by the corresponding constiutive ribosomal protein gene product in order to give the ratios seen in the tables above.

Figure 6.3.

WESTERN BLOT ANALYSIS OF ADULT AND MICROFILARIAL ANTIGEN WITH ANTI-rASCUT-1.

Adult and microfilarial DOC extracts were run out on 12.5% SDS PAGE minigels, and immunoblotted onto nitrocellulose paper. The blots were incubated in either affinity-purified or whole anti-rASCUT-1 antiserum at several dilutions. Control lanes were incubated with pre-immune rabbit serum. The second antibody was goat anti-rabbit alkaline phosphatase conjugate diluted 1/6,000. BCIP/NBT substrate was used to develop the blot.

The filled arrow indicates the prominent 34KDa band which is recognized by the antiserum in both adult and mf extracts.

A. Adult DOC extracts.

lane 1	affinity-purified anti-rASCUT-1	1/20
lane 2	affinity-purified anti-rASCUT-1	1/40
lane 3	affinity-purified anti-rASCUT-1	1/80
lane 4	affinity-purified anti-rASCUT-1	1/160
lane 5	whole anti-rASCUT-1	1/500
lane 6	whole anti-rASCUT-1	1/1000
lane 7	pre-immune rabbit serum	1/500
lane 8	pre-immune rabbit serum	1/1000

B. Microfilarial DOC extracts.

lane 1	affinity-purified anti-rASCUT-1	1/20
lane 2	affinity-purified anti-rASCUT-1	1/40
lane 3	affinity-purified anti-rASCUT-1	1/80
lane 4	affinity-purified anti-rASCUT-1	1/160
lane 5	whole anti-rASCUT-1	1/500
lane 6	whole anti-rASCUT-1	1/1000
lane 7	pre-immune rabbit serum	1/500
lane 8	pre-immune rabbit serum	1/1000

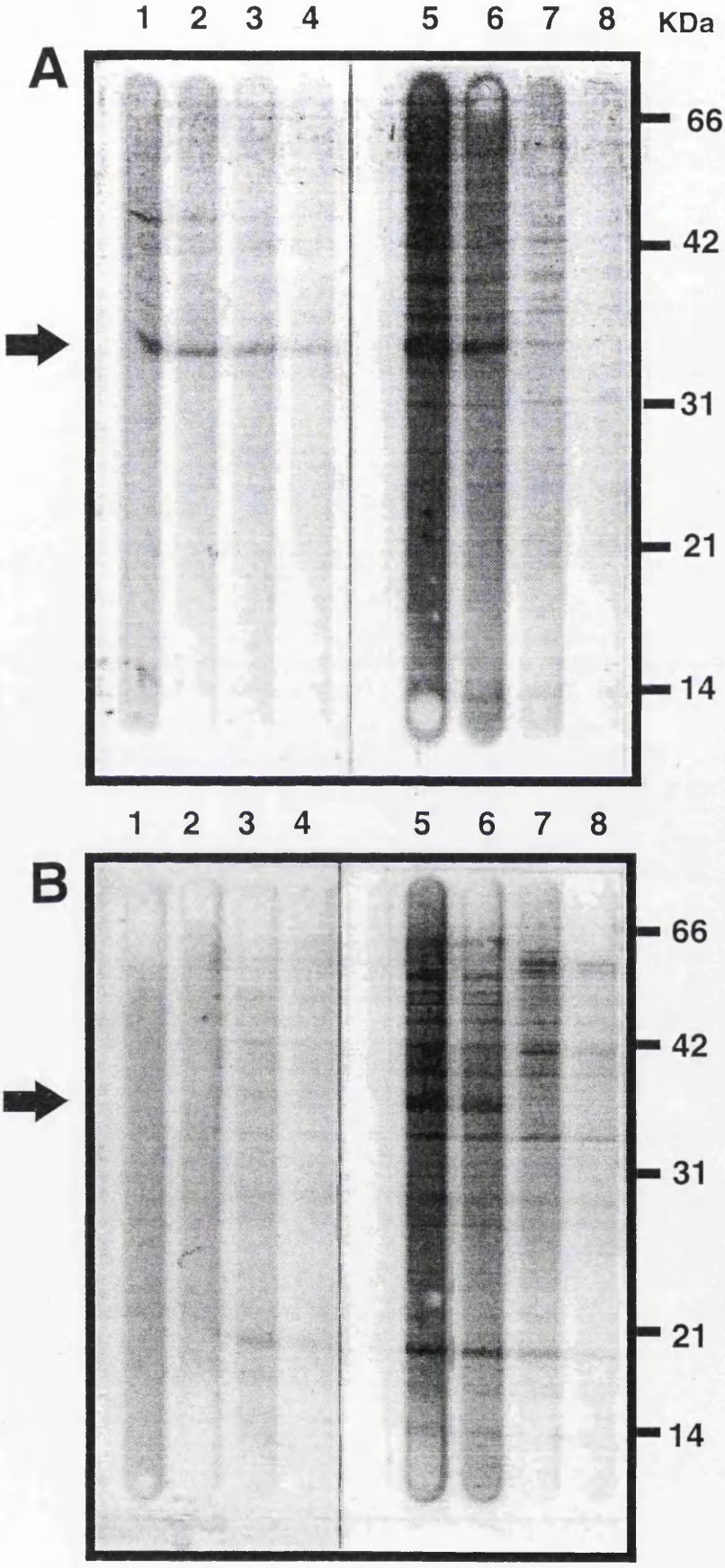


Fig. 6.3

Figure 6.4.

This figure shows a cryostat cross-section of an adult *B. pahangi* worm reacted with affinity-purified anti-rASCUT-1, using FITC-conjugated goat anti-rabbit antiserum as the secondary antibody. The image was viewed on a Zeiss axial plan microscope. Both dic (differential interference contrast) images and fluorescent images were viewed using filter set 15 (BP 546/12, FT 580, LP 590).

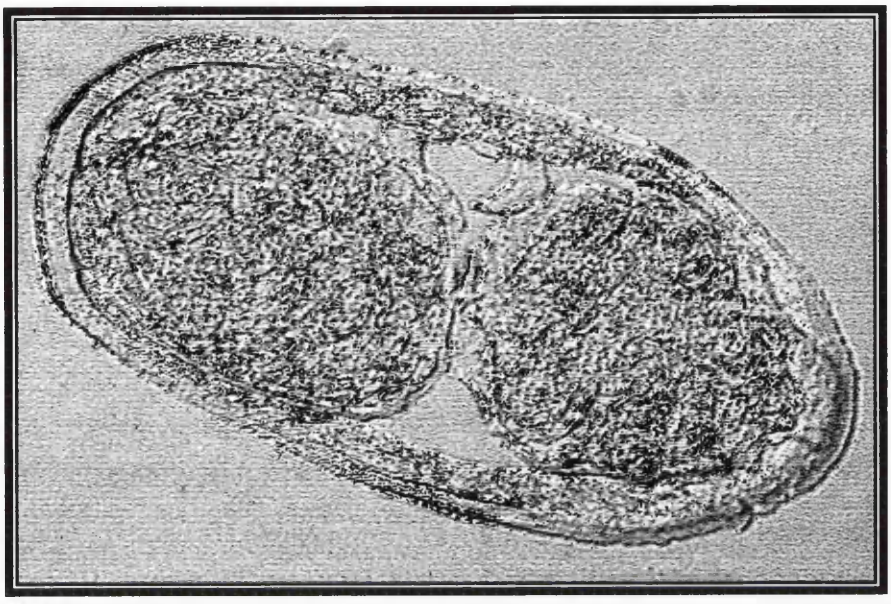
A - dic image

B - IFAT image

To Note:

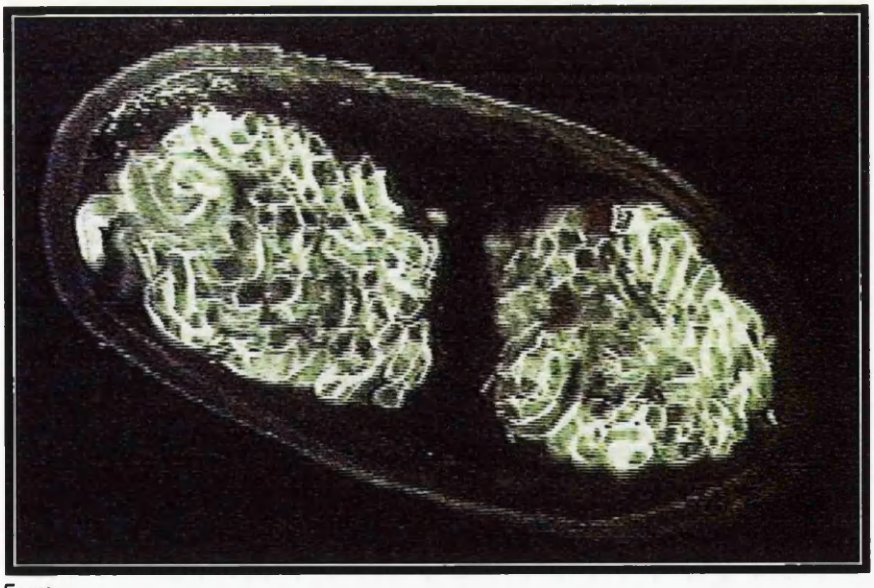
- Apparent double layer of epitope recognition in the cuticle.
- The antiserum appears to recognize epitopes on the mf surface more strongly than those on the adult cuticle.

A



5μm

B



5μm

Fig. 6.4.

Figure 6.5.

This figure shows a cryostat cross-section through the anterior of an adult female *B. pahangi* worm reacted with affinity-purified anti-rASCUT-1, using FITC-conjugated goat anti-rabbit antiserum as the secondary antibody. The image was viewed on a Zeiss axial plan microscope. Both dic (differential interference contrast) images and fluorescent images were viewed using filter set 15 (BP 546/12, FT 580, LP 590).

A - dic image
B - IFAT image

To Note:

- The mf in both uteri appear to be mature, and are recognized by the antiserum.

I Intestine

M Muscle

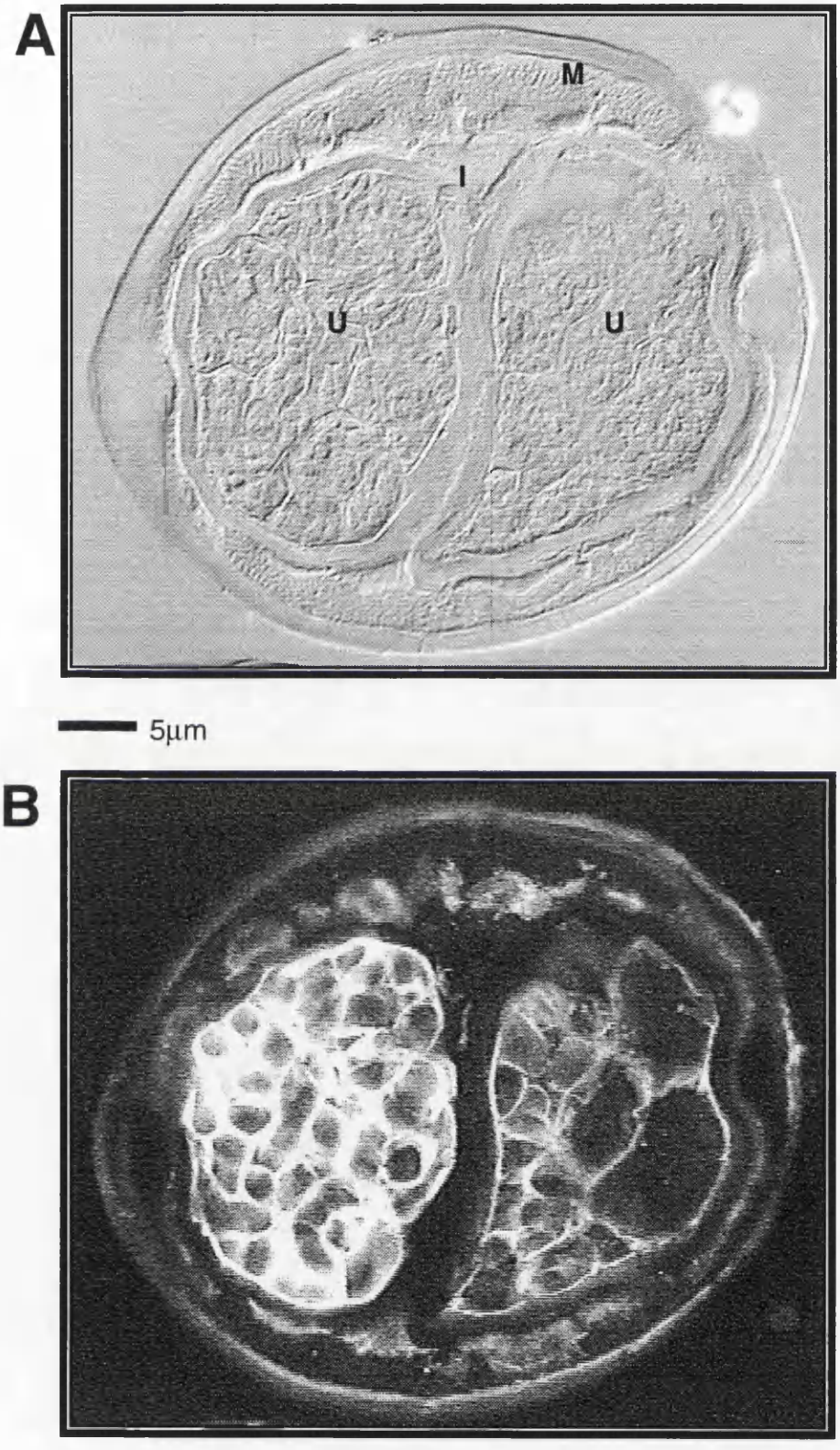


Fig. 6.5.

Figure 6.6.

This figure shows a cryostat cross-section through the posterior of an adult female *B. pahangi* worm reacted with affinity-purified anti-rASCUT-1, using FITC-conjugated goat anti-rabbit antiserum as the secondary antibody. The image was viewed on a Zeiss axial plan microscope. Both dic (differential interference contrast) images and fluorescent images were viewed using filter set 15 (BP 546/12, FT 580, LP 590).

A - dic image
B - IFAT image

To Note:

- The mf in both uteri appear to be immature, and are not recognized by the antiserum.
- Apparent double layer of epitope recognition in the cuticle.

I Intestine

U Uterus

M Muscle

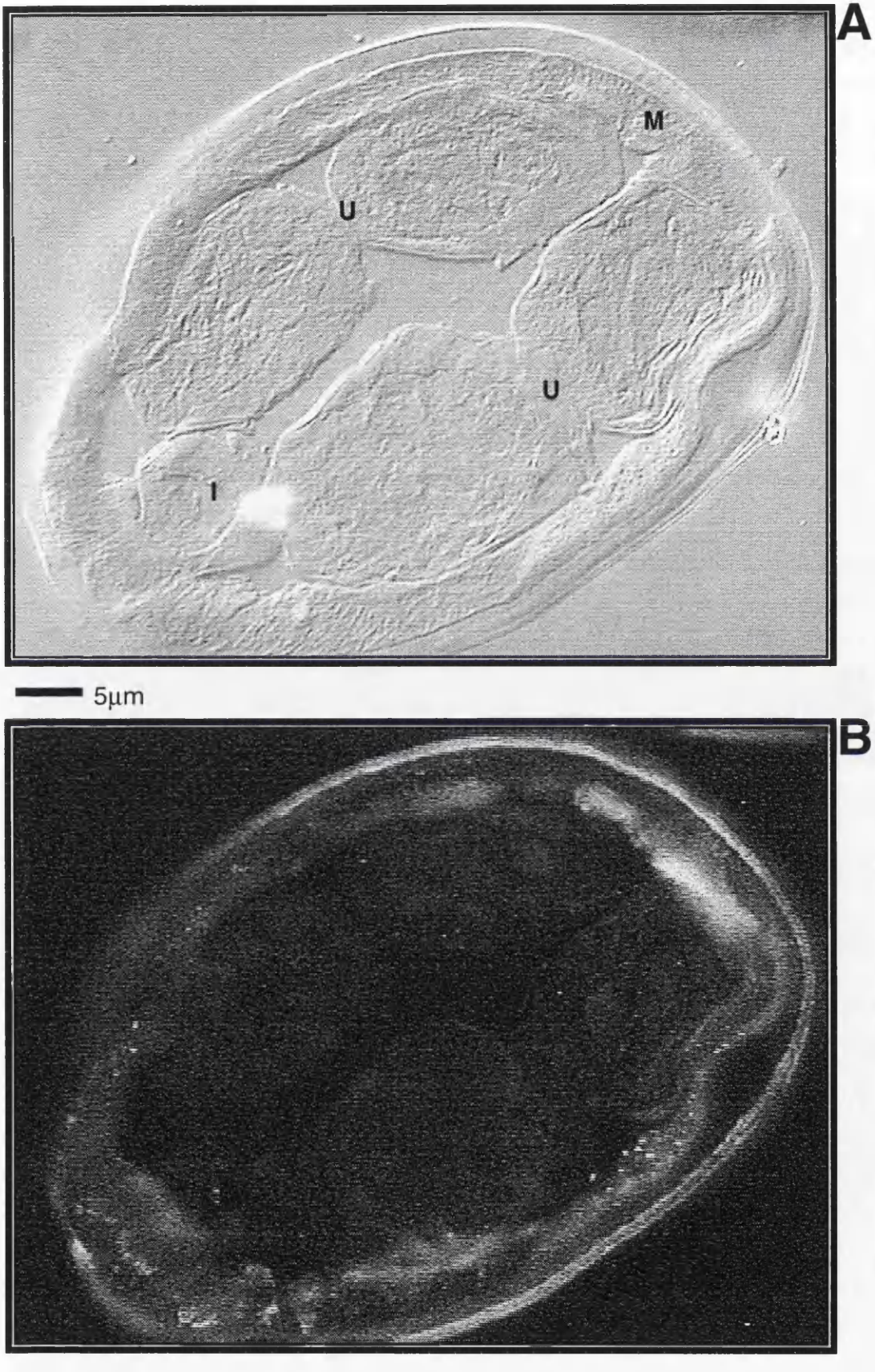


Fig. 6.6.

Figure 6.6.

This figure shows a cryostat cross-section through the posterior of an adult female *B. pahangi* worm reacted with affinity-purified anti-rASCUT-1, using FITC-conjugated goat anti-rabbit antiserum as the secondary antibody. The image was viewed on a Zeiss axial plan microscope. Both dic (differential interference contrast) images and fluorescent images were viewed using filter set 15 (BP 546/12, FT 580, LP 590).

A - dic image
B - IFAT image

To Note:

- The mf in both uteri appear to be immature, and are not recognized by the antiserum.
- Apparent double layer of epitope recognition in the cuticle.

I Intestine

U Uterus

M Muscle

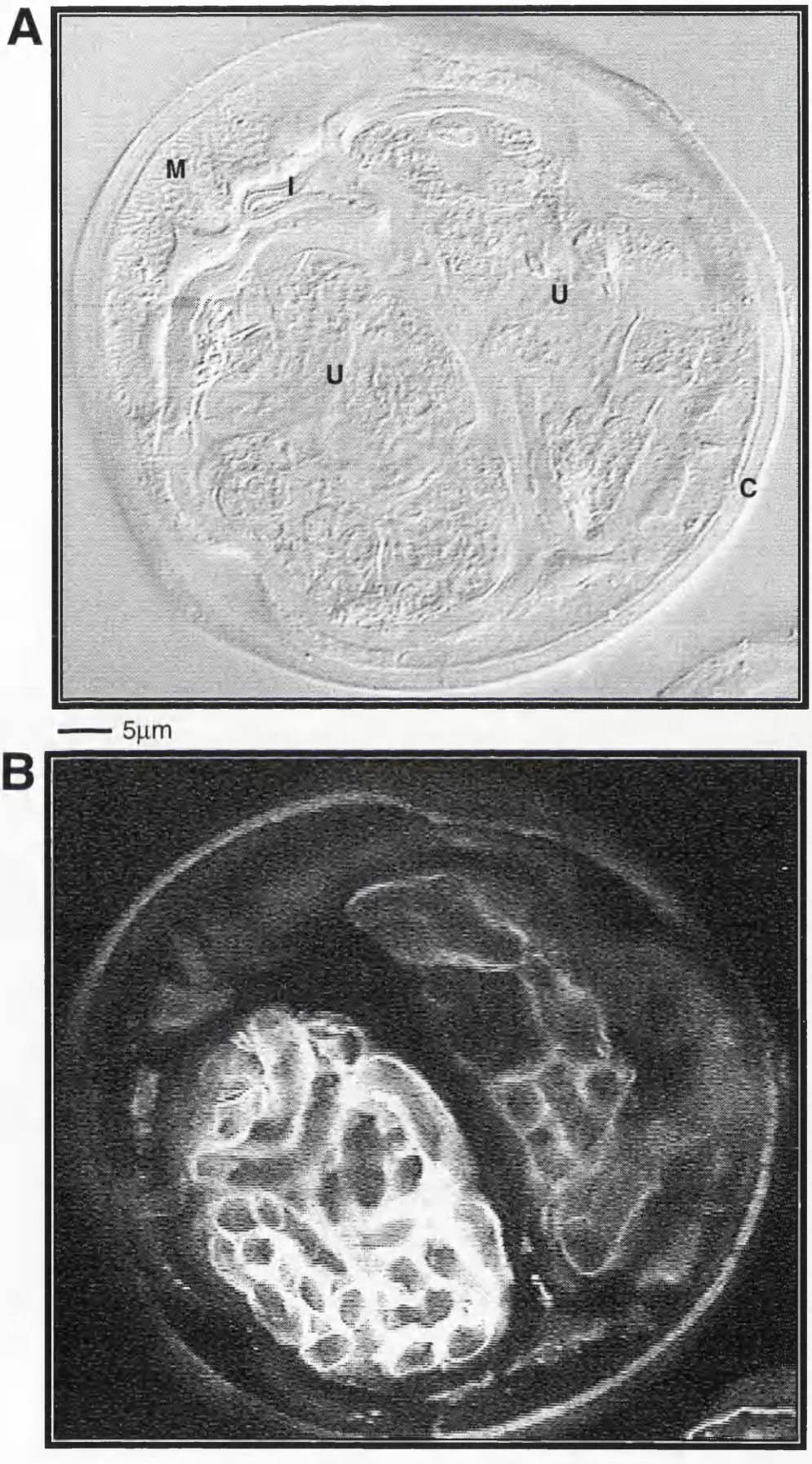


Fig. 6.7

Figure 6.8.

Immunofluorescence anti-body tests (IFAT) using the anti-*cut-1* anti-serum anti-rASCUT-1 on *B. pahangi* extruded microfilariae and L4s. All experimental details are included in Materials and Methods. Worms were fixed, either whole or sectioned, onto glass slides, and reacted with anti-rASCUT-1. Labelling was achieved by reaction with a secondary antibody: FITC-conjugated goat anti-rabbit antiserum. Fluorescence was viewed and photographed using a LEITZ microscope.

A. Extruded microfilaria x4000 from the second segment of an adult female. The mf is exsheathed, demonstrating that the anti-rASCUT-1 anti-serum is recognising epitopes on the worm cuticle, rather than on the sheath.

B. Extruded mf from the second segment of an adult female x 1000, showing reactivity of the anti-rASCUT-1 on the surface of the mf. In the photograph it can be noted that there is a third, non-fluorescent mf.

C. Cross-section through a *B. pahangi* L4 x1000, showing anti-rASCUT-1 labelling of the larval cuticle. There is no internal labelling.

D. As above.

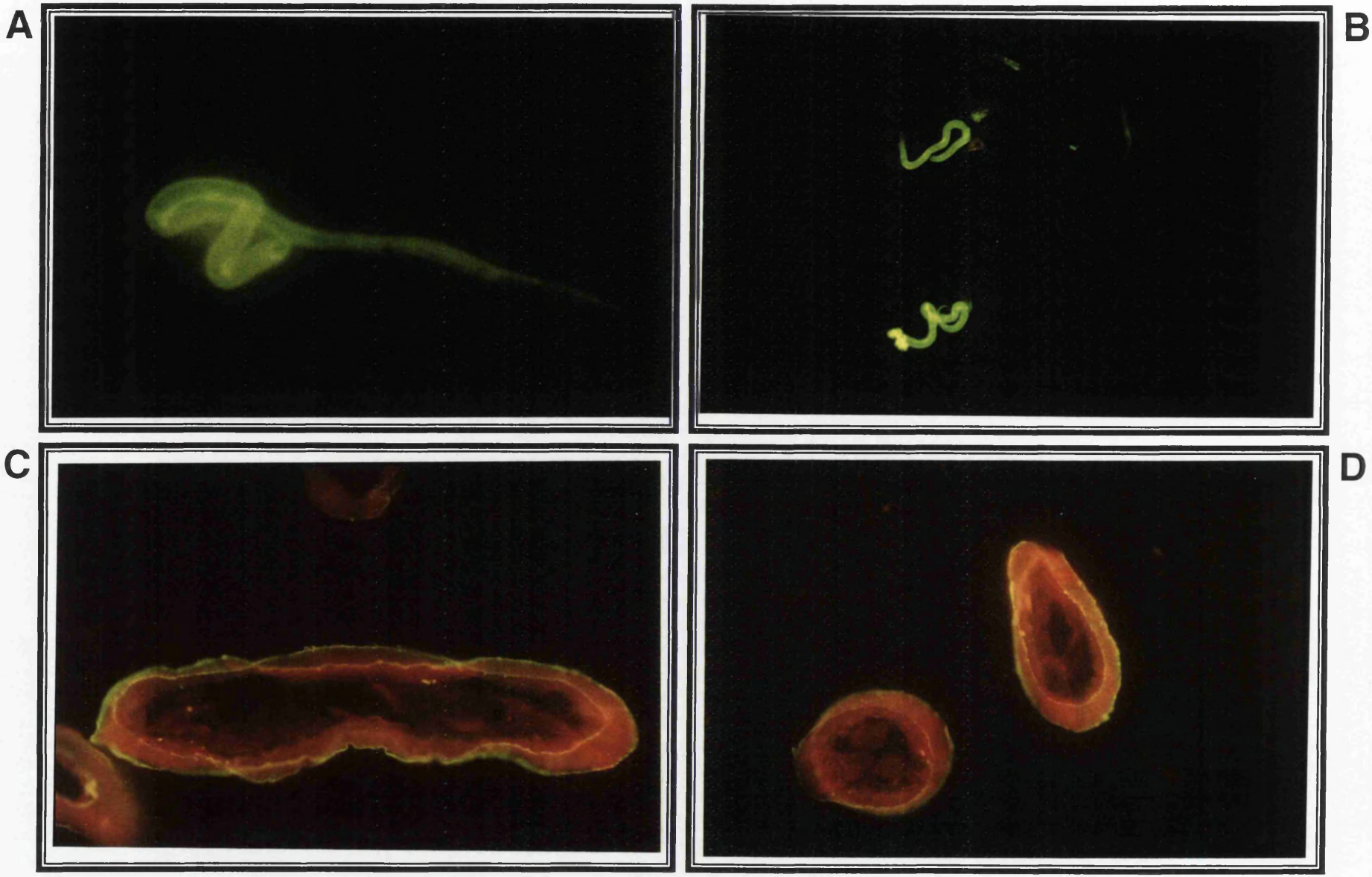

A
B
C
D

Figure 6.9.

This figure shows a cryostat cross-section through the anterior of an adult female *B. pahangi* worm reacted with affinity-purified anti-rASCUT-1, using FITC-conjugated goat anti-rabbit antiserum as the secondary antibody. The image was viewed on a Zeiss axial plan microscope. Both dic (differential interference contrast) images and fluorescent images were viewed using filter set 15 (BP 546/12, FT 580, LP 590).

A - IFAT image
B - dic image

To Note:

- Recognition by the antiserum is specifix to epitopes on the mature mf surface; there is no internal labelling of the embryos.

C Cuticle

M mf

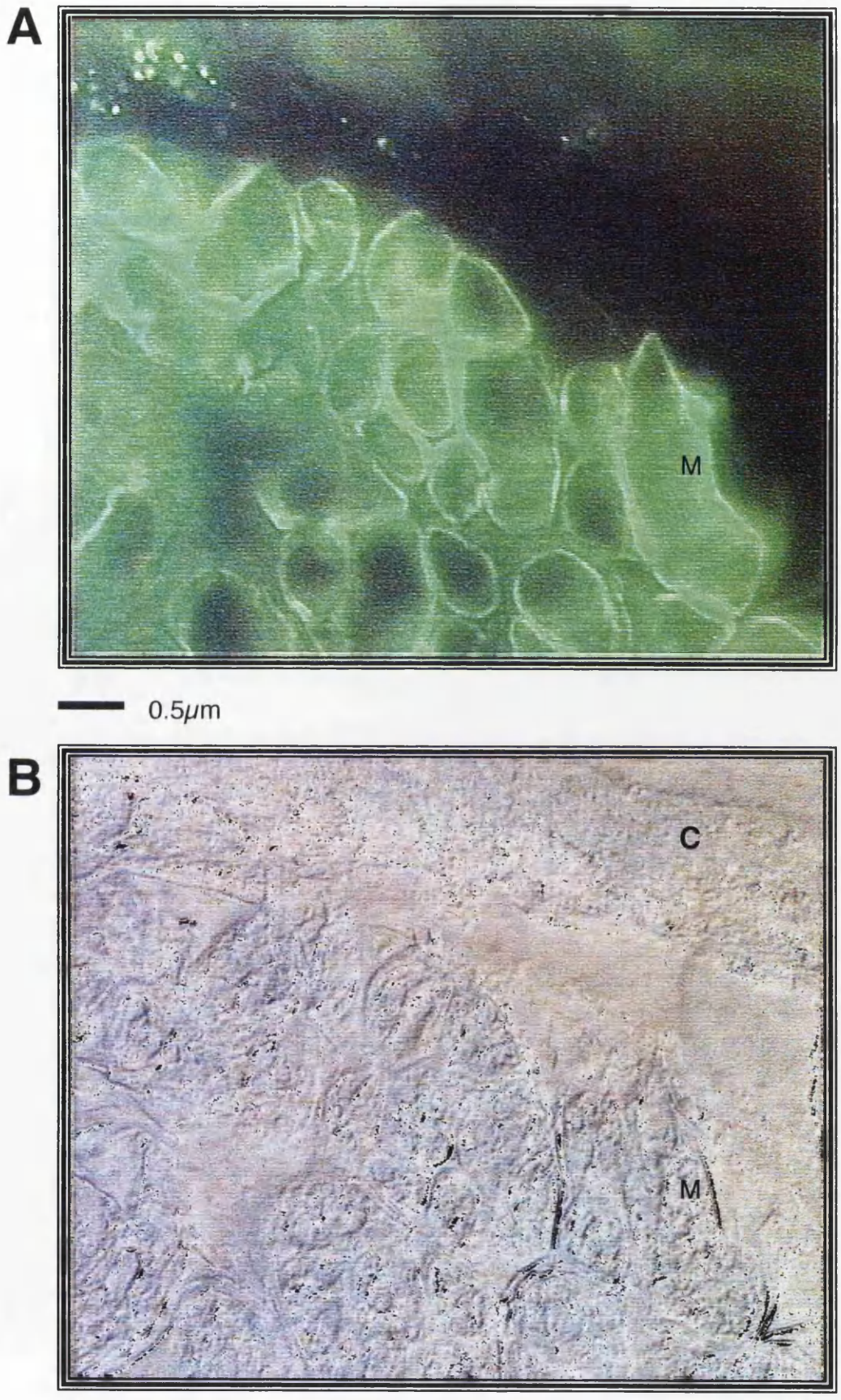


Fig. 6.9.

Figure 6.10.

This figure shows a cryostat cross-section through the anterior of an adult female *B. pahangi* worm reacted with affinity-purified anti-rASCUT-1, using FITC-conjugated goat anti-rabbit antiserum as the secondary antibody. The image was viewed on a Zeiss axial plan microscope. Both dic (differential interference contrast) images and fluorescent images were viewed using filter set 15 (BP 546/12, FT 580, LP 590).

A - dic image
B - IFAT image

To Note:

- The antiserum is recognizing epitopes on the embryos in the lower, and not the upper, uterus.

I Intestine

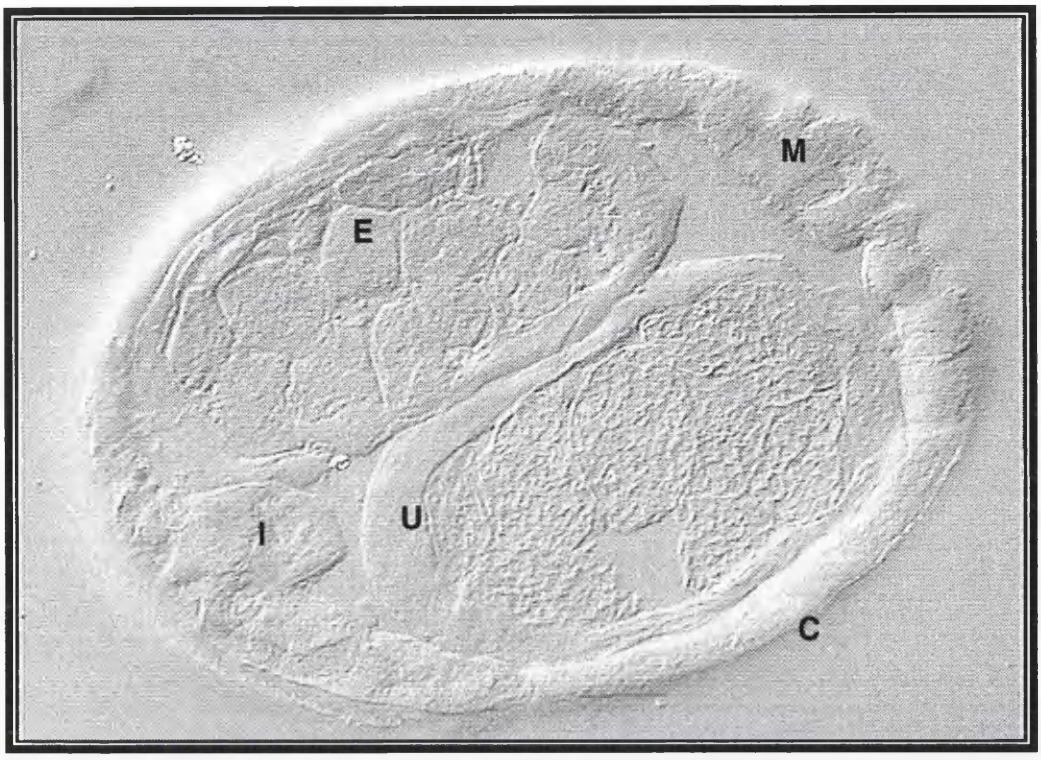
C Cuticle

M Muscle

E Embryo

U Uterus

A



5µm

B

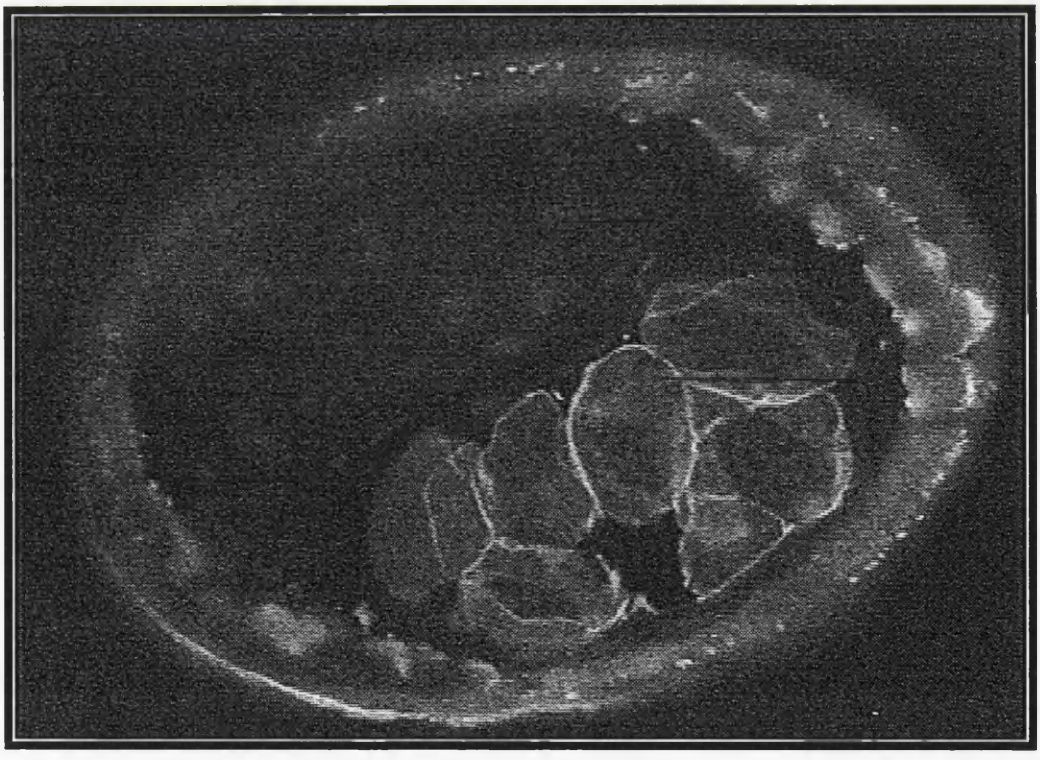


Fig. 6.10

Figure 6.11

This figure shows an ultrathin section through an adult female *B. pahangi* worm, reacted with either affinity-purified anti-rCUT-1 antiserum or pre-immune rabbit serum. The secondary antibody was goat anti-rabbit anti-IgG conjugated to 10nm Au. The image was viewed by electron microscopy. Full experimental details are given in Materials and Methods.

A Negative control: reacted with pre-immune rabbit serum.

B Reacted with a 1/10 dilution of affinity-purified anti-rCUT-1.

Magnification : X24,000

C Cortical layer of the cuticle

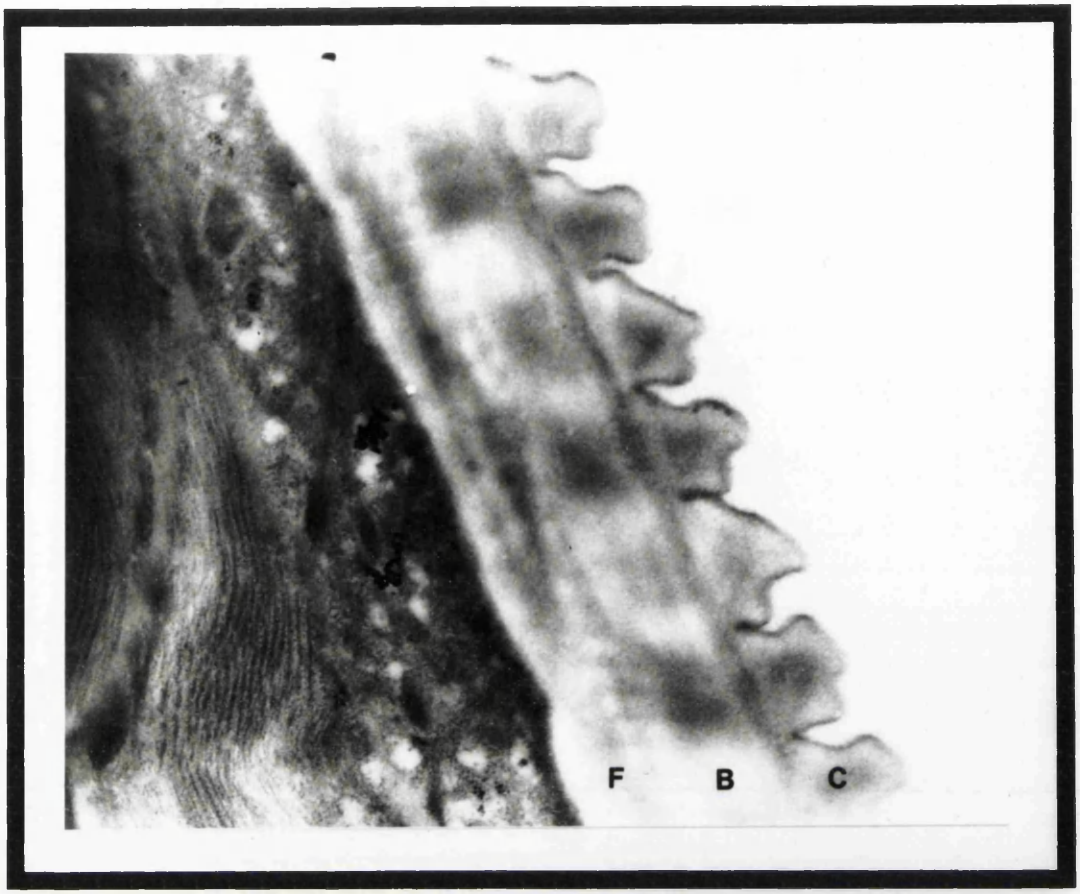
B Basal layer of the cuticle

F Fibrous layer of the cuticle.

To note:

- The epitopes recognised by the antiserum localise to a discrete layer of the nematode cuticle, the basal layer.
- There is no recognition of epitopes on the external surface of the cuticle.
- There appears to be recognition of epitopes in the cells of the hypodermis, which underlies the cuticle.

A



B

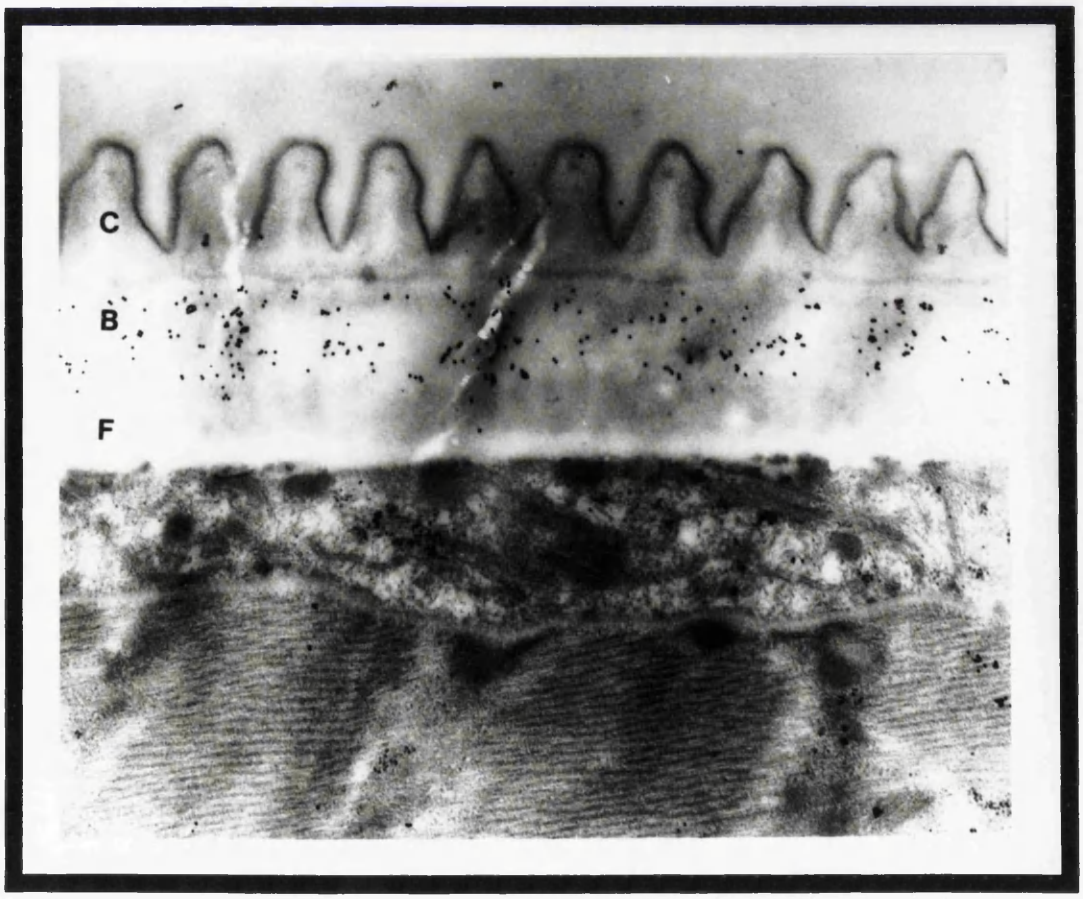


Figure 6.12

This figure shows an ultrathin section through an adult male *B. pahangi* worm, reacted with either affinity-purified anti-rCUT-1 antiserum or pre-immune rabbit serum. The secondary antibody was goat anti-rabbit anti-IgG conjugated to 10nm Au. The image was viewed by electron microscopy. Full experimental details are given in Materials and Methods.

A Negative control: reacted with pre-immune rabbit serum.

B Reacted with a 1/10 dilution of affinity-purified anti-rCUT-1.

Magnification : X 24,000

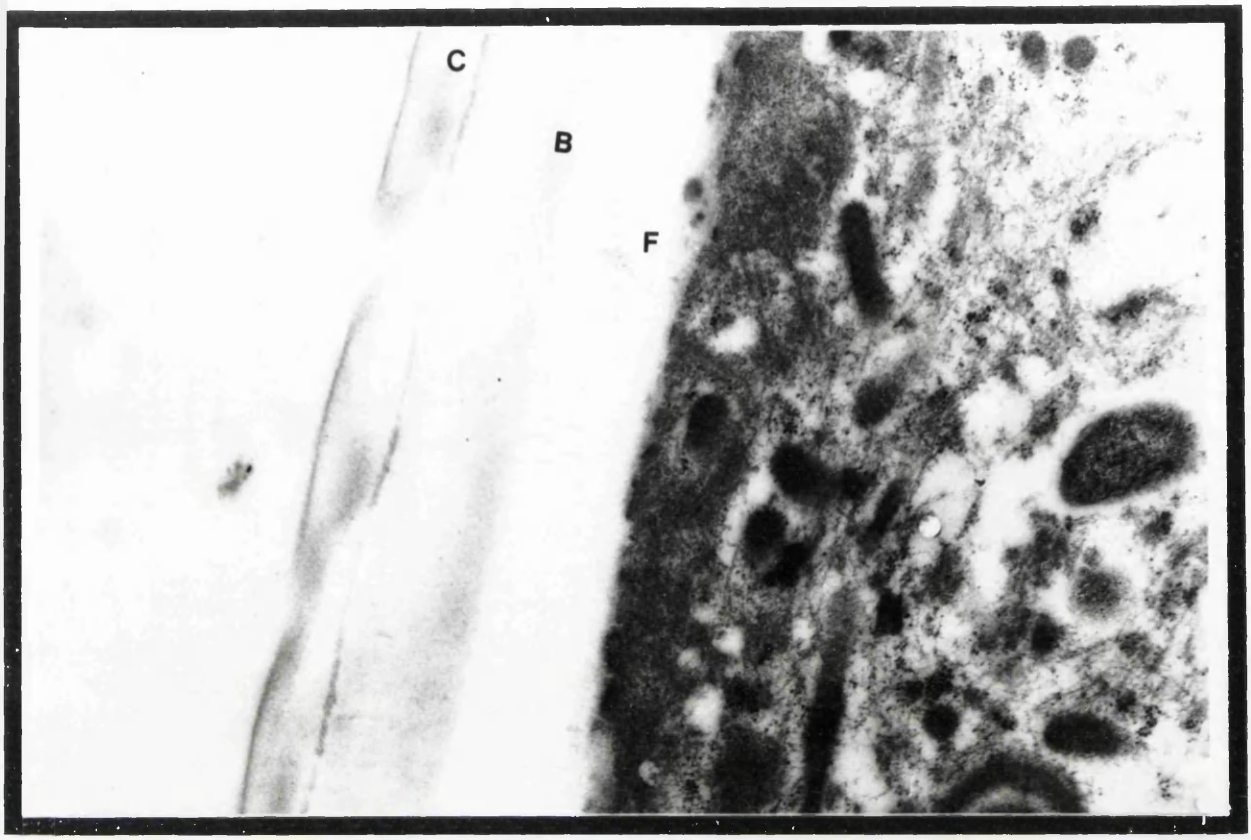
C Cortical layer of the cuticle

B Basal layer of the cuticle

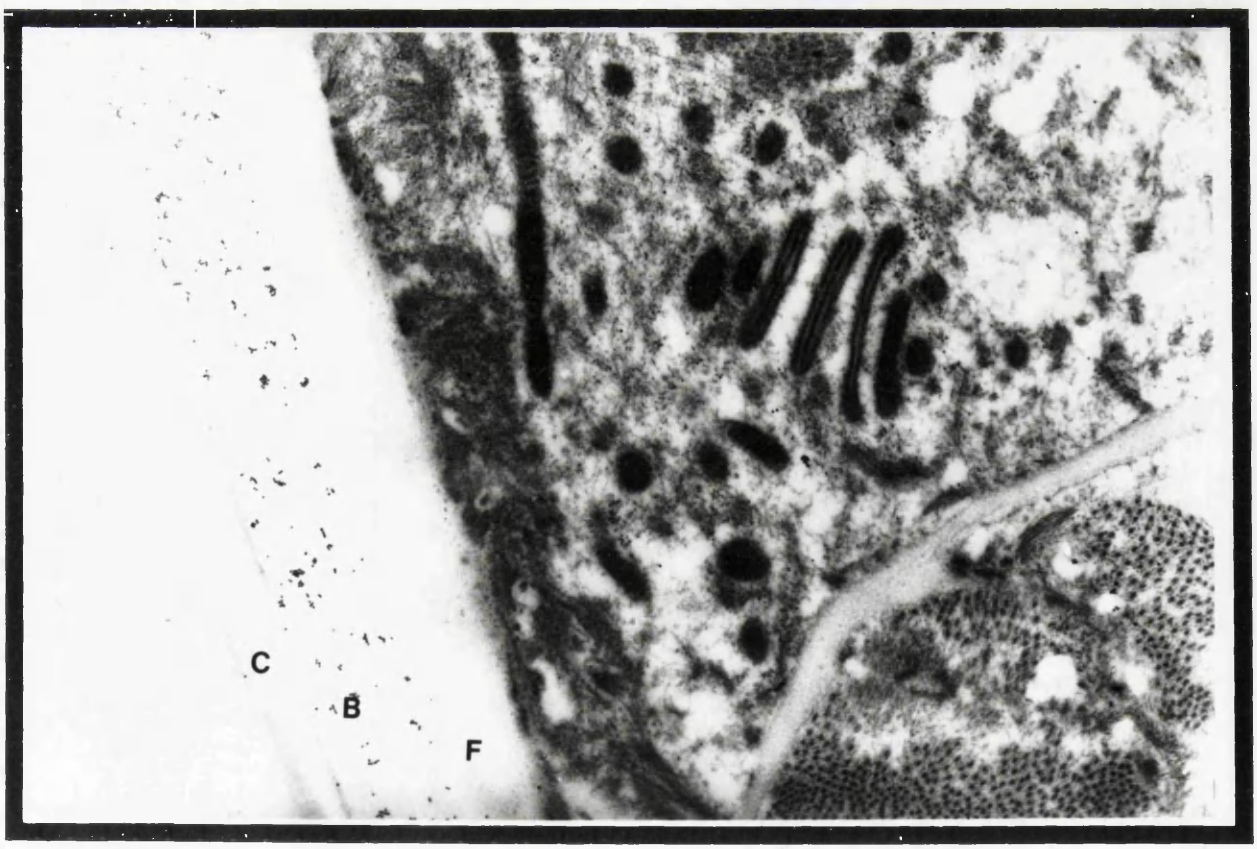
F Fibrous layer of the cuticle.

To note:

- The epitopes recognised by the antiserum localise to a discrete layer of the nematode cuticle, the basal layer.
- There is no recognition of epitopes on the external surface of the cuticle.



A



B

Figure 6.13

This figure shows ultrathin sections through *B. pahangi* mf *in utero,* showing the localisation of the epitopes recognised by the affinity-purified anti-rCUT-1 antiserum mf cuticle. The secondary antibody was goat anti-rabbit anti-IgG conjugated to 10nm Au. The image was viewed by electron microscopy. Full experimental details are given in Materials and Methods.

A Immature mf reacted with a 1/10 dilution of affinity-purified anti-rCUT-1.

B Mature mf reacted with a 1/10 dilution of affinity-purified anti-rCUT-1.

Magnification : X40,000

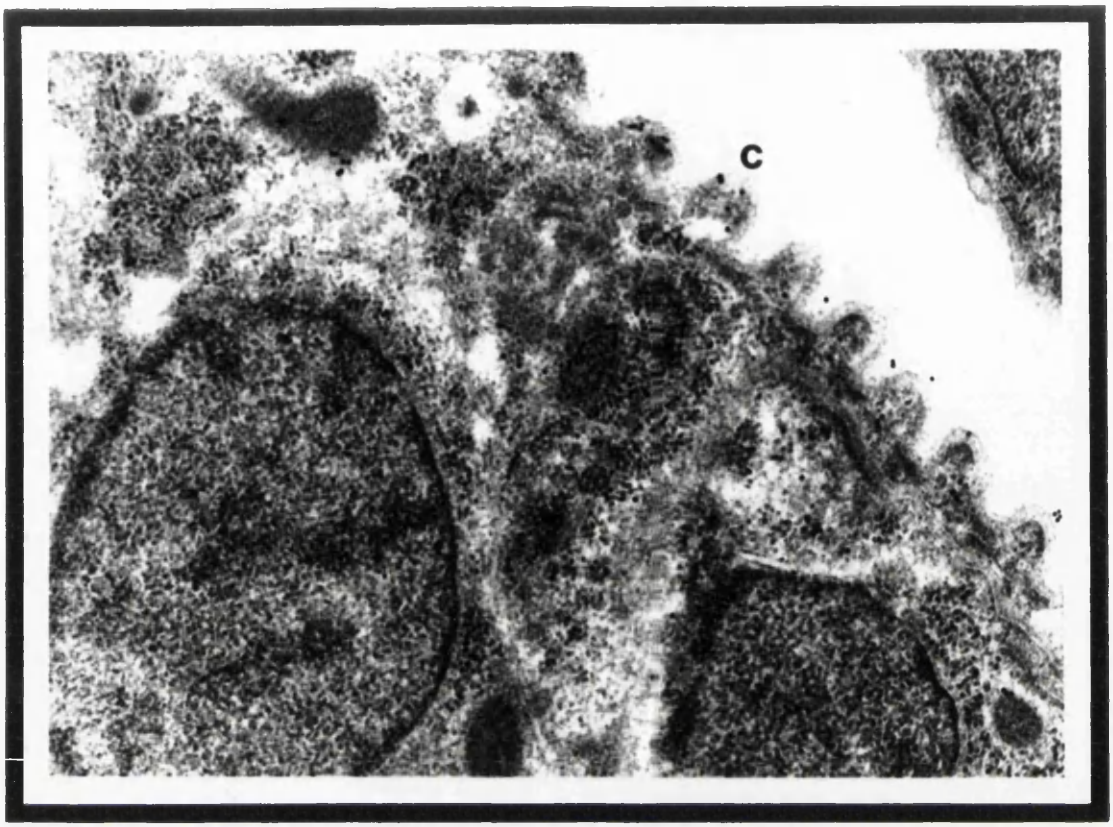
C Cuticle of the mf

S mf sheath

To note:

- The epitopes recognised by the antiserum localise to the mf cuticle.
- There is no recognition of epitopes on the mf sheath.
- There is no recognition of epitopes on the cuticle of the immature mf.

A



B

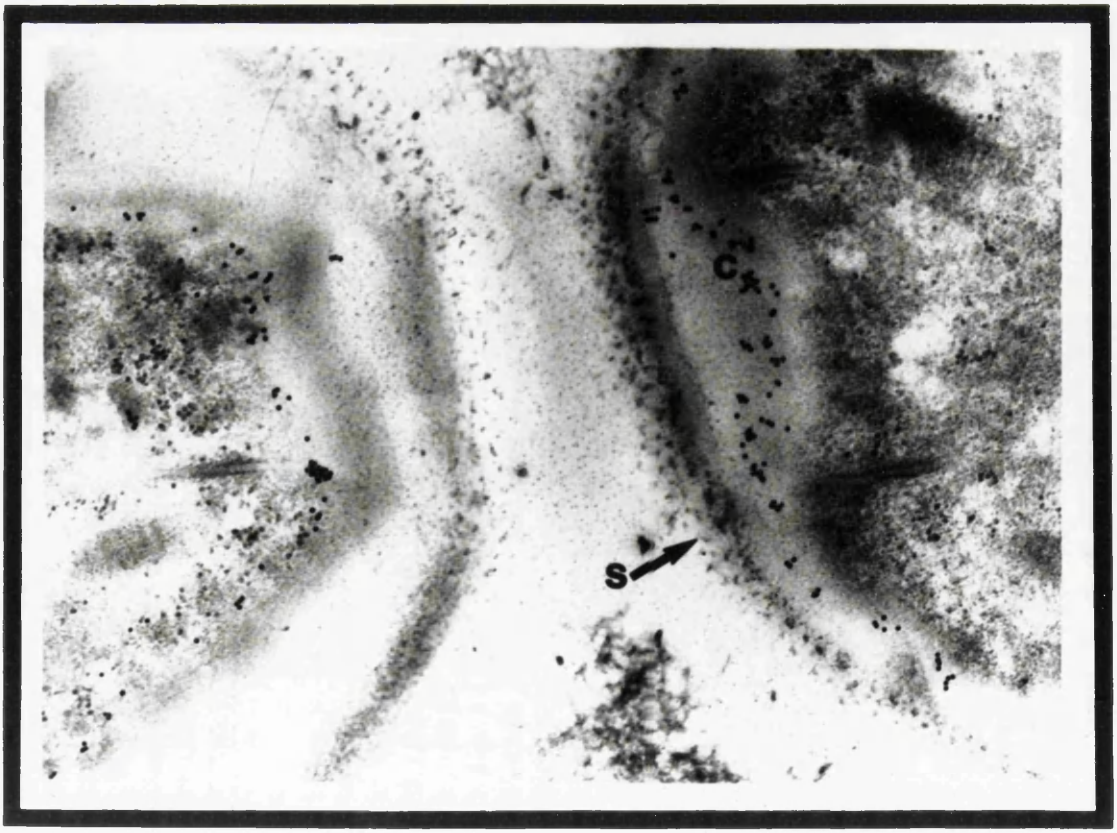


Figure 6.14

This figure shows an ultrathin section through peritoneal-derived (mature)*B. pahangi* mf, showing the localisation of epitopes recognised by the affinity-purified anti-rCUT-1 antiserum The secondary antibody was goat anti-rabbit anti-IgG conjugated to 10nm Au. The image was viewed by electron microscopy. Full experimental details are given in Materials and Methods

A Negative control: reacted with pre-immune rabbit serum.

B Reacted with a 1/10 dilution of affinity-purified anti-rCUT-1.

Magnification : X60,000

C Cortical layer of the mf cuticle

B Basal layer of the mf cuticle

F Fibrous layer of the mf cuticle

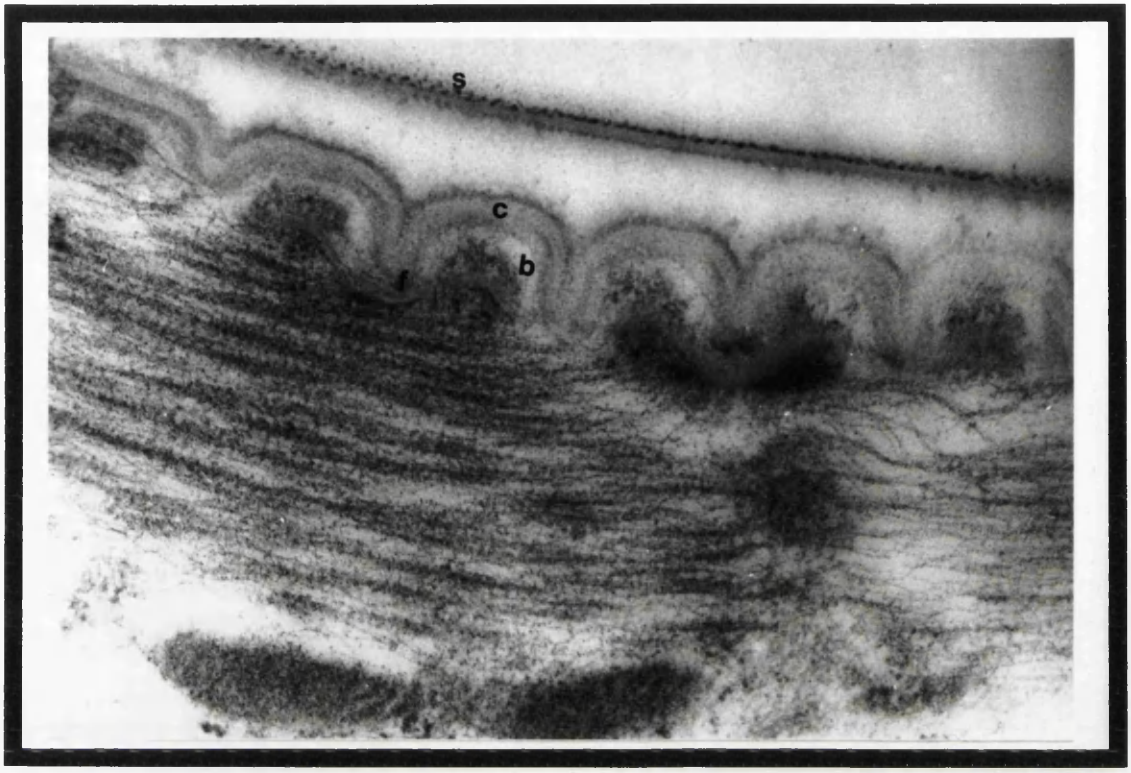
S mf sheath

To note:

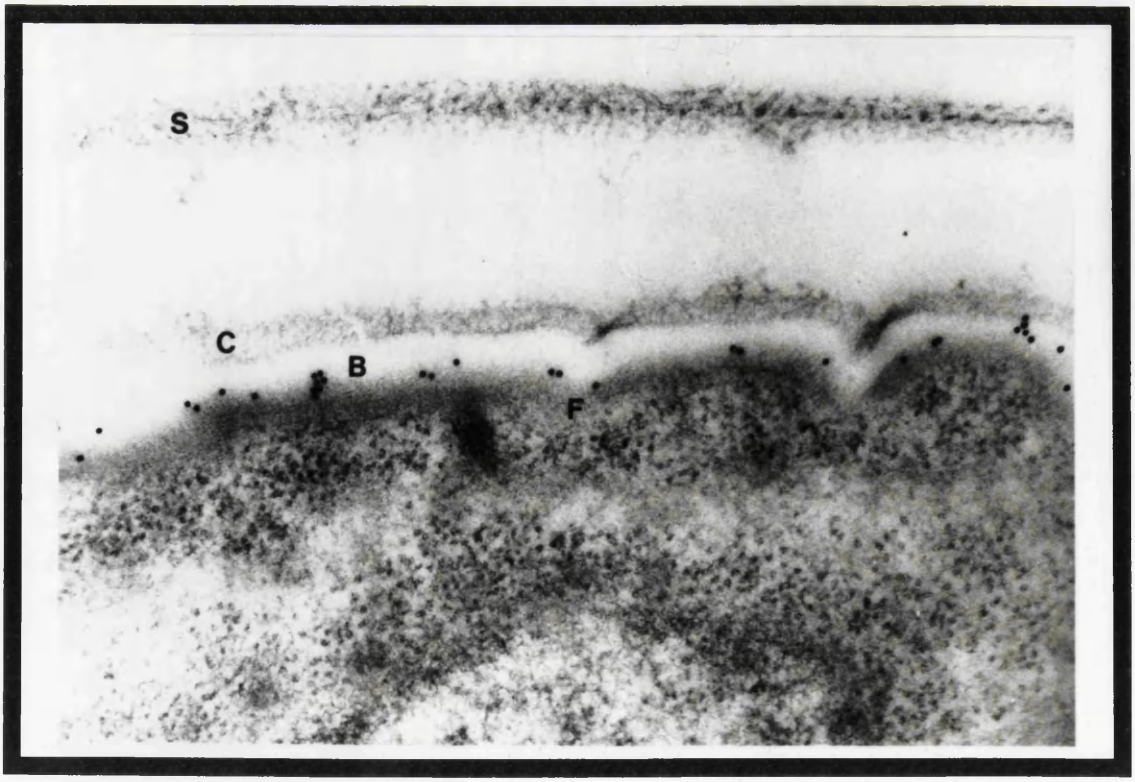
- The epitopes recognised by the antiserum appear to be located in the basal layers of themf cuticle.

- There is no recognition of epitopes on the mf sheath.

A



B



CHAPTER SEVEN: CONCLUDING REMARKS.

The aim of the discussions in Chapters Three to Six was to deliberate the possible meanings and significance of the data presented in each experimental chapter. Additionally, the practical methodologies used at each stage were discussed in the context of the results obtained. Ambiguities and conflicting observations were presented and, hopefully, addressed. Conversely, the aim of this chapter is to draw together the most meaningful points which have emerged in the course of the project as a whole, and show how they could be used to further the study of nematode cuticlin genes.

Comment is also made on areas of the project which yielded results which were confusing and which could interfere with future studies, or which require further investigation.

The first of these is the cloning of ELBP3, isolated during the course of an antibody screening of the *B. pahangi* adult cDNA library using antisera raised against recombinant *C.elegans* CUT-1 and CUT-2. ELBP3 was shown to be the *Brugia* homologue of a *C. elegans* dead-box RNA helicase gene, f01f1.7, which was identified in the course of the *C. elegans* Genome Sequencing Project. The recognition of the clone by the antisera was specific, as indicated by the fact that the clone was independently recognised by the antisera four times. The most likely explanation for the presence of an antibody against a nematode RNA helicase is that the rabbit used to raise the antisera against recombinant CUT-1 or recombinant CUT-2 already had anti-RNA helicase antibodies, due to a previous natural nematode infection. Germ-line specific RNA helicases have been shown to be highly immunogenic, and pre-immune rabbit sera has been shown to contain anti-RNA helicase antibodies (Strome & Wood, 1983; Gruidlet al, 1996) The fact that the same cDNA species was recognised four times relates to the fact that RNA helicase mRNAs would be expected to be highly represented in an adult cDNA library, due to the fact that maternally derived embryonic proteins are being synthesised at high levels, and RNA helicases play an important role in translation. If ELBP3 was a germ-line-specific RNA helicase, there is an argument that it would be worthwhile to continue with its study as part of a project concerned with nematode development. However, in the context of the current project, ELBP3 represented a dead-end in the research process.

Southern blots of cleaved genomic DNA from a variety of parasitic nematodes of medical and veterinary importance were probed with ^{32}P-labelled *Ascaris cut-1.* The results of these experiments revealed the presence of at least one hybridising band in each of the nematodes analysed, implying the presence of at least one *cut-1*-like gene in each of the genomes. The probe hybridised to several bands in the *B. pahangi* digests. These results demonstrated that the *cut-1*-like gene is ubiquitous throughout the nematode phylum, that there is probably more than one *cut-1*-like gene in the *B. pahangi* genome, and that hybridisation between the species is easily detectable using the *ascut-1* probe.

A fragment (named LS2) of the *B. pahangi* cuticle collagen gene *bpcol-1* was isolated by RT-PCR on day 16 p.i. first strand cDNA using a *cut-1*-specific primer in combination with an oligo (dT) primer. This was an intriguing finding, because *bpcol-1* had been originally isolated during the course of another project, the aim of which was the isolation of *Brugia* cuticlin genes. The gene was isolated by screening a *B. pahangi* cDNA library using antisera raised against the insoluble residue of the *B. pahangi* cuticle i.e. the cuticlin residue (Bisoffi & Betschart, 1996). It is known that none of the nematode cuticlin genes so far isolated have any similarity, at the nucleotide or amino acid levels, to any known nematode cuticle collagen. So at first glance it seems an extraordinary coincidence: the independent isolation of the same collagen gene by two completely different cuticlin-based methods: antibody recognition using anti-cuticlin antisera; and PCR using a gene-specific primer based on the sequence of a cuticlin gene. However, there is a rational explanation for both. Bisoffi & Betschart (1996) hypothesise that, even following extensive chemical and enzymatic treatment to remove collagen from the insoluble material used for immunisation, some collagen remains which masks the immunogenicity of the cuticlin components. Consequently they report that using the resultant antiserum to screen the *B. pahangi* cDNA resulted in the isolation of several collagen-like clones and no cuticlin-like clones. The amplification of LS2, a fragment of *bpcol-1*, using a *bpcut-1*-specific primer, is probably attributable to a combination of two factors: the first is that the 3' primer was oligo (dT), notorious for non-specific priming, as has been exhaustively covered in Chapters Four and Six; and the second is that it is very likely that *bpcol-1* is highly represented in cDNA from day 16 p.i., as this time point occurs immediately before a moult, when the structural components of the nematode cuticle would be expected to be synthesised.

The aim of the project was to identify a *Brugia* cuticlin homologue, which has been accomplished from both *B. pahangi* and *B. malayi*. The two genes, named *bpcut-1* and *bmcut-1*, are the *Brugia* homologues of the *C. elegans cut-1* gene, and as such share homology with the *cut-1*-like genes isolated from *Ascaris* and *M. artiella*. The cDNA corresponding to *bpcut-1* was obtained by PCR on day 7p.i. cDNA using primers based on the *cut-1* sequences of *Brugia* and *Ascaris*. Comparison of the genomic and cDNA sequences revealed the existence of a *bpcut-1*-specific intron.

The pattern of *bpcut-1* mRNA abundance throughout the mammalian stages of the life-cycle of the parasite strongly implies that it encodes a component of the nematode cuticle: peaks of abundance occur before the L3-L4 and the L4-adult moults, when the components of the cuticle are being maximally synthesised. The mRNA abundance profile also shows that the *bpcut-1* transcript can be detected during the intermoult period, but at a very much lower abundance. There seems to be little or no *bpcut-1* transcript present in the adult mRNA, implying that the protein encoded by *bpcut-1* is incorporated into the insoluble component of the cuticle at the time of cuticle synthesis, and that it is not continuously renewed throughout the adulthood of the parasite.

The localisation studies carried out using the anti-rASCUT-1 antiserum in *B. pahangi* firstly confirmed that the protein encoded by *bpcut-1* is present exclusively within the cuticle of the worm, as would be expected for a structural component of the cuticle. Furthermore, the antiserum showed strong reactivity to the surface of mature mf, revealing the probable existence of a second *Brugia cut-1*-like gene which encodes a component of the embryonic cuticle. The anti-rASCUT-1 antiserum was raised against the '*cut-1* box' region of the CUT-1 protein, known to show a high homology to all the CUT-1-like proteins predicted from the isolated *cut-1*-like genes of parasitic nematodes, and also from the *C. elegans cut-1*-like genes identified by the *C.elegans* Genome Sequencing Project (this has already been discussed at length in the Introduction). Consequently, the antiserum would be expected to cross-react with the proteins encoded by any other *cut-1*-like genes present in the *B. pahangi* genome. The RT-PCR data shows clearly that the *bpcut-1* transcript is not present in the adult-derived mRNA, which includes embryonic mRNA species. Therefore it would seem reasonable to conclude that there is a second, embryo-specific *cut-1*-like gene, which also appears to encode a structural component of the embryonic cuticle. This assumption is reinforced by the fact that 3' RACE carried out on *B. pahangi* adult polyA RNA results in the amplification of a fragment of cDNA which hybridises very strongly to a *cut-1* probe, and is the correct size, as predicted from the *ascut-1* cDNA sequence, to represent the 3' end of a second *Brugia cut-1*-like gene. Unfortunately this product has not been cloned and sequenced, due to the limitations of time.

Screening a *B.pahangi* adult genomic library resulted in the isolation and partial sequencing of four clones, all of which represent the same region of the *B. pahangi* genome. The presence of the *bpcut-1*-specific intron in all four clones allowed them to be identified as *bpcut-1*, rather than *bpcut-1*-like genes. PCR on one of the genomic clones using a 5' primer based on the sequence of the EMBL3 polylinker and a 3' primer based on the *bpcut-1* sequence allowed the direction of transcription of the gene to be determined, as well as enabling the gene to be positioned on the λ clone. From this information it can be seen that, using the gene length of the previously characterised nematode *cut-1* genes as a guide-line to predict the size of the *bpcut-1* gene, the full-length genomic sequence could almost certainly be obtained by cloning and sequencing the central, hybridising restriction fragment of the clones, which is only 3kb long. Screening an adult *B. pahangi* cDNA library resulted in the isolation of a clone (ned) which sequencing and analysis revealed to represent a mis-transcription of the *bpcut-1* gene: the resultant cDNA appears to be the result of transcription of the non-coding strand of the *bpcut-1* mRNA. However, using primers made on the sequence of the cDNA clone in PCR with adult *B. pahangi* genomic DNA resulted in the amplification of a fragment (ngen) which was subsequently cloned and sequenced and shown to correspond to *bpcut-1.*

A combination of the facts that *bpcut-1* does not appear to be trans-spliced, is expressed for limited periods of time in the *B. pahangi* life-cycle, and seems to be expressed at relatively

low levels (undetectable by northern blot) have made the isolation of a full-length cDNA clone extremely difficult. RT-PCR shows that the transcript is not present in adult mRNA, and screening of the conventional adult cDNA library resulted in the isolation of ned, pointing to the conclusion that the *bpcut-1* cDNA will not be represented in an adult library. It is extremely difficult to obtain sufficient parasite material with which to make larval cDNA libraries, and the apparent low expression of *bpcut-1* could make screening such a library difficult and unproductive. The production of SL1 cDNA libraries from small amounts of RNA from individual time-points in the parasite life-cycle overcomes the problem of lack of parasite material, and as it is now thought that the majority of nematode genes are trans-spliced, these libraries are likely to prove very useful in the isolation of transiently expressed cDNA clones. Unfortunately *bpcut-1* is almost certainly not trans-spliced, so this option was not available. It would seem that the use of 3' and 5' RACE is the most likely method with which to obtain the *bpcut-1* cDNA ends.

FURTHER WORK.

At this stage there are three further pieces of work required to enhance the study of the *cut-1*-like genes of *B. pahangi*. The first is the sequencing of the central, hybridising fragment of the genomic clone 4b, which would give information on the structure of the gene. This is interesting with respect to comparison with other nematode *cut-1*-like genes from an evolutionary point of view, and also could provide information on the region upstream of the gene which controls transcription. Secondly it is important to obtain a full-length cDNA clone of *bpcut-1*, or at least the 3' end of the cDNA, because it is in the 3' region that the *cut-1*-like genes of nematodes appear to differ. Therefore the character of the product encoded by the 3' end of *cut-1*-like genes is likely to define the function of the protein within the worm, whereas the homologous region serves only to define the proteins they encode as CUT-1-like. And finally it would be interesting to obtain the embryonic *cut-1*-like gene of *B. pahangi*, in order to compare its structure and mRNA abundance profile with that of *bpcut-1*.

The work done so far in isolating and characterising cuticlin genes from parasitic nematodes has been valuable in demonstrating that the genes are ubiquitous throughout the nematode phylum, and that they almost certainly represent gene families in which individual genes are specialised to encode proteins which serve specially adapted purposes throughout the life-cycle of the worm. An example of this has been shown in this project: it would appear that *B. pahangi* has at least two *cut-1*-like genes which encode different larval and embryonic cuticle components. This would imply that the *cut-1*-like genes have been highly conserved throughout evolution and serve an important function or functions in the nematode life-cycle. The *cut-1*-like genes of *B. pahangi* are likely to encode structural components of the cuticle, and their study could prove interesting in comparison with the study of cuticlins of other nematodes. The filarial cuticle is very different from that of the free-living *C.elegans* and the partially free-living *Ascaris*, and it would be interesting to see if the differences between the

proteins encoded by the cuticlin genes of these nematodes reflect, or even define, the cuticle differences.

The potential that the nematode cutlclins offer as a field of study has been covered in the Introduction, and at this stage it is my opinion that the most interesting work to be done on the nematode *cut-1*-like genes will be done in *C. elegans*, simply because the techniques required have been pioneered and optimised in this organism, and because the Sequencing Project has already resulted in the discovery of nineteen cuticlin-like genes, the number of which will no doubt increase before the completion of the project. The following are some ideas about further work on the cuticlins which have been suggested from what we already know about the genes, and inspired by the huge amount that we do not know.

- At this stage it would obviously be very interesting to raise antibodies against the gene-specific regions of the various CUT-1-like proteins, so as to compare the localisation patterns with those of the antibodies raised to the conserved regions of the proteins. This would give an idea of the variation in function of the proteins which are encoded by the *cut-1*-like genes.
- The techniques available in the *C. elegans* system are ideal for studying the *cut-1* gene family. As has already been described for *cecut-1*, the expression of the genes could be studied by transforming worms with fusions of the gene-specific promoters to the β-galactosidase gene, inducing expression of the gene with X-gal, and staining whole worm mounts to reveal the cells in which the protein is synthesised. Because the sequence of the genes are known, there are various techniques for obtaining mutants for the genes. One method is by the use of anti-sense RNA; it has been shown that the function of a gene can be inactivated by the microinjection into the gonads of a transgene carrying a gene segment in inverted orientation with respect to the promoter (Fire et al, 1991). This presumably works by binding to the mRNA of the gene, blocking transcription and subsequent expression of the gene. Mutants can also be found by searching 'libraries' of mutated DNA from individual worms by PCR using gene-specific primers. The most widely used method is to mutagenise populations via the insertion of the Tc1 transposon, and then screen mutants by PCR, using a gene-specific primer and a primer based on the transposon sequence (Zwaal et al, 1993; Rushforth et al 1993). Once mutants have been found for the genes, the phenotypes can be established, the relationship between genotype and phenotype understood, and the *cut-1* gene family consequently defined.
- A knowledge of the three dimensional structure of the CUT-1 proteins would yield more consistent information about the residues which may be important in the functional proteins.
- A more detailed picture of which features of the *cut-1* genes are conserved within the family would provide information which could be used to search for similar or potential ancestral genes or gene families in other organisms.

REFERENCES

Aeschbach, R., Amad, R. & Neukom, H. (1976) Formation of dityrosine cross-links in proteins by oxidation of tyrosine residues. Biochim. Biophys. Acta 439, 292-301.

Agrios, G.N. (1988) Plant Pathology (3rd Edition) Academic Press, San Diego.

Albertson, D.G. & Thomson, J.N. (1976) The pharynx of *C. elegans*. Philos. Trans. R. Soc. Lond. Biol. Sci. 275: 299-325.

Alfonso, A., Grundahl, K., McManus, J.R., Ashbury, J.M. & Rand, J. (1994B) Alternative splicing leads to two cholinergic proteins in *C. elegans*. J. Mol. Biol. 241: 627-630.

Ambrose, V. (1997) Heterochronic genes In *C. elegans* II (Eds. Riddle, Blumenthal, Meyer & Priess) Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Ambrose, V., & Horwitz, H.R. (1984) Heterochronic mutants of the nematode *C. elegans*. Science 226, 409-416.

Anderson, R.C. (1992) Nematode parasites of vertebrates. C.A.B. International.

Anderson, S.O. (1964) The cross-links in resilin identified as di-tyrosine and tri-tyrosine. Biochim. Biophys. Acta 93, 213-215.

Anderson, S.O. (1966) Covalent cross-links in a structural protein, resislin. Acta Physiol.Scand. Suppl. 263: 1-18.

Araujo, A., Souto-Padron, T. & De Souza, W. (1994) An ultrastructural, cytochemical and freeze-fracture study of the surface structures of *B. malayi* mf. Int. J. Para. 24/6, 899-907.

Archer, G.T., Coulits, N., Jindra, J. & Robson, J.E. (1985) Eosinophilia, mast cell hyperplasia, and antibody production in rats following injection of *Ascaris* cuticle including *in-vitro* studies of immune eosinophil granule lysis. Pathology 17(1), 101-107.

Barron, G.L. (1977) The nematode-destroying fungi. Canadian Biological Publications Ltd, Guelph, Canada.

Betshart, B. & Wyss, K. (1990) Analysis of the cuticular collagens of *Ascaris suum*. Acta Tropica. 47, 297-305.

Betshart, B., Marti, S. & Glaser, M. (1990) Antibodies against the cuticlin of *Ascaris suum* cross-react with epicuticular structures of filarial parasites. Acta Trop. 47, 255-268.

Bird, A.F. & Bird, J. (1991) The structure of nematodes. 2nd Edition. Academic press, San Diego.

Bird, A.F. (1971) The structure of nematodes. Academic press, New York.

Bird, A.F.(1980) The nematode cuticle and its surface in Nematodes as biological models vol. II (ed. B.M. Zuckerman). Academic Press, New York.

Birnboim, H.C. & Doyly, J. (1979) Rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids. Res. 7, 1513-1523.

Bisoffi, M. & Betshart, B. (1996) Identification and sequence comparison of a cuticular collagen of *Brugia pahangi*. Parasitology 113, 145-155.

Bisoffi, M., Marti, S. & Betschart, B. (1996) Repetitive peptide motifs in the cuticlin of *Ascaris suum*. Mol. Biochem. Para. 80, 55-64.

Blaxter, M., Page, A.P., Rudin, W., & Maizels, R.M. (1992) Nematode surface coats: actively evading immunity. Para. Today 8(7), 243-247.

Blaxter, M.L. (1993) Cuticle surface proteins of Wild Type and Mutant *C. elegans*. J. Biol. Chem. 268/9, 6600-6609.

Blumenthal, T. & Steward, K. (1997) RNA and Gene Structure. In *C. elegans* II (Eds. Riddle, Blumenthal, Meyer & Priess) Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Bonner, T.P. & Weinstein, P.P. (1972) Ultrastructure of cuticle formation in the nematodes *Nippostrongylus brasiliensis* & *Nematospiroides dubius.* J. Ultrastructural Res. 40, 261-271.

Bonner, T.P., Menebee, M.G. & Etges, F.J. (1969) Ultrastructure of cuticle formation in a parasitic nematode, *Nematospiroides.* Z. Zelforsch. 104, 193-204.

Bonner, T.P., Weinstein, P.P. & Saz, H.J. (1971) Synthesis of cuticular protein during the third moult in the nematode *N. brasiliensis.* Comp. Biochem. & Phys. 40B, 121-127.

Brenner, S. (1974) The genetics of *C. elegans.* Genetics 77, 71-94.

Briza, P., Ellinger, A., Winkler, G. & Breitenbach, M. (1990) Characterization of a DL-tyrosine-containing macromolecule from yeast ascospore walls. J. Biol. Chem. 265, 15118-15123.

Bundy, D.A.P., Grenfell, B.T., & Rajagopalan, P.K. (1991) Immunoepidemiology of lymphatic filariasis: the relationship between infection and disease. Immunoparasitology Today 3, A71-A75.

Cassada, R.C. & Russell, R.L. (1975) The dauer larva, a post-embryonic developmental variant of the nematode *C. elegans.* Dev. Biol. 46, 326-342.

Chanteau, S., Luquiaud, P., Failloux, A-B. & Williams, S.A. (1994) Detection of *W. bancrofti* larvae in pools of mosquitos by the Polymerase Chain Reaction. Trans. R. Soc. Trop. Med. & Hyg. 88, 665-666.

Chen, C. & Howells, R. (1981) The up-take of monosaccharides, disaccharides and nucleic acids precursors by adult *Dirofilaria immitis.* Ann. Trop. Med. & Para.75, 329-334.

Chitwood, B.G. & Chitwood, M.B. (1974) An introduction to Nematology. University Park Press, Baltimore, Maryland.

Clark, R.B. (1964) In Dynamics of metazoan evolution. Clarendon press, Oxford.

Collin, W.K. (1971) Ultrastructural morphology of the oesophageal region of the infective larvae of *B. pahangi.* J. Para. 57, 449-468.

Conrad, R., Lion, R.F. & Blumenthal, T. (1993) Conversion of a *trans*-spliced *C. elegans* gene into a conventional gene by introduction of a splice donor site. EMBO J. 12, 1249-1255.

Conway Morris, S. (1993) The fossil record and the early evolution of the metazoa. Nature 361: 219-225.

Cookson, E., Blaxter, M.L. & Selkirk, M.E. (1992) Identification of the major soluble cuticular protein of lymphatic parasitic nematodes as a secretory homologue of glutathione peroxidase. Proc. Natl. Acad. Sci. USA. 89, 5837-5844.

Cox, G.N. & Hirsh, D. (1985) Stage-specific patterns of collagen gene expression during development of *C. elegans.* Mol. Cell. Biol. 5, 363-372.

Cox, G.N. (1990) Molecular biology of the cuticle collagen gene families of *C. elegans* and *Haemonchus contortus.* Acta Tropica 47, 269-281.

Cox, G.N. (1992) Molecular and biochemical aspects of nematode collagens. J.Para. 78/1, 1-15.
Cox, G.N. Kramer, J.M. & Hirsh, D. (1984) Number and organization of collagen genes in *C. elegans.* Mol. & Cell. Biol. 4, 2389-2395.

Cox, G.N., Carr, S., Kramer, J.M. & Hirsh, D. (1985) Genetic mapping of *C. elegans* collagen genes using DNA polymorphisms as phenotypic markers. Genetics 109, 513-528.

Cox, G.N., Carr, S., Kramer, J.M. & Hirsh, D. (1985) Genetic mapping of *C. elegans* collagen genes using DNA polymorphisms as phenotypic markers. Genetics 109, 513-528.

Cox, G.N., Fields, C., Kramer, J.M, Rosenweig, B. & Hirsh, D. (1989) Sequence comparisons of developmentally regulated collagen genes of *C. elegans.* Gene 76, 331-344.

Cox, G.N., Kramer, J.M. & Hirsh, D. (1984) Number and organisation of collagen genes in *C. elegans*. Mol. Cell Biol. 4, 2389-95.

Cox, G.N., Kusch, M. & Edgar, R.S. (1981A) Cuticle of *C. elegans*: its isolation and partial characterization. J. Cell Biol. 90, 7-17.

Cox, G.N., Staprans, S. & Edgar, R.S. (1981b) The cuticle of *C. elegans* II: Stage-specific changes in ultrastructure and protein composition during post-embryonic development. Dev. Biol. 86, 456-470.

Cox, G.N., Kusch, M., De Nevi, K. & Edgar, R.S. (1981c) Temporal regulation of cuticle synthesis during development of *C. elegans*. Dev. Biol. 84, 277-285.

Cox, G.N., Laufer, J.F., Kusch, M. & Edgar, R.S. (1980) Genetic and phenotypic characterization of roller mutants of *C. elegans*. Genetics 95, 317-339.

Cox-Singh, J., Paine, M.J.I., Martin, S.A.M. & Devaney, E. (1994) Stage-specific differences in steadt-state levels of mRNA encoding the major surface glycoprotein of *B. pahangi*. Trop. Med. Para. 45, 352-354.

Cross, J.H., Partono, F., Hsu, M.K., Ash, L.R. & Oemijati, S. (1979) Experimental transmission of *W. bancrofti* to monkeys. Am. J. Trop. Med. & Hyg. 28, 56-68.

Day, K.P., (1991) The endemic normal in lymphatic filariasis: a static concept. Para. Today 7, 341-343.

Day, K.P., Grenfell, B., Spark, R., Kazura, J.W. & Alpers, A. (1991A) Age-specific patterns of change in the dynamics of *W. bancrofti* infections in P.N.G. Am. J. Trop. Med. Hyg. 44, 518-527.

Day, K.P., Gregory, W.F., & Maizels, R.M. (1991C) Age-specific acquisition of immunity to infective larvae in a bancroftian filariasis endemic area of PNG. Parasit. Immunol. 13, 277-283.

De Giorgi, C., De Luca, F. & Lamberti, F. (1996) A silent *trans*-splicing signal in the cuticlin-encoding gene of the plant-parasitic nematode *Meloidogyne artiella*. Gene, 170, 261-265.

De Giorgi, C., De Luca, F., Di Vito, M. & Lamberti, F. (1997) Modulation of expression at the level of splicing of *cut-1* RNA in the infective second stage juvenile of the plant parasitic nematode *Meloidogyne artiella*. Mol. Gen. Genetics 253, 589-598.

De Luca, F., De Giorgi, C. & Lamberti, F. Cuticlin gene organization in *Meloidogyne artiella*. In Advances in Molecular Plant Nematology. (Eds. Lamberti, F., De Giorgi, C. & Bird, D.M.) Plenum Press, New York, 1994) pp. 85-89.

Delves, C.J. Howells, R.E. & Post, R.J. (1986) Gametogenesis and fertilization in *Dirofilaria immitis*. Parasitology 92, 181-197.

Delves, C.J., Rees, H.H. & Howells, R.E. (1989) Egg production in *B. pahangi*. Parasitology 98, 105-113.

Denham, D. A. & McGreevy, P.B. (1977) Brugian filariasis: epidemiological and experimental studies. Advances in Parasitology 15, 243-309.

Devaney, E. (1988) The biochemical and immunochemical characterization of the 30 kDa surface antigen of *B. pahangi*. Mol. & Biochem. Para. 27, 83-92.

Devaney, E., Martin, S.A.M. & Thompson, F.J. (1996) Stage-specific gene expression in lymphatic filarial nematodes. Para. Today 12/11 418-424.

Dissanayake, S. & Piessens, W.F. (1992) Identification of filarial larvae in vectors by DNA hybridisation. Para. Today 8/2, 67-69.

Dodemont, H., Reimer, D., Ledger, N. & Weber, K. (1994) Eight genes and alternative RNA processing pathways generate an unexpectedly large diversity of cytoplasmic intermediate filament proteins in the nematode *C. elegans*. EMBO J. 13(11), 2625-2638.

Donelson, J.E. & Zeng, W. (1990) A comparison of *trans*-RNA splicing in trypanosomes and nematodes. Para Today 6/10 327-334.

Douvres, F.W. & Urban, J.F. (1983) Factors contributing to the *in vitro* development of *A. suum* from 2nd stage larvae to mature adults. J. Parasitol. 69: 3 539-548.

Edeson, J., Wilson. T., Wharton, R.H., Liang, A.B.G. (1960) Experimental transmission of *B. malayi* and *B. pahangi* to man. Trans. R. Soc. Trop. Med. Hyg. 54, 229-234.

Ewart, A. & Ho, B.C. (1967) The fate of *Brugia pahangi* larvae immediately after feeding by infective vector mosquitos. Trans. R. Soc. Trop. Med. Hyg. 61, 659-662.

Ewart, A., & El Bihari, S. (1971) Rapid recovery of *B. malayi* larvae following experimental infection in cats. Trans. R. Soc. Trop. Med. Hyg. 65, 364-368.

Favre, R., Hermann, R., Cermola, M., Hohenberg, H., Muller, M. & Bazzicalupo, P. (1995) Immunogold labelling of CUT-1, CUT-2, and cuticlin epitopes in *C. elegans* and *Heterorhabditis sp.* processed by high pressure freezing and freeze substitution. J. Submicroscop. Cytol. Pathol. 27, 341-347.

Fetterer, R.H & Urban, J.F. (1988) Developmental changes in cuticular proteins of *A. suum.* Comp. Biochem. & Physiol. 90B, 321-327.

Fetterer, R.H. & Hill, D.E. (1994) Localization of phenol oxidase in female *Trichuris suis.* J. Parasitol. 80, 952-959.

Fetterer, R.H. & Rhodes, M.L. (1990) Tyrosine-derived cross-linking amino acids in the sheath of *Haemonchus contortus* infective larvae. J. Parasitol. 76/5, 619-624.

Fetterer, R.H., Hill, D.E. & Urban, J.F. (1990) The cuticular biology in developmental stages of *Ascaris suum.* Acta Tropica 47, 289-295.

Fetterer, R.H., Rhodes, M.L. & Urban, J.F. (1993) Synthesis of tyrosine-derived cross-links in *Ascaris suum* cuticular proteins. J. Para. 79, 160-166.

Fire, A. & Waterston, H. (1989) Proper expression of myosin genes in transgenic nematodes. EMBO J. 8, 3419-3428.

Fire, A., Harrison, S. & Dixon, D. (1990) A modular set of lacZ fusion vectors for studying gene expression in *C. elegans.* Gene 93, 189-198.

Fitch D.H.A. & Thomas, W. K. (1997) Evolution In *C. elegans* II (Eds. Riddle, Blumenthal, Meyer & Priess) Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Foerder, C.A. & Shapiro, B.M. (1977) Release of ovoperoxidase from sea-urchin eggs hardens the fertilization membrane with tyrosine cross-links. Proc. Nat. Acad. Sci. USA. 74, 4214-4218.

Fraser E.M. & Kennedy, M.W. (1991) Heterogeneity in the expression of surface-exposed epitopes among larvae of *A. lumbricoides.* Para. Immunol. 13 (2): 219-225.

Frohman, M.A., Dusch, M.K. & Martin, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Nat. Acad. Sci. USA. 85, 8998-9002.

Fujimoto, D. & Kanaya, S. (1973) Cuticlin: a non-collagen structural protein from *Ascaris* cuticle. Arch. Biochem. Biophys. 157, 1-6.

Fujimoto, D. (1975) Occurrence of di-tyrosine in cuticlin, a structural protein from *Ascaris* cuticle. Comp. Biochem. Physiol. 51b, 205-207.

Fujimoto, D., Horiuchi, K. & Hirama, M. (1981) Iso-trityrosine, a new cross-linking amino acid isolated from *Ascaris* cuticle collagen. Biochem. & Biophys. Res. Comm. 99/2 637-643.

Gigliotti, S., Graziani, F., De Ponti, L., Rafti, F., Manzi, A., Lavorgna, G., Gargiulo, G. & Malva, C. (1988) Sex-, tissue- and stage-specific expression of a vitelline membrane protein gene from region 32 of the second chromasome of *Drosphila melanogaster.* Dev. Genet. 10, 33-41.

Golden, J.W. & Riddle, D.L. (1984a) A pheromone-induced developmental switch in *C.elegans*: temperature sensitive mutants reveal a wild-type temperature-dependent process. Proc. Natl. Acad. Sci. 81: 819-823.

Greenwald, I.S. & Horwitz, H.R. (1980) *unc-93-(e1500)*: a behavioural mutant of *C. elegans* that defines a gene with a wild-type null phenotype. Genetics 96, 147-164.

Gregory, W.F., Blaxter, M.L. & Maizels, R.M. (1997) Differentially-expressed, abundant *trans*-spliced cDNAs from larval *B. malayi*. Mol. Biochem. Parasitol. 87, 85-95.

Gross, A.J. & Sizer, I.W. (1959) J. Biol. Chem. 234, 1611-1614.

Gruidle, M.E., Smith, P.A., Kuzuicki, K.A., McKrone, J.S., Kirchner, J., Roussel, D.L., Strome, S. & Bennett, K.L. (1996) Multiple potential germ-line helicases are components of the germ-line-specific P-granules of *C. elegans*. Proc. Natl. Acad. Sci. U.S.A. 93, 13837-13842.

Guo, S. & Kemphues, K.J. (1995) *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative ser/thr kinase that is asymmetrically distributed. Cell 81, 611-620.

Guo, X. & Kramer, J.M. (1989) The two *C. elegans* basement membrane (Type IV) collagen genes are located on different chromasomes. J. Biol. Chem. 264, 17574-17582.

Haung, X.Y. & Hirsh, D. (1989) A second *trans*-spliced RNA leader sequence in the nematode *C. elegans*. Proc. Natl. Acad. Sci. U.S.A. 86, 8640-8644.

Hawking, F. (1967) The 24 hour periodicity of mf: biological mechanisms responsible for its production and control. Proc. R. Soc. B. 169, 59-76.

Hay, B., Yeh Jan, L. & Nung Jan, Y. (1988) A protein component of *Drosophila* polar granules is encoded by *vasa* and has extensive sequence similarities to ATP-dependent helicases. Cell 55, 577-587.

Hemmer, R.M., Donkin, S.G., Chin, K.J., Grenache, D.G., Bhatt, H. & Politz, S.M. (1991) Altered expression of an L1-specific, O-linked cuticle surface glycoprotein in mutants of the nematode *C. elegans*. J. Cell Biol. 115, 1237-1247.

Hewitt, R.F., Kushner, S., Stewart, H.W.,White, E., Wallace, W.S. & Subbarow, Y. (1947) Experimental chemotherapy of filarisis III. Effect of 1-Diethyl carbamyl-4-methyl piperazine hydrochloride against naturally acquired filarial infections in cotton rats and dogs. J. Lab. Clin. Med. 32, 1314-1329.

Hodgkin, J., Plasterk, R.H.A. & Waterson, R.H. (1995) The nematode *C. elegans* and its genome. Science 270, 410-414.

Hodgkin, J.A., Horwitz, H.R. & Brenner, S. (1979) Non-disjunction mutanys of the nematode *C. elegans*. Genetics 91, 67-94.

Hojrup, P., Anderson, S.O. & Roepstorff, P. (1986) Isolation, characterization and N-terminal sequence studies of cuticular proteins from the migratory locust, *Locusta migratoria*. Eur. J. Biochem. 154, 153-159.

Holton, T.A. & Graham, M.W. (1990) A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. Nucl. Acids Res. 19(5), 1156-1159.

Howells, R. & Chen, C. (1981) *Brugia pahangi*: feeding and nutrient up-take *in vitro* and *in vivo*. Exp. Para. 51, 42-48.

Howells, R.I. (1980) Filariae: dynamics of the surface in The Host Invader Interplay (Ed. H. van den Bosshe) Elsevier/ North Holland Biomedical Press, Amsterdam.

Inglis, W.G. (1964) The structure of the nematode cuticle. Proc. Zool. Soc. London 143, 465-502.

Jenkins, M.K. & Schwartz, R.H. (1987) Antigen presenation by chemically modified splenocytes induces antigen-specific t-cell unresponsiveness *in vitro* and *in vivo*. J. Exp. Med. 165, 302-308.

Johnstone, I.L. (1994) The cuticle of the nematode *C. elegans*: a complex collagen structure. BioEssays 16/3, 171-178.

Johnstone, I.L.& Barry, J.D. (1996) Temporal reiteration of a precise gene expression pattern during nematode development. EMBO J. 15/14, 3633-3639.

Johnstone, I.L., Shafi, Y. & Barry, J.D. (1992) Molecular analysis of mutations in the *C. elegans* collagen gene *dpy-7*. EMBO J. 11, 3857-3863.

Joshua, G.W.P. & Hsieh, C.Y. (1995) Stage-specifically expressed genes of *Angiostrongylus cantonensis*: identification by differential display. Mol. Biochem. Parasitol. 71, 285-289.

Kan, S.P. & Davey, M.G. (1968) Moulting in a parasitic nematode *Dipetalonema decipiens III*. The histochemistry of cuticle deposition and protein synthesis. Canadian J. Zool. 46, 723-727.

Kawasaki, E.S. & Wang, A.M. (1989) Detection of gene expression. In (Ehrlich, H.A. ed.) PCR Technology: principles and applications for DNA amplification. Stockton Press, New York, NY pp. 89-97.

Kay, E.S., Eddy, E.M. & Shapiro, B.M. Assembly of the fertilization membrane of the sea urchin: isolation of a divalent cation-dependent intermediate and its cross-linking *in vitro*. (1982) Cell 29, 867-875.

Kingston, I.B. & Pettit, J. (1990) Structure and expression of *Ascaris suum* collagen genes: a comparison with *C. elegans*. Acta Tropica 47, 283-287.

Kingston, I.B. (1991) Nematode collagen genes. Para. Today 7/1 11-15.

Kingston, I.B., Wainwright, S.M. & Cooper, D. (1989) Comparison of collagen gene sequences in *Ascaris suum* and *C. elegans*. Mol. & Biochem. Para. 37, 137-146.

Klei, T.R., Enright, F.M., Blanchard, D.P. & Uhl, S.A. (1981) Specific hyporesponsive granulomatous tissue reactions in *B. pahangi*-infected jirds. Acta Trop. 38, 267-276.

Konsolaki, M., Komitopoulou, K., Tolias, P.P., King, D.L., Swimmer, C. & Kafatos, F.C. (1990) The chorion genes of the medfly, *Ceratitis capitata* I: structure and regulatory conservation of the S36 gene relative to two *Drosophila* species. Nuc. Acids Res. 18, 1731-1737.

Kozek, W.J. (1971) The moulting pattern in *Trichinella spiralis*. II: an electron microscopic study. J. Parasitol. 5, 1029-1038.

Kramer, J.M (1997) Extracellular Matrix In *C. elegans* II (Eds. Riddle, Blumenthal, Meyer & Priess) Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Kramer, J.M. & Johnson, J.J. (1993) Analysis of mutants in the *sqt-1* and *rol-6* collagen genes of *C. elegans*. Genetics 135: 599-606.

Kramer, J.M. (1994) Structures and functions of collagens in *C. elegans* . FASEB J. 8, 329-336.

Kramer, J.M., Cox, G.N. & Hirsh, D. (1982) Comparisons of the complete sequences of two collagen genes from *C. elegans*. Cell 30, 599-606.

Kramer, J.M., Cox, G.N. & Hirsh, D. (1985) Expression of the *C. elegans* collagen genes *col-1* and *col-2* is developmentally regulated. J. Biol. Chem. 260, 1945-1951.

Kramer, J.M., French, R.P., Park, E.C. & Johnson, J.J. (1990) The *C. elegans rol-6* gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen. Mol. Cell Biol. 10, 2081-2089.

Krause, M. & Hirsh, D. (1987) A *trans*-spliced leader sequence on actin mRNA in *C. elegans*. Cell 49, 753-761.

Krause, M. (1995) Transcription and translation. In Epstein, H.F. & Shakes, D.C. (Eds.) Methods in Cell Biology 48: *C. elegans*: modern biological analysis of an organism 483-512.

Kreil, G. (1981) Transfer of proteins across membranes. Annu. Rev. Biochem. 50: 317-348.

Kurniawan, A., Yasdanbakhsh, M., van Ree, R., Aalberse, R., Selkirk, M.E., Partono, F. & Maizels, R.M. (1993) Differential expression of IgE & IgG4-specific antibody responses. J. Immunol. 150, 3942-3950.

Kusch, M. & Edgar, R.S. (1986) Genetic studies of unusual loci that affect body shape of the nematode *C. elegans* and may code for cuticle structural proteins. Genetics 113, 621-639.

Kwan-lim, G-E., Forsyth, K.P. & Maizels, R.M. (1990) Filarial-specific IgG4 response correlates with active *W. bancrofti* infections. J.Immunol.145, 4298-4305.

Laing, A.B.G.,Edeson, J. & Wharton, H.R. (1961) Studies on filariasis in *B. Malayi:* further experiments on the transmission of *B. malayi* and *W. bancrofti.* Ann. trop. Med. Parasitol. 55, 86-92.

Lammie, P.J., Hightower, W., Richards, F.O., Bryan, R-T, Spencer, H.C., McNeele, D.F., Mc Neele, M.B. & Eberhard, M.L. (1992) Alterations in filarial antigen-specific immunologic reactivity following treatment with ivermectin and DEC. Am. J. Trop. Med. Hyg. 46, 292-295.

Lammie, P.J., Hitch, W.L., Walker-Allen, E.M., Hightower, W. & Eberhard, M. (1991) Maternal filarial infection as risk factor for infection in children. Lancet 337, 1005-1006.

Lassandro, F., Sebastiano, M., Zei, F. & Bazzicalupo, P. (1994) The role of dityrosine formation in the cross-linking of CUT-2, the product of a second cuticlin gene of *C. elegans.* Mol. & Biochem. Para. 65, 147-159.

Laurence, B.R. & Simpson, M.G. (1974) The ultrastructure of the mf of *Brugia*, *Nematoda*: Filaroidea. Int. J. Para. 4, 523-536.

Lee, D.L. (1966) Structure and composition of the helminth cuticle. Adv. Para. 4/65, 214-238.

Lee, D.L. (1970) Moulting in nematodes: the formation of the adult cuticle during the final moult of *Nippostrongylus brasiliensis.* Tissue & Cell 2, 139-153.

Lee, Y.H., Huang, X-Y., Fox, G.E. & Hecht, R.M. (1992) Conservation of gene organization and *trans*-splicing in the glyceraldehyde-3-phosphate dehydrogenase encoding genes of *C. briggsae*. Gene121, 227-235.

Leiva, L.E. & Lammie, P.J. (1989) Modulation of lymphocyte activation by soluble *B. pahangi* extracts. Trop. Med. Parasitol. 40(3) 327-331.

Leushner, J.R.A. & Pasternak, J.P. (1975) Programmed synthesis of collagen during post-embryonic development of the nematode *Panagrellus silusiae.* Devel. Biol. 47, 68-80.

Leushner, J.R.A., Semple, N.E. & Pasternak, J.P. (1979) Isolation & characterization of the cuticle from the free-living nematode *Panagrellus silusiae.* Biochim. Biophys. Acta 580, 166-174.

Leushner, J.R.A., Semple, N.E. & Pasternak, J.P. (1979) Isolation and characterization of the cuticle from the free-living nematode *Panagrellus silusiae.* Biochem. et Biophys. Acta 580, 166-174.

Levy, A.D. & Kramer, J.M. (1993) Identification, sequence and expression patterns of the *C. elegans col-36* & *col-40* collagen-encoding genes. Gene. 137(2), 281-285.

Levy, A.D., Yang, J. & Kramer, J.M. (1993) Molecular and genetic analysis of the *C. elegans dpy-10* & *dpy-2* collagen genes: a variety of alterations affect organismal morphology. Mol. Biol. Cell 4, 261-266.

Lewin, B. (1990) Genes IV. Oxford, O.U.P.

Link, C.D., Silverman, M.A., Breen, M. & Watt, K.E. (1992) Characterization of *C. elegans* lectin binding mutants. Genetics 131, 867-881.

Liu, Z. & Ambrose, V. (1991) Alternative temporal control systems for hypodermal cell differentiation in *C. elegans.* Nature 350, 162-165.

Liu, Z., Kirch, S. & Ambrose, V. (1995) The *C. elegans* heterochronic gene pathway controls stage-specific transcription of collagen genes. Dev. 121: 2471-2478

Lustigman, S., Brotman, B., Huima, T., Castelhano, A.L., Singh, R.N., Mehta, K. & Prince, A.M. (1995) Transglutaminase-catalysed reaction is important for moulting of *Onchocerca volvulus* third-stage larvae. Antimicrob. Agents Chemother. 39(9), 1913-1919.

Lustigman, S., Brotman, B., Huitman, T., Prince, A.M. & McKerrow, J. (1992) Molecular cloning and characterization of onchocystatin, a cysteine protease inhibitor of *O.volvulus.* J. Biol. Chem. 267, 17339-17346.

MacDonald, W.W. & Sheppard (1965) Cross-over values in the sex chromasome of the mosquito *Aedes aegypti* and evidence of the presence of conversion. Ann. Trop. Med. Parasit. 59, 74-87.

Mahowald, A.P. (1968) Polar granules of *Drosophila* II: Ultrastructural changes during early embryogenesis. J. Exp. Zool. 67, 237-262.

Maizels, R.M., Partono, F., Oemejati, S., Denham, D.A. & Ogilvie, B.M. (1983) Cross-reactive surface antigens on three stages of *B. malayi*, *B. pahangi* & *B. timori.* Parasitology 87, 249-263.

Maizels, R.M., de Savigny, D. & Ogilvie, B.M. (1984) Characterization of surface and excretory-secretory antigens of *Toxocara canis* infective larvae. Para. Immunol. 6, 23-37.

Maizels, R.M., Gregory, W.F., Kwan-Lim, G-E & Selkirk, M.E. (1989) Filarial surface antigens: the major 29 kDa glycoprotein and a novel 170-200 kDa complex from adult *B. malayi* parasites. Mol. & Biochem. Para. 32. 213-218.

Maizels, R.M. & Lawrence, R.A. (1991) Immunological tolerance: the key feature in human filariasis. Para. Today 7/10: 271-276.

Maizels, R.M., Bundy, D.A.P, Selkirk, M.E., Smith, D.F., & Anderson, K.M. (1993) Immunological modulation and evasion by helminth parasites in human populations. Nature 365, 797-805.

Maizels, R.M., Sartono, E., Kurniawan, A., Partono, F., Selkirk, M.E. & Yasdanbaksh, M. (1995) T-cell activation and the balance of antibody isotypes in human lymphatic filariasis. Para. Today 11/2, 50-56.

Mak, J.W. (1987) Epidemiology of filariasis in Filariasis, Wiley, Chichester (CIBA Foundation Symposium 127) 5-14.

Mak, J.W., Cheong, W.H., Yen, P.K.F, Lim, P.K.L. & Chan, W.C. (1982) Studies on the epidemiology of sub-periodic *Brugia malayi* in Malaysia: problems in its control. Acta Trop. 39, 237-245.

Malakhov, V.V. (1994) Nematodes: structure, development, classification and phylogeny. Smithsonian Institution Press, Washington D.C.

Marchuk, D. (1990) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucl. Acids. Res. 19(5), 1154-1156.

Maroney, P.A., Denker, J.A., Darzynkiewitz, E., Laneve, R. & Nilsen, T.W. (1995) Most mRNAs in the nematode *Ascaris lumbricoides* are *trans*-spliced: a role for spliced-leader addition in translational efficiency. RNA 1/7: 714-723.

Martin, S.A.M., Thompson, F.J. & Devaney, E. (1995) The construction of spliced-leader cDNA libraries from the filarial nematode *B. pahangi.* Mol. & Biochem. Parasitol. 70, 241-245.

McBride, O.W. & Harrington, W.P. (1967) *Ascaris* cuticle collagen: on the disulphide cross-linkages and the molecular properties of the sub-units. Biochemistry 6, 1484-1489.

McCarthy, J.S. Zhong, M., Gopinath, R., Otteson, E.A., Willliams, S.A. & Nutman, T.B. (1996) Evaluation of a PCR based assay for the diagnosis of *W. bancrofti* infection. J. Inf. Dis. 173, 1510-1510.

McKim, K.S., Matheson, C., Marra, M., Wakarchink & Baillie, D.C. (1994) The *C. elegans unc-60* gene encodes proteins homologous to a family of actin-binding proteins. Mol. Gen. Genetics 242, 346-357.

McLaren, D. (1973) Oogenesis and fertilization in *Dipetalonema viteae.* Parasitology 66, 465-472.

Mello, C.C., Kramer, J.M., Stinchcomb, D. & Ambrose, V. (1992A) Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10, 3959-3970.

Meyrowitsch, D.W., Simonsen, P.E. & Makunde, W.H. (1996) Mass DEC chemotherapy for control of bancroftian filariasis: comparitive efficacy of four strategies two years after start of treatment (1996) Trans. R. Soc. Trop. Med. Hyg. 90/4, 423-428.

Morgan, T.M., Sutanto, I, Purnomo, Sukartono, Partono, F. & Maizels, R.M. (1986) Antigenic characterization of adult *Wuchereria bancrofti* filarial nematodes. Parasitology 95, 559-569.

Nakai, K. & Kanehisa, M. (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics 14, 897-911.

Nelson, R.E., Fessler, L.I., Takagi, Y., Blumberg, B., Keene, D.R., Olson, P.F., Parker, C.G. & Fessler, G.H. (1994) Peroxidasin: a novel enzyme-matrix protein of *Drosophila* development. EMBO J. 13, 3438-3447.

Nilsen, T.W. (1989) *Trans*-splicing in nematodes - a minireview. Exp. Parasitol.69, 413-416.

Nilsen,T.W., Shambaugh, J., Denker, J.A., Chubb, G., Faser, C., Putnam, L., & Bennett, K. (1989) Characterization and expression of a spliced leader RNA in the parasitic nematode *Ascaris lumbricoides* var. *suum*. Mol.Cell.Biol. 9, 3543-3547.

Ortega-Pierres, G., Mackenzie, C.D., & Parkhouse, R.M. (1984) The occurrence of antibodies to hidden and exposed antigens of the parasitic nematode *Trichinella spiralis*. Parasitology 88/2: 359-369

Otteson, E., A. (1984) Immunological aspects of lymphatic filariasis and onchocerciasis in man. Trans. R. Soc. Trop. Med. Hyg. 78, (suppl) 9-18.

Otteson, E.A. & Ramachandran, C.P. (1995) Lymphatic filariasis infection and disease: control strategies. Para. Today 11(4), 129-131.

Otteson, E.A. (1980) Immunopathology of lymphatic filariasis in man. Springer Semin. Immunopathol. 2, 373-385.

Otteson, E.A. (1985) Efficacy of DEC in eradicating infection with lymphatic-dwelling filariae in humans. Rev. Inf. Dis. 7, 341-356.

Otteson, E.A. (1992) Infection and disease in lymphatic filariasis - an immunological perspective. Parasitology 104, 571-579.

Otteson, E.A., Weller, P.F, and Heck, L. (1977) Specific cellular immune unresponsiveness in human filariasis. Immunology 33, 413-421.

Otteson, E.A., Weller, P.F., Lunde M.N. and Hussain, R. (1982) Endemic filariasis on a Pacific island. II Immunological Aspects: immunoglobulin, complement, and specific antifilarial IgG, IgM and IgE antibodies. Am. J. Trop. Med. Hyg. 31, 953-961.

Ouzana, R. & Herbage, D. (1981) Biochemical characterization of the cuticle collagen of the nematode *C. elegans*. Biochim. Biophys. Acta 669, 236-243.

Palmieri, J.R., Ratiwayanto, S., Masbar,S., Tirtokusomo, S., Rusch, J. & Marmoto, H.A. (1985) Evidence of possible natural infections of man with *B. pahangi* in South Kalimantan (Borneo), Indonesia. Trop. Geogr. Med. 37, 239-244.

Parise, G. & Bazzicalupo, P. (1996) Assembly of nematode cuticle: role of hydrophobic interactions in CUT-2 cross-linking. Biochim. Biophys. Acta. Prot. Sruct. Mol. Enzym. 1337, 295-301.

Park, Y-S. & Kramer, J.M. (1994) The *C. elegans sqt-1* and *rol-6* collagen are co-ordinately expressed during development, but not at all stages that display mutant phenotypes. Dev. Biol. 163: 112-124.

Park, Y. & Kramer, J.M. (1990) Tandemly duplicated *C. elegans* collagen genes differ in their modes of splicing. J.Mol. Biol. 211, 395-406.

Parkhouse, R.M.E., Phillip, M. & Ogilvie, B.M. (1981) Characterization of surface antigens of *T. spiralis* infective larvae. Para. Immunol. 3, 339-352.

Partono, F., Maizels, R.M. & Purnomo, (1989) Towards a filariasis-free community: evaluation of filariasis control over an eleven year period in Flores, Indonesia.Trans. R. Soc. Trop. Med. & Hyg. 83, 821-826.

Pettit, J. & Kingston, T.B. (1994) Developmentally regulated alternative splicing of a nematode Type IV collagen gene. Dev. Biol. 161(1), 22-29.

Piessens, W.F., Ratiwayanto, S., Piessens, P.W. Tuti, S. & McGreevy, P.B. (1981) Effect of treatment with DEC on immune responses to filarial antigens in patients infected with *B. malayi*. Acta Trop. 38, 227-234.

Plasterk, R.H.A. & Groenen, J.T.M. (1992) Targeted alterations of the *C. elegans* genome by transgene instructed DNA double strand break repair following Tc1 excision. EMBO J. 11, 287-290.

Politz, J.C. & Edgar, R.S. (1984) Overlapping stage-specific sets of numerous small collagenous polypeptides are translated *in vitro* from *C. elegans* RNA. Cell 37, 853-860.

Politz, S.M., Chin, K.J. & Herman, D.L. (1987) Genetic analysis of adult-specific surface antigenic differences between varieties of the nematode *C. elegans*. Genetics 117, 467-476.

Politz, S.M., Philipp, M., Estevez, M., OíBrien, P.J., Chin. K.J. (1991) Genes that can be mutated to unmask hidden antigenic determinants in the cuticle of the nematode *C. elegans*. Proc. Natl. Acad. Sci. USA. 87, 2901-2905.

Popodi, E., Minoo, P., Burke, T. & Waring, G.L. (1988) Organization and expression of a second chromasome follicle cell gene cluster in *Drosophila*. Dev. Biol. 127, 247-252.

Prockop, D.J. & Kivirikko, K.I. (1995) Collagens: molecular biology, diseases and potential for therapy. Annu. Rev. Biochem. 64: 403-434.

Prockop, D.J. (1992) Seminars in medicine of the Beth Israel Hospital, Boston. Mutations in collagen genes as a cause of connective tissue diseases. N. Engl. J. Med. 326 (8), 540-546.

Proenca, R., Muljo, S. & Hedgecock, E.M. (1997) The *mig-4* gene is an extracellular matrix molecule involved in gonad primordium adhesion. *C. elegans* Bi-annual Conference Abstract 292.

Proudfoot, L., Kusel, J.R., Smith, H.V. & Kennedy, M.W. (1991) Biophysical properties of the nematode surface in Parasitic Nematodes - membranes, antigens and gene (Ed. M.W. Kennedy) pp 1-26 Taylor & Francis, London.

Proudfoot, L.J., Kusel, J.R., Smith, H.V. & Kennedy, M.W. (1993B) Rapid changes in the surface of parasitic nematodes during transition from pre- to post-parasitic forms. Parasitology 107, 107-117.

Rajkovic, A. (1990) A spliced leader is present on a sub-set of mRNAs from the human parasite *S. mansoni*. Proc. Natl.Acad. Sci. USA 87(22)8879-8883.

Ray, B.K., Lawson, T.G., Jramer, J.C., Cladaras, M.H., Gufo, J.A., Abramson, R.D., Merrick, W.C. & Thach, R.E. (1995) ATP-dependent unwinding of mRNA structure by eukaryotic initiation factors. J. Biol. Chem. 260, 756-768.

Reddigari, S.R., Jansma, P.L., Premachandran, D. & Hussey, R.S. (1986) Cuticular collagenous proteins of 2nd stage juveniles and adult females of *Meloidogyne incognita*: isolation and partial characterization. J. Nematol. 18, 294-302.

Ristoratore, F., Cermola, M., Nola, M., Bazzicalupo, P. & Favre, R. (1994) Ultrastructural immunolocalization of CUT-1 and CUT-2 antigenic sites in the cuticles of the nematode *C. elegans*. J. Submicrosc. Cytol. Pathol. 26(3), 437-443.

Rogers, R., Denaham, D.A. & Nelson, G.S. (1974) Studies with *B. pahangi* 5. Structure of the cuticle. J. Helminthol. 48, 113-117.

Rogers, R., Ellis, D.S. & Denham, D.A. (1976) Studies with *B. pahangi* 14. Intrauterine development of the microfilariae, and a comparison with other species. J.Helminth. 50/4, 251-257.

Roussel, D.L. & Bennet, K.L. (1993) *glh-1*, a putative germ-line RNA helicase from *C. elegans*, has four zinc fingers. Proc. Natl. Acad. Sci. U.S.A. 90, 9300-9304.

Rushforth, A.M., Saari, B. & Anderson, P. (1993). Site-selected insertion of the transposon Tc1 into a *C. elegans* myosin light chain gene. Mol. Cell. Biol. 13: 902-910.

Rutherford, T.A. & Webster, J.M. (1974) Transcuticular up-take of glucose by an entomophilic nematode *Mermis nigrescens*. J. Para. 60, 804-808.

Sakura, S. & Fujimoto, D. (1984) Absorbtion and fluorescence studies of tyrosine-derived cross-linking amino acids from collagen. Photochem. & Photobiol. 40, 731-734.

Sakwe, A.M. & Titanje, V.P. (1997) Evidence for increased hydroxylation of pyrrolidone amino acid residues in the cuticle of mature *Onchocerca volvulus*. Biochim. Biophys. Acta 1360(3), 196-202.

Salz, H.K., Maine, E.M., Keyes, L.N., Samuals, M.E., Cline, T.W. & Scheld, P. (1989) The *Drosophila* female-specific sex-determination gene, sex-lethal, has stage-, tissue-, and sex-specific RNAs, suggesting multiple modes of regulation. Genes & Development 3, 708-719.

Sambrook, J., Frisch, E.F. & Manniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Sanger, F., Nicklen, S. & Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74, 5463-5467.

Sartono, E., Kruize, Y.C.M., Kurniawan, A., van der Meide, P.H., Partono, F., Maizels, R.M. & Yasdanbakhsh, M. (1995) elevated cellular immune responses and g-IFN release after long-term DEC treatment of patients with human lymphatic filariasis. J.Inf.Dis. 171, 1683-1687.

Sasser, J.N. & Freckman, D.W. (1987) A world perspective on nematology: the role of the Society. In Vistas on Nematology (Eds. Veech, J.A. & Dickson, D.W.) Painter Printing Company, DeLeon Springs. pp 7-14.

Schacher, J.F. & Sahyoun, P.F. (1967) A chronological study of the histopathology of filarial disease in cats and dogs caused by *B. pahangi*. Trans. R. Soc. Trop. Med. & Hyg. 61, 234-243.

Schacher, J.F. (1962) Developmental Studies of *B. pahangi* in the final host. J. Parasitol. 48/5, 693-706.

Scherer, L.J., Harris, D.H. & Petrie, W.H. (1988) *Drosophila* vitelline membrane genes contain a 114 b.p. region of highly conserved coding sequence. Dev. Biol. 130, 786-788.

Schwartz, R.H. (1990) A cell-culture model for t-lymphocyte clonal anergy. Science 134, 1349-1356.

Scott, A.L., Yenbutr, P., Eisinger, S.W. & Raghavan, N. (1995) Molecular cloning of the cuticular collagen gene *bmcol-2* from *B. malayi*. Mol. & Biochem. Para. 70, 221-225.

Sebastiano, M., Lassandro, F. & Bazzicalupo, P. (1991) *cut-1* a *C. elegans* gene coding for a dauer-specific non-collagenous component of the cuticle. Dev. Biol. 146, 519-530.

Selkirk, M.E., Nielsen, C., Kelly, C., Partono, F., Sayers, G. & Maizels, R.M. (1989) Identification, synthesis and immunogenicity of cuticular collagens from the filarial nematodes *B. malayi* and *B. pahangi*. Mol. & Biochem. Para. 32, 229-246.

Selkirk, M.E. (1991) The nematode cuticle in Parasitic nematodes – membranes, antigens and genes. Ed M.W. Kennedy, Taylor and Francis, London.

Shamansky, L.M., Pratt, D., Boisvenue, R.J. & Cox, G.N. (1987) Cuticle collagen genes of *Haemonchus contortus* and *C. elegans* are highly conserved. Mol. & Biochem. Para. 37, 73-86.

Sibley, M.H., Johnson, J.J. Mello, C.C. & Kramer, J.M. (1993) Genetic identification, sequence and alternative splicing of the *C. elegans alpha 2 (IV)* collagen gene. J. Cell. Biol. 123(1), 255-264.

Sidow, A. & Thomas, W.K. (1994) A molecular evolutionary framework for eukaryotic model organisms. Curr. Biol. 4: 596-603.

Singh, R.N., & Sulston, J.E. (1978) Some observations on moulting in *C. elegans*. Nematologica 40, 63-71.

Smith, L.T., Wertelecki, W., Milstone, L.M., Petty, E.M., Seashore, M.R., Braverman , I.M., Jenkins, T.G. & Byers, P. H. (1992) Human dermatosparaxis: a form of Ehlers-Danlos syndrome that results from failure to remove the amino terminal propeptide of type 1 pro-collagen. Am J. Hum. Genet. 51: 235-244.

Somers, C.E & Shapiro, B.M. (1991) Functional domains of proteoliaisin, the adhesive protein that orchestrates fertilization envelope assembly. J. Biol. Chem. 266, 16870-16875.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Spieth, J., Brooke, G., Kuersten, S., Lea, K. & Blumenthal, T. (1993) Operons in *C. elegans*: polycistronic mRNA precursors are processed by *trans*-splicing of SL2 to down-stream coding regions. Cell 73, 521-532.

Srivasta, A.K. (1985) A method for isolation and purification of the sheath of the mf of *B. malayi*. J. Parasitol. 71, 257-258.

Steele, C., Guinea, A., McCarthy, J.S. & Otteson, E.A. (1994) Long-term effect of pre-natal exposure to maternal mf on immune responsiveness to filarial parasite antigens. Lancet 343, 890-893.

Stonor, R.D. & Hankes, L.V. (1958) In vitro metabolism of DL-tyrosine-2-C-14 & DL-tryptophan-2-C-14 by *Trichinella spiralis* larvae. Exp. Para. 7, 145-151.

Strome, S. & Wood, W.B. (1982) Immunofluorescence visualization of germ-line specific cytoplasmic granules in embryos, larvae and adults of *C. elegans*. Proc. Nat. Acad. Sci. U.S.A. 79, 1558-1562.

Strome, S., & Wood, W.B. (1983) Generation of assymetry and segregation of germ-line granules in early *C. elegans* embryos. Cell 35, 15-25.

Sulston, J., Horwitz, H.R. & Kimble, J. (1988) Appendix 3, Cell lineage in The nematode *C. elegans* (Ed. W.B. Wood and the community of *C. elegans* researchers) pp 457-489. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Sulston, J.E. & Horwitz, H.R. (1981) Abnormal cell lineages in mutants of the nematode *C. elegans*. Dev. Biol. 82: 41-55.

Tabor, S. & Richardson, C.C. (1987) DNA sequencing analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA. 84, 4767-4771.

Tackacs, A.M., Denker, J.A., Perrine, K.G., Maroney, P.A. & Nilsen, T.W. (1988) A 22-nucleotide spliced-leader sequence in the human parasitic nematode *B. malayi* is identical to the *trans*-spliced leader exon in *C. elegans*. Proc. Natl. Acad. Sci. 85:7932-7936. .

Tang, L. Gonnaris, R., Griffiths, C. & Selkirk, M.E. (1995) Heterologous expression and enzymatic properties of a selenium-independent glutathione peroxidase from the parasitic nematode *B. pahangi*. J. Biol. Chem. 270, 18313-18318.

Tang, L., Ou, X., Henkle-Dhurseen, K.J. & Selkirk, M.E. (1984) Extracellular and cytoplasmic CuZn superoxide dismutase from the *Brugia* lymphatic filarial parasites. Infect. Immun. 62: 961-967.

Terry, A., Terry, R.J. & Worms, M.J. (1961) *Dipetalonema viteae*, filarial parasite of the jird II. The reproductive system, gametogenesis and development of the mf. J. Para. 47, 703-711.

Tessier, L.H. (1991) Short leader sequences may be transferred from small mRNAs to pre-mature RNAs by trans-splicing in *Euglena*. EMBO J. 10(9), 2621-2625.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nuc. Acids Res. 22, 4673-4680.

Timinouni, M., & Bazzicalupo, P. (1997) *cut-1*-like genes of *Ascaris lumbricoides*. Gene 193(1), 81-87.

Urban, J.F., Douvres, F.W. & Tromba, F.G. (1981) A rapid method of hatching of *Ascaris suum* eggs *in vitro*. Proc. Helm. Soc. Wash. 48: 241-243.

Vanamail, P., Ramaiah, K.D., Pani, S.P., Das, P.K., Grenfell, B.T. & Bundy, D.A.P. (1996) Estimation of the fecund life-span of *Wuchereria bancrofti* in an endemic area. Trans. R. Soc. Trop. Med. & Hyg. 90, 119-121.

Vandereycken, W.J.D., Engler,J.D., Vanmontagu, M. & Gheysen, G. (1994). Identification and analysis of a cuticular collagen-encoding gene from the plant parasitic nematode *M. incognita.* Gene 151: 237-242.

Varkey, J.P., Jansma, P.L., Minniti, A.N. & Ward, S. (1995) The *C. elegans spe-26* gene is necessary to form spermatids and encodes a protein similar to the actin-associated proteins KELCH & SCRUIN. Genes Dev. 9, 1074-1086.

Vincent, A.L., Ash, L.R. & Frommes, S.P. (1975) Ultrastructural study of adult *B. malayi.* J. Para. 61(3), 499-512.

Vogelstein, B. & Gillespie, D. (1979) Preparitive and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA. 76/26:165-619.

von Heijne, G. (1986) A new method for predicting signal sequence cleavage sites. Nuc. Acids Res. 14(11), 4683-4690.

von Mende, N., Bird, D.M., Albert, P.S. & Riddle, D.L. (1988) *dpy-13*, a nematode collagen gene that affects body shape. Cell 55: 567-576.

Vuorio, E. & De Crombrugghe, B. (1988) The family of collagen genes. Ann. Rev. Biochem. 59, 837-872.

Waffenschmidt, S., Woessner, J.P., Beer, K. & Goodenough, U.W (1993) Iso-dityrosine cross-linking mediates insolubilization of cell walls in *Chlamydomonas.* Plant Cell 29, 809-820.

Wahle, E. & Keller, W. (1992) The biochemistry of 3'-end cleavage and polyadenylation of mRNA pre-cursors. Ann. Rev. Biochem. 61, 419-440.

Waterstone, H., Sulston, J.E. & Coulson, A.R. (1997) The Genome in *C. elegans* II pp 23-45.

Watson, B.D. (1965) The fine structure of the body wall and the growth of the cuticle in the adult nematode *Ascaris lumbricoides.* Quarterly J. Micro. Sci. 106, 83-96.

Weil, G.J, Hussein, R., Kumaraswami, V., Tripathy, S.P., Phillips, K.S. & Otteson, E.A. (1983) Prenatal allergic sensitization to helminth antigens in off-spring of parasite-infected mothers. J. Clin. Invest.71, 1124-1129.

Weil, G.J., Sethumadhavan, K.V., Santhanam, S., Jain, D.C. & Ghosh, T.K. (1988) Persistence of parasite antigenaemia following DEC treatment in bancroftian filariasis. Am. J. Trop. Med. Hyg. 38, 589-595.

Wharton, D.A. & Murray, D.S. (1990) Carbohydrate-lectin interactions between the nematophagous fungus *Arthrobotrys oligospora* and the infective juvenile of *Trichostrongylus colubriformis.* Parasitology 101:101-106.

Wharton, H.R. (1963) Adaptation of *Wuchereria* and *Brugia* to mosquitos and vertebrate hosts in relation to the distribution of filarial parasites. Zoonosis Res. 2, 1-12.

WHO 1984. Lymphatic filariasis. 4th report of the WHO Expert Committee on filariasis, Geneva. WHO Tech. Report. Ser.702.

Williams, S.A., Nicholas, L., Lizotte-Waniewski, M., Plichart, C., Luquiard, P., Nguyen, L.N. & Moulia-Pelat, J-P. (1996) A PCR assay for the detection of *W. bancrofti* in blood samples from French Polynesia. Trans. R. Soc. Trop. Med. & Hyg. 90. 384-387.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M. et al (1994) The *C. elegans* genome sequencing project: contiguous nucleotide sequence of over two megabases from chromosome III. Nature 368, 32-38.

Wood W.B. (1988) Preface in The Nematode *C. elegans.* Ed. W.B. Wood. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Wright, K.A. (1987) The nematode cuticle - its surface and the epidermis: function, homology, analogy- a current consensus. J. Para. 73, 1077-1083.

Yang, J., & Kramer, J.J. (1994) *In vitro* mutagenesis of *C. elegans* cuticle collagens identifies a potential subtilisin-like protease cleavage site and demonstrates that carboxyl domain disulphide bonding is required for normal function but not assembly. Mol. Cell. Biol. 14: 2722-2730.

Zahner, H., Hobom, G. & Stirm, S. (1995) The microfilarial sheath and its proteins. Para. Today 11/3, 116-120.

Zaman, V. (1987) Ultrastructure of the *B. malayi* egg-shell and its comparison with the microfilarial sheath. Parasitol. Res. 73, 281-283.

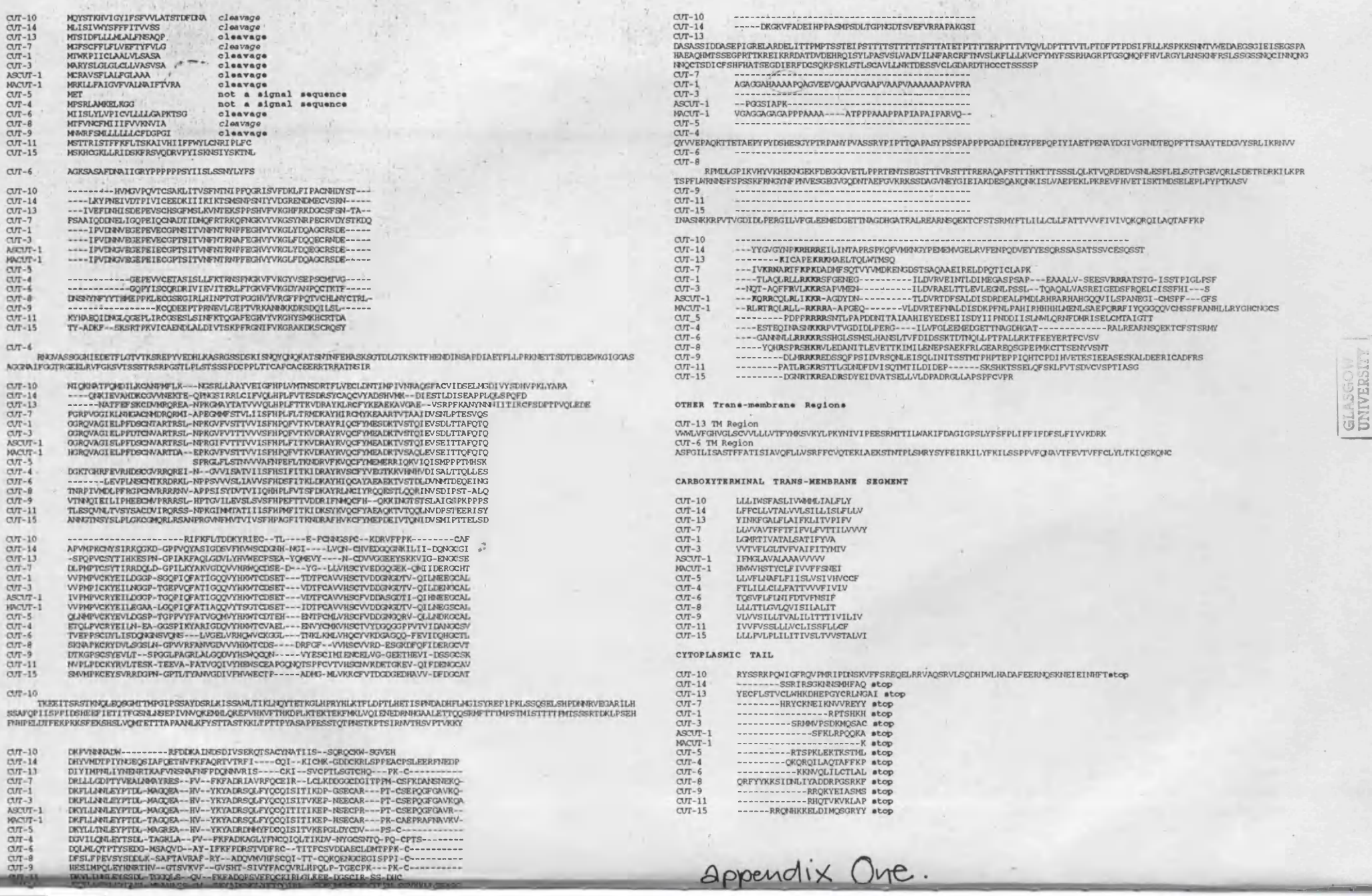
Zorio, D.A.R., Chen, N.N., Blumenthal, T. & Speith, J. (1994) Operons represent a common form of chromosomal organization in *C. elegans*. Nature **372**: 270-272.

Zuckerman, B.M., Himmelhoch, S. & Keisel, M. (1973) Nematologica 19, 109-112.

Zuckerman, B.M., Kahane, I. & Himmelhoch, S. (1979) *C. Briggsiae* and *C. elegans*: partial characterization of the cuticle surface carbohydrates. Exp. Para. 47, 419-424.

Zvelebil, M.J., Tang, L., Cookson, E., Selkirk, M.E. & Thornton, J. M. (1993). Molecular modelling and epitope prediction of gp29 from lymphatic filariae. Mol. & Biochem. Para. 58, 145-154.

Zwaal, R.R., Broeks, A., van Meurs, J., Groenen, J.T.M. & Plasterk, R.H.A. (1996) Target-selected gene activation in *C. elegans* by using a frozen transposon insertion mutant bank. Proc. Natl. Acad. Sci. 90: 7431-7435.



Appendix One.