IDENTIFICATION OF GENES ENCODING SECRETED PROTEINS OF SCHISTOSOMES

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Submitted for the degree
of
Doctor of Philosophy

The University of York
Department of Biology

March 2000

ABSTRACT

In mice given a single vaccination with optimally irradiated *S. mansoni* cercariae, cell-mediated mechanisms operating against lung-stage larvae are highly effective in reducing challenge worm burden by 60-70%, relative to those in control mice. Therefore, the proteins secreted from the live schistosomula are a potential source of novel candidates for a recombinant vaccine. Only minute quantities of protein are secreted by the larval parasites making their identification difficult. Therefore, several methods to identify and clone the genes encoding such proteins, based on recombinant DNA technology, were developed in this study.

Initially, 106 expressed sequence tags (ESTs) were produced from a lung-worm cDNA library. By analysis of the sequences in the schistosome protein database, using the computer algorithm, SignalP, 27 distinct open reading frames encoding membrane and/ or secreted (M/S) proteins were identified. Following this, all the *S. mansoni* ESTs were translated into protein sequences, the longest open reading frame selected, and analysed by SignalP. Out of a total of 7102 ESTs, 218 were found to encode for putative M/S proteins; only five were derived from the lung-worm. Three of these five ESTs and the one larval ORF, CL1, were transferred to high level expression vectors. Recombinant protein was purified for only CL1 and antiserum was raised against it. Analysis of this recombinant protein indicates that the protein is secreted from the developing schistosomulum.

To identify those proteins that are stage-specifically expressed by the lung-worm, the technique of random arbitrarily-primed PCR (RAP-PCR) was used. The mRNAs of cercariae, lung and adult-worms were compared in parallel. In total 9 stage-specific bands and 4 differentially expressed bands were excised from gels. Several of these represent single copy genes as determined by Southern blotting; the gene-expression profiles remain to be verified.

Finally, a functional assay that selects ORFs based on the ability of the corresponding proteins to direct secretion of invertase in yeast was established. The system was validated using schistosome cDNAs coding for known M/S proteins, namely p48, superoxide dismutase and antigen 10-3. Schistosome cDNAs coding for cytosolic proteins, calpain and phosphoglycerate kinase, were used as negative controls. These experiments show that the system readily discriminates between schistosome M/S and cytosolic proteins and can now be applied to cDNA mixtures that have been generated by random-hexamer primed reverse-transcription.

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ACKNOWLEDGEMENTS

I would like to thank the following:

Prof. D. S. C. White and Prof. A. Fitter in whose department this work was carried out.

The BBSRC and The Dept. of Zoology, The Natural History Museum, London for providing funding for the project.

Friends and colleagues at the Biology Dept., The University of York and at The Natural History Museum.

The CRU and NHM sequencing facilities for the endless numbers of gels that had to be run.

I would like to extend special thanks to the following people:

Alan for all his scientific guidance, enthusiasm and perseverance.
David for all his helpful advice and confidence.

Rich, Gui, David J, Matthias, Adrian and Rachel for putting up with my sometimes incessant questioning and for their technical expertise.

Benika and Andrea for their friendship over the last three years.
Sue and Mike for all those much needed evening tipples.
My family, especially Jagumasi and Rekhamasi, for all their support through some trying times.
Finally, my parents, for being so patient, and it is to them that this thesis is dedicated.

DECLARATION

All of the work contained in this thesis is my own except for the following:

Chapter 2: Perfusion of mice to obtain adult worms was carried out by Dr. A. Mountford and Miss C. Sadler

Chapter 3: All injections to immunise mice were done by Dr. P. Coulson

ABBREVIATIONS

γ	gamma
λ	lambda
°C	degrees centigrade
μMT	B-cell deficient
0-3h RP	0-3 hour released proteins
1-D	one dimensional
A	absorbance
aa	amino acids
ADCC	antibody dependent cell-mediated cytotoxic
APCs	antigen-presenting cells
BCG	*Mycobacterium bovis* bacillus Calmette-Guerin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLASTN	basic local alignment search tool for nucleotides
BLASTX	basic local alignment search tool for peptides
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CFA	Complete Freunds Adjuvant
Ci	curies
cm	centimetres
d6-8 RP	days 6-8 released proteins
DD	differential display
DEPC	diethylpyrocarbonate
DLC	dynein light chain
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
EST	expressed sequence tag
FACS	fluorescence activated cell sorter
g	gravitational force
gDNA	genomic DNA
GPCR	G-protein coupled receptor
GST	glutathione-S-transferase
IFNγ	interferon-γ
IFN$\gamma^{-/-}$	IFNγ gene disrupted
IFNγR$^{-/-}$	IFNγ receptor knockout
IL	Interleukin
IMAC	immobilised metal affinity chromatography
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodaltons
krad	kilorads
M/S	membrane and/or secreted
MAb	monoclonal antibody
MAP	multiple antigenic peptide
Mb	megabase pairs
M-MLV	Moloney murine-leukaemia virus
MPL^{M}	thrombopoietin receptor mutant

mRNA	messenger RNA
Mw	molecular weight
NBT	nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
nm	nanometers
NO	nitric oxide
nt	nucleotides
OCT	optimum cutting compound
OD	optical density
OMP	outer membrane protein
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PLAP	placental alkaline phosphatase
r.p.m.	revolutions per minute
RA	radiation-attenuated
RAP-PCR	random-arbitrarily primed PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse-transcription
SAGE	serial analysis of gene expression
SCAP	soluble cercarial antigen preparation
sdH_2O	sterile distilled water
sdLNs	skin draining lymph nodes
SDS	sodium dodecyl sulphate
SEA	soluble egg antigen
SLAP	soluble lung-worm antigen preparation
SOD	superoxide dismutase
SRP	signal recognition particle
SS	signal sequence
SST	signal sequence trap
SUC^-	invertase gene deleted
SWAP	soluble adult-worm antigen preparation
TAE	tris-acetate electrophoresis buffer
TALEST	tandem arrayed ligation of expressed sequence tags
TBE	tris-borate electrophoresis buffer
TBS	tris buffered saline
TBS-T	tris-buffered saline with Tween
Th	T-helper
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
TPI	triose-phosphate isomerase
U	units
V	volts
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
YSST	yeast signal sequence trap

L	litre	mg	milligram	M	molar
ml	millilitre	µg	microgram	mM	millimolar
µl	microlitre	ng	nanogram	µM	micromolar
		pg	picogram	pM	picomolar

CHAPTER 1

INTRODUCTION

1.1 Background

Infection by blood dwelling flukes belonging to the family Schistosomatidae results in a debilitating disease known as schistosomiasis. Of the genera in this family, seven are confined to birds and five to mammals, but only the genus *Schistosoma* is associated with humans. The greatest geographical distribution and diversity of the definitive hosts parasitised, has been achieved by this genus.

With three hundred million individuals at risk of infection and around 200 000 deaths each year (WHO, 1993), schistosomiasis is one of the most widespread parasitic diseases of humans, and is currently endemic in 76 countries. Seven species of *Schistosoma* parasitise humans; *S. mansoni, S. haematobium* and *S. japonicum* are the most widespread, while *S. intercalatum* and *S. mekongi* have a more localised distribution. In humans, symptoms range from fever, anaemia and diarrhoea to hepatosplenomegaly; eventually death may result.

Due to the relative ease with which the life-cycle of *S. mansoni* is maintained in the laboratory, this species has received most attention. Thus, this thesis will refer almost exclusively to *S. mansoni*.

1.2 The Life-cycle of *S. mansoni*

The life-cycle of this parasite is complex, involving a number of morphological changes and including two hosts, the intermediate mollusc and the definitive mammal. The miracidium is the free larval stage ensuring transmission between the vertebrate and the snail. After penetration, the miracidium develops into the mother sporocyst, which reproduces asexually to produce daughter sporocysts. Further amplification of parasite numbers occurs following the differentiation of daughter sporocysts into cercariae. Therefore, the penetration and development of one miracidium leads to the production of several thousand cercariae (Theron, 1986) thereby counterbalancing the significant reduction in parasite number during snail location. The development process of cercariae from daughter sporocysts takes up to one week, after which mature cercariae, consisting of a head and a bifurcated tail, leave the snail in response to sunlight.

Under optimal conditions, the free swimming cercariae have 5 to 8 hours after shedding, in which to find and penetrate an appropriate host before infectivity is impaired (Lawson and Wilson, 1983). Penetration of host skin requires the co-ordination of

muscular contraction and secretory processes in the cercaria. Coincident with the release of proteases, the cercaria sheds its outer glycocalyx and tail, thereby allowing it to penetrate the host and concurrently transform into a schistosomulum.

The vast majority of information concerning the migratory pathway of *S. mansoni*, and the accompanying biochemical and morphological changes undergone by the parasite in the mammalian host comes from studies in laboratory rodents. This is described in more detail in section 1.6.2. The parasites continue to move through the host skin and eventually the majority penetrate a blood vessel. A combination of active migration through capillary beds and passive carriage in larger blood vessels takes a proportion of the schistosomula to the liver via the lungs. Once in the liver, the schistosomula undergo further biochemical and morphological changes transforming them into liver worms. Pairing of the adult worms takes place in the liver followed by their migration to the mesenteric veins where the female begins egg-laying. The majority of the eggs, which are deposited in blood vessel walls, pass through to the lumen of the gut and are eventually voided in the faeces. When the environmental conditions are appropriate, the eggs hatch in fresh water releasing the miracidia which are capable of infecting snails, and the life-cycle is thus perpetuated.

The life-span of an adult worm, in humans, is estimated to average between 3.5 to 12 years. Therefore, schistosomiasis is a chronic disease and the host immune response to the eggs can cause severe pathology. Approximately 300 eggs are produced per day by an adult female but many are never voided from the host; instead they are washed into, and lodge in, the liver. The host delayed-type hypersensitivity (DTH) reaction to these trapped eggs results in the formation of granuloma. This eventually leads to disruption of hepatic tissue and can result in portal hypertension and splenomegaly.

1.3 Current Control Measures

The provision of safe and adequate water supplies and sanitation is a major control strategy for schistosomiasis. This can be effective, as water is the medium through which the free-living stages of the parasite life-cycle locate and infect their respective hosts. Latrines reduce faecal and urinary contamination of water, preventing the miracidia locating the snail host, and thereby lowering transmission levels. Clean water for drinking and washing reduces the level of human contact with parasite-contaminated water and again this reduces the levels of parasite infection and transmission. Any such

modifications have to be accompanied by a health education programme aimed at not only informing communities about the biology of the parasite but also emphasising the role of the individual in spreading schistosomiasis. However, such measures require substantial amounts of money, which at present is not forthcoming in most endemic countries.

Control of the intermediate host snail populations using molluscicides, such as niclosamide, has not always produced the desired effects. Furthermore, the eradication of snail populations has only been possible in extreme situations such as in oases in Tunisia (Klumpp and Chu, 1987). This approach, as well as being expensive and impractical, is potentially damaging to the environment and could lead to the evolution of molluscicide-resistant snails. In addition, snail populations exhibit an impressive potential for regeneration and re-invasion of an area, re-establishing thriving populations in a period of months.

Chemotherapy plays a leading role in the control of schistosomiasis with the use of three basic treatment strategies. Mass treatment refers to the administration of drugs to entire populations without prior individual diagnosis. In selective treatment, only those individuals that are infected (determined by either serum sampling or faecal egg counts) are chosen for chemotherapy, whereas in targeted therapy only those individuals with high egg counts are treated. Although the mass treatment of infected human populations with chemotherapeutic drugs has been effective in reducing the prevalence and incidence of disease (Mahmoud *et al.*, 1983), diagnosis followed by chemotherapy is presently the control measure of choice. Praziquantel is the main drug used for such treatment programmes as it has few side effects, is administered as a single dose and is relatively cheap (WHO, 1985). However, the drug does not reverse pathological damage in patients; this can be a major problem as infection with schistosomes can go undetected for many years by which time the internal organs of the patient are badly damaged. In addition, the high chance of re-infection in endemic areas means that repeated treatment is necessary. This is not only costly but increases the risk of the evolution of a drug-resistant strain of parasite. Indeed, reduced parasite susceptibility to praziquantel has been reported both in experimental animals (Fallon *et al.*, 1994) and in populations living in endemic areas (Ismail *et al.*, 1996; Fallon *et al.*, 1995).

Relatively successful control, through the integration of complementary strategies, has been achieved in several countries, e.g., Brazil (WHO, 1993). However, recent environmental changes, closely linked to the development of water resources and

increases in population densities, have led to the spread of disease to previously low or non-endemic areas, e.g., Senegal (Talla *et al.*, 1990). Here, the first cases of schistosomiasis were reported one and a half years after completion of the Diama Dam on the Senegal river. Such water development programmes, together with the increase in drug-resistant strains of the parasite, have major implications for control of transmission throughout populations. Given the socio-economic climate of the endemic countries, it seems unlikely that the control strategies currently available will be effectively implemented and/or sustained.

All of the control measures discussed so far have shortcomings, therefore, the development of a schistosome vaccine would provide a durable, long-term control measure, relieving the suffering of many millions of people. A single-shot vaccine, composed of recombinant molecules and presented in the appropriate manner, that provided total immunity would be ideal. If administered before exposure to the parasite, such a vaccine would prevent egg-induced pathology associated with schistosomiasis and consequently reduce the prevalence, morbidity and rates of transmission and infection. However, as schistosomes do not replicate in the mammalian host, the requirement for a vaccine that induces sterile immunity is not absolute. Partial protection, which resulted in reduced worm burdens, would in turn reduce the number of people developing severe clinical disease and hence morbidity, as well as decreasing transmission levels.

1.4 Vaccine Development

An ideal vaccine is considered to be a modified, non-pathogenic form of an infectious agent, not as capable of replication and spread as the wild type but still able to stimulate the immune system, e.g. the Sabin polio vaccine. By following the same route of infection as the pathogen, the live vaccine encounters the same host defence systems and cells as the infectious agent and thus stimulates them to react in a manner similar to that seen in a natural infection. Unfortunately it is not always possible to develop such a vaccine for all pathogens, for many reasons, e.g. reversion to virulence, appropriate attenuation, storage and transport costs due to required refrigeration. One alternative is to use killed organisms as vaccines, but these, while useful in the short term, often fail to induce long-lasting immunity. In addition, there are numerous other disadvantages such as potential side effects due to the considerable amount of material administered, incomplete inactivation of the organism, and potential hazard posed to personnel and

environment. Therefore, to induce immunity to eukaryotic parasites it is necessary to generate vaccines based on subunits of the organism e.g. peptides, DNA, synthetic fragments or purified components. It is in this context that the majority of schistosome vaccine research is being directed, with emphasis on defining candidate antigens for a vaccine and determining the mechanisms of protective immunity. In addition, as the disease is associated with egg induced pathology, some workers have focused on the manipulation of the host immune response to parasite eggs and/or anti-fecundity vaccines. The different approaches being pursued are discussed here briefly. The research falls into two areas; cytokine intervention and identification of candidate antigens to reduce pathology and determining factors that influence anti-fecundity effects.

Anti-pathology vaccines

In mice, the immune response to schistosome eggs results in the formation of eosinophil-rich granulomas around them. $CD4^+$ T cells are crucial for the development of these granulomas (Mathew and Boros, 1986). However, conflicting views exist regarding the role of the two T helper (Th) cell subsets. On the one hand Th1 cells have been shown to mediate granuloma formation (Chikunguwo *et al.*, 1991). On the other, studies indicate that Th2 cells are the principal mediators of granuloma formation (Wynn and Cheever, 1995). Nevertheless, Cheever *et al.* (1994) showed that the co-administration of interleukin-4 (IL-4) and parasite eggs leads to an increase in granuloma size, while anti-IL-4 suppresses granuloma formation. Moreover, administration of IL-12 to egg-injected mice leads to an impaired granulomatous response on intravenous egg challenge (Wynn *et al.*, 1994a) as does the neutralisation of interferon gamma (IFNγ; Wynn *et al.*, 1995a). Subsequently, Wynn *et al.* (1995a) showed that sensitisation with eggs and IL-12 reduces granuloma formation and tissue fibrosis induced by a natural *S. mansoni* infection. This last result indicates that it might be possible to reduce pathology by downmodulation of a subset of immune responses to the parasite egg.

Although the $CD4^+$ T cell response to total soluble egg antigens (SEA) has been well studied, there is very limited information about the individual components of SEA. Those molecules that have been identified include SmE16 (Moser *et al.*, 1992), Smp40 (Nene *et al.*, 1986), the 44.7/56.8 kDa antigens (Hirsch *et al.*, 1997) and a 62kDa antigen (Asahi *et al.*, 1999). The most abundant component of SEA is Smp40 and consequently this

molecule has been studied extensively. Upon sequencing Smp40 fully, it was found to be a small heat shock protein with homologies to α-crystallins (Nene *et al.*, 1986). Cai *et al.* (1996) demonstrated that Smp40 is a potent immunogen, which elicits Th1 type responses. The T-cell epitopes of this antigen have been mapped; one of these epitopes is immunodominant (Chen and Boros, 1998) and is important for granuloma formation.

Anti-fecundity vaccines

An alternative approach is to formulate an anti-fecundity vaccine that would act by preventing/reducing egg production by the female schistosome and thereby decrease the level of pathology in infected individuals. In the long term this would reduce the rate of transmission throughout the treated population. Such an anti-fecundity effect was demonstrated using the 28kDa recombinant antigen glutathione-S-transferase (GST) to immunise baboons (Boulanger *et al.*, 1991) and Patas monkeys (Boulanger *et al.*, 1995; see section 1.7.1).

A multifactorial vaccine designed to polarise a beneficial immune response would comprise of larval antigens, which elicit partial protection, and egg antigens, which reduce the egg induced pathology caused by the worms that escape the protective response. The vaccine would also contain appropriate adjuvants that would enhance/neutralise specific cytokines, thereby optimising the method of presentation to the immune system. The recent advances in molecular biology techniques, the identification of cytokines and their functions, and in antigen presentation and delivery, makes the development of a vaccine against schistosomes more feasible.

1.5 Can naturally acquired immunity form the basis of a protective vaccine?

Much effort has been directed towards understanding the immune responses associated with infection in the hope that a vaccine may be developed based on naturally acquired immunity. Evidence for the development of acquired resistance to schistosomiasis comes from studies monitoring intensity of reinfection after curative chemotherapy at the same time as measuring the levels of exposure to infected water in humans (Fulford *et al.*, 1992; Kabatereine *et al.*, 1999). The data showed a decreased prevalence and intensity of infection in adults compared to children, independent of water

contact. The authors attribute these differences to the development of acquired immunity with age. Furthermore, adult newcomers to an endemic area are as susceptible to infection as young children, in contrast to adults resident in the same area (Kloetzel and daSilva, 1967). This observation supports the idea that immunity to schistosomiasis can develop in humans, as do studies on the immunology (Rihet *et al.*, 1991; Dunne *et al.*, 1992) and genetics (Marquet *et al.*, 1996) of infection. However, the age-intensity data has been reinterpreted to suggest that other factors associated with puberty, such as hormonal changes, may be responsible for the reduced rate of infection in older individuals (Fulford *et al.*, 1998). More recent studies of acquisition of infection with age in a previously unexposed population have been carried out in Senegal (Stelma *et al.*, 1993) and in Kenya (Ouma *et al.*, 1998). Both of these studies showed that acquired immunity was not the only factor effecting a reduction in infection intensities with age.

The inherent difficulties in investigating and describing immunological events in the human host has necessitated the use of animal infection models. The most commonly used models are the mouse and rat and, to a lesser extent, the guinea-pig and non-human primates. Each animal model has its advantages and disadvantages. Primates provide good experimental models but, due to the high maintenance costs and lack of specific reagents, not much is known about schistosome infections in such hosts. The laboratory rat clears a primary schistosome infection after four to six weeks before the majority of worms have matured. This makes the rat model inappropriate for the study of egg-induced pathology. The self-cure phenomenon seen in laboratory rats has been attributed to increased levels of parasite-specific IgE and the subsequent activation and degranulation of mast cells (Cutts and Wilson, 1997). Dunne *et al.* (1992) showed a similar correlation in human resistance to reinfection in which the IgE reactivity was directed against adult worm antigens. Thus, the schistosome infection in the rat could be a model for human immunity. However, it is difficult to envisage the design of a vaccine based on this protective effector response that does not carry the risk of dangerous side-effects such as increased allergy.

S. mansoni infection in the mouse has been the focus of attention of most schistosomiasis researchers. This is because the murine model provides good parallels with human infections in that mice develop egg-induced liver pathology and develop resistance to infection. Also mice are widely available and relatively cost-effective. Chronically infected mice are totally resistant to challenge infection (reviewed by Dean, 1983). The immune mechanism operating in this model appears to be directed against the

migrating larvae of the challenge infection, whilst the adult worms from the primary exposure remain unharmed. The term 'concomitant immunity' was coined to describe this phenomenon (Smithers and Terry, 1969). However, it has been demonstrated that a major component of this mechanism is a result of pathological changes in the hepatic portal vasculature due to egg deposition by adult worms of the primary infection (Wilson, 1990). Therefore, it is difficult to attribute resistance to reinfection entirely to an acquired immune response.

Overall, there is no one model of a normal schistosome infection that provides any clear immune mechanisms upon which a vaccine can be based.

1.6 The radiation-attenuated (RA) vaccine model

An alternative route would be to design a vaccine based on a novel host immune response not normally encountered by the parasite and against which it has no defence. Optimally irradiated cercariae induce consistently high levels of acquired immunity to challenge infection in mice, rats and primates (Coulson, 1997). Gamma (γ) or UV irradiation of infective larvae attenuates the parasites such that, depending on the radiation dose, they fail to develop to maturity (discussed in more detail in section 1.6.3). Therefore, there is no egg deposition and hence no egg-associated pathology. Due to practical and ethical reasons this attenuated vaccine cannot be used in humans. However, it does provide a paradigm upon which a vaccine can be designed (Wilson and Coulson, 1998). As the majority of information available on schistosome infections has been obtained from studies in mice, the rest of this thesis will refer to the RA vaccine model in mice, unless otherwise stated.

1.6.1 Conditions for immunisation with irradiated parasites

Several methods have been used to attenuate parasites and induce resistance in laboratory hosts. These include the use of γ-irradiation (Villella *et al.*, 1961), UV light (Dean, 1983), X-rays (Hsü *et al.*, 1981) and chemicals (Bickle and Andrews; 1985). However, the use of γ-radiation is most widespread.

The dose of radiation applied to the parasite strongly influences the extent of migration of, and the level of protection induced by the attenuated larvae. Over the years

there has been a dispute over the optimal amount of irradiation. Bickle *et al.* (1979a) demonstrated that parasites irradiated with 20 kilorads (krad) induced greater levels of protection than higher or lower doses of radiation. However, Minard *et al.* (1978) found that the highest levels of resistance were induced when a radiation dose of 50 krad was used. This disagreement is thought to reflect differences in parasite maintenance and/or irradiation conditions (James and Dobinson, 1985). More recently, irradiation doses of 15-20 krad have consistently resulted in higher levels of protection than doses of 50 krad or more (Richter and Harn, 1993). At York, the optimal irradiation dose was found to be 20 krad (Coulson, unpublished observations).

If mice are vaccinated once, the number of applied parasites does not appear to affect the levels of resistance (Bickle *et al.*, 1979b), with optimal levels of immunity achieved using as few as 50 attenuated cercariae (Dean *et al.*, 1983). In addition, the resistance stimulated by vaccination with irradiated parasites seems to be species-specific. Mice vaccinated with irradiated *S. mansoni* cercariae and subsequently challenged with other *Schistosoma* species are not protected (Agnew *et al.*, 1989). However, geographically distinct isolates of *S. mansoni* are able to cross-protect indicating that the antigens relevant to protection are common to these isolates (Hackett *et al.*, 1987). A single dose of optimally γ-irradiated cercariae reduces worm burdens from challenge infections by 60-80% as compared to non-vaccinated control mice.

Before the mechanisms of immunity in this model can be discussed, and particular antigens pinpointed, the development of the normal parasite and its interaction with the immune system must be considered.

1.6.2 The normal infection in laboratory mice.

Migration and development

Upon penetration of the mouse skin, the cercarial tail is lost and the parasite undergoes several biochemical and further structural changes as it transforms into the next larval stage, the schistosomulum. The parasite then negotiates the epidermis, basement membrane and dermis. On average, the skin phase of migration takes between two and five days (Miller and Wilson, 1978). During the first three hours post-transformation the trilaminate outer membrane of the cercaria is replaced by a heptalaminate membrane.

The new membrane is formed as membranous vesicles, which originate in the subtegumental cells, pass into the tegument (Hockley and McLaren, 1973). The parasite continues its migration until it eventually enters a blood vessel aided by lytic secretions from the head gland (Crabtree and Wilson, 1985). Whilst most of the schistosomula leave via the blood, 10-20% exit via the lymphatic vessels (Mountford *et al.*, 1988). The parasites migrate in the direction of blood/lymph flow, using a combination of active migration and passive carriage, via the pulmonary artery to the lungs. There is a period of development of three to four days in the lungs, during which the parasites elongate (Wilson *et al.*, 1978). Additionally, the mid-body spines are lost progressively (Crabtree and Wilson, 1980), accompanied by an increase in tegumental pits, which are responsible for extending the surface area of the membrane. A new inclusion body, the homogeneous body, is also first seen at this stage (Crabtree and Wilson, 1986a). These changes are thought to adapt the parasite for onward migration.

The parasites that successfully migrate through the lungs pass into the left side of the heart and are distributed, in the same proportion as cardiac output, to the systemic organs of the body. Those parasites that gain access to the splanchic organs negotiate capillary beds, enter the hepatic portal system and are eventually retained in the liver. Those distributed to the other systemic organs traverse the capillaries and re-enter the heart to be carried back to the lungs. Trapping of schistosomula in the liver is not totally efficient and an estimated 14-30% may return to the lungs (Wilson *et al.*, 1986). Wilson and Coulson (1986) speculated that three circuits of parasites around the pulmonary-systemic vasculature were required to recruit the entire hepatic population. The high nutrient content of the hepatic portal system, lower blood pressure or oxygen tension may be the triggers required for parasite shortening and thus termination of migration (Wilson *et al.*, 1978). Accumulation of parasites in the liver levels off by day 21 after infection (Wilson *et al.*, 1986). The parasites begin to feed on blood and increase in mass, and between days 28 and 35 post-penetration they pair and migrate up the hepatic vessels to the mesenteric veins where egg laying commences.

Elimination of parasites during a primary infection

Only 30-50% of the parasites that penetrate the skin mature to adult worms. The failure of the remaining skin penetrants to reach the hepatic portal system has been the focus of much debate (Wilson and Coulson, 1989) and was only clarified by the autoradiographic tracking of isotopically labelled schistosomula in the compressed

organs of infected mice (Georgi, 1982). It was found that up to 78% of penetrants had migrated as far as the lungs of mice by day 7 (Mangold and Dean, 1983). Also, by day 14 post-infection more than 90% of the parasites detected in the skin at day 0 were present in other sites (Wilson *et al.*, 1986). Mangold *et al.* (1986) demonstrated that the number of maturing parasites did not differ significantly when lung-stage schistosomula were injected directly to the lungs intravenously, compared to a normal percutaneous infection. Therefore, it would seem that only a very small proportion of parasites are eliminated at the skin stage of migration.

The transit time of the parasite through the lungs is considerably longer than through other organs (Wilson and Coulson, 1986), suggesting that the larvae find this a difficult organ to negotiate. Ultrastructural examination of infected lungs supports this hypothesis as the lung worms were seen to cause considerable distension of the vessels (Crabtree and Wilson, 1986a). It was found that 80% of parasites present in the lungs had been diverted into the alveoli by day 20 post-infection (Crabtree and Wilson, 1986b). It has also been shown that schistosomula delivered to the alveoli, via the trachea, had a limited capacity to re-enter the tissues and mature, whereas parasites removed from the alveoli and transferred to the hepatic portal system matured normally (Coulson and Wilson, 1988). This suggests that despite adaptive changes, the parasites find the pulmonary capillaries difficult to negotiate, and a large proportion of them rupture the narrow blood-air barrier and enter alveoli. It is assumed that the schistosomula starve to death here as they are unable to re-enter tissues. Thus, taken together, these studies indicate that the majority of parasite loss occurs in the lungs. It seems that parasite attrition in the naïve mouse is a consequence of physical constraints in the lungs rather than immunological mechanisms.

1.6.3 The effect of radiation on the parasites and their ability to migrate.

The migratory pattern of attenuated larvae is affected by the radiation dose applied. A dose of 2-10 krad results in decreased worm recoveries from the lungs; 20 krad reduces this even further, while 40 krad results in almost no parasites reaching the lungs (Mangold and Dean, 1984). Most 90 krad irradiated parasites fail to leave the skin indicating that irradiation diminishes the parasite's ability to migrate. Optimally-irradiated parasites (20 krad) migrate more slowly through the host compared to their normal counterparts. Only 13% of normal parasites are found in the skin by day 5

whereas 43% of applied parasites remain in the skin of vaccinated mice (Mountford *et al.*, 1988). Migration through the skin-draining lymph nodes (sdLNs) was also more rapid for normal parasites. Mastin *et al.* (1983) showed that the migration of the attenuated parasites terminated in the lungs of vaccinated mice.

Irradiation of larvae does not seem to cause a change in their ultrastructure (Mastin *et al.*, 1985) or in the surface antigens of the larvae (Simpson *et al.*, 1985). Vierra *et al.* (1987) demonstrated that there was no difference in the ability of normal or irradiated larvae to induce proliferation of peripheral blood mononuclear cells from human patients with a chronic infection. Wales *et al.* (1992) showed that protein synthesis in the first 24 hours post-irradiation is inhibited but is completely normal by 72 hours. Thus, no difference can be detected between irradiated and normal cercariae. However, examination of the morphological differences between irradiated and normal larvae at the lung stage of development showed impaired neuromuscular co-ordination in the former (Harrop and Wilson, 1993a). The irradiated parasites exhibited random constrictions at intervals along the length of the body, probably resulting from over-constriction of muscle fibres. The authors suggest that this may account for the truncated migration by γ-irradiated parasites.

1.6.4 Induction of protective immunity

There is ample evidence to suggest that resistance in the irradiated cercaria model is the result of a specific immune response. For example, the immunity generated in vaccinated mice, but not those with chronic infections, was transferred to naïve animals across a parabiotic union (Dean *et al.*, 1981). Also, athymic mice fail to develop resistance following exposure to irradiated cercariae, indicating that T cells are important (Sher *et al.*, 1982).

The precise reason why exposure to attenuated cercariae elicits protection in mice is not fully understood. However, their altered migration kinetics, as compared to normal parasites, may provide one explanation. Attenuated parasites undergo a protracted migration from the site of skin penetration to the lungs where they die between days 14 and 21 post-vaccination. Following vaccination, a proportion (10-15%) of parasites enter and persist in the skin-draining lymph nodes. In addition greater amounts of parasite-released material were detected in the sdLNs of vaccinated mice than in those from

control mice (Mountford *et al.,* 1988). Thus, greater quantities of parasite proteins are exposed to the host's immune system than after infection with normal cercariae.

The removal of the draining lymph nodes before vaccination reduces the level of resistance. The same is true when draining lymph nodes are removed a week after vaccination, whereas lymphadenectomy at later times does not abrogate resistance (Mountford and Wilson, 1990). Furthermore, Constant *et al.* (1990) showed that lymphocytes derived from the draining lymph nodes of vaccinated mice proliferate more than those derived from the spleens of the same mice, thereby confirming that parasite persistence in the sdLNs is essential for the induction of protective immunity. Taken together, these studies suggest that optimally attenuated parasites persist in the lymph nodes, where antigenic processing is intense, and release greater amounts of material, thus priming those lymphocytes required for successful vaccination.

Incomplete ablation of resistance when the draining lymph nodes are removed suggests that there is another site important for the induction of protection. Coulson and Mountford (1989) found that intra-dermal administration of attenuated 8 day old schistosomula results in high levels of protection, comparable to percutaneous vaccination. However, only intermediate levels of protection were elicited by delivery of parasites directly to the lungs (intra-tracheal), whilst intravenous injection yielded little or no resistance. This indicates that the arrested migration of irradiated schistosomula in the lungs and their subsequent death there is also important for the induction of immunity. Thus, it seems that the irradiated larvae must persist in both the lungs and in the draining lymph nodes to elicit optimal levels of protection. The arrival of attenuated parasites in the lungs triggers an inflammatory response which recruits $CD4^{+}$ T cells to the pulmonary parenchyma (Aitken *et al.,* 1988). These cells are important for the initiation of the host response to a subsequent challenge infection (Smythies *et al.*, 1992b). The immune response in the lymph nodes involves the expansion of $CD4^{+}$ Th cells, specifically of the Th1-cell subset as determined by the secretion of abundant IFNγ after *in vitro* stimulation (Pemberton *et al.*, 1991; Mountford *et al.*, 1992). This is supported by the finding that the co-administration of recombinant IL-12 with the attenuated cercariae boosts both the secretion of IFNγ and the levels of protection (Wynn *et al.*, 1995b; Anderson *et al.*, 1998). The Th1 bias of the immune response in the sdLNs and lungs is corroborated by cytokine mRNA expression patterns in these tissues (Betts and Wilson, 1998; Wynn *et al.*, 1995b).

1.6.5 The effector response

It is now generally accepted that the majority of challenge parasites are eliminated in the lungs via a Th1-cell mediated mechanism (reviewed by Coulson, 1997). A rapid inflammatory response is triggered when normal challenge schistosomula reach the pulmonary vasculature of a vaccinated mouse. The schistosomulum is stationary for 2-3 days as it undergoes morphological adaptations for onward migration and it is within this time that the individual challenge parasites become surrounded by a dense cellular focus, characteristic of a DTH response (Crabtree and Wilson, 1986b). In contrast, parasites in a non-vaccinated lung do not attract a focus unless they enter alveoli.

The mechanism of parasite elimination remains unresolved. Macrophage-mediated killing, involving the production of nitric oxide (NO) has been suggested (James and Nacy, 1993). Wynn *et al.* (1994b) demonstrated that levels of inducible nitric oxide synthase (iNOS) mRNA were elevated in the lungs of vaccinated mice suggesting that NO activity was increased. However, studies using mice genetically deficient for iNOS showed only ~30% abrogation of protection, indicating that NO is not a major component in parasite elimination (Coulson *et al.*, 1998; James *et al.*, 1998).

As mentioned above, many of the parasites arriving in the lungs attract a focus of inflammation. By day 11 post-challenge the foci have increased in size and surround the parasites trapped in capillaries and alveoli. Ultrastructural studies show no visual damage to the schistosomula (Crabtree and Wilson, 1986a). Moreover, trapped schistosomula recovered from the lungs of vaccinated mice are able to migrate and mature fully when introduced into a naïve host (Coulson and Wilson, 1988). This suggests that the effector mechanism does not harm the parasites but in fact acts to block onward migration of the challenge worms.

The pro-inflammatory cytokine IFNγ has been shown to play a key role in the effector response (Sher *et al.*, 1990). More recently *in vivo* ablation studies (Smythies *et al.*, 1992a) and experiments using IFNγ gene disrupted mice (IFNγ $^{-/-}$) (Wilson *et al.*, 1996) have confirmed these data, as protection levels were reduced in both studies. Indeed, the pulmonary foci formed in the IFNγ $^{-/-}$ mice were different in their architecture and cellular composition; they were larger, contained more giant cells and were not as compact as foci from genetically intact mice (Wilson *et al.*, 1996). The authors suggest that the larger, more diffuse inflammatory foci do not prevent the parasites from migrating

through the lungs. Further studies using gene-disrupted mice have shown that the lack of tumour necrosis factor (TNF) completely abrogates protection (Street *et al.*, 1999). However, the mechanism through which TNF acts is unknown.

Although the precise mechanisms responsible for parasite elimination in the lungs remain unclear, it has been demonstrated that the radiation-attenuated vaccine induces a Th1 biased immune response which is protective. The serum from singly-vaccinated mice confers only low levels of protection on transfer into naïve mice, supporting the hypothesis that antibodies are not important in this vaccine model. More recently, two studies using B-cell deficient (μMT) mice have been carried out to investigate the role of B cells in the 1x vaccinated mouse model (Anderson *et al.*, 1999; Jankovic *et al.*, 1999). Although the data in the two studies is similar the authors reach very different conclusions. Anderson *et al.* (1999) found that two-thirds of the μMT mice had protection levels similar to their wild type counterparts, indicating that cell-mediated mechanisms alone are adequate for protection. In contrast, the remaining μMT mice had worm burdens identical to the challenge control group. The authors suggest that there is a genetic basis for the observed differences between individual μMT mice and that cell-mediated immunity confers substantial protection against challenge infection. However, Jankovic *et al.* (1999) found that protection levels were lower in μMT mice than in wild type controls suggesting that the protective mechanism in operation in the 1x vaccinated mouse is likely to involve humoral antibodies.

In contrast, serum from multiply vaccinated mice is capable of transferring a degree of resistance (Mangold and Dean, 1986). Multiple vaccination has been shown to result in modest increases in the level of immunity compared to that elicited by a single vaccination (Caulada-Benedetti *et al.*, 1991). The latter authors also show that multiple vaccination predisposes towards a Th2 response, suggesting that there may be qualitative and quantitative changes in antibody titres as compared to those in 1x vaccinated mice. This was demonstrated by Dalton and Strand (1987), as antibodies from singly-vaccinated mice were directed against glycoproteins of molecular weight (Mw) from >300 to <10kDa while antibodies from twice-vaccinated mice were directed towards high Mw glycoproteins. Additionally, multiple vaccination of IFNγ$^{-/-}$ mice, in which Th1 responses are absent, results in increased protection with each successive vaccination and serum from these animals will confer up to 60% protection on naïve C57BL/6 recipients (Wilson *et al.*, 1999). Furthermore, triple vaccination of μMT mice fails to increase the

protection levels above those reached after the primary vaccination, indicating that antibody-mediated responses are critical in multiply vaccinated mice (Anderson *et al.*, 1999; Jankovic *et al.*, 1999). It should also be noted that these experiments show that the cell-mediated effector response is not boosted by multiple vaccination with irradiated parasites. The site and mechanism of challenge parasite elimination in the multiple vaccination model is unclear.

1.6.6 What kind of antigens make good vaccine candidates?

Given that the radiation-attenuated vaccine will elicit either Th1 or Th2 mediated responses, depending on the vaccination protocol, several deductions can be made about the antigens involved.

When normal challenge parasites reach the lungs of a 1x vaccinated mouse they provoke an inflammatory response which is mediated by Th1 cells. Therefore, the antigens involved in the induction phase of immunity must by processed by antigen-presenting cells (APCs) and presented to the T cells in the context of MHC class II. Antigens released from the schistosomulum (as opposed to somatic or membrane-associated molecules) are the most likely candidates as they are the most readily available material for uptake by APCs. Although it can be argued that dead or dying schistosomula can also prime immune cells, the target of the immune response is the live schistosomulum and it would therefore be logical to assume that only those antigens derived from the live parasite are important. It has been suggested that irradiation may modify antigen conformation which means that the immune system is primed by abnormal or non-native antigens (Wales and Kusel, 1992). However, this would not help as the T cells primed against such antigens would not be able to recognise the unmodified peptides from challenge larvae. Thus, antigens released by the lung-stage parasites are a rich source of potential vaccine candidates. More recently, Riengrojpitak *et al.*, (1998) have shown that there is contact between immune cells, which have characteristics of macrophages and dendritic cells, and the parasite surface in the sdLNs. This indicates that proteins found on the surface of the parasite tegument are a key source of protective antigens.

Biosynthetic labelling of larvae grown to the lung-stage *in vitro* has revealed a pattern of proteins that are synthesised and secreted during this stage of development (Harrop and Wilson, 1993b). Approximately 15 proteins of diverse molecular weight were

detected *in vitro* between days 6-8. The day 6-8 larval secretions, in comparison with soluble parasite preparations, were more potent stimulators of proliferation of lymphocytes from vaccinated mice (Mountford *et al.*, 1995). Therefore, one or more of these 15 proteins is likely to be the key antigen(s) in this model of protective immunity. The minute amount of material released from 6-8 day schistosomula makes the isolation of these proteins difficult. Indeed, there has only been one published study directed specifically at identifying these antigens (Harrop *et al.*, 1999). This is discussed in more detail in section 1.7.7.

1.7 Candidate vaccine antigens

Although it is possible to protect mice, rats and primates against a challenge infection by prior immunisation with radiation-attenuated cercariae, this vaccine strategy cannot be used in humans for ethical and safety reasons. Therefore, much attention has focused on the identification of particular parasite antigens that may be responsible for inducing the protective immune responses. Following the recent advances in molecular biology technologies and vaccinology, a vast number of schistosome antigens have been described and their potential as vaccine candidates tested. To discuss all of these antigens is beyond the scope of this thesis; I have therefore concentrated on those antigens which have received most attention. In 1991 the WHO selected a panel of six antigens for independent vaccine trials in experimental animals (Bergquist, 1995); each of these is discussed in more detail.

1.7.1 Glutathione S-Transferases

The 28kDa glutathione S-transferase of *S. mansoni* (GST) is by far the best characterised vaccine candidate. This antigen was first described by Balloul *et al.* (1985). A 28kDa fraction of *S. mansoni* soluble adult worm antigens was found to elicit a protective antibody response in Fischer rats and BALB/c mice. In addition, the antigen could be detected in the *in vitro* translation products of adult worm mRNA and in ^{125}I labelled surface antigens of schistosomula. The purified antigen could elicit protective immunity in rats (50-70%) and mice (40-43%) by direct immunisation (Balloul *et al.*, 1987a). At the same time the cDNA encoding the antigen was cloned, sequenced and expressed in *E. coli* and yeast (Balloul *et al.*, 1987b). The recombinant protein also

elicited protective immunity in rats, hamsters (Balloul *et al.*, 1987b) and mice (Boulanger *et al.*, 1991). The major epitopes eliciting T and B cell responses in mice and rats have been identified (Wolowczuk *et al.*, 1989; Auriault *et al.*, 1988). Subsequently a peptide (115-131) from GST was shown to have both T and B-cell epitopes and, when delivered as an octameric construct, resulted in 40-50% protection in rats (Wolowczuk *et al.*, 1991).

Taylor *et al.* (1988) have characterised the 28kDa protein as a glutathione S-transferase and have demonstrated that the enzyme is present in the adult worm tegument, protonephridial cells and subtegumental parenchymal cells. GSTs form a large multigenic family of isoenzymes, which play a major role in detoxifying various electrophiles and oxidative free radicals in eukaryotes. There is no cross-reactivity between parasite and human and rat GSTs thus, it was thought that inhibition of enzyme activity might have a protective effect. To investigate this, a monoclonal antibody (MAb) against GST (S13) was passively transferred into rats and mice. Whilst treatment significantly reduced worm burden in rats, there was no effect on worm burden in mice but tissue egg load was decreased (Xu *et al.*, 1991). The authors suggest that this difference may be due to distinct effector mechanisms in operation against GST in the two animal models. The response in rats generates high levels of IgE antibodies, whereas in the mouse model the protective immunity involves antibody-independent cellular mechanisms (Wolowczuk *et al.*, 1989; Damonneville *et al.*, 1988). In addition, Boulanger *et al.* (1991) demonstrated a decrease not only in worm burden but also in faecal egg output (by 66%) and female worm fecundity (by 33%) in baboons immunised with GST. Taking all these data together, it was proposed that GST might function as part of an anti-fecundity vaccine. More recently, the anti-fecundity effect of *S. haematobium* GST was also seen in Patas monkeys infected with *S. haematobium* (Boulanger *et al.*, 1999). The S13 MAb was shown to recognise the C-terminal (190-211) and N-terminal (10-43) peptides of GST. Immunisation of mice with the C-terminal peptide reduced worm burdens and egg deposition (Xu *et al.*, 1993). The authors postulate that a peptide construct containing the C-terminal and N-terminal peptides, together with the 115-131 amino acids, could be used to decrease both worm burden and female fecundity.

The data reviewed so far emphasise the role of antibodies in anti-fecundity but it should be noted that IFNγ produced by $CD4^+$ and $CD8^+$ T cells also has an effect on

levels of hepatic tissue damage (Pancré *et al.*, 1994). Grezel *et al.* (1993) have suggested that IgA antibodies are important for influencing parasite fecundity, whereas IgE is responsible for affecting protection against challenge worms. With regard to the design of a vaccine strategy, enhanced secretory IgA production may be achieved using mucosal immunisation. Consequently, various mucosal vaccine delivery systems, which increase the immunogenicity of the antigen and stimulate the required immune profile, have been investigated.

Ivanoff *et al.* (1996) delivered SmGST, incorporated into liposomes, orally to mice and found elevated levels of IgA in gut washes, and IgG1 and IgG2b antibodies in serum, as well as decreases in worm burdens. Similarly, single dose nasal administration of biodegradable microparticles containing recombinant SmGST (rGST) gave a specific mucosal immune response (Baras *et al.*, 1999). Experiments by Mielcarek *et al.* (1997), in which mice had been immunised intranasally with *Bordetella pertussis* expressing rGST fused to filamentous haemagglutinin, showed high levels of IgA in the lungs and IgG in serum as well as a decrease in worm burdens. Other live expression systems such as *Salmonella typhimurium* (Khan *et al.*, 1994a+b) and *Mycobacterium bovis* bacillus Calmette-Guerin (BCG; Kremer *et al.*, 1996) have also been used successfully to induce anti-GST antibodies. Most recently, Sun *et al.* (1999) have demonstrated that administration of rGST conjugated to cholera toxin B subunit induces strong mucosal IgA immune responses and T cell hyporesponsiveness, as well as consistently suppressing granuloma formation and inhibiting parasite development.

Experiments have also been carried out using plasmid DNA encoding SmGST (Dupre *et al.*, 1997). Following intradermal administration to rats, the antigen could be detected in skin cells by immunohistochemistry; long-lasting IgG2a and IgG2b antibodies were also detectable in sera. These sera were able to mediate antibody dependent cell-mediated cytotoxic (ADCC) killing of larvae *in vitro,* and a parasite challenge of immunised mice induced a strong and rapid boost of specific IgG antibodies. No protection data was reported. The *S. japonicum* GST has also been tested for immunogenicity using a similar plasmid construct but failed to elicit specific antibodies (Waine *et al.*, 1997).

1.7.2 Paramyosin (Sm97)

Intradermal vaccination of mice with thawed adult worms, using BCG as an adjuvant, produced unusually monospecific antibodies to a 97kDa antigen (Pearce *et al.*, 1986). The antigen was identified as the myofibrillar protein paramyosin (Lanar *et al.*, 1986) and localised to the tegument and muscle of adult worms (Matsumoto *et al.*, 1988). Immunisation of mice with either native or recombinant paramyosin conferred significant resistance (26-33%) against challenge infection (Pearce *et al.*, 1988). In addition, paramyosin was shown to stimulate T-cells from vaccinated mice to produce IFNγ in response to living schistosomula (Pearce *et al.*, 1988; Flanigan *et al.*, 1989). Paramyosin is a non-surface protein, in contrast to the other vaccine candidates that are thought to be surface expressed or secreted. It has been suggested that cell-mediated killing of the parasites could be due to paramyosin that was released from the parasite as part of normal protein turnover (Gobert, 1998).

DNA vaccination with plasmid encoding paramyosin has also been tested; although antibodies to paramyosin were generated, the mice were not protected against cercarial challenge (Waine *et al.*, 1997).

1.7.3 Triose-phosphate Isomerase (TPI)

This protein was identified using a MAb (M1) from mice vaccinated with detergent extracts of mechanically transformed schistosomula (Harn *et al.*, 1985). The 28kDa protein is found in all developmental stages of the parasite; perhaps most intriguingly it is transiently expressed on the surface of newly transformed schistosomula. Immunisation of mice with immune complexes containing the 28kDa antigen bound to M1 gave a 38% reduction in worm burden (Harn *et al.*, 1992).

Shoemaker *et al.* (1992) cloned the full length cDNA, and the recombinant protein was found to have enzymatic activity that was typical of a TPI; the T and B cell epitopes have been mapped and four-armed multiple antigenic peptides (MAP) have been constructed. The recombinant protein induces lymph node cells from infected mice to secrete IL-2 and IFNγ and immunisation with the MAP-4 construct leads to specific T cell proliferation and IFNγ production by lymph node cells upon restimulation with full length TPI *in vitro* (Reynolds *et al.*, 1994).

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Ferru *et al.* (1997) constructed a MAP, termed MAP DA, containing 3 peptide sequences, two from TPI and one from GST. Subcutaneous immunisation of mice with MAP DA elicited a T cell response against the TPI components and a B cell response against the GST peptide. This study is the first to demonstrate the use of one construct to immunise a host against two distinct antigens.

1.7.4 Sm23

This molecule was first reported as the target of a MAb that bound to the surface of cultured lung-stage schistosomula (Harn *et al.*, 1985) and the cDNA clone was identified by several groups (Dalton *et al.*, 1987; Wright *et al.*, 1990). Sm23 is part of a superfamily of membrane proteins all of which have unknown function. The structure of Sm23 is believed to consist of four transmembrane domains and two extracellular domains. The T and B cell epitopes in the predicted external domains have been mapped and shown to be highly immunogenic (Reynolds *et al.*, 1992). Although this antigen was tested in the WHO vaccine trials there is no other published data on its protective efficacy.

1.7.5 IrV-5

As discussed earlier, mice exposed to radiation-attenuated cercariae are highly resistant to challenge infection, and sera from multiply vaccinated mice can confer partial resistance when transferred to naïve recipients (see section 1.6.5). A polyclonal serum from twice vaccinated mice recognises a restricted subset of antigens. Hence, a polyclonal serum raised in rabbits against this subset of antigens was used to screen a cDNA library. A cDNA clone encoding a fragment of one of these glycoproteins was isolated, sequenced and designated IrV-5 (Soisson *et al.*, 1992). The clone shows a high degree of homology to mammalian β-myosin heavy chain and the native protein homologous to IrV-5 is a 200kDa antigen.

Immunisation of mice with recombinant IrV-5 (rIrV-5) complexed to outer membrane protein of meningococcus (OMP) gave up to 72% protection in mice, 97% in rats (Soisson and Strand, 1993) and up to 54% in baboons (Soisson *et al.*, 1993).

1.7.6 Sm14

This antigen was identified and cloned following the analysis of the antigens in a protective saline extract of adult worms (Moser *et al.*, 1991). The recombinant molecule protected mice by up to 67% against a challenge infection, even in the absence of an adjuvant (Tendler *et al.*, 1996). The recombinant Sm14 also protected mice against a challenge with *Fasciola hepatica*, suggesting that similar parasite proteins could mediate immune reactivity and therefore represent a basis for a vaccine against both species.

Surprisingly, despite the induction of protective responses by each of the antigens in the stipulated formulations in the originators' laboratories "the stated goal of consistent induction of 40% protection or better was not reached" in the independent WHO trials (TDR News, 1995). In addition, detailed analyses of the responses of peripheral blood leukocytes from infected humans, stimulated *in vitro* with each of the candidates, failed to highlight any obvious correlation between resistant and susceptible individuals (Bergquist and Colley, 1998). This has prompted a re-evaluation of the antigen formulations in use. It is in this context that most research on these six antigens is being directed, with GST, paramyosin, MAP-4/TPI and Sm14 being moved towards Phase I clinical trials, and IrV-5 and MAP-3/Sm23 being developed for DNA vaccination (Bergquist and Colley, 1998).

1.7.7 Other Vaccine Candidates

Although the antigens discussed so far represent the most intensively studied schistosome proteins, a number of other antigens have been proposed as vaccine candidates. Some of these are potent stimulators of the immune system and warrant further discussion.

Most of the antigens currently being investigated were originally identified under the premise that proteins presented at the surface, or associated with the tegument, of the parasite would be the best vaccine candidates as such molecules are in close contact with the host immune system. Two main approaches have been used to identify such proteins. One method made use of surface radio-labelling techniques, followed by membrane isolation, electrophoresis and autoradiography. Sera from vaccinated or infected mice were then used to assess the immunogenicity of the labelled proteins. The second

method identified antigens by immunoprecipitation, affinity chromatography or Western blotting of surface components with monoclonal or polyclonal sera.

Two labelling techniques, lactoperoxidase (Dissous *et al.*,1981) and Iodogen (Knight *et al.*, 1984) have been used, and both gave identical results. Human or animal infection sera precipitate six major polypeptide surface antigens of Mr 92, 38, 32, 20, 17 and 15kDa (Simpson and Smithers, 1985). Since then many more surface antigens have been described, several of which can be precipitated by either chronic mouse serum or vaccinated mouse serum.

Gryzch *et al.* (1982) produced a rat monoclonal antibody which transferred significant levels of protection to rats (53-61%) and which immunoprecipitated the 38kDa surface antigen seen by Dissous *et al.* (1981). Many other studies have shown that monoclonal antibodies that recognise schistosomula surface antigens are protective (Zodda and Phillips, 1982; Harn *et al.*, 1984; Bickle *et al.*, 1986) suggesting that these antigens can mediate immunity. Further evidence that surface antigens induce protection was provided by Smith and Clegg (1985). They vaccinated mice with a surface antigen, purified using a protective monoclonal antibody, and demonstrated low but significant levels of protection. Furthermore, Tarrab-Hazdai *et al.* (1985) purified antigen 9B using a highly protective monoclonal antibody and achieved 45% protection in mice immunised with the antigen in Complete Freunds Adjuvant (CFA). This level of protection was elevated to 65% when the antigen was delivered within proteosome vesicles. One protective B cell epitope of 9B has been identified (Tarrab-Hazdai *et al.*, 1998) and shown to give ~40% protection in mice when delivered either subcutaneously conjugated to bovine serum albumin (BSA), or intranasally, expressed in flagella of *Salmonella dublin* (Ben-Yedidia *et al.*, 1999).

Sera from mice protectively vaccinated with adult worm surface membranes precipitated a 25kDa glycoprotein (Sm25); this protein has been characterised independently in several laboratories (Knight *et al.*, 1989; Ali *et al.*, 1991) and is principally located subtegumentally in adult worms. Although there are currently no protection data available for this antigen, vaccination with the recombinant protein induces high antibody titres (Suri *et al.*, 1997).

Yet another tegument associated protein, Sm10, has been identified as a putative vaccine candidate based on its properties as a potent stimulator of T cells from adults living in an endemic area (Couissinier-Paris *et al.*, 1995). Significant protection is induced by the recombinant antigen in mice (Henri *et al.*, 1998). The authors describe

the molecule as a dynein light chain (DLC) and there is indeed significant sequence identity to the DLC described by Hoffmann and Strand (1996). These molecules seem to be members of a family of tegumental proteins as they both display sequence similarity to the 20.8KDa tegumental antigen (Hoffmann and Strand, 1997) which is in turn related to Sm22.6 (Webster *et al.*, 1996; Jeffs *et al.*, 1991), and Sm21.7 (Francis and Bickle, 1992). A comparison of the levels of anti-recombinant Sm22.6 (rSm22.6) antibodies with intensities of reinfection after treatment of human patients showed a negative correlation between IgE responses to rSm22.6 and infection intensity (Webster *et al.*, 1996); this is similar to protection data obtained with recombinant Sm10 in mice (Henri *et al.*, 1998).

Human responses correlated with resistance to reinfection have been used to identify other protective antigens. Thus, a study of a Brazilian population showed that the susceptibility of those individuals who became rapidly reinfected after treatment could be correlated to the absence/reduced levels of anti-37kDa IgG antibodies (Dessein *et al.*, 1988). The cDNA encoding the antigen, Sm37, has been cloned and sequenced (Goudot-Crozel *et al.*, 1989). Of the five B cell and two T cell epitopes found on the molecule, one B cell epitope, 37-5, was shown to be highly antigenic in human infections (Argiro *et al.*, 1999). A synthetic peptide corresponding to 37-5 coupled to ovalbumin and administered in CFA to mice induced 25% protection.

Several studies have attempted to characterise the proteins released by the parasite (McKerrow and Doenhoff, 1988; Harrop and Wilson, 1993; Lewis and Strand, 1991; Atkinson and Atkinson, 1982). However, the serine protease, elastase, is the only molecule from these studies that has been postulated as a potential vaccine candidate. Elastase was described by several groups simultaneously (Marikovsky *et al.*, 1988, Newport *et al.*, 1988) and has been characterised as crucial for skin penetration by cercariae (McKerrow *et al.*, 1985). The enzyme elicits a transient immune response during a natural infection. Darani *et al.* (1997) immunised mice with material that contained the protease as the main constituent and showed that in animals that did produce antibodies to elastase there was a 40% reduction in worm burden. Doenhoff (1998) has suggested that this protease, or recombinant derivatives of the enzyme, could be used as an effective vaccine in accordance with Waksman's postulate which states that "rather than examining parasite antigens recognised by infected animals and patients as potential vaccine immunogens, we should focus an those molecules against which little or no response was directed" (Sher, 1989).

All of the vaccine candidates discussed so far have been identified on the basis of their reactivity with antibodies. However, as mice given a single immunisation with radiation attenuated cercariae develop protective immunity associated with Th1-type responses it would be logical to identify antigens based on their ability to stimulate Th1 reactivity. This has proved to be a difficult task, and to date the only antigen thus identified is calpain (Jankovic *et al.*, 1996). A protective Th1 cell clone was derived from mice that had been vaccinated with freeze-thawed adult worms using BCG as an adjuvant. An adult worm cDNA expression library was screened with the clone and its target identified. The antigen was found to be the large subunit of calpain, surface expressed and immunogenic in natural infections (Andresen *et al.*, 1991). The recombinant protein elicited 29-39% protection and DNA vaccination using gene gun vectors expressing the protein gave up to 60% protection (Hota-Mitchell *et al.*, 1999). More recently, a screening system to identify antigens based on their T cell reactivity has been developed (Eberl *et al.*, 1999). Initial results have led to the identification of a fragment of β-myosin heavy chain. This fragment does not overlap with IrV-5, but is adjacent to it in the DNA sequence of myosin heavy chain.

All of the vaccine candidates discussed above are intracellular except for elastase and Sm23. These intracellular antigens are unlikely to come into contact with the host immune system unless the parasite is first damaged by a non-immune process. As the proposed immune effector mechanisms for the elimination of schistosomes require the secretion/excretion of antigens from the intact parasite it is essential to continue the search for such antigens. The dearth of secreted antigens is highlighted by the fact that by October 1996 only 9 open reading frames (ORFs), of the 300 deposited on the schistosome databases, had been annotated as having a characteristic secretory signal peptide at the N-terminus.

As mentioned earlier, Harrop *et al.* (1999) have attempted to identify proteins released from developing schistosomula by antibody screening of cDNA libraries but have had limited success. The serum raised against the released proteins identified predominantly genes encoding somatic antigens from the cDNA libraries screened, for example, paramyosin, fructose 1-6-bisphosphate aldolase and myosin. This may be due to the presence, in the sera, of high titre and high affinity antibodies to these immunogenic molecules. It is difficult to envisage that these proteins (given their functions) are

released from the parasite as part of its normal biological processes. These proteins are probably derived from dead/damaged larvae and therefore do not represent the true secretions of the parasite. In addition, random sequencing of the cDNA libraries used in these studies has revealed that the genes encoding calcium binding protein and aldolase are highly represented (Franco *et al.*, 1997; Santos *et al.*, 1999). Therefore, both random sequencing and antibody screening of cDNA libraries are biased towards the isolation of abundant, immunogenic, somatic constituents and are ineffective approaches for the identification of putative vaccine candidates.

1.8 Aims of this study

From the preceding introduction it should be evident that T cells play a major role in the protective immune response seen in mice vaccinated once with optimally irradiated cercariae. One can infer from this that the immune response must be triggered by antigen(s) expressed on the surface of, or released from the live schistosomula to enable processing by APCs and subsequent presentation to T cells. It should also be clear that the antigens involved are most likely to be released from the developing schistosomula as these correspond to the challenge larvae eliminated in the lungs of vaccinated mice. As conventional approaches to identify these proteins have been inefficient the aim of this study was to devise strategies that would isolate the genes coding for the membrane and/or secreted (M/S) proteins of *S. mansoni.* The work presented here describes three distinct approaches that have been developed.

Chapter 3 describes a computational biology approach. The large number of expressed sequence tags (ESTs) in the schistosome genome database represents a significant resource which was exploited to identify M/S proteins. As there was a relatively small number of ESTs derived from the lung worm approximately 100 ESTs were initially generated from a lung worm cDNA library. Following this, an algorithm was used to screen the entire database for cDNAs encoding M/S proteins. Those ESTs potentially encoding M/S proteins and derived from the larval stages of development were analysed further. A number of clones were transferred into a high level expression system. Antibodies were raised against a purified recombinant protein and immunocytochemistry used to localise the antigen to the parasite.

In the second approach, the technique of RNA arbitrarily primed PCR (RAP-PCR) has been used to identify genes expressed either uniquely of preferentially by lung-stage

schistosomula. Chapter 4 describes the technique leading to the identification, cloning and sequencing of several cDNAs. Analysis of these cDNAs by Southern and Northern blotting was carried out.

Finally, Chapter 5 describes a screening system which identifies cDNAs encoding M/S proteins based on a single-step genetic selection in a mutant strain of yeast. To establish the system, several controls to test that the system could recognise and process schistosome cDNAs were carried out.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Preparation of parasite material

A Puerto Rican isolate of *S. mansoni* was maintained by passage through MF1 mice and albino *Biomphalaria glabrata* water snails.

Cercariae: Infected, patent snails were exposed to bright light inducing them to shed cercariae. The parasites were concentrated by sedimentation on ice followed by brief centrifugation. The pellet of parasites was then washed several times in sterile phosphate buffered saline (PBS; 150mM NaCl, 8mM Na_2HPO_4, 2.6mM KCl, 1.5mM KH_2PO_4) to remove any traces of snail material.

Lung Schistosomula: Eight day schistosomula were obtained by *in vitro* culture essentially as described by Harrop and Wilson (1993b). Briefly, a concentrated cercarial suspension was taken up in 2-3mls RPMI medium (Gibco BRL, Life Technologies, Paisley, UK) and the parasites mechanically transformed by vortexing for 90 seconds. The resulting heads and tails were incubated at 37°C for 2-3 hours before the heads were isolated by centrifugation on a discontinuous percoll gradient (70-40%). The heads were washed six times in 10ml RPMI medium to remove any remaining tails and then resuspended in medium 169 (M169; Basch, 1981) for eight days at 37°C, 5% CO_2. Those cultures containing more than 85% viable larvae were then pooled, the parasites pelleted and washed several times in sterile PBS to remove any traces of M169.

Adult Worms: MF1 mice were infected with approximately 200 cercariae. Adult worms were obtained by perfusion of the hepatic portal system of six week-infected mice, with RPMI medium containing heparin. Any contaminating debris and mouse tissue was removed by hand and by washing the worms several times in sterile PBS.

Eggs: The livers and small intestines of mice that had been infected with 200 cercariae six weeks previously were removed, homogenised in a small volume of buffer (0.06M Na_2HPO_4, 0.003M KH_2PO_4) and digested with trypsin for 3 hours at 37°C. the trypsinised tissues were passed through a 300μm mesh, followed by a 180μm mesh to remove any undigested tissue debris. The filtrate was allowed to settle and the liquid then gently poured off leaving behind the eggs. These were then washed in 0.9% saline, collected into a small crystallising dish, and pelleted by gentle agitation of the dish. Any visible debris was removed using a Pasteur pipette and the eggs washed very briefly in sterile PBS prior to storage.

All the parasite material was pelleted by brief centrifugation and stored at -70°C until required.

Soluble Parasite Antigen Preparations: The soluble proteins of cercariae (SCAP), lung worms (SLAP) and adult worms (SWAP) were obtained by thawing the parasites to room temperature and sonicating for 2 minutes on ice (21kHz at 6.5μm amplitude). The sonicates were then centrifuged at 100 000g for 1 hour at 4°C. The resulting supernatant was aliquoted and stored at -20°C.

0-3 hour Released Proteins (0-3h RP): A concentrated cercarial suspension was mechanically transformed as described above and the resulting heads and tails incubated in 30ml RPMI for 3 hours. After this time the culture supernatant was centrifuged at 1000 r.p.m. to remove any remaining parasite bodies and then concentrated using an Ultrafree-15 centrifugal filter column (Millipore, Watford, UK).

2.2 Microbiological techniques

2.2.1 Growth, maintenance, selection and preservation of *E. coli*

E. coli strains were grown in LB liquid medium (1% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract, 1% w/v NaCl per litre; pH 7.0) at 37°C with agitation (200 r.p.m.) for preparative procedures. Isolation of single colonies was achieved by streaking cells onto LB-agar plates (as for LB medium with the addition of 1.5% w/v agar) followed by incubation at 37°C.

Antibiotic selection of cells was achieved by supplementing the growth medium with the appropriate antibiotic (Carbenicillin, 50μg ml^{-1}; Chloramphenicol, 34μg ml^{-1}; Kanamycin, 25μg ml^{-1}; Tetracycline, 12μg ml^{-1}). For long term preservation, glycerol stocks (15% v/v) were prepared from overnight cultures and stored at -70°C.

2.2.2 Transformation of *E. coli* by heat shock

E. coli cells were prepared for transformation by inoculation of fresh LB medium with 0.01 volume of an overnight culture and grown to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6. The cells were pelleted by centrifugation at 3000g for 5 minutes at 4°C and

the resulting pellet resuspended in 0.5 volume ice-cold 100mM $CaCl_2$. The cells were incubated on ice for 30 minutes followed by centrifugation at 1000g for 5 minutes. The pellet was resuspended evenly in 0.05 volume ice-cold 100mM $CaCl_2$ and subsequently the cells were aliquoted and used directly. For long term storage the cells were diluted with an equivalent volume of 20% (v/v) glycerol for storage at -80°C.

The DNA to be transformed into the cells was added to the competent cells and the mixture incubated at 4°C for 30 minutes. The DNA/cell mixture was then placed in a 42°C water bath for exactly 45 seconds, followed immediately by a two minute incubation on ice. The cells were incubated at 37°C with gentle shaking in 1ml of SOC medium (0.5% w/v yeast extract, 2% w/v tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose) for 1 hour before plating onto LB agar plates containing the appropriate antibiotics.

2.2.3 Transformation of *E. coli* by electroporation

E. coli cells were prepared for transformation by inoculation of fresh LB medium with 0.01 volume of an overnight culture and grown to an OD_{600} of 0.5 to 0.6. The cultures were chilled on ice for 15 minutes prior to harvesting by centrifugation at 5000g at 4°C for 20 minutes. The pellets were washed twice in ice-cold sterile distilled water (sdH_2O). After the final wash the cells were resuspended evenly in a volume of ice-cold sdH_2O equivalent to the estimated packed cell volume. Aliquots of the cells were used directly or diluted with an equivalent volume of 20% (v/v) glycerol and stored at -70°C. Electroporation of competent cells was carried out using the Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The DNA was added to the cells and the mixture incubated on ice for 20 minutes before pulsing. A field strength of 25kV cm^{-1} was applied to 40µl of cells in a 0.1 cm gap electroporation cell with the instrument resistance set at 200Ω and a capacitance setting of 25 µF. After the pulse, cells were incubated at 37°C with shaking in 1ml of SOC medium for 1 hour before plating onto LB agar plates containing the appropriate antibiotics.

2.3 Preparation, manipulation and analysis of nucleic acids

2.3.1 Standard nucleic acid purification

Organic extraction and alcohol precipitation

Nucleic acids were deproteinised by single or multiple rounds of extraction with buffered phenol: chloroform: iso-amyl alcohol (25:24:1) as described in Current Protocols in Molecular Biology (1997).

Precipitation with ethanol or isopropanol in the presence of monovalent cations was used routinely to recover nucleic acids from aqueous solution. In all cases 0.3M sodium acetate (pH 5.2) was the salt of choice, and for very dilute solutions commercially available glycogen was used as a carrier. Precipitated nucleic acids were washed in 70% ethanol to remove residual salts and/or phenol. Pellets were dried under a mild vacuum and dissolved in sdH_2O or TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0).

Non-organic extraction

The QIAquick PCR purification kit (Qiagen, Crawley, UK) was used, according to the manufacturer's instructions, to purify up to 10μg of single or double stranded DNA between 50 base pairs (bp) and 10 kilo base pairs (kb).

2.3.2 Isolation of DNA

Isolation of Genomic DNA (gDNA)

S. mansoni gDNA was extracted from cercariae, and mouse gDNA from mouse spleens. Approximately 5ml minced tissue/pelleted parasites were resuspended in 10ml extraction buffer (10mM Tris-Cl pH 8.8, 0.1M EDTA, 20μl ml^{-1} RNase, 0.5% sodium dodecyl sulphate; SDS) and incubated at 37°C for 1 hour with shaking. Proteinase K was added to a final concentration of 100μg ml^{-1} and the suspension incubated at 50°C for 3 hours. Following this, several extractions with phenol: chloroform: iso-amyl alcohol (25:24:1) were carried out. The DNA was then ethanol precipitated and resuspended in TE buffer.

Isolation of plasmid DNA from E. coli

Plasmid mini-preparations (5ml overnight cultures) were made using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions.

Large scale, high purity plasmid preparations were made using the QIAfilter plasmid kit (Qiagen) following the manufacturer's guidelines.

In all cases the plasmid DNA was dissolved in an appropriate volume of TE buffer.

2.3.3 Isolation of RNA

When working with RNA all glass and plasticware used was sterile and RNase free and all solutions and reagents were treated with diethylpyrocarbonate (DEPC) to inhibit any RNase activity.

All samples from which RNA was to be extracted were frozen in liquid nitrogen as soon as they were obtained, and stored at -70°C. The samples were thawed on ice immediately prior to extraction and homogenised by passing through various hypodermic needles of increasing gauge several times.

Total RNA was extracted from parasites and mouse tissues using TRIzol reagent (Gibco BRL) as per the manufacturer's instructions. Total RNA was stored in 20μl aliquots at -70°C.

The Micro-Fast Track mRNA isolation kit (Invitrogen, Groningen, The Netherlands) was used, according to the manufacturers instructions, to extract mRNA from parasites and mouse tissues. As with total RNA the mRNA was stored as 10μl aliquots at -70°C. All RNA samples were treated with DNase directly after extraction.

2.3.4 Analysis of nucleic acids by gel electrophoresis

Agarose gel electrophoresis of DNA

DNA was size-fractionated on agarose to determine the quality and success of its isolation and manipulation, or for further analysis by Southern blotting. Molecular

biology grade agarose was dissolved in Tris-Acetate electrophoresis buffer (TAE; 0.04M Tris acetate, 0.001M EDTA, pH 8.1). The concentration of the gel used was determined by the size of the DNA fragments of interest, and varied between 0.5 and 1.5%. Samples were diluted with 0.2 volume loading buffer (20% Ficoll 400 X, 0.1M EDTA pH 8.0, 1.0% SDS, 0.25% bromophenol blue) prior to loading into preformed wells. Gels were run at 5V cm^{-1} in TAE. The DNA was then stained by soaking the gels in TAE containing ethidium bromide at 0.5μg ml^{-1} and subsequently visualised by exposure to UV transillumination. Images were recorded digitally using an AlphaImager (Alpha Innotech Corporation, California, USA). The size of the DNA was estimated by comparison with commercially available marker DNA of known size.

Agarose-formaldehyde gel electrophoresis of RNA

Total RNA was size fractionated on 1% agarose-formaldehyde gels as described in Current Protocols in Molecular Biology (1997) to determine the quality and success of its isolation.

2.3.5 Recovery of DNA from agarose gels

The QIAquick gel extraction kit was used routinely, according to the manufacturers instructions, for fragments between 100 to 5000bp.
For larger fragments the QIAEX II Gel extraction kit (Qiagen) was used according to the manufacturer's instructions.

2.3.6 Quantification of nucleic acids

Nucleic acid concentrations were calculated from absorbance readings at 260nm (A_{260}) determined by UV spectroscopy using a Beckman DU 650 spectrophotometer (Beckman, High Wycombe, UK). In all cases the A_{260}/A_{280} ratio was also calculated to ensure that the preparation was clean (values between 1.7 and 2.0 were acceptable for DNA and >1.8 for RNA).

Alternatively, DNA concentrations were estimated for samples separated on agarose gels by comparison to known concentrations of standard DNAs.

2.3.7 Enzymatic manipulation of nucleic acids

Restriction enzyme digests

Digests of DNA were carried out in the appropriate minimum volume, for 2 hours at the defined temperature, with an excess of enzyme at 2 units per μg of DNA. All of the restriction endonucleases were supplied by New England Biolabs (NEB, Hitchin, UK), and the recommended buffers used as directed. When two enzymes were used in combination, a buffer was selected in which both enzymes would have high activity. When a compatible buffer could not be found for double digests, buffer exchange was achieved by ethanol precipitation of DNA. Reactions were terminated by the addition of Na_2EDTA to a final concentration of 10mM, heat inactivation for thermolabile enzymes, or by the addition of DNA loading dye if the samples were to be analysed by electrophoresis.

Ligation of DNA fragments

Ligation reactions were carried out in the minimum volume possible. Inserts (0.1-1.5kb) were added at 2 to 5-fold molar excess over the vector and T4 DNA ligase (Promega, Southampton, UK) was present at a final concentration of 5-20 units μl^{-1} in the supplied buffer. The ligation reactions were incubated at 16°C for up to 15 hours.

To increase ligation efficiencies, the 5' termini of linearised vectors were dephosphorylated using calf intestinal alkaline phosphatase (Promega). The reaction was carried out as per the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

Taq polymerase (Promega), a thermostable polymerase, was used routinely to amplify specific DNA sequences by repeated rounds of primer extension. Matched primer pairs were designed with the aid of the Williamstone Enterprises Primer Design package (www.williamstone.com/primers), and synthesised by Genosys Biotechnologies Ltd (Pampisford, UK). Reactions (10, 20 or 50μl volume, overlaid with mineral oil) were carried out in the supplied buffer observing the manufacturer's guidelines for use of the enzyme. Generally, dNTPs (Promega) were at a final concentration of 200μM and the primers at 0.5-1pM each depending upon the application. Thermal cycling conditions

were determined with the following parameters in mind: in each cycle 1 minute at 95°C was allowed for template denaturation; primer annealing was 45 seconds at 5°C below the lowest calculated melting temperature of the primers; 1 minute at 72°C for polymerisation was allowed for each kilobase pair of the anticipated product. The number of cycles was varied between 20 and 35 depending on the nature of the target sequence and all reactions were terminated by a 5 minute incubation at 72°C. A Perkin Elmer 480 Thermal cycler (Perkin Elmer, Warrington, UK) was used throughout this study. In general the PCR products were resolved on agarose gels and the enzyme and excess nucleotides and primers removed using the QIAquick PCR purification kit (Qiagen) before any further molecular modifications/analyses were carried out.

Reverse transcription (RT) of RNA

Total RNA was denatured by heating to 65°C for 10 minutes before being added to a reverse transcription reaction containing 100μg ml^{-1} of oligo $(dT)_{15}$ (Promega), 100μM dNTPs, 10mM dithiothreitol (DTT) and 100 units Moloney murine leukaemia virus reverse transcriptase (M-MLV; Promega) in the supplied buffer to give a final volume of 20μl. This was incubated at 37°C for 45 minutes and the resulting single strand DNA stored at -20°C.

Double stranded cDNA was prepared by adding an aliquot of the single-stranded DNA to a 10μl PCR mixture containing 1pM of each of two sequence specific primers and amplifying through a number of cycles.

2.3.9 DNA Sequencing

DNA was sequenced by the dideoxynucleotide termination method (Sanger *et al.*, 1977) using the Dye Terminator sequenase sequencing system (Perkin Elmer). Templates were prepared by either PCR or mini-preparation of plasmids from *E. coli*. The manufacturer's guidelines were observed and the resulting reactions were run on a 373A automated sequencer (Perkin Elmer).

The quality of each sequencing reaction was checked by analysis of the chromatogram from the automated sequencer. The sequences were then manually edited to remove vector and/or primer regions and subsequently compared to all DNA and protein sequences at the National Centre for Biotechnology Information (NCBI) using the Basic

Local Alignment Search Tool for nucleotides (BLASTN) and peptides (BLASTX) comparisons.

2.4 Screening for recombinant bacterial colonies

PCR, using gene or vector specific primers, was used as a rapid screening method for correct insert prior to growing colonies for plasmid isolation. Colonies were picked using sterile 10μl pipette tips, concomitantly re-streaked onto LB agar plates and inoculated into 20μl sdH_2O. The tubes were vortexed to disperse the bacteria, heated to 95°C for 5 minutes and chilled rapidly on ice. An aliquot of this suspension was used for PCR amplification. All putative recombinants were further characterised by isolation of plasmid DNA and DNA sequencing.

2.5 Analysis of proteins

2.5.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein preparations were analysed by size fractionation under reducing conditions within polyacrylamide gels based on the method of Laemmli (1970). The samples were diluted with 0.25 volume of 4x sample buffer (250mM Tris-Cl pH 6.8, 20% β-Mercaptoethanol, 40% glycerol, 8% SDS, 0.1 % methylene blue) and boiled for 5 minutes before being loaded into pre-formed wells of the stacking gel (4.5% acrylamide, 0.12% bis-acrylamide in 0.125M Tris-Cl pH 6.8 containing 0.1% SDS) which overlaid the 15-18% separating gel (15-18% acrylamide, 0.4%-0.48% bis-acrylamide in 0.375M Tris-Cl pH 8.8 containing 0.1% SDS). Gels were run in electrode buffer (25mM Tris, 192mM glycine, 0.1% SDS) at 100V constant voltage. The proteins were visualised by staining in Coomasie Blue [0.1% (w/v) Coomasie Brilliant Blue, 40% (v/v) methanol, 10% (v/v) glacial acetic acid] for 1 hour and then destaining in 40% methanol, 7.5% glacial acetic acid. Molecular weight standards (NEB) were treated in the same manner and included on all gels to allow protein molecular weight to be estimated.

2.5.2 Western Blotting

Aliquots of protein preparations were separated by SDS-PAGE and transferred to Immobilon membranes (Millipore). Transfers were generally for 1 hour at 100V constant voltage, using the Bio-Rad Mini Trans-blot cell in transfer buffer (25mM Tris, 192mM glycine, 12% methanol) at 4°C. The non-specific sites on the membranes were blocked with 5% non-fat dried milk (Marvel) in PBS containing 0.05% Tween-20 (v/v) for 2 hours at room temperature or overnight at 4°C prior to incubation with the primary serum, diluted as required in 0.01% dried milk in PBS-T for 2 hours at room temperature. Membranes were then washed in a large excess of PBS-T for 30 minutes with several changes of buffer before incubation, for 2 hours at room temperature, in an appropriate peroxidase labelled antibody, diluted as required in 0.01% dried milk in PBS-T. The membranes were washed as before and developed by adding the peroxidase substrate, 3,3', 5,5'-tetramethylbenzidine (TMB; Kirkegaard & Perry Laboratories, Dynex Technologies, Billingshurst, UK). The reaction was stopped by washing the membrane in a large excess of H_2O.

2.5.3 Quantification of proteins

All protein concentration measurements were determined by the Bio-Rad protein assay which is based on the Bradford assay (Bradford, 1976).

CHAPTER 3

THE USE OF COMPUTATIONAL BIOLOGY AND THE *S. MANSONI* GENOME DATABASE TO IDENTIFY M/S PROTEINS

3.1 INTRODUCTION

3.1.1 The *S. mansoni* genome and the generation of ESTs

The DNA of mammalian schistosomes is contained within eight pairs of chromosomes of which one pair is heterologous in females (Short, 1983). Characterisation of schistosome DNA by Simpson *et al.* (1982) showed that the haploid genome is approximately $2.7x10^8$ bp in size with a base composition of 64% AT. This makes the schistosome genome approximately ten times larger than that of protozoan parasites (*Plasmodium falciparum* ~30Mb; *Trypanosoma brucei* ~53Mb) and a tenth of the size of the human genome (~3000 Mb). C_0T analysis predicts that about 40% of the genome is composed of repetitive sequences. Only 30% of the genome is protein coding and it is estimated to contain 20 000 expressed genes (Franco *et al.*, 1995). By October 1998, largely as a result of conventional cDNA library screening, 427 ORFs had been deposited in the *S. mansoni* database. These ORFs represent approximately 1% of the total number of genes (taking into account redundant ORFs). Therefore, the remaining 99% of the genetic information must be unravelled before the parasite biology and host-parasite relationships can be understood and drug and vaccine development advanced effectively. Thus, genome analysis is an important aspect of schistosome research (Johnston *et al.*, 1999).

The generation of ESTs by sequencing of cDNA libraries has been described as a fast and efficient approach to the discovery of new coding regions within genomes (Adams *et al.*, 1991) and the method is effective for *S. mansoni* (Franco *et al.*, 1995). From the initiation, by WHO, of the Schistosome Genome Project to 1998 approximately 7000 cDNA sequences were deposited in the databases (Genbank, October 1998). Cluster analysis of this data set showed that approximately 18-25% of the predicted genes have been tagged in this manner (Williams and Johnston, 1999). However, a large proportion of these ESTs has no significant homology to genes encoding proteins of known structure/function (Rollinson and Johnston, 1999). Therefore, there now exists a large database of sequences to be analysed if the full potential of genome sequencing is to be realised.

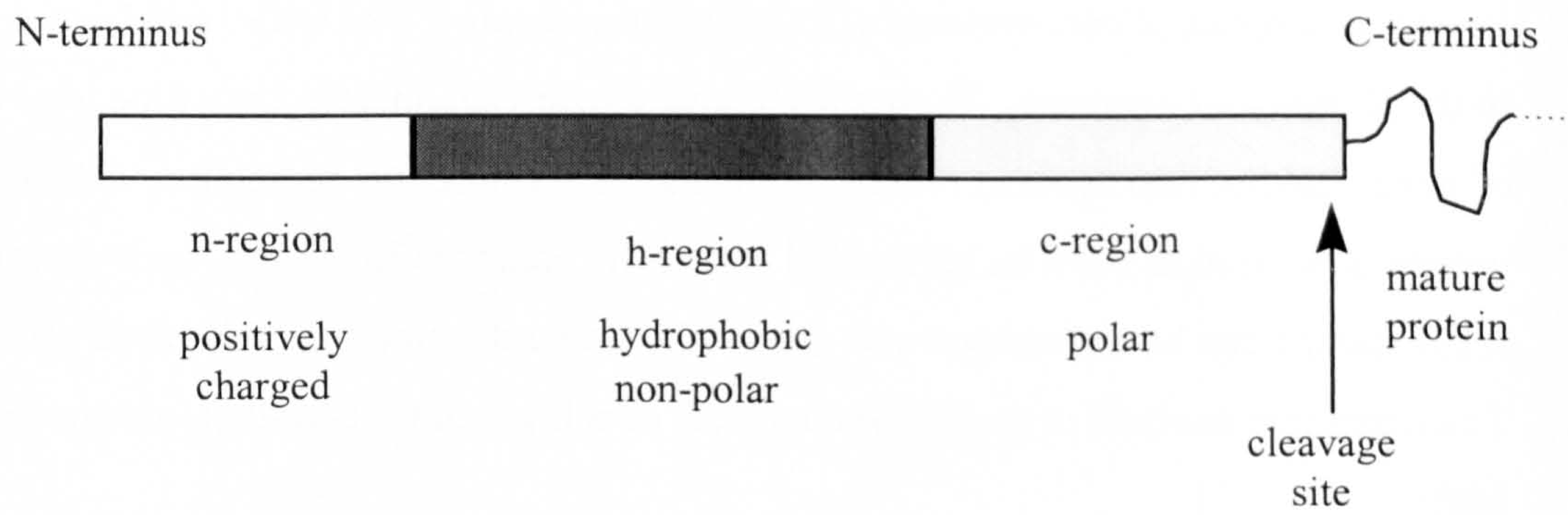


Figure 3.1: Schematic diagram of a signal peptide.

3.1.2 Membrane/secreted (M/S) proteins and signal peptides

As discussed in Chapter 1, those proteins that are released (i.e. secreted) from the larval stages of development as part of normal biological processes or those exposed on the parasite surface (designated earlier as M/S proteins), are the most likely targets of the protective immune response elicited by the radiation-attenuated vaccine. Entry of proteins into the secretory pathway in eukaryotic cells usually requires the translocation of the protein across the membrane of the endoplasmic reticulum (ER). In all eukaryotes there are two pathways by which most M/S proteins are transported across the ER, the co- and post-translational pathways. Both translocation modes require that polypeptides destined for the secretory pathway be specifically targeted to the ER. This is mediated by a N-terminal signal sequence on the nascent polypeptide and can be dependent on the signal recognition particle (SRP) or be SRP-independent (Rapoport *et al.*, 1996). Transport to the cell surface then proceeds via the 'ER to Golgi intermediate compartment' followed by the typically stacked Golgi apparatus and *trans*-Golgi network. Therefore, the vast majority of M/S proteins, which are very diverse in structure and function, have a N-terminal signal peptide (Blobel and Dobberstein, 1975). This short peptide sequence is the only factor common to M/S proteins.

Signal peptides lack primary sequence homology (Watson, 1984) which renders more traditional molecular methods of searching for M/S proteins ineffectual. However, they share a common physical structure, shown in Figure 3.1, divided into a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c region which also contains the cleavage site for a signal peptidase (von Heijne, 1985).

Analysis of signal sequence mutants showed that the h-region, comprising six to fifteen amino acids, is the most essential part of the leader peptide, required for targeting and membrane insertion (von Heijne, 1985). The polar c-region often contains helix-breaking proline and glycine residues as well as small uncharged residues in positions -3 and -1 (relative to the first amino acid of the mature protein) that determine the site of signal peptide cleavage (von Heijne, 1990). Comparative analysis of a large number of signal peptides shows that there is substantial variability in their length, ranging from 15 to more than 50 amino acids; the n-region contributes most to the overall variation in length (von Heijne, 1986).

3.1.3 Identification of signal peptides

Over the past few years the complete genome sequence has been determined for a number of organisms e.g. *Haemophilus influenzae* (Flieschmann *et al.*, 1995), *Mycobacterium tuberculosis* (Cole *et al.*, 1998) and *S. cerevisiae* (Mewes *et al.*, 1997). This means that a large number of sequences, which encode putative proteins with unknown functions, are being entered onto databases. Computational biology using various algorithms and statistical tools is the most efficient method to interpret all these sequence data.

Several protein sorting prediction methods have been developed recently. Some workers have attempted to predict subcellular localisation of proteins on the basis of amino acid composition (Cedano *et al.*, 1997; Reinhardt and Hubbard, 1998). This is based on the study by Nakashima and Nishikawa (1994) who showed that it is possible to discriminate between intra- and extracellular proteins using amino acid composition and residue-pair frequencies. Others have developed methods based on prediction of sorting signals (McGeoch, 1985; Von Heijne, 1983). This is possible because the majority of proteins destined for locations other than the cellular cytoplasm have a characteristic signal peptide. The secretory signal peptide is the most intensively studied of all such signal sequences.

The SignalP algorithm (Nielsen *et al.*, 1997) has been designed for the identification of secretory signal peptides. The algorithm relies on the shared physical characteristics of signal peptides to identify putative signal peptides using a combined neural network approach. One network is trained to recognise the cleavage site at the C-terminal end of the signal peptide and the other network is trained to classify each amino acid as either belonging or not belonging to a signal peptide. Output from the algorithm consists of one score from each neural network and one combined score. There are three versions of the algorithm, each trained on different data sets (eukaryotes, Gram-negative and Gram-positive bacteria). These three versions account for the differences in the characteristics of signal peptides from these groups of organisms. To date, the SignalP algorithm is the most accurate prediction method for signal peptides publicly available.

The aim of this chapter was to use the SignalP algorithm to putatively identify M/S proteins from the sequences deposited on the schistosome genome database. Initially, sequences of proteins of known localisation and/or function would be run through the

algorithm to test its ability to discriminate between M/S and non-M/S schistosome proteins. Following a successful outcome, the schistosome EST database would then be analysed to identify novel M/S proteins. In order to do this, candidate cDNA clones would be transferred to high level expression vectors and recombinant protein produced. This would then be used to raise antiserum, which would allow for immunocytochemical localisation of the protein with the parasite, which in turn would verify the data output from the SignalP algorithm. As there were only 166 lung-stage ESTs on the database when this work was initiated (October 1996), it was necessary to generate ESTs from a lung-worm cDNA library. This life-cycle stage was of particular interest as it is the target of protective immunity in the radiation attenuated-vaccine model.

3.2 MATERIALS AND METHODS

3.2.1 Generation of ESTs

A lung-stage schistosomular cDNA library (provided by Dr Harrop), constructed in the λZAP II vector (Stratagene, Amsterdam, The Netherlands), was diluted in SM buffer (100mM NaCl, 50mM Tris-Cl pH 7.5, 5mM $MgSO_4$) to give a titre of approximately 1x 10^5 plaque forming units per ml (pfu ml^{-1}). An overnight culture of *E. coli* XL-1 Blue cells (Stratagene) was pelleted and resuspended in 10mM $MgSO_4$. A 100 µl aliquot of cells was mixed with 1µl of diluted cDNA library and incubated at 37°C for 30 minutes. To this was added 1.4ml top agar (LB medium with 0.7% agar) and after brief mixing, the agar was poured evenly onto pre-warmed agar plates containing tetracycline (12µg ml^{-1}). The plates were incubated at 37°C until phage plaques were approximately 2mm in diameter. Well separated plaques were picked at random using a sterile toothpick and each one was placed in 500µl SM buffer for 2 hours at room temperature or overnight at 4°C.

The phage insert was amplified by PCR using 1µl of the phage suspension as the template and primers to the flanking regions of the vector, M13R and T7 (see Appendix 1 for primer sequences). The products were analysed on 1% agarose gels and sequenced from the 5' end.

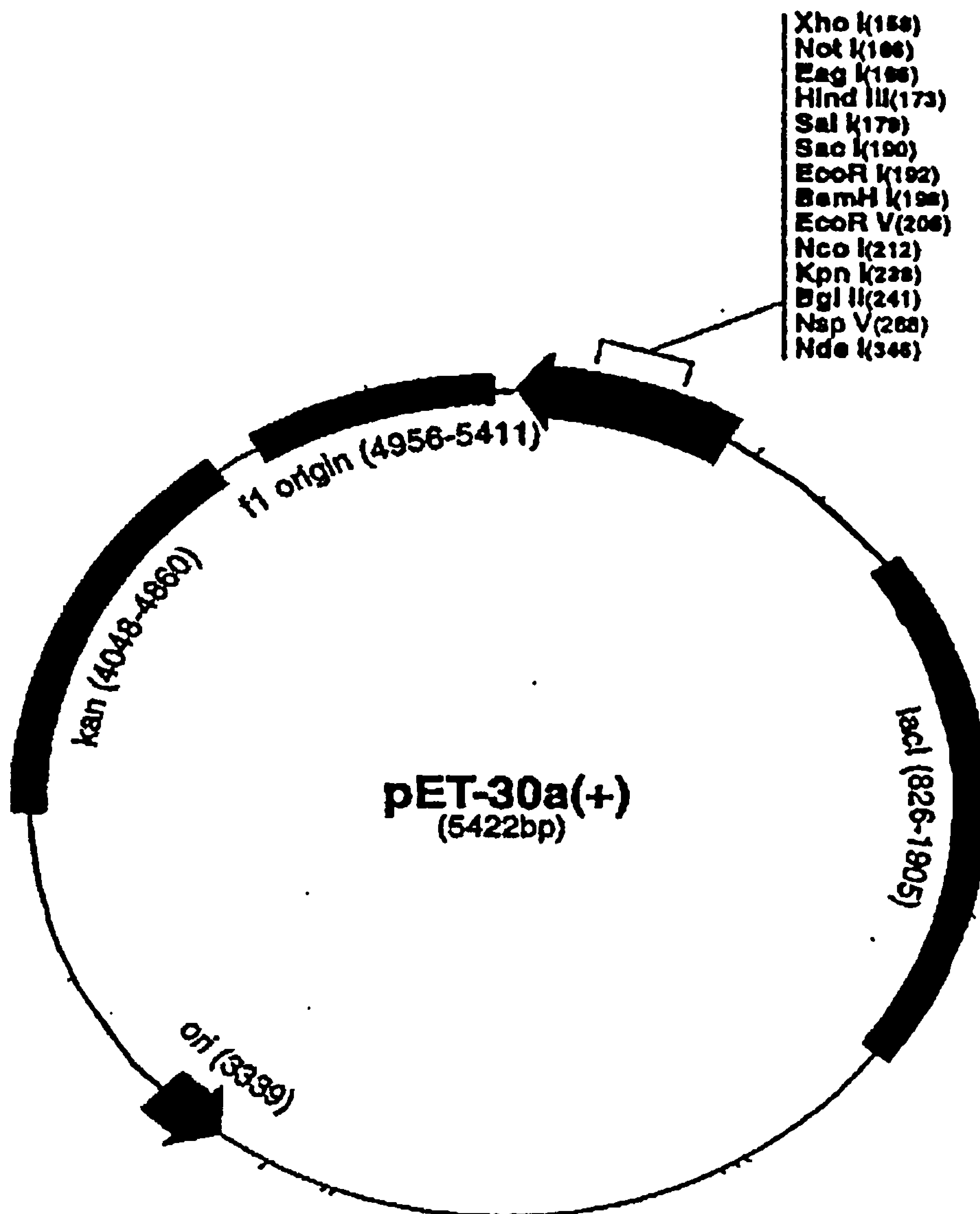


Figure 3.2: Prokaryotic Expression Vector pET-30a

The prokaryotic expression vector pET30a has the bacteriophage T7 promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG), followed immediately by the *lac* operator. The operator sequence binds the *lac* repressor which is provided on the same plasmid. Foreign ORFs can be inserted in frame downstream of the promoter into the multiple cloning site. The vector contains both a N-terminal and a C-terminal 'His-Tag' which is a poly-histisdine tag that facilitates protein purification by immobilised metal affinity chromatography (IMAC). The presence of a C-terminal tag is important for the purification of proteins whose N-terminal sequences may be processed during expression.

3.2.2 Searching the *S. mansoni* database for putative signal peptides using the SignalP algorithm

In all cases the prediction method was applied using networks trained on the eukaryotic data set.

All the ORFs in the *S. mansoni* database were run through SignalP and the data analysed. Only those sequences that gave positive results for all three scores were classified as having a typical cleavable signal peptide.

All the ESTs in the database were first translated in all six reading frames, the longest ORF selected using specifically written computer programs (P. Ashton), and the first 70 amino acids of each selected ORF run through the algorithm. The sequences without an initiator methionine were removed by screening all SignalP-positive sequences manually.

3.2.3 Sub-cloning of *S. mansoni* ORFs into the prokaryotic expression vector, pET30.

The expression vector, pET30 (Novagen, Beeston, UK) and the T7 expression system are described in Figure 3.2.

Oligonucleotide primers were designed to amplify the sequences containing the required *S. mansoni* ORFs. The primers incorporated restriction enzyme sites to facilitate molecular manipulations and were designed to ensure that appropriate translation of the ORF would be maintained when ligated into the vector (see Appendix 1 for primer sequences).

Inserts were generated by PCR amplification using primers at 0.5pM, dNTPs at 200μM and the plasmid template at 0.5ng μl^{-1}. The enzyme and excess dNTPs were removed after successful amplification and the inserts digested with the appropriate restriction enzymes followed by ligation into the appropriate vector that had been linearised with the corresponding restriction enzymes. Competent HB101 *E. coli* cells (Stratagene) were transformed with the ligation products and transformants were selected on LB agar plates containing kanamycin (25μg ml^{-1}).

3.2.4 Expression and purification of recombinant proteins

The host *E. coli* strain BL21(DE3)(pLysS) was used for recombinant protein production as it contains the bacteriophage T7 RNA polymerase gene as a λ lysogen under the control of the *lac* operon which is inducible by IPTG (Studier and Moffat, 1986). In addition, as an *E. coli* B strain, BL21 lacks the lon protease and the ompT outer membrane protease that can degrade proteins during purification. Therefore, target proteins should be more stable in BL21 than in K12 strains of *E. coli*. The presence of the plasmid, pLysS, provides a small amount of T7 lysozyme which inhibits T7 RNA polymerase and so increases the tolerance of BL21(DE3) for expression plasmids with toxic inserts.

Growth, induction and optimisation of protein expression

Plasmid pET30 and its derivatives containing genes encoding *S. mansoni* ORFs were used to transform BL21(DE3)(pLysS) cells. Single colonies were grown in liquid culture and glycerol stocks prepared for storage at -70°C. Bacterial cultures for protein expression were inoculated with 0.01 volume of an overnight culture and grown at 37°C to an OD_{600} of 0.5 prior to induction by the addition of IPTG to a final concentration of 1mM. For evaluation of optimum expression, protein production was monitored as a function of time at various different temperatures (25, 30, 37°C). Total protein expression was analysed at each time point by resuspending 50µl of cells in SDS-PAGE sample buffer for electrophoresis. In addition, a crude purification of the recombinant protein was carried out at each time point. Briefly, 5ml of culture was centrifuged, the pelleted cells resuspended in 200µl GHC (6M guanidine hydrochloride, 100mM Na_2HPO_4, pH 8.0), vortexed and incubated for 10 minutes at room temperature. The suspension was centrifuged for 5 minutes in a microfuge and 25µl TALON metal affinity resin (Clontech) was added to the supernatant. TALON is an IMAC resin that uses cobalt ions for purifying recombinant polyhistidine tagged proteins. After a 30 minute incubation with gentle agitation at room temperature, the resin was washed once with 8M urea (8m urea, 100mM Na_2HPO_4, 50mM NaCl, pH 8.0). Subsequently 25µl SDS-PAGE sample buffer was added to the resin and the sample prepared for electrophoresis.

A duplicate gel was transferred to Immobilon membrane (Millipore) by Western blotting and probed using a Ni-NTA alkaline phosphatase conjugate (Qiagen). As this

was an alkaline phophatase conjugate it was necessary to use Tris-buffered saline (TBS-T; 20mM Tris-Cl, pH 7.5, 500mM NaCl, 0.05% Tween-20) and the 5-bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium (BCIP/NBT) substrate solution (Sigma-Aldrich, Poole, UK).

Preparative fermentation of E. coli

Bacterial cultures were scaled up to 500ml in 2.5L baffled conical flasks for those clones expressing recombinant *S. mansoni* proteins. Bacterial fermentation and the induction of protein expression was carried out under optimal conditions as determined previously. Cells were harvested by centrifugation (5000g at 4°C for 20 minutes) and the supernatant removed completely. The cells were resuspended in 50ml GHC, vortexed and incubated at room temperature for 30 minutes with agitation. The suspension, containing lysed cells was centrifuged at 5000g for 10 minutes at 4°C and 1.25ml TALON matrix added to the supernatant. This was incubated at room temperature for 1.5 hours with gentle agitation. The matrix was allowed to settle, washed 3 times in an excess of 8M urea, pH 8.0, followed by 2 washes with 8M urea pH 7.5. The recombinant protein was then eluted by washing the matrix with 2ml 8M urea containing 50mM Na_2EDTA pH 7.5. The elution step was repeated four times. Protein samples were analysed by SDS-PAGE electrophoresis.

3.2.5 Production of antisera

Antiserum to recombinant protein was raised in IFNγ$R^{-/-}$ mice. Prior to the first immunisation, a tail-bleed was taken to serve as a control. For the primary immunisation, 10μg of the recombinant protein was mixed, by sonication, with an equal volume of Titremax gold adjuvant (Sigma) and injected subcutaneously at two sites on the back of the mouse. Subsequent immunisations were given at four week intervals and consisted of antigen alone. In all cases subsequent injections were given at the same place as the primary immunisation. Small tail-bleeds were taken before every injection to monitor changes in antibody titres. Each blood sample was allowed to clot for 1 hour at room temperature, ringed with a mounted needle, left to stand at 4°C for several hours, centrifuged at 10 000g for 10 minutes, the serum pipetted off, and stored at -20°C. When sufficient antibody titres had been reached, a final bleed was obtained by cardiac puncture. The serum was prepared as for the test bleeds and stored in aliquots at -20°C.

3.2.6 Enzyme-linked immunosorbant assay (ELISA)

The antibody titres of sera to their respective recombinant proteins or to various antigenic fractions of *S. mansoni* were determined by ELISA. In order to identify the appropriate antigen and antibody dilutions to be used, a matrix was set up in which sera were diluted down the plate and the antigen across the plate. The antigen concentration that gave the most clearly defined dilution curve was used to coat microtitre plates in all subsequent assays. The serum dilution which gave an optical density reading that fell on the linear part of the dilution curve was used. Microtitre immuno-plates (Nunc, Life Technologies, Paisley, UK) were coated with an appropriate concentration of antigen in carbonate coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$) by incubation at 4°C overnight in a humid chamber. Subsequently, the plates were washed 3 times with PBS-T, the serum to be analysed (diluted in PBS-T) was added to each well and the plates were incubated at room temperature for 2 hours. Plates were washed as before and the appropriate peroxidase-labelled secondary antibody, diluted in PBS-T, added to each well. After a 2 hour incubation at room temperature the plates were washed as before and the enzyme substrate, TMB (Kirkegaard & Perry Laboratories) added to each well. When sufficient colour had developed spectrophotometric readings were taken at 690nm in a Dynex DIAS Microplate Reader.

3.2.7 Immunocytochemistry on parasite sections

Parasites were prepared for immunocytochemistry based on the method developed by Riengrojpitak *et al.* (1989). Briefly, the cercariae or lung-schistosomula were pelleted in Eppendorf tubes by low speed centrifugation. Ten pairs of adult worms were hand-counted and placed in Eppendorf tubes. The parasites were mixed into optimum cutting compound (OCT; Tissue-Tek, London, UK) such that each parasite was coated in it. The mixture was placed on a cork disc and plunged into thawing isopentane. Once frozen, the discs were stored at -70°C. The samples were sectioned (7μm thickness) onto Superfrost slides at -20°C using a cryostat (Slee, London, UK), and air dried overnight. The sections were fixed for 5 minutes in cold, absolute acetone and washed for 5 minutes in PBS three times before treatment. Sections were blocked by incubating with undiluted normal goat serum for 1 hour. The antibody was then applied at three different

concentrations (1:10; 1:20 and 1:40) diluted in PBS with 10% normal goat serum. 4B1 (culture supernatant from hybridoma MBL-Sm-4B1; Pearce *et al.*, 1986) and anti-adjuvant serum were used as positive and negative controls respectively. The slides were incubated at room temperature for 1 hour. Following two washes in PBS the secondary antibody, FITC-conjugated goat anti-mouse Ig (Sigma) diluted 1:30 in PBS, was applied and incubated in the dark for 1 hour. The slides were washed twice in PBS and mounted in Citifluor (Agar Scientific, Stanstead, UK). The sections were examined under a Nikon fluorescence microscope.

3.3 RESULTS

3.3.1 The generation of lung worm ESTs.

As only sequences greater than 150bp in length and with less than 5% ambiguities were considered useful for analysis, a total of 106 ESTs was generated. On the basis of homology searches with all sequences in current DNA and protein databases the composition of the lung-stage cDNA library was evaluated. The ESTs were classified into the six groups shown in Table 3.1.

E. coli, rRNA and mitochondrial sequences made up 19% of the sequences. From the remaining 86 sequences, 25 (29.0%) could be putatively identified, based on matches to *S. mansoni* entries, 56 (65.1%) matched non-schistosome entries on databases and 10 (11.6%) had no significant matches. Nine ESTs matched known *S. mansoni* genes with more than 85% identity (Table 3.2). These represent mainly genes encoding enzymes (glutathione peroxidase, phosphoglycerate kinase, cathepsin) and cytoskeletal/structural proteins (tropomyosin, fibrillin). Of the 16 ESTs with database matches to other *S. mansoni* ESTs (Table 3.3), 13 matched ESTs derived from adult worm cDNA libraries. Table 3.4 lists the 19 ESTs which have database matches to other organism entries. Further analysis of the remaining ESTs showed that there was no redundancy in this dataset implying that there is little redundancy in the library.

EST Category	No. of Clones	(%)
Putatively Identified		
(a) S. m. match	25	23
(b) Non-S.m. match	19	18
Not Identified		
(a) Non-S.m. partial match	32	30
(b) No database match	10	10
E. coli	17	16
rRNA and mitochondrial	3	3
Total	106	100

Table 3.1: EST categories of the *S. mansoni* lung-schistosomulum library.
Average size of the ESTs: 847bp Average sequence length: 373 bases
All useful ESTs have been deposited on public databases (Accession numbers: AA568097-100; AA629470-516; AW466234-265; AA499464-466).

EST (Accession number)	Identification (Accession number)	Length[a] (nt)	Identity [b] (%)
2004 (AW466254)	Glutathione peroxidase (L14328)	366	89
2011 (AW499465)	phosphoglycerate kinase (SCMPGK)	470	89.6
2030 (AW466265)	Cathepsin L (SMCATHL)	26	100
019 (AW499464)	Elongation initiation factor 5A (SME1F5A)	103	100
054 (AW466236)	Cyclophillin (SCMCYC)	201	86.5
063 (AW466237)	Fibrillin 2 (SMU54588)	238	98
071 (AW466239)	LGG (SMLGGR)	277	99
075(AW466240)	Tropomyosin (SMTROPO)	204	98
077 (AW466241)	LGG (SMU10431)	312	95

Table 3.2: Database match of ESTs to known *S. mansoni* genes.
a: refers to the match lengths (nucleotides)
b: indicates percentages of identical nucleotides.

EST (Accession number)	Identification (Accession number)	Length [a] nt	Identity [b] (%)
2001 (AW466252)	Adult worm (T14349)	326	100
2009 (AW466257)	Adult worm, similar to ribosomal protein S4 (T14380)	204	99
2010 (AW466258)	Adult worm (N33710)	206	88
001 (AA56897)	Adult worm (N28069)	228	98
025 (AW466234)	Adult worm (AA218451)	258	96
038 (AW466235)	Adult worm (AA140599)	272	89
070 (AW466238)	Adult worm, similar to myosin light chain (T14468)	324	99
090 (AW466243)	Adult worm (H85669)	266	89
103 (AW466244)	Adult worm (AA218457)	185	94
113 (AW466246)	Adult worm, similar to cysteine-rich intestinal protein (AA218481)	148	100
121 (AW466248)	Adult worm, similar to glutamine synthase (T14539)	165	87
125 (AW466249)	Adult worm (N21946)	144	85
145 (AW466251)	Adult worm (N25397)	239	85
2019 (AW466262)	Cercaria (AA143844)	246	92
088 (AW466242)	Lung stage, similar to 40S ribosomal protein (AA125707)	257	100
109 (AW466245)	Lung stage, similar to RNA polymerase subunit II (AA125704)	273	95

Table 3.3: Database match of ESTs to *S. mansoni* ESTs
a: refers to match lengths (nucleotides)
b: indicates the percentages of identical nucleotides

EST (Accession number)	Identification (Accession number)	Length [a] aa	Length [a] nt	Similarity [b] (%)	Identity [c] (%)
2012 (AW466259)	Calothrix D253 gDNA (CSPAG13)		223	-	75
2021 (AW466264)	Human MrNA for ribosomal protein L32 (HSRPL32)		232	-	62
014 (AA568099)	Human ATP synthetase Beta (HSATPB)		344	-	75
037 (AA629484)	*D. discoideum* phosphatitidylinositol (DDU23478)		113	-	65
046 (AA629486)	Barstead mouse pooled organs (cDNA sequence) (AA266199)	171		62	62
056 (AA629491)	XENLA 26S protease regulatory subunit (P46470)	113		91	86
059 (AA629492)	human 60S ribosomal protein S27 (metallopan-stimulin 1) (HAU86019)	56		83	71
061 (AA629494)	Human SET protein (Q01105)	82		83	67
072 (AA629496)	CAEEL hypothetical 9.8 KD protein (CELZK652)		100	-	85
089 (AA629503)	Human retinoblastoma-binding protein (HSU35143)	128		95	85
100 (AA629470)	Human phosphatase 2A inhibitor (HSU51924)		374	-	63
106 (AA629473)	*Soares* retina N2Bhr cDNA clone (AA020798)	194		61	61
110 (AA629506)	60S ribosomal protein (P39023)		377	-	87
116 (AW466247)	Human ribosomal protein S25 mRNA (HUMPRS25)		279	-	78
123 (AA629510)	*D. discoideum* protein kinase (DDIPRKNGPK)		307	-	67
131 (AA629512)	Human DNA sequence from cosmid 203C2 (HS203C2)		200	-	60
132 (AA629512)	Human ankyrin, brain variant 2 (Q01485)		185	-	87
133 (AA629514)	Human Xq28 genomic DNA (HSU52112)		86		63
135 (AA629515)	D. discoideum DNA (DDCPRO2U)		297	-	73

Table 3.4: Database match of ESTs to non-*S. mansoni* genes.

a: match lengths are given in nucleotides (nt) for DNA and amino acid residues (aa) for peptide alignments.

b: similarity indicates percentages of identical aa plus conservative substitutions.

c: identity indicates the percentage of identical nt for DNA or identical aa for peptides.

3.3.2 Identification of putative signal peptides using SignalP

The 427 ORF sequences in the *S. mansoni* genome database were run through the SignalP algorithm and only 79 sequences (18.5%) fulfilled the criteria set for the identification of signal peptides. However, once all the redundant sequences had been removed, of the 173 distinct proteins remaining, only 27 possessed signal peptides. Of these sequences, 23 have been identified by various researchers on the basis of sequence homology with known proteins (Table 3.5). There are 8 secreted, 6 plasma-membrane, 3 lysosomal and 6 endoplasmic reticulum/Golgi associated proteins, all of which can be expected to have a leader sequence. The four remaining signal peptides identified by the algorithm are associated with proteins of no known homology. They are:

CL1 (gi: 263832): cercarial library
ORF-RF2 (gi: 951431): A female-specific cDNA, adult library
Unknown (gi: 1002620): Trans-spliced mRNA, adult library
Female specific protein 800 (gi: J03999): adult library

As the algorithm was able to distinguish *S. mansoni* signal peptides from non-signal peptides the entire EST data set, comprising 7102 sequences (October, 1998), was subjected to SignalP analysis. A total of 446 ESTs (6.3%) had putative signal peptides but this number was reduced to 218 (3.0%) when those without an appropriate initiator methionine were removed. Table 3.6 shows the distribution of the putative signal peptides according to different life cycle stages, bearing in mind that sequences occurring in more than one life cycle stage have not been removed from the data set. Thus, there are 62 signal peptides from the infective cercarial stage, only 1 from the sporocyst stage (where the proteins used in host invasion are synthesised), and 5 from the migrating lung schistosomulum, the target for the protective immune responses in mice vaccinated with irradiated cercariae. As a consequence of this exercise we now have 68 larval ESTs potentially coding for M/S proteins versus only 2 ORF M/S sequences, cercarial elastase and CL1.

Identification	No. of clones	Accession No. of most complete clone	Function
Hemoglobinase (S.m. 32)	3	M21308	secreted enzyme
Elastase	7	U31768	secreted enzyme
Superoxide Dismutase	4	M27529	secreted enzyme
Antigen 10-3	2	M22346	oesophageal gland secretion
Eggshell protein (chorion)	10	M21607	vitelline cell secretion
Eggshell protein	2	J03982	vitelline cell secretion
p48	2	M74170	vitelline cell secretion
LGG	1	U10431	secreted protein
Epidermal growth factor receptor	8	M86399	membrane receptor
Glucose transport protein 2	2	L25067	membrane transporter
Glucose transport protein 4	2	L25066	membrane transporter
S.m. 13	2	AF072886	membrane protein
gp 18-22	2	M62811	membrane glycoprotein
S.m. 23	5	M34453	membrane protein
Cathepsin C	3	Z32531	lysosomal enzyme
Cathepsin B	2	M21309	lysosomal enzyme
Cathepsin L	1	U07345	lysosomal enzyme
Calreticulin	3	L24159	ER luminal protein
Disulphide isomerase	2	Z22933	ER luminal protein
Cyclophillin B	2	U30874	ER luminal protein
S.m. IrV1	1	L08641	ER luminal protein
ER luminal cysteine proteinase ER 60	1	Z22934	ER luminal protein
Fucosyltransferase	1	AF016899	Golgi protein

Table 3.5: Summary of M/S proteins identified by SignalP analysis

Life Cycle Stage	No. of ESTs with signal peptides (% of total)	No. of ESTs in the database	% of ESTs with signal peptides
Cercaria	62 (31.3%)	2632	2.6
Lung schistosomulum	5 (2.5%)	254	2.0
Adult worm	127 (64.1%)	3855	3.2
Egg	3 (1.5%)	268	1.1
Sporocyst	1 (0.5%)	32	3.1
Miracidium	0 (0.0%)	61	0
Total	198[a] (100%)	-	-

Table 3.6: Summary of signal peptides identified by SignalP analysis of ESTs

a: 20 clones were removed from this analysis as they were nucleotide sequences corresponding to peptide sequences listed in Table 3.5.

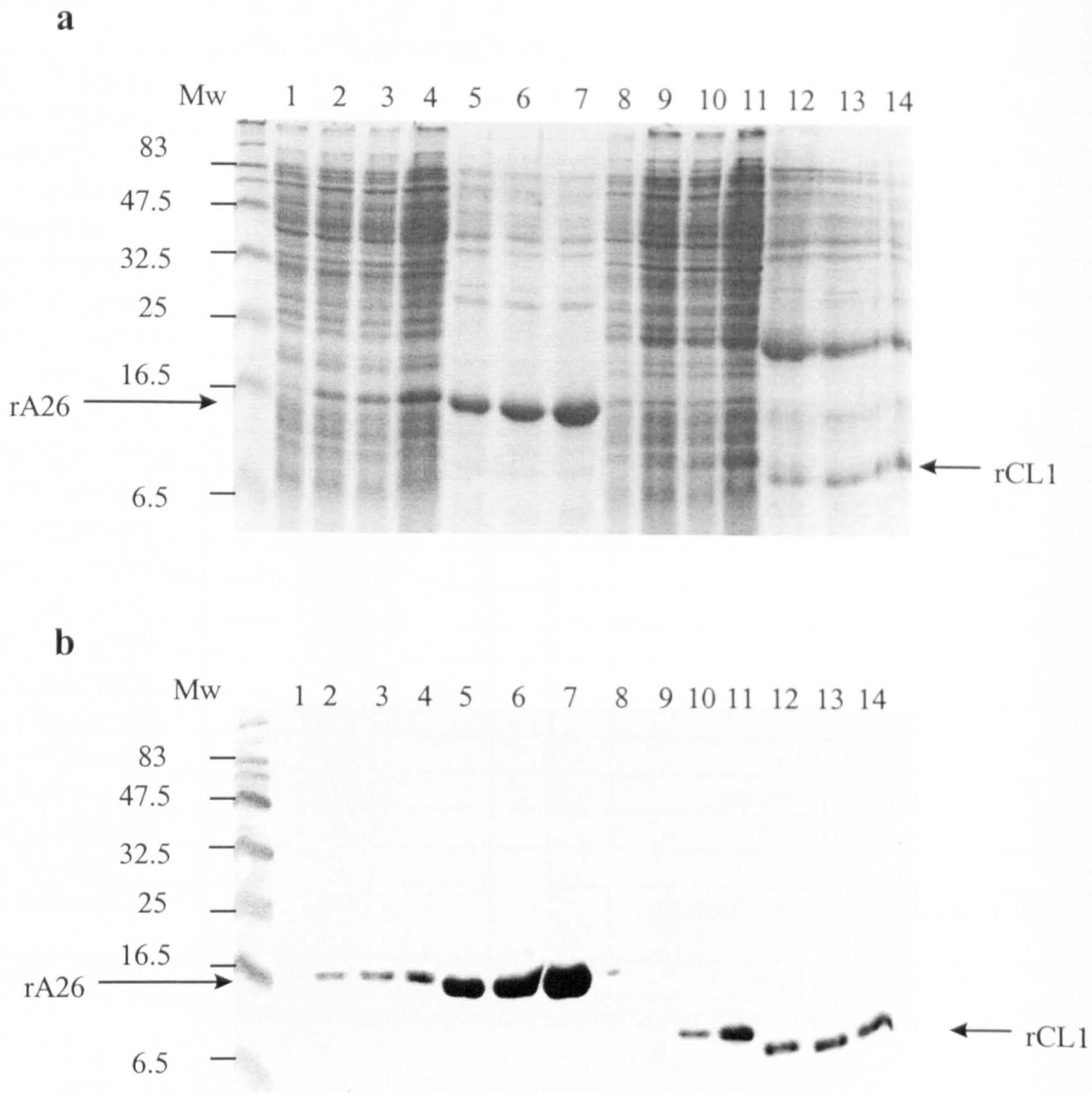


Figure 3.3: High Level Expression of CL1; Coomasie stained SDS-PAGE gel (a) and Western Blot probed with Ni-NTA alkaline phosphatase conjugate (b).

Lanes 1-7: Expression of A26 (positive control for expression)
Lanes 8-14: Expression of CL1

Lanes 1 and 8: uninduced crude lysates
Lanes 2 and 9: crude lysates 1hr after induction of expression
Lanes 3 and 10: crude lysates 2hrs after induction of expression
Lanes 4 and 11: crude lysates 3hrs after induction of expression
Lanes 5 and 12: crude purification of recombinant protein 1hr after induction
Lanes 6 and 13: crude purification of recombinant protein 2hrs after induction
Lanes 7 and 14: crude purification of recombinant protein 3hrs after induction

3.3.3 High level expression and analysis of recombinant proteins

As M/S proteins from schistosomula are the most likely candidates of protective immunity, three of the lung schistosomula ESTs with putative signal peptides (001, 046, 123) and the one unidentified larval ORF (CL1) were chosen for further investigation. Each clone was sequenced in its entirety and the sequences analysed. None of the clones showed significant homology to any DNA or protein sequences in Genbank/SwissProt that could give any clues to the function of the proteins they each encode. The cluster analysis data (Williams and Johnston, 1999) was used to check if any of the chosen sequences formed part of any contigs. Clones 001 and CL1 were not orphan sequences; CL1 was at the 5' end of contig 0885 whereas clone 001 appeared to be in the middle of contig 0383 and was thus discarded.

Clones CL1, 046 and 123 were transferred to the prokaryotic high level expression vector, pET30, recombinant protein expression induced and expression levels monitored over 3 hours. High level expression was obtained only for CL1 as determined by Coomassie blue staining of SDS-PAGE gels and by probing of Western blots with the Ni-NTA alkaline phosphatase conjugate (Figure 3.3). The expression levels of CL1 are much lower than that of the positive control A26 (a lung-stage antigen; Harrop *et al.*, 1999). Recombinant CL1 (rCL1) cannot be seen clearly in the crude lysates until 3 hours after induction (Figure 3.3A, lane 11) and it is not detectable 1 hour after induction even by Western blotting (Figure 3.3B, lane 9). Separation of the bacteria into the membrane component, the periplasmic fraction and the cytoplasmic fraction revealed that some of the rCL1 was being directed into the periplasm (data not shown).

The recombinant protein was purified from 2 litres of culture medium using TALON affinity matrix resin (Clontech). Serum was raised against the purified recombinant CL1 protein (rCL1) in IFNγR$^{-/-}$ mice and the antibody titer was analysed by ELISA (Figure 3.4). All sera raised against mixtures of parasite proteins were produced by Dr. R Harrop. The serum raised against the recombinant protein recognised rCL1 strongly, whereas serum raised against the adjuvant alone showed only background reactivity. As a control for these ELISAs, serum raised against a different *S. mansoni* lung-worm antigen, 118 (B. Raybould, unpublished) was used. The serum raised against antigen 118 showed weak recognition of rCL1 (3x more than anti-adjuvant serum). Both the 12 week chronic infection serum and the anti-SLAP serum recognised rCL1 weakly. In contrast, there was little or no recognition of the recombinant protein by sera either raised against

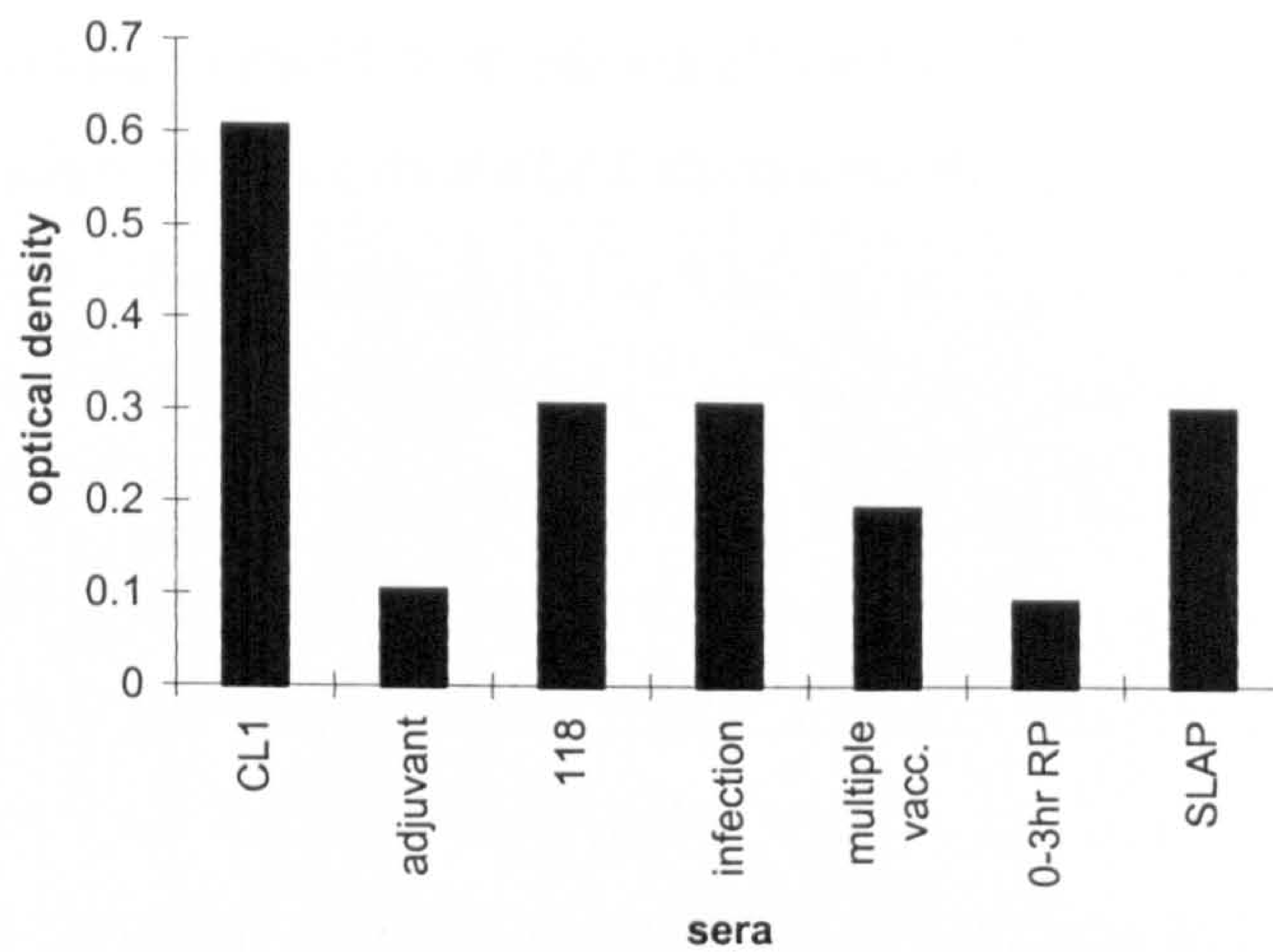


Figure 3.4: Antibody titers of various antisera vs rCL1 determined by ELISA

Microtitre plates were coated with 50μl of rCL1 at 0.4μg ml^{-1} and probed with various sera diluted 1:400. The secondary antibody used was goat anti-mouse IgG horseradish peroxidase conjugate diluted 1:1000.

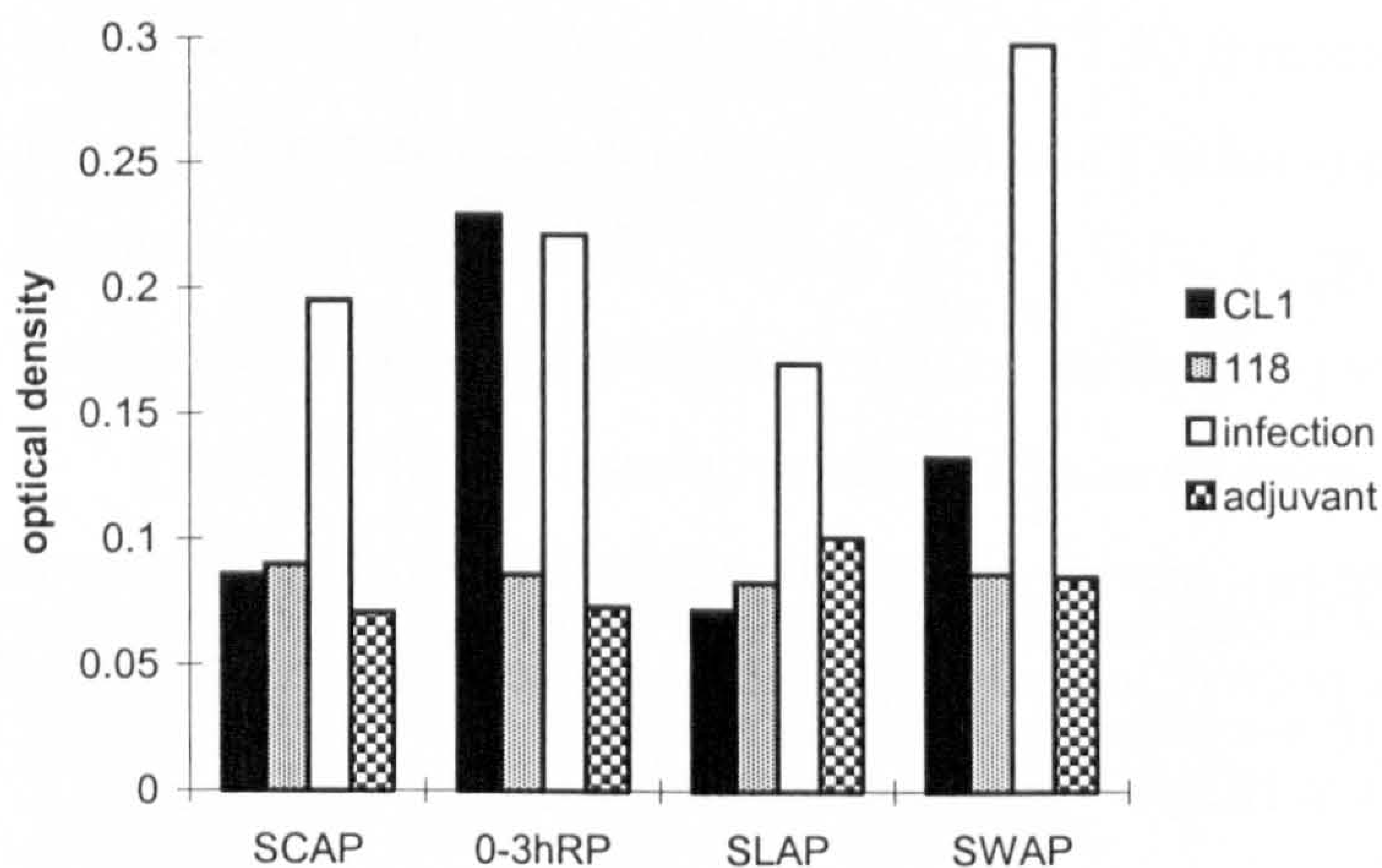


Figure 3.5: Recognition of parasite preparations by various antisera as determined by ELISA.

The microtitre plate wells were coated with 50μl of SCAP, 0-3h RP, SLAP or SWAP at 5μg ml^{-1}. Each antigen preparation was probed with the four antisera (CL1, 118, infection, adjuvant) at a 1:400 dilution.

0-3h RP or from mice vaccinated 5x with irradiated cercariae (5x vaccination serum provided by Dr P Coulson).

The anti-rCL1 serum was used to probe ELISA plates coated with SCAP, SLAP, SWAP or 0-3h RP (Figure 3.5). The serum showed strong recognition of the 0-3h RP but did not recognise any component of SCAP, SLAP or SWAP. Similar data were obtained when the serum was used to probe Western blots of SCAP, SLAP, SWAP and 0-3h RP (Figure 3.6). The serum bound to the recombinant protein (approximately 8kDa) and what seems to be a dimer of the recombinant as well, but only to a single band in the 0-3h RP at approximately 12kDa. The intensity of the recognised band varied with different preparations of 0-3h RP (data not shown). The serum did not bind to any proteins in the three soluble parasite preparations, even at high protein concentrations (up to 60μg per lane). Each of these experiments has been done in triplicate. The anti rCL1 serum was used to probe cryostat-cut sections of cercariae, lung- and adult-worms but there was no recognition of the protein in any of the parasite stages (data not shown).

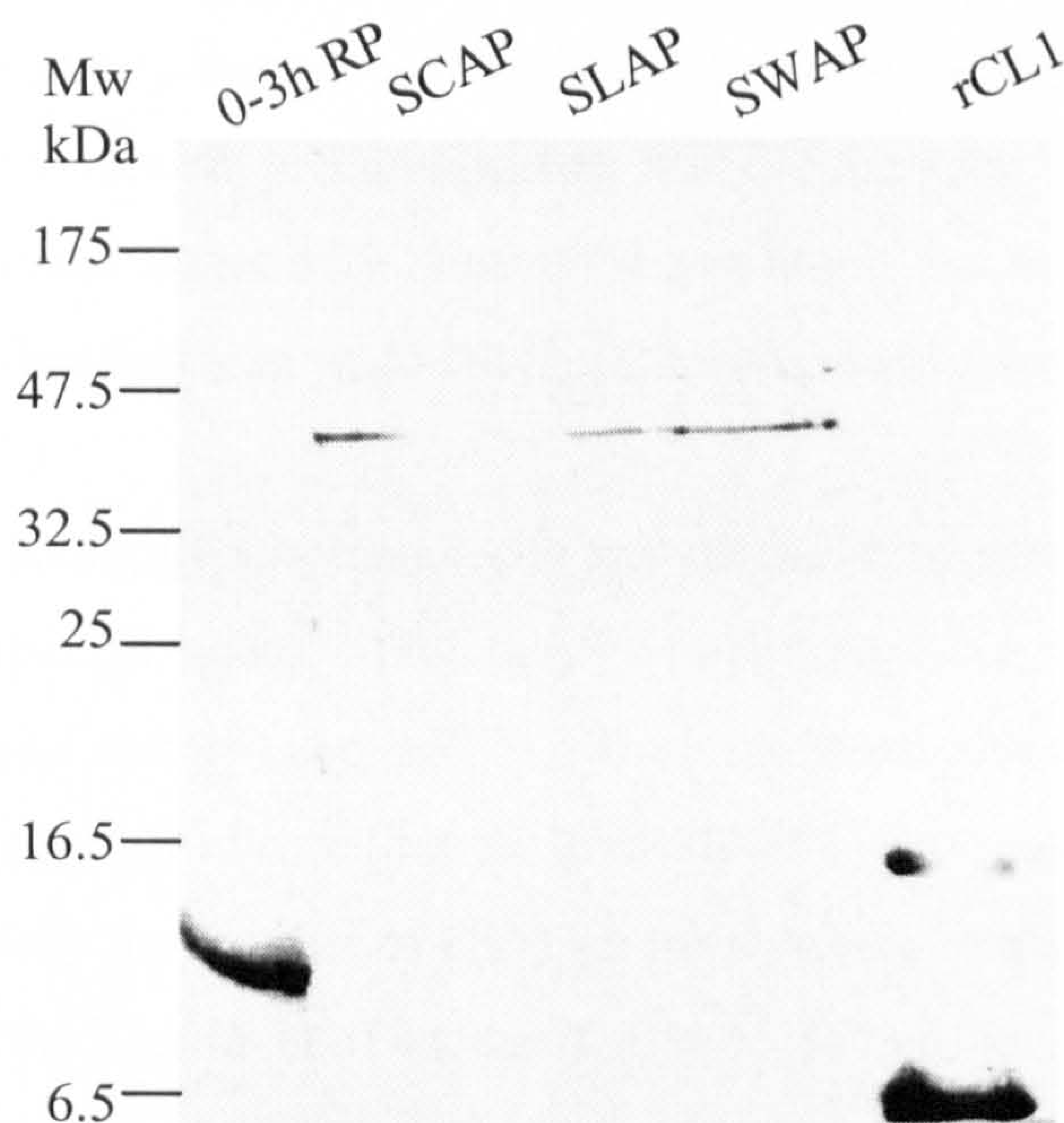


Figure 3.6: Western blot of the target recombinant alongside various parasite preparations. 0-3h RP (lane 1), SCAP (lane 2), SLAP (lane 3) and SWAP (lane 4) rCL1 (lane 5) probed with serum against rCL1 (1:400). Samples of 10μg soluble parasite protein preparations and 1μg recombinant protein were loaded per lane.

3.4 DISCUSSION

3.4.1 The generation of ESTs

Adams *et al.* (1995) state that a useful cDNA library should contain no more than 20% useless sequences, at least 50% new genes and a broad variety of transcripts. The sequencing of 100-200 ESTs is sufficient to assess the quality of the library based on these criteria. Overall, with respect to these parameters, the lung worm cDNA library is of good quality as there is no redundancy within the ESTs and the sequences have homology to genes encoding proteins of diverse function (enzymes from metabolic pathways, DNA-binding proteins, structural and regulatory proteins). The only drawback is the large number of *E. coli* sequences in the library, probably introduced by contamination during its construction. These sequences could be removed by preabsorbing the library with *E. coli* cDNA thereby increasing the efficiency of gene discovery by EST generation.

Many of the ESTs have significant homology to previously characterised *S. mansoni* genes (23%). The majority of these genes (21/25; 84%) have been identified from different developmental stages of the parasite. Therefore, they are likely to represent genes which are necessary throughout the life-cycle, such as structural and/or housekeeping genes. Indeed, in a preliminary study of the gene expression profile in different developmental stages of *S. mansoni* it was found that several genes, including some of unknown function, can be found in all developmental stages of the parasite (Franco *et al.*, 1997).

The ESTs with no significant matches to any entries in current databases represent either *S. mansoni* specific genes, which may be important in the development of the parasite, or regions of untranslated mRNA. The generation of ESTs from this lung-stage cDNA library has not only allowed for the analysis of its composition but has also increased significantly the number of ESTs on the database from this developmental stage. Apart from the high level of contaminating *E. coli* sequences the library consists of inserts with short poly (A) tails, no chimeric clones and a high proportion of distinct genes. This analysis reflects results obtained by Franco *et al.* (1997) in an independent study of the same cDNA library.

SignalP analysis

The 23 well characterised proteins identified by SignalP as containing putative signal peptides are indeed all proteins that are targeted to the secretory pathway, as determined by their function (Table 3.5). It should be noted that of the six W.H.O. selected vaccine candidates only Sm23 features in this list, and of the other vaccine candidates discussed in Chapter 1 only elastase and gp18-22 appear. As *S. mansoni* glucose transporter protein 4 localises to the tegument apical membrane and is involved in glucose uptake (Skelly and Shoemaker, 1996) this may represent a putative therapeutic target in maturing schistosomula.

SignalP analysis of the *S. mansoni* protein database suggests that M/S coding sequences represent 16% of the genes expressed by schistosomes. This is comparable to the estimated frequency of secreted proteins in *S. cerevisiae* (15%; Mewes *et al.*, 1997) and *Helicobacter pylori* (23%; Tomb *et al.*, 1997). This result is also similar to that obtained when the prediction method was applied to the putative coding regions of the completely sequenced *Haemophilus influenzae* genome (Nielsen *et al.*, 1997). Here 11% of the sequences contained 'typical' signal sequences. The authors note that the parameters set for 'typical' signal sequences are likely, on the one hand, to include signal-anchor like sequences of type II (single-spanning) or type IV (multispanning) membrane proteins. On the other hand, those signal sequences that are too long are likely to be missed out. Therefore, Nielsen *et al.* (1997) state that the data output from the algorithm should be analysed several times, adjusting the defined parameters each time to account for such variations and after such analyses of the *H. influenzae* genome they estimate 15-20% M/S proteins.

The frequency of positive hits of ESTs (3.0%) was much lower than that of ORFs (16%). One explanation may be that reverse transcription of mRNA with an oligo dT primer begins at the 3' end and frequently truncates before reaching the 5' end which codes for the signal peptide and is therefore likely to be under-represented (in contrast ORFs tend to be deposited on databases only after sequencing of longer length clones). In addition, a proportion of the ESTs was sequenced from the 3' end only. Thus the sequence data obtained from these is unlikely to extend to the 5' end of each clone due to limitations of current sequencing technologies. Therefore, the lower percentage of intact 5' ends in the EST data set may account for the reduced frequency of hits in comparison with the ORF data set. The percentage of hits is approximately the same for each life-

cycle stage (2-3%) irrespective of absolute numbers of ESTs analysed. Unfortunately, the distribution of ESTs reflects the effort made in obtaining sequences from the various life-cycle stages of the parasite, resulting in very few signal peptides derived from larval ESTs.

Both Schneider *et al.* (1999) and Nielsen *et al.* (1999), using different prediction methods, found very few sequences encoding signal peptides in the genome of *Methanonccocus jannaschii.* In both cases, the authors state that the number found is unexpectedly small and suggest that this may have arisen because the secreted protein precursors from the archaebacterium might contain signal peptide features that were not taken into account in the training sets. Therefore, to get an accurate estimation of the number of secreted proteins, the algorithm would have to be trained using a set of signal peptides that had been experimentally determined specifically from the organism of interest. Therefore, other schistosome signal sequences in the dataset may have been missed altogether due to the set of sequences used to train the predictive algorithm.

Those ESTs that had been predicted to contain signal peptides but that lacked an appropriate initiator methionine may correspond to an entirely untranslated region of RNA or to an internal stretch of a protein. In the latter case, this can lead to false positive predictions as non-cytoplasmic ends of transmembrane helices are often rather similar to signal peptide cleavage sites. In the former, wrong start codon assignments can produce false negatives, since the resulting sequence may contain only a partial signal peptide or a signal peptide plus a stretch of an irrelevant amino acid sequence (derived from RNA that is untranslated *in vivo*). The N-terminus of the preproteins is often difficult to assign and where protein coding regions are predicted by gene-finding algorithms there are often numerous errors. For example, in *Caenorhabditis elegans* automatic assignment methods alone predict less than 70% of the start codons correctly (Reinhardt and Hubbard, 1998). This is exacerbated by the fact that ESTs are obtained by single pass sequencing and errors in the DNA sequence which generate false stop codons (or conversely, remove true stop codons) are possible, leading to the selection of the wrong reading frame for analysis.

As discussed by Bhattacharya *et al.* (2000), many gene identification tools exist, but these would all require modification using information derived from individual parasite species before being applied to the sequence data generated by the genome projects of many of these organisms. Thus, the results of this first analysis of the schistosome EST database using the SignalP algorithm represent an initial screen. Further analyses using

other prediction tools have to applied to the sequences to take into account the possible occurrence of signal anchor sequences, transmembrane helices and incorrect reading frame selection. A further refinement may be to use the schistosome contigs that have been constructed. This would be more efficient as redundant sequences would be removed, and also more accurate as sequence errors would be reduced and ORF prediction would be more accurate.

3.4.3 High level expression of recombinant proteins

As none of the clones (CL1, 046, 123) showed any significant homology to any informative entries in Genbank or SwissProt to date, they are likely to represent novel schistosome genes. It is possible to infer that each clone codes for a M/S protein as all of them have a cleavable signal peptide sequence as determined by the SignalP algorithm.

Difficulty was experienced in getting expression of the three clones. Recombinant protein was purified only for CL1. The presence of a signal peptide can be problematic in some bacterial expression systems with the recombinant protein accumulating in the periplasm, often as an inclusion body (Stüber *et al.*, 1990). This may account for the low expression levels of rCL1 in comparison with rA26, which does not have a signal sequence (Harrop *et al.*, 1999), particularly as rCL1 was detected in the periplasmic fraction of the bacteria. Expression levels might be improved by removing the CL1 signal peptide. Alternatively, a eukaryotic high level expression system might process schistosome signal peptides more efficiently leading to higher productivity of recombinant proteins, e.g. a baculovirus expression system. Insect cells, as well as directing proteins to the appropriate subcellular compartments, utilise many of the post-translational modifications present in eukaryotic cells allowing production of protein that is antigenically, immunogenically and also functionally similar to the native protein (Miller, 1988). Eukaryotic expression systems also offer the advantage that any secreted proteins are exported into the culture medium making purification of the recombinant protein relatively simple.

There may be many reasons, other than the presence of a signal peptide, for the lack of recombinant protein production from clones 046 and 123, including toxic gene products, rapid breakdown of protein by cellular proteases, excessive rare codon usage, no translation due to a region of sequence that interferes (at the RNA level) with the interaction between *E. coli* ribosomes and the ribosome binding site. To circumvent

these problems it would be necessary to clone the cDNAs without their signal sequences and try using various bacterial host strains and expression vectors. Failing this, a eukaryotic expression system might be considered. If no recombinant protein can be produced an alternative would be to generate a synthetic peptide which was designed to include the most antigenic portion of the protein sequence of interest.

3.4.4 Analysis of the recombinant CL1 protein

The anti-CL1 serum contains antibodies specific to rCL1 as determined by ELISA and Western blotting. However, the control serum, anti-118, cross-reacts with rCL1 as determined by ELISA. This is possibly due to trace amounts of some *E. coli* proteins that co-purify with recombinant proteins during IMAC. This cross-reactivity is likely to be more prominent in cases where the recombinant protein expression levels were low. This necessitates the use of much larger culture volumes to produce sufficient quantities of recombinant protein and in turn leads to higher levels of contaminating proteins. In this case both rCL1 and r118 expression levels were relatively low, so accounting for the cross-reactivity in the sera. One solution to this problem would be to absorb the sera with *E. coli* proteins thereby removing the common antibodies.

A difference in molecular weight between the recombinant CL1 protein (~8kDa) and the native molecule (12kDa) was observed; this may be attributed to the glycosylation of the native molecule. The cDNA clone encoding CL1 appears to be complete, with a start codon surrounded by the appropriate sequence signatures and a poly A tail preceded by the appropriate stop codon. Although there is only one predicted N-glycosylation site (O-linked glycosyaltion sites are very difficult to predict) glycosylated proteins do not run to their true molecular weight on SDS-PAGE gels, often running slightly higher (Hames and Rickwood, 1990), thus providing an explanation for the discrepancy in size.

The data obtained using the anti-rCL1 serum indicates that CL1 is a component of the 0-3h RP. This suggests that it is a secreted protein that is released from the parasite during transformation. In addition, CL1 was originally identified by screening of a cercarial cDNA library with anti-d6-8 RP serum (Harrop *et al.*, 1999). This implies that CL1 is also a constituent of the d6-8 RP (the d6-8RP were not used in the analyses reported here due to the difficulty in obtaining sufficient quantities of material). This is not surprising as the majority of the proteins in the 0-3h RP are also seen in culture supernatants taken at daily intervals up to day 7 post-transformation (Harrop and Wilson,

1993b). CL1 is not detected in SCAP and SLAP as would be expected, because the majority of proteins in the released protein preparations are a subset of the proteins in the soluble parasite preparations. However, as there are many more proteins in these soluble preparations CL1 may be present but at a much lower relative concentration and therefore be undetectable.

The data obtained by probing rCL1 with various sera does not correlate with the above findings. Only chronic infection serum and that raised against SLAP recognised the recombinant antigen. The fact that the anti-0-3h RP serum showed no recognition of the recombinant protein was surprising. However, the variation in concentration of CL1 in different 0-3h RP preparations may provide an explanation. If the concentration of CL1 was low in the particular preparation used to raise the antiserum it is unlikely that sufficient antibodies to CL1 would have been produced. This varying concentration of CL1 in 0-3h RP may have arisen as there are several random variables in the protocol used to produce the protein preparation. For example, the time that the cercariae are left on ice, in order to concentrate the parasites after shedding, is arbitrary. It is possible that such inconsistencies may cause subtle differences in gene expression levels leading to variation of relative protein concentrations. This is unlikely to affect the general pattern of bands seen on a Coomassie stained gel as only the abundant proteins are visualised, and slight changes in their concentrations would not be detectable. However, the concentration of proteins expressed at low levels, which are not seen on the gels but that are detectable by Western blotting, are likely to be affected to a greater extent.

It was hoped that immunolocalisation of the protein to the parasite would provide more information regarding the expression pattern of CL1. However, as the protein was not recognised in any of the parasite sections this remains open to debate. An explanation for the lack of detection of CL1 may be that the protein is secreted from a particular structure in the parasite through which no section was obtained e.g. small secretory vesicles.

Overall, although the results obtained are contradictory, there is some indirect evidence to suggest that CL1 is released from developing schistosomula post-transformation. These experimental results also show that the use of algorithms such as SignalP can be used to mine the schistosome genome database effectively.

CHAPTER 4

THE IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES USING RANDOM-ARBITRARILY PRIMED PCR (RAP-PCR)

4.1 INTRODUCTION

As discussed in Chapter 1 a single immunisation of mice with optimally-irradiated cercariae results in the elimination of a subsequent challenge infection in the lungs, via a cell-mediated effector mechanism. As the targets of the effector response are the lung-schistosomula, the antigens derived from this developmental stage must be essential for restimulation of the relevant immune cells. Therefore, such antigens represent a rich source of potential vaccine candidates. It is also implicit that the antigens derived from the vaccinating and challenge parasites must be identical.

As irradiated parasites die before or at the lung-stage of migration (Mountford *et al.*, 1988), antigens produced only by the adult worm are unlikely to be involved in the induction of immunity in the RA vaccine model. In addition, Coulson and Mountford (1989) showed that high levels of protective immunity are induced when *ex vivo* lung-stage larvae (derived from irradiated cercariae) are administered intradermally, indicating that antigens specific to the cercariae, or newly-transformed larvae are not crucial. Indeed, Mountford *et al.* (1995) showed that the highest levels of IFNγ secretion were obtained by restimulation of lymph nodes cells of 1x vaccinated mice with SLAP in comparison to SCAP and SWAP. In fact, the released proteins from the lung-worms (d6-8 RP) were the most potent inducers of a Th1-type immune response.

The use of one-dimensional (1-D) and two-dimensional (2-D) SDS-PAGE analysis of protein preparations from various developmental stages has revealed sex and stage-specific proteins (Atkinson and Atkinson, 1982; Yuckenberg *et al.*, 1987). A comparison of the proteins in SCAP, SLAP and SWAP by 1-D SDS-PAGE showed that whilst many proteins were common to all preparations, several appear to be specific to one life cycle stage (Mountford and Harrop, 1998). In addition, a comparison of the 0-3h RP and d6-8 RP reveals significant differences in protein composition (Harrop and Wilson, 1993b) suggesting that stage-specific proteins are also released from the parasite.

Several genes encoding differentially expressed proteins have been reported, for example, p14 (Bobek *et al.*, 1986), fs800 (Reis *et al.*, 1989) and F-10 (Giannini *et al.*, 1995), that are specific to the female worm. These genes are of interest as the proteins they encode are thought to be involved in the sexual maturation of the female worm. Other differentially expressed genes include those coding for elastase (Pierrot *et al.*, 1996), 8kDa calcium binding protein (Ram *et al.*, 1989), hsp70 (Neumann *et al.*, 1993)

and SmSPO-1 (Ram *et al.*, 1999). The expression of each of these genes is thought to be transcriptionally regulated with the amount of protein being directly proportional to the levels of the corresponding mRNA in each of the developmental stages studied. However, the number of stage-specific/differentially expressed genes described is small in comparison with the number of differentially expressed proteins seen on SDS-PAGE gels. In addition, considering the biochemical changes that the parasite undergoes throughout its life-cycle, many more differentially expressed genes would be expected. Therefore, the aim of the work described in this chapter was to develop a method to identify those schistosome genes whose transcription is developmentally regulated, without prior knowledge of their sequence and/or protein product. In this way, a subset of lung-worm specific proteins could be isolated from which putative vaccine candidates might be identified.

Traditional methods to identify differentially expressed genes include differential screening of cDNA libraries (Okubo *et al.*, 1992) and subtractive hybridisation (Sargent, 1987). However both of these techniques require large amounts of starting material which is a limiting factor when working with certain life-cycle stages of schistosomes. Analysis of the ESTs in the *S. mansoni* genome database by Franco *et al.* (1997) suggests that several of these may represent stage-specific genes. However, this cannot be confirmed until a representative number of ESTs is generated from all of the life-cycle stages of the parasite. The study by Santos *et al.* (1999) showed that the ESTs derived from two different cercarial cDNA libraries corresponded to quite different sets of genes, with the exception of the highly abundant genes related to energy metabolism. This highlights the fact that the quality of the cDNA libraries used for EST generation is important, especially when looking for stage-specifically expressed genes.

The techniques of differential display (DD, Liang and Pardee, 1992) and RAP-PCR (Welsh *et al.*, 1992) have been used to successfully isolate differentially expressed genes in a wide range of organisms and experimental systems (McClelland *et al.*, 1995; Sager, 1997). The main advantage of DD and RAP-PCR over techniques such as subtractive hybridisation and differential screening, in addition to being technically relatively simple and low cost, is that more than two RNA samples can be compared in parallel using significantly less starting material. The basis of DD and RAP-PCR is as follows:

(i) pools of cDNA fragments are produced from the RNA samples being compared.

(ii) the analogous pools obtained are resolved side by side on a polyacrylamide gel giving fingerprints.

(iii) fragments that are present in only one sample (or are much more abundant in one than in others) are excised from the gel and investigated.

(iv) many different pools of cDNA fragments are produced by changing the parameters of pool generation, thereby allowing many RNA species to be compared.

The only difference between DD and RAP-PCR is the primer used for reverse transcription of RNA. An oligo d(T)-based primer is used in DD whereas an arbitrary primer is used in RAP-PCR. This means that for DD, during reverse transcription priming occurs mainly at the 3' end of mRNA molecules. After reverse transcription, denaturation and second-strand synthesis, the majority of the cDNAs are derived from the 3' end of mRNAs. However, the RAP-PCR protocol samples anywhere in the RNA molecules as the arbitrary primer used for reverse transcription can anneal to regions of RNA that have 6-8 base matches with its 3' end. Therefore, the advantage of RAP-PCR is that regions of mRNA that are towards the 5' end are more likely to be sampled. This represents an added bonus when looking for genes which encode M/S proteins as the signal sequence is usually located at the 5' end of coding regions.

Neto *et al.* (1997) showed that arbitrarily primed cDNA can be used to generate ESTs from very small amounts of starting material. The ESTs generated in this way showed little redundancy and a large proportion represented new schistosome genes. The fact that several of the RAP-ESTs had homology to other schistosome ESTs in the databases shows that the bands in each profile do in fact represent different genes and are not simply lots of fragments of the same gene. This chapter describes the use of RAP-PCR, based on the protocol of Neto *et al.* (1997), to compare the mRNA profiles of cercariae, lung-schistosomula and adult worms. A general outline of the method used in this study is shown in Figure 4.1.

Extract mRNA from target
life-cycle stage

Reverse transcribe mRNA
with an arbitrarily selected primer

Amplify cDNA, by PCR, with a second arbitrary primer

Resolve PCR products are on
polyacrylamide minigels

C L A

Differentially
expressed band

Lung-stage
specific band

Excise differentially expressed bands, elute and
reamplify DNA

Confirm differential expression by
Northern blotting

Figure 4.1: A general outline of RAP-PCR

4.2 MATERIALS AND METHODS

4.2.1 RAP-PCR

For each set of fingerprints generated, a 10μl aliquot of mRNA (equivalent to 1μg) from each of the life cycle stages (cercariae, lung-schistosomula and adult worms) was denatured by heating to 65°C for 10 minutes and then added to a reverse transcription reaction containing 1.28pM of an arbitrarily selected primer (see Appendix 1), 100μM dNTPs, 25mM Tris-HCl, 3mM $MgCl_2$, 10mM DTT and 100 units M-MLV RT (Promega) to give a final volume of 20μl. This was incubated at 37°C for 45 minutes and the resulting single-stranded DNA stored at -20°C.
Double stranded cDNA was prepared by adding an aliquot of single-stranded DNA into a 10μl PCR mixture containing 1pM of a second arbitrary primer and incubating at 95°C for 3 minutes, 50°C for 2 minutes and 72°C for 1 minutes. The cDNA was then PCR-amplified under the following conditions: 33 cycles of 95°C for 45 seconds, 50°C for 1 minute, 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes.

4.2.2 Polyacrylamide gel electrophoresis of DNA

DNA mixtures were resolved on 6% polyacrylamide gels (19:1 acrylamide: bisacrylamide; National Diagnostics) as described by Sanguinetti *et al.* (1994). Generally gels were cast in Tris-Borate electrophoresis buffer (TBE; 0.9M Tris-borate, 20mM EDTA, pH 8.1) and allowed to polymerise at room temperature and run at 10V cm^{-1} in TBE. The DNA was visualised by silver staining. Briefly, the gel was fixed in 10% ethanol, 0.5% acetate for five minutes followed by a five minute incubation in staining solution (0.2% $AgNO_3$, 10% ethanol, 0.5% acetate). The gel was then washed in sdH_2O for 2 minutes before the developer was added (1.1M NaOH, 0.75% formalin). Once sufficient colour had developed the reaction was stopped by washing the gel in H_2O several times.

4.2.3 Recovery of DNA from acrylamide gels

A sterile scalpel blade was used to excise the specific band of interest, the gel slice transferred to an eppendorf and rinsed several times in sdH_2O. The slice was the boiled

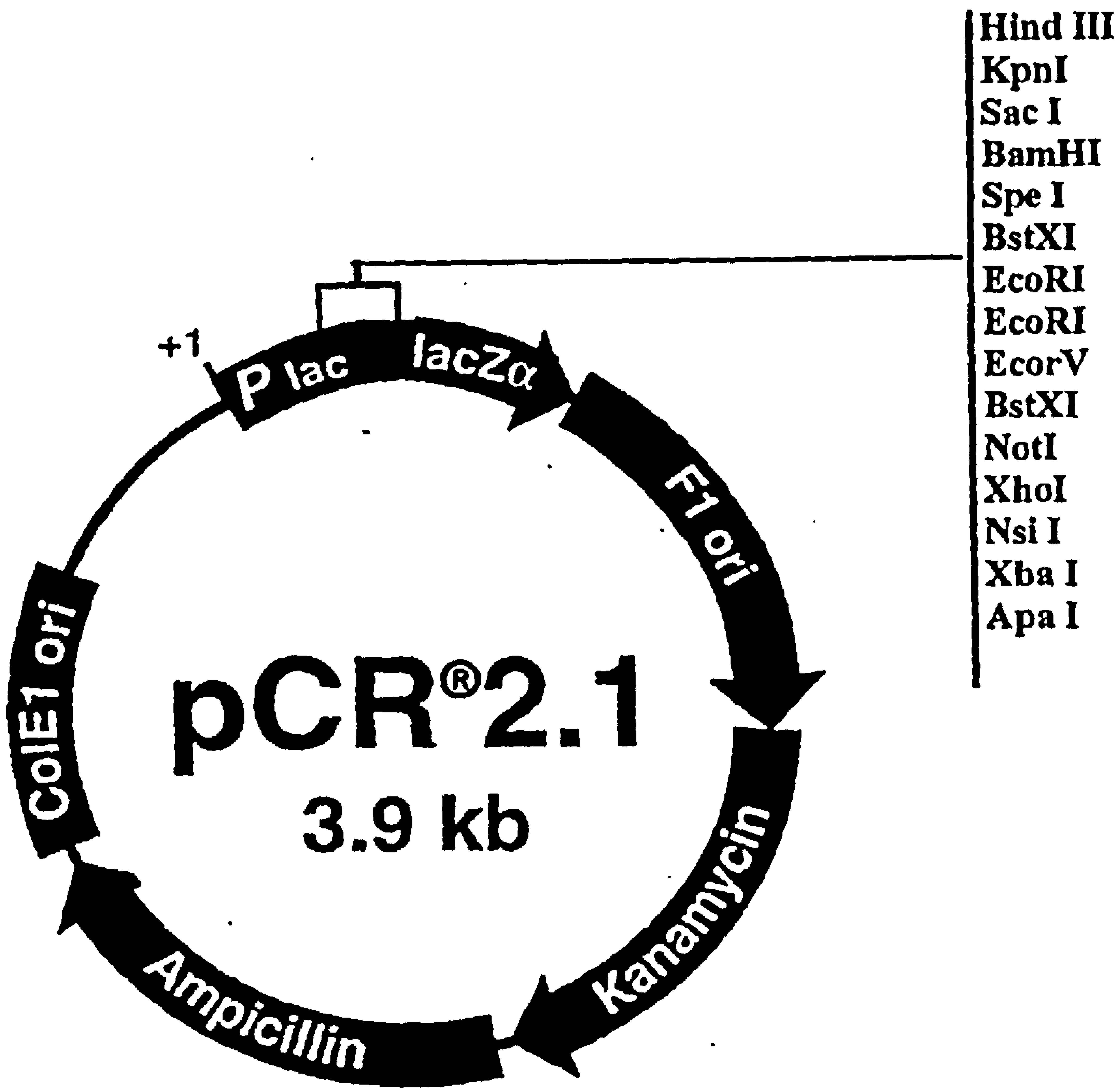


Figure 4.2: The pCR 2.1 TA cloning vector (Invitrogen)

for 20 minutes in 50µl elution buffer (10mM Tris-Cl, 50mM KCl, 1.5mM $MgCl_2$, 0.1% Triton X-100), followed by a 2 hour incubation at 37°C. Once cooled to room temperature, the solution was spun briefly to remove any remaining pieces of gel and the DNA ethanol-precipitated and amplified by PCR.

4.2.4 Cloning of PCR products into the vector pCR2.1

PCR products were cloned directly into the vector, pCR2.1 (Figure 4.2), after their purification and the ligation reaction was transformed into INVαF' *E. coli* cells (Invitrogen) using the TA cloning kit (Invitrogen). The transformants were selected on LB agar plates containing Carbenicillin. In addition, the plates also contained 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside/IPTG (X-gal/IPTG) to allow for selection on the basis of the blue/white colony phenotype.

4.2.5 Southern Blotting

Digested genomic DNA was resolved by agarose gel electrophoresis and an image recorded. Gels were incubated sequentially in an excess of depurinating solution (0.25M HCl) for 15 minutes, denaturing solution (1.5M NaCl, 0.5M NaOH) for 30 minutes, and neutralising solution (1.5M NaCl, 0.5M Tris-Cl, 1mM EDTA, pH 8.0) for 30 minutes. The DNA was then transferred from the gel to a nylon membrane (Hybond N^+, Amersham, Little Chalfont, UK) by capillary blotting as described by Southern (1975) using 20x SSC (3M NaCl, 0.3M tri-sodium citrate) as buffer. The DNA was fixed onto the membrane by exposure to a UV crosslinker for 5 minutes.

Labelling of DNA probes

Radioactively labelled DNA probes were prepared by random-primer labelling using the Megaprime DNA labelling system (Amersham) following the manufacturer's instructions. The target template was a defined restriction fragment containing the sequence of interest at a final concentration of 25ng μl^{-1} and the radiolabelled dNTP used was generally α-^{32}P dCTP (specific activity: 3000Ci mol^{-1}). Excess dNTPs were removed by passing the probes through a G-50 sephadex spin column (Boehringer Mannheim, Lewes, UK). Probes were thermally denatured and snap cooled before use.

Membrane hybridisation and washing

Membranes were incubated in hybridisation buffer (5x Denhardts solution, 5x SSC, 1% w/v SDS, 100μg ml^{-1} denatured herring sperm DNA) for 2 hours at 65°C prior to addition of the probe. Once the probe was added the membrane was incubated at 65°C overnight. Any unhybridised probe was removed by washing the membrane in 2x SSC, 0.1 % (w/v) SDS for 20 minutes at room temperature. The stringency of the washes was increased by reducing the salt concentration of subsequent washes to 0.1x SSC, 0.1% (w/v) SDS and increasing the temperature of each wash to a final temperature of 60°C. The membrane was then prepared for autoradiography.

Autoradiography

Autoradiography film (Kodak BioMax MS-1 film) was placed in contact with hybridised membrane in a X-ray cassette incorporating an intensifying screen. The cassettes were stored at -70°C for a suitable exposure time. The films were then developed using X-ograph Compact X2 (Xograph Imaging Systems which uses Rapid X-ray developer and fixer from Photosol).

4.2.6 Northern Blotting

Total RNA (10μg) from cercariae, lung-schistosomula and adult worms was resolved by agarose-formaldehyde gel electrophoresis (see section 2.3.4). Gels were incubated sequentially in an excess of denaturing solution (1.5M NaCl, 0.05M NaOH) for 30 minutes, neutralising solution (0.5M Tris-Cl pH 7.4, 1.5M NaCl) for 20 minutes and 20x SSC for 45 minutes. The RNA was then transferred from the gel to a nylon membrane by capillary action as described by Southern (1975) using 20X SSC as buffer. The RNA was fixed onto the membrane by exposure to a UV crosslinker for 3.5 minutes.

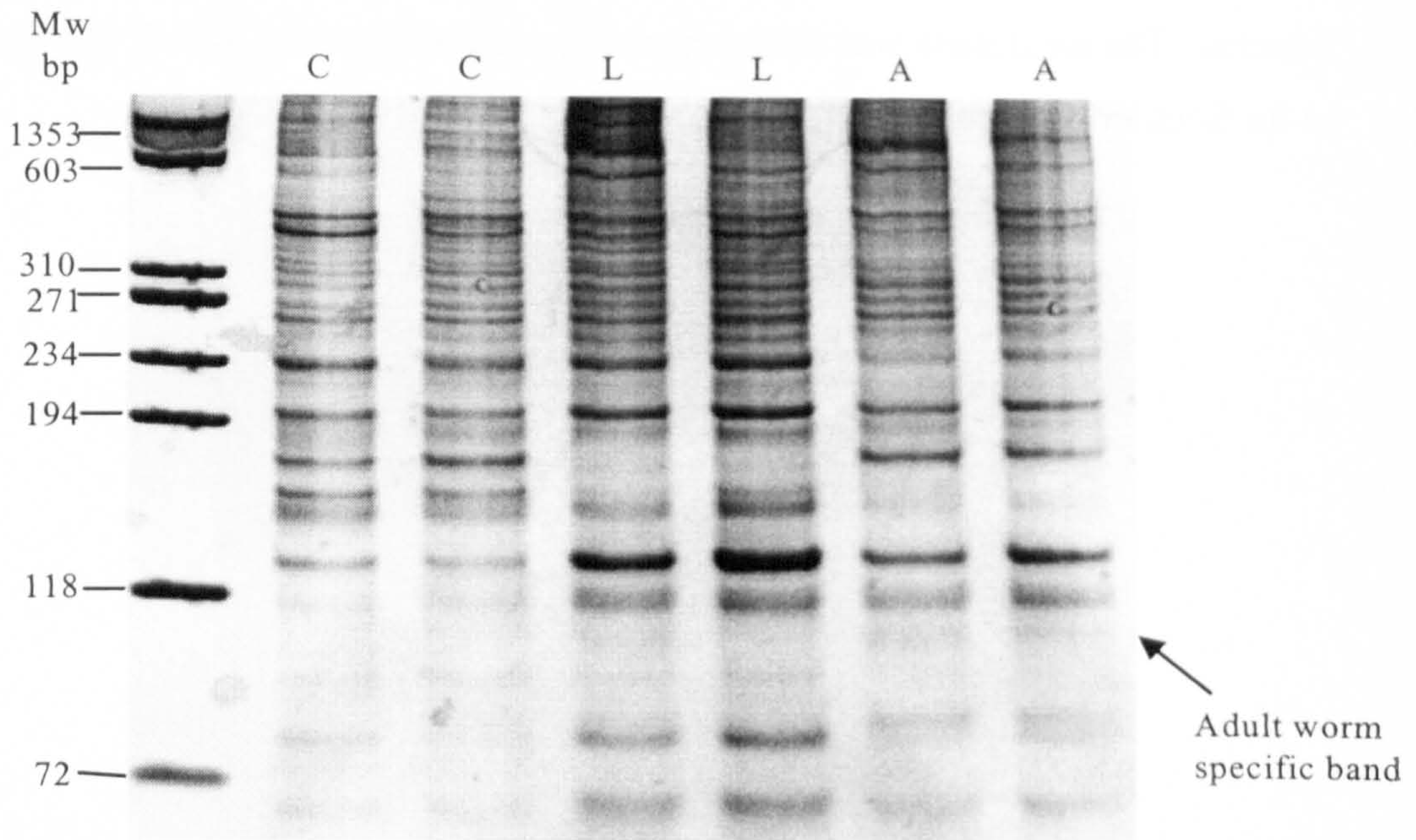
DNA probes were prepared as described for Southern blotting (4.2.5).

Membrane hybridisation and washing

The membrane was wetted in 6x SSC prior to washing in formamide hybridisation buffer (5X Denhardt's solution, 5x SSC, 50% formamide, 1% w/v SDS, 100μg ml^{-1} denatured herring sperm DNA) for 3 hours at 50°C, with rotation, after which the probe

was added and incubated overnight. Any unhybridised probe was removed by washing the membrane in 2x SSC, 0.1% SDS for 10 minutes at room temperature. This was repeated twice, followed by two higher stringency washes in 0.2X SSC, 0.1% SDS for 5 minutes. The membrane was then prepared for autoradiography, which was carried out as for Southern blotting (4.2.5).

a



b

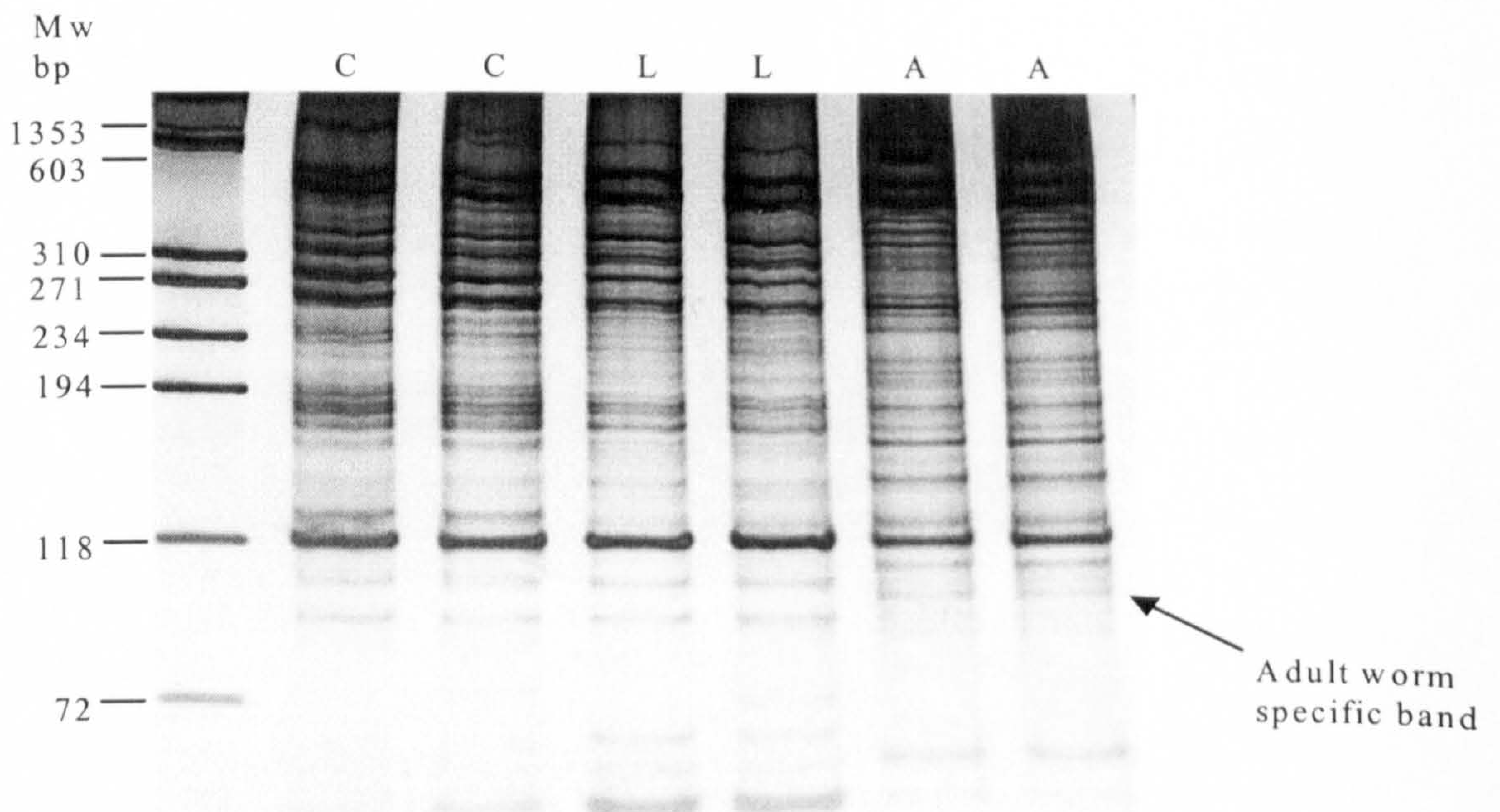


Figure 4.3: Comparison of RAP-PCR products of mRNA from cercariae (C), lung-worms (L) and adult-worms (A) using the primer combination C2 for reverse transcription and SI for subsequent PCR. The two gels (a and b) represent amplification of the same cDNAs on two separate occasions.

4.3 RESULTS

4.3.1 RAP-PCR

To identify genes that are differentially regulated between cercariae, lung-schistosomula and adult worms, RNA fingerprints of the three life-cycle stages, generated by RAP-PCR, were compared. Twenty-four different primer combinations were used with each reaction being performed in duplicate for each life-cycle stage. Only those reactions that gave reproducible profiles were considered useful for analysis. Based on this, seventeen primer combinations gave analysable data; the other primer pairs gave smears or non-reproducible fingerprints. A typical gel is shown in Figure 4.3a. To further ensure that each profile was reproducible, second-strand synthesis and PCR amplification of the cDNA was carried out again under the same conditions. The profiles obtained from the repeat RAP-PCR were compared to those from the original and only those bands that were differentially amplified in both sets were considered useful for analysis (Figure 4.3). On average, one differentially expressed product, out of a total of approximately 100 bands, was found for every primer combination that gave reproducible data. A total of 13 differentially expressed bands was excised from the gels, as well as 6 bands that were common to all three life-cycle stages (Table 4.1). The latter bands were taken to serve as controls and to verify that they did represent mRNAs that were constitutively expressed.

4.3.2 Reamplification and cloning of RAP-PCR products

Out of the 19 bands that were cut out from the gels, only twelve were successfully reamplified by PCR. However, on reamplification the PCR products were not homogeneous necessitating their cloning and sequencing (Figure 4.4). Approximately ten clones were sequenced for each band (sequencing of all the clones was done by Dr D Johnston at the Natural History Museum, London). Only those sequences with less than 5% ambiguous bases were considered useful for analysis. The most frequently occurring sequence from each set of successfully sequenced clones was taken as that corresponding to the excised band. In the case of band 56, three different sequences occurred with similar frequencies making it impossible to decide which sequence corresponded to the

original differentially expressed product. Therefore, clones representing each of the three sequences were investigated further. Only three of the 10 clones (3ALL, 10, 53) had any significant sequence identity to any entries in the current databases (last homology searches done 17/02/00). Figure 4.5 shows the 10 sequences that were obtained in this manner.

4.3.3 Southern blot analysis with cloned fragments

Southern blots of schistosome and mouse genomic DNA were probed with the inserts of the sequenced clones (Figure 4.6). This showed that several of the clones were derived from the schistosome genome (bands 62, 63, 3ALL, 4ALL, 53). These all seem to be single copy genes. Bands 1AL and 10 failed to give any signal, this might be due to inefficient probe preparation. The probe for GST (provided by Dr. R Pierce) used as a positive control, gave a much stronger signal than any of the test probes, most probably because it was longer.

The three different clones derived from band 56 (56.15, 56.16 and 56.20) gave three different results on the Southern blot (Figure 4.7). The insert in 56.15 gave a strong signal comprising multiple bands, characteristic of a repetitive element, whereas 56.20 and 56.16 hybridised to different fragments.

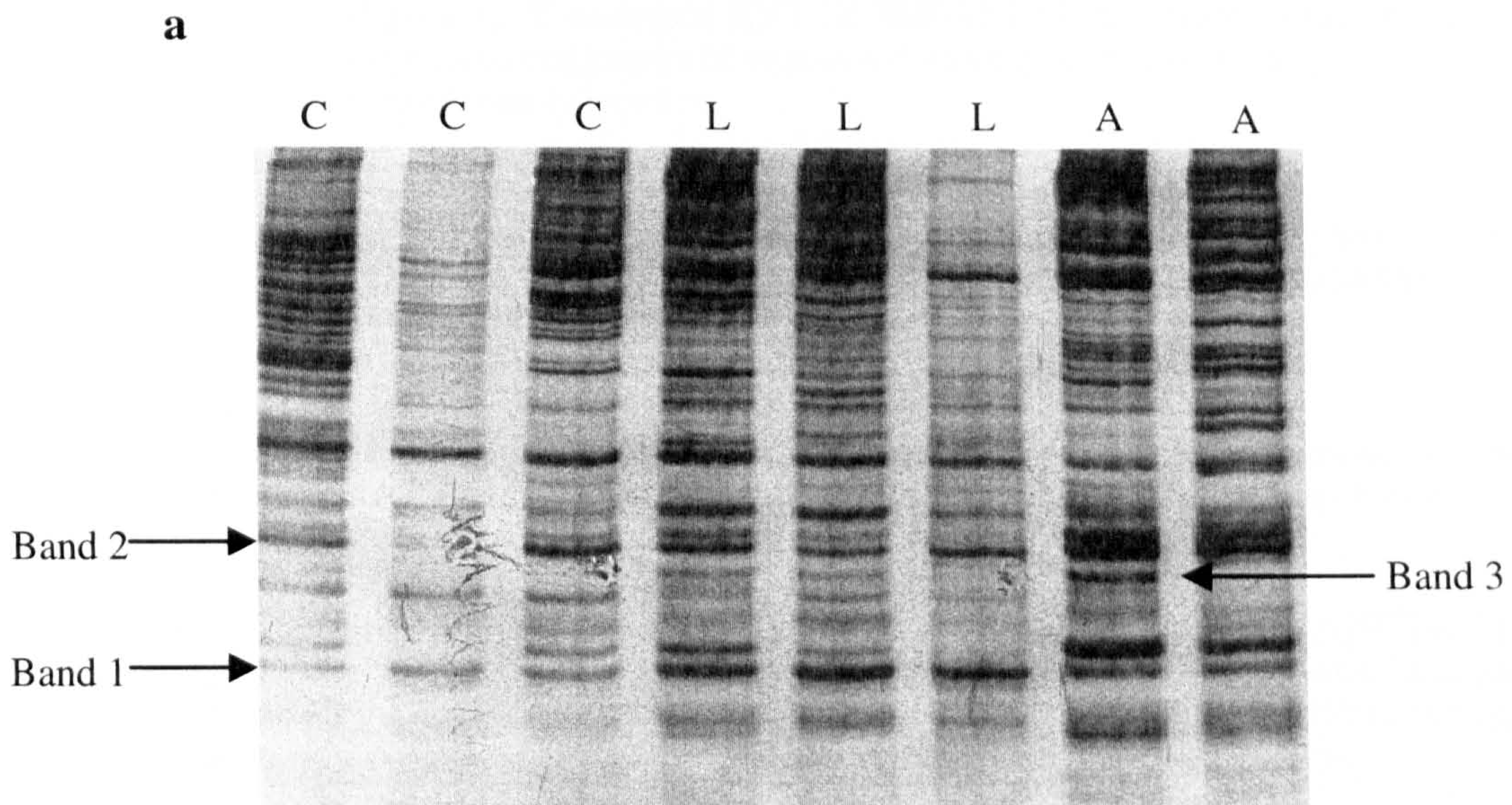
4.3.4 Verification of differential expression of cloned fragments

The probes that hybridised to schistosome gDNA were used to probe total RNA from the three life-cycle stages being investigated in order to confirm the expression patterns of the genes they represent. However, a signal was obtained only for actin, the positive control (Figure 4.8).

As the amount of RNA available for study was limiting, further optimisation of Northern blots was not pursued. An RT-PCR approach was taken with primers based on the sequence data obtained previously. However, no product was visible for any of the RAP-PCR products even though the control reactions using primers for actin were successful (data not shown).

Band	Primers used	~Mw (bp)	Present in (life-cycle stage)
1C	C2, S1	234	Cercariae
2C	SP6, C2	180	"
1L	SP6, C2	180	Lung-worms
2L	SP6, C2	190	"
3L	SP6, S4	200	"
56	G2, SK	180	"
1A	C2, S1	107	Adult worms
62	G1, G2	150	"
63	G1, G2	155	"
1CL	C2, S6	230	Cercariae and lung worms
1AL	KS, S1	150	Lung and adult worms
2AL	C2, S6	250	"
53	G2, G1	135	"
1ALL	C2, S1	118	All
2ALL	C2, S1	286	"
3ALL	KS, S1	130	"
4ALL	KS, S1	160	"
10	C2, G1	90	"
58	G2, G1	120	"

Table 4.1: The bands that were excised from the RAP-PCR gels.



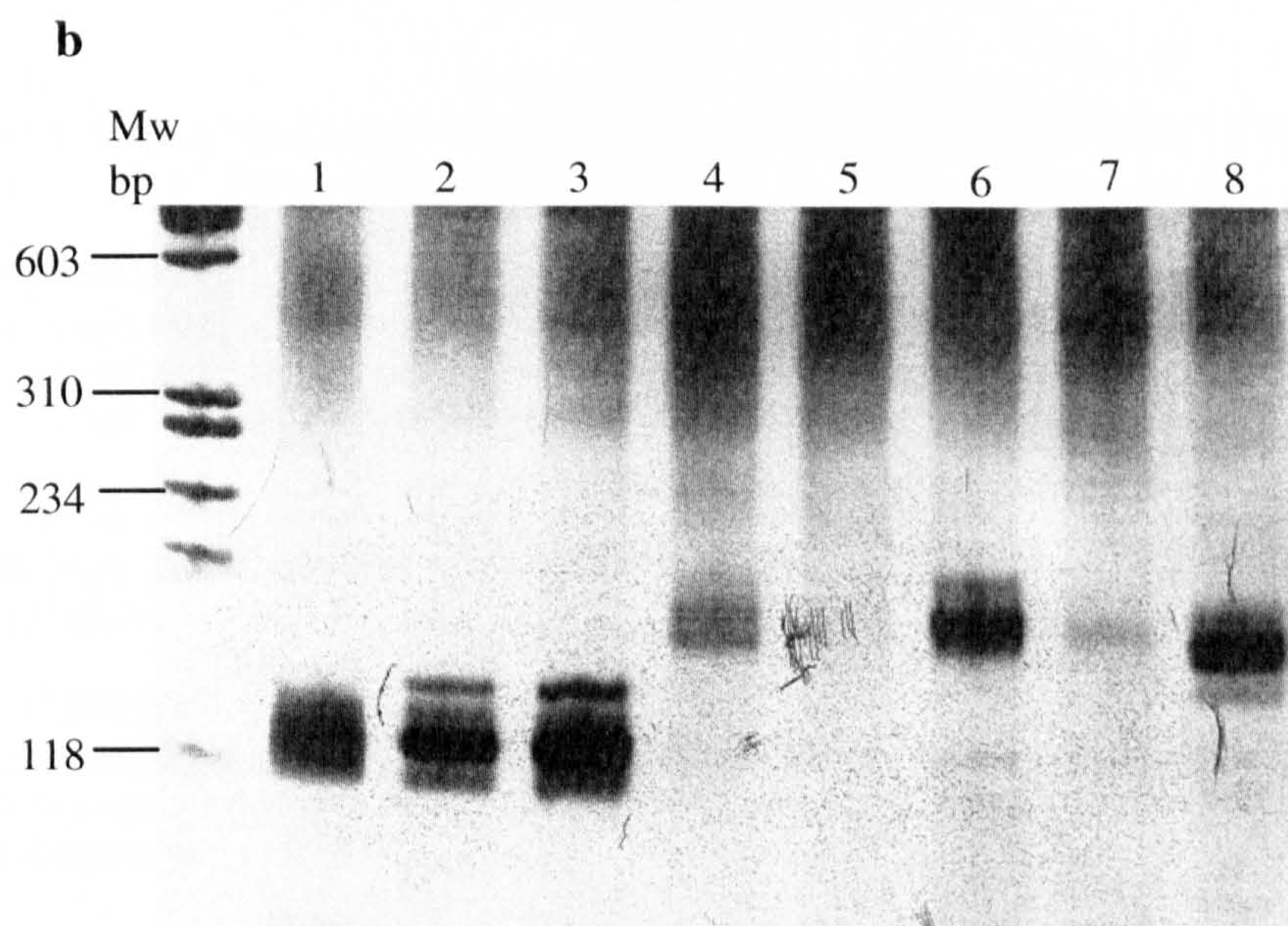


Figure 4.4: Reamplification of DNA from excised bands

(a) Original RAP-PCR gel from which bands 1,2 and 3 were excised for further investigation.
Bands 1 and 2: common to all life-cycle stages; Band 3: lung- and adult-worms only
(b) polyacrylamide gel of the reamplification products:
Band 1 in lanes 1,2 and 3 from C, L and A respectively
Band 2 in lanes 4,5, and 6 from C,L and A respectively
Band 3 in lanes 7 and 8 from L and A respectively
C: cercariae L: lung-worms A: adult-worms

3ALL (126bp) Homologous to *S. mansoni* EST208943 (accession number: AI067268)
atgacgctggccttattagtgtttctatttgatatacgcaggatccgggttaaaacctcggtattcatgg
ccgaaactttgatgcaaaagaccggaggatagttataggataccgtcgacctcga

10 (102bp) Homologous to *S. mansoni* EST SCMRAP139 (accession number: L47052)
gaatcgctgtataaaagttatcctgtagaattcgaaaatatttgcatttcgctcgtggtctagcttttga
cagccttccgacctttgtttaccagccttc

4ALL (176bp)
atgacgctggccttattagttcaaacgtatcttatatatgtggctactctgaacccattattttacatca
gattggtttaacattgactgtttttttgttttaacatcttgaatgtaaactatatatatattcttgagtaa
ccgaaacagtcttctgataccgtcgacctcga

1AL (158bp)
atgacgctggccttattagttttatgagtcagatattaataaataagagtgaatatttaactttcaatatc
cagacttatctcataatttaacttaagggtcggaagttgtaggctcattggtcagatattataagcgatac
cgtcgacctcga

53 (156bp) Homologous to *S. mansoni* EST272077(accession number: AI977483)
ggaaaacaaatggtcgggaagggaacttatgagtgaatgttttaattaatgaattcaattcacagctaatc
tttaattgttcaatgaaaatgatagatgataccaatctaaatggtttctccccnacttcntgtttactttt
atncngcgattcag

62.16 (115bp)
ggaaaacaaatggtcggaagtacagtaggttttagagcctcagcatcatctaacaatccagtaattgctcc
agtaaggaatccgatttgtgctccacttttatacagcgattcag

63 (99bp)
ctgaatcgctgtataaaagtataaaatcagtatattaagaaatcttctcggcctgtgtaagatacagcaaa
atctgcgccatttgcagttggttcggaa

56.15 (198bp)
ggaaaacaatggtcggaagcgttatccactagatgattttgatttgatttaaccaactctctttctttatt
atttgtacacgtttcaacgaanaaatcttcaatttcttcttcaacttcagtatcatcatcagcagcagtag
aaattgtagtttcggtagtaccaacatttccttcatgatccactagttctagagcg

56.16 (87bp)
agaaactagtggatcaacagctcgtaattcgaatactcagtcattgtcatcgttgtcatcatcatcttccg
accatttgttttccaa

56.20 (156bp)
ggaaaacaaatggtcggaagtggaaaacgacccatgaaatttactcaaagggttccgagtgttttcgaaag
ggtattccaaaatgaagaataaatccaggtatttaaatgtgtagtaatattacactaggacttcgatccac
tattctagagcg

Figure 4.5: The DNA sequence of each of the excised bands.
The regions corresponding to RAP-PCR primers are underlined.
See: Table 4.1 for individual primer pairs used for generation of each band
Appendix 1 for complete primer list

Several of the sequences shown represent complementary sequence of the original cDNAs. This is because the cloning into the TA vector is non-directional.

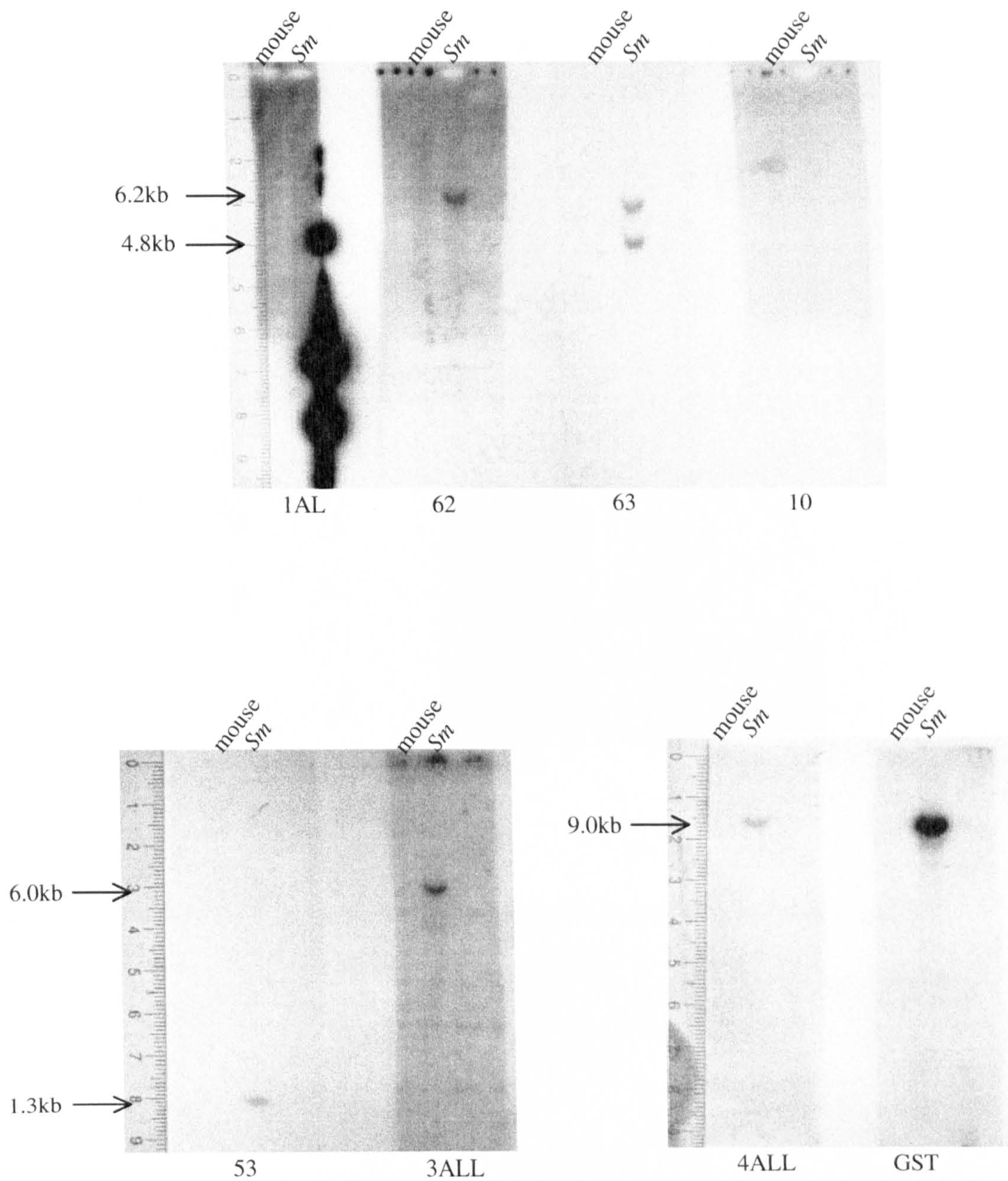


Figure 4.6: Southern blot analysis using the cloned inserts of the excised bands

Bands can be seen for 62, 63, 53, 3ALL, 4ALL.

GST was used as a positive control.

The signal seen on 1AL is carry over from the molecular weight marker that was used.

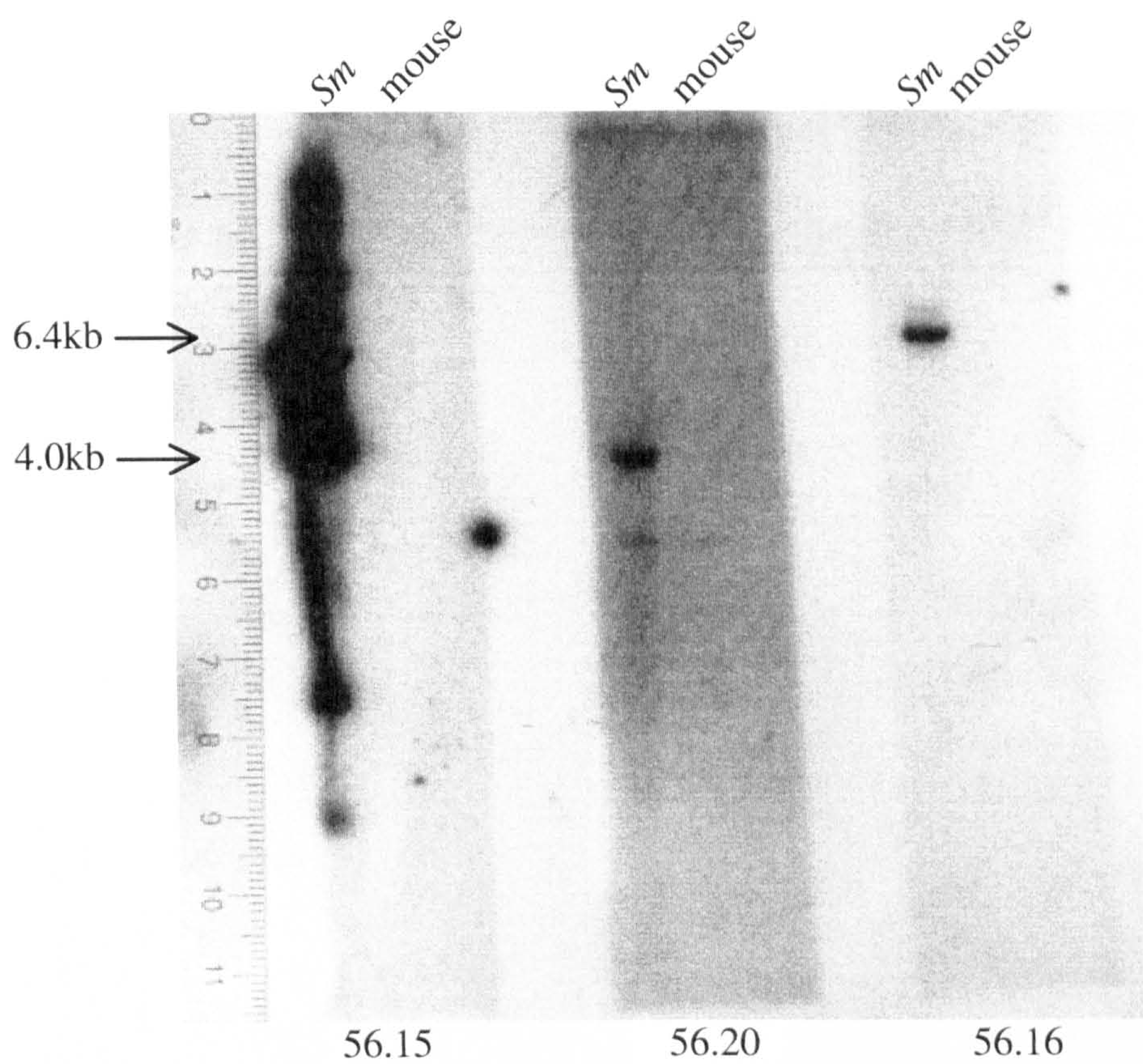


Figure 4.7 Southern blot analysis of the three different clones from band 56.

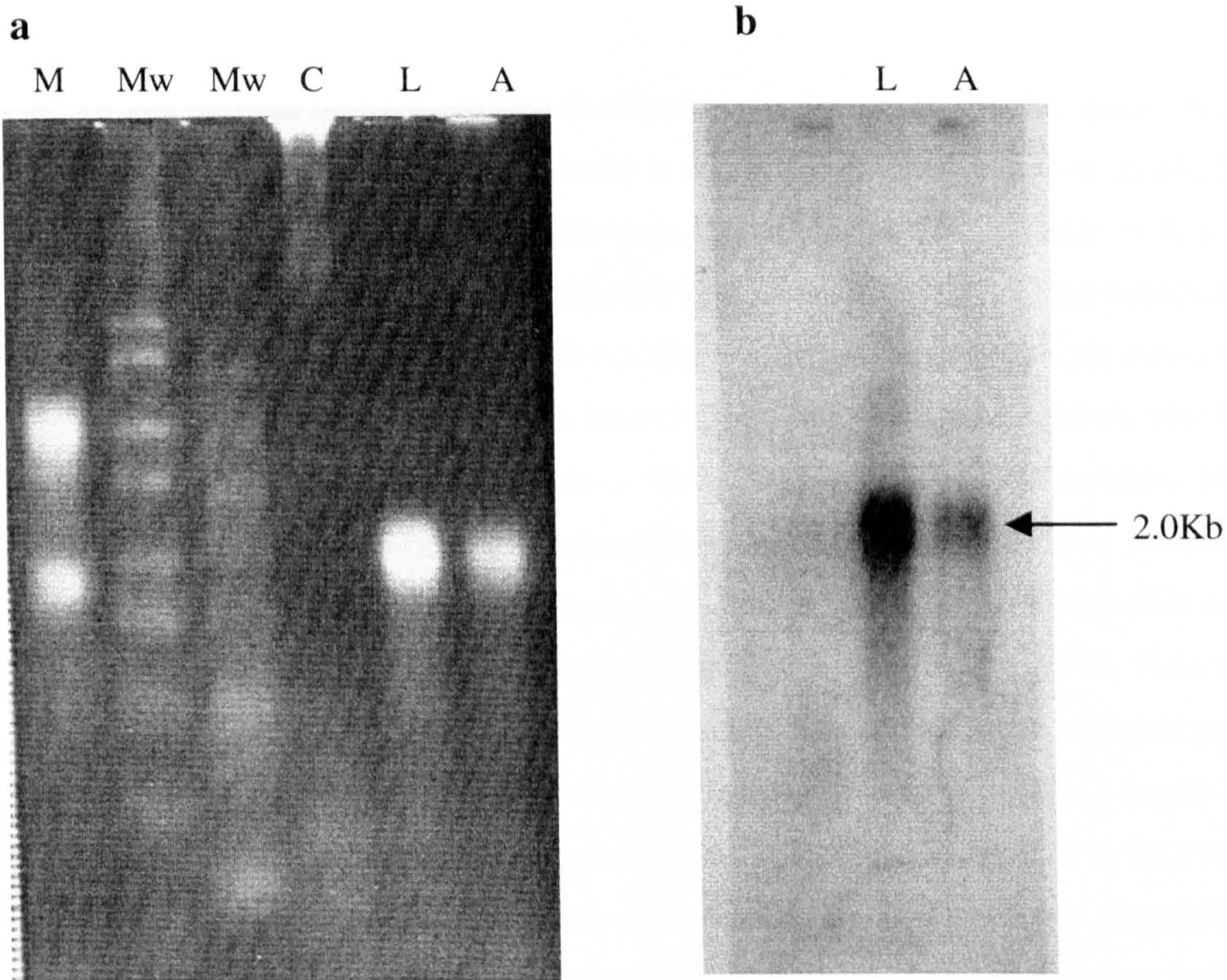


Figure 4.8: Northern blot analysis of actin expression.

10μg total RNA from cercariae, lung-schistosomula and adult-worms were loaded side by side and the gel stained with ethidium bromide prior to transfer, to check both the integrity of the RNA and to ensure that equivalent amount were loaded onto the gels (a). Mouse RNA was also run and stained as a control (M), but was not transferred. No cercarial RNA entered this particular gel. After transfer, the membrane was probed with a fragment of the actin gene (b). The levels of actin in lung-schistosomula and adult-worms are the same (taking into account the difference in amounts of RNA loaded onto the gels).

4.4 DISCUSSION

4.4.1 RAP-PCR

The purpose of the work described in this chapter was to identify genes that were differentially expressed in the parasite as it matured from a cercaria to an adult worm, with particular interest in those that were upregulated at the lung-stage of development. RAP-PCR was used to compare the mRNA profiles of cercariae, lung-schistosomula and adult worms using pairs of arbitrary primers. Each of the primers used was a non-degenerate 20-mer. The rationale behind using long primers was simply that they would anneal to many sequences along the template with a variety of mismatches. It has been noted, when using 10-mers for differential display, that the majority of primer-binding sites on the template match only 6 out of 10 bases exactly at the 3' end with up to 4 mismatches at the 5' end (Liang and Pardee, 1995; Bauer *et al.*, 1993). Neto *et al.* (1997) and Welsh and McClelland (1991) have shown that the use of two different primers for RT and PCR results in shorter products although the number of cDNA bands is similar to that obtained with the use of a single primer. This result is expected as the average distance between adequate matches on the template is less when two oligonuleotides are used as primers. In addition, smaller PCR products might be expected to amplify more efficiently than longer molecules, suggesting that using two primers might give clearer results. Furthermore, PCR products that use a single primer can produce hairpins during renaturation, putting them at a possible disadvantage relative to PCR products amplified using two primers.

A major concern with RAP-PCR is the number of false positives that arise (Debouck, 1995). PCR amplification of each of the cDNA pools was carried out in duplicate in order to reduce the number of false positives identified. Only those bands present in both samples were considered. Although the majority of the primer combinations used gave reproducible profiles, some yielded smears in all of the lanes. The application of a few initial low stringency annealing PCR cycles followed by cycles of higher stringency annealing might have reduced the problem of smears and produced a greater number of reproducible profiles. Such an approach has been used in many DNA fingerprinting applications (Zhao *et al.*, 1995).

The number of differentially expressed products that were detected in this study was relatively low in comparison to a similar study using *F. hepatica* (Reed *et al.*, 1998). This is probably due to the fact that full-length sequencing gels were used in the latter study. This allows for better separation of the fragments and thus more individual fragments can be visualised than on the minigels used here. The use of minigels may also provide an explanation for the fact that no differentially expressed products were seen when using some primer combinations. Alternatively, those primer sets might just not have annealed to any differentially expressed product. In fact, the use of a longer format gel (Bio-Rad midigel system) did allow for the visualisation of a significantly larger number of fragments. Therefore, this may represent an improvement to the protocol reported here. As in studies with *F. hepatica* (Reed *et al.*, 1998), *Leishmania mexicana amazonensis* (Heard *et al.*, 1996) and *Eimeria bovis* (Abrahamsen *et al.*, 1995) the number of amplified cDNA products generated by DD was limited in comparison to fingerprints from mammalian cell mRNAs. This may either reflect a difference in the complexity of mRNA expression or be due to subtle differences in the methodologies used. Overall, the RAP-PCR profiles generated were reproducible and several differentially expressed products were identified.

4.4.2 Selection and isolation of differential bands

The DNA from several of the excised bands was not reamplified by PCR. This seemed to reflect the intensity of the band on the RAP-PCR gel; those bands that were more prominent amplified more easily, whereas very faint bands did not. The DNA from several of these faint bands gave products that were visible on silver-stained polyacrylamide gels after two amplification rounds each consisting of 40 PCR cycles. However, these products were not pursued due to the increased likelihood that PCR artefacts had been generated after so many rounds of PCR. Therefore, the reamplification of cDNAs is limited by the concentration of material that is excised from the RAP-PCR gel. This might be a drawback of the protocol used here if rare transcripts are identified.

The PCR products of those bands that did reamplify after excision from RAP-PCR gels invariably contained more than one species of DNA. This has been reported repeatedly in the literature and several different approaches to identify the correct

differentially amplified product have been described (Mathieu-Daudé *et al.*, 1996 and references therein).

The fact that no significant matches to entries on current databases were obtained for the majority of the sequences is not surprising given that there is only limited sequence information available for parasitic trematodes. This is also similar to the data obtained from differential display studies on *F. hepatica* (Reed *et al.*, 1998), *Angiostronylus cantonensis* (Joshua and Hsieh, 1995) and *Giardia lamblia* (Que *et al.*, 1996). Indeed, one of the differentially expressed products identified by Tawe *et al.* (1998) from *C. elegans* had no sequence homology even though over 75% of the *C. elegans* genome had been sequenced at that time.

4.4.3 Verification of differential expression

Northern blot analysis is the best way to determine unequivocally if the expression of a particular gene is differentially regulated (Liang and Pardee, 1995). In addition, it offers information regarding the transcript size and presence of multiple species. However, as no signals were seen in this study it can only be assumed that the technique is not sensitive enough to detect the mRNAs corresponding to the differentially expressed bands identified (the same probes were used successfully on a Southern blot). The insensitivity of Northern blotting has been reported by several workers (Liang *et al.*, 1993; Reed *et al.*, 1998; Rivas *et al.*, 1997; Dimopoulos *et al.*, 1996). All of them suggest that it is likely that the mRNAs of the genes being investigated are expressed at levels below the detection limits of Northern blot analysis. For example, Reed *et al.* (1998) were only able to detect one of their 5 differentially expressed clones by Northern blot analysis. Indeed, Pierrot *et al.* (1996) found low levels of elastase mRNA transcripts in adult worms by RT-PCR whereas Newport *et al.* (1988) had not been able to detect the transcript by Northern blotting in the same life-cycle stage. Several alternative approaches which are more sensitive than Northern blots, including semi-quantitative PCR, RNase protection assay and *in situ* hybridisation can be used to verify expression patterns of differentially expressed genes. However, due to limiting amounts of parasite material, especially lung worms, it was not possible to optimise any of these approaches for any of the *S. mansoni* probes.

There is considerable debate in the literature about the possible bias of DD and RAP-PCR towards abundant transcripts. Bertioli *et al.* (1995) found that in a model system,

where the target mRNA was added externally, the differentially expressed target became undetectable when it constituted less than 1% of the total sample. In contrast, Wan *et al.* (1996) found that of the 23 differentially expressed products they identified 13 were rare mRNA species (i.e. each comprised less than 0.01% of total mRNA) and the expected abundant differentially expressed transcripts were not found. There are many variables including the competition for PCR primers between RNAs of different abundance, the length of the primers, the number of PCR cycles and the amount of starting material that may account for such differences. Each would have to be tested individually to optimise the method to identify all possible differentially expressed products in any one experimental system.

Adaptations to the original protocol to enrich for products of a particular gene family, e.g. for zinc finger proteins in mammalian systems, by designing primers for the conserved amino acid region of such proteins have been reported (Stone and Wharton, 1994; Johnson *et al.*, 1996). Similarly, to identify transcripts with intact 5' ends from trypanosomes, the second-strand synthesis primer was derived from the shared 5' mini-exon sequence, which is common to most trypanosome mRNAs (Murphy and Pelle, 1994). A set of primers based on the analysis of available coding regions of mammalian G-protein coupled receptors (GPCRs) was used to amplify 7 known GPCRs from 30 randomly isolated bands (Lopez-Nieto and Nigam, 1996). GPCRs possess very few conserved motifs making their isolation very difficult. However, the results presented by Lopez-Nieto and Nigam (1996) show that this approach is viable for proteins with few similarities. With this in mind, the signal sequences of the eight schistosome proteins known to be secreted prior to the work presented in chapter 3 were analysed. As expected no common DNA sequence was found but the amino acids leucine, isoleucine and valine occurred in repetitive patterns. Some primers were designed, based on these patterns, taking into account the codon bias of schistosomes (Ellis and Morrison, 1994) and used for RAP-PCR (see Appendix 1 for primers, S1-S6). Although these primers would not be expected to amplify signal sequences exclusively, it was hoped that they would skew the bias towards hydrophobic regions, and hence M/S proteins. The RAP-PCR fingerprints obtained using these primers were no better or worse than those obtained using totally arbitrary primers. It remains to be seen if this approach was successful as it is impossible to tell if any of the clones identified in this study represent M/S proteins based on the limited sequence data obtained. A more suitable approach may be to modify the system such that the 5' end of mRNAs is selected for, thereby

allowing for the identification of those cDNAs with a signal sequence as soon as clones are sequenced. This might be achieved by using a degenerate primer for the Kozak sequence, which occurs, at the 5' end of most genes. Martinez *et al.* (1991) showed that this is a feasible approach using total RNA from rat embryo fibroblast cells and its *ras/p53* transformed derivatives.

Fischer *et al.* (1995) described a variation of the differential display technique which combined restriction fragment length polymorphism technology with domain-directed DD. This method not only allows all members of a multi-gene family to be identified but also distinguishes each member of the family. This may prove to be a more sensitive technique for the identification of members of gene families and analysis of their expression patterns. Several other variations on the basic technique of DD have been reported including the use of class II S restriction enzymes (Kato, 1995), the PCR amplification of 3' end restriction fragments of biotinylated cDNA that are isolated with avidin beads (Ivanova and Belyavsky, 1995) and ordered DD (Matz *et al.*, 1997). Each of these approaches has clear advantages over DD when a complete characterisation of an RNA sample is required. However, these techniques are more complicated and require more skill and specialised materials to produce a reproducible fingerprint in comparison to classical DD.

Although the stage-specificity of the cDNAs identified in this study remains to be proved, comparison of the mRNA from cercariae, lung- and adult-worms by simple RAP-PCR has revealed that there are several genes that are transcriptionally regulated as the parasite matures from a cercaria to an adult worm. If any of the genes identified here is truly lung stage-specifically expressed, it could provide a useful marker for future studies of differential gene expression in schistosomes.

CHAPTER 5

THE SIGNAL SEQUENCE TRAP

5.1 INTRODUCTION

The use of the SignalP algorithm (Chapter 3) revealed how few M/S antigens have been deposited on the schistosome database. Additionally the number of sequenced genes that have been identified from the larval stages of the parasite (especially the sporocyst and lung-schistosomulum) is relatively small in comparison with those derived from the adult worm. This is a drawback as it is the larval stages that are the targets of the protective immune responses seen in the irradiated vaccine model and hence it is the genes expressed by these stages that are of interest. The use of algorithms such as SignalP is limited to only those genes that have already been tagged (e.g. by EST generation) and by the quality of the sequence data being analysed. Therefore, a method to identify M/S proteins, which is independent of the sequences on the genome database, would probably be more successful.

The traditional biochemical approach for the identification of M/S proteins is the isolation of the proteins, usually by either electrophoresis and elution or by liquid chromatography, followed by peptide sequencing techniques. This, however, often turns out to be very challenging as the separation and sequence analysis by Edman degradation of peptides derived from hydrophobic proteins can be difficult. Although the sequencing problems may be circumvented by using proteomic technologies such as mass spectrometry to determine amino acid composition of peptides (Shevchenko *et al.*, 1996), the amount of material available for study remains a problem, especially for larval stages of schistosomes.

Several genomic approaches aimed specifically at the identification of M/S proteins from various organisms have been described. These include the use of a subtractive antibody screening system in which a polyclonal antiserum is depleted of antibodies that recognise non-target proteins (Scherer *et al.*, 1998) or the creation of a cDNA library enriched for genes encoding extracellular proteins followed by the use of high throughput *in situ* hybridisation (Kopczynski *et al.*,1998). Although there are several potential sources from which schistosome M/S proteins could be derived, e.g. acetabular and head glands, the homogeneous body, the gut and the nephridial system, it would be impossible to produce a cDNA library enriched for M/S proteins. This is because schistosomes are acoelomate platyhelminths, which means their different tissues cannot be separated. Additionally, as antibody screens are biased towards the identification of the most abundant or highly immunogenic proteins it is likely that only a small subset of proteins

would be isolated. The third approach has been coined the "signal sequence trap" (SST). This is a more robust method, based on the ability of signal peptides to redirect the expression of an export-defective reporter protein to the cell surface or medium. This approach relies on the fact that signal peptides can direct the secretion of heterologous proteins and are also interchangeable between diverse organisms (Hitzeman *et al.*, 1990; Rapoport *et al.*, 1996). For example, β-lactamase from *E. coli* can be secreted by *Xenopus* oocytes (Wiedmann *et al.*, 1984) and prepro-α-mating factor of *S. cerevisiae* can be imported co-translationally by mammalian microsomes (Garcia and Walter, 1988).

In the first SST system described (*Tac* SST), a mixture of cDNAs was cloned into a vector such that the foreign DNA was directly upstream of a signal sequence-deficient IL-2 receptor gene (Tashiro *et al.*, 1993). After transformation of the plasmid mixture into COS cells, surface expression of the receptor was detected by antibody staining. The isolation of several mammalian M/S proteins, including putative growth factors, receptors or adhesion molecules, has been reported (Nakamura *et al.*, 1995; Shirozu *et al.*, 1996; Kimura *et al.*, 1998).

Recently, the *Tac* SST system has been used to identify secretory proteins from the salivary glands of the insect *Anopheles gambiae* (Arcà *et al.*, 1999). The authors reported the identification of 15 distinct cDNAs of which six were expressed specifically in the salivary glands. A variation of the *Tac* SST system has been used to isolate various plant M/S proteins (Kristofferesen *et al.*, 1996). These last two studies represent the only reports in which *Tac* SST-based systems have been used to identify non-mammalian M/S proteins and reinforce the fact that that secretion systems are able to process heterologous signal peptides.

The immunostaining step described in the *Tac* SST strategy has several drawbacks including low sensitivity, the requirement for large, expensive pieces of equipment (e.g. Fluorescence activated cell sorter; FACS) and being time-consuming and labour intensive. In order to improve the detection system, several modifications have been developed. Chen and Leder (1999) have described the use of human placental alkaline phosphatase (PLAP) as the reporter. PLAP is only functional when it is brought to the cell surface (cytoplasmic PLAP is inert) and a simple colourimetric assay is used to identify positive clones. This reduces the number of false positives that arise when using

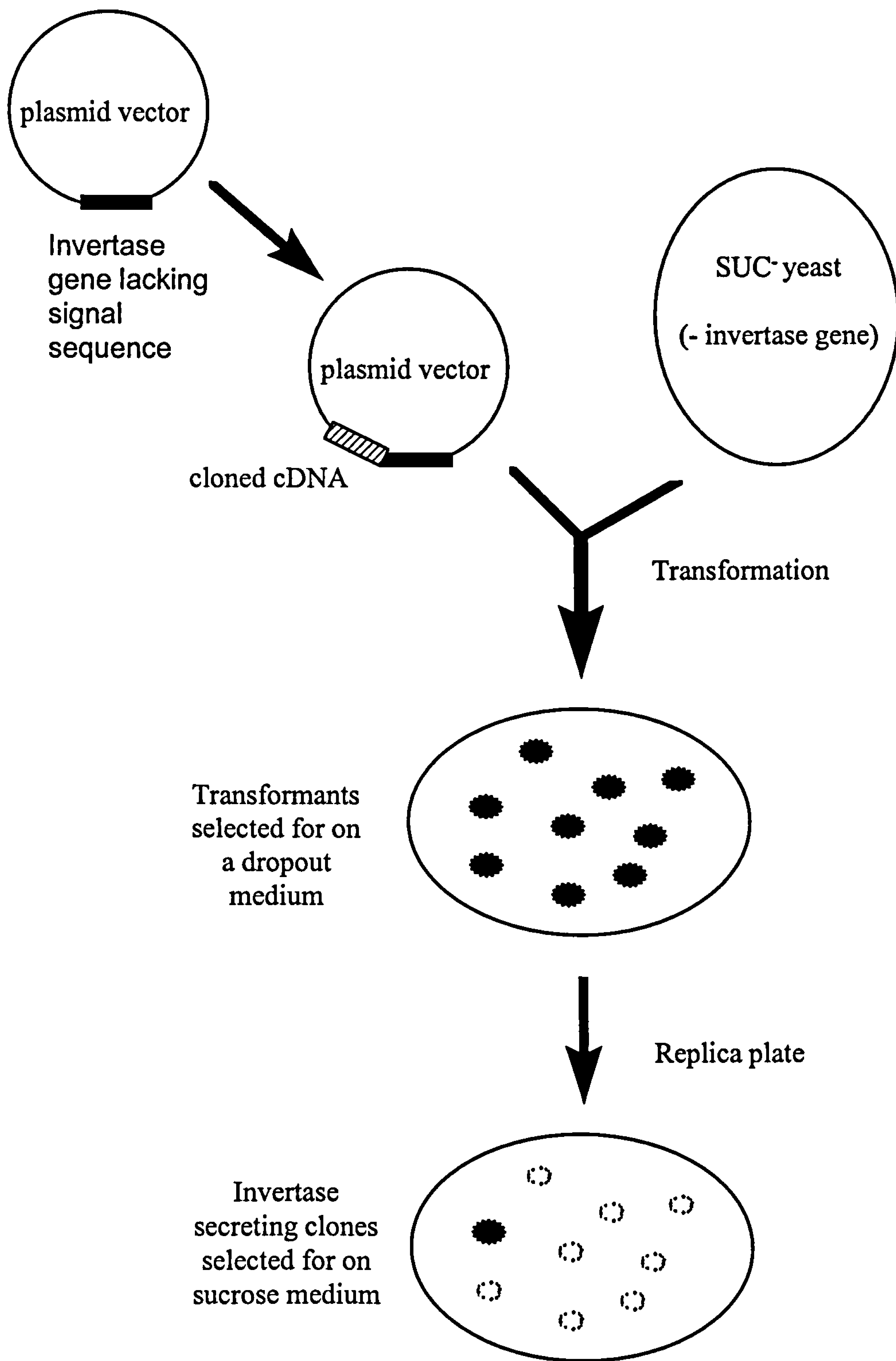


Figure 5.1: A schematic representation of the yeast-based signal sequence trap.

the previous detection system. However, before a single positive clone is isolated, several rounds of screening and rescreening have to be carried out.

Kojima and Kitamura (1999) have described a retro-virus mediated expression system that detects signal sequences based on their ability to redirect a mutant form of the thrombopoietin receptor, MPL^M, to the cell surface. A constitutively active MPL^M permits IL-3 independent growth of cell lines, which are normally IL-3 dependent. Therefore, this removes the need for multiple rounds of screening for the identification and isolation of positive clones as is necessary in the previous systems.

An alternative, yeast-based SST (YSST) has also been developed (Klein *et al.*, 1996; Jacobs *et al.*, 1997). This system uses a single-step genetic selection to identify the requisite cDNAs, relying on the principle that secretion of the enzyme invertase is essential for cell survival in certain environments. The invertase gene codes for two forms of the enzyme; a constitutive cytoplasmic enzyme and a secreted, glycosylated enzyme. Expression of the two forms of the enzyme is differentially regulated during transcription (Carlson and Botstein, 1982) with the cytoplasmic form being constitutively expressed. Glucose concentration is thought to regulate the expression of the secreted form, with synthesis being upregulated in the absence of glucose (Perlman and Halvorson, 1981). Therefore, it is imperative for yeast that invertase is secreted when growing in environments where glucose is not available as a carbon source (Carlson *et al.*, 1983). Hence, invertase secretion represents a simple and sensitive selection upon which the yeast-based SST has been established.

A strain of *S. cerevisiae*, with its chromosomal copy of the invertase gene deleted (SUC^-), is used in conjunction with a plasmid containing a truncated invertase gene lacking both its signal sequence and the initiator methionine for cytoplasmic invertase. Foreign cDNA is cloned directly upstream of the truncated gene and the plasmid transformed into the mutant yeast. Only those transformants that acquire a cDNA encoding a functional signal peptide will grow when sucrose is the sole carbon source. Figure 5.1 shows a general outline of the YSST.

The stringent requirement for both a signal sequence and initiator methionine means that the invertase protein is not expressed in the empty vector. Although invertase secretion is essential for growth under selective conditions, as little as 0.6% of wild type expression levels is sufficient to allow for growth on sucrose medium and 4.7% results in growth rates indistinguishable from that supported by a normal invertase protein (Kaiser and Botstein, 1986). This means that even if non-yeast signal sequences facilitate only a

low level of invertase export, growth will still be permitted. Kaiser *et al.* (1987) showed that 20% of the random fragments generated by the digestion of human genomic DNA can function, at least minimally, as signal peptides. Indeed, Jacobs *et al.* (1997) observed that many cDNAs encoding different lengths of IFNγ peptides fused to invertase were readily isolated using the selection system. A further advantage of yeast is that, unlike mammalian cells, its ribosomes do not require specific nucleotide sequences to initiate translation thereby increasing the efficiency of heterologous gene expression (Yoon and Donahue, 1992).

Although Kaiser *et al.* (1987) demonstrated that invertase is tolerant of amino-terminal extensions, Jacobs *et al.* (1997) have modified the plasmid used for the SST. A sequence encoding a Kex2 proteolytic site has been incorporated into the plasmid between the multiple cloning site and the invertase coding region. Kex2 is a yeast protease which functions in the late Golgi (Redding *et al.*, 1991). This allows the expressed fusion protein to be cleaved so that the foreign fusion partner does not interfere with the function of the secreted invertase.

This aim of this chapter was to establish this yeast based signal sequence trap for the identification of schistosome M/S proteins. Initially the system would be validated using schistosome cDNAs encoding proteins whose localisation within the parasite was known. Following this, a cDNA mixture derived from the parasite would be cloned into the vector and screened using the SST for novel M/S proteins.

5.2 MATERIALS AND METHODS

5.2.1 Growth, maintenance and preservation of *S. cerevisiae*

S. cerevisiae cells were grown in YPD liquid medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose) at 30°C with agitation (100 r.p.m.) for preparative procedures. Isolation of single colonies was achieved by streaking cells onto YPD-agar plated (YPD medium with the addition of 2% agar) followed by incubation at 30°C. For long term preservation, glycerol stocks (15% v/v) were prepared from liquid cultures and stored at -70°C.

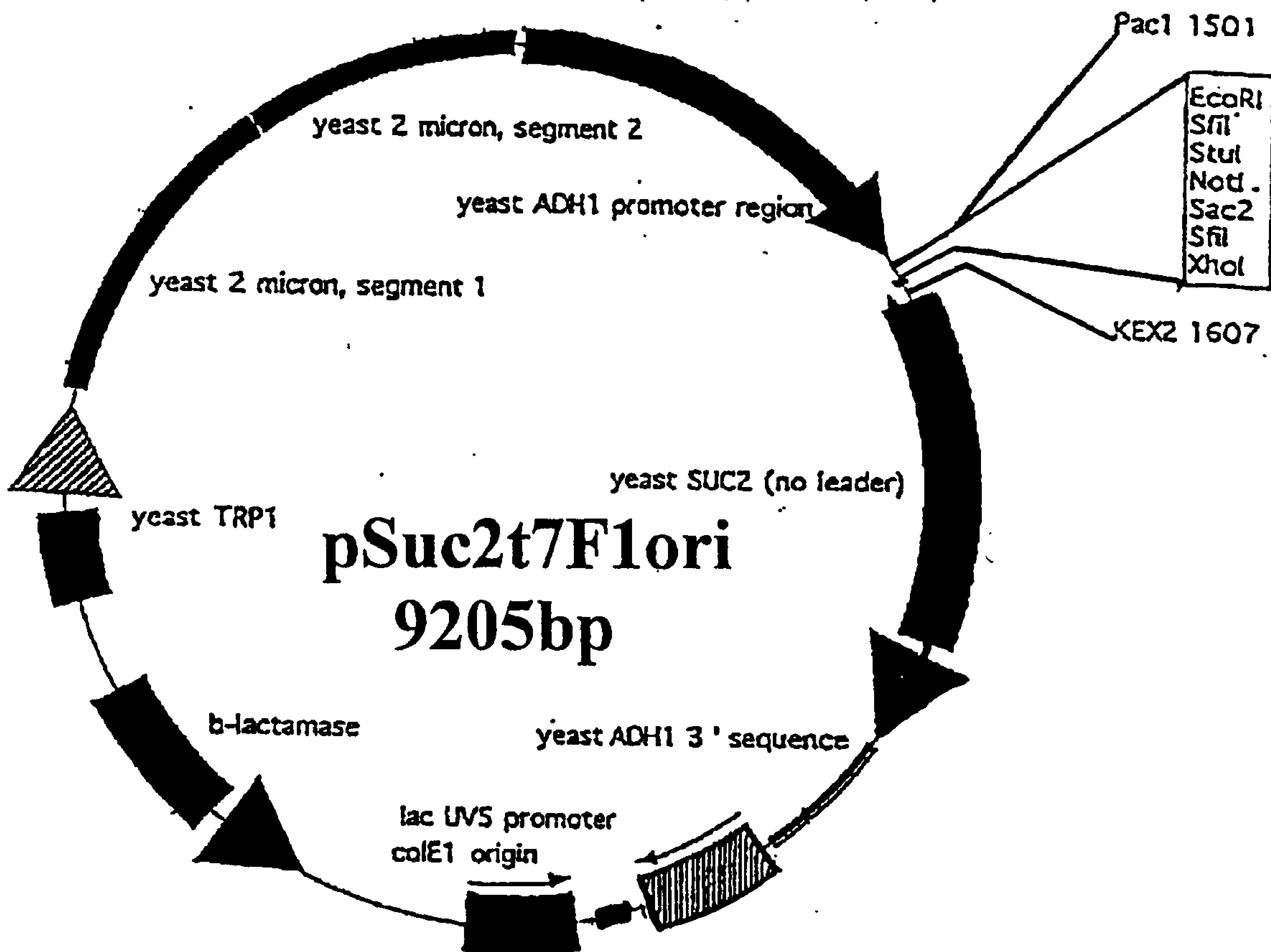


Figure 5.2: The yeast/.*E. coli* shuttle vector pSuc2t7F1ori.

This vector is designed to identify sequences in cloned cDNAs that mediate synthesis and transport of invertase into the endoplasmic reticulum and hence into the secretory pathway. The vector replicates episomally both in *E. coli* (colE1 origin, F1 origin) and yeast (2 micron segments 1 and 2) and carries the ampicillin resistance (β-lactamase gene) and tryptophan (Trp1 gene) selectable markers for *E. coli* and yeast respectively. The invertase reporter gene (lacking both its signal sequence and initiator methionine) is trancribed from the alcohol dehydrogenase promoter, a strong promoter in *S. cerevisiae*, and foreign ORFs can be cloned directly upstream of the invertase gene into the multiple cloning site.

5.2.2 Sub-cloning of ORFs into the yeast/*E. coli* shuttle vector, pSuc2t7F1ori

The yeast/*E. coli* vector and the invertase selection system are described in Figure 5.2.

Oligonucleotide primers were designed to amplify the sequences containing the required ORFs. The primers incorporated restriction enzyme sites to facilitate molecular manipulations and were designed to ensure that appropriate translation of the ORF would be maintained when ligated into the vector. All primers are shown in Appendix 1. Inserts were generated by PCR amplification using primers at 0.5pmol, dNTPs at 200μmol and the plasmid template at 0.5ng μl^{-1}. The enzyme and excess dNTPs were removed after successful amplification and the inserts digested with the appropriate restriction enzymes followed by ligation into the appropriate vector that had been linearised with the corresponding restriction enzymes. Competent HB101 *E. coli* cells (Stratagene) were transformed with the ligation products and transformants were selected on LB agar plates containing the kanamycin (25μg ml^{-1}).

5.2.3 Lithium Acetate Transformation of *S. cerevisiae*

Fresh cells were transformed essentially as described by Becker and Guarente (1991). Briefly, 1ml of an overnight culture (approximately $5x10^6$ cells ml^{-1}) was used to inoculate 25ml warm YPD and the culture grown at 30°C, with shaking, to an OD_{600} of 0.8. The cells were washed twice in 10ml sdH_2O after having been pelleted by centrifugation for 5 minutes at 5000 r.p.m. and the supernatant discarded. A final wash was carried out using 10ml LiAc/TE buffer (1M Lithium acetate, 100mM Tris-Cl pH 7.5, 1mM Na_2EDTA) after which the cells were resuspended in 300μl LiAc/TE and incubated at 30°C for 15min.

To each 50μl aliquot of cells was added 400μl PEG/LiAc/TE buffer (40% PEG, LiAc/TE), 1-5μg plasmid DNA and 50μg herring sperm DNA (heated to 95°C and snap cooled just prior to use). The cells were then incubated at 30°C for 30 minutes and then for a further 20 minutes at 42°C before being pelleted and the supernatant removed. Finally the cells were resuspended in 300μl sdH_2O. The *S. cerevisiae* strain YTK12 was used for all experiments described in this thesis. This strain contains a chromosomal mutation that makes it a tryptophan auxotroph. Therefore, this was used as the selectable marker for transformation. Transformants were selected for by plating onto solid

tryptophan dropout plates (CMD-W;YPD medium lacking tryptophan) and grown for 3 days at 30°C.

5.2.4 Selection of transformants able to secrete invertase.

The yeast transformants were replica-plated onto solid sucrose selective medium (YPD-agar without glucose, with 2% sucrose) and grown for a further 8 days at 30°C. The resulting colonies were patched onto solid sucrose medium lacking tryptophan (CMD-W without glucose, with 2% sucrose) and grown for a further 8 days. Those still growing were then taken as invertase-secreting clones.

5.2.5 Isolation of plasmid DNA from *S. cerevisiae*

Plasmid preparations were made by pelleting cells from an overnight culture by brief centrifugation and resuspension in 100µl lysis buffer (2.5M LiCl, 50mM Tris-Cl pH 8.0, 6.5M EDTA, 4% (w/v) Triton X-100). An equal volume of phenol: chloroform (1:1) and 0.5g acid-washed glass beads (Sigma, 0.5mm) were added and the mixture vortexed vigorously for 2 minutes. After centrifugation the aqueous layer was taken, the DNA ethanol-precipitated, and resuspended in TE.

5.2.6 Generation of a schistosome SST library

A mixture of cDNA was generated from 10µg schistosome egg mRNA using the Stratagene cDNA synthesis kit, following the manufacturer's instructions.
First strand cDNA synthesis: The following components were added, in order, into a RNase-free microfuge tube:

- 5µl 10X first-stand buffer
- 3µl first-strand methyl nucleotide mixture
- 2µl linker-primer (as provided by manufacturers)
- 3.5µl DEPC-treated water
- 1µl RNase block ribonuclease inhibitor

All the reagents were vortexed and spun briefly in a microfuge before 34µl egg mRNA (0.13µg ml^{-1}) was added. The reaction mixture was left to stand at room temperature for

10 minutes after which time 1.5μl MMLV-RT (50U μl^{-1}) was added. The first strand-synthesis reaction was incubated at 37°C for 1 hour.

Second-strand cDNA synthesis: The following reagents were added, in order, to the first-strand synthesis reaction: 20μl second-strand synthesis buffer

6μl second-strand dNTP mixture

114μl sterile, distilled water

2μl RNase H (1.5U μl^{-1})

11μl DNA polymerase I (9.0U μl^{-1})

The reaction mixture was vortexed gently, spun in a microcentrifuge and incubated for 2.5 hours at 16°C.

Blunting the cDNA termini: The following reagents were added to the second-strand synthesis reaction: 23μl blunting dNTP mix

2μl cloned *Pfu* DNA polymerase (2.5U μl^{-1})

The reaction mixture was vortexed, spun briefly in a microcentrifuge and incubated at 72°C for exactly 30 minutes. The reaction mixture was then subjected to a phenol-chloroform extraction, followed by one chloroform extraction and the DNA recovered by ethanol precipitation. The resulting DNA pellet was resuspended in 9μl of EcoRI adaptors.

Ligation of EcoRI adaptors and phosphorylation of the EcoRI ends: The following reagents were added to the cDNA and EcoRI adaptors:

1μl 10x ligase buffer

1μl 10mM rATP

1μl T4 DNA ligase (4U μl^{-1})

The reaction was incubated at 8°C overnight and was stopped by heating to 70°C for 30 minutes. The following components were added to the reaction mix once it had cooled to room temperature:

1μl 10x ligase buffer

2μl 10mM rATP

6μl sterile water

1μl T4 polynucleotide kinase (10U μl^{-1})

The reaction was incubated for 10 minutes at 37°C followed by a 30 minute incubation at 70°C.

Digesting with XhoI: The following components were added to the reaction:

28μl XhoI buffer supplement

3μl XhoI (40U μl^{-1})

The reaction was incubated for 1.5 hours at 37°C after which 5μl STE buffer and 125μl 100% ethanol was added. The DNA was recovered after precipitation and resuspended in 14μl STE buffer.

This cDNA solution was used for ligation into the SST plasmid. Ligations were performed using a 1:1 molar ratio of cDNA: plasmid using T4 DNA ligase (Promega) as described in section 2.3.7.

5.3 RESULTS

To test the YSST system using schistosome sequences, the 5' ends of cDNAs encoding well characterised secreted proteins (p48, superoxide dismutase (SOD) and antigen 10-3), and cytoplasmic proteins (calpain and clone 11, an EST with homology to the schistosome phosphoglycerate kinase gene) were cloned into the vector pSuc2t7F1ori, in frame with the invertase coding region in the plasmid. The 5' end of the *S. cerevisiae* invertase cDNA was also cloned into the vector to serve as a positive control for the initial trial experiments. The DNA sequence and the corresponding peptide sequence of each of the inserts is shown in Figure 5.3. SUC^- yeast transformed with vector containing the homologous invertase signal peptide grew with a plating efficiency of 100% after 3 days under selection for invertase secretion. Equivalent growth rates and plating efficiencies were obtained with the clones transformed with plasmids containing each of the three schistosome signal sequences (p48, SOD, 10-3). In contrast, SUC^- yeast transformed with vector alone or with constructs containing the 5' ends of schistosome cDNAs for cytoplasmic proteins (calpain, clone 11) produced no visible colonies even after 7 days under selection (Figure 5.4). These experiments were repeated three times; it should be noted that on one occasion 1% of the clones capable of producing only cytoplasmic invertase (i.e. containing calpain or clone 11 cDNA) started to grow after 8 days under selection. Following selection on sucrose medium the cDNA inserts were amplified by PCR directly from the yeast clone and subsequently sequenced to ensure that the correct clone had been transformed (Figure 5.5).

The cDNA prepared from the egg mRNA contained fragments ranging from 100bp to 800bp, with the majority being ~100bp. This cDNA mixture was ligated into the SST plasmid. After transformation of the recombinant plasmids into SUC^- yeast and selection of transformants for invertase secretion, 4 clones were isolated out of a total of approximately 500 transformants. The four clones took eight days to appear on the selective plates. The amino acid sequence corresponding to the DNA sequence of each of the cDNA inserts is shown in Figure 5.6. The inserts were very short (5-11 amino acids), only one contained a hydrophobic amino acid and one even lacked a methionine.

S. cerevisiae invertase: (Genbank Accession number: E00284); Insert: 387bp (111aa)

Plasmid, pRB58, provided Dr C Kaiser (Kaiser *et al.*, 1987)

```
ttc tct cag aga aac aag caa aac aaa aag ctt ttt ctt tca cta acg tat atg
atg ctt ttg caa gct ttc ctt ttc ctt ttg gct ggt ttt gca gcc aaa ata tct
 M   L   L   Q   A   F   L   F   L   L   A   G   F   A   A   K   I   S
gca tca atg aca aac gaa act agc gat aga cct ttg gtc cac ttc aca ccc aac
 A   S   M   T   N   E   T   S   D   R   P   L   V   H   F   T   P   N
aag ggc tgg atg aat gac cca aat ggg ttg tgg tac gat gaa aaa gat gcc aaa
 K   G   W   M   N   D   P   N   G   L   W   Y   D   E   K   D   A   K
tgg cat ctg tac ttt caa tac aac cca aat gac acc gta tgg ggt acg cca ttg
 W   H   L   Y   F   Q   Y   N   P   N   D   T   V   W   G   T   P   L
ttt tgg ggc cat gct act tcc gat gat ttg act aat tgg gaa gat caa ccc att
 F   W   G   H   A   T   S   D   D   L   T   N   W   E   D   Q   P   I
gct atc gct ccc aag cgt aac gat tca ggt gct ttc tct ggc tcc atg gtg gtt
 A   I   A   P   K   R   N   D   S   G   A   F   S   G   S   M   V   V
gat tac aac
 D   Y   N
```

S. mansoni p48: (Genbank Accession number: M74170); Insert: 139bp (27aa)

Plasmid provided by Dr P LoVerde (Chen *et al.*, 1992)

```
gcttttgtataaacaatcgtagtgtattcgaaaatttctgttgttcaggattgaaaaa atg
                                                            M
aat tta tta gta ttc agt ata cta ata act tgt tta ctg aac agc gtt tat agt
 N   L   L   V   F   S   I   L   I   T   C   L   L   N   S   V   Y   S
gga tac aat gga tat act aat gga
 G   Y   N   G   Y   T   N   G
```

S. mansoni SOD: (Genbank Accession number:M28543); Insert: 240bp (80aa)

Plasmid, pBABY-SP-SOD, provided by Dr P LoVerde (Hong *et al.*, 1993)

```
atg aca gta tat tcc tat tta gtt ata tta ttt att ctt ctt gat aat tat tgt
 M   T   V   Y   S   Y   L   V   I   L   F   I   L   L   D   N   Y   C
tcc gca tat gga tat ggt tac tca tat tat cat cgt aga cat ttt gat ccg gct
 S   A   Y   G   Y   G   Y   S   Y   Y   H   R   R   H   F   D   P   A
att gct tca ttt acg aag gaa cca tat ata ggt gcg gtg tgg ttc aca caa cat
 I   A   S   F   T   K   E   P   Y   I   G   A   V   W   F   T   Q   H
gga gat tat atg tac gtt aat gga agc gtg gca gga ctc cca cct gga aaa ctg
 G   D   Y   M   Y   V   N   G   S   V   A   G   L   P   P   G   K   L
ttg ggt aca cat gtt cat cgt tat
 L   G   T   H   V   H   R   Y
```

S. mansoni 10-3: (Genbank Accession number:M22346); Insert: 300bp (89aa)

Plasmid, pUR288, provided by Dr W Kunz (unpublished)

```
cattatacaggacggaagttgaaattcaacaat atg aat atc tac ctt att ggt
                                   M   N   I   Y   L   I   G
ata tta tgt att gtg ggt ttg att att tca caa ggg tct act gct aac gga agt
 I   L   C   I   V   G   L   I   I   S   Q   G   S   T   A   N   G   S
cca ctt gac gat cga ttc aat gat gtt aat acc ata aac aag aaa caa ttc acc
 P   L   D   D   R   F   N   D   V   N   T   I   N   K   K   Q   F   T
gaa gag gaa ttt tcc aga ctg ata aat tcc atg tta aag aag tat atc gaa gac
 E   E   E   F   S   R   L   I   N   S   M   L   K   K   Y   I   E   D
aaa aac gtt gat atc cgc ata att gga aat aag aag gat aaa cag cct act cag
 K   N   V   D   I   R   I   I   G   N   K   K   D   K   Q   P   T   Q
aaa aca act ccc aaa cct aca aca cca aag
 K   T   T   P   K   P   T   T   P   K
```

S. mansoni calpain: (Genbank Accession number:); Insert: 279bp (74aa)

Plasmid, pRizk1C, provided by Dr M Eberl (Andresen *et al.*, 1991)

```
tgg tgt aga aaa ctt gaa aaa acc cta att cat tca tta taa ttt cta aga ata
ata atg gga cga ata caa att gtt tat tct cct gat gaa aat gta tct gga cga
     M   G   R   I   Q   I   V   Y   S   P   D   E   N   V   S   G   R
act aac cgt cct ggt aaa gag gta gtg gat cca cgt act ggt aga ata atc aaa
 T   N   R   P   G   K   E   V   V   D   P   R   T   G   R   I   I   K
gtg aaa cga gaa act cca gat gat tac ttg aat gta tta aaa ccc atc aaa ggt
 V   K   R   E   T   P   D   D   Y   L   N   V   L   K   P   I   K   G
cca aag cgt atg gaa ttt aat cca tat ctt cct aaa aca ctt act cca aaa gga
 P   K   R   M   E   F   N   P   Y   L   P   K   T   L   T   P   K   G
tat gct aag
 Y   A   K
```

Clone 11: (lung-schistosomulum EST; Genbank Accession number: AW499465); Insert: 141bp (41aa)

```
ggaattgccgatggttgg atg gga tta gat att ggt cca aaa aca att gaa gaa ttc
                    M   G   L   D   I   G   P   K   T   I   E   E   F
agt aaa gtg att agt cgt gcc aaa act att gtt tgg aac ggt ccg atg ggt gtt
 S   K   V   I   S   R   A   K   T   I   V   W   N   G   P   M   G   V
ttt gaa atg gac aaa ttt gca aca gga aca
 F   E   M   D   K   F   A   T   G   T
```

Figure 5.3: DNA and peptide sequences of each of the inserts cloned into the plasmid pSuc2t7F1ori.

The signal peptides are underlined.

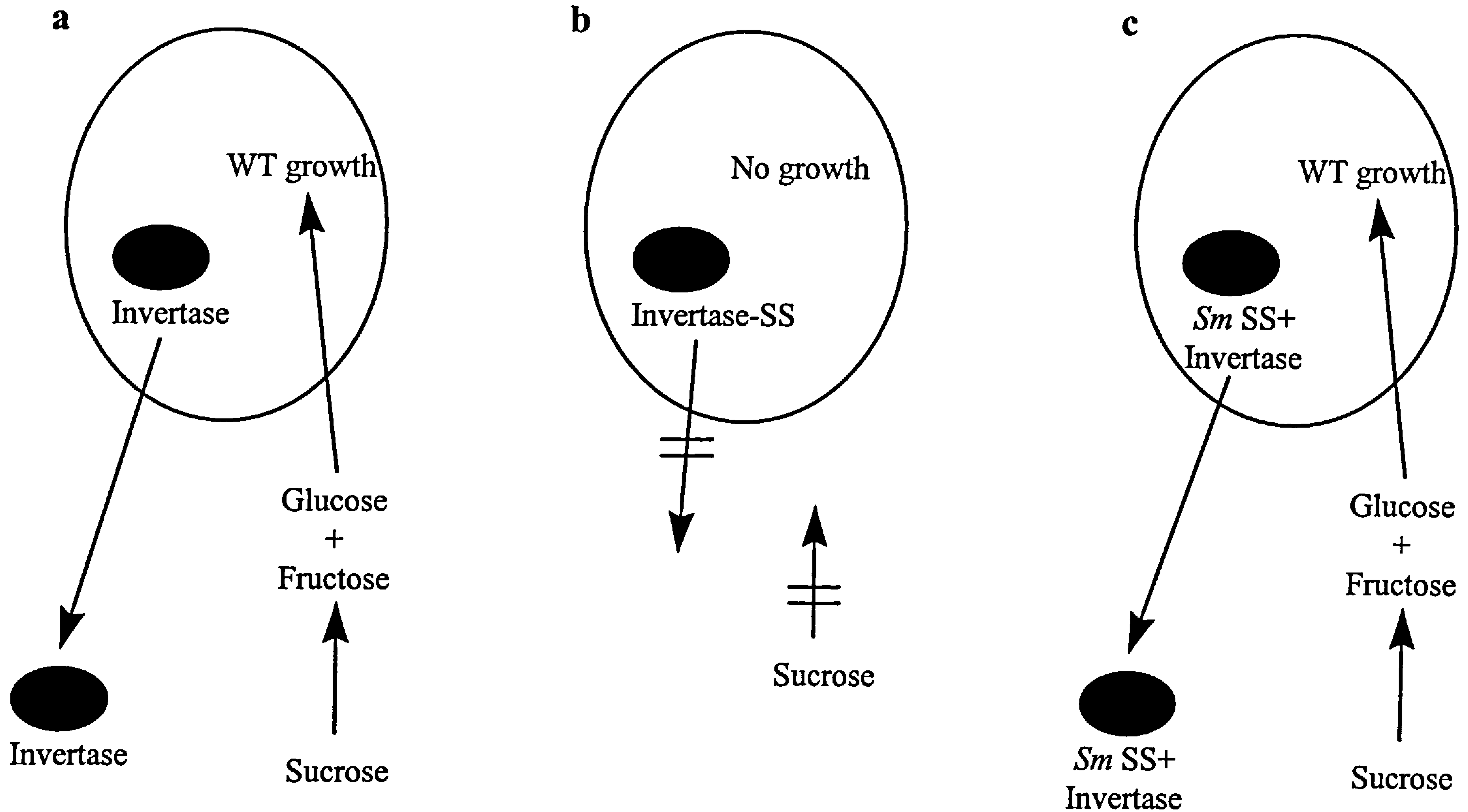


Figure 5.3: Schematic representation of the validation of the signal peptide selection system.

Each panel shows the result of transformation of the yeast strain, YTK12, with the plasmid, pSUC2T7M13ORI, into which were cloned the 5' ends of the genes encoding *S. cerevisiae* invertase including the signal sequence (a), *S. mansoni* calpain, clone 11 (b), *S. mansoni* p48, SOD, 10-3 (c). Panel b also represents the result obtained when no cDNA was cloned into the plasmid prior to transformation.

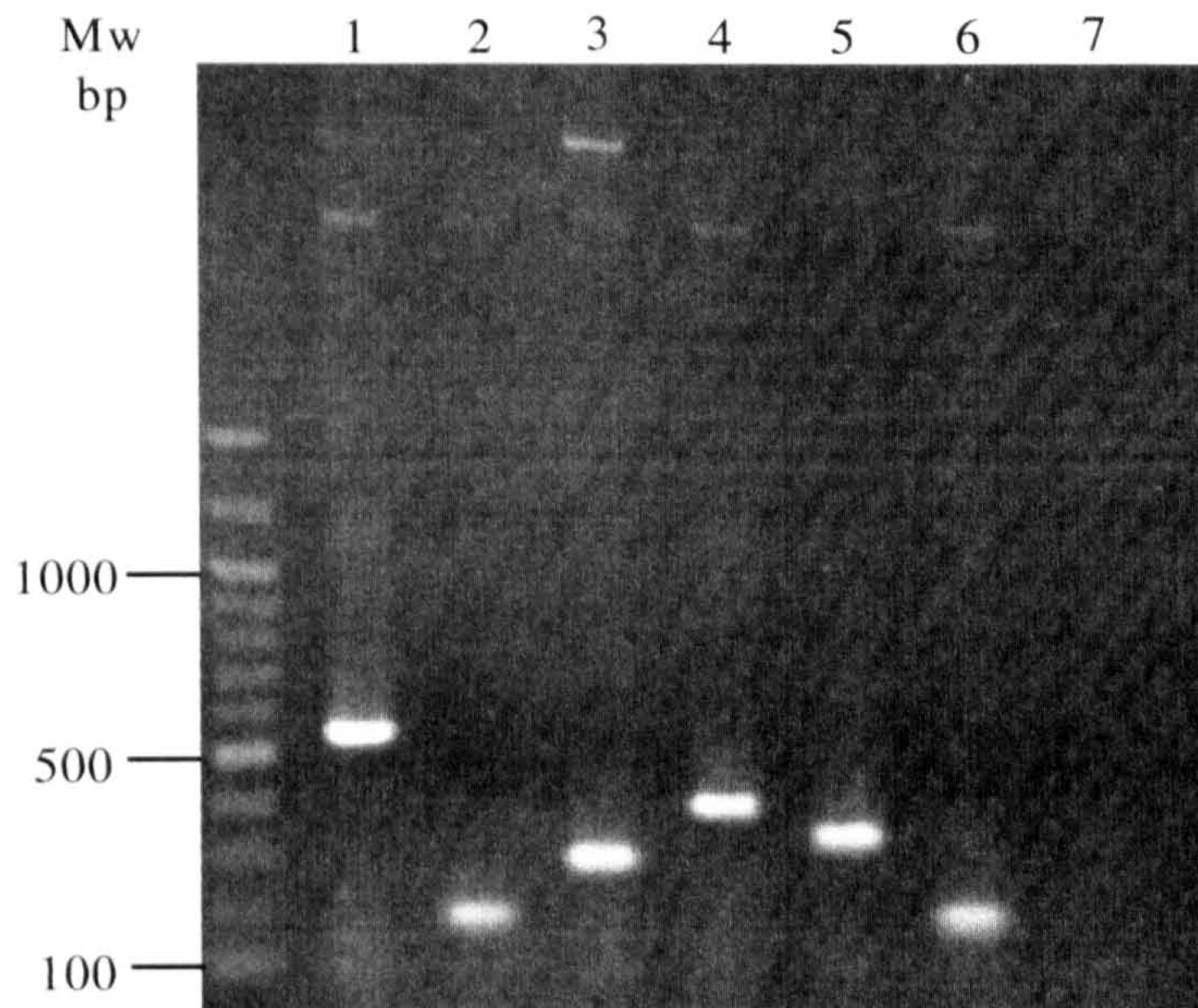


Figure 5.5: PCR of insert cDNAs directly from yeast clones.

Invertase (lane 1), p48 (lane 2), SOD (lane 3) and 10-3 (lane 4) cDNAs were amplified from their respective clones after selection on sucrose medium. Calpain (lane 5) and clone 11 (lane 6) cDNAs were amplified from their respective clones after selection on Trp^- medium. Lane 7 is the PCR contamination control into which no template DNA was added.

Clone 5.1

ATG	AAT	TCG	GCA	CGA	GAG
M	**N**	**S**	**A**	**R**	**E**

Clone 5.2

ATG	ATT	CGG	CAC	GAG
M	**I**	**R**	**H**	**E**

Clone 5.3

GAA	TTC	GGC	ACG	AGA
E	**F**	**G**	**T**	**R**

Clone 5.4

ATG	GAA	TTC	GGC	ACG	AGC	TGG	TGC	CGA	ATT	CGG
M	**E**	**F**	**G**	**T**	**S**	**W**	**C**	**R**	<u>**I**</u>	**R**

Figure 5.6: Sequence of the cDNA PCR amplified from the four clones positively selected on sucrose medium.
The hydrophobic amino acid is underlined.

5.4 DISCUSSION

The results presented in this chapter demonstrate that the YSST is effective for the selection of schistosome cDNAs encoding M/S proteins as clones with intact signal sequences were able to grow on sucrose medium whereas those clones without did not grow. These results also show that the YSST is not affected by the 5' UTR of schistosome sequences (p48, 10-3) and that it is able to process relatively long cDNA fragments encoding schistosome proteins efficiently (SOD, 10-3). The occurrence of 1% of clones that are able to grow after 8 days, when producing only cytoplasmic invertase, is similar to the results shown by Jacobs *et al.* (1997) where 2% of such clones grew after selection on sucrose medium for 8 days.

The four cDNAs isolated from the egg cDNA mixture using the YSST code for none of the hallmark features of signal peptides as described in Chapter 3. However, the growth of the four clones was slow (8 days on sucrose medium); equivalent to that of the 1% spurious clones containing calpain or 11 cDNA. Short fragments of DNA that do not code for hydrophobic peptide regions have been trapped in all of the SST systems so far described. The reason for this remains unknown but this highlights the importance of size fractionation of the cDNA being screened. Simply reducing the number of short cDNA fragments can reduce the number of such false positives. Kaiser *et al.* (1987) noted that such slow growing transformants produce high levels of non-glycosylated invertase (i.e. cytoplasmic invertase). Therefore, taking only those colonies that are able to grow before day 5 post-replica-plating could also reduce the number of false positives. True signal sequences, fused in frame to the invertase gene, should be able to direct secretion of the enzyme and allow growth of the colony within this time.

As the cDNA mixture used in this study was oligo d(T) primed, it is likely that the four cDNA clones identified represent schistosome 3' UTRs. This can be deduced as every cDNA cloned into the YSST vector would have contained an in-frame stop codon before the poly(A) tail, that is present on most mRNAs. Therefore, the yeast translation machinery would have stopped before reaching the invertase transcript further downstream, resulting in no invertase protein being produced. However, those cDNAs which fortuitously have an in-frame methionine codon in the 3' UTR (after the stop codon) will allow for translation of the invertase mRNA to produce functional, cytoplasmic enzyme. Therefore, it is essential to use cDNA that has been synthesised from mRNA that was reverse transcribed by random-hexamer priming as this reduces the

frequency of stop codons and 3'UTRs. This also increases the number of cDNAs with intact 5' ends in the library. Screening was carried out using the oligo d(T) primed egg cDNA before the importance of random-hexamer priming had been fully appreciated. All of the schistosome cDNA libraries available to the scientific community have been constructed using oligo d(T) primed reverse transcription (Johnston *et al.*, 1999). Therefore, before the YSST can be applied effectively to schistosomes, new cDNA libraries will have to be constructed using random-hexamer priming.

More recently, there have been further publications reporting the use of the YSST to identify M/S proteins from *Arabidopsis* (Goo *et al.*, 1999a) and from *Drosophila* (Goo *et al.*, 1999b). These reports show that the yeast system is versatile and can be used for the identification of M/S proteins from a diverse range of organisms. A comparison of the data from these two papers and the Klein *et al.* (1996) and Jacobs *et al.* (1997) papers reveals that of the total number of unique clones identified, on average, 88% represent M/S proteins. This percentage seems to vary with the quality and/or source of the starting mRNA used for library construction. However, in comparison to random sequencing or antibody screening, this represents a much more efficient method for the isolation of M/S proteins. In addition, the number of redundant clones reported in the four publications was relatively small with no one molecular species predominating. This is in contrast to antibody screening where several highly immunogenic molecules are identified repeatedly (Harrop *et al.*, 1999). If when applied to schistosomes, a few cDNAs predominate after the first transformation, then a simple screen to remove these before sequencing can be used. For example, PCR amplification of the inserts from all positive clones, followed by gridding onto nylon membranes and probing with the previously identified abundant cDNAs.

Goo *et al.* (1999a) state that only 0.1% of the total cDNAs are isolated by the YSST. Although the percentage of cDNAs encoding M/S proteins identified was much lower than the expected frequency ratio (10%) of M/S proteins among total expressed proteins, the study by Jacobs *et al.* (1997) demonstrated that particular genes are isolated partly as a function of sample size. As the majority of genes coding for M/S proteins are likely to be expressed at relatively lower levels than those genes coding for proteins involved in protein metabolism, the number of cDNAs corresponding to M/S proteins in the library is likely to be small. Therefore, this suggests that the YSST would allow for the identification of rarely expressed genes. Another reason for the low percentage might be that many of the cDNAs cloned into the vector were out of frame with the invertase gene.

A simple modification to the current vector to create a series of three plasmids, each of which allows for the fusion of cDNAs in a different reading frame, would increase the probability of a particular cDNA being in-frame with the invertase gene. This might increase the total number of cDNAs encoding M/S proteins identified.

Finally, it will be necessary to determine the localisation, in the parasite, of each M/S protein identified before deciding which ones to pursue as vaccine candidates. *In situ* hybridisation, using the cDNAs isolated by the YSST as probes, is one such option. However, past experience in the laboratory has shown that the ProteinaseK treatment step damages the tissues to such an extent that individual structures cannot be identified (R A Wilson, pers. comm.). Therefore, an immunocytochemical approach will be used. Before this can be done, expression and purification of each protein would have to be carried out in order to obtain sufficient protein to permit antibodies to be raised in mice. However, this is a lengthy and often difficult process as recombinant protein expression may fail for many reasons (see section 3.4.3). A more direct way to purify the expressed proteins would be modify the yeast plasmid further by adding a sequence encoding an affinity purification tag in front of the Kex2 recognition site, i.e. at the C-terminus of each expressed schistosome polypeptide. This would allow the secreted protein to be captured from the culture medium either using monoclonal antibodies or by IMAC. As only 10μg recombinant protein are needed to raise antibodies in mice using TitreMax Gold (Sigma) as adjuvant, this seems a feasible approach. However, yeast has a cell wall and most secreted proteins remain in the periplasmic space between the plasma membrane and the cell wall itself. Therefore, even though the invertase is secreted into the culture medium in this system, there is no guarantee that the schistosome polypeptides will be treated in the same way. If the schistosome polypeptides do remain in the periplasm, it should be possible to purify adequate amounts of protein by isolating the periplasmic fraction first by removing the cell wall only, leaving an intact spheroplast.

Overall, the YSST has been shown to identify secreted proteins, various different types of membrane proteins and proteins that are present in the ER, or other structures downstream of the ER, from an evolutionary diverse set of organisms. Application of this system to schistosomes should lead to the isolation of a diverse group of M/S proteins, many of which have not been described previously.

CHAPTER 6

CONCLUDING DISCUSSION

This chapter summarises the achievements of this investigation, the application of alternative approaches and the implications for future work.

In contrast to the large body of information available on the protective mechanisms which operate against *S. mansoni* in the murine host, there is still very little known about the antigens involved. Given that the schistosome genome contains 15 000 to 20 000 expressed genes, there is an enormous pool of potential vaccine candidates, making the task of pinpointing the relevant antigens difficult. The problem is compounded by the fact that many somatic antigens are highly immunogenic but are unlikely to be involved in protection (Harrop *et al.*, 1999). Based on detailed information available on the induction and effector phases of immunity in the R-A vaccine model, it has been hypothesised that the proteins released from schistosomula (i.e. M/S proteins) would provide a source of potential vaccine candidates. The limited amount of parasite material available for study, particularly for the larval stages, makes M/S antigen characterisation by conventional biochemistry difficult. Immunoscreening of cDNA libraries with serum raised against the released proteins of schistosomula has been carried out, but unfortunately, the majority of clones isolated encoded the same somatic antigens identified in other studies, and of the few unknowns only one coded for a putative signal peptide (Harrop *et al.*, 1999). Therefore, the aim of this thesis was to use several strategies to identify and clone M/S antigens from schistosomes.

Computational Biology

As the schistosome genome project gathers momentum an increasing number of genes are being sequenced without any further characterisation of the products they encode. Many computational methods have been developed to predict protein function. The two main approaches are sequence alignments (Altschul *et al.*, 1990; Pearson *et al.*, 1996) and sequence-motif methods (Bairoch *et al.*, 1996; Henikoff and Henikoff, 1994; Attwood *et al.*, 1997). Several of these programmes have been used to identify protein functions in those organisms whose complete genome sequence is available, e.g. *C. elegans* (Hutter *et al.*, 2000) and *Mycoplasma genitalium* (Koonin *et al.*, 1996). One such sequence-motif algorithm, SignalP, was applied to all of the 427 sequences in the schistosome protein database (Chapter 3). This revealed how little information is available for M/S proteins from schistosomes (only 23 well characterised proteins). The method clearly pinpoints those schistosome proteins that are targeted to the secretory pathway, plasma membrane,

or for export. It has not identified any non-M/S proteins, and as far as I am aware, it has not missed any published M/S sequences.

The subsequent application of SignalP to the schistosome EST dataset (Chapter 3) has revealed 218 DNA sequences that may encode putative signal peptides, thereby increasing dramatically the number of novel DNA sequences from schistosomes that code for M/S proteins (approximately 10 fold). Therefore, this approach represents a relatively simple, cost-effective method to screen large amounts of sequence data for putative M/S encoding genes. However, as discussed by Schneider *et al.* (1999) and Nielsen *et al.* (1999), atypical signal peptides will not be detected by such algorithms. In order to ensure that all schistosome signal peptides are detectable it would be necessary to train the algorithm on a large set of schistosome signal peptides that had been experimentally determined. At present, with only 23 schistosome signal peptides a large enough set is not available to train a predictive algorithm.

Although this approach works very well when analysing full-length cDNA sequences, the data obtained from EST analysis has to be considered carefully. As ESTs are usually obtained by single-pass sequencing any errors in the DNA sequence could lead to the generation of false stop codons and hence to the selection of the wrong reading frame for analysis. Therefore, the use of other prediction methods such as those for start codons (to eliminate trans-membrane fragments) and ORFs (to eliminate untranslated regions) in tandem with SignalP should reduce the number of false annotations. This should allow for effective analysis of the schistosome ESTs prior to the availability of the complete genome sequence.

Differential gene expression

Experimental approaches to analyse gene function are currently based on the study of the transcriptome (DeRisi *et al.*, 1997), the proteome (Blackstock and Weir, 1999) and the metabolome (Oliver *et al.*, 1998). Currently, the analysis of the transcriptome is the most easily achieved, as the technology to manipulate DNAs and RNAs is much simpler than that for proteins. Subtractive hybridisation, differential screening and DD were the first techniques that made it possible to identify differentially expressed genes by comparison of the levels of mRNAs found in various cell types or developmental stages of an organism. As DD and RAP-PCR are based on PCR, small amounts of mRNA are required. This is an important advantage when working with parasites, as they are often only available in small quantities, especially the larval stages of schistosomes.

Therefore, RAP-PCR was used to identify those genes that were stage-specifically-expressed in schistosomes (Chapter 4). This study showed that several transcripts are differentially expressed as the parasite matures from a cercaria to an adult worm. A total of 9 stage-specific cDNAs and 4 differentially expressed cDNAs (i.e. those that were present in only 2 of the 3 life-cycle stages) were excised from gels. Several of these cDNAs were used to probe Southern blots of schistosome DNA. The majority of them represent genes derived from the schistosome genome as they gave single bands on the Southern blot. However, the expression pattern of each one remains to be verified by Northern blotting or quantitative RT-PCR. However, the application of RAP-PCR is not without technical problems and practical limitations. The numbers, sizes and intensities of amplified fragments are extremely sensitive to small changes in reaction conditions. Cycling parameters, specifically the number of amplification cycles and annealing temperatures may also affect banding patterns. This makes the technique very labour-intensive to ensure that all variable parameters have been controlled and/or optimised and the possibility of generating PCR-artefacts kept to a minimum. In addition, a considerable amount of time and resources often has to be expended before any information about the function of the differentially expressed transcripts is found.

More recently, techniques such as suppression-subtractive hybridisation (Diatchenko *et al.*, 1996), serial analysis of gene expression (SAGE, Velculescu *et al.*, 1995) and its variants e.g. tandem-arrayed ligation of expressed sequence tags (TALEST, Spinella *et al.*, 1999) have been described. Such techniques now represent more efficient, high-throughput methods for the determination of expression profiles of various genes and as such may be the methods of choice for the future. Although these techniques offer much, the cost of sequencing the required number of fragments/genes is very high; hence these are not presently viable for individual research groups. However, these techniques could be incorporated into the schistosome genome network programme together with the cDNA microarray approach that is already being used (Williams and Johnston, 1999). Limitations remain with respect to the number of genes that can be analysed in a single microarray, and of course the method is applicable only to those genes whose sequence is already available. An additional drawback of all of these applications, as with the computational approach (described in Chapter 3), is that once a cDNA is identified a lot of work has to be done to characterise the protein function before its potential as a vaccine candidate can be assessed. Therefore an experimental method that allows for

isolation of clones based on protein function would probably be more fruitful in the hunt for genes coding for M/S proteins.

Signal sequence trap

Genomic assays which select ORFs from a pool of genes based on the function of the encoded protein are another possible route to identify those of particular interest. Two basic types of functional assay exist: (i) those based on purified, expressed proteins and their effect in an extrinsic assay (ii) those based on the transformation of a host cell, with protein expression of each cloned gene allowing for selection on the basis of a suitable assay of the host cell.

The YSST described in Chapter 5 is a genomic assay, which selects ORFs based on the ability of the corresponding protein to direct secretion in yeast. Therefore, this functional assay overcomes the major limitation of the two previously discussed approaches, as any clone identified by YSST can be annotated as encoding a M/S protein without any further verification. In addition, only enough parasite material to make one random-primed cDNA library from each life-cycle stage is required. This overcomes the problem of obtaining large amounts of parasite material repeatedly. Therefore, the YSST should allow for the efficient isolation of M/S proteins from schistosomes. Also, it may be possible to purify recombinant protein directly from clones, thus removing the need to sub-clone each cDNA into a high-level expression vector (a significant saving of time and money).

The results presented in Chapter 5 show that the yeast secretion machinery recognises the schistosome signal peptides and is able to discriminate between secreted and cytosolic proteins. As the classical secretion pathway is conserved, even between prokaryotes and eukaryotes (reviewed by Walter and Johnson, 1994), this system should also allow for the isolation of those M/S proteins with atypical signal sequences, unlike the computational approach. The sequences obtained from the YSST would also provide a set of schistosome signal peptides that could be used to train computational prediction methods such as SignalP in the future.

It is thought that the majority of M/S proteins are targeted to their final destinations by the N-terminal signal sequence via the classical pathway described in this thesis. However, it must be noted that both prokaryotes and eukaryotes have a small number of M/S proteins that do not have classical signal peptides and reach their final destinations via other pathways (Rapoport *et al.,* 1996 and references therein). Therefore, it is

unlikely that the YSST or SignalP will identify every single M/S protein from schistosomes. At present very little is known about the alternative secretion pathways. Therefore no suitable methods to identify those proteins that are secreted via these pathways can be developed. Overall, of the three approaches used in this study, the YSST represents the most efficient method to isolate the majority of the schistosome genes encoding M/S proteins.

Proteomic techniques could be used to identify and characterise the subset of proteins of interest directly. However, as M/S proteins tend to have hydrophobic regions, which means that such proteins often do not go into solution, prior to separation by 2-D electrophoresis. Therefore, there are no suitable techniques available at present that allow for the enrichment of this subset of proteins from total parasite proteins. Additionally, the amount of parasite material available for study is still a limiting factor for such analyses. For those proteins that are identified by 2-D electrophoresis and mass spectrometric analysis, their subsequent analysis is hindered by the lack of DNA sequence available for schistosome genes.

The application of YSST to schistosomes and the use of several computer algorithms to analyse the schistosome DNA sequences should lead to the identification of many genes coding for M/S proteins. Characterisation of each of these proteins, in terms of its function and expression pattern, may lead to a better understanding of basic parasite biology, including signalling pathways in the parasite, parasite-parasite and host-parasite relationships. In order to focus on those proteins most likely to be involved in the induction and priming of the relevant immune responses, a preliminary screen will have to be carried out, prior to their evaluation as potential vaccine candidates. Immunolocalisation studies, to identify only those proteins that localise to parasite tissues from which such proteins could be derived (e.g. the head gland, larval tegument), are likely to be the most informative.

In summary, this thesis describes the development of several different approaches to identify the antigens that have been implicated as important stimulators of the immune response elicited by the RA vaccine model. It is hoped that the YSST will allow for the identification of M/S proteins and that further characterisation of some of those proteins will eventually lead to trial vaccination experiments in mice.

APPENDIX 1

Primers used for generation of ESTs

M13R	CAGGAAACAGCTATGAC	17mer
T7	TTARACGACTCACTATAGGG	20mer

Primers used for PCR to generate inserts for high level expression vector, pET30

CL15'	CCGGAATTCACAACTTCATATGGACTAAAGATGAAT	36mer
CL13'	CCGCTCGAGTTCGTTATAATGTCCTCT	27mer
0465'	CGGGGTACCAATGTCTGAATTTTTCGGA	28mer
0463'	CCGCTCGAGTTCTGAGTGATTTAAATTGGC	30mer
1235'	CCGGAATTCACGAGATACTTAACAATCTTCTTACAAACT	39mer
1233'	CCGCTCGAGTTTATTTATTTGTTTAAGTCCCCACTTTC	39mer

Primers used for RAP-PCR

C2	GAAGGCTGGTAAACAAAAGG	20mer
G1	CTGAATCGCTGTATAAAAGT	"
G2	GGAAAACAAATGGTCGGAAG	"
KS	TCGAGGTCGACGGTATC	"
SK	CGCTCTAGAACTAGTGGATC	"
SP6	GATTTAGGTGACACTATAG	"
M13R	CAGGAAACAGCTATGAC	17mer
T7	TTARACGACTCACTATAGGG	20mer

Signal sequence primers

S1	ATGACGCTGGCCTTATTAGT	20mer
S2	ATGCTTATTAGTGTTTTAGT	20mer
S3	ATGACGTTAATTTCATTAGT	20mer
S4	ATGGCTTTGTTAGTTATTTC	20mer
S5	AGCTTATTA	9mer
S6	ATGAATACTCTGGTTATTAT	20mer

Primers used for PCR to generate fragments for plasmid pSUC2t7F1ori

SUCSIG5'	CGGAATTCCGTTCTCTCAGAGAAACAAGCA	30mer
SUCSIG3'	CCGCTCGAGCGGTTGTTGTAATCAACCACCAT	32mer
p48SIG5'	CGGAATTCCGGCTTTTGTATAAACAATCGTAGTGT	35mer
P48SIG3'	CCGCTCGAGCGGCATTGTATCCACTATAAACGCTGT	36mer
SODSIG5'	GGAATTCCATGACAGTATATTCCTATTTAG	30mer
SODSIG3'	CCGCTCGAGCGGCATAACGATGAACATGTGTA	32mer
10-3SIG5'	GGAATTCCCATTATACAGGACGGAAGTT	28mer
10-3SIG3'	CCGCTCGAGCGGAGTAGGCTGTTTATCCTTCT	32mer
CalpainSIG5'	CGGAATTCCGTGGTGTAGAAAACTCGAAAAAACC	34mer
CalpainSIG3'	CCGCTCGAGCGGACTTAGCATATCCTTTT	29mer
Clone11SIG5'	CGGAATTCCGCCGATGGTGGATGGGAT	27mer
Clone11SIG3'	CCGCTCGAGCGGGAGTACTTCATCCAT	27mer

Sequencing primers for yeast signal sequence trap

SUC2a	TTCTGCACAATATTTCAAGA	20mer
SUC2b	GCTTCAGCTTCTCTTCTA	18mer

APPENDIX 2

Genotypes of *E. coli* strains

XL1-Blue (Stratagene): recA1 end A1 gyrA96 thi- 1 hsdR17 supE44 relA1 lac {F'*pro*AB *lacI*^{q}ZΔM15 Tn10 (Tet^r)}^{c}

BL21(DE3)pLysS (Novagen): F$^-$ *omp*T *hsd*S_B (r_B^- m_B^-) *gal dcm* (srl-recA) 306:: Tn 10 (Tc^R) (DE3) pLysS (Cm^R)

INVαF' (Invitrogen): F' *end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169

HB101 (Statagene): supE44 ara14 galK2 lacY1 Δ(*gpt-proA*)62*rpsL*20 (Str^r) *xyl-5 mtl-1 recA13* Δ*(mcrC-mrr)* HsdS$^-$(r$^-$ m$^-$)

Genotype of *S. cerevisiae* strain

YTK12: suc2Δ9 ade2-101 trp2-52

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