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MOLECULAR CHARACTERIZATION OF MEMBRANE PROTEINS OF SCHISTOSOMA MANSONI

by

Steven Robert Karcz

Department of Zoology

Submitted in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

April, 1990

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ABSTRACT

Schistosomiasis is one of the most prevalent human diseases and is caused by the long-term survival of parasitic blood flukes. Membrane constituents at the surface of these parasites play a major role in parasite metabolism and elaborate sophisticated mechanisms for evasion of the host immune response. Despite this fact, little is known about the molecular properties or functions of individual membrane proteins. This study provides information regarding two polypeptides associated with the apical plasma membrane (APM) of Schistosoma mansoni.

An in vitro system for studying protein phosphorylation in the isolated APM was developed and the protein substrates of endogenous protein kinase activity were described. A 24 kilodalton (kDa) phosphoprotein was characterized in detail. Analysis of the structure of this polypeptide by lectin affinity chromatography, endoglycosidase digestion and phase separation in Triton X-114 demonstrated that the 24 kDa molecule was an integral membrane protein with N-linked oligosaccharides. In addition, the 24 kDa phosphoprotein was shown to be a major APM immunogen by immunoprecipitation with anti-APM antisera and with antibodies affinity purified from the 24 kDa region of preparative Western blots.

In order to isolate cDNA clones encoding APM polypeptides, comprehensive cDNA expression libraries were constructed in λ bacteriophage

vectors and were screened with anti-APM antisera. Two cDNA clones were isolated and their nucleotide sequences determined. One cDNA was 141 base pairs in length and was shown to encode antigenic determinants shared with the 24 kDa phosphoprotein antigen. This cDNA was not full length since the homologous mRNA was approximately 800 residues in length. A possible open reading frame from this cDNA however, contained a signal for N-linked glycosylation. A second near full-length cDNA of 2621 base pairs was also cloned and sequenced. An open reading frame deduced from this cDNA predicted a protein of 702 amino acids with a molecular weight of 76 kD. The deduced protein sequence was shown to be similar to the known sequences of vertebrate calpains.

This study provides the basis for a detailed structure/function analysis of the membrane-associated polypeptides encoded by the cloned cDNAs.

DEDICATION

This thesis is dedicated to my parents and grandparents

because they always understood

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I would like to thank my supervisor, Ron Podesta for stimulating my initial interest in schistosomes and for allowing a great deal of freedom of expression. His patience, guidance and encouragement throughout the years have helped me in many ways. I would also like extend my gratitude to Dr. G.A. Mackie. Much of the early work in this thesis could not have been done without his guidance. Special thanks also to Michael Clarke for many hours of helpful discussion.

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Chapter 1

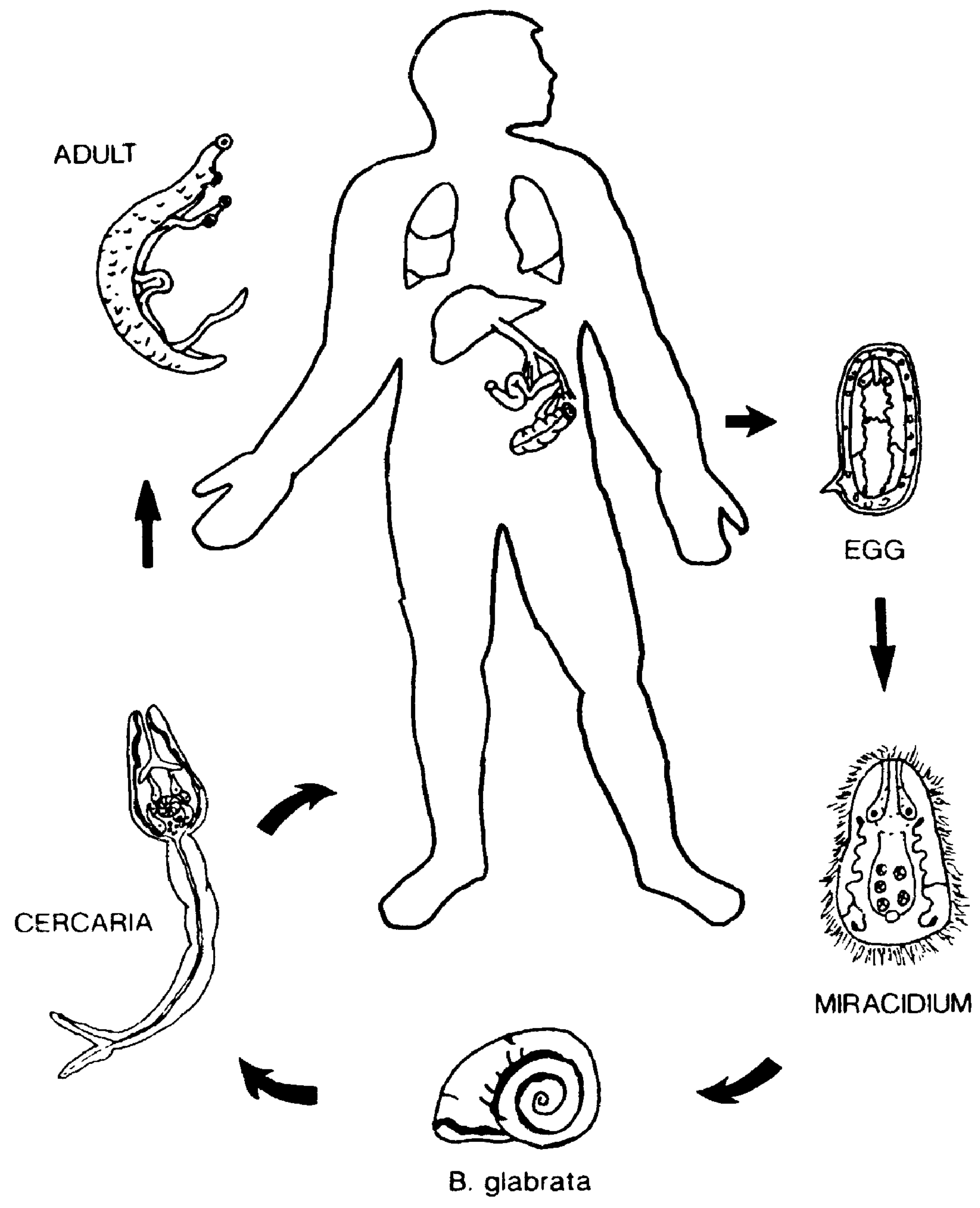
1.1 General Introduction

Schistosomiasis, as a human disease, poses unique and far-reaching challenges to governments, health care organizations and researchers whose goal is the control of the disease and its manifestations in man. Seventy-five tropical and sub-tropical countries have regions where schistosomiasis is endemic, with two hundred million individuals currently infected and millions more at risk of infection (Mott, 1987). The etiological agents of human schistosomiasis are three blood-dwelling helminth parasites of the genus Schistosoma, namely, S. japonicum, S. mansoni, and S. haematobium. Schistosoma japonicum is found in regions of the Far East and the Philippines, whereas S. haematobium and S. mansoni are distributed primarily throughout sub-Saharan Africa. In addition, S. mansoni is also found in three South American countries and a number of Caribbean islands.

By far the best studied of the human schistosomes (and the subject of this thesis) is S. mansoni, whose life cycle is shown in Figure 1. Infection is initiated by penetration of the cercariae through the skin of a potential host which has come into contact with infested fresh water. The cercariae, upon penetration, lose their tails, and reside in the sub-cutaneous skin layers for about one day. The schistosomula, as the newly penetrated juveniles are known, then begin a period of migration within the bloodstream of the host

Figure 1.1: The Life Cycle of Schistosoma mansoni.

Human schistosome infection is initiated following penetration of the free-living cercariae directly through the skin. The resultant juveniles, called schistosomula, then migrate through the bloodstream via the lungs and eventually come to reside within the hepatic portal system or venules of the mesenteric plexus. It is here that male and female parasites pair and the female begins to lay eggs at about 30-35 days post-infection. Eggs deposited within the bloodstream either become lodged within the liver and other tissues giving rise to the pathology characteristic of schistosomiasis, or are voided into the environment with the faeces. Upon contact with freshwater, the eggs hatch releasing the miracidia, which seek out and penetrate an appropriate molluscan host (Biomphalaria glabrata). After a period of asexual reproduction within the snail, the cercariae are released to continue the life cycle.


ADULT
EGG
MIRACIDIUM
B. glabrata
CERCARIA

which eventually carries them to the lungs at or around seven days after infection. A number of morphological and biochemical changes occur while the worms reside in the lungs which presumably prepare the parasites for further migration Approximately two to three weeks post-infection, the worms leave the lungs and are carried by the flow of blood to the liver. Within the liver sinusoids the worms begin to feed on host red blood cells, and continue to grow and develop. After thirty to thirty five days within the vertebrate host, the worms migrate into the hepatic portal and mesenteric veins, male and female parasites pair, and the female becomes patent. Eggs deposited by the female can either penetrate through the wall of the intestines or be carried back to the liver with the flow of blood. Eggs which become lodged in the liver give rise to an immune response which is largely responsible for the pathology observed in infected hosts. On the other hand, eggs which are voided into the environment and contact fresh water subsequently hatch to release the ciliated miracidia, which seek out and penetrate the tissues of the molluscan intermediate host (Biomphalaria glabrata). Within the snail tissues, the parasites reproduce asexually, giving rise to many thousands of clonally-derived fork-tailed cercariae. After a period of four to six weeks within the snail, the cercariae are released and continue the life cycle.

1.2 The Pathology of Schistosomiasis mansoni

As mentioned earlier , the pathology of schistosomiasis results from the cumulative effects of long-term oviposition by the female parasite while residing within the hepatic portal and intestinal vasculature. It has been estimated that a mature female from of S. mansoni may produce an average of 300 embryonated eggs per day. This process continues for the life span of the parasite, which has been estimated to be about three to five years but may be as long as thirty years (Vermund et al., 1983). Approximately half of the eggs pass into the lumen of the intestine where they are voided with the feces while the remainder become trapped within liver tissue and vasculature, lung and other sites (von Lichtenberg, 1987). Despite the seemingly relentless deposition of eggs, the disease spectrum of chronic schistosomiasis is surprisingly diverse ranging from mild abdominal cramps, fever, nausea and diarrhea to full blown hepatosplenomegaly (enlargement of the liver and spleen) and portal pipestem fibrosis with the development of collateral circulation and esophageal varices. The disease can be fatal in the most severe cases as a result of the rupturing of esophageal varices with attendant haemorrhagic shock. While the factors which predispose individuals for disease are at present not fully understood, it has been estimated that 5-10% of heavily infected patients ultimately progress to severe hepatosplenic schistosomiasis (Butterworth, 1988).

Generally, the migrating larvae themselves elicit little more than inflammatory reactions during migration and development within the mammalian host. The adult parasites too, once having achieved their definitive location within the host, appear to elicit little in the way of direct pathological consequence. In contrast, eggs immobilized within the liver, spleen and intestine and products released from the developing miracidium elicit a wide variety of reactions characteristic of granulomatous hypersensitivity. The eggs gradually become encapsulated with the resultant lesion being called a granuloma. In cross-section the granuloma is found to contain a cellular infiltrate consisting of neutrophils, eosinophils, monocytes, plasma cells, and fibroblasts and may grow to several times the size of the egg (350 μm or more) (von Lichtenberg, 1987). The consequences of granuloma formation within the target tissues are occlusion of the vasculature and organomegaly, particularly when large egg masses become embolized. Thus hepatomegaly and hepatosplenomegaly are among the most common clinical manifestations of severe cases of chronic schistosomiasis. Progression to the more advanced stages of disease is characterized by severe vascular encroachment known as portal pipestem fibrosis in which fibrotic lesions form within the hepatic vasculature, contributing further to portal hypertension, dilation of the hepatic arteries and possibly the development of vascular "shunt" pathways and ultimately variceal haemorrhage in the most severe forms.

There is good evidence to suggest that both murine and human hosts are able to modulate granuloma formation through a complex variety of immunological interactions (Kayes and Colley, 1979; Doughty et al., 1984). Specifically, a given host may respond briskly to egg antigens at the humoral and cellular levels following initial infection, and subsequently down-regulate the response thereafter (von Lichtenberg, 1987). Hence responses to egg antigens persist with the onset of chronic infection in an attenuated form resulting in a steady state of egg turnover within the tissues in the majority of subclinical schistosomiasis cases. Thus, some evidence suggests that the gradual path to severe hepatosplenic disease may be expressed in host genotypes which fail to efficiently modulate granuloma formation (Colley et al., 1986). The plethora of immunological mechanisms which give rise to the phenomenon of granuloma modulation might be regarded as beneficial to both the long-term survival of the parasite and the infected host.

1.3 An Overview of Schistosomiasis Control

Given the complexity of the life cycle with its absolute requirement for passage through a snail intermediate host, it should, in theory, be possible to halt or reduce parasite transmission with an attendant decrease in disease manifestations in man. Several strategies have been devised and applied in a systematic manner in the field, in an attempt to deal with the problem of parasite transmission. The elimination of the intermediate host by application

of molluscicides has been attempted in a number of instances, with some success (McCullough, 1986). Although expensive, labour-intensive and potentially harmful to the environment, particularly after repeated molluscicide application, dramatic decreases in snail population have been observed in foci of high parasite transmission. The population of *B. glabrata* has also been reduced by the introduction of robust snails of the genus *Thiara*, which have a shorter generation time and appear to compete effectively for the same environmental niche (Pointier and McCullough, 1989). This biological control strategy appears less harsh on the environment but the long-term ecological effects of the introduction of foreign species have yet to be evaluated.

There is currently much emphasis on health education and water supply programs (Giguemde, 1989). These programs serve to stress the importance of maintaining higher standards of personal hygiene and the role of water contact in disease transmission (Teesdale, 1986). This also has obvious benefits for the transmission of other water-borne and fecally-transmitted diseases. In addition to the high costs of maintaining such programs and the requirement for skilled personnel, it is clear that the efficacy of such programs is absolutely dependent on full involvement of the community. Since humans are the primary reservoir of infection and human behaviour sustains parasite transmission, the role of behavioural change is widely appreciated (Gillet, 1985). Paradoxically, it is often far-reaching behavioural change and human activities in the form of

freshwater manipulation schemes which contribute to enhanced parasite transmission by bringing human hosts and snails into contact through a common body of freshwater.

Among the most effective current tools for schistosomiasis control is drug therapy. A number of drugs including metrifonate, oxamniquine, and praziquantel are effective against one or more species of schistosome (Marshall, 1987). One of the primary benefits of chemotherapy is the ability to partially or completely eliminate the parasite burden in already infected individuals. As a result, some disease symptoms and complications are reduced in parallel (Doehring et al., 1985). This effect, however, is often not permanent as drug-cured individuals often become reinfected. Indeed, drug treatment/reinfection studies have formed the basis of experiments designed to examine the relationship between epidemiological factors such as water contact patterns and age to immunologically-based resistance in previously infected individuals (Butterworth et al., 1985). As a result of the reinfection problem, retreatment programs are often necessary. Heavy drug pressure, however, may give rise to drug resistant parasite populations, a phenomenon which has already been documented in some endemic regions (Coles et al., 1986). Nonetheless, the relative cost-effectiveness, safety, ease of administration (orally and in one dose for praziquantel) and lack of a requirement for refrigeration are important benefits of chemotherapy which cannot be overlooked when considered against

the backdrop of the economic and logistical realities of developing countries (Gutteridge, 1989).

Currently, a major focus of schistosomiasis research is geared towards the development of an efficacious vaccine. In contrast to drugs, an effective vaccine may have no effect on a resident parasite population in an already infected individual, but may be useful for the reduction or prevention of parasite establishment in children who have yet to be exposed to the parasite, i.e. a prophylactic vaccine. Alternatively, a vaccine which would function to alleviate the immunologically based pathology and reduce morbidity might also be useful, i.e. an anti-pathology effect. Thus, while no vaccine is currently available for schistosomiasis (or any other human parasitic disease), it is a potentially powerful adjuvant to the already existing tools for schistosomiasis control.

1.4 An Overview of Schistosome Immunity and Vaccine Development

In addition to the multiplicity of immune responses elicited by schistosome eggs, the long term survival and metabolic activities of adult parasites also give rise to a range of immune reactions within the infected host. It is of significance to evaluate the relationships between immune responses to different developmental stages and to determine if any of these responses are host-protective in nature and therefore serve to limit the intensity of infection. Since schistosomes do not replicate within the vertebrate host, as is the case for a variety of parasitic protozoa, the parasite burden is determined by the

equilibrium established between the attrition of resident parasites and the acquisition of new parasites. The question of whether host-protective immunity can be brought to bear on the invading parasites of secondary infections has been the focus of a great deal of research over the past number of years. Indeed, this question has provided the impetus for further immunological investigations since it is thought to be of direct relevance to the feasibility of vaccine development.

While immunity to schistosome reinfection under field conditions has important implications in terms of host-parasite interaction and parasite transmission, there is increasing evidence that the immune system of an immunocompetent host can be experimentally manipulated such that protective immunity to primary infection can be induced (James, 1987). In other words, both crude (James et al., 1985; Smithers et al., 1989) and purified (Smith and Clegg, 1985; Pearce et al., 1988; Balloul et al., 1987) non-living antigen preparations have been shown to possess vaccinating potential, lending further credence to the feasibility of vaccine design. It is in this regard that animal models of infection and immunity have played and will continue to play an important role, particularly in the definition of candidate vaccines and in the elucidation of protective immune mechanisms. While each animal model is unique in itself and may not emulate the characteristics of human infection completely, they nonetheless play an indispensable role in the evaluation of

potential schistosome vaccines.

1.4.1 Concomitant Immunity

The historical precedent for vaccine development evolved from the concept of concomitant immunity or resistance to homologous reinfection, initially proposed by Smithers and Terry (1969). Accordingly, a resident parasite population harboured within an experimental host is thought to elicit a specific immunological response which is capable of eliminating challenge parasites, while remaining ineffective against the resident population. Indeed, the failure of challenge parasites to increase the existing parasitemia has been experimentally demonstrated (Dean et al., 1981). As a result, a number of studies examining the stage specificity of the immune-mediated attrition were designed. Most of these studies focused on the very young schistosomulum (0-3 hours) which can be prepared and cultured relatively easily in vitro. It has been exhaustively demonstrated that these young organisms can be killed in the presence of antibody from chronically infected animals and a variety of effector cells (with or without complement) including macrophages (McLaren and James, 1985), eosinophils (Butterworth et al., 1975) or neutrophils (Incani and McLaren, 1981). This susceptibility to antibody-dependent cellular cytotoxicity (ADCC) mechanisms continues until development proceeds to the lung stage. Lung and post-lung schistosomula appear to be completely resistant to ADCC

(Tavares et al., 1980). These observations, coupled with the demonstration that juvenile schistosomes could be killed in the skin in vivo (Smithers and Gammage 1980) led to the suggestion that the susceptible stage of development was the young schistosomulum and that immunity to challenge infection took place in the skin. Thus the prevailing evidence seemed to indicate that induction of an effective immune response against the surface of the young schistosomulum was an absolute prerequisite for an efficacious vaccine.

Recently, however, a re-examination of the fate of migrating schistosomes of primary and secondary infections in the mouse using autoradiographic tracking and microsphere injection techniques has challenged the very nature of concomitant immunity in this model (Wilson et al., 1983; Dean and Mangold, 1984). Studies using ^{75}Se-labelled cercariae (a radiolabel which is apparently retained by the organism for relatively long periods of time) suggest that the majority of invading challenge parasites indeed progress to the lung stage (Dean and Mangold, 1984). During the hepatic phase of migration, however, changes in the hepatic vasculature (which are related to egg-induced pathology in the chronically infected host) apparently cause challenge parasites to be "shunted" to the vena cava and eventually to become trapped and killed in sites unfavourable for development (McHugh et al., 1987). Thus, it has been suggested that the resistance observed upon perfusion of these mice may lack

immunological specificity and instead, be related to pathological alterations within the hepatic vasculature as a result of chronic infection. In a broader sense, it appears as though the susceptibility of the very young schistosomulum to in vitro killing has no in vivo correlate.

It is perhaps not surprising that mice harbouring a low-dose chronic infection manifest these vascular changes which might be interpreted as immunologically-based resistance. It has been estimated that one worm pair in a small animal such as a mouse is equivalent to 3000 parasites in a human (McLaren and Smithers, 1987) . As such, the pathology which ensues may become quite severe in a relatively short period of time. However, the concept of immunity in humans has been supported by a number of field studies carried out in endemic regions of different parts of the world (Butterworth et al., 1985; Hagan et al., 1987). These studies have examined the intensities of reinfection of previously infected subjects following elimination of the worm burden with drug treatment. Thus, the acquisition of new infections could be measured and related to age, sex, and exposure (ie. water contact patterns). The results of these studies provide evidence for the slow development of and age-dependent acquired resistance to reinfection that cannot be explained exclusively on the basis of reduced exposure (Butterworth et al., 1985). One of the immunological correlates of resistance in older individuals and in highly resistant individuals within younger age group cohorts appears to be reflected in the levels of IgM

and IgG_2 antibodies to highly immunogenic egg carbohydrates which cross react with the surface of larval schistosomes. Accordingly, low levels of these cross-reactive antibody isotypes correlate with high levels of in vitro killing of juvenile schistosomes and high levels of resistance (Butterworth et al., 1988). It has been suggested that high levels of IgM and IgG_2 surface and egg carbohydrate specific antibodies in young susceptible individuals may block the expression of potentially protective immune mechanisms. The gradual decline in the level of these "blocking" antibody specificities with age may thus permit the expression of immunity in older individuals.

1.4.2 Immunization with Irradiated Cercariae

An animal model of immunity in which significant resistance to challenge infections can be consistently induced is the irradiated vaccine model. Mice and other rodents which have been exposed to gamma-irradiated cercariae appear to develop high levels of resistance to a viable cercarial challenge (Dean, 1983). The irradiated organisms die at some point along the migratory pathway and thus do not establish a patent infection. Therefore, this model offers the distinct advantage of studying potentially protective antigens and immune mechanisms in the complete absence of egg-induced pathology.

Investigation of the factors involved in irradiated vaccine resistance have revealed a relatively restricted spectrum of immunological mediators. Specifically, studies demonstrating passive transfer of immunity (Mangold and

Dean, 1986), and μ suppression (which reduces humoral responses) (Sher et al., 1982) have indicated a role for IgG antibody. In addition, a role for lymphokine-activated macrophages has been elucidated using P strain mice which have defects in macrophage function (James et al., 1984). However, mast cells, IgE, IgM and complement do not appear to be involved in protection in the mouse model (Sher et al., 1982; Sher et al., 1983). It is clear that the observed resistance induced by irradiated cercariae is both B cell and T cell dependent. With respect to the cellular arm of the immune system, recent work has demonstrated that in vivo depletion of the $CD4^+$ T cell subset, but not the $CD8^+$ population, is capable of abrogating resistance (Kelly and Colley, 1988; Vignali et al., 1989a).

Despite the clear involvement of specific immune effector mechanisms in the irradiated vaccine model, the target of immune attack is still somewhat controversial (Wilson and Coulson, 1989; McLaren, 1989). This controversy may be due at least in part, to differences in parasite and mouse strain, or to variation in experimental protocol. In addition, it appears as though different animal models (eg. mouse vs guinea pig) differ from one another in the main site of immune elimination as well as in the mechanisms involved (McLaren, 1989). Nonetheless, autoradiographic tracking experiments have been crucial in elucidating the site of attrition of challenge organisms (Dean et al., 1984; Wilson et al., 1986). Emerging from these studies is the notion of a so-called

"window" of susceptibility, spanning from three to four day old parasites to approximately 2 week liver parasites, during which challenge organisms could be eliminated (McLaren, 1989). Regardless of the peculiarities of any given experimental host, the elucidation of immune mechanisms and the temporal definition of susceptibility provides a background upon which molecular vaccines based on the irradiated vaccine model can be developed.

1.4.3 Immunization Regimens Using Non-living Antigens and Prospects for Vaccine Development

A wide range of experimental approaches have been used to demonstrate that both active and passive immunity to schistosomes can be induced in experimental hosts using non-living material. Passive transfer experiments using serum from mice exposed to irradiated cercariae as well as monoclonal antibodies have shown that naive animals can be substantially protected from challenge infection (Mangold and Dean, 1986; Zodda and Phillips, 1982; Hazdai et al., 1985). Active immunization trials using crude soluble worm extracts (James, 1985), membrane fractions (Smithers et al., 1989) and anti-idiotypic antibodies (Kresina and Olds, 1989) have revealed similar levels of resistance. In addition, purified molecules isolated from schistosomes (Smith and Clegg, 1985; King et al., 1987; Flanigan et al., 1989) or from recombinant sources (Balloul et al., 1987) have also been used in studies of protective immunity in animal models and as such, have provided the basis for optimism with respect

to the development of a defined molecular vaccine.

The study of infection and immunity in animal models of schistosomiasis has revealed some common features which currently pose challenges to vaccine development. For example, the presence of complete sterilizing immunity is very rarely observed on a wide scale (Colley and Colley, 1989). Thus, animals are only partially protected by the various vaccination protocols, with partial protection being expressed as the percentage reduction of the adult worm burden compared to a control group. Currently, the most effective vaccination regimens are capable of eliciting reductions of 40-60% (Colley and Colley, 1989). This is viewed as significant, however, since schistosomes are non-replicating and since they present a stable antigenic repertoire to the host (Sher, 1988). Since the goal of vaccine development is the prevention or reduction of severe disease, the stimulation of sterile immunity may not be an absolute prerequisite. It should also be remembered that most studies of protective immunity have been carried out in inbred rodent strains and that the possible effects of host genetic variation has not been subject to a systematic analysis, although this is beginning to change (Mendlovic et al., 1989).

Another feature which merits some consideration is the incredible diversity of antigenic moieties capable of eliciting protection, in terms of their biochemical, functional and topographical properties. Both carbohydrate and polypeptide epitopes have been implicated in mediating protection (Omer Ali et

al., 1988; Grzych et al., 1987). Soluble proteins, structural cytoskeletal proteins and polypeptides from the tegument, musculature and gut have been similarly implicated (King et al., 1987; Pearce et al., 1988; Taylor et al., 1988), although the protective capacity of each of these moieties has not yet been subject to detailed mechanistic analysis. It is likely that the list of protective antigens will continue to increase. Enhanced levels of immunity beyond those currently attainable may therefore depend on the induction of the appropriate type of immunity acting swiftly at the appropriate site within the host (Vignali et al., 1989b). The development of vaccination regimens, incorporating potent and selective adjuvants to stimulate T and B cell mediated immunity in the absence of adverse immunological consequences (eg. autoimmunity, induction of blocking antibody isotypes) is currently the focus of research on a number of infectious diseases (Mitchell, 1989). This goal is requisite for the ultimate development of a schistosome vaccine. Nonetheless, it is likely that in the coming decades, vaccination will likely play a major role alongside current drug and community-based programs in the control of schistosomiasis.

1.5 Immune Evasion by Schistosomes - A Functional Perspective

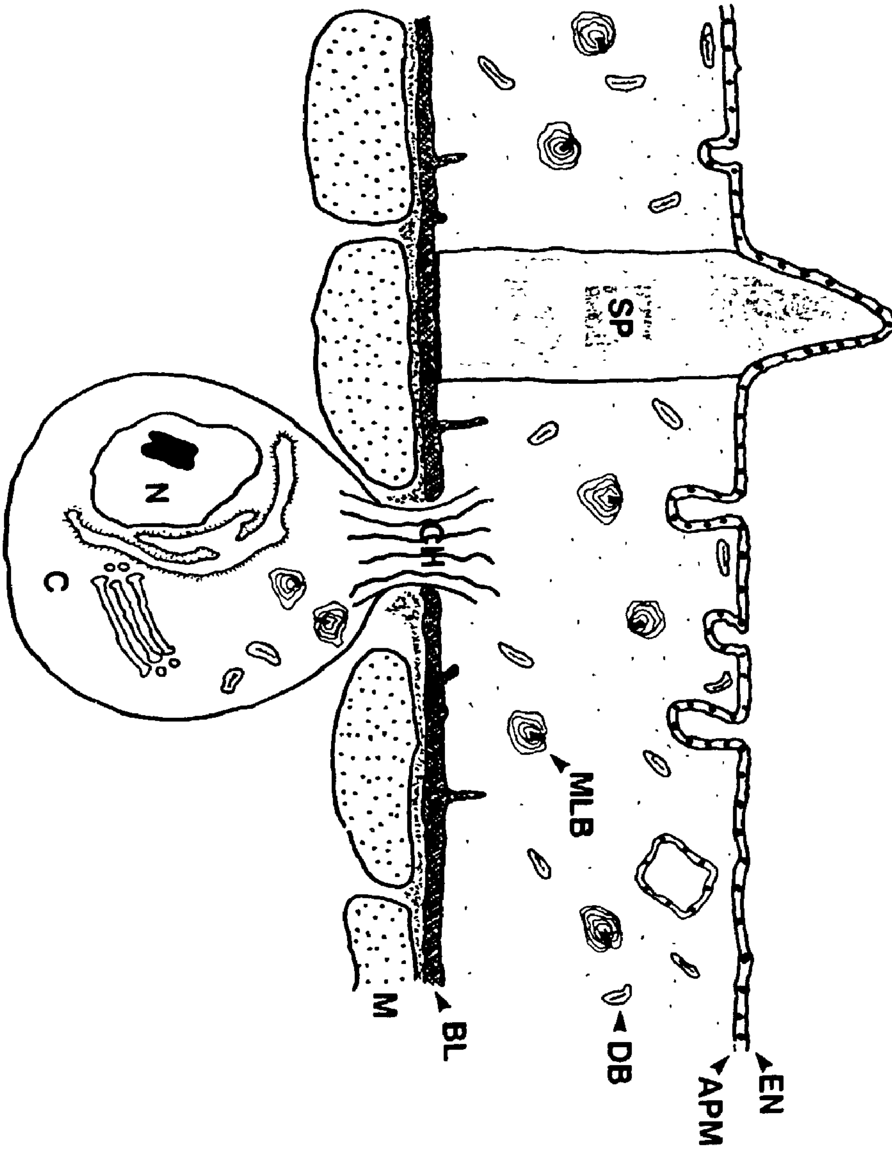
The above discussion has referred to the remarkable capacity of the developing and adult schistosome to persist in an immunologically hostile milieu. Not surprisingly, immune evasion by schistosomes is complex and multifactorial and is likely to be due to a variety of mechanisms. The

hypotheses which have been proposed to explain evasion strategies have all been supported by correlative experimental evidence but the adaptive significance of these mechanisms, in many cases, remains obscure. However, it is becoming clear that knowledge accumulated through investigation of immune evasion mechanisms will provide the basis for novel methods of immunological or pharmacological intervention in schistosomiasis (Mitchell, 1989).

Maintenance of the integrity of the host-parasite interface is of crucial importance to parasite survival. The chemotherapeutic effect of praziquantel, for example, in disrupting the overall structure of the schistosome surface underscores this suggestion (Bricker et al., 1983). Consequently, most studies have focused on the properties of the surface membrane complex of the schistosome which render it refractile to immune effector components. The surface epithelial layer of the adult schistosome (illustrated in Figure 1.2) is an anucleate syncytium bounded by apical and basal membranes. The apical membrane complex actually consists of two membranes; the apical plasma membrane (APM) and the envelope (En). The APM is thought to be analogous to the plasma membrane of other cells and constitutes the primary osmotic permeability barrier (Podesta, 1982). The En, on the other hand, is a unique feature of schistosomes and may be a major adaptation to parasitism in the blood. This membrane is formed shortly after cercarial penetration and is

Figure 1.2: The Surface Epithelial Layer of Schistosoma mansoni.

The surface epithelium of S. mansoni is a syncytium characterized by the lack of transverse membrane barriers. The surface is limited on the apical face by two membranes (the apical plasma membrane (APM) and the Envelope (En)) and on the basal aspect by a basal membrane under which lies the basal lamina (BL). The syncytium is continuous with cell bodies (C), which lie beneath muscle bundles (M) and contain a nucleus (N) and the membrane synthetic apparatus. Membrane precursors (multilamellar bodies (MLB) and discoid bodies (DB)) are synthesized within the cell bodies and are transported to the syncytium through channels (CH) lined with cytoskeletal elements. The MLB and DB are proposed to be the precursors of the En and APM respectively. Another prominent structure within the syncytium is the spine (SP) which is composed largely of cross-linked actin filaments.



SP
N
C
CH
MLB
M
BL
DB
APM
EN

preceded by the appearance of large numbers of multilamellar bodies (MLB) within the syncytium (Cousin et al., 1981). This observation led to the suggestion that the MLB are the precursors of the En (Podesta et al., 1987; McLaren, 1980). The discoid bodies (DB), also found in the tegument, are ubiquitous in parasitic flatworms and due to their biochemical and structural properties are likely to be the precursors of the APM (Hockley, 1973; Podesta et al., 1987). The tegument is connected via cytoskeletal elements to cell bodies (C) underlying the syncytium within which are located the nucleus (N), membrane synthetic apparatus (endoplasmic reticulum and Golgi complex) and DB and MLB. Hence, the precursors of the apical membrane complex may be synthesized in the subtegumental cell bodies and transported to the surface in a cytoskeleton-dependent fashion (Zhou and Podesta, 1989a). In addition, the cytoskeletal structures within the tegument itself may be more complex than previously envisaged (Zhou, Y. pers. comm.). Another prominent feature is the presence of spines (SP) which have an unknown function but are known to be composed of actin filament bundles (Zhou and Podesta, 1989b).

The absence of transverse membrane barriers and distinct cellular compartments in the plane of the tegument of parasitic flatworms including schistosomes suggests that the sacrifice of a cellular for a syncytial epithelial layer is a major adaptation to parasitism. Podesta (1982) has argued that ion and water regulation is unlikely to be the major advantage of a syncytium since

most parasitic flatworms inhabit an essentially isotonic environment. Rather, the presence of the syncytium may favour survival in hostile environments by virtue of its "design" for efficient membrane biogenesis and turnover. The degree of metabolic coupling in the syncytial field is self-evident as is the relative energy-efficiency of syncytial membrane turnover in contrast to the turnover of whole cells. As such, the cells underlying the syncytium function cooperatively in the synthesis of membrane precursors for a single large expanse of membrane. Thus, the hypothesis of membrane turnover as an immune evasion strategy employed by schistosomes is supported on a theoretical basis by the functional properties of a syncytial versus a cellular epithelial layer (Podesta, 1982).

A variety of other hypotheses to explain immune evasion which do not depend on the presence of a syncytial epithelium have also been proposed. The disguise hypothesis (Smithers et al., 1969), for example, suggests that the parasite passively adsorbs a coating of host antigens and therefore evades immune surveillance by not being "seen" as foreign (McLaren, 1984). This hypothesis also suggests that the vulnerability of the young schistosomulum to in vitro ADCC is a result of not having acquired a disguise (McLaren, 1984). There is little doubt that the parasite can adsorb host antigenic material. A wide range of host antigens including blood group antigens (Clegg et al., 1971), major histocompatibility antigens (Gitter et al., 1982; Sher et al., 1978), α2-

macroglobulin (Damian et al., 1973) and immunoglobulins (Sondgares Bernal, 1976; Kemp et al., 1976) have all been demonstrated on the surface of S. mansoni. Although some of these host antigens may be ligands for parasite receptors (Kemp et al., 1980; Tarleton and Kemp, 1981), the adaptive significance of the adsorption of host antigens remains obscure. In addition, the observation that resistance to in vitro ADCC mechanisms can develop in schistosomula cultured in the absence of host macromolecules (Dessein et al., 1981; Samuelson et al., 1980) suggests that alternative mechanisms may be involved. Indeed, proponents of the disguise hypothesis acknowledge that other "intrinsic" parasite mechanisms also operate (McLaren, 1984).

Another potential evasion strategy involving host antigens suggests that the parasite synthesizes host-like determinants (antigen mimicry) (Damian, 1964). This strategy proposes that the capacity to synthesize host-like antigens is fixed in the parasite genome by natural selection acting on coincidental antigenic cross-reactivity in the host-parasite relationship. Although it has been shown that S. mansoni can synthesize antigenic structures which resemble those found in the snail (Dissous and Capron, 1989) and potentially vertebrate (Nyame et al., 1989) hosts, it is unlikely that the parasite genome has the capacity to synthesize a comprehensive array of host antigens from all potential hosts in which schistosomes could develop. In addition, the genes encoding MHC glycoproteins have not been found within the parasite genome (Simpson

et al., 1983).

Both the disguise and antigen mimicry hypotheses suggest that immune evasion is accomplished by virtue of the fact that the parasite is not "seen" as foreign by the host. By extension, parasite antigens at the host-parasite interface may not be immunogenic. This is clearly not the case since the immunogenicity of schistosome surface structures can be demonstrated by a variety of techniques (Omer Ali et al., 1988; Karcz et al., 1988). The exposure of parasite surface epitopes in vivo is also implied by the resistance which can be passively transferred with monoclonal antibodies or serum from mice vaccinated with irradiated cercariae. Thus the presence of host molecules on the parasite surface, whether adsorbed or mimicked, may afford a somewhat less than complete escape from immune surveillance mechanisms.

In terms of membrane physiology, perhaps the most attractive hypothesis to explain immune evasion is membrane turnover, the theoretical/functional basis of which is discussed above. Accordingly, immune responses directed against the surface are thwarted by rapid membrane shedding/recycling and renewal before the integrity of the cytoplasm is compromised. Membrane renewal as an intrinsic parasite mechanism accords little significance to the presence of host macromolecules on the schistosome surface and as such, may provide alternative explanations for the susceptibility of young schistosomula to in vitro ADCC and the apparent "non-antigenicity" of the surface of developing

schistosomula (Pearce et al., 1986).

Estimates of membrane turnover rates in schistosomes have been obtained by both external and biosynthetic labelling of parasite surface components. Turnover of schistosomular glycolipids and glycoproteins occurred with a half-time of 8-10 hours in vitro (Samuelson and Caulfield, 1982; Samuelson et al., 1982). There is also accumulating evidence that re-introduction of surface-labelled schistosomula into mice can enhance the loss of label from the surface (Ruppel and McLaren, 1986). Pearce et al., (1986) have estimated that shedding of surface-labelled macromolecules can occur after a 30 minute in vivo pulse. Adult parasites apparently have an increased rate of surface turnover in vitro with a half-time of 2-4 hours (Wilson and Barnes, 1977). Dean and Podesta (1984) have measured the renewal of metabolically labelled membrane proteins and estimated rates of 3 and 6 hours for the En and APM, respectively, under control conditions. Stimulation of membrane turnover has also been observed in vitro. Kemp et al., (1980) and Tarleton and Kemp (1981) have reported the complete loss of a specific surface label (fluorescently labelled Staphylococcus aureus) within 20 minutes. The latter observations may have been mediated via signal transduction mechanisms involving F_c (carboxyl terminus of immunoglobulin molecule) and C_3 (complement component 3) receptors, both of which have been demonstrated on S. mansoni (Torpier et al., 1979; McGuiness and Kemp, 1981; Tarleton and Kemp, 1981).

An investigation of the factors which modulate surface membrane phospholipid biosynthesis has revealed that the indoleamine 5-hydroxytryptamine (5-HT), the third component of complement (C_3) and a calcium ionophore (A23187) can stimulate synthesis and incorporation of the predominant phospholipid (phosphatidylcholine, PC) into the apical membranes (Young and Podesta, 1986). Specifically, 5-HT stimulates synthesis and incorporation of PC into the APM, whereas C_3 and A23187 stimulate the synthesis and incorporation of PC into the En (Young and Podesta, 1986). These findings have been extended by Zhou and Podesta (1989a), who have shown that 5-HT stimulates the synthesis of DB while C_3 stimulates the synthesis of the MLB. The DB and MLB both appear to be transported to the syncytium in a cytoskeleton-dependent fashion (Zhou and Podesta, 1989a). Taken together, these results support the hypothesis that the DB are the precursors of the APM while the MLB are the precursors of the En.

S. mansoni is known to possess a 5-HT (Mansour, 1984) and a C_3 receptor although they have yet to be isolated or biochemically characterized. The 5-HT receptor is coupled to an active adenylate cyclase (Kasschau and Mansour, 1982) and is likely to function in a cAMP-dependent signal transduction system (Podesta et al., 1987). C_3, on the other hand, appears to effect changes in intracellular calcium levels (Young, 1984) and thus may be involved in a predominately calcium-mediated signalling mechanism (Podesta et

al., 1987). The definition of signal substances and a response element involving membrane synthesis has important implications for the elucidation of the signal transduction mechanisms mediating an important parasite survival strategy. However, much remains to be learned with respect to the membrane components involved in these signalling systems and individual membrane proteins in general.

1.6 Rationale and Thesis Objectives

The use of recombinant DNA methodology has had and will continue to have a large impact on the schistosome field. Parasite proteins which have been previously described only in relatively crude immunochemical or serological terms, can now be precisely characterized through the cloning of their genes and complementary DNAs (cDNAs) and the determination of their primary sequences. Most schistosome membrane-associated molecules, which appear to be of low abundance, have been characterized by immunological rather than intrinsic functional means and have been studied using sensitive labelling techniques and relatively small quantities of schistosome tissue (Simpson and Smithers, 1985). There is good reason for this since biochemical analysis of almost any functional aspect of *S. mansoni* is severely hampered by the inability to produce large quantities of schistosomes by laboratory maintenance of the life cycle. It is clear that the continued development of biochemical "micromethods" and molecular cloning technology will be central to the study of a variety of

schistosome functions, including membrane physiology as it relates to immune evasion (discussed above). This thesis attempts to address the gap in our knowledge of schistosome membrane proteins using an approach based on standard biochemical, immunological and molecular cloning techniques. Information from this study will be useful for stimulating further functional analysis of membrane polypeptides and their potential role in mediating essential survival functions (Podesta et al., 1987) or in the induction of protective immunity (Smithers et al., 1989; Huebert, 1990). The present study was designed to address the following objectives:

1) To examine the biochemical properties of a limited number of S. mansoni membrane proteins using small quantities of a defined APM fraction with the emphasis on immunogenic proteins in this fraction.

2) To establish comprehensive cDNA libraries from S. mansoni poly A+ RNA and isolate clones corresponding to immunogenic polypeptides of the APM fraction.

3) To determine the primary sequence of the polypeptides identified in objective 2 to provide insight into possible biological function.

Chapter 2- Characterization of a 24 kDa Phosphoprotein Antigen

2.1- Introduction

During the course of their life cycle, schistosomes must adapt to a variety of different environments and possess the ability to locate and migrate to appropriate sites within their vertebrate and molluscan hosts, as well as resist immune attack. It is clear, therefore, that parasite survival is dependent upon the ability to "perceive" and respond to environmental signals. The transition between the free-living cercariae and the parasitic schistosomulum, for example, is accompanied by a change in the temperature and osmolality of the ambient milieu (i.e. freshwater to vertebrate host). It has been suggested that these signals give rise to events associated with schistosomular transformation including the attendant synthesis of the envelope overlying the apical plasma membrane (Wiest et al., 1989). It is likely that schistosomes can respond to a wide range of signals resulting in the modulation of metabolic processes affecting many aspects of parasite physiology. Of particular interest are the signalling mechanisms influencing membrane biogenesis and turnover, since membrane modulation has been suggested to be an important mechanism favouring survival in an immunologically hostile environment (Podesta, 1982).

Signalling mechanisms in a variety of cells usually involves external signal ligands (eg. hormones, neurotransmitters, growth factors), membrane receptors of a variety of types, a limited number of second messengers (eg.

cyclic nucleotides, Ca^{2+}, inositol phosphates), phosphorylation cascades (eg. protein kinases, phosphoprotein phosphatases, protein substrates) and physiological responses (Huganir and Greengard, 1987; Shenolikar, 1987; Cohen, 1988). Schistosomes have been shown to possess receptors for the F_c portion of immunoglobulins (Tarleton and Kemp, 1981), the C_3 component of complement (Tarleton and Kemp, 1981), 5-hydroxytryptamine (5HT) (Kasschau and Mansour, 1982), and low density lipoprotein (Rumjanek et al., 1988). In addition, the F_c, C_3, and 5HT receptors have been implicated in a variety of physiological responses involving membrane modulatory phenomena (Kemp et al., 1980; Young and Podesta, 1986; Zhou and Podesta, 1989b). Thus, these receptor-mediated signalling systems may prove to be useful models for the study of signal transduction pertaining to an important immune evasion strategy utilized by schistosomes. In this sense, it is of interest to define and characterize the components of signal transduction systems at the schistosome surface. Studies of this nature may also be crucial for the rational design of novel immunological or pharmacological intervention strategies.

While ambient signal molecules and their resultant physiological responses have been documented in schistosomes, the intermediate steps between receptor binding and the response element have not been subjected to extensive analysis. Specifically, the existence of second messengers and the nature of phosphorylation reactions are only now being investigated (Estey and

Mansour, 1987; Kalopothakis et al., 1987; Kawamoto et al., 1989). For example, the 5HT receptor is known to be coupled to adenylate cyclase via a G protein, resulting in cAMP synthesis (Kasschau and Mansour, 1982). Calcium has also been implicated in response to binding of C_3 (Young, 1984; Podesta et al., 1987). Moreover, the machinery involved in phosphorylation of polypeptides associated with the epithelial syncytium of S. mansoni has been demonstrated (Edwards, 1986; Podesta et al., 1987; Kalopothakis et al., 1987). These studies, although not exhaustive, do support the existence of complex regulatory mechanisms for mediating the transduction and amplification of signals across the surface of S. mansoni.

In order to further identify and characterize protein phosphorylation associated with the epithelial syncytium of S. mansoni, an in vitro system using the apical plasma membrane (APM) isolated according to McDiarmid et al. (1983) was developed. This work was prompted by investigations carried out with a variety of isolated cell membrane systems (Huganir and Greengard, 1983; Moore et al., 1983; Caro et al., 1987; Beliveau et al., 1988; Thiel and Soling, 1988) which have led to the identification of specific membrane-associated protein kinase activity and the protein substrates of such activity. The objectives of the work outlined in this chapter are as follows:

1) To determine if protein kinase activity is associated with the isolated APM fraction and to examine endogenous substrates of the phosphorylation

reactions.

2) To determine if *in vitro* phosphorylation can be modulated.

3) To examine and characterize immunogenic polypeptides identified in Objective 1.

4) To examine the biochemical properties of the polypeptides identified in Objective 1 and 3.

These objectives were pursued with the goal of developing useful biochemical assays with small quantities of schistosome tissues and sensitive analytical techniques. The methods described herein may therefore prove useful for the characterization of many other schistosome polypeptides and antigens for which no specific immunochemical probes are currently available.

2.2 Materials and Methods

2.2.1 Chemicals and Biochemicals

All chemicals were from standard sources and were reagent or analytical grade or higher. Electrophoresis chemicals, blotting materials, nitrocellulose (0.2μm pore size), bromochloroindolyl phosphate (BCIP), and nitro-blue tetrazolium (NBT) were obtained from Bio Rad. Alkaline phosphatase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Jackson Immunoresearch. Concanavalin A (Con A) agarose, lentil lectin agarose, wheat germ agglutinin (WGA) agarose, Triton X-114 (TX-114), sodium meta-periodate, neuraminidase, 5-hydroxytryptamine (5-HT), cyclic 3'-5' adenosine monophosphate (cAMP) and protease inhibitors were supplied by Sigma. Endoglycosidase H (Endo H) and Endoglycosidase F (Endo F) were products of Boehringer Mannheim. N-glycanase was from Genzyme. Pre-stained molecular weight markers from Bethesda Research Laboratories were used throughout the study and were visualized directly in gels or on blots. [γ^{32}P] adenosine triphosphate ([γ^{32}P]ATP, 3000 Ci/mmol) was purchased from New England Nuclear.

2.2.2 Isolation of Parasites

Syrian hamsters (Mesocricetus auratus) were infected with approximately 1400 S. mansoni cercariae (Puerto Rican strain) obtained from light-stressed infected Biomphalaria glabrata which were supplied by the Centre for Tropical

Diseases, University of Lowell, MA. Adult parasites were eluted from the cut portal and mesenteric veins of infected hamsters (40-45 days post infection) by cardiac perfusion with Krebs-Ringer phosphate (KRP) buffer pH 7.4 (120mM NaCl, 16 mM Na_2HPO_4, 5 mM KCl, 1mM $MgSO_4$, 1mM KH_2PO_4, 1mM $CaCl_2$, pH 7.4) and maintained on ice in KRP for one hour or less.

2.2.3 Membrane Isolation

All manipulations involving membrane isolation were carried out at 4°C or on ice. Fractions enriched in either the Envelope (En) or the apical plasma membrane (APM) of the apical membrane complex of the schistosome were obtained as previously described (McDiarmid et al., 1983; Dean and Podesta, 1984). Removal of the envelope was accomplished using a 5 minute incubation with gentle agitation in 20 mL of 0.1M Tris, pH 7.4, 12 mM sucrose, 0.1% digitonin (TSD). The parasites were then rinsed with an equal volume of 0.1M Tris, pH 7.4, 0.2M sucrose (TS). These two membrane removal fluids were pooled and stored on ice. The APM of the partially denuded parasites was removed using the same procedure except that the incubation in TSD was for 25 minutes. The two fractions obtained, enriched in En and APM respectively, were then centrifuged at 35,000 X g for 1 hour. Membrane pellets were resuspended in TS and washed by three cycles of resuspension and centrifugation at 15000 X g for 15 minutes each at 4°C in an Eppendorf microcentrifuge. Only the APM fraction was used in the present study.

Membranes were either used directly or stored at -70°C. Protein was estimated by the Coomassie blue dye-binding method (Bradford, 1976), using bovine serum albumin (BSA) as standard. Alkaline phosphatase activity was determined colorimetrically using a kit supplied by Sigma and para-nitrophenyl phosphate as substrate.

2.2.4 Preparation of Antisera

Anti-APM antisera were raised in both rabbits and mice. All blood was collected, incubated 1 hour at 37°C, then incubated overnight at 4°C. The sera were separated from the clotted blood and stored in aliquots at -70°C.

2.2.4.1 Preparation of Rabbit Anti-APM Antiserum (RαAPM)

New Zealand white rabbits were immunized with APM protein which was denatured by boiling with 2% (w/v) sodium dodecyl sulphate (SDS), 25mM dithiothreitol (DTT) in 100mM Tris pH 6.8, and precipitated with five volumes of acetone. An initial injection of 1 mg of protein in Freund's complete adjuvant was administered followed by three subsequent injections of 500 μg protein in Freund's incomplete adjuvant after 3, 5, and 7 weeks. The animals were bled 7 days after the final boost. This serum was called rabbit anti-APM serum (RαAPM).

2.2.4.2 Preparation of Mouse Anti-APM Antiserum (MαAPM)

Balb/c and C57Bl/6 mice were immunized following the same schedule as the rabbits. In this case, Freund's adjuvants were omitted, the proteins were

not denatured or precipitated and the initial injection was 50μg while subsequent injections contained 25μg protein. These sera were called mouse anti-APM sera (MαAPM).

2.2.4.3 Preparation of Infected Mouse Serum (IMS)

Infected mouse serum (IMS) was collected from Balb/c and C57Bl/6 mice which were infected 11 weeks previously with 40 to 100 cercariae.

2.2.4.4 Preparation of Normal Mouse Serum (NMS) and Normal Rabbit Serum (NRS)

Normal rabbit and normal mouse serum (NRS and NMS, respectively) were collected from animals which were injected with buffer alone, buffer in Freund's adjuvants or a control protein (rat transferrin) in Freund's adjuvants according to the same immunization schedule.

2.2.5 Electrophoresis of Proteins

APM membrane protein samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (1971). The protein sample buffer for SDS-PAGE was 100 mM Tris, pH 6.8, 2 % (w/v) SDS, 5 % (v/v)β-mercaptoethanol, 20 % (v/v) glycerol, 0.05 % (w/v) bromphenol blue (SDS sample buffer). Resolving gels were composed of 10-12% polyacrylamide while stacking gels were composed of 3-5% polyacrylamide. Two-dimensional electrophoresis was performed using an isoelectric focusing gel in the first dimension and SDS-PAGE in the second dimension as described by O'Farrell

(1975). All protein electrophoresis and blotting methods were carried out in mini format using a system supplied by, and methods described by the manufacturer (Bio-Rad). In most experiments, electrophoretically separated polypeptides were transferred to nitrocellulose. Some gels were stained directly in 0.1% Coomassie Blue in 50% methanol, 10% acetic acid and destained with 50% methanol, 10% acetic acid. The gels were then stored in 10% acetic acid.

2.2.6 Electroblotting of Proteins Resolved by Gel Electrophoresis

Electroblotting of SDS-PAGE fractionated material to nitrocellulose was carried out at 4°C using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (Towbin et al., 1978) for 1 hour at a constant voltage of 100 volts. The post-transfer gel was often stained as described in section 2.2.5 to assess the degree of transfer. In some cases blots were stained in 0.1% Amido Black in 50% methanol, 10% acetic acid for 5 minutes and destained in 50% methanol, 10% acetic acid.

2.2.7 Detection of Nitrocellulose-bound Antigen

Nitrocellulose blots containing immobilized schistosome membrane proteins were blocked in 3% (w/v) gelatin in Tris buffered saline (TBS; 20mM Tris pH 7.4, 500mM NaCl). Blots were then washed in TBS containing 0.05% Tween 20 (TTBS), and reacted with either a 1/200 dilution of RαAPM, a 1/500 dilution of MαAPM, a 1/100 dilution of IMS or affinity purified antibodies prepared as described below. Dilutions of these antibodies were prepared in

TTBS containing 1% gelatin. After washing the blots in TTBS, bound antibody was detected with a 1/5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse IgG and the bror ochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate system. The enzymatic colour reaction was developed in 0.1M Na_2CO_3 pH9.8, 1mM $MgCl_2$ containing 300μg/mL NBT and 150μg/mL BCIP. The procedure involving electrophoretic transfer of proteins to nitrocellulose and subsequent antigen detection using antibodies is hereafter referred to as immunoblotting.

2.2.8 Affinity Purification of Antibodies Specific for Smgp24

Antibodies specific for a 24 kilodalton antigen called Smgp24 (Karcz et al., 1988) were prepared using a modification of published methods (Beall and Mitchell, 1986; Olmsted. 1981) involving low pH elution of antibodies from preparative Western blots. Samples containing 10μg APM protein were electrophoresed on preparative 1-dimensional 12% polyacrylamide gels. The region of the gels containing proteins of approximately 30-15 kDa (as judged by the migration of co-electrophoresed pre-stained markers) was removed and electroblotted to nitrocellulose. Blots were blocked, washed, and incubated in a 1/200 dilution of RαAPM or a 1/500 dilution of MαAPM. The precise location of Smgp24 was determined by processing the detached edges of the blot with the appropriate second antibody conjugate and BCIP/NBT. Antibodies binding to Smgp24 were then eluted by a 1 minute incubation in a solution of 50 mM

glycine pH 2.8 which was agitated vigorously. The blot strips were removed and the eluates were neutralized by the addition of 2M Tris pH 8 to a final concentration of 50 mM. The antibody solutions were then adjusted to 500mM NaCl, 0.05% Tween 20, 1% gelatin, and 0.05% sodium azide and stored at 4°C. Affinity purified antibodies prepared in this way were used directly without concentration or dilution. Typically, antibodies prepared from five preparative blots were stored in a final volume of 25 mL. These antibodies are referred to as rabbit or mouse affinity purified antibodies.

2.2.9 Lectin Affinity Chromatography

Absorption of solubilized membrane glycoproteins to immobilized lectin affinity matrices was carried out on a small scale in batch fashion. APM membrane proteins (2μg) were solubilized in 200μL of lectin equilibration buffer (20mM Tris pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1mM $CaCl_2$, 1mM $MnCl_2$) for 15 minutes at room temperature. Insoluble material was removed by centrifugation at 15000xg for thirty minutes at 4°C. Fifty μL of a 50% slurry of either Con A agarose, lentil lectin agarose or WGA agarose beads in equilibration buffer was added to the solubilized protein and agitated at room temperature for 30 minutes. The resins were collected by centrifugation at 300x g in a clinical centrifuge and the unbound fractions removed. The beads were then washed three times with 10mL equilibration buffer. Bound material was then specifically eluted by a 30 minute incubation at room temperature in

200μL 0.5M α-methylmannoside (for Con A and lentil lectin) or 0.5 M N-acetylglucosamine (for WGA). The sugar eluates and the unbound fractions from the lectin resins, all of which were in a volume of 200μL, were precipitated with an equal volume of 20% trichloroacetic acid (TCA) for 30 minutes on ice in the presence of 5μg BSA as carrier. Precipitates were collected, rinsed with acetone, dried, solubilized in SDS sample buffer (100mM Tris pH 6.8, 2% SDS, 25mM dithiothreitol, 20% glycerol, 0.05% bromphenol blue) and analyzed by SDS-PAGE and immunoblotting using affinity purified antibodies.

2.2.10 Treatment of Membranes with Carbohydrate Modifying Agents

All membrane samples prepared as described below were analyzed by SDS-PAGE and immunoblotting using affinity purified antibodies as a probe.

2.2.10.1 Carbohydrate Digestion with Endo H, Endo F and N-glycanase

Digestion of membrane glycoconjugates with Endo H, Endo F and N-glycanase was carried out in 0.1M sodium phosphate pH 6.5, 10mM EDTA, 0.5% Triton X-100, 0.05% SDS, 1% β-mercaptoethanol. Membrane samples containing 2 μg protein in a volume of 20 μL were boiled in this buffer for 2 minutes and cooled to room temperature. Insoluble material was removed from the reaction mixture by centrifugation at 15000xg for 15 minutes before adding enzyme and incubating at 37°C. Optimal enzyme concentrations and incubation times were determined empirically for each enzyme. In initial experiments, the reaction mixture was supplemented with pepstatin, leupeptin, antipain, and

aprotinin (each at $5\mu g/mL$) and 1mM phenylmethylsulfonyl fluoride. These protease inhibitors had no effect on the product profile and were omitted in subsequent experiments. Digestions were stopped with the addition of twice concentrated SDS sample buffer, boiled and then stored for further analysis. In some experiments, membrane samples which had been treated with Endo F or N-glycanase in an initial volume of 20 μL were boiled to inactivate the enzymes, diluted up to 200 μL with lectin equilibration buffer and adsorbed to lectin affinity matrices as described above.

2.2.10.2 Digestion of Membrane Glycoconjugates With Neuraminidase

Neuraminidase treatment was carried out on intact membranes ($2\mu g$) in 50 μL of 50mM sodium acetate pH 5.1. Enzyme concentration and incubation time was titrated over a tenfold range. Following digestion, membranes were collected by centrifugation at 15000xg for 15 minutes at 4°C and solubilized in SDS sample buffer.

2.2.10.3 Treatment of Membranes with Sodium Periodate

Treatment with sodium meta-periodate was performed in incubation mixtures containing $2\mu g$ APM protein in 100 μL of 50mM sodium acetate buffer. Periodate concentrations were titrated up to 100mM and oxidations were performed for 1 hour at room temperature in the dark at pH 4.5 and 5.8. The membranes were collected by centrifugation and then treated with 100 μL of 10mM sodium borohydride in 50mM sodium phosphate pH 7.4, 150 mM NaCl

.. ɒS) for 30 minutes at room temperature in the dark. The membranes were then washed twice with PBS and solubilized in SDS sample buffer.

2.2.11 Phase Separation of APM Proteins in Triton X-114

Stock TX-114 was pre-condensed as described by Bordier (1981). APM membrane proteins (2μg) were solubilized in 1% TX-114 in 20mM Tris pH 7.4, 150mM NaCl on ice for 10-30 minutes with occasional agitation. Insoluble material was removed by centrifugation at 15000 x g for 30 minutes at 4°C. The detergent extract was then layered onto a 6% sucrose cushion in 20 mM Tris pH 7.4, 150mM NaCl, 0.06% TX-114 and incubated at 30°C for 3 minutes. Detergent-enriched and detergent-depleted phases were then separated by centrifugation at 1000x g for 3 minutes at room temperature. The detergent-depleted phase then received fresh TX-114 to a final concentration of 0.5% and was layered onto the same sucrose cushion. Phase separation was carried out as before. The detergent-depleted phase was rinsed with 2% TX-114, recondensed and collected. Proteins partitioning into the detergent-depleted phase were then precipitated with TCA in the presence of 5 μg BSA, while those in the detergent-enriched phase were precipitated with acetone also in the presence of carrier BSA. Protein samples were then solubilized and analyzed by SDS-PAGE and immunoblotting using affinity purified antibodies against the Smgp24 complex.

2.2.12 In Vitro Phosphorylation of S. mansoni Membrane Proteins

The standard phosphorylation assay was carried out using 1 μg membrane protein in 20mM Tris, 50mM NaCl, 10 mM $MnCl_2$, and 10μCi [γ-^{32}P]ATP in a final volume of 20 μL for 5 minutes at 25°C. The reaction was terminated by the addition of an equal volume of 10% TCA and incubation on ice for 30 minutes. The precipitate was collected by centrifugation for 15 minutes at 16000 xg in an Eppendorf microcentrifuge, rinsed with acetone, dried and solubilized in SDS sample buffer. The samples were analyzed by SDS-PAGE. Gels were fixed in 50% methanol, 10% acetic acid, dried and were exposed to Kodak X-ray film (X-Omat AR) with intensifying screens for up to 24 hours. Ion substitution experiments were carried out by replacing $MnCl_2$ with either $CaCl_2$, $ZnCl_2$, or $MgCl_2$. The effects of modulators of the phosphorylation reaction were studied by supplementing the standard reaction with either sodium vanadate (Na_3VO_4), 5-HT, or cAMP to final concentrations of 1 mM, 1 μM and 1 μM, respectively. In some experiments, phosphorylated polypeptides were subjected to lectin affinity chromatography or phase partitioning in TX-114 as described above in sections 2.2.9 and 2.2.11, respectively.

2.2.13 Immunoprecipitation

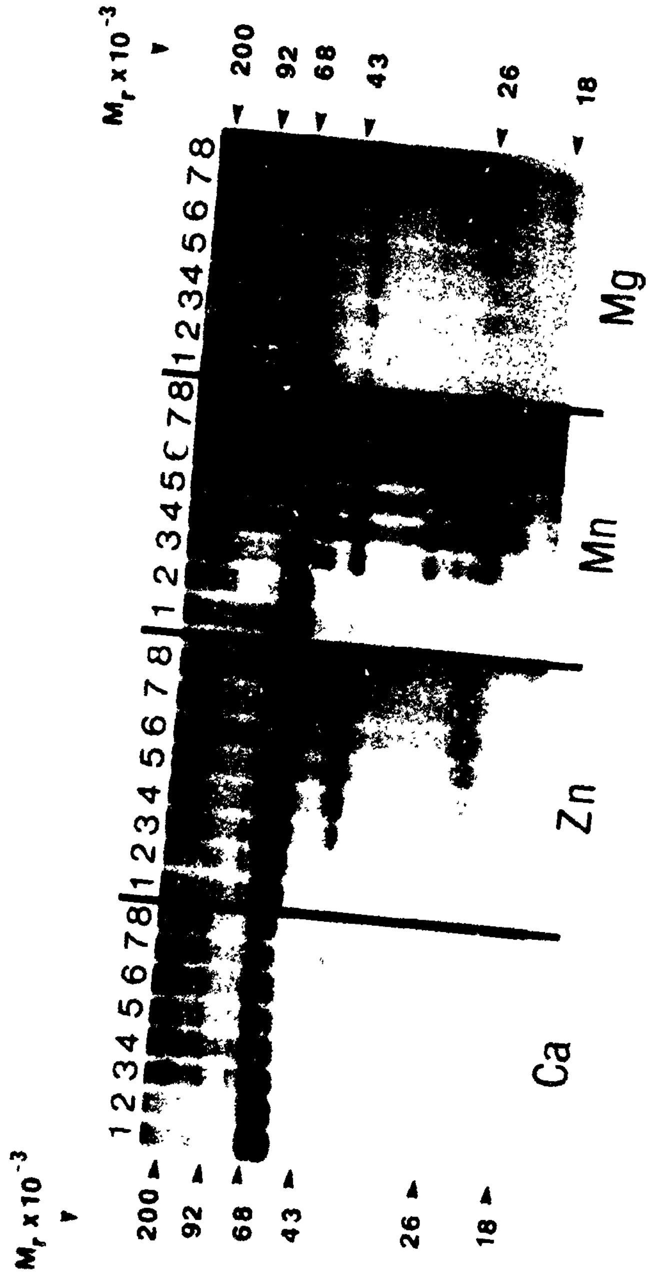
A phosphorylation reaction prepared in the presence of Mn^{2+} and sodium vanadate was terminated by the addition of SDS and DTT to a final concentration of 1% and 20 mM, respectively. The reaction was boiled for 3

minutes and insoluble material was removed by centrifugation at 15000 X g for 15 minutes at 4°C. The solubilized membrane phosphoproteins were then diluted with a 10-fold volume excess of immunoprecipitation buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2mM EDTA, 2mM EGTA, 1% Triton X-100, 1% sodium deoxycholate). The diluted reactions were then supplemented with 2 μL of either normal rabbit serum (NRS) or rabbit anti-APM serum (RαAPM) and incubated at 4°C for 1 hour. Immune complexes were then adsorbed onto Protein A Sepharose (which was previously equilibrated with immunoprecipitation buffer) using a 1 hour incubation at 4 °C with agitation and 50 μL of a 50% slurry of Protein A Sepharose. The beads were then gently centrifuged in a clinical centrifuge and washed 5 times with 5 mL of immunoprecipitation buffer. Immune complexes were then eluted from the beads by boiling in 50 μL of twice concentrated SDS sample buffer and analyzed by SDS-PAGE. Immunoprecipitation with affinity purified antibodies was carried out exactly as described above except that the Protein A slurry was incubated overnight at 4°C with 4 mL of affinity purified antibody solution prepared as described in section 2.2.8.

2.3 Results

The polypeptide profiles illustrated in Figure 2.1 demonstrate that a range of APM proteins are phosphorylated when isolated membranes are incubated in a buffer containing a divalent cation and [γ^{32}P]ATP, indicating that isolated membranes possess endogenous protein kinase activity and substrates for this reaction. The highest incorporation of label into the widest range of polypeptides was observed in the presence of 10 mM Mn^{2+} (Mn panel, lane 8). This formed the basis for the standard reaction (Chapter 2.2.12) as well as subsequent experiments. Phosphorylated polypeptides of 160, 82, 65, 54, 50, 38, 28 and 24 kDa were observed at 500 μM Mn^{2+} (lane 4) and their specific activity increased as the concentration of Mn^{2+} increased (lanes 5 to 8). Ion substitution experiments in which Ca^{2+}, Zn^{2+} and Mg^{2+} replaced Mn^{2+} in the standard reaction, showed that in each case a subset of the Mn^{2+}-stimulated phosphoproteins were detected. An exception to this occurred in the case of Ca^{2+} in which a 110 kDa polypeptide was detected at Ca^{2+} concentrations in excess of 100 μM (Ca panel, lanes 3-8). At low concentrations ($\leq$ 100 μM) for each of the cations, two prominent bands of 65 and 58 kDa were consistently observed. In the case of Ca^{2+}, Mn^{2+} and Mg^{2+}, the specific activity of the 58 kDa species decreased as divalent cation concentrations exceeded 100 μM (Ca, Mn and Mg panels, lanes 4-8). In the presence of Zn^{2+}, however, the intensity of the 58 kDa band appeared to increase with increased Zn^{2+} concentrations

Figure 2.1: SDS-PAGE analysis of APM polypeptides of Schistosoma mansoni phosphorylated in vitro in the presence of different divalent cations (see section 2.2.12). The cation used in the phosphorylation reaction (either Ca^{2+}, Zn^{2+}, Mn^{2+} or Mg^{2+}) is indicated under the panel of the respective autoradiogram. Cation concentrations were 0μM, 10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM and 10mM in lanes 1 through 8, respectively, for each of the cations. The relative molecular masses (M_r) of pre-stained protein markers are indicated at the side of the figure, and M_r is used as a notation for relative molecular mass in subsequent figures.



$M_r \times 10^{-3}$
200
92
68
43
26
18
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
Ca
Zn
Mn
Mg

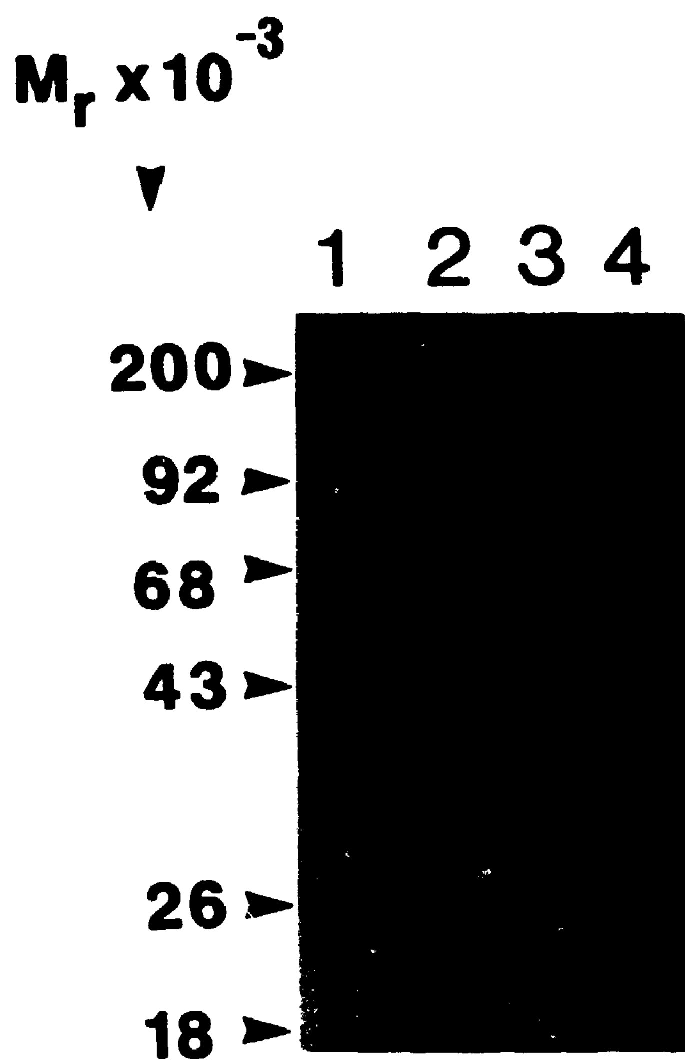
(Zn panel, lanes 4-8).

These results prompted an examination of whether phosphorylation in vitro could be modulated by agents implicated in signal transduction mechanisms in S. mansoni (eg. 5-HT and cAMP). The results depicted in Figure 2.2 suggest that the presence of vanadate (lane 2) and 5-HT (lane 3) in the standard phosphorylation assay, had little effect on the phosphorylation of the membrane polypeptides Cyclic AMP, appeared to specifically stimulate the phosphorylation of the 82 kDa molecule (lane 4).

Further investigation of the properties of APM polypeptides phosphorylated in vitro demonstrated that the 160 and 24 kDa molecules were glycoproteins based on their specific elution from lectin affinity matrices (Figure 2.3). Both of these molecules were found primarily in the eluates from chromatography on Con A and lentil lectin agarose (lanes 5 and 7), but are evident in both the unbound and eluted fraction derived from chromatography on WGA agarose (lanes 2 and 3). There appeared to be no other major phosphorylated proteins which were bound and specifically eluted from the lectin affinity matrices. The 82, 65, 54, 32 and 28 kDa molecules were evident in the unbound fractions (lanes 2,4 and 6) and therefore are unlikely to possess carbohydrate structures which bind to these immobilized lectins.

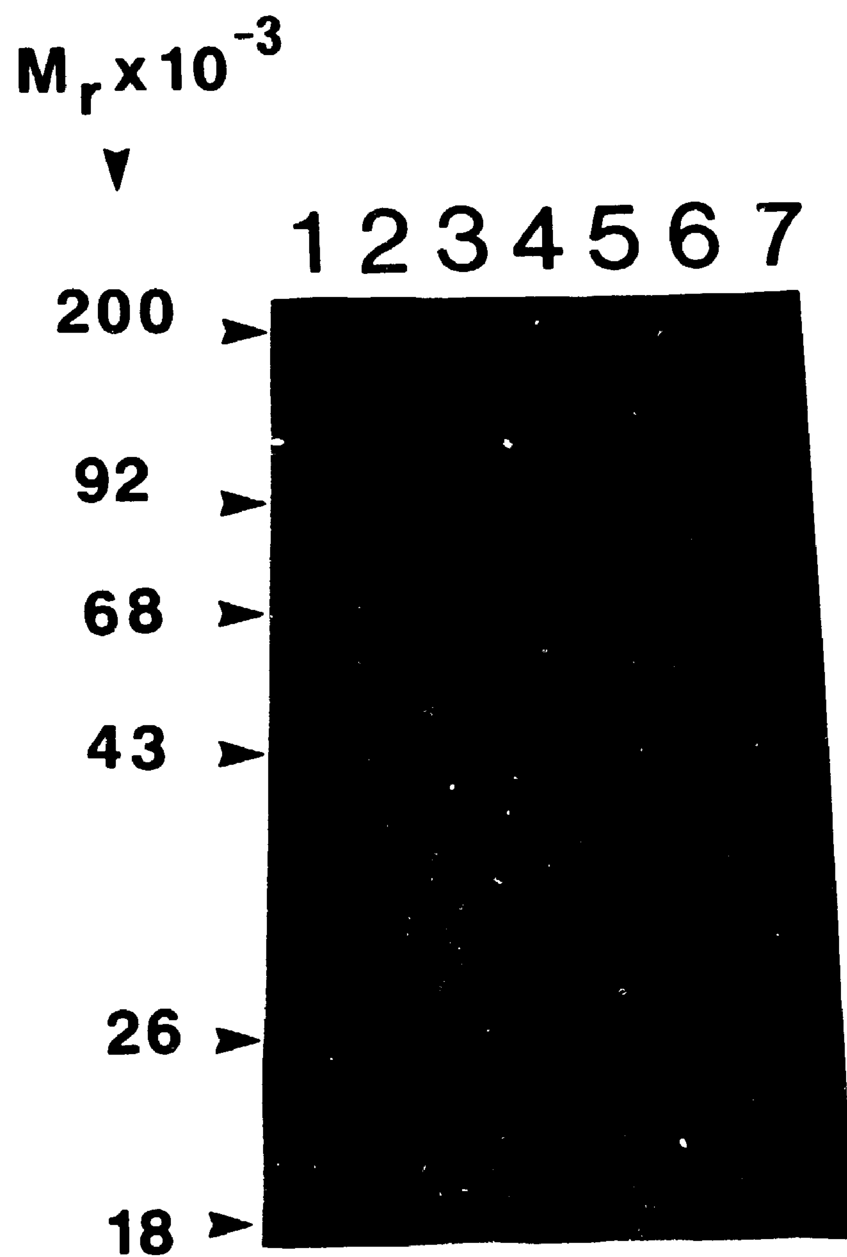
The phosphoproteins were also fractionated on the basis of their behaviour in solutions of TX-114 (Figure 2.4). Aqueous solutions of TX-114

Figure 2.2: SDS-PAGE analysis of the modulation of Schistosoma mansoni APM protein phosphorylation in vitro. Lane 1 illustrates a standard phosphorylation reaction carried out in the presence of 10 mM Mn^{2+} (section 2.2.12). Lanes 2 through 4 are standard phosphorylation reactions supplemented with 1 mM sodium vanadate. In addition to vanadate, reactions were supplemented with 1 μM 5-hydroxytryptamine (5-HT), (lane 3) and 1 μM cAMP (lane 4).



M_r x 10^{-3}
1 2 3 4
200
92
68
43
26
18

Figure 2.3: SDS-PAGE analysis of Schistosoma mansoni APM polypeptides phosphorylated in vitro and adsorbed onto lectin affinity matrices. Lane 1 illustrates a standard phosphorylation reaction which was diluted, centrifuged and subjected to room temperature incubations, as described in section 2.2.9 for the lectin affinity chromatography experiments . Lanes 2 and 3 represent the unbound and 0.5M N-acetylglucosamine eluate, respectively, from chromatography on WGA agarose. Lanes 4 and 5 represent the unbound and 0.5M α-methylmannoside eluate, respectively, from chromatography on lentil lectin agarose. Lanes 6 and 7 are the unbound and 0.5M α-methylmannoside eluate, respectively, from chromatography on Con A agarose.


$M_r \times 10^{-3}$
1 2 3 4 5 6 7
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92
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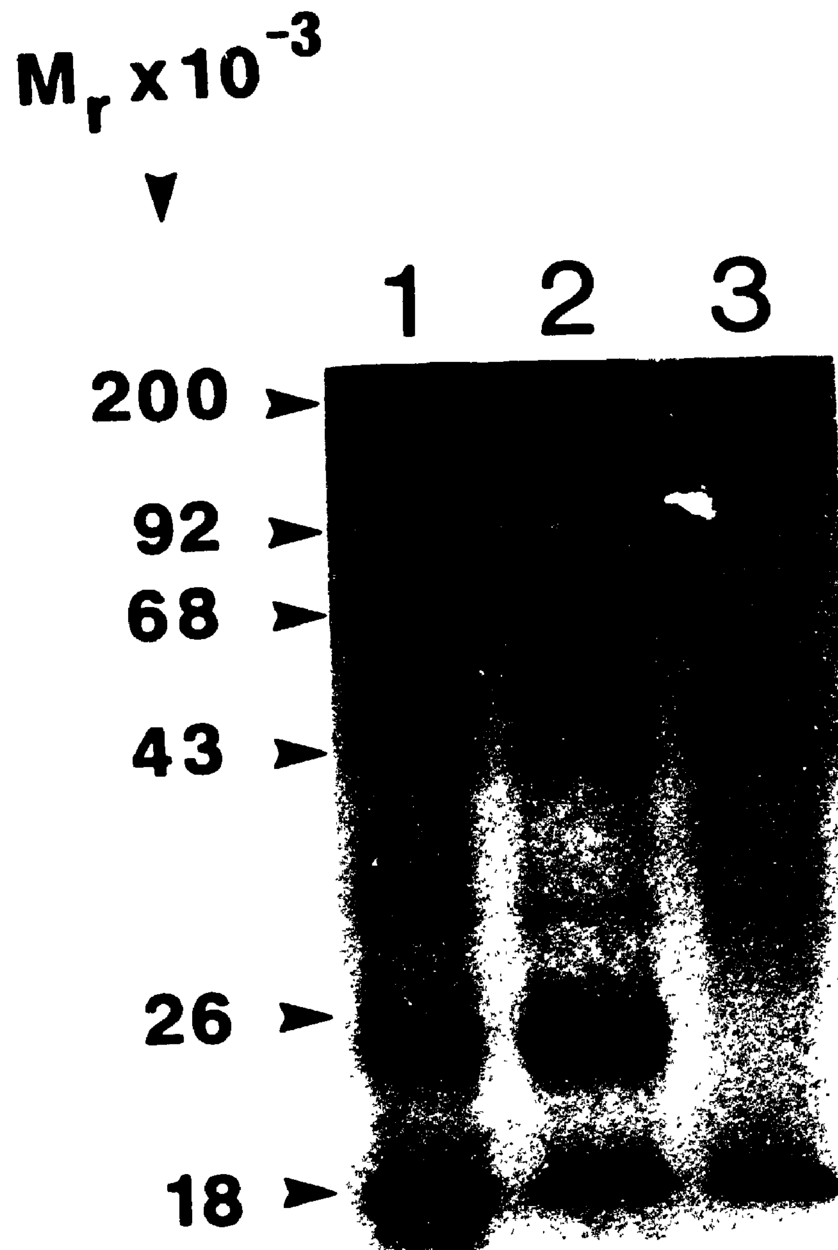
43
26
18

undergo a temperature-induced phase transition (see Figure 2.11) in which polypeptides which interact with the detergent partition into a detergent-enriched phase and polypeptides which do not bind detergent partition into a detergent-depleted phase (Bordier, 1981). The 65, 50, 28 and 24 kDa polypeptides were found primarily in the detergent-enriched phase (Figure 2.4, lane 2) indicating that they bind significant quantities of TX-114. The 54 kDa polypeptide appeared to partition primarily into the detergent-depleted phase (lane 3). The 160 and 38 kDa phosphoproteins appeared in both phases (lanes 2 and 3).

In light of some of the biochemical properties of in vitro phosphorylated APM polypeptides, it was of interest to determine if any of the identified phosphoproteins corresponded to parasite-specific antigens recognized by antibodies produced during infection or by an experimental immunization protocol known to elicit protective immunity. Figure 2.5 illustrates a Western blot analysis of APM antigens recognized by IgG antibodies in the serum of chronically infected mice (lane 1), mice immunized with the APM (lane 2) and normal mice (lane 3). A number of common and unique polypeptide antigens recognized by chronically infected and APM-immunized mice ranging in molecular weight from 200 to 24 kDa were revealed. The 24 kDa antigen was of particular interest, since its electrophoretic properties seemed to parallel those of the 24 kDa phosphoprotein previously characterized and it was a dominant

Figure 2.4: SDS-PAGE analysis of *Schistosoma mansoni* APM polypeptides phosphorylated *in vitro* and fractionated by phase separation in TX-114. Lane 1 illustrates a standard phosphorylation reaction. Lanes 2 and 3 represent the detergent-enriched and detergent-depleted phases, respectively, following phase separation in TX-114 as described in section 2.2.11.


M_r x 10^{-3}
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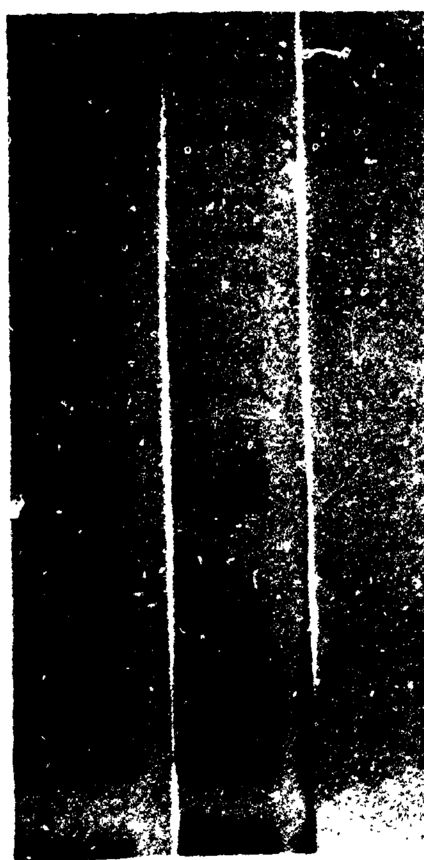
3
200
92
68
43
26
18

Figure 2.5: Immunoblot detection of APM polypeptides of Schistosoma mansoni recognized by different mouse antisera. APM polypeptides were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with the following antisera: Lane 1, infected mouse serum (IMS), 1/100 dilution; lane 2, mouse anti-APM serum, 1/500 dilution; lane 3, normal mouse serum (NMS), 1/100 dilution. Each lane of the gel was loaded with 2 μg protein.


$M_r \times 10^{-3}$
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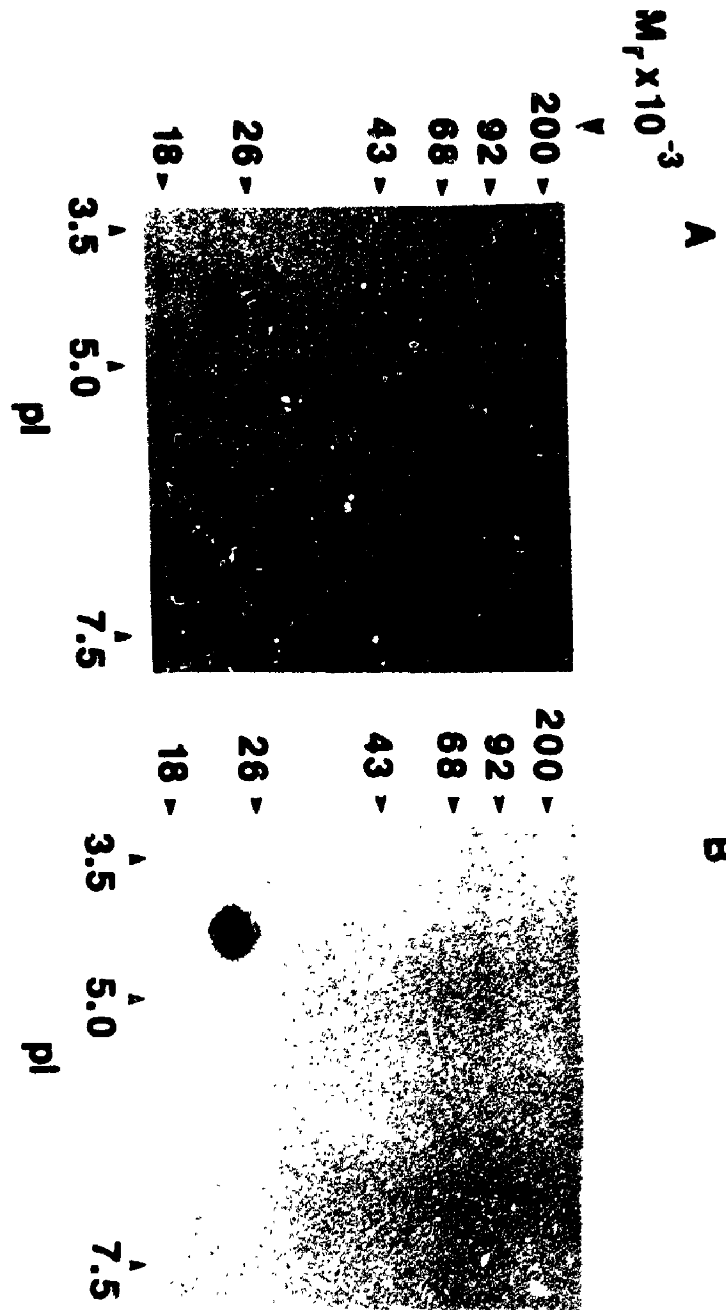
immunogen. Antibodies were subsequently affinity-purified by low pH elution from the 24 kDa region of preparative Western blots of immobilized APM probed with RαAPM, MαAPM, NMS and NRS (section 2.2.8). These antibodies were used in a variety of immunoprecipitation and blotting analyses to determine if the 24 kDa antigen corresponded to the 24 kDa phosphoprotein.

The specificity of these antibodies was first tested by two dimensional electrophoresis of APM proteins followed by immunoblotting (Figure 2.6b). Both rabbit and mouse affinity purified antibodies bound exclusively to a single antigen complex with approximate isoelectric points between 4.2 and 4.6 which corresponded to a faintly stained polypeptide complex with the same mobility properties (Figure 2.6a). This complex exhibited migrational heterogeneity in both the isoelectric focusing (IEF) dimension and the SDS dimension. Nonetheless, no other antigenic 24 kDa molecules were detected using antibodies prepared as described in section 2.2.8.

The affinity-purified antibodies were then used to immunoprecipitate APM polypeptides phosphorylated in vitro (Figure 2.7). RαAPM was shown to precipitate a prominent 24 and a faint 28 kDa phosphoproteins whereas NRS failed to precipitate anything. Antibodies eluted from the 24 kDa region of preparative APM Western blots and shown to be specific for an acidic 24 kDa antigen (Figure 2.6b) specifically immunoprecipitated a 24 kDa phosphoprotein (Figure 2.7, lane 4). No phosphoproteins were precipitated using material

Figure 2.6: Two-dimensional blot analysis of Schistosoma mansoni APM polypeptides using antibodies affinity purified from the 24 kDa region of preparative Western blots (section 2.2.8). APM polypeptides were resolved by two dimensional electrophoresis followed by immunoblot detection (sections 2.2.5 and 2.2.6). A) Amido Black stained blot of APM-associated polypeptides. B) Two-dimensional immunoblot probed with rabbit affinity purified antibodies. The first dimensional gel in A) was loaded with 40 μg protein while that in B) was loaded with 5 μg protein.



$M_r \times 10^{-3}$
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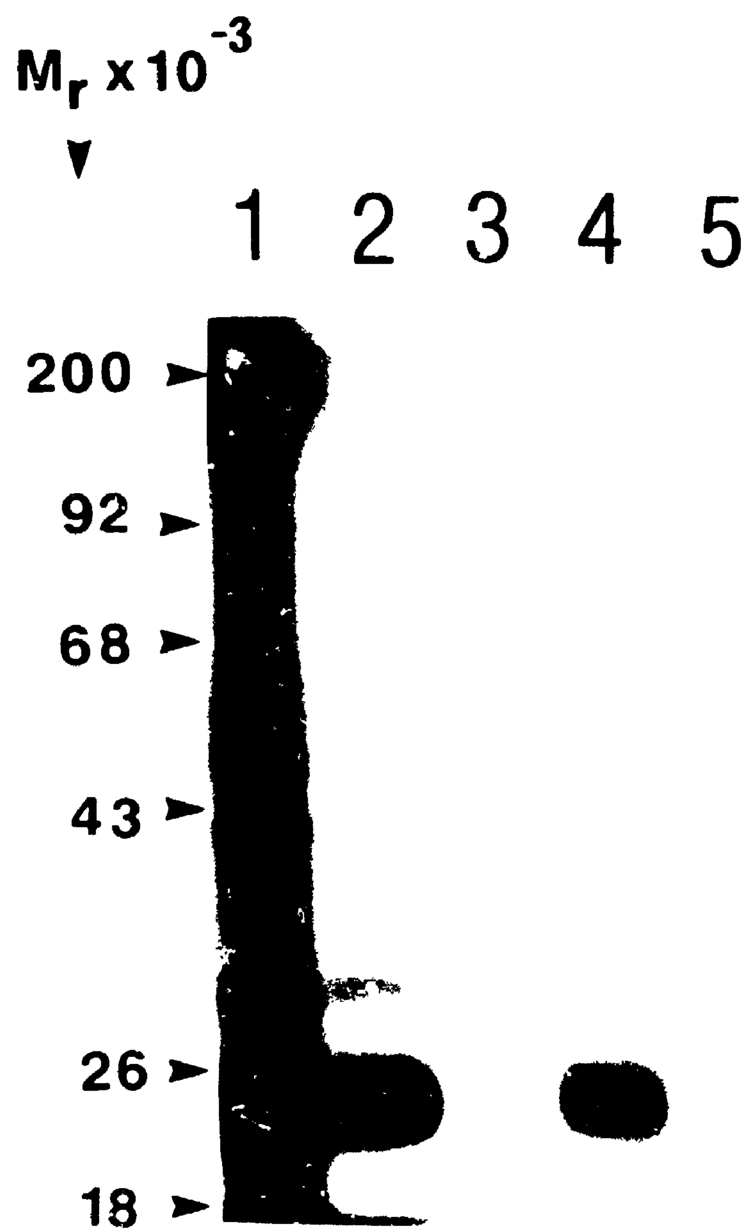
18
3.5
5.0
7.5
pI
B
200
92
68
43
26
18
3.5
5.0
7.5
pI

Figure 2.7: SDS-PAGE analysis of _Schistosoma mansoni_ APM polypeptides phosphorylated _in vitro_ and immunoprecipitated with rabbit anti-APM antiserum (RαAPM) and rabbit affinity purified antibodies. Lane 1 illustrates a standard phosphorylation reaction. Lanes 2 and 3 represent the immunoprecipitation of APM phosphoproteins with RαAPM and normal rabbit serum (NRS), respectively. Lane 4 represents immunoprecipitation with rabbit antibodies affinity purified from the 24 kDa region of preparative Western blots probed with RαAPM. Lane 5 represents immunoprecipitation with rabbit antibodies affinity purified from the 24 kDa region of preparative Western blots probed with NRS.



M_r x 10^{-3}
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43
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18

eluted from the 24 kDa region of preparative APM Western blots which were probed with NRS. These results support the suggestion that the 24 kDa antigen identified by immunoblot analysis corresponded to the 24 kDa polypeptide phosphorylated in vitro.

Analysis of lectin binding followed by immunoblotting using affinity-purified antibodies (Figure 2.8), demonstrated that the 24 kDa polypeptide bound and was specifically eluted from both lentil lectin and Con A agarose affinity matrices (Figure 2.8, lanes 3 and 5). Again, this result paralleled the findings obtained using lectin affinity chromatography of the 24 kDa phosphoprotein. These results were extended by an examination of the nature of the carbohydrate residues associated with the 24 kDa molecule (Figure 2.9). These studies were carried out following treatment with carbohydrate modifying agents and assaying for a shift in electrophoretic mobility. Experiments using membranes treated with sodium meta-periodate (Figure 2.9A) followed by the detection of the 24 kDa complex with affinity purified antibodies indicated that the mobility pattern of the complex was condensed and shifted slightly to a more rapidly migrating position. Treatment with neuraminidase or Endo H (Figure 2.9B, lanes 1-4), did not induce a mobility shift. Identical results were obtained with these enzymes even upon extended incubation for up to 18 hou However, incubation of membranes with Endo F or N-glycanase resulted in the rapid appearance of a discrete 20 kDa

Figure 2.8: Detection of Smgp24 in fractions from lectin affinity chromatography (section 2.2.9) by immunoblotting using mouse affinity purified antibodies. Lane 1 shows total APM protein. Lane 2 shows the unbound fraction and lane 3 the α-methylmannoside eluate from affinity chromatography on Con A agarose. Lane 4 shows the unbound fraction and lane 5 the α-methylmannoside eluate from chromatography on lentil lectin agarose. Each lane of the gel was loaded with approximately 500 ng APM protein.

$M_r \times 10^{-3}$

1 2 3 4 5

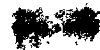
200 ►

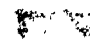
92 ►

68 ►

43 ►

26 ►



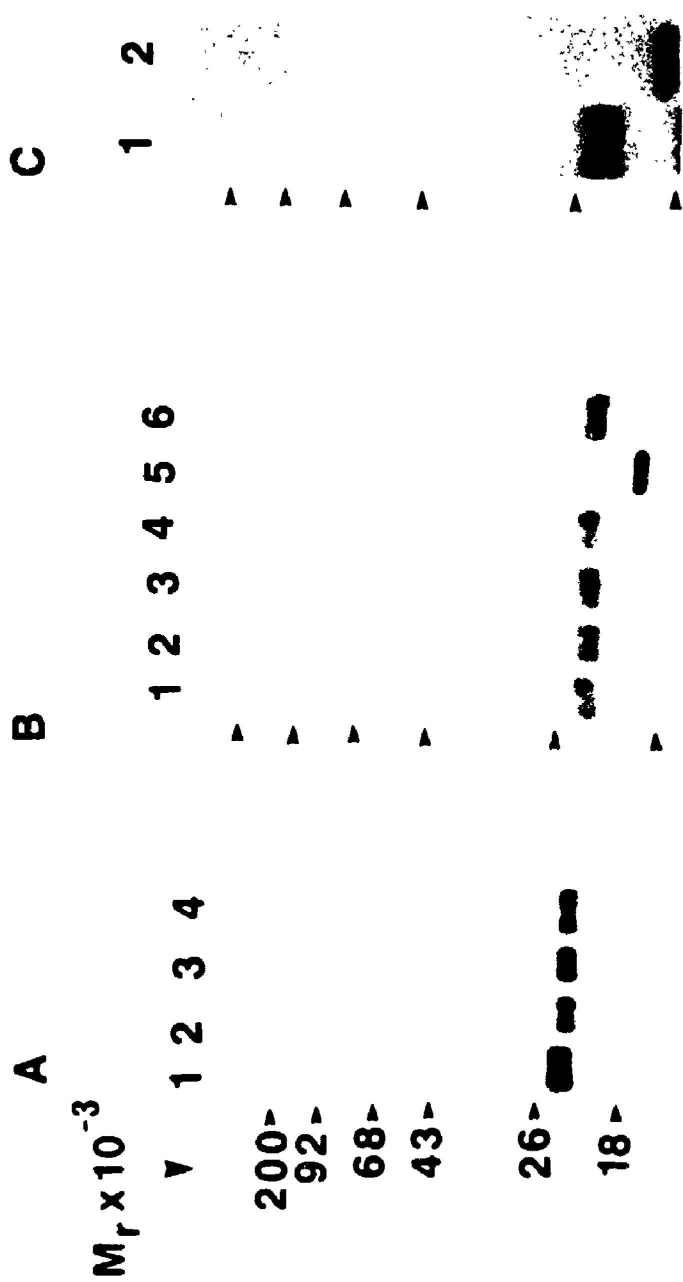


18 ►

component (Figure 2.9B, lane 5; Figure 2.9C, lane 2). The appearance of the 20 kDa species correlated with the disappearance of the 24 kDa complex under the indicated conditions. The presence of protease inhibitors in the incubation mixtures did not inhibit the appearance of the 20 kDa polypeptide during digestion with Endo F or N-glycanase. Moreover, the 20 kDa polypeptide produced by Endo F or N-glycanase treatment no longer bound to Con A agarose and was found primarily in the unbound fraction following lectin affinity adsorption experiments (Figure 2.10, lanes 4 and 6). The 20 kDa species was also a minor component of the Con A eluates (Figure 2.10, lanes 5 and 7).

A final experiment to confirm the identity of the 24 kDa antigen was carried out by examination of the properties of the antigen in solutions of TX-114 (Figure 2.11). This experiment demonstrated that the 24kDa molecule could not be completely solubilized from the membrane by treatment with 1% TX-114 for 10-30 minutes on ice (Figure 2.11B, lane 2). However, the rest of the 24 kDa complex could be almost quantitatively recovered in the detergent-enriched phase (Figure 2.11B, lane 3) suggesting that it bound significant quantities of the detergent. In addition, no 24 kDa antigen could be detected in the detergent-depleted phase (Figure 2.11B, lane 4). Thus, the 24 kDa antigen appeared to possess all the properties described for the 24 kDa phosphoprotein.

Figure 2.9: Immunoblot analysis of the effect of carbohydrate modifying agents on the electrophoretic mobility of the Smgp24 complex using mouse affinity purified antibodies. A) An examination of the effect of sodium meta-periodate. Membranes were treated with 0, 10, 50, or 100 mM periodate in lanes 1-4, respectively and analyzed by immunoblotting. B) An examination of the effects of neuraminidase, Endo H, and Endo F. Lane 1 shows treatment of membranes with 1 unit/mL neuraminidase and lane 2 shows the undigested control. Lane 3 illustrates the digestion of membranes with 0.25 units/mL Endo H and lane 4 shows the undigested control. Lane 5 shows treatment of membranes with 1 unit/mL Endo F and lane 6, the undigested control. C) An examination of the effect of N-glycanase. Lane 1 shows the undigested control and lane 2 shows treatment of membranes with 1 unit/mL N-glycanase. All enzyme digestions were carried out as described in section 2.2.10. Each lane of the gel was loaded with approximately 500 ng APM protein.



A
B
C
Mr x 10-3
200
92
68
43
26
18
1 2 3 4
1 2 3 4 5 6
1 2

Figure 2.10: Immunoblot analysis of the effect of Endo F and N-glycanase on the Con A binding properties of the Smgp24 complex using mouse affinity purified antibodies. APM proteins were digested with glycohydrolase as described in section 2.2.10.1 and chromatographed on lectin affinity matrices as described in section 2.2.9. Lane 1 shows total APM protein. Lane 2 represents the unbound fraction and lane 3 the α-methylmannoside eluate from Con A chromatography of APM proteins incubated in the absence of glycohydrolase. Lane 4 shows the unbound fraction and lane 5 the α-methylmannoside eluate from Con A chromatography using APM proteins treated with 1 unit/mL Endo F. Lane 6 represents the unbound fraction and lane 7 the α-methylmannoside eluate from Con A chromatography using APM treated with 1 unit/mL N-glycanase. Each lane of the gel was loaded with approximately 500 ng APM protein.

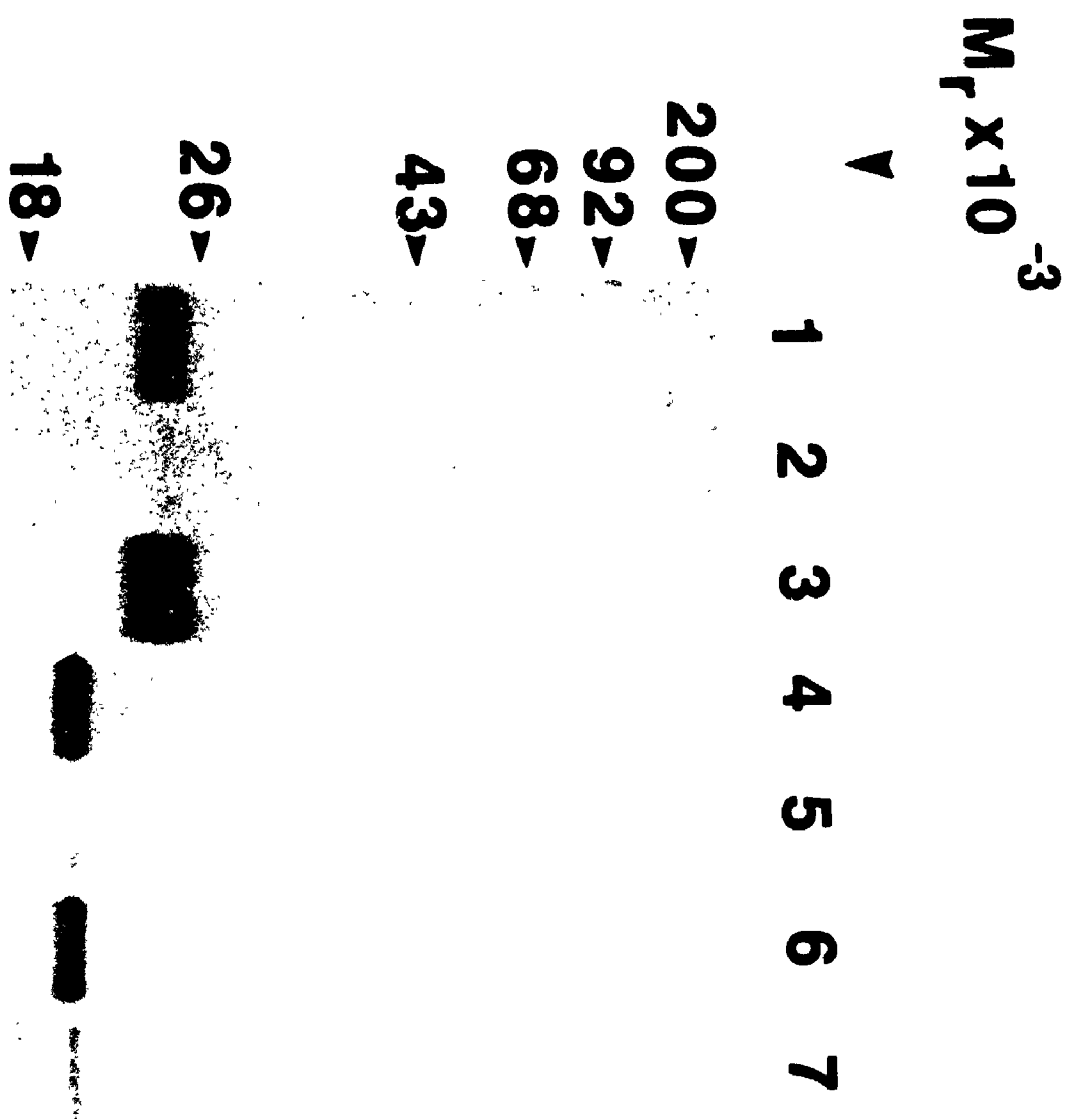
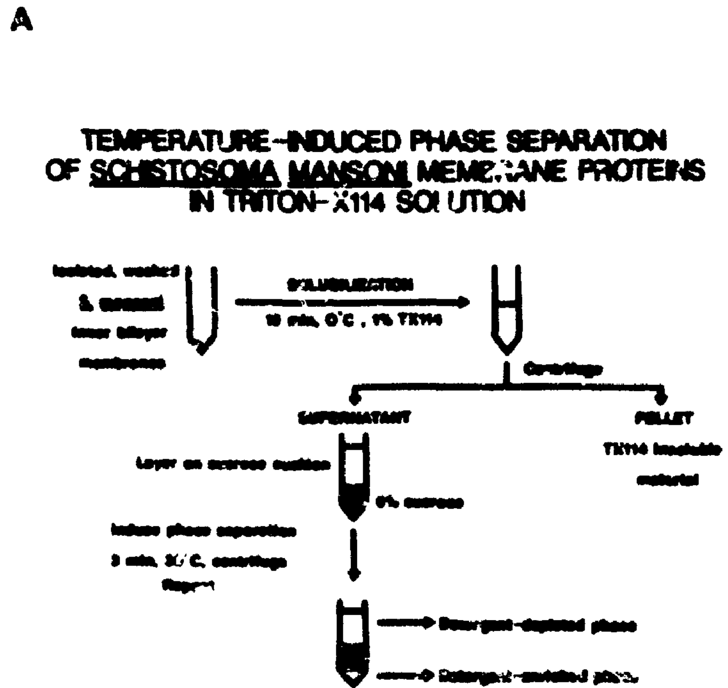
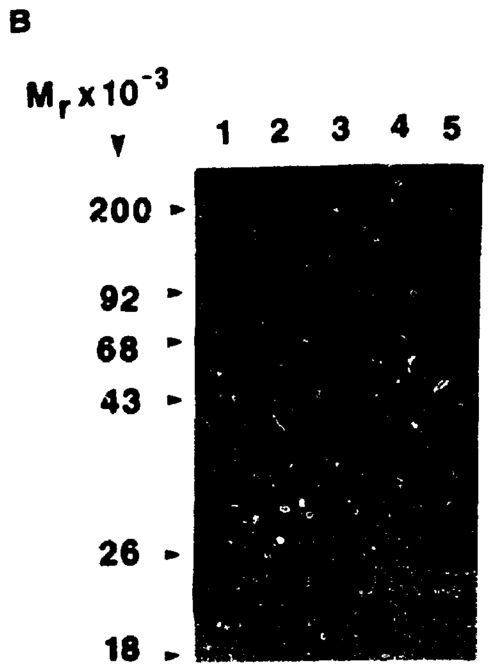

$M_r \times 10^{-3}$
200
92
68
43
26
18
1 2 3 4 5 6 7

Figure 2.11: Phase partitioning of the Smgp24 complex in solutions of Triton X-114. A) Flow diagram illustrating the method as applied to *Schistosoma mansoni* membrane proteins. B) Immunoblot detection of Smgp24 in Triton X-114 phases using rabbit affinity purified antibodies. Lane 1 shows total APM proteins. Lane 2 represents Triton X-114 insoluble material, lane 3, the detergent-enriched phase from TX-114 phase separation and lane 4 the detergent-depleted phase from TX-114 phase separation. Lane 5 shows total APM proteins probed with normal rabbit serum (NRS). Each lane of the gel was loaded with the entire fraction derived from phase separation using 2 μg APM protein as starting material.


A
TEMPERATURE-INDUCED PHASE SEPARATION
OF SCHISTOSOMA MANSONI MEMBRANE PROTEINS
IN TRITON-X114 SOLUTION
SUPERNATANT
PELLET


B
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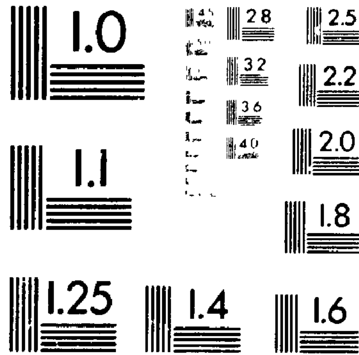


2.4 Discussion

Polypeptide phosphorylation is widely accepted as a mechanism which alters the properties of uniquely situated proteins responsible for a diverse range of regulatory events within cells (Shenolikar, 1987; Cohen, 1988). Interest in the characterization of phosphoproteins in our laboratory stems from the importance of these polypeptides in signalling mechanisms and the lack of biochemical information regarding this family of polypeptides in schistosomes. We postulate that investigation of the components of signalling mechanisms will provide new opportunities for the rational development of anti-schistosome vaccines and drugs. These components may represent ideal candidates as functionally important molecules or "Achilles heels", a concept discussed by Sher (1988). The emphasis in this chapter was the biochemical characterization of membrane-associated polypeptides phosphorylated in vitro and to determine if any of these phosphoproteins were immunogenic. In this light, a major antigen of 24 kDa has been shown to be phosphorylated and additional biochemical properties of this molecule have been described.

The experiments outlined in this chapter describe for the first time, the presence of endogenous protein kinase activity and protein substrates in the isolated APM of S. mansoni. This in vitro system makes use of small quantities of APM (1 μg per assay) and results in the phosphorylation of a limited number of polypeptides with distinct biochemical properties which can be readily

detected following SDS-PAGE and autoradiography. Although I was unable to discern what type(s) of kinase activity was present in the isolated APM fraction, the identification and characterization of endogenous substrates clearly indicate that further characterization of the kinase activity in this fraction is desirable.

Maximal phosphorylation was observed when isolated membranes were incubated in the presence of [$\gamma^{32}P$]ATP and 10 mM Mn^{2+}. The other ions examined induced phosphorylation of only a subset of the polypeptides observed in the presence of Mn^{2+}. The exception to this was the phosphorylation of a 110 kDa polypeptide found only in the phosphoprotein profile produced in the presence of Ca^{2+}. The phosphorylation of this polypeptide may thus be mediated by kinase activity requiring Ca^{2+}, such as protein kinase C or Ca^{2+}/calmodulin dependent protein kinase, although no attempt was made to optimize the conditions for these enzymes. However, both of these activities are known to be associated with membranes in other cell systems (Huganir and Greengard, 1987; Bazzi and Nelsestuen, 1988).

The Mn^{2+}-stimulated activity is somewhat unusual since many membrane-associated protein kinases (eg. casein kinase II, cAMP-dependent protein kinase) exhibit a preference for Mg^{2+} as cofactor (Huganir and Greengard, 1983; Hirato and Suzuki, 1987). A Mn^{2+} requirement is characteristic of some tyrosine protein kinases (Pang et al., 1988) although phosphotyrosine was not detected in schistosome membranes. Studies carried out in conjunction with Dr. E.H.

Ball of the Department of Biochemistry, U.W.O., suggest that the major phosphorylated residue in acid hydrolysates of S. mansoni APM proteins phosphorylated in vitro is phosphoserine (data not shown).

The effects of three possible modulators of phosphorylation were also examined when the standard reaction was supplemented with vanadate, 5HT, and cAMP. Vanadate is a known inhibitor of the Na^{+}-K^{+}-ATPase of schistosomes (Noel and Sauto-Pardon, 1989) and therefore, vanadate may simply increase the amount of ATP available for the phosphorylation. Cyclic AMP on the other hand, was observed to increase the phosphorylation of an 82 kDa polypeptide, but had no effect on the phosphorylation of the other polypeptides. This result points to the possible presence of a cAMP-dependent protein kinase activity in the APM fraction. Two of the membrane phosphoproteins identified here (with molecular masses of 160 and 24 kDa) are glycoproteins since they bind to and specifically elute from lectin affinity matrices. The behaviour of both of these species following chromatography on WGA, however, merits some comment. The results indicate that these two glycoproteins could be found in both the unbound and bound fraction using WGA. At least two possibilities may explain this behaviour. First, it is possible that the amount of WGA used for adsorption of APM glycoproteins was insufficient to bind all the potential WGA-binding moieties (i.e. insufficient column capacity). Secondly, it is also possible that in both the 160 and 24 kDa

molecules, only a fraction of either of these species possess the N-acetylglucosamine residue on their carbohydrate chains. This might be expected if the polypeptides exhibit carbohydrate microheterogeneity (differential glycosylation). This is manifested clearly in the case of the 24 kDa species where a more rapidly migrating population of this complex is found in the unbound fraction.

The behaviour of the phosphoproteins in solutions of Triton X-114 was also examined. This technique has been shown to fractionate polypeptides based on their interaction with the detergent (Bordier, 1981; Pryde, 1986). Triton X-114 solutions undergo a phase separation into a detergent-enriched phase and a detergent-depleted phase when warmed above 20°C. Integral membrane proteins are by definition polypeptides which require detergent for their solubilization and accordingly are found in the detergent-enriched phase after warming (Bordier, 1981). Conversely, polypeptides which do not interact with the detergent are found in the detergent-depleted phase. With respect to the schistosome APM phosphoproteins, the 65, 50 and 24kDa molecules appeared to partition preferentially into the detergent-enriched phase indicating properties consistent with integral membrane proteins. Only the 54 kDa polypeptide was found exclusively in the detergent-depleted phase. Both the 160 and 38 kDa polypeptides were found in both fractions. This may indicate that these two polypeptides are components of a noncovalent multi-component

complex whose individual components have both hydrophilic and hydrophobic characteristics. Often, integral membrane proteins which are strongly associated with cytoskeletal elements exhibit this type of behaviour in TX-114 solutions (Pryde, 1986).

One of the main objectives of the present study was to identify if any of the membrane-associated phosphoproteins described here were major immunogens either in a chronic infection or by an immunization protocol capable of eliciting protective immunity (ie. immunization with adult membrane proteins, Smithers et al., 1989; Huebert, 1990). This approach therefore integrates an immunological approach to membrane antigen characterization with one based on potentially important biochemical function. In this light, a 24 kDa antigen has been characterized and shown to be identical to the 24 kDa phosphoprotein by immunoprecipitation and analysis of lectin binding and Triton X-114 phase separation. The relationship between the 24 kDa antigen and the 24 kDa phosphoprotein was investigated by making extensive use of antibodies affinity purified from the 24 kDa region of preparative Western blots (Olmsted, 1981). I have demonstrated that antibodies prepared in this manner from polyspecific, polyclonal antisera (RαAPM, MαAPM), can be used to determine the properties of individual antigens. In addition, the nature of the carbohydrate moieties of the 24 kDa glycoprotein has been investigated further by endoglycosidase digestion. The results with respect to this polypeptide,

taken together, suggest that it is an immunogenic integral membrane glycoprotein with an acidic isoelectric point which is also phosphorylated in vitro. The oligosaccharide residues present on this molecule are primarily of the "complex" type since the polypeptide is sensitive to Endo F and N-glycanase and insensitive to Endo H (Trimble and Malley, 1984). Treatment of the 24 kDa polypeptide with Endo F or N-glycanase yields a 20 kDa species which is largely deglycosylated since the 20 kDa species does not bind appreciably to lectin affinity resins. Thus, the 20 kDa species may represent the protein backbone of the molecule which is post-translationally modified with carbohydrate chains to form the mature 24 kDa protein.

In summary, an in vitro system has been developed to study protein phosphorylation using the isolated APM from S. mansoni. Phosphorylation was not appreciably altered by vanadate and 5HT, while cAMP specifically stimulated the phosphorylation of an 82 kDa polypeptide. Two phosphoproteins (160 and 24 kDa) were also glycoproteins with the 24 kDa protein being a dominant immunogen. Further characterization of the 24 kDa protein is desirable for two reasons. Firstly, the phosphorylation of the 24 kDa protein implies that it may play an important regulatory role in cell signalling. Secondly, an immunogenic protein with properties similar to those described here for the 24 kDa protein has recently been implicated in p tective immunity by three different research groups (Smithers et al., 1989; Wright et al., 1988;

Huebert, 1990). Thus, the emerging candidacy of this molecule as an experimental vaccine will also need to be evaluated.

Chapter 3- Production and Partial Characterization of cDNA Clones

3.1 Introduction

Over the past number of years, recombinant DNA technology has played a leading role in many aspects of schistosomiasis research. From the first description of schistosome cDNA libraries (Cordingley et al., 1983) to the more recent characterization and expression of potentially protective antigens (Smith et al., 1988; Balloul et al., 1987), this methodology has provided new insights into the nature of the host-parasite relationship. This is particularly true in studies concerned with schistosome speciation (Walker et al., 1989), sexual differentiation (Koster et al., 1988; Reis et al., 1989), development (Newport et al., 1988; Ram et al., 1989), serodiagnosis (Klinkert et al., 1988; Klinkert et al., 1989), invasion and penetration mechanisms (Newport et al., 1988; Davis et al., 1987) and antigenic complexity (Lanar et al., 1985; Havercroft et al., 1988; Dalton et al., 1987), including antigens with demonstrable potential as protective vaccines (Balloul et al., 1987; Smith et al., 1987; Lanar et al., 1986). In addition to furnishing new information of direct relevance to applied control strategies, such as vaccine development, this information explosion has provided a new perspective on long-standing fundamental biological, metabolic and evolutionary questions. It is clear that continued analysis of structure-function relationships at the molecular level will enhance our perception of the host-parasite relationship.

The interest in the characterization and analysis of membrane proteins in our laboratory led me to adapt a similar molecular approach to the identification of membrane protein cDNAs. In particular, it was hoped when this study was initiated that proteins involved in potential signalling mechanisms, could be characterized. In this regard, particular emphasis was placed on the isolation of cDNAs encoding the 24 kDa phosphoprotein antigen, described in Chapter 2, such that an analysis of the antigenic and functional properties could be initiated.

The approach which was ultimately adapted might be described as a 'brute force' method for the identification of membrane protein cDNAs. Since no sequence information was available at the time for screening of cDNA libraries with nucleic acid probes, the use of anti-membrane antibodies to screen for antigenically related recombinant expression products, seemed to represent a useful strategy. The development of lambda bacteriophage expression vectors such as λgt11 (Young and Davis, 1983) suggested that isolation of membrane protein cDNAs, by screening of cDNA expression libraries, was a distinct possibility.

This chapter outlines the construction and screening of recombinant S. mansoni expression libraries and the preliminary characterization of immunoreactive clones. The use of the polyspecific anti-membrane antisera prepared as described in Chapter 2 resulted in the isolation of apparently

distinct clones from a λgt11 and a λZAP expression library. This approach remains unique in the schistosome field and provides a basis for a detailed analysis of individual adult parasite membrane-associated proteins.

3.2 Materials and Methods

3.2.1 Chemicals and Biochemicals

All general laboratory chemicals and biochemicals were obtained from commercial sources. Guanidinium isothiocyanate was a product of Fluka Fine Chemicals. Oligo-dT cellulose, Type 7, was purchased from P-L Biochemicals (now Pharmacia). Enzymes for the manipulation of DNA fragments (restriction enzymes, polymerases, ligases, kinases) were obtained from either Boehringer Mannheim, Pharmacia or Promega Biotec (BioCan Scientific) and used according to the manufacturer. AMV Reverse Transcriptase was obtained from Life Sciences, Inc., St. Petersburg, Fla. Oligonucleotides (oligo $dT_{12\text{-}18}$ and Eco RI linkers), random-primed DNA labelling kits and DNA and RNA molecular weight markers were purchased from Boehringer Mannheim. Media for the growth of bacteria and bacteriophage were obtained from Gibco/BRL Life Sciences. Placental ribonuclease inhibitor and dephosphorylated λgt11 DNA were purchased from Promega Biotec. In vitro λ packaging extracts were obtained from Stratagene Cloning Systems. Materials for electrophoresis of nucleic acids and proteins and Biogel A 1.5m (for gel filtration) were products of Bio Rad. A kit for the isolation of DNA from agarose gels (Geneclean™) was obtained from Bio 101 (BioCan Scientific). Nitrocellulose filter discs for phage screening were obtained from Millipore, whereas diethylaminoethyl (DEAE) membranes were purchased from Schleicher and Schuell. All radiochemicals were purchased

from New England Nuclear.

3.2.2 Bacterial Strains and Bacteriophage

The bacteriophage λgt10 and Escherichia coli strains C600 and C600 hfl were obtained from Dr. C. Parfett of the Cancer Research Laboratory at the University of Western Ontario. Strain C600 was used for the routine plating, growth and propagation of λgt10 whereas C600 hfl was used for the amplification of recombinant λgt10 libraries.

E. coli strains Y1088, Y1089 and Y1090 and C600 harbouring λgt11 as a lysogen were purchased from the American Type Culture Collection. These strains are described in Young and Davis (1983b). Y1088 was used for plating and amplification of λgt11 and λgt11 recombinant libraries. Y1090 was used for screening of λgt11 libraries while Y1089 was used for the production of recombinant lysogens.

In addition a λZAP recombinant cDNA library, E. coli strains BB4 and XL1-blue and phage M13 strain R408 were obtained from Stratagene Cloning Systems.

3.2.3 Growth Conditions for Bacteria and Bacteriophage

Bacterial cells used for plating of bacteriophage were grown overnight at 37°C in LB medium (10 g/L tryptone or peptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with 0.2% maltose and were used directly. Bacteriophage were grown on standard 85 mm or 150 mm diameter plastic petri plates

containing 1.5% agar in LB medium (bottom agar). Cells and phage were mixed in 0.6% agar in LB (top agar). For plating and phage titration, dilutions of bacteriophage solution prepared in λ diluent (10mM Tris, 10mM $MgCl_2$) were mixed with an equal volume of a saturated culture of fresh "overnight" cells and incubated for 20 minutes at room temperature. Following absorption of the phage, top agar prewarmed to 43°C was added and the mixture was poured onto bottom agar plates prepared two days previously. The plates were allowed to stand at room temperature for 5 minutes and incubation was continued at 37°C for a period of six hours to overnight.

Bacteria harbouring recombinant plasmids were grown overnight in LB medium in the presence of 100μg/mL ampicillin.

3.2.4 Isolation of Nucleic Acids

3.2.4.1 Isolation of λgt10 DNA

Stock λgt10 vector DNA was prepared using 10-30 85 mm "plate stocks". Approximately 10^6 phage were mixed with 0.15 mL of fresh saturated overnight culture of E. coli C600 and plated on freshly prepared LB agar plates supplemented with 0.2% glucose. The plates were incubated right side up in a humidified chamber at 37°C until confluent lysis was evident (5-6 hours). The plates were removed to 4°C and overlaid with 5 mL λ diluent and allowed to stand overnight. The liquid was then harvested, mixed with a few drops of chloroform and centrifuged for 2 hours at 20000 rpm in a Sorvall SS-34 rotor

at 4°C. The supernatants were decanted and the pellets were resuspended in 1 mL λ diluent. The phage solution was then layered onto a CsCl block gradient consisting of 1 mL of 5M CsCl in λ diluent underneath 3 mL 3M CsCl in λ diluent in a SW 50.1 ultracentrifuge tube. The gradients were centrifuged 1 hour at 35000 rpm in a Beckman SW 50.1 rotor. The bluish phage band was recovered from the 3M-5M CsCl interface in a volume of 0.5 mL and mixed with an equal volume of 7.2 M CsCl and placed in a fresh ultracentrifuge tube. The phage were then overlaid with 3 mL of 5M CsCl and 1mL 3M CsCl and were centrifuged to the interface as above. The final phage band was again removed, placed in a dialysis sac and dialysed overnight against 20 mM Tris pH 7.5, 50 mM NaCl, 2mM EDTA. The partially disrupted phage were removed from the dialysis bag and supplemented with SDS to a final concentration of 0.5%. The mixture was heated to 55°C for 10 minutes. Potassium acetate was then added to a final concentration of 0.5M and the tubes were placed on ice for 5 minutes. The flocculent precipitate was removed by centrifugation in an Eppendorf microcentrifuge at 15000 x g for 10 minutes and the supernatant was then removed and dialysed extensively against 10 mM Tris acetate pH 8, 0.5 mM EDTA. The final phage DNA preparation was quantitated by measuring the absorbance at 260 nm assuming 1 $O.D._{260}$ unit corresponded to 50 μg/mL DNA. The yield of vector DNA using this procedure was approximately 5-10 μg DNA per plate lysate.

3.2.4.2 Isolation of λgt11 DNA

Bacteriophage λgt11 was obtained by induction of a C600[λgt11] lysogen. A small 2 mL culture was grown in LB medium overnight at 30°C and used to inoculate 4 flasks containing 125 mL each of enriched growth medium (16 g/L tryptone, 8 g/L yeast extract, 5 g/L NaCl). The cells were grown to an optical density at 600 nm ($O.D._{600}$) of 0.5 (mid-log phase). The temperature of the culture was then rapidly increased to 42°C and incubation continued for 20 minutes at this temperature. The temperature of the culture was then lowered to 37°C and incubation was continued for 2 hours. The cells harbouring the λgt11 phage were collected by centrifugation at 7000 rpm in a Sorvall GSA rotor for 15 minutes at 4°C. The cells in each centrifuge bottle were then taken up sequentially in 10 mL λ diluent and were lysed with 1 mL chloroform. The tube containing the cell lysate was supplemented with a few crystals of bovine pancreatic DNAse and was turned end over end for 2 minutes. The lysate was then centrifuged at 10000 rpm in a Beckman JS-13 rotor for 10 minutes at 4°C and loaded onto a CsCl block gradient prepared in a SW 40 ultracentrifuge tube. The gradient consisted of three 1 mL blocks of CsCl solutions in λ diluent of density 1.7 g/cc, 1.5 g/cc and 1.3 g/cc. The blocks were overlaid with 0.5 mL of 20% sucrose in λ diluent followed by the crude cell lysate. The gradients were centrifuged at 22000 rpm for 2 hours in an SW 40 rotor. The phage band was removed from the tube in a volume of 1 mL,

and made up to a final volume of 5 mL with 1.5 g/cc CsCl and centrifuged to equilibrium for 16.5 hours at 30000 rpm in a Beckman 50 Ti rotor. The phage band was removed from the tube and CsCl was removed by dialysis against 20 mM Tris pH 7.5, 50 mM NaCl, 2 mM EDTA. DNA was prepared from the λgt11 phage exactly as described above for λgt10.

3.2.4.3 Isolation of Plasmid DNA

Plasmid DNA was recovered from recombinant bacteria by the alkaline lysis method described by Maniatis et al. (1982) based on the method of Birnboim and Doly (1979).

3.2.4.4 Restriction Enzyme Digestion of DNA

λ DNA isolated as described above or commercially available λ or plasmid DNA was digested with restriction endonucleases essentially as described by the manufacturer using the concentrated buffer solution provided with the enzyme. A standard 20 μL reaction contained 1-2 μg λ or plasmid DNA in the supplied restriction buffer and 3-5 units of restriction enzyme. The reaction was incubated for 1-3 hours at 37°C. This basic method was scaled up or down when greater or less DNA was to be analyzed or prepared.

3.2.4.5 Isolation of RNA from Schistosoma mansoni

Total RNA was isolated from fresh adult male and female S. mansoni. Parasites were extensively washed in KRP and blotted dry on four layers of cheesecloth. The wet weight of the worms was estimated and the parasites

were transferred to a tube containing 4M guanidinium isothiocyanate, 0.1M Tris pH 7.4, 10 mM EDTA, 1% β-mercaptoethanol and immediately homogenized for 20 seconds using a polytron mixer (Brinkman Instruments). Five mL of the guanidinium isothiocyanate solution was used per gram wet weight of parasite material. The schistosome homogenate was then centrifuged at 10000 rpm in a Beckman JS-13 rotor at 4°C. The supernatants were then extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (PCI, 25:24:1). The aqueous phases containing parasite nucleic acids were then pooled and precipitated with 2.5 volumes of ethanol at -20°C overnight. Precipitated material was collected by centrifugation at 10000 rpm in the JS-13 rotor for 30 minutes at 4°C. The supernatants were decanted and the pellets were rinsed with 70% ethanol and dried under vacuum. The dried samples were then resuspended in 0.2 mL deionized double distilled water (ddH_2O) and made up to 3.5 mL with guanidinium isothiocyanate solution. The nucleic acid solution was then supplemented with 1.4 g CsCl and layered onto a 1.2 mL cushion of 5.7 M CsCl in 0.2 M EDTA (pH 7.5) and centrifuged for 18 hours at 30000 rpm in a Beckman SW 50.1 rotor at 22.5 °C. The supernatants were carefully removed and the RNA pellets were resuspended in sterile ddH_2O, adjusted to 0.3 M sodium acetate and precipitated with 2.5 volumes of ethanol. The pellet was rinsed with 70% ethanol, dried and dissolved in sterile ddH_2O. The RNA was quantitated by measuring the absorbance of a diluted RNA sample at 260

nm assuming that 1 O.D.$_{260}$ unit corresponded to 40 μg/mL RNA. Total RNA yields using this procedure were between 0.75 and 1 mg total RNA per gram wet weight of parasite tissue. Where possible, all glassware and solutions used in the manipulation of RNA were autoclaved or autoclaved and baked at 80°C for two hours. RNA solutions were stored at -70°C directly in ddH$_2$O for short-term storage or as a precipitate under 70% ethanol at -70°C for longer term storage.

3.2.4.6 Isolation of polyadenylated mRNA

Polyadenylated mRNA was isolated from total S. mansoni RNA by affinity chromatography on Oligo-dT cellulose, Type 7. 100mg oligo-dT cellulose was extensively equilibrated with binding buffer (20 mM Tris pH 7.4, 0.5 M NaCl 2 mM EDTA, 0.1% SDS). Microfine particulates were removed by several cycles of settling and resuspension of the cellulose in binding buffer. The cellulose slurry was poured into a 10 mL syringe equipped with a 20 gauge needle and a Whatman GF/A filter in the bottom to prevent loss of the matrix. The cellulose was further washed in the column with 5 to 10 bed volumes of binding buffer before loading of the total RNA. S. mansoni RNA was first heated to 60°C for 5 minutes in binding buffer and applied to the column. In different experiments, between 1 and 10 mg of total RNA was loaded. Unbound material which passed thr-ugh the column on the first pass was reapplied to the column. The cellulose was washed sequentially with 5 bed

volumes of binding buffer, 3 volumes of 20 mM Tris pH 7.4, 0.1M NaCl, 2 mM EDTA, 0.1% SDS (low salt wash) and poly A+ mRNA was eluted with 20 mM Tris pH 7.4, 2 mM EDTA, 0.1% SDS (elution buffer). The poly A+ mRNA was then supplemented with twice concentrated binding buffer, reheated and reapplied to the same column which was re-equilibrated with binding buffer. The entire procedure was repeated and the final eluate in elution buffer (approximately 3 mL) was adjusted to 0.3M sodium acetate and precipitated with 3 volumes of ethanol at -70°C overnight in a siliconized glass tube. Poly A+ RNA was recovered by centrifugation for 30 minutes at 15000 x g. The pellet was rinsed with 70% ethanol , dried and dissolved in sterile ddH_2O, quantitated and stored at -70°C. The yield of poly A+ RNA was 1-5% of the total RNA originally applied to the column. The RNA fractions were analyzed by agarose gel electrophoresis as described in Section 3.2.5.6

3.2.5 Gel Electrophoresis of Nucleic Acids and Proteins

3.2.5.1 Agarose Gel Electrophoresis of DNA

Intact or restricted DNA was routinely analyzed by submarine agarose gel electrophoresis using an apparatus constructed in-house or purchased from a commercial supplier. Agarose gels were prepared and run in 40 mM Tris acetate, pH 8, 1 mM EDTA (TAE buffer) or 89 mM Tris borate, pH 8, 2mM EDTA (TBE buffer). Gels of 0.5-1.2% agarose were cast depending on the size of the DNA to be analyzed. Samples were prepared by two to five fold dilution

in loading buffer (0.2 M EDTA, pH 8, 20% glycerol, 0.05% bromphenol blue). Samples were loaded into the preformed pockets of the gel and run using a voltage gradient of about 10 V/cm. DNA molecular weight markers were either purchased commercially or provided by restriction enzyme digestion of λ DNA with Hind III or pBR322 with Rsa I or Hinf I. The length of the gel run was determined by the migration of the co-electrophoresed marker dye and was usually between 45 minutes to three hours. DNA was visualized in the gel by staining in a solution of running buffer containing 0.5 μg/ mL ethidium bromide for 30 minutes to 1 hour followed by viewing on an ultraviolet transilluminator.

3.2.5.2 Isolation of DNA from Agarose Gels

DNA electrophoresed on agarose gels was isolated using a method based on electroelution onto diethylaminoethane (DEAE) anion exchange membrane. The DNA fragment or size class to be isolated was first visualized with ethidium bromide staining. A small slit was then made to the anode side of the DNA in the gel and the gel was replaced in the electrophoresis tank. Electrophoresis was continued until the band(s) of interest were clearly visible on the DEAE membrane using a hand-held UV lamp. Care was taken to avoid contamination with other DNA bands migrating close to the band of interest. The DEAE membrane was then removed from the slit and washed with two changes of 0.2 M NaCl in 20 mM Tris, pH 7.4, 1mM EDTA. The DNA was then eluted by incubating the membrane in 1 M NaCl in 20 mM Tris, pH 7.4, 1 mM EDTA at

60°C for 10 minutes. The eluate was then extracted once with an equal volume of 2-butanol, twice with an equal volume of PCI, and once with chloroform. Two volumes of ethanol were added, the DNA was chilled at -20°C overnight and then recovered by centrifugation at 15000 x g for 15 minutes at 4°C in an Eppendorf microcentrifuge. The DNA pellet was then rinsed with 70% ethanol, dried and resuspended in a small volume (5-20 μL) of TE (10mM Tris, pH 7.5, 0.1mM EDTA). Often, 1-5 μg of E. coli tRNA was added prior to ethanol addition to enhance the recovery of the DNA of interest if such an addition did not interfere with subsequent manipulation or analysis.

Alternatively, DNA was isolated directly from agarose gel slices using a commercially available kit (Geneclean™), using the manufacturers instructions. This method involves the dissolution of agarose at 50 °C using the chaotrope NaI. The DNA was then adsorbed onto fine glass beads, washed and eluted in water or TE.

3.2.5.3 Polyacrylamide Gel Electrophoresis of DNA

DNA between 20-600 bp in size was analyzed by non-denaturing polyacrylamide gel electrophoresis. Gels of 6% polyacrylamide were prepared and run in TBE buffer. Samples and molecular weight markers were loaded in the same buffer as described for agarose gels (Chapter 3.2.5.1). Electrophoresis was carried out at a voltage gradient of approximately 5 V/cm using polyacrylamide slab gels of dimensions 10 X 20 cm for about 3 hours. DNA

was visualized by ethidium bromide staining and ultraviolet transillumination or by autoradiography of the frozen gel.

3.2.5.4 Isolation of DNA from Polyacrylamide Gels

DNA in polyacrylamide gel was isolated by excision of the ethidium-bromide stained band from the gel and subsequent maceration of the gel slice in a flame-sealed 1 mL pipette tip plugged at the bottom with siliconized glass wool. 600μL of elution buffer (0.5M ammonium acetate, pH 8, 10mM $MgCl_2$, 0.1% SDS) was added to the gel pieces, the top was sealed with parafilm and the DNA fragments were allowed to elute passively overnight at 37°C. The eluate was then recovered by centrifugation at 400 xg in a clinical centrifuge following removal of the tip and placement within a siliconized glass centrifuge tube. The gel pieces were then rinsed with a further 600 μL elution buffer and recentrifuged. The pooled eluates were then extracted twice with 2-butanol, twice with PCI and once with chloroform. The DNA was then precipitated with two volumes of ethanol, centrifuged, rinsed with 70% ethanol, dried and resuspended in 5-20 μL of TE.

3.2.5.5 Alkaline Agarose Gel Electrophoresis of DNA

Alkaline agarose gels were used for the analysis of the radiolabelled products of first and second strand cDNA synthesis. 1.2 % agarose gels were cast in 50 mM NaCl, 1 mM EDTA. The gel was then soaked in alkaline running buffer (30mM NaOH, 1mM EDTA) for 1 hour at room temperature. Samples

containing approximately 10000 TCA-precipitable cpm were loaded in 50mM NaOH, 1mM EDTA, 2.5% Ficoll, 0.025% bromocresol green. The gel was then electrophoresed for approximately 1 hour at 45 volts. Following electrophoresis, the gel was neutralized in 0.5 M Tris, pH 7.4 for 20 minutes, placed on several layers of filter paper and placed in an oven for 45 minutes at 75°C. The gel was then dried flat on a gel dryer. The dried gel was then exposed to Kodak X-Omat AR film with an intensifying screen.

3.2.5.6 Agarose Gel Electrophoresis of RNA

RNA was analyzed by denaturing agarose gel electrophoresis using glyoxal as described by McMaster and Carmichael (1977). RNA samples in sterile ddH_2O were denatured for 1 hour at 55°C in 10 mM sodium phosphate, pH 7, 50% v/v dimethyl sulfoxide, 16% v/v deionized glyoxal. The samples were then supplemented with one fifth volume of 10 mM sodium phosphate, pH 7.0, 20% v/v glycerol, 0.1% bromphenol blue and loaded onto a 1.2% agarose gel prepared in 10 mM sodium phosphate. RNA molecular weight markers were provided by Dr. G.A. Mackie (U.W.O.) and Phillip Wong (U.W.O.) and consisted of E. coli (23S and 16S) or human (28S and 18S) ribosomal RNA. Alternatively, markers were purchased from Boehringer Mannheim. Electrophoresis was carried out using 10mM sodium phosphate as running buffer for 2 to 3 hours at 75 volts or until the dye was 2-3 cm from the end of the gel. The buffer was recirculated during the run to prevent the formation

of a pH gradient. Gels were stained by first pre-soaking the gel in 50 mM NaOH for 20 minutes followed by incubation for 1 hour in 0.5% ethidium bromide in 0.5 M ammonium acetate. RNA was visualized in the gel by UV transillumination.

3.2.5.7 Electrophoresis of Proteins

Analysis of proteins by SDS-PAGE was carried out as described in Chapter 2, section 2.2.5.

3.2.6 Synthesis of Double-stranded, Blunt-ended Complementary DNA

3.2.6.1 Synthesis of First Strand complementary DNA (cDNA)

First and second strand cDNA was synthesized using S. mansoni poly A+ RNA as template using the conditions of Gubler and Hoffman, (1983) as a basis. First strand synthesis was carried out using avian myeloblastosis virus reverse transcriptase (AMV-RT) and 7.5 μg poly A+ RNA in a final volume of 50 μL. The reaction mixture contained 50 mM Tris pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 1.25 mM each of deoxyguanosine triphosphate (dGTP), deoxyadenosine triphosphate (dATP) and thymidine triphosphate (TTP), 0.5 mM deoxycytidine triphosphate (dCTP), 20 μCi [α^{32}P]dCTP (3000 Ci/mmol), 100 μg/mL oligo dT_{12-18}, 30 units placental ribonuclease inhibitor and 20 units AMV-RT. The reaction was incubated for 2 hours at 43°C. These conditions were elucidated following a series of experiments designed to optimize the synthesis of the first strand product with respect to both yield and integrity. The yield

of first strand cDNA was estimated from the specific radioactivity of dCTP in the reaction mixture and from the amount of radioactivity incorporated into cDNA. The latter was determined by precipitation of aliquots of the reaction taken throughout the incubation with trichloroacetic acid (TCA) in the presence of 100 μg/mL bovine serum albumin (BSA) carrier. The TCA precipitates were collected onto glass microfibre filters by vacuum filtration and radioactivity determined by liquid scintillation counting or by Cerenkov counting. The integrity of the first strand product was monitored by alkaline agarose gel electrophoresis as described in section 3.2.5.5. Following optimization of the conditions for first strand synthesis, reactions to be used for further analysis were prepared either in the absence of a radioactive tracer or by substitution of [^{3}H]dCTP (100 mCi/mmol). The first strand reaction was then stopped by the addition of EDTA to 20 mM and was routinely precipitated twice from 2 M ammonium acetate with two volumes of ethanol in preparation for second strand synthesis.

3.2.6.2 Synthesis of Second Strand cDNA

Second strand cDNA synthesis was carried out in a final volume of 250 μL containing 0.5 to 1 μg first strand cDNA (1-2 μg of cDNA-mRNA hybrid) in 20 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 150 μM β-nicotinamide dinucleotide (β-NAD), 40 μM each dGTP, dATP, TTP and dCTP, 10 μCi [α^{32}P]dCTP , 3units E. coli RNAse H, and 50 units E. coli DNA

polymerase I. The reaction was incubated for 2 hours at 15 °C and for a further 1 hour at 22°C. The second strand reaction was also assayed for yield and integrity as described for the first strand reaction. Samples used for analysis by alkaline agarose gel electrophoresis were prepared with unlabelled first strand reaction product. Samples for further use were prepared in the presence of [^{3}H]dCTP. The final double stranded cDNA preparation was precipitated twice from 2 M ammonium acetate using two volumes of ethanol.

3.2.6.3 Preparation of Radiolabelled Marker DNA

Marker DNAs for alkaline agarose gel electrophoresis were prepared following double digestion of pBR322 with Bam HI/Eco RI and Bam HI/Ava I. Aliquots of the digests were analyzed on agarose and acrylamide gels as described in sections 3.2.5.1 and 3.2.5.3. Digestion with Bam HI/ Eco RI produced fragments of 0.38 kilobases (kb) and 3.9 kb, whereas Bam HI/AvaI digestion produced fragments of 1 and 3.3 kb. These fragments were then labelled by repair of the recessed ends with Klenow fragment of DNA polymerase I. Restriction digests containing 2 μg pBR322 DNA in 20 μL of restriction buffer (50mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 100 mM NaCl) were supplemented directly with 20 μCi [α^{32}P]dATP (for the Bam/Eco digest) or 20 μCi [α^{32}P]dCTP (for the Bam/Ava digest). The remaining unlabelled deoxynucleotide triphosphates were added to the corresponding reactions to a final concentration of 0.125 mM. Two units of Klenow

polymerase was then added and the reactions were incubated at room temperature for 15 minutes. Unlabelled dATP and dCTP were then added to the corresponding reactions until their final concentrations were also 0.125 mM and incubation was continued for a further 15 minutes. The reactions were stopped by adding EDTA to a final concentration of 20 mM and the radioactivity incorporated into DNA was determined by TCA precipitation. Aliquots of approximately 2000 cpm were co-electrophoresed with the first and second strand reaction products on alkaline agarose gels.

3.2.6.4 Desalting of Double Stranded cDNA

The double stranded cDNA precipitate was resuspended in 100 μL 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA and was applied to a 1 mL "spun column" of Sephadex G-25 previously equilibrated with the same buffer and prepared in a 1 mL syringe plugged with siliconized glass wool (Maniatis et al., 1982). The cDNA was then recovered by a 4 minute centrifugation at 1300 xg in a clinical centrifuge in a final volume of 100 μL. Recovery of the double stranded material was estimated by liquid scintillation counting following precipitation of the cDNA with ethanol.

3.2.6.5 Treatment of Double Stranded cDNA with T4 DNA Polymerase

The double stranded cDNA was treated with T4 DNA polymerase to maximize the number of blunt-ended molecules in the preparation. 200 ng-1 μg cDNA was processed in a 50 μL reaction containing 33 mM Tris pH 7.8, 66

mM potassium acetate, 10mM magnesium acetate, 1 mM DTT, 100μg/mL BSA and 1 unit T4 DNA polymerase. The reaction was allowed to proceed for 3 minutes at room temperature followed by addition of all four deoxynucleotide triphosphates to a final concentration of 100 μM. The reaction was then incubated at 37°C for a further 30 minutes, extracted with PCI and precipitated with ethanol.

3.2.7 Cloning of Double-stranded DNA

3.2.7.1 Phosphorylation of Eco RI linkers

200 pmol Eco RI linkers (5'-GGAATTCC-3') were phosphorylated in a 10 μL reaction containing 10 μCi [γ^{32}P]ATP (3000 Ci/mmol) in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, and 2.5 units T4 polynucleotide kinase. The reaction was allowed to proceed for 30 minutes at 37°C. The incubation mixture was then supplemented with unlabelled ATP to a final concentration of 0.5 mM and the reaction was incubated for a further 30 minutes at 37°C. Phosphorylated linkers were then stored at -20°C or used directly. An aliquot of 10 pmol linkers was assayed for the ability to self-ligate by incubation in 66 mM Tris, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP (ligation buffer) containing 2 units T4 DNA ligase. The ligation was incubated for 2 hours at 14°C. Two μL of the ligation was electrophoresed on a 6% polyacrylamide gel (section 3.2.5.3). The gel apparatus was disassembled, the gel was wrapped in Saran wrap and then autoradiographed at -70°C.

3.2.7.2 Ligation of Phosphorylated Eco RI linkers to Double Stranded cDNA

Phosphorylated Eco RI linkers were ligated to double stranded cDNA in a final volume of 20 μL in a reaction mixture containing ligation buffer, 125 pmol [^{32}P] linkers, 0.5-1 μg cDNA and 5 units T4 DNA ligase. The reaction was incubated at 14°C for 4 hours. An aliquot of the reaction was analyzed by polyacrylamide gel electrophoresis and autoradiography as described in section 3.2.5.3.

3.2.7.3 Digestion of Linker-cDNA Ligation

The linker-cDNA ligation reaction was then treated for 10 minutes at 70°C to heat-inactivate the ligase enzyme. The reaction was made up to 200 μL and its composition was adjusted to Eco RI digestion buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 5mM DTT, 100mM NaCl). 100 units Eco RI was added and the reaction incubated at 37°C for 2 hours. A further 50 units Eco RI was added and incubation was continued for 1 hour at 37°C. An aliquot of the digest was analyzed by polyacrylamide gel electrophoresis and autoradiography to assay for the completeness of digestion.

3.2.7.4 Separation of Linkered cDNA from Free Linkers and ATP

Linkered cDNA was then separated from free linkers and labelled ATP by gel filtration chromatography on Bio Gel A 1.5m in a column (1 x 30 cm) equilibrated in 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mg/ml yeast RNA. The column was washed with 5 volumes of this buffer followed by

10 volumes of the same buffer without yeast RNA. The cDNA-linker digest was loaded onto the column and fractions of approximately 250 μL were collected in Eppendorf microcentrifuge tubes. The radioactivity of each of the fractions was determined by Cerenkov counting. The fractions containing the cDNA (in the column void volume) were pooled and precipitated with ethanol in the presence of 1 μg E. coli tRNA.

3.2.7.5 Ligation of Linkered cDNA to Eco RI-digested λgt10 DNA

Aliquots of approximately 50 ng of linkered cDNA were ligated to 1 μg of Eco RI-digested λgt10 DNA in ligation buffer in a final volume of 5 μL using 2 units T4 DNA ligase. The ligation was incubated at 14 °C for 8 hours.

3.2.7.6 In vitro Packaging of Recombinant DNA

The cDNA-λgt10 recombinants were packaged into phage heads in vitro using commercially available λ packaging extracts. Approximately 2.5 μL of ligation was used per packaging extract. Packaged phage were then titred on E. coli C600 and the ratio of recombinants to non-recombinants was determined.

3.2.7.7 Amplification of λgt10 cDNA Library

10^6 recombinant phage were amplified on E. coli C600 hfl using 10 plate stocks of 10^5 recombinant phage per plate. 10^5 recombinants were mixed with 0.3 mL of an overnight culture of E. coli C600 hfl and plated on fresh LB plates containing 0.2% glucose. Following 6 hours of growth in a humidified

chamber, the plates were overlaid with λ diluent. These plate stocks were then titred and equal quantities of each individual plate stock were mixed (10^{10} phage from each plate). This mixture then constituted the λgt10 S. mansoni cDNA library. 10^6 recombinants from the λgt10 S. mansoni cDNA library were subsequently amplified and the DNA was isolated using the CsCl block gradient method as described for λgt10 (section 3.2.4.1).

3.2.7.8 Isolation of Amplified cDNA from λgt10 S. mansoni cDNA Library

DNA from the recombinant λgt10 S. mansoni cDNA library (200 μg) was digested with 300 units Eco RI in final volume of 400 μL and the products separated on 3 preparative agarose gels with well sizes of 3 cm long by 1 cm deep. The regions of the gels corresponding to cDNA of 100 base pairs to greater than 3 kilobase pairs were localized by ethidium bromide staining (section 3.2.5.1) and electroeluted onto DEAE membranes as described in section 3.2.5.2. The recovered cDNA was subsequently used for cloning into λgt11.

3.2.7.9 Ligation of Amplified S. mansoni cDNA to λgt11 DNA

λgt11 DNA restricted with Eco RI and treated with alkaline phosphatase was obtained from Promega Biotec. 1μg of this DNA was coprecipitated in ethanol with approximately 100 ng amplified S. mansoni cDNA . The DNA pellet was rinsed and dried, then ligated and packaged as described for the S. mansoni λgt10 cDNA library (sections 3.2.7.4 and 3.2.7.5). Recombinant λgt11

phage were titred on E. coli Y1088.

3.2.8 Construction of a cDNA Library in λZAP

A cDNA library was also constructed from S. mansoni poly A+ RNA in the expression vector λZAP by personnel at Stratagene Cloning Systems in La Jolla, CA. S. mansoni poly A+ RNA was shipped to Stratagene on dry ice and cDNA synthesis and cloning was carried out using a protocol similar to that described in section 3.2.7. The cDNA, however, was ligated to Xba I linkers (5'-TTCTAGAA-3') and cloned into the Xba I site of λZAP. In addition, the cDNA was size fractionated on a Sepharose CL-4B column and only cDNA larger than 500 bp was used for cloning.

3.2.9 Screening of Recombinant Expression Libraries

3.2.9.1 Preparation of Antiserum for Screening of Recombinant λ Expression Libraries

Rabbit anti-APM antiserum was prepared and diluted as described in Chapter 2, section 2.2.3. The serum was diluted 1/200 in 1% gelatin in TTBS (50 mM Tris pH 7.5, 500 mM NaCl, 0.5% Tween 20). Before use, the antiserum was pre-adsorbed with an E. coli lysate to remove antibodies in the serum reacting with E. coli proteins. A 100 mL culture of E. coli Y1088 was grown in LB medium to an $O.D._{600}$ of 1.0. The culture was centrifuged at 7000 rpm in a Sorvall SS-34 rotor, resuspended in 10 mL of TTBS and quick frozen in liquid nitrogen. The cell suspension was sonicated using a Branson sonifier

with 6 fifteen second bursts. The cell lysate was then refrozen and resonicated. The final crude lysate was then mixed with 200 mL of the 1/200 dilution of rabbit anti-membrane serum and incubated at room temperature for 1 hour. The adsorbed antiserum was then centrifuged to remove particulate material and supplemented with 0.05% merthiolate.

The antiserum used to screen the λZAP library was a mouse anti-membrane antiserum (MαAPM, section 2.2.3) diluted 1/1000 in TTBS with 1% gelatin. A preliminary screen of λZAP phage demonstrated that reactivity with E. coli proteins was not a problem using this dilution. Therefore, this serum was used directly and was not pre-adsorbed with E. coli lysate.

3.2.9.2 Preparation of Nitrocellulose Plaque Lifts for Screening

Nitrocellulose filter discs (137 mm, Millipore HATF) were prepared for use by soaking for 2 hours at room temperature in 10 mM isopropyl β-D-thiogalactoside (IPTG). The filters were then air dried and numbered.

S. mansoni λgt11 recombinants were grown on 150 mm LB agar plates at a density of 10^5 phage per plate using E. coli Y1090 as the host cell. The phage were then grown for 4 hours at 42°C. IPTG saturated filters were then overlaid on the recombinant phage to induce fusion protein expression. The plates were removed to 37°C and incubated for a further 4-6 hours. Just prior to removal of the filter discs from the plates, three asymmetric marks were made in the filter using a 26 g needle. These orientation marks were recorded

on the bottom of each plate to assist in the alignment of the filters and the localization of immunoreactive phage.

λZAP recombinants were grown in exactly the same manner except that E. coli BB4 was used as the host cell. For both the λZAP and λgt11 libraries, approximately 10^6 recombinants were screened.

3.2.9.3 Immunoscreening of Recombinant Expression Libraries

The method used for screening of recombinant phage was analogous to that described in Chapter 2, section 2.2.7 for the processing of Western immunoblots. The nitrocellulose filters with bound material from the phage plaques were peeled from the top agar layer and blocked with 300 mL 3% gelatin in TBS. A large volume of this solution was used to ensure complete blocking of the filters and removal of debris adhering to the filter. The filters were then washed twice for 5 minutes each in TTBS and immersed in primary antibody solution for 1 hour. The discs were washed again and further incubated for 1 hour at room temperature in secondary antibody (1/5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse IgG). Following washing, immunoreactive phage were then revealed using the BCIP/NBT substrate system.

3.2.9.4 Secondary Screening of Immunoreactive Phage

Positive phage were localized on their corresponding plates by aligning the nitrocellulose filters according to the location of orientation marks. A 1 cm

diameter agar plug was removed from the plate corresponding to the vicinity of the signal producing phage. The plugs were placed in 1 mL λ diluent containing a drop of chloroform. The phage titre of the agar plug eluates were determined for each potential positive signal. A second round of screening was then carried out using approximately 500 phage from each of the primary phage eluates on 85 mm plates. The phage were grown, overlaid with IPTG-saturated filters and rescreened. Signal producing plaques were located and the procedure was subsequently repeated using less than 100 phage per plate. Usually, three rounds of screening were required before the signal producing plaque could be unambiguously identified.

3.2.9.5 Preparation of Immunoreactive Recombinant Phage Stocks

Immunoreactive phage were identified, and were transferred to plates containing a lawn of host E. coli cells in top agar using a sterile toothpick. The toothpick was used to inoculate the top agar with phage such that a single 2 cm diameter plaque was produced following overnight growth. The plaques were then removed from the plates and the agar plugs were placed in λ diluent with a few drops of chloroform. These phage stocks were retested for immunoreactivity by rescreening a nitrocellulose filter containing 500 plaques from each isolate. Less than 0.1% of the phage from each isolate failed to produce a signal and at this point were considered clones. In some cases, plate stocks were prepared from these stocks for long term storage at 4°C.

3.2.10 Analysis of Recombinant Phage

3.2.10.1 Preparation of Recombinant λgt11 lysogens

E. coli Y1089 were grown to an $O.D._{600}$ of 0.5 (1.5 x 10^8 cells/mL) and infected for 20 minutes with λgt11 recombinants at a multiplicity of infection of 5. The cell suspension was diluted with LB and aliquots containing about 200 cells were plated on LB plates and grown overnight at 32 °C. Twenty colonies were selected from each plate and transferred to separate 1 mL aliquots of LB. The diluted cell suspension was vortexed and 10 μL of each isolate was spotted in ordered arrays onto duplicate LB plates. One set of plates was incubated at 32 °C and the other was incubated at 42 °C. Following overnight growth, those colonies showing no growth at 42 °C were selected for further analysis.

3.2.10.2 Preparation of Lysogen Lysates

Potential lysogens were grown in LB at 32 °C to an $O.D._{600}$ of 1.0. Aliquots of each culture were then dispensed into an equal volume of LB, with one culture remaining at 32 °C and one shifted to 42 °C for 20 minutes. These cultures represented the uninduced and induced cultures for each lysogen, respectively. Uninduced cultures were incubated a further 1 hour at 32 °C. Thermally induced cultures were shifted down to 37 °C, supplemented with IPTG to 10 mM and incubated for 1 hour. The uninduced and induced cultures were collected by centrifugation and lysed directly by boiling in SDS sample

buffer. Proteins were analyzed by SDS-PAGE and immunoblotting (Chapter 2, sections 2.2.5 to 2.2.7).

3.2.10.3 Rescue of Recombinant pBluescript Plasmids from λZAP Recombinants

E. coli XL1-blue was grown to an $O.D._{600}$ of 1.0. Twenty μL of these cells were then superinfected with 10^6 λZAP recombinants and 10^9 m13 R408 helper phage. The cell suspension was incubated for 15 minutes at 37 °C and supplemented with 5 mL of 2X LB medium followed by growth at 37 °C for a further 5 hours. The cultures were then heated to 70 °C for 20 minutes and cells were removed by centrifugation. The supernatants were used to infect 200 μL fresh $O.D._{600}$ 1.0 XL1-blue cells and dilutions were plated on LB agar with 100 μg/mL ampicillin. Colonies transformed to ampicillin resistance were selected and restreaked on LB/ampicillin plates

3.2.11 Nucleic Acid Hybridization

3.2.11.1 Preparation of Radiolabelled Probe

DNA isolated from acrylamide gel or by the Geneclean^{TM} procedure was radiolabelled using a random-primed DNA labelling kit according to the instructions of the manufacturer. Incorporation of [α^{32}P]dCTP into nucleic acid was estimated by TCA precipitation. Unincorporated label was removed by two sequential ethanol precipitations from 2M ammonium acetate.

3.2.11.2 Immobilization of Nucleic Acids

DNA in phage plaques was lifted onto nitrocellulose discs by overlaying

the discs onto the phage plates for 1 minute. The discs were then peeled from the top agar layer and placed sequentially onto Whatman 3MM paper saturated with 0.5 M NaOH (5 minutes), 0.5 M Tris, pH 7.4 (5 minutes), and 6X SSC (standard saline citrate, Maniatis et al. (1982)). Filters were then exposed to U.V light for 3-4 minutes (Khandjian, 1987), and used for hybridization.

3.2.11.3 Hybridization Analysis

Immobilized nucleic acids were pre-hybridized at 43 °C for 4 hours or more in 50 % (v/v) formamide, 6X SSC, 5X Denhardt's solution, 0.1% SDS, 200 μg/mL sheared calf thymus or herring sperm DNA (Maniatis et al., 1982). Filters were then hybridized with radiolabelled probe at a concentration of 5 x 10^5 to 1 x 10^6 TCA-precipitable cpm/mL at 43 °C for 16 hours. The filters were then washed at 65 °C with 4 changes of 2X SSC, 0.1% SDS over a period of 1 hour. Bound probe was detected by autoradiography of the filters.

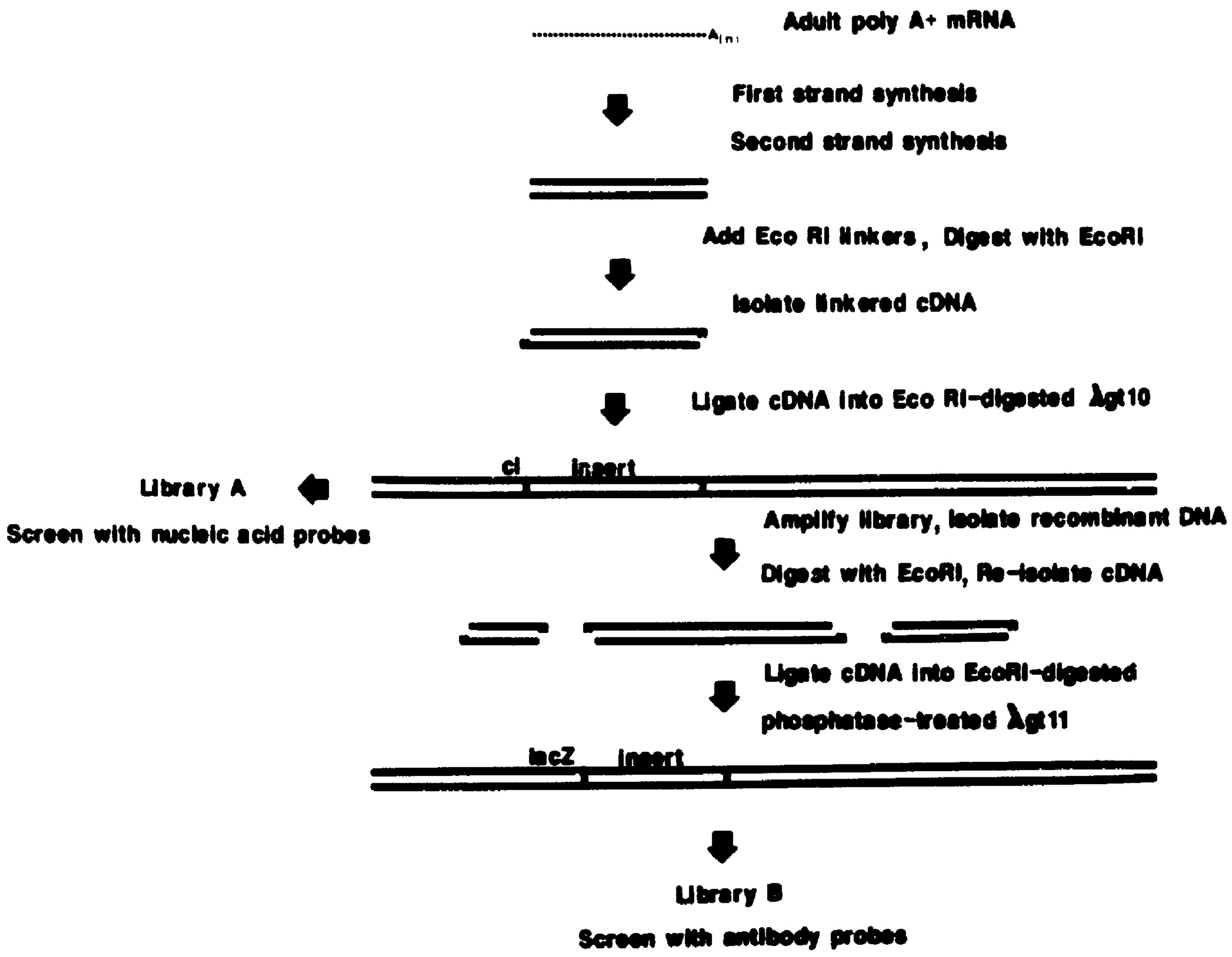
Chapter 3.3--Results

3.3.1 Construction of S. mansoni cDNA libraries

The molecular cloning of cDNA into bacteriophage λ expression vectors, particularly λgt11 (Young and Davis, 1983a) has become a popular and powerful method for the characterization of cDNAs and genes encoding proteins of biological interest. The strategy employed in this study (outlined in Figure 3.1) involved the synthesis, 'pre-cloning' and amplification of cDNA in λgt10, followed by cloning of the amplified cDNA into λgt11. This approach has been described (C. Farfett, pers. comm.; Kemp et al., 1984) and is apparently well suited for cDNA preparations which clone with low efficiency in λgt11 directly. The resultant cDNA libraries, however, were suitable for two purposes. λgt10, because of its robust growth characteristics is an ideal vector for screening of cDNA libraries by hybridization, while λgt11 libraries can be immunologically probed due to the efficiency of fusion protein production.

In summary, two recombinant expression libraries were ultimately screened using antibody probes. The λgt11 library was screened with RαAPM. During the course of this work, a λZAP library was also obtained from Stratagene. This library was screened with MαAPM, since it was the only antiserum available at the time. Similar screening strategies were used for both expression libraries, such that two independent panels of clones were assembled for further analysis.

Figure 3.1: Flow chart illustrating the strategy used for synthesis and cloning of complementary DNA using S. mansoni poly A+ RNA as a template. The resultant cDNA libraries constructed in λgt10 and λgt11 could thus be screened with nucleic acid and antibody probes, respectively. λgt11 recombinants expressing S. mansoni cDNA fragments as C-terminal β-galactosidase fusion proteins were screened with antisera prepared as described in Chapter 2, section 2.2.3.

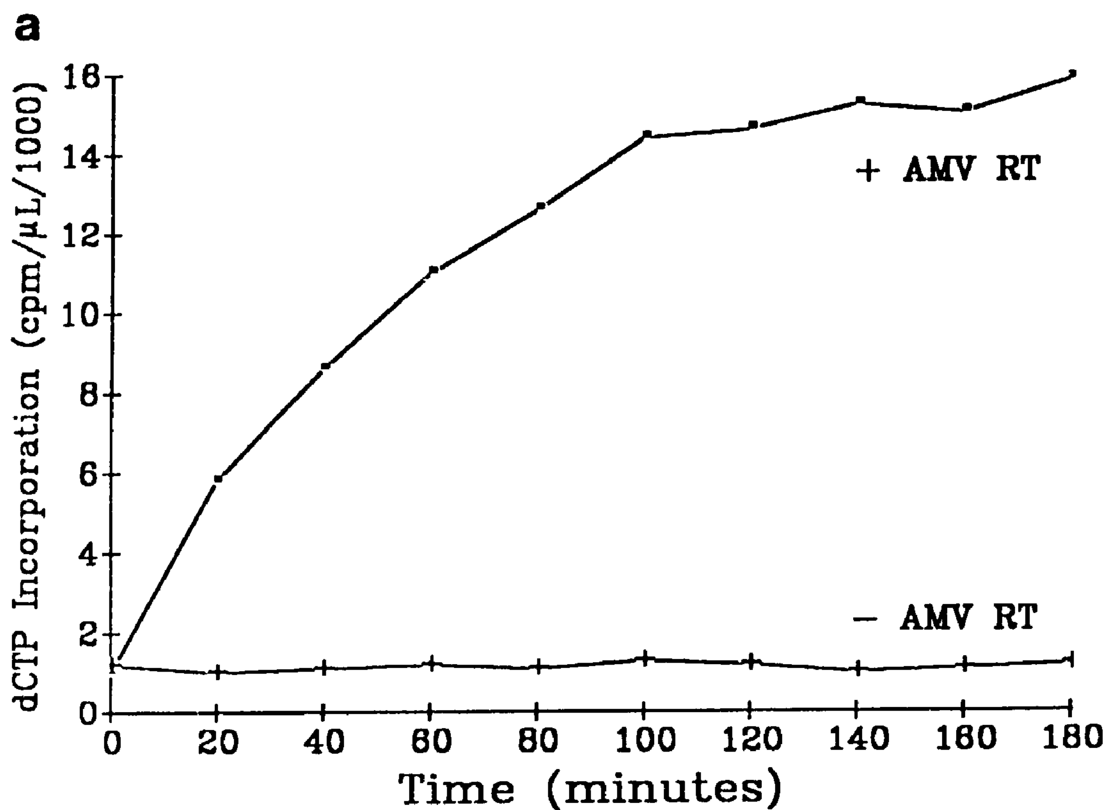


MOLECULAR CLONING OF cDNA FROM SCHISTOSOMA MANSONI
A(n)
Adult poly A+ mRNA
First strand synthesis
Second strand synthesis
Add Eco RI linkers, Digest with EcoRI
Isolate linkered cDNA
Ligate cDNA into Eco RI-digested λgt10
cI
insert
Library A
Screen with nucleic acid probes
Amplify library, isolate recombinant DNA
Digest with EcoRI, Re-isolate cDNA
Ligate cDNA into EcoRI-digested phosphatase-treated λgt11
lacZ
insert
Library B
Screen with antibody probes

3.3.1.1 Synthesis of double-stranded cDNA

The ultimate success of any multi-step procedure, such as cDNA synthesis and cloning, described above, is dependent on the efficiency of each of the component steps. This is particularly true of the first step in the procedure. In this regard, several experiments were carried out in order to optimize the synthesis of the first strand reaction products, with respect to both yield and integrity, using the method of Gubler and Hoffman (1983) as a starting point. Figure 3.2 shows the results of time course and enzyme titration experiments for the first strand synthesis reaction. The incorporation of dCTP into first strand reaction product increased for up to 2 hours of incubation in the presence of 3 U AMV-RT/μg poly A+ RNA (450 U/ml. After 2 hours, there was little increase in dCTP incorporation. The effect of increasing AMV-RT concentration on dCTP incorporation was also examined. Following a 2 hour incubation, incorporation of dCTP was found to be increased as the concentration of AMV-RT increased. The yield of first strand cDNA (determined from dCTP incorporation, specific activity and mass of template RNA) was approximately 20% in the presence of 450 U/ml AMV-RT but increased to almost 50% in the presence of 1800 U/ml AMV-RT. This increased incorporation, however, was found to be accompanied by a decrease in the integrity of the first strand transcripts as judged by alkaline agarose gel electrophoresis (Figure 3.3A). As the concentration of AMV-RT in the reaction

Figure 3.2: Incorporation of $[\alpha^{32}P]$dCTP into oligo-dT-primed first strand cDNA using S. mansoni poly A+ RNA as a template. A) Time course of incorporation of $[\alpha^{32}P]$dCTP into first strand cDNA. Using the reaction conditions outlined in Chapter 3.2.6.1, first strand cDNA primed with oligo-dT was synthesized from S. mansoni poly A+ RNA in the presence or absence of avian myeloblastosis virus reverse transcriptase (AMV-RT). Aliquots of the reaction mixtures were removed at the indicated time points up to 3 hours, precipitated with TCA and incorporated radioactivity determined by Cerenkov counting. B) Effect of AMV-RT concentration on the incorporation of $[\alpha^{32}P]$dCTP into first strand cDNA. S. mansoni first strand cDNA was synthesized in parallel reactions containing AMV-RT concentrations ranging from 3 U/μg template RNA (450 U/mL) to 12 U/μg RNA (1800 U/mL) for 2 hours. Radioactivity incorporated into cDNA was determined by TCA precipitation and Cerenkov counting.


a
16
14
12
10
8
6
4
2
0
dCTP Incorporation (cpm/μL/1000)
+ AMV RT
− AMV RT
0
20
40
60
80
100
120
140
160
180
Time (minutes)

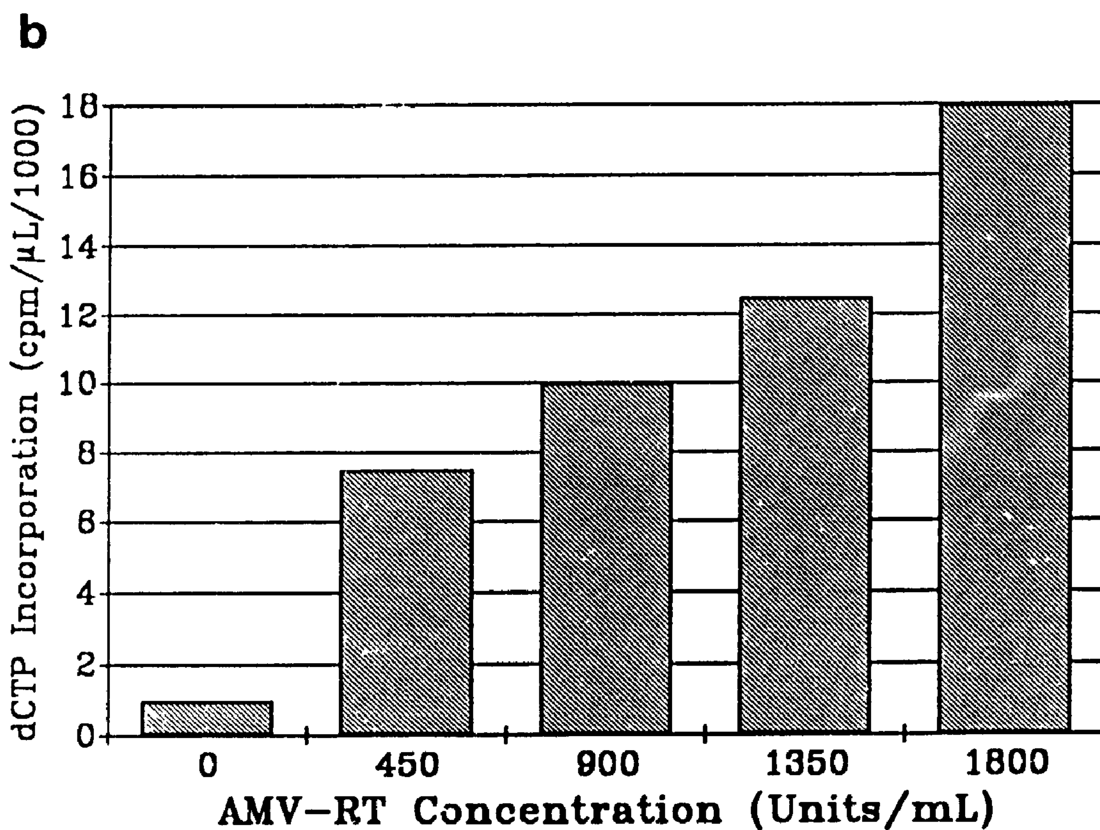
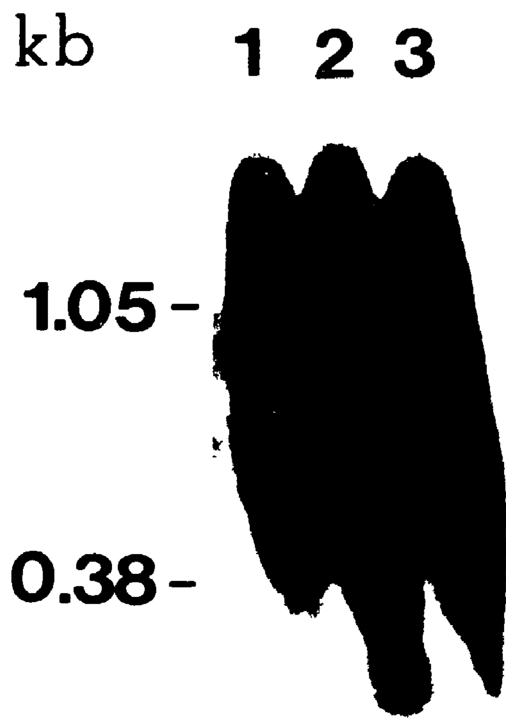

b
18
16
14
12
10
8
6
4
2
0
dCTP Incorporation (cpm/μL/1000)
0
450
900
1350
1800
AMV−RT Concentration (Units/mL)

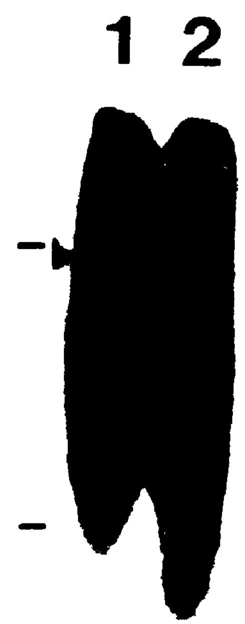
Figure 3.3: Analysis of first and second strand S. mansoni cDNA reaction products by alkaline agarose gel electrophoresis. A) Effect of AMV-RT concentration on the integrity of oligo-dT-primed first strand cDNA product. Alkaline agarose gel electrophoresis (section 3.2.5.5) of approximately 10000 TCA-precipitable cpm of first strand cDNA prepared in the presence of [α^{32}P]dCTP and the following AMV-RT concentrations expressed as U/μg of template poly A+ RNA: 3U/μg (lane 1), 6U/μg (lane 2)and 9U/μg (lane 3). The size of end-labelled marker DNA in kilobases (kb), prepared as described in section 3.2.6.3 is indicated to the left of the panel. Markers were prepared by enzymatic repair of recessed ends of restriction fragments of pBR 322 in the presence of radioactive deoxynucleotide triphosphates.

B) Analysis of first and second strand S. mansoni cDNA by alkaline agarose gel electrophoresis. Lane 1 shows the oligo-dT-primed first strand product synthesized in the presence of 3 U/μg template RNA and [α^{32}P]dCTP. Lane 2 shows the second strand product, which was synthesized in the presence of RNAse H and DNA polymerase I. For this experiment, first strand cDNA was synthesized in the absence of α- ^{32}-P-dCTP and the resultant cDNA/mRNA hybrid was used as a template for the synthesis of second strand product in the presence of [α^{32}P]dCTP as described in section 3.2.6.2). End-labelled markers were the same as in panel A.

A



B



increased from 450 U/ml (Figure 3.3A, lane 1) to 900 U/ml and 1350 U/ml (Figure 3.3A, lanes 2 and 3 respectively), increased quantities of low molecular weight transcripts were produced. Since first strand cDNA transcript integrity was a high priority, a concentration of 450 U/ml AMV-RT and a first strand incubation time of 2 hours were used in all subsequent experiments. This resulted in the production of cDNAs ranging in size from 0.3 to > 2.5 kb. The yields of first strand cDNA in individual experiments ranged from 14-22%.

The second strand reaction was found to be considerably more efficient. Conditions established by Gubler and Hoffman (1983) were found to be suitable for S. mansoni cDNA. The yield of second strand cDNA in individual experiments ranged from 60-80%. Moreover, the size distribution of the second strand cDNA closely paralleled that of the first strand transcripts (Figure 3.3B). Overall, approximately 1 to 2μg of double-stranded cDNA could routinely be prepared from 7.5μg of S. mansoni poly A+ RNA using the methods described in section 3.2.6.

An absolute requirement for the cloning of DNA fragments using synthetic linkers is that the DNA possess blunt-ended termini. The original Gubler and Hoffman (1983) protocol has been used successfully for cloning of cDNA using a method involving tailing catalyzed by terminal deoxynucleotidyl transferase. However, it is unclear what proportion of the cDNA produced using the method is blunt-ended at both ends. In particular, one can envision

potential problems at the 5' end of mRNAs, where small regions of DNA/RNA hybrids might still exist. As such, in the present study, I have found (by trial and error) that treatment of cDNA with T4 DNA polymerase was required for cloning of cDNA using linkers. This strategy (section 3.2.6.4) exploited the 3'-5' exonuclease activity of this enzyme in the absence of deoxynucleotide triphosphates followed by repair activity in their presence.

3.3.1.2 Cloning of S. mansoni cDNA

The cDNA was subsequently ligated to phosphorylated Eco RI linkers (Figure 3.4). Linkers prepared in this manner were shown to ligate into multimers in the absence (Figure 3.4, lane 1) or presence of S. mansoni cDNA (Figure 3.4, lane 2). The ligation of linkers to cDNA resulted in an apparent decrease in the efficiency of linker self-ligation, concomitant with the appearance of radiolabel in a high molecular weight fraction which barely entered the 6% acrylamide gel.

The linker-modified cDNA was then digested with Eco RI and chromatographed on a Biogel A 1.5m column (Figure 3.5). cDNA ligated to Eco RI linkers eluted in the column's void volume, clearly resolved from the bulk of the label, which probably represented small linker fragments, linker monomers, and unincorporated [γ^{32}P]ATP. The column void volume fractions (Fractions 4-8 in Figure 3.5) were pooled and precipitated and used for ligation into λgt10.

Figure 3.4: Analysis of ligation of Eco RI linkers to blunt-ended double-stranded S. mansoni cDNA. Double-stranded cDNA prepared in the presence of [^{3}H]dCTP was treated with T4 DNA polymerase and ligated with [^{32}P] labelled Eco RI linkers (5'-GGAATTCC-3') as outlined in section 3.2.7.2. Ligation reactions carried out in the absence (lane 1) or presence (lane 2) of S. mansoni cDNA were analyzed on a 6% polyacrylamide TBE gel followed by autoradiography. The positions of linker monomers and multimers are indicated to the left of the panel.

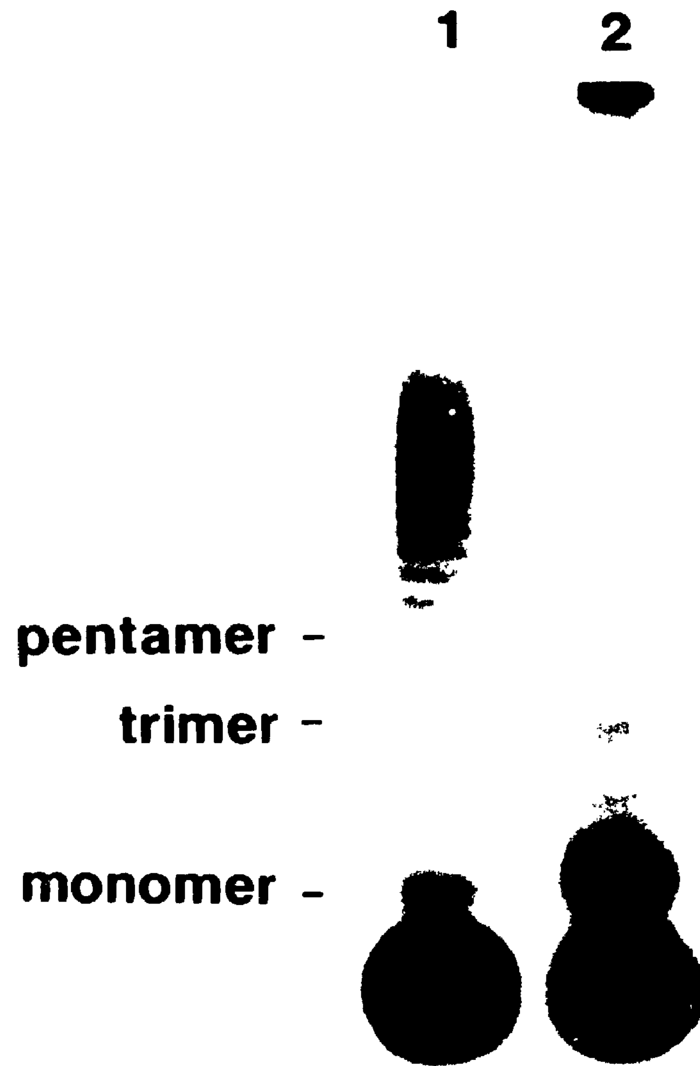
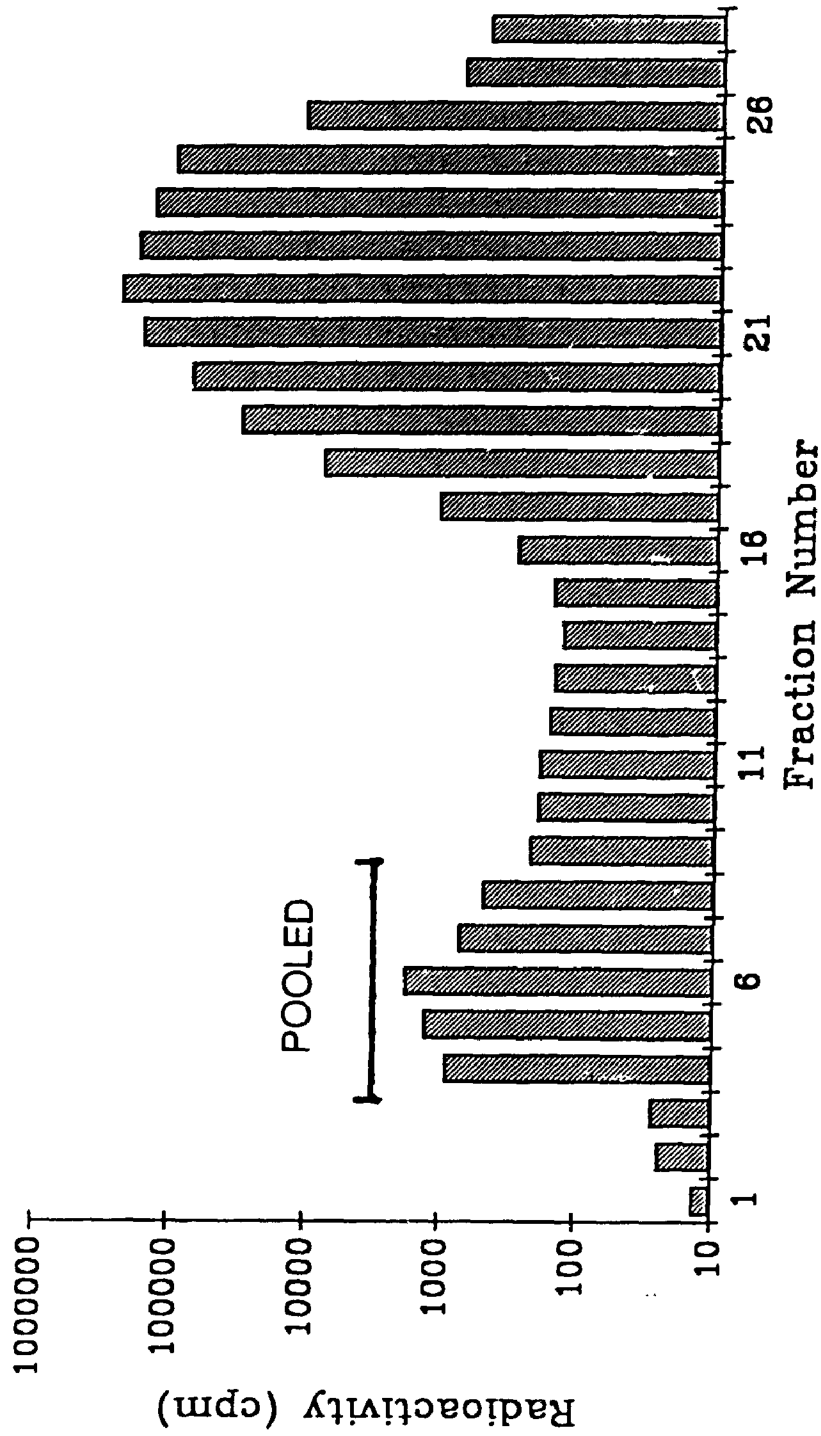

1
2
pentamer -
trimer -
monomer -

Figure 3.5: Elution profile of linker-modified S. mansoni cDNA digested with Eco RI from Bio gel A 1.5m. Double-stranded, blunt-ended S. mansoni cDNA labelled with [^{3}H]dCTP was ligated [^{32}P] linkers and digested exhaustively with Eco RI. The resultant digest was loaded onto a Bio gel A 1.5m column and separated by gel filtration chromatography as described in section 3.2.7.4. Fractions 4 to 8 were pooled, precipitated and ligated into the Eco RI-digested arms of λgt10.


Radioactivity (cpm)
1000000
100000
10000
1000
100
10
1
6
11
16
21
26
Fraction Number
POOLED

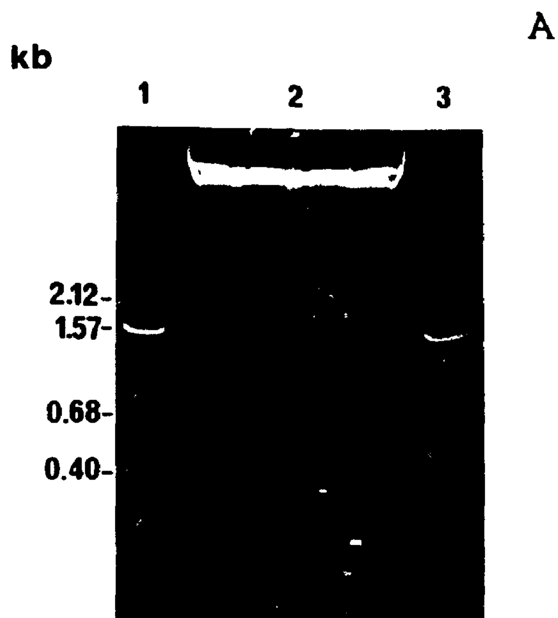
The linker-modified S. mansoni cDNA was ligated into the unique Eco RI site of λgt10, and recombinant phage DNA was packaged in vitro. The λgt10 DNA was not phosphatase-treated, and as a consequence the resultant cDNA library contained about 90% non-recombinants (religated vector)(data not shown). This was not a problem, however, since plating and amplification of the library on E. coli C600 hfl provided a positive selection for recombinants. The λgt10 S. mansoni cDNA library consisted of 4.3 x 10^6 recombinant phage. This was derived from 15μg S. mansoni poly A+ RNA; the overall cloning efficiency was approximately 2.9 x 10^5 recombinants/ μg poly A+ RNA.

Approximately 10^6 recombinant phage from this library were amplified and about 200μg total recombinant phage DNA was isolated from 20 plate stocks. This DNA was restricted with Eco RI and electrophoresed on an agarose gel, from which the amplified DNA was recovered (Figure 3.6). The size distribution of the amplified DNA revealed that, like the first and second strand cDNA products (Figure 3.3), the bulk of the population migrated between 0.3 and 3 kilobase (kb) pairs. As shown in Figure 3.6A, the cDNA was then electroeluted onto a DEAE membrane (Figure 3.6B). The cDNA was evident on the DEAE membrane as a thin ethidium bromide-stained line, following electroelution. This amplified cDNA was re-isolated and used for cloning into λgt11.

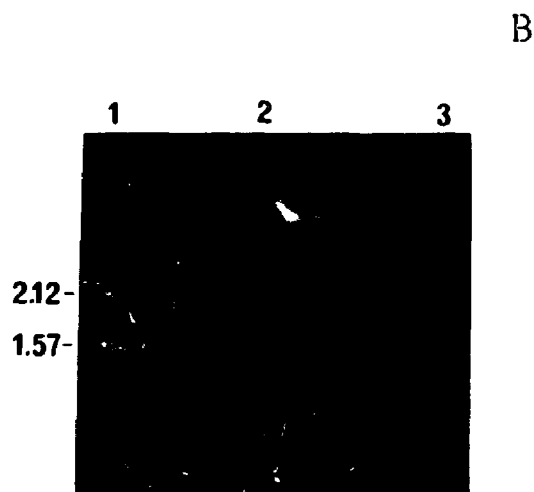
λgt11 DNA also has a unique Eco RI site. However, since there is no

Figure 3.6: Isolation of amplified cDNA from an S. mansoni cDNA library constructed in λgt10. The S. mansoni λgt10 library was amplified, processed for DNA isolation (section 3.2.4.1) and digested with Eco RI (section 3.2.7.8). The digest was electrophoresed in agarose gels and cDNA localized by ethidium bromide staining. Panel A shows the stained gel with the Eco RI digested cDNA in lane 2. Molecular weight markers were an Rsa I digest and a Hinf I digest of pBR 322 in lanes 1 and 3 respectively. The size of the marker DNAs (in kilobase pairs (kb)) are indicated to the left of the panel. A slit was made near the bottom of the gel illustrated in panel A and the intensely stained vector material at the top of the gel was removed with a razor blade. A DEAE membrane was inserted into the slit and the cDNA was recovered on the membrane (panel B) by electroelution (section 3.2.5.2).

A



B



positive selection for recombinants, λgt11 DNA which was phosphatase-treated, was used. Early experiments using amplified cDNA for cloning into non-phosphatase-treated vectors, resulted in libraries in which >75% of the packaged phage were non-recombinants. Phosphatase treatment, while lowering the overall cloning efficiency, resulted in cDNA libraries in which <10% of the phage were non-recombinants. The resultant λgt11 library consisted of $4x10^6$ recombinants. An S. mansoni cDNA expression library, constructed in λZAP, was also obtained from Stratagene Cloning Systems. This library was constructed in a similar fashion to that described above. However, Xba I linkers were used, the cDNA was size fractionated (only cDNA > 500 bp was used for cloning) and cloning was accomplished directly into the Xba I site within the polylinker of λZAP. Approximately $3x10^6$ recombinants were generated with 10% of the phage representing non-recombinants.

In summary, two cDNA expression libraries were constructed from S. mansoni cDNA; one which was made with Eco RI linkers, using λgt11, and the other constructed with Xba I linkers in λZAP. These libraries were then screened for immunogenic S. mansoni APM polypeptides, using the sera produced as described in Chapter 2, section 2.2.3.

3.3.2 Screening of S. mansoni cDNA expression libraries

The screening strategy used in the present study was to isolate clones expressing antigens (in the context of recombinant fusion proteins) recognized

by antibodies in the sera of rabbits and mice, exposed to S. mansoni APM polypeptides. Since these antisera were polyspecific, it was hoped that clones encoding several different membrane antigens could be isolated.

The λgt11 library was initially screened with RαAPM. However, with time, this antiserum became unsuitable for screening and Western blot analysis. When the λZAP library became available, only MαAPM was available and suitable for screening. Thus, while the original intent was to screen both expression libraries with the same antiserum, the two libraries were ultimately screened with two different antisera. This approach, however may have been useful for the isolation of distinctly different clones.

3.3.2.1 Screening of the S. mansoni λgt11 library with RαAPM

The λgt11 library was screened with RαAPM as described in section 3.2.9. In a preliminary screen of 10^6 recombinants, 32 potential positives were detected, of which 22 survived three rounds of screening. Thus, immunoreactive phage arose at a frequency of about 0.002%.

3.3.2.2 Screening of the S. mansoni λZAP library with MαAPM

The λZAP library was screened in a similar manner, with MαAPM. In this experiment, 27 putative positives were detected in the first round, 21 of which survived 3 rounds of screening, resulting in approximately the same frequency of positive clones as the λgt11 library. Figure 3.7 shows the primary, secondary and tertiary filters, resulting in the isolation of a λZAP recombinant.

Figure 3.7: Immunoscreening of S. mansoni cDNA expression libraries constructed in λ expression vectors. A λZAP libraries was screened with antisera raised against S. mansoni adult membrane proteins as described in section 3.2.9. Illustrated here are the primary (panel 1), secondary (panel 2), and tertiary (panel 3) screens resulting in the isolation of a λZAP recombinant. Approximately 10^5, 500 and 100 plaques (panels 1, 2 and 3 respectively) were on the plates from which these nitrocellulose filters were lifted. Following incubation in the primary antiserum, positive phage were revealed using phosphatase-conjugated second antibody and the BCIP/NBT substrate system. Dark spots indicate plaques producing fusion proteins recognized by the anti-membrane antiserum (MαAPM).

1

2

3

The proportion of positive phage on each of these plates increased, indicating enrichment of the clone of interest. In general, phage stocks could be prepared following identification of the clones on the tertiary plates.

3.3.3 Preliminary characterization of the λgt11 and λZAP clone panels

An important objective of the present study, was to identify clones encoding the 24 kDa phosphoprotein antigen, characterized in Chapter 2. The 2 independent panels of immunoreactive clones were thus screened with affinity-purified antibodies, against the 24 kDa protein which were prepared as described in Chapter 2.2.9. Of the 22 λgt11 clones, only one (clone 12), specifically bound antibodies against the 24 kDa antigen (Figure 3.8). None of the λZAP clones reacted with these antibodies. Clone 12 was renamed λSK2 and was used to construct lysogens in E. coli Y1089, in order to examine the β-galactosidase fusion protein, encoded by this clone. While a thermally and IPTG-induced lysate of a λSK2 lysogen, produced a barely detectable fusion protein (Figure 3.9A, lane 2; arrow), the product could be readily detected following immunoblotting (Figure 3.9B). These results show that although the λSK2 lysogen produced a fusion protein of about the same size as a λgt11 lysogen (Figure 3.9A, compare lanes 2 and 4), the immunoreactive moiety is associated with the S. mansoni cDNA fragment, since antibodies did not bind to β-galactosidase encoded by λgt11 (Figure 3.9B, compare lanes 2 and 4). The results suggest that the fusion protein encoded by λSK2 and the 24 kDa

Figure 3.8: Screening of 22 λgt11 recombinants with affinity-purified antibodies against Smgp24. The S. mansoni λgt11 library was screened with RαAPM and 22 immunoreactive phage were isolated and phage stocks prepared. One μL of each of these stocks was dotted in an ordered array on a lawn of E. coli Y1090. The phage were grown for immunoscreening (section 3.2.9) and reacted with affinity-purified antibody against Smgp24 prepared as described (Chapter 2, section 2.2.8). Positive phage were revealed using phosphatase-conjugated second antibody and BCIP/NBT. The numbers of individual clones and λgt11 are indicated. Clone X is a non-immunoreactive recombinant.

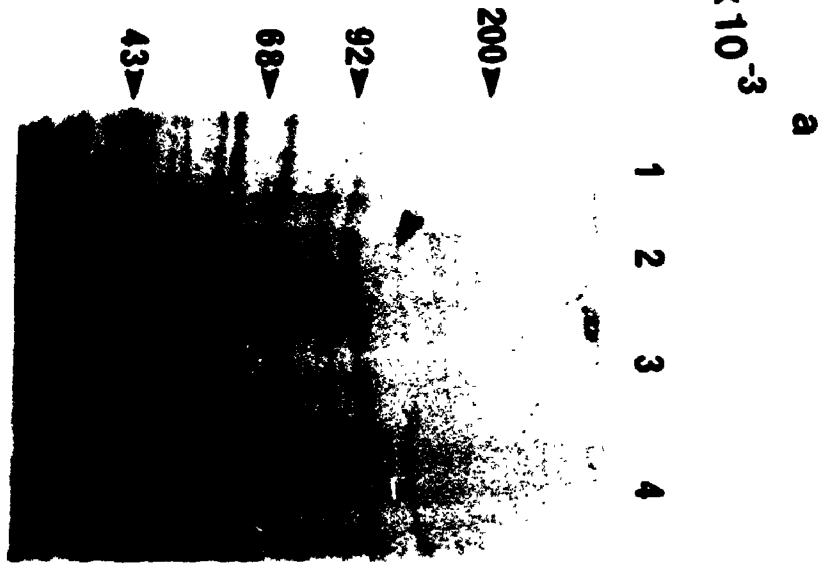
1 2 3 4 5 6

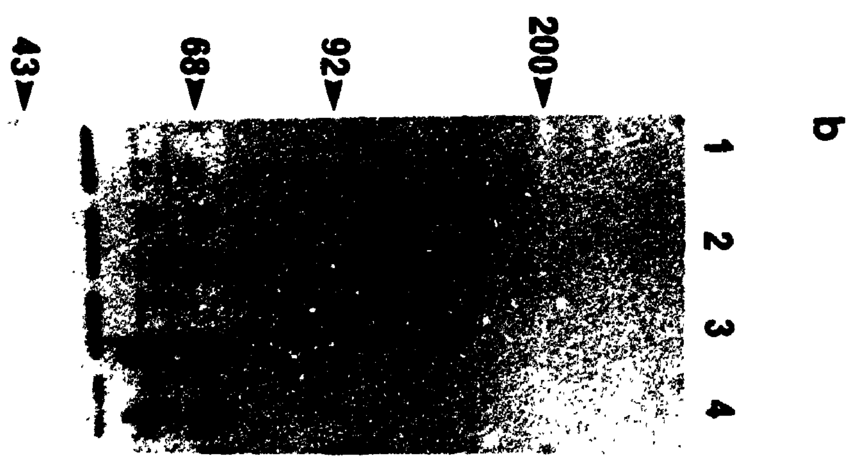
7 8 9 10 11 12 13 14

15 16 17 18 19 20 21

22 X gt11

Figure 3.9: Immunoblot analysis of recombinant λgt11 lysogens. Lysogen lysates were prepared from the clone (λSK2) which bound affinity-purified antibodies specific for Smgp24 and from λgt11 as described in section 3.2.10.2. Panel A illustrates a Coomassie blue stained SDS gel loaded with 10 μL of SDS-solubilized lysates of uninduced and induced (heat and IPTG) lysogens. Lanes 1 and 2 show uninduced and induced lysates of a λSK2 lysogen, whereas lanes 3 and 4 show the uninduced and induced lysates of a λgt11 lysogen. The arrow shows the position of the fusion protein encoded by λSK2. The relative molecular masses of the co-electrophoresed markers are indicated to the left of panel A. Panel B shows an immunoblot of the same samples illustrated in panel A probed with affinity-purified antibodies against Smgp24. The gel used to produce the blot in panel B was loaded with 10% of the sample used in panel A.


a
$M_r \times 10^{-3}$
200
92
68
43
1
2
3
4


b
200
92
68
43
1
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3
4

phosphoprotein antigen share antigenic determinants.

In order to evaluate the lack of anti-24 kDa immunoreactivity in the λZAP panel of clones, the cDNA inserts were rescued into a pBluescript plasmid vector, using the automatic excision procedure outlined as described in section 3.2.10.3. Inserts harboured by these clones were released by Xba I digestion and sized on an agarose gel (7 of which are shown in Figure 3.10). The pBluescript vector band of 2.96 kb is evident in all lanes. The presence of additional high molecular weight bands may be indicative of partial digestion. Moreover, some of these clones were of the same size. The sizes of the inserts shown in this figure were 1.5 kb (lane 6, clone 23), 1.8 kb (lanes 2 and 3, clones 3 and 4), 2.3 kb (lane 7, clone 27) and 2.6 kb (lanes 1, 4 and 5, clones 1, 9 and 11). Indeed, the inserts from each of the λZAP clones fell into these four size classes. The insert of the smallest of the subclones (clone 23) was purified (using the Geneclean method), radiolabelled and hybridized using high stringency conditions, with the λZAP phage panel (Figure 3.11). Each of the λZAP recombinants hybridized detectably with this probe but not with λZAP. These results therefore indicate that the clones comprising the λZAP panel are all related to clone 23.

Figure 3.10: Restriction Enzyme analysis of immunoreactive λZAP clones. Rescue of recombinant pBluescript plasmids from λZAP was carried out as described in section 3.2.10.3. The cDNAs were released by digestion with Xba I and analyzed by agarose gel electrophoresis. Seven clones (clones 1,3,4,11,19,23,27) are shown here in lanes 1 through 7. Lane m shows the position of DNA molecular weight markers in kilobase pairs (kb).

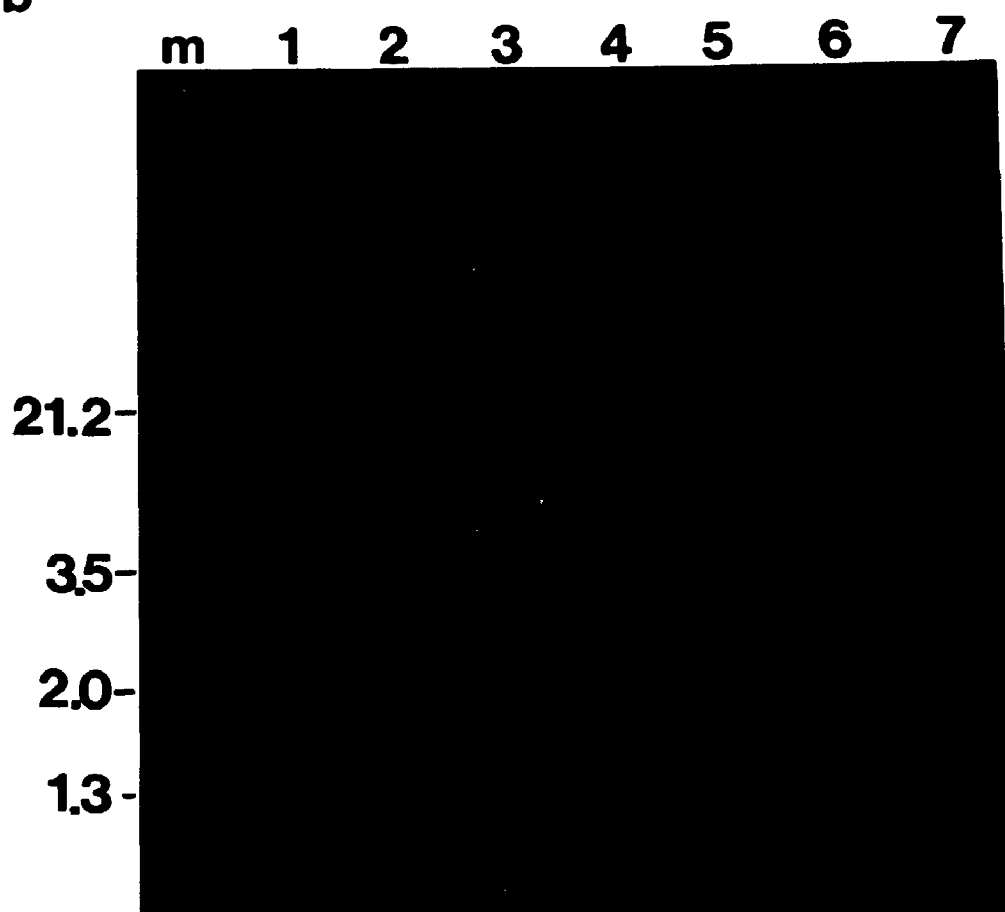
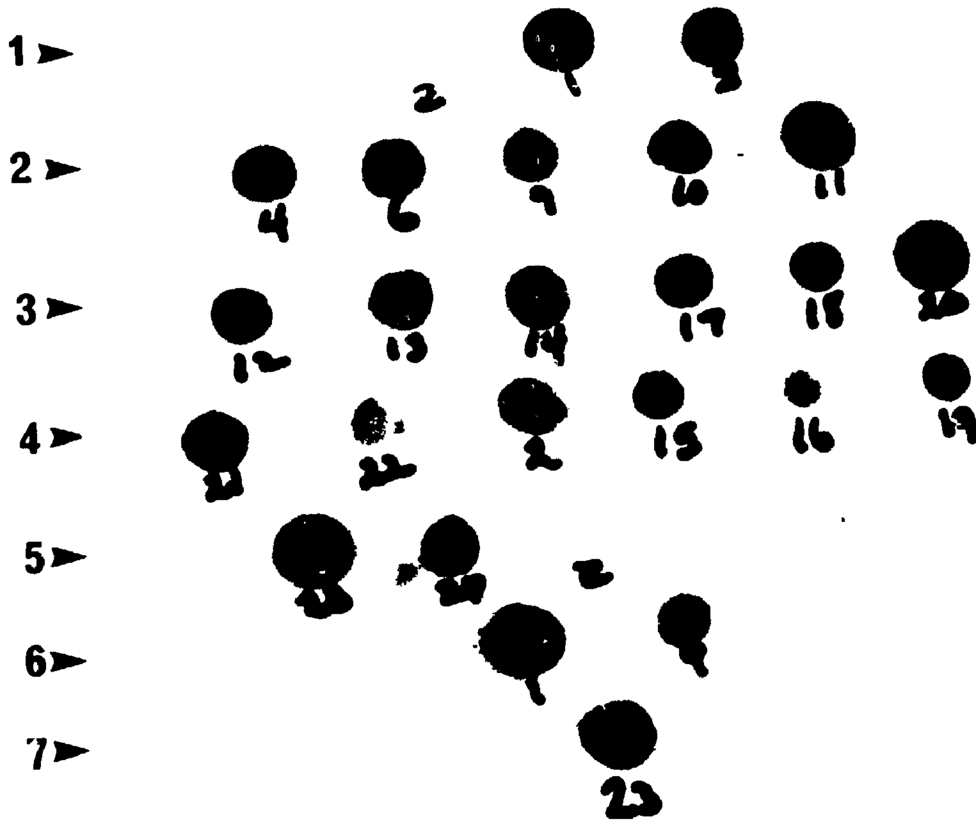

kb
m
1
2
3
4
5
6
7
21.2-
3.5-
2.0-
1.3 -

Figure 3.11: Hybridization analysis of λZAP clones. Immunoreactive clones isolated by screening the λZAP library were spotted in an ordered array as described in Figure 3.8. Phage DNA was lifted onto a nitrocellulose filter and hybridized with the insert from clone 23 (labelled and hybridized as described in section 3.2.11). The clones are: Row 1, λZAP, clone 1, clone 3; Row 2, clones 4,6,9,10,11; Row 3, clones 12,13,14,17,18,20; Row 4, clones 21,22,2,15,16,19; Row 5, clones 23,27 and λZAP; Row 5, clones 1 and 9; Row 7, clone 23.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3.4 Discussion

The results presented in this chapter document the construction and successful screening of S. mansoni cDNA expression libraries. Two panels of clones have been isolated; one panel was derived from screening of a λgt11 library with RαAPM and one panel derived from screening of a λZAP library with MαAPM. One clone from the λgt11 panel, λSK2, synthesized a fusion protein, which binds antibodies specific for the 24 kDa phosphoprotein antigen described in Chapter 2. Clones from the λZAP panel do not bind these antibodies but rather appear to be related to the 1.5 kb insert harboured by Clone 23 of this panel. Thus λSK2 shares antigenic determinants with the previously characterized 24 kDa membrane antigen, while all the λZAP clones appear to encode a distinct and as yet uncharacterized membrane antigen.

The λ bacteriophage expression vectors used in this chapter have proven to be quite popular for the efficient isolation of cDNAs and genes (Young and Davis, 1983a; 1983b). λgt11, in particular, has been almost universally used in the isolation of schistosome genes. The advantage of using these phage vectors, stem from the ability to clone cDNAs with high efficiency into a unique site of the vector and the relatively high levels of expression of recombinant fusion proteins. Thus, cDNAs corresponding to antigens recognized by antibodies in a variety of antisera can be efficiently isolated from large, comprehensive cDNA libraries. The availability of anti-schistosome antisera

from chronic infections in animal models or humans, or from a variety of immunization protocols, therefore makes possible a systematic analysis of schistosome antigenic complexity.

The stages leading up to the construction of a S. mansoni cDNA library were described in some detail. A crucial step in this protocol was found to be the treatment of double-stranded cDNA with T4 DNA polymerase. Although cDNA which was not T4 polymerase treated behaved in much the same way as T4 polymerase treated material on acrylamide gels and by gel filtration following linker addition, such cDNA was simply unclonable. The T4 polymerase step was absolutely essential for high efficiency cloning. The proliferation of commercial cDNA synthesis kits incorporating a T4 polymerase treatment attests to this observation.

The use of polyspecific antisera in the screening of these cDNA libraries was a deliberate attempt to maximize the probability of obtaining membrane protein cDNAs. Thus, panels of clones reacting with the whole antisera could be assembled and subsequently screened with enriched antibody preparations. Only a single clone in one million λgt11 recombinants was selected, which reacted with antibodies specific for the 24 kDa phosphoprotein antigen. This might have been due to a 'scrambling effect' induced by the amplification of the λgt10 library, or it might reflect the low abundance of the mRNA encoding the antigen. However, a 24 kDa clone was not selected by immunoscreening of the

λZAP library. This is likely due to the nature of the antiserum used for the screening of the individual libraries. That is, the RαAPM used to screen the λgt11 library was raised against denatured and precipitated APM, whereas the MαAPM was raised against APM polypeptides in a more 'native' conformation. The failure of 'native' antisera to detect desired cDNAs within libraries has been described, despite the presence of cognate clones in the libraries (Timmins et al., 1985). Thus, the use of antisera against denatured proteins has been suggested to be more suitable for screening of expression libraries since it is enriched in antibodies reacting with non-topographically assembled epitopes which might be expected to predominate in the context of recombinant fusion proteins. Nonetheless, although the use of MαAPM did not result in the isolation of a comprehensive panel of cDNAs encoding distinct membrane antigens, the isolation of related λZAP clones suggests that the MαAPM reproducibly recognized different sized versions of cDNA encoding the same protein.

The clone selected by the affinity purified anti-24 kDa antibodies (λSK2) and selected clones from the λZAP panel (in particular clone 1) were chosen for further analysis. A major objective which is outlined in the next chapter, was the isolation of potentially full length cDNAs hybridizing with these clones for the determination of their nucleotide sequence.

Chapter 4- Sequence Analysis of cDNA Clones

4.1 Introduction

Perhaps not surprisingly, several cloned schistosome cDNAs have been shown to exhibit similarity in their deduced amino acid sequence with better-studied proteins with known sequences. For example, schistosome sequences encoding polypeptides similar to mammalian glutathione S-transferase (Smith et al., 1987), myosin (Newport et al., 1987), heat shock protein 70 (Hedstrom et al., 1987), cathepsin B (Klinkert et al., 1989), tropomyosin (Xu et al., 1989) and superoxide dismutase (Simurda et al., 1988) have all been recently described. Analyses of sequence similarities has several important implications which have opened new investigative avenues. In many cases, some insight into the biological function of individual schistosome proteins and antigens has been gained for the first time. In addition, the structural similarities of schistosome proteins with host proteins raises many issues pertaining to the structural constraints of immunological specificity and vaccine design.

A major goal of the studies outlined in the thesis was the identification and sequence analysis of cDNAs encoding antigens associated with the APM fraction of adult S. mansoni. In this light, the nucleotide sequence of two cDNAs has been determined. I have described the isolation of both of these cDNA clones in Chapter 3. The first cDNA clone (λSK2) encodes a fusion protein which shares antigenic determinants with the 24 kDa membrane antigen

characterized in Chapter 2. The sequence of this cDNA shows no appreciable similarity with known nucleic acid or protein sequences. The second cDNA clone (pSKZ-1, the longest cDNA from the λZAP panel of clones) appears to encode a homologue of vertebrate calcium-activated neutral protease (calpain; E.C. 2.3.22.17). While the sequence of the first cDNA has been recently published (Knight et al., 1989), the calpain-like molecule has not previously been described in schistosomes. The implications of the characterization of these cDNAs is discussed.

4.2 Materials and Methods

4.2.1 Chemicals and Biochemicals

Sources of standard laboratory chemicals, biochemicals, restriction and modifying enzymes, radiochemicals and electrophoresis and blotting materials were obtained as outlined in Chapters 2 and 3, sections 2.2.1 and 3.2.1. In vitro transcription was carried out using an in vitro transcription kit obtained from Stratagene. Promega Biotec supplied components for small scale λDNA isolation (LambdaSorb^{TM} phage adsorbent), in vitro translation, generation of deletion subclones (Erase-A-Base^{TM} kit), and DNA sequencing in pGEM plasmids (GemSeq^{TM} K/RT kit). DNA sequencing components were also purchased from U.S. Biochemical (Sequenase^{TM} v. 2.0). All of these kits were used according to th manufacturers protocols. DNA sequencing was carried out in an apparatus obtained from American BioNuclear or Bio-Rad. Nylon membranes were obtained from Pall (Biodyne A) or Amersham (Hybond-N). Human poly A+ RNA was a gift from Phillip Wong.

4.2.2 Bacteria, Phage and Plasmids

The cloning vectors pGEM3Z and pGEM4Z were purchased from Promega Biotec. These plasmids and recombinant derivatives were grown in E. coli JM109 available in the laboratory of Dr. G.A.Mackie. Transformation-competent E. coli XL-1 blue was obtained from Stratagene Cloning Systems. Other bacterial strains and phage were obtained from sources indicated in section

3.2.2.

4.2.3 Growth of Recombinant Phage and Bacteria

Recombinant λgt10, λgt11 and λZAP were grown on their respective host cells as described in Chapter 3, section 3.2.3. λgt10 and λgt11 recombinants were prepared for DNA isolation using a plate lysate method (Chapter 3, section 3.2.4). Bacteria harbouring recombinant plasmids were routinely grown in liquid culture or selected on agar plates containing LB medium supplemented with 100 μg/mL ampicillin. E. coli JM109 was made transformation-competent using the $CaCl_2$ method outlined in Maniatis et al. (1982).

4.2.4 Isolation of Nucleic Acids

The isolation of RNA from S. mansoni and rat liver was carried out as outlined in Chapter 3, sections 3.2.4.5 and 3.2.4.6. Similarly, the large-scale isolation of λ DNA was performed using the CsCl block gradient method described in Chapter 3, section 3.2.4.1. Isolation of DNA fragments from acrylamide and agarose gel pieces has also been described (Chapter 3, sections 3.2.5.4 and 3.2.5.2, respectively).

4.2.4.1 Small Scale Isolation of λ Phage

λgt10 and gt11 recombinants were prepared for DNA isolation by a plate stock method as described in Chapter 3, section 3.2.4.1. Bacteriophage particles were eluted from the plate lysates overnight in λ diluent containing 0.1 M NaCl. The titre of the phage eluate was determined and only those containing greater

than 10^{10}/mL were used for further analysis. The phage suspension was supplemented with a few drops of chloroform and centrifuged at 10000 x g for 10 minutes. The phage were then adsorbed to LambdaSorb phage adsorbent (Promega Biotec) using 50 μL adsorbent per 5 mL plate stock. The phage-adsorbent mixture was incubated on ice for 30 minutes and then centrifuged at 5000 x g for 10 minutes. The adsorbent pellet was washed three times using 1 mL of λ diluent with 0.1 M NaCl and transferred to an Eppendorf microcentrifuge tube. The phage were then disrupted by resuspension of the pellet in 400 μL of 10 mM Tris pH 7.5, 10 mM EDTA and heating at 70 °C for 5 minutes. The suspension was centrifuged to remove the adsorbent and the supernatant was extracted once with phenol/chloroform/isoamyl alcohol (PCI) and once with chloroform. The aqueous phase was then supplemented with ammonium sulfate to a final concentration of 2.5 M, and precipitated with ethanol. The phage DNA pellet was then rinsed with 70 % ethanol, dried and resuspended for subsequent analysis in 20 μL of TE.

4.2.4.2 Small Scale Isolation of Plasmid DNA

Recombinant plasmid DNA was isolated on a small scale using the alkaline lysis method described in Maniatis et al. (1982). The final DNA preparation was then resuspended in ddH_2O and precipitated with 8% polyethylene glycol 8000 from 0.8 M NaCl. Plasmid DNA prepared in this manner was suitable for sequence analysis.

4.2.4.3 Large Scale Isolation of Plasmid DNA

Plasmids were isolated from 250 mL cultures using a scaled up version of the small scale method.

4.2.5 Electrophoresis of Nucleic Acids and Proteins

Electrophoresis of proteins, transfer of proteins to nitrocellulose and immunological detection of nitrocellulose-bound proteins were carried out as described in Chapter 2, sections 2.2.5 through 2.2.7. Similarly, electrophoretic analysis of DNA and RNA followed methods previously described in Chapter 3, section 3.2.5.

4.2.5.1 Denaturing Polyacrylamide Gel Electrophoresis of DNA

DNA sequencing reactions were analyzed on 6% polyacrylamide gels containing 7M urea which were prepared and run in TBE. The dimensions of the gel were approximately 20 cm X 50 cm. Gels were cast using either 0.4 mm spacers or 0.2-0.45 mm wedge spacers. Gels were run at 1800 volts at a temperature of about 50 °C. Samples were loaded using a sharkstooth comb and electrophoresed until either the bromphenol blue or xylene cyanol marker dyes reached the bottom of the gel. Each set of sequencing reactions was reloaded in lanes adjacent to those of the first run in order to maximize the amount of sequence information obtained from a single gel. Following electrophoresis, the gels were fixed for 15 minutes in 10% ethanol, 10% acetic acid, rinsed for 10 minutes in ddH_2O, dried and autoradiographed using Kodak

AR film.

4.2.6 Northern Blot Analysis

RNA was denatured with glyoxal, electrophoresed on a 1.2% agarose gel and stained as described in Chapter 3, section 3.2.5.6. The gel was then treated with 0.5 M Tris, pH 7.4 for 30 minutes and placed in a capillary transfer setup according to Maniatis et al. (1982). Transfer of RNA to Biodyne A or Hybond-N was carried out overnight using 10X SSC. The filter was then treated with U.V. light and hybridized with radiolabelled probes as described in Chapter 3, section 3.2.11.

4.2.7 DNA Sequence Analysis

4.2.7.1 Subcloning of cDNA Into pGEM Plasmid Sequencing Vectors

cDNA inserts from immunopositive λgt11 phage purified from polyacrylamide as described (Chapter 3, section 3.2.5) were subcloned into plasmid sequencing vectors by ligation of the insert fragment (in ligation buffer) with phosphatase-treated vector DNA and subsequent transformation into E. coli JM109. Vector DNA was prepared by digestion with Eco RI and treatment with 2 U calf intestinal alkaline phosphatase (Maniatis et al., 1982). Transformation competent JM109 was treated with approximately 50 ng of the vector-cDNA ligation.

The rescue of cDNA inserts from λZAP recombinants into pBluescript vectors was carried out as described previously (Chapter 3, section 3.2.10.3).

4.2.7.2 Generation of Deletion Subclones from pBluescript Recombinants

Long cDNAs were prepared for DNA sequencing using the Exonuclease III method of Henikoff (1984) for the generation of deletion subclones as described in the methods accompanying the Promega Erase-A-Base^{TM} kit. Restriction sites within the polylinker of the pBluescript vector were selected on either side of the Xba I site which would be suitable for Exonuclease III digestion and which did not result in the internal cleavage of the insert of interest. In this sense, Xho I and Kpn I were suitable for sequencing from the T7 promoter while Sac I and Not I were selected for sequencing from the T3 promoter. Five μg of double cut plasmid DNA was suspended in 40 μL Exo III buffer (66 mM Tris, pH 8, 0.66 mM $MgCl_2$), and incubated at 35 °C. Fifteen tubes were then assembled on ice, each containing 7.5 μL S1 nuclease mix (40 mM potassium acetate, pH 4.6, 338 mM NaCl, 1.35 mM $ZnCl_2$, 6.7 % glycerol and 2.5 U S1 nuclease). The Exo III reaction was initiated by the addition of 300 U Exo III to the plasmid digest in Exo III buffer. Aliquots of 2.5 μL Exo III digest were then removed to the tubes containing S1 mix at 30 second intervals. DNA from each Exo III time point was then digested with S1 nuclease by placing the S1 tubes at room temperature for 30 minutes. The S1 digest was terminated by the addition of 1 μL of 0.3 M Tris, pH 8, 50 mM EDTA. Aliquots of the S1 digest were then analyzed by agarose gel electrophoresis. The S1 digests were then supplemented with 1 μL 20 mM Tris,

pH 7.5, 100mM $MgCl_2$ containing 0.2 U Klenow DNA polymerase, and incubated for 3 minutes at 37 °C. The reactions were further supplemented with 1 μL of 0.125 mM of each deoxynucleotide triphosphate and incubated a further 5 minutes. The blunt-ended deletion subclones were then ligated by the addition of 40 μL 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 5% polyethylene glycol, 1 mM DTT and 0.1 U T4 DNA ligase. Aliquots of the ligation mixture were then used to transform competent XL1-blue cells followed by selection of transformants on LB/ampicillin plates. A number ot clones from each time point were selected and the si. - of their plasmids estimated by agarose gel electrophoresis.

4.2.7.3 DNA Sequencing

Plasmid templates were sequenced using the dideoxy chain termination method (Sanger et al., 1977) and protocols provided with the Promega GemSeq™ or USB Sequenase™ kits. The Sequenase™ method outlined briefly here.

Plasmid templates were denatured in alkali and precipitated from 2 M ammonium acetate. The pellets were rinsed with 70 % ethanol, dried and resuspended in a 10 μL annealing mixture containing 1 pmol sequencing primer (T3 or T7) in 40 mM Tris, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl. The plasmid-primer annealing reactions were heated to 70 °C for 5 minutes and slowly cooled to room temperature over a period of 1 hour or more. For each

template, 4 termination tubes were prepared and pre-incubated at 37 °C. Each tube contained 2.5 μL of termination mixture consisting of 80 μM of each deoxynucleotide triphosphate and 8 μM of one of either dideoxy GTP, ATP, TTP or CTP, resulting in sequencing reactions terminated at G, A, T or C residues, respectively.

Oligonucleotides were then labelled by supplementing the annealing reaction for each template with 5 mM DTT, 0.5 μM each of dGTP, TTP and dCTP, 5 μCi [^{35}S]dATP and 2 U T7 DNA polymerase followed by incubation at room temperature for 3 minutes. This labelling reaction was distributed in 3.5 μL aliquots to each of the termination tubes at 37 °C and incubation continued for 5 minutes. The reactions were then stopped by the addition of 4 μL of stop buffer (95% formamide, 20 mM EDTA, 0.05% each of bromphenol blue and xylene cyanol) followed by heating at 70 °C for 3 minutes. Reactions were either analyzed directly on denaturing polyacrylamide gels (section 4.2.3.1) or stored at - 70 °C.

4.2.8 Analysis of Protein Products by coupled in vitro transcription / in vitro translation

pBluescript plasmids were linearized with Xho I or Not I for analysis of *in vitro* transcription initiated from the T7 and T3 promoters, respectively. Plasmids were treated with 1 μg proteinase K directly in the restriction buffer. The digests were then extracted with PCI and precipitated with ethanol.

Linearized, protease-treated plasmids (1 μg) were resuspended in a 25 μL reaction containing 40 mM Tris, pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 30 mM DTT, 40 μM each of rUTP, rCTP, rATP, rGTP, 20 U placental ribonuclease inhibitor, and 10 U T7 or T3 RNA polymerase. The reaction was incubated at 37 °C for 30 minutes and then supplemented with 10 U RNAse-free DNAse and incubated a further 5 minutes. Aliquots of the transcription reactions (2 μL) were added directly to rabbit reticulocyte translation reactions. The translation reactions contained 35 μL reticulocyte lysate, 7 μL sterile ddH_2O, 10 U ribonuclease inhibitor, 1 μL 1 mM amino acid mixture (without methionine), 2 μL RNA and 40 μCi [^{35}S] methionine. Samples of the translation mixtures were analyzed by SDS-PAGE.

4.2.9 Computer Analysis of Nucleotide Sequences

Nucleotide sequences were analyzed, assembled and manipulated using the public domain program, Seqaid II, which was obtained from Dr. M. Clarke of the Department of Microbiology and Immunology, U.W.O. Nucleotide and deduced protein sequences determined in this chapter were used as query sequences to search nucleic acid and protein sequence databases. Programs and databases were part of the Canadian Scientific Numeric Database (CAN/SND) prototype in Ottawa, Canada. Nucleotide query sequences were used to search the EMBL and GenBank nucleotide sequence databases for sequence homology using the program FASTA. Similarly, protein query sequences were used to

search the Protein Identification Resource (PIR) database of the National Biomedical Research Foundation, also using FASTA.

Chapter 4.3- Results

4.3.1- Characterization and nucleotide sequence analysis of λSK-2 cDNA

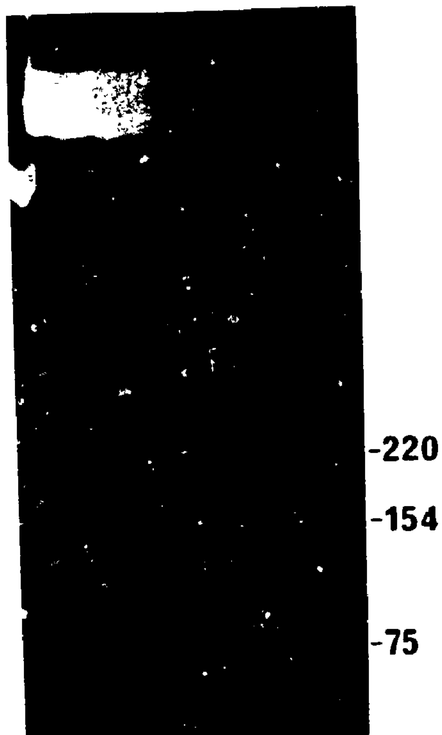
The recombinant phage λSK-2 which bound antibodies specific for the 24 kDa antigen was isolated and the cDNA insert harboured by this recombinant was characterized. Figure 4.1A shows that this cDNA is approximately 150 base pairs in length. The cDNA was then isolated from polyacrylamide gel, radiolabelled with ^{32}P and used to probe a Northern blot of S. mansoni Poly A+ RNA. The RNA complementary to the λSK-2 cDNA was approximately 800 bases in length (Figure 4.1B). Thus the λSK-2 cDNA was not full length and was missing approximately 600-700 base pairs.

In order to isolate a full length cDNA homologous to the λSK-2 cDNA, the λgt10 library was screened with the λSK-2 cDNA probe. In a screen of 10^6 recombinant phage, 15 primary signals were observed of which 8 remained positive after 3 rounds of plaque purification. An example of the screening procedure is outlined in Figure 4.2. The DNA was isolated from each of the recombinants hybridizing from λSK-2, digested with Eco RI and analyzed by polyacrylamide gel electrophoresis (Figure 4.3). All 8 clones harboured an insert which was the same size as that of the probe (approximately 150 bp). The failure to obtain longer cDNA clones is discussed in section 4.4.

The nucleotide sequence of the λSK-2 insert was subsequently determined following subcloning of the insert into the Eco RI site of pGEM3Z. This vector

Figure 4.1: Length of the insert in λSK-2 and the size of the corresponding mRNA. A) DNA from λSK-2 was isolated, digested with Eco RI and analyzed by electrophoresis on a 6% polyacrylamide gel (Panel A, lane 1). The size in base pairs (bp) of co-electrophoresed markers provided by a Hinf I digest of pBR322 (lane 2) are indicated to the right of the panel. B) Five μg of poly A+RNA from rat liver (Panel B, lane 1) or S. mansoni (lane 2) was denatured with glyoxal, electrophoresed on a 1.2% agarose gel, transferred to nylon, and hybridized with the radiolabelled insert from λSK-2. The RNA complementary to the probe was visualized by autoradiography. The numbers to the left of the panel indicate the positions of the marker RNAs co-electrophoresed on the original gel (in Svedberg units). The markers were human (28S and 18S) and E. coli (23S and 16S) ribosomal RNAs. The size of the markers in descending order in kilobases is 4.7, 2.9, 1.8 and 1.5.

A



B

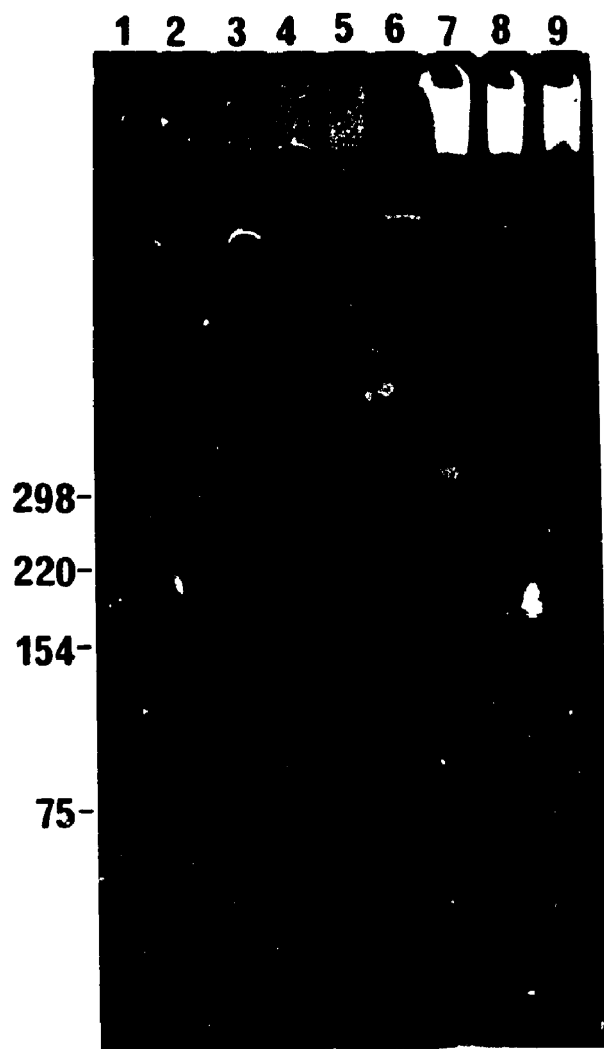
Figure 4.2: Isolation of cDNA clones from the λgt10 S. mansoni cDNA library which hybridize with the λSK-2 insert. Approximately 10^6 recombinants from the S. mansoni λgt10 library were plated at a density of 10^5 phage per plate and nitrocellulose plaque lifts were prepared. The filters were then hybridized with the radiolabelled insert from λSK-2. Positive phage were revealed by autoradiography. Panel 1 shows an example of the results of a primary screen of one plate revealing 2 positive clones (arrows). These phage were isolated, plated at a density of about 500 plaques per plate and hybridized with the λSK-2 insert (panel 2). One clone was selected and the procedure was repeated with about 50 phage per plate (panel 3). A single hybridizing plaque could be unambiguously identified and a phage stock was prepared.

1

2

3

Figure 4.3: Analysis of the inserts of S. mansoni λgt10 cDNA clones hybridizing with the λSK-2 insert. Eight independent recombinants were isolated from the λgt10 library and were processed for small scale λ DNA isolation (section 4.2.4.1). The cDNAs were released by Eco RI digestion and analyzed on a 6% polyacrylamide gel. The size of marker DNAs in base pairs (bp) from a pBR322 Hinf I digest are indicated to the left of the panel.


bp
1 2 3 4 5 6 7 8 9
298-
220-
154-
75-

was chosen because sequence analysis could be easily carried out in both directions by priming from the SP6 and T7 promoters in this vector. The nucleotide sequence was 141 base pairs in length including the Eco RI termini. The amino acid sequence of a possible open reading frame derived from the nucleotide sequence illustrated in Figure 4.4, demonstrates the presence of a signal for N-linked glycosylation (indicated with an asterisk). One particularly noteworthy feature of this sequence is that the Eco RI termini do not display the extra nucleotides present in the Eco RI linkers used to construct the library. The Eco RI sites are defined by the sequence GAATTC rather than GGAATTCC as might be expected if they were derived from the linkers.

4.3.2 Characterization and nucleotide sequence analysis of clones isolated from the λZAP library

I have shown in Chapter 3 that a clone harbouring the smallest insert from the original panel of immunoreactive λZAP clones (pSKZ-23), hybridized detectably with each of the recombinants but not with λZAP. Moreover, the λZAP panel contained clones which were represented by four distinct size classes. The largest of these clones was pSKZ-1, whose insert was analyzed by agarose gel analysis and is shown in Figure 4.5, lane 1. The size of this insert was about 2.6 kb. Digestion of pSKZ-1 with either Xho I or Kpn I showed a single band of approximately 5.6 kb (Figure 4.5, lanes 3, 4) . This demonstrated that the plasmid contained only a single site for either of these enzymes i.e. the

Figure 4.4: Nucleotide and deduced amino acid sequence of the λSK-2 insert. The λSK-2 insert was subcloned into the EcoRI site of pGEM-3Z and sequenced in both directions using the SP6 and T7 promoter primers by the dideoxy chain termination method (section 4.2.7.3). The position of a potential signal for N-linked glycosylation is indicated with an asterisk. The nucleotides are numbered beginning with the first G in the Eco RI site at the 5' end of the sequence.

GAATTC 6

TTGAATGAAA[illegible]TCTATTGAAATTAAAGAA 36
LeuAsnGluThrSerIleGluIleLysGlu 10
*

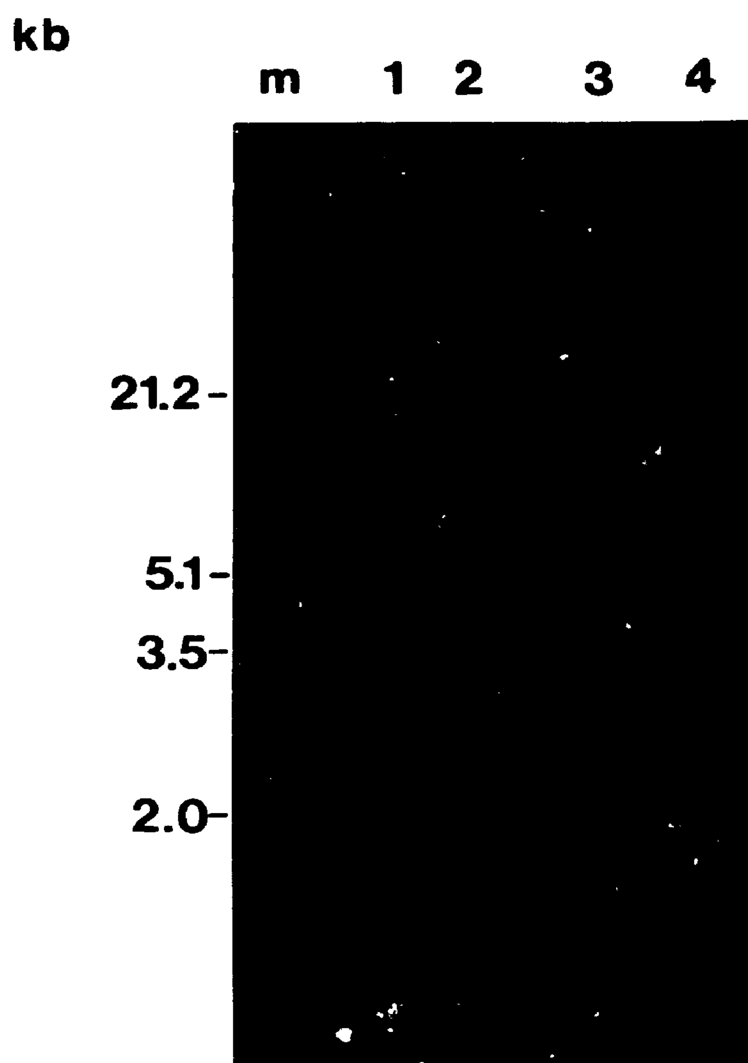
GAATTAGGCCAAGAACTTCATCAATTACAA 66
GluLeuGlyGlnGluLeuHisGlnLeuGln 20

CTTATATTAGATGAATTAAGTAGAAGAATA 96
LeuIleLeuAspGluLeuSerArgArgIle 30

AGGGCAACTCCAAATTCAGCAAATAAATAT 126
ArgAlaThrProAsnSerAlaAsnLysTyr 40

ATGAAAAATGAATTC 141
MetLysAsnGluPhe 44

Figure 4.5: The size of the insert in pSKZ-1. The plasmid was isolated, restricted with Xba I (lane 1), Xho I (lane 3), and Kpn I (lane 4) and analyzed by agarose gel electrophoresis. Lane 2 shows intact pSKZ-1. The size of DNA markers (lane m) in kilobase pairs is indicated to the left of the panel.


kb
m
1
2
3
4
21.2-
5.1-
3.5-
2.0-

pSKZ-1 insert was not cleaved internally by these enzymes This experiment was carried out to define restriction sites within the pBluescript polylinker which were suitable for the generation of deletion subclones for sequence analysis (see below).

The size of the mRNA homologous to the insert from pSKZ-1 was determined by Northern blot analysis (Figure 4.6). This experiment showed that an RNA of approximately 2.8 kilobases hybridized with the pSKZ-1 insert but did not detect any homologous human sequences (Figure 4.6, lane 2). The close correspondence between the sizes of the pSKZ-1 insert and the mRNA suggested that the insert was near full length.

The strategy used for determination of the nucleotide sequence of the pSKZ-1 insert was based on a method described by Henikoff (1984). This method uses Exonucleas III (Exo III) for the generation of a set of unidirectional nested deletions which can subsequently be used to generate overlapping nucleotide sequences throughout the length of the sequence of interest. Exo III preferentially digests DNA with 5' overhangs at their termini. Thus, digestion of plasmids with two restriction enzymes, one leaving a 5' overhang and one leaving a 3' overhang, enables deletions to be generated in one direction. The enzyme leaving a 3' overhang must protect the site from which sequence reactions are primed. Moreover, neither enzyme should cleave the insert internally.

3 OF/DE 3

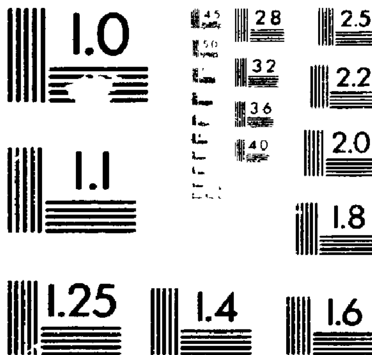
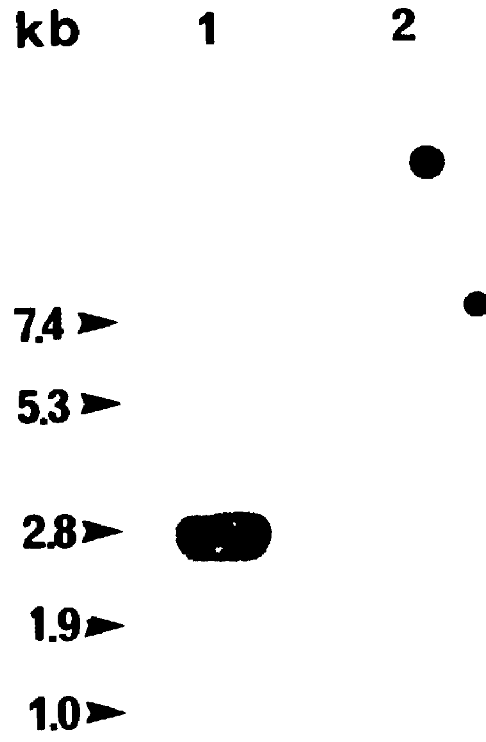


Figure 4.6: The size of the mRNA complementary to the insert of pSKZ-1. The insert from pSKZ-1 was isolated, radiolabelled and used to probe a Northern blot containing 5 μg of S. mansoni poly A+ RNA (lane 1) and human poly A+ RNA (lane 2). The size of co-electrophoresed marker RNAs in kilobases (kb) is indicated to the left of the panel.

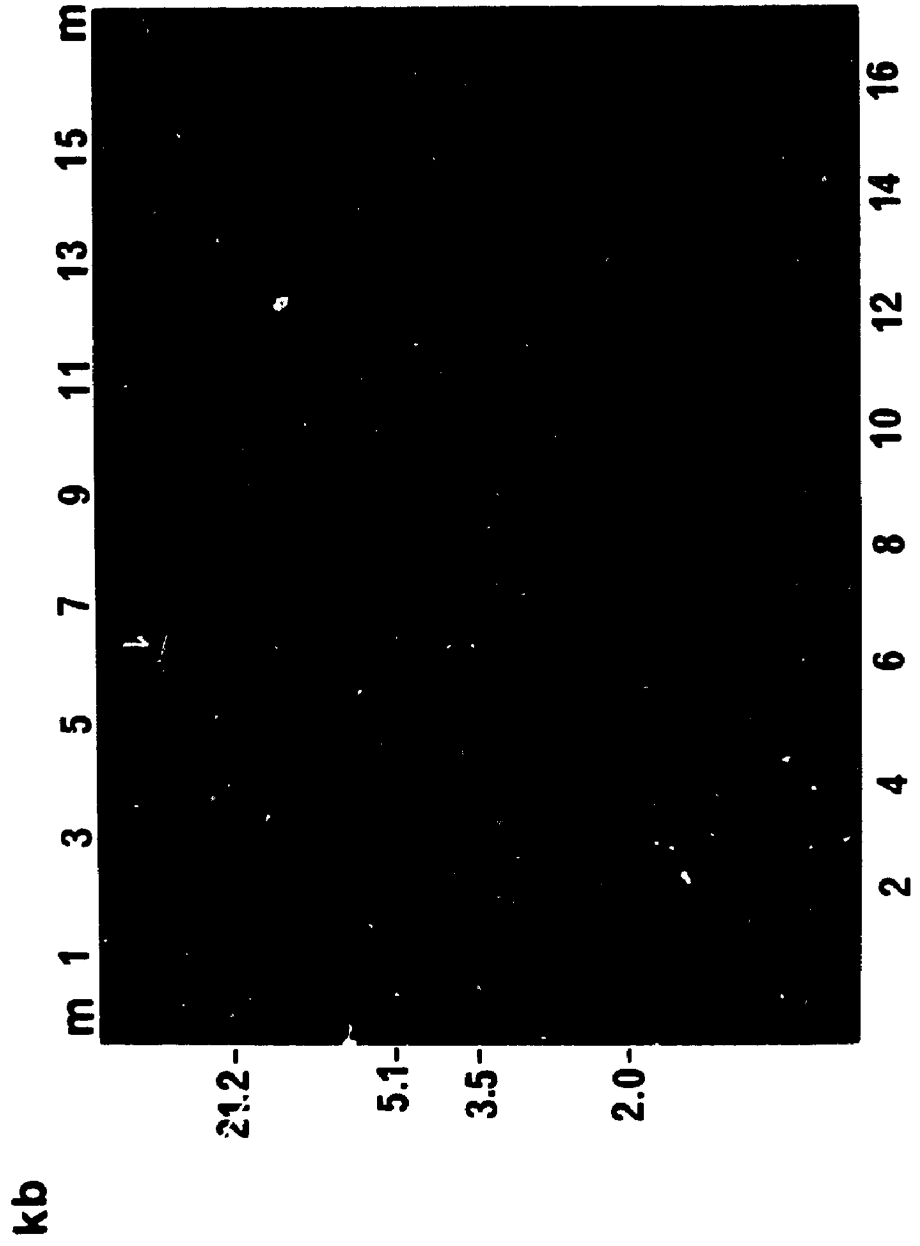

kb
1
2
7.4
5.3
2.8
1.9
1.0

pSKZ-1 was first restricted with a variety of enzymes which were known to cleave within the polylinker of pBluescript. Enzymes which were found to cleave the insert internally included Cla I, Bam HI and Hind III. However, 4 enzymes which appeared to be suitable for the generation of deletion subclones were Xho I and Kpn I (for sequence analysis from the T7 promoter primer) and Sac I and Not I (for sequences generated from the T3 promoter).

pSKZ-1 was digested with Xho I/Kpn I and Not I/Sac I and used for deletion experiments as described in section 4.2.7.2. The results of a time course analysis of Exo III/S1 nuclease treatment of pSKZ-1 DNA digested with Xho I/ Kpn I is illustrated in Figure 4.7. The bulk of the DNA population was observed to become successively smaller as the experiment proceeded. Lane 14 shows that the size of the bulk DNA population after 6.5 minutes incubation with Exo III was almost the same size as the pBluescript vector (lane 16) indicating that almost all of the insert had been deleted.

The deletion reactions from each time interval were then repaired with Klenow polymerase, recircularized and transformed into competent XL1-blue cells. Between 20 and 200 clones were obtained from each time interval. Four transformants from each time interval were then selected and sized on a 1% agarose gel. There was remarkable variability in the plasmid size of transformants from any given time interval. For example, some transformants from early time points harboured plasmids which were unexpectedly small while

Figure 4.7: Time course of Exonuclease III (Exo III) digestion of pSKZ-1 restricted with Kpn I and Xho I. Plasmid was isolated and digested with Kpn I and Xho I (lanes 1 and 15) and subsequently digested with Exo III and S1 nuclease as described in section 4.2.7.2. Lanes 2 through 14 show aliquots of the ExoIII/S1 digest taken at successive 30 second intervals. Marker DNAs were run in lanes labelled m and lane 16 shows the pBluescript vector cut with Xba I.


m 1 3 5 7 9 11 13 15 m
2 4 6 8 10 12 14 16
kb
21.2
5.1
3.5
2.0

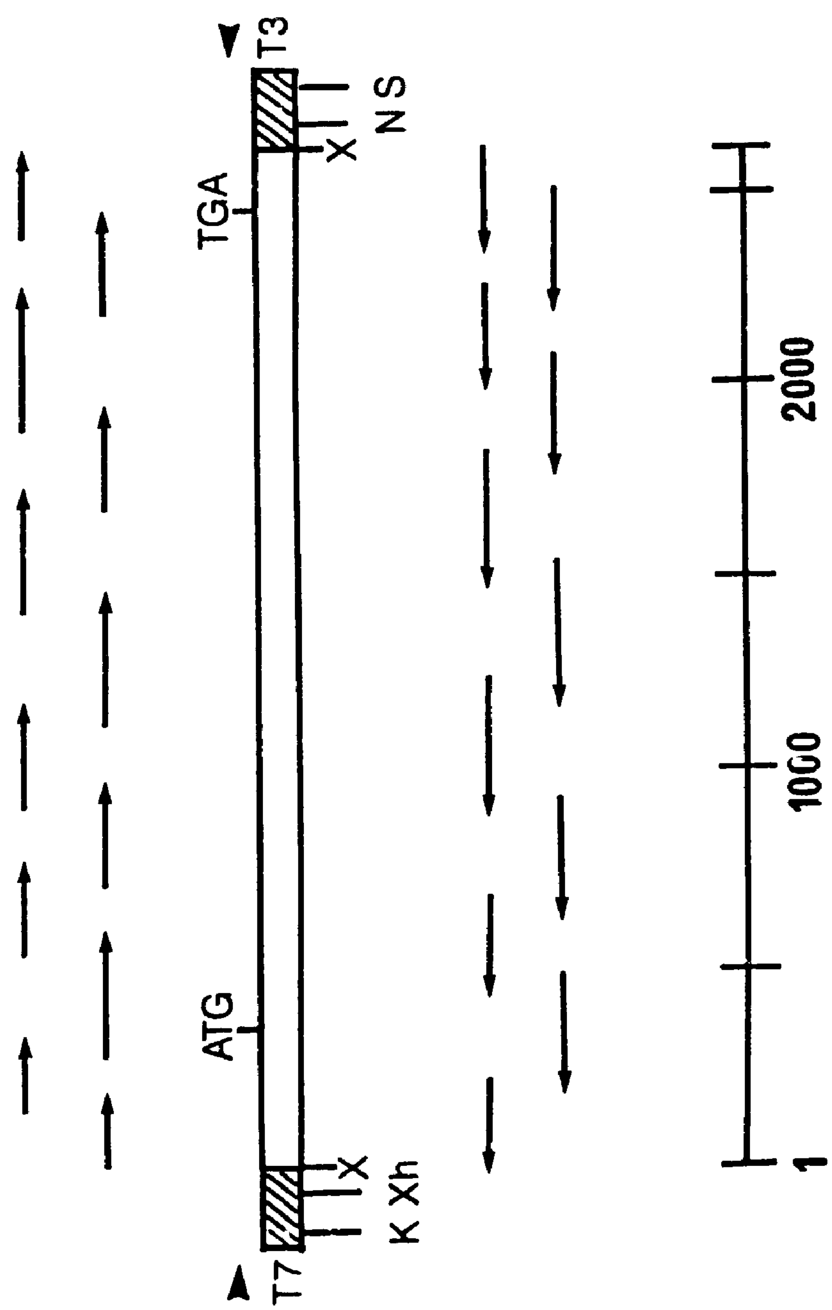
some from later time points appeared to be hardly deleted at all. From these preliminary transformants, clones harbouring plasmids with approximately equidistant deletions were assembled and re-analyzed on a 1% agarose gel (Figure 4.8). In all, 11 deletion clones (and the pSKZ-1 parent) were used to determine the sequence of the pSKZ-1 cDNA from the T7 primer site while 10 deletion clones (and the parent pSKZ-1 plasmid) were used to sequence from the T3 primer site. Following sequence analysis, the deletion subclones were found to be between 150 and 350 nucleotides apart. The sequencing strategy and the position of the deletion subclones is summarized in Figure 4.9. Sequences comprising both strands were independently analyzed, compared and used to assemble the sequence given in Figure 4.10.

The sequence shown in Figure 4.10 spans 2621 nucleotides with an open reading frame of 2106 nucleotides predicting a protein of 702 amino acids with a molecular mass of about 78 kDa. The nucleotide residues depicted in Figure 4.10 are numbered 1 through 2621 with residue 1 being the first nucleotide following the Xba I site at the 5' end. The amino acids are numbered beginning with the methionine at nucleotide 363. This methionine codon was chosen since it was the only one which resulted in a significant reading frame of 702 amino acids. Other ATG codons in the region of nucleotide 363 resulted in short potential open reading frames. The sequence shown predicts 362 nucleotides of a 5' untranslated region and 150 nucleotides of a 3' untranslated

Figure 4.8: Agarose gel analysis of deletion subclones derived from pSKZ-1. Plasmid DNA from the deletion time series illustrated in Figure 4.7 was repaired with Klenow polymerase, ligated with T4 DNA ligase and transformed into competent E. coli XL1-blue. The size of the plasmids from four representative clones derived from each time point were analyzed by electrophoresis on a 1 % agarose gel. Selected clones were then assembled according to their size and a panel of successively smaller plasmids was electrophoresed as shown. Lane 1 shows intact pSKZ-1 (approximately 5.8 kilobase pairs (kB)), while lane 17 shows uncut pBluescript (3.0 kb). Lanes 2 through 16 show the intact deletion plasmids spanning the length of the insert of pSKZ-1 assembled in order of decreasing size. These clones were used for direct sequence analysis.

kb
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
5.8-
3.0-

Figure 4.9: Strategy for the determination of the nucleotide sequence of pSKZ-1. The pSKZ-1 sequence is represented by an open box with the proposed translational start (ATG) and stop (TGA) codons indicated. The bar at the bottom of the figure indicates the scale in nucleotides. The shaded boxes on either side of the pSKZ-1 sequence show the polylinker regions and the associated T7 and T3 promoter sites within the pBluescript vector (not to scale). Restriction sites within the polylinker which were used to generate deletion clones are as follows: X, Xba I; K, Kpn I; Xh, Xho I; N, Not I; S, Sac I. The positions and sequencing directions of the two sets of deletion subclones are indicated above and below the sequence.


T3
N S
X
TGA
2000
1000
ATG
X
K Xh
T7
1

region. There was no poly A tail nor a polyadenylation signal within the 3' untranslated sequence suggesting that the pSKZ-1 cDNA does not encode the complete 3' end of the corresponding mRNA. The nucleotide and deduced amino acid sequence were used as query sequences to search the EMBL and GenBank nucleotide sequence and the PIR protein sequence databases running under the CAN/SND implementation in Ottawa, Canada, for sequence homology to any known sequences. While there were no significant homologies revealed at the nucleic acid level, the deduced amino acid sequence clearly resembled previously determined protein sequences corresponding to vertebrate calcium-activated neutral protease (calpain; E.C.3.4.22.17). The deduced schistosome sequence showed high levels of homology over its entire length to both the human and chicken enzyme sequences (both of which were determined from nucleotide sequences). Individual comparisons between the schistosome and the chicken sequences and between the schistosome and human sequences both demonstrated levels of amino acid identity of approximately 40%. The overall similarity was close to 70% based upon comparison of identical amino cids and "conservative replacements". The residues which have been proposed to be involved in catalysis (Cysteine-108, Histidine-264 in the chicken sequence) are conserved in the schistosome sequence and are indicated with an asterisk in Figure 4.10 (Cysteine-98 and Histidine-256). The sequences in the vicinity of the catalytic residues are also highly conserved. In addition, since calpains are

known to bind and be activated by calcium, two sequences were located within the schistosome sequence which conform to the concensus sequence for E-F hand structure (Goodman et al., 1979). These two sequences are underlined in Figure 4.10 and are localized within the C-terminal region of the protein sequence.

A summary of the sequence similarities among the schistosome, human and chicken sequence, at the protein level, is shown in Figure 4.11. The asterisks denote only those amino acids which are identical in all three sequences. The amino acid numbers are derived from the schistosome sequence and are compared with corresponding residues of the human and chicken sequences. Gaps which were introduced to maximize sequence similarity are indicated with the + symbol. These data show that 32% of the amino acids are identical in all three sequences. Several contiguous blocks of sequence identity are particularly evident between amino acids 90 to 360.

The pSKZ-1 cDNA was also used for the production of runoff RNA transcripts using the RNA polymerase promoters of T3 and T7 RNA polymerase promoters in the pBluescript vector. RNA transcribed from the T3 promoter (Figure 4.12, lane 1) resulted in the labelling of faint 45 and 25 kDa bands which were also seen in the absence of template. In contrast RNA transcribed with T7 RNA polymerase (Figure 4.12, lane 2) coded for a more strongly labelled protein of approximately 76 kDa. This confirmed the identity of the

Figure 4.10: Nucleotide and deduced amino acid sequence of the insert of pSKZ-1. The nucleotide sequence was determined by the dideoxy chain termination method from panels of overlapping deletion subclones, and example of which is shown in Figure 4.8. Sequencing reactions were analyzed by denaturing polyacrylamide gel electrophoresis (section 4.2.3.1). Nucleotide residues were numbered 1 through 2621 with residue 1 being the first nucleotide following the Xba I site on the coding strand. Amino acids were numbered beginning with the methionine at nucleotide 363 and are indicated in their one-letter code. The proposed catalytic residues are indicated with an asterisk while potential calcium binding sites are underlined.

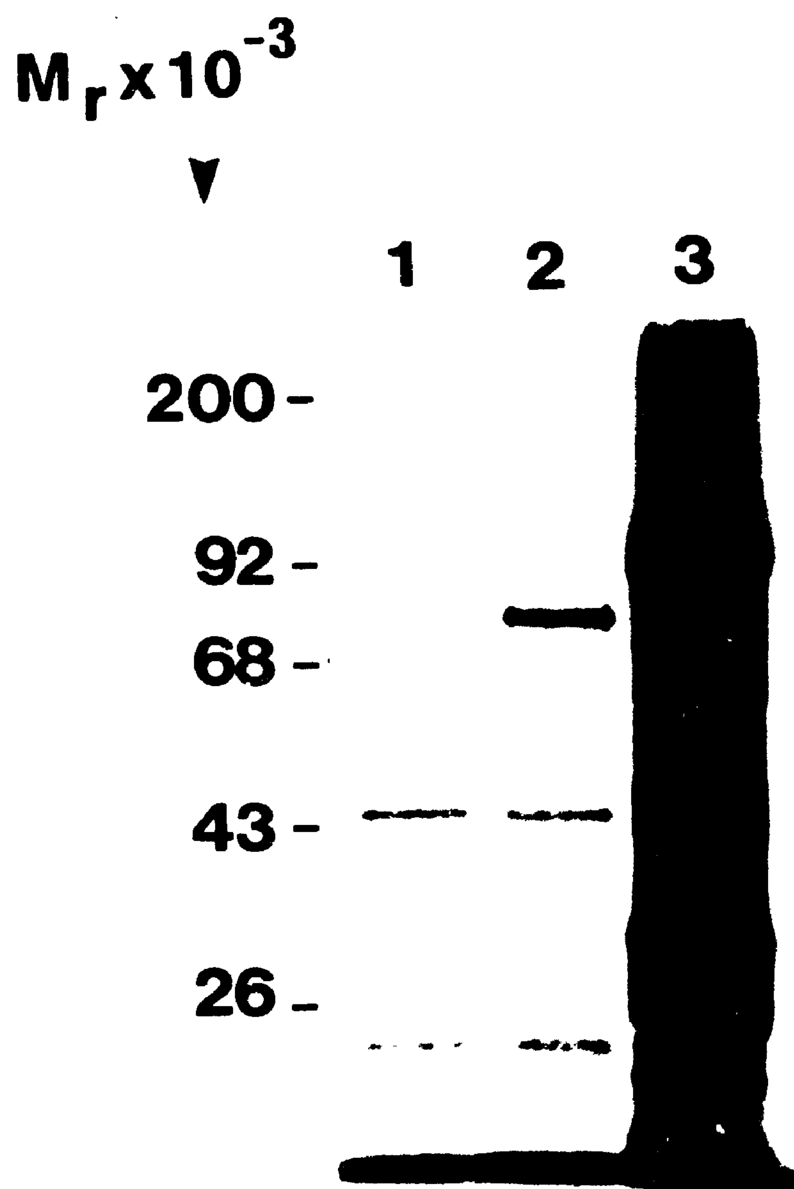
```
                                                                                          GG     2

CTACTTGATAATCCGCGTACAAATTAAAAATCTTTTCAAATAATGTCGGATACACATATATATGAGTGTATTTGACAGAGAGAGAGAAAG    92

TTCTCTTATTATCATTCCAATCAGTTTTAGTGAACGATTGAAGTTTGGTGTAGAAAACTTGAAAAAACCCTAATTCATTCATTATAATTT   182

CTAAGAATAATAATGGGACGAATACAAATTGTTTATTCTCCTGATGAAAATGTATCTGGACGAACTAACCGTCCTGGTAAAGAGGTAGTG   272

GATCCACGTACTGGTAGAATAATCAAAGTGAAACGAGAAACTCCAGATGATTACTTGAATGTATTAAAACCCATCAAAGGTCCAAAGCGT   362

ATGGAATTTAATCCATATCTTCCTAAAACACTTACTCCAAAAGGATATGCTAAGTTTAAACTAATGATGAATGTTGCAAGTAAACAATAT   452
M  E  F  N  P  Y  L  P  K  T  L  T  P  K  G  Y  A  K  F  K  L  M  M  N  V  A  S  K  Q  Y     30

GAAACGCTAGTTAAACGTTTAAAAACTGAACGAACACTCTGGGAAGATCCTGACTTTCCAGCTAATGATAAAGCAATTGGGAATCTGCCA   542
E  T  L  V  K  R  L  K  T  E  R  T  L  W  E  D  P  D  F  P  A  N  D  K  A  I  G  N  L  P     60

GATTTCAGAGAGAGAATTGAATGGAAAAGACCACATGAAATTAATCCAAATGCTAAATTTTTCGCTGGTGGAGCAAGTCGTTTCGATATT   632
D  F  R  E  R  I  E  W  K  R  P  H  E  I  N  P  N  A  K  F  F  A  G  G  A  S  R  F  D  I     90

GAACAAGGAGCACTTGGTGACTGCTGGTTACTTGCAGTAGTGGCTTCCATATCAGGATACCCACAGCTATTTGATCAAGTGGTGCCTAAA   722
E  Q  G  A  L  G  D  C  W  L  L  A  V  V  A  S  I  S  G  Y  P  Q  L  F  D  Q  V  V  P  K    120
                     *
GATCAAGAATTGAAAGGACCAGAATATGTTGGTGTTGTACGTTTTCGGTTTTGGCGTTTTGGTCACTGGGTGGAAGTGCTTATAGATGAT   812
D  Q  E  L  K  G  P  E  Y  V  G  V  V  R  F  R  F  W  R  F  G  H  W  V  E  V  L  I  D  D    150

CGTCTTCCGGTTCGTCAAGGACGTAATACACTCGTTTTTATGCATTCTAATGATCCCACGGAATTCTGGTCAGCTCTGTTGGAAAAAGCT   902
R  L  P  V  R  Q  G  R  N  T  L  V  F  M  H  S  N  D  P  T  E  F  W  S  A  L  L  E  K  A    180

TATGCAAAATTGAATGGTTGTTATGCTCATCTGAGTGGAGGATCACAAAGTGAAGCTATGGAAGATTTAACTGGTGGAATTTGTTTATCT   992
Y  A  K  L  N  G  C  Y  A  H  L  S  G  G  S  Q  S  E  A  M  E  D  L  T  G  G  I  C  L  S    210

CTTGAACTGAACCAAAAAGAACGTCCAAGTGATCTTATAGATCAGCTTAAAATTTATGCTCAACGTTGTTGCCTTATGGGTTGTAGTATT  1082
L  E  L  N  Q  K  E  R  P  S  D  L  I  D  Q  L  K  I  Y  A  Q  R  C  C  L  M  G  C  S  I    240

GATTCATCAGTTATGGAACAAAAAATGGATAATGGACTTATAGGTAGTCACGCATACAGTTTAACAGGTGTATATCCCGTCAACTACCGA  1172
D  S  S  V  M  E  Q  K  M  D  N  G  L  I  G  S  H  A  Y  S  L  T  G  V  Y  P  V  N  Y  R    270
                                                 *
GGTCGCACTCAATGGTTAATGCGTTTACGTAATCCATGGGGTGACAGTCATGAATGGAAAGGTGCATGGTGTGATGGTTCACCTCAATGG  1262
G  R  T  Q  W  L  M  R  L  R  N  P  W  G  D  S  H  E  W  K  G  A  W  C  D  G  S  P  Q  W    300

CGTGAAATAAGTGAACAAGAAAAGAAAAATATTAACTTGAGCTTCACTGCAGATGGAGAATTTTGGATGTCATATGAAGATTTTGTTACT  1352
R  E  I  S  E  Q  E  K  K  N  I  N  L  S  F  T  A  D  G  E  F  W  M  S  Y  E  D  F  V  T    330

TGTTTTTCACGAGTTGAAGTATGTCATTTAGGTCTTGAAAGTTTAGAGTATAATCAAAATTTCCATGGGAAAAGAAGATTAGACGAAGCT  1442
C  F  S  R  V  E  V  C  H  L  G  L  E  S  L  E  Y  N  Q  N  F  H  G  K  R  R  L  D  E  A    360

ATATTTTCTGGTCAATGGCAACGTAACGTTAATGCTGGTGGTTGTATTAATAATCGCACTACCTATTGGACGAATCCACAATTTCGCATC  1532
I  F  S  G  Q  W  Q  R  N  V  N  A  G  G  C  I  N  N  R  T  T  Y  W  T  N  P  Q  F  R  I    390
```

```
ACAGTTGAAGATCCAGATCCTGATGACGATGATAATAAGTGTTCAGTACTCATTGGTTTAATGCAAACAGATATCAGGAAGAAAGTCGGA   1622
T  V  E  D  P  D  P  D  D  D  D  N  K  C  S  V  L  I  G  L  N  Q  T  D  I  R  K  K  V  G      420

GCAGATTTTCAACCTATAGGTTTTATGGTTTATAATGCACCTGATGATTTAAACACTTTATTATCACGTGCACAACTTTTAACTAGATCT   1712
A  D  F  Q  P  I  G  F  M  V  Y  N  A  P  D  D  L  N  T  L  L  S  R  A  Q  L  L  T  R  S      450

CCAATAGCTAAATCACAATTTATTAATACACGTGAAGTTACTGCACAATTTCGTGTACCACCAGGATCATATGTTGTTATTCCGAGTACA   1802
P  I  A  K  S  Q  F  I  N  T  R  E  V  T  A  Q  F  R  V  P  P  G  S  Y  V  V  I  P  S  T      480

TTCGATCCAAATATTGAAC   AATTTTATATTACGTGTATTTTCACAAACATCTATTACAGAACAAGAACTTGATGAAGACAATACTAAC   1892
F  D  P  N  I  E     N  F  I  L  R  V  F  S  Q  T  S  I  T  E  Q  E  L  D  E  D  N  T  N      510

CAAGGTCTACCAGATGATGTGATTGAAGCTTTGAAATTAGAAGATACTTTGTTAGATGAAGATCAAGAAATTGAACAGAAATTTTTAGCT   1982
Q  G  L  P  D  D  V  I  E  A  L  K  L  E  D  T  L  L  D  E  D  Q  E  I  E  Q  K  F  L  A      540

ATCCGTGATCCAAAAACTAATGCTATAAACGCTGTTAAATTGGGTGAACTTTTAAATAATAGTACATTACAAGATATCCCTAACTTCCAA   2072
I  R  D  P  K  T  N  A  I  N  A  V  K  L  G  E  L  L  N  N  S  T  L  Q  D  I  P  N  F  Q      570

GGATTTAATAAAGAATTATGTCGTAGTATGGTTGCATCTGTAGATAATAACTTAACAGGTCATGTTGAATTAAACGAATTTATGGATCTT   2162
G  F  N  K  E  L  C  R  S  M  V  A  S  V  D  N  N  L  T  G  H  V  E  L  N  E  F  M  D  L      600

TGGATACAAGCTAAAGGATGGAAACATATATTTATAAAACATGATGTTGATCAAAGTGGTTATTTCAGTGCATATGAATTTCGTGAAGCA   2252
W  I  Q  A  K  G  W  K  H  I  F  I  K  H  D  V  D  Q  S  G  Y  F  S  A  Y  E  F  R  E  A      630

CTGAATGATGCAGGTTATCATGTGAGTAATCGATTAATCAATGCTATTATAAATCGATATCAAGATCCTGGCACAGATAAAATAAGTTTT   2342
L  N  D  A  G  Y  H  V  S  N  R  L  I  N  A  I  I  N  R  Y  Q  D  P  G  T  D  K  I  S  F      660

GAAGATTTTATGTTGAGCATGGTACGACTTAAAACTGCCTTTGAAACAATTGAAGCACATCCGAAAAATATTGAAGGCACATCACTTTTC   2432
E  D  F  M  L  S  M  V  R  L  K  T  A  F  E  T  I  E  A  H  P  K  N  I  E  G  T  S  L  F      690

AGTGCTGAAGACTATCTACGATTTTCAGTTTATCTCTGATTCTATCAAGAAACAGTTATTGAATTGGATGAACCACTGACAATTTACTTC   2522
S  A  E  D  Y  L  R  F  S  V  Y  L  >        702

ACACTATCTTTTCTTGTTACAATCATATGTTGACTTATTTTTTCAATTTAGAAATCTACAAATATTTAAACAAAGAAGTATATTGTACTT   2612

TTTTGTTTT   2621
```

Figure 4.11: Sequence similarity amongst the deduced amino acid sequence from the open reading frame of pSKZ-1 and human and chicken calpains (E.C. 3.4.22.17). The amino acids are numbered according to the numbering system outlined in Figure 4.10. Gaps in the sequences were introduced to maximize sequence similarity and are indicated by the symbol +. Amino acids which are invariant in all three sequences are labelled with an asterisk. The chicken and human sequences are those reported by Ohno et al. (1984) and Aoki et al. (1986).

```
                               30                              60
no                              v                               v
sm MEFNPYLPKTLTPKGYAKFKLMMNVASKQYETLVKRLKTERTLWEDPDFPANDKAIG++NLPDFRER+++IEWKRPNEINPNAKFFLGGASRF
hu GVSAQVQKQRARELGLGRHENAIKYLGQDYEQLRVRCLQSGTLFRDEAFPPVPQSLGYKDLGPNSSKTYGIKWKRPTELLSNPQFIVDGATRT
ch GIAARLQRDRLRAEGVGEHNNAVKYLNQDYEALKQECIESGTLFRDPQFPAGPTALGFKELGPYSSKTRGVEWKRPSELVDDPQFIVGGATRT
id               *              ** *        **  *  **          *           **** *      *   **

90                           120                           150                           180
 v                             v                             v                             v
DIEQGALGDCWLLAVVASISGYPQLFDQVVPKDQELKGPEYVGVVRFRFWRFGHWVEVLIDDRLPVRQGRNTLVFMHSNDPTEFWSALLEKAYAK
DICQGALGDCWLLAAIASLTLNDTLLHRVVPHGQSFQN+GYAGIFHFQLWQFGEWVDVVVDDLLPIKDGK++LVFVHSAEGNEFWSALLEKAYAK
DICQGALGDCWLLAAIGSLTLNEELLHRVVPHGQSFQE+DYAGIFHFQIWQFGEWVDVVVDDLLPTKDG++ELLFVHSAECTEFWSALLEKAYAK
** ***********   *      *   ***  *      * *   *  * ***** *  ** **   *   * * **    *************

                        210                           240                              270
                          v                             v                                v
LNGCYAHLSGGSQSEAMEDLTGGICLSLELNQKERPSDLIDQLKIYAQRCCLMGCSID+SSVMEQKMD++NGLIGSHAYSLTGVYPVNYRGRTQW
VNGSYEALSGGSTSEGFEDFTGGVTEWYEL++RKAPSDLYQIILKALERGSLLGCSIDISSVLDMEAITFKKLVKGHAYSVTGAKQVNYRGQVVS
LNGCYESLSGGSTTEGFEDFTGGVAEMYDL++KRAPRNMGHIIRKALERGSLLGCSIDITSAFDMEAVTFKKLVKGHAYSVTAFKDVNYRGQQEQ
 ** *  *****  *  ** ***      *     *               * *****  *           *   **** *    *****

                      300                           330                           360
                        v                             v                             v
LMRLRNPWGDSHEWKGAWCDGSPQWREISEQEKKNINLSFTADGEFWMSYEDFVTCFSRVEVCHLGLESLEYNQNFHGKRRLDEAIFSGQWQRNV
LIRMRNPWGEV+EWTGAWSDSSSEWNNVDPYERDQLRVKM+EDGEFWMSFRDFMREFTRLEICNLTPDAL++++KSRTIRKWNTTLYEGTWRRGS
LIRIRNPWGQV+EWTGAWSDGSSEWDNIDPSDREELQLKM+EDGEFWMSFRDFMREFSRLEICNLTPDALTKDE++++LSRWHTQVFEGTWRRGS
* * *****   ** *** * *  *                 *******  **   *   * * *    *                 *  * *

                  390                                420                               450
                    v                                  v                                 v
NAGGCINNRTTYWTNPQFRITVEDPDPDDD++++DNKCSVLIGLMQTDIR++KKVGADFQPIGFMVYNAPDDL++++NTLLSRAQLLTRSPIAKS
TAGGCRNYPATFWVNPQFKIRLDETDDPDDYGDRESGCSFVLALMQKHRRRERRFGRDMETIGFAVYEVPPELVGQPAVHLKRDFFLANASRARS
TAGGCRNNPATFWINPQFKIKLLEEDDDP++GDDEVACSFLVALMQKHRRRERRVGGDMHTIGFAVYEVPEEAQGSQNVHLKKDFFLRNQSRARS
 **** *   * * **** *     *           **    ***   *     * *   *** **  *          *     *     * *

                       480                           510                           540
                         v                             v                             v
+QFINTREVTAQFRVPPGSYVVIPSTFDPNIEVNFILRVFSQTSITEQELDEDNTNQGLPDDVIEALKLEDTLLDEDQEIEQKFLAIRDPKTNAI
EQFINLREVSTRFRLPPGEYVVVPSTFEPNKEGDFVLRFFSEKSAGTVELD+DQIQANLPDEQV++++LSEEEIDEN++FKALFRQLAGEDME+I
ETFINLREVSNQIRLPPGEYIVVPSTFEPHKEADFILRVFTEKQSDTAELDEE+ISADLADE++EEITEDDI++++EDGFKNMFQQLAGEDME+I
  *** ***    * *** * * ***  *  *  * **          ***       * *                      *

                    570                           600                           630
                      v                             v                             v
NAVKLGELLNN+STLQDIPNFQGFNKELCRSMVASVDNNLTGHVELNEFMDLWIQAKGWKHIFIKHDVDQSGYFSAYEFREALNDAGYHVSNRLI
SVKELRTILNRIISKHKDLRTKGFSLESCRSMVNLMDRDGNGKLGLVEFNILWNRIRNYLSIFRKFDLDKSGSMSAYEMRMAIESAGFKLNKKLY
SVFELKTILNRVIARHKDLKTDGFSLDSCRNMVNLMDKDGSARLGLVEFQILWNKIRSWLTIFRQYDLDKSGTMSSYEMRMALESAGFKLNNKLH
    *   **            **    ** **   *        * **  **       *     * * **  * ** * *   **      *

               660                           690
                 v                             v
NAIINRYQDPGTDKISFEDFMLSMVRLKTAFETIEAHPKNIEGTSLFSAEDYLRFSVYL
ELIITRYSEPDLA+VDFDNFVCCLVRLETMFRFFKTLDTDLDGVVTFDLFKWLQLTMFA
QVVVARYADAETG+VDFDNFVCCLVKLETMFRFFHSMDRDGTGTAVMNLAEWLLLTMCG
     **         *  *    * * * *           *         *
```

Figure 4.12: Coupled in vitro transcription/translation analysis of the protein encoded by pSKZ-1. The plasmid was linearized by digestion with either Xho I or Not I for production of runoff RNA transcripts initiated from the T3 and T7 promoters, respectively, within the pBluescript vector. Transcripts were translated directly in a rabbit reticulocyte lysate in vitro translation system as described in section 4.2.8. Translation products produced from the T3 (lane 1) and T7 (lane 2) transcripts were analyzed by SDS-PAGE and autoradiography. Lane 3 illustrates translation programmed by brome mosaic virus RNA. Molecular weight markers are indicated to the left of the panel.


$M_r \times 10^{-3}$
1
2
3
200
92
68
43
26

coding strand and resulted in a protein of approximately the same size as that predicted from the protein sequence encoded by the open reading frame of pSKZ-1.

4.4 Discussion

The work outlined in this chapter details the partial characterization and nucleotide sequence analysis of two cDNAs isolated from S. mansoni cDNA libraries. One (λSK2) was isolated from a λgt11 expression library and shown to synthesize a fusion protein which shares antigenic determinants with a 24 kDa membrane protein (Chapter 3). The other (λSKZ-1) was shown, following sequence analysis, to encode a polypeptide homologue of vertebrate calcium-activated neutral protease (calpain; E.C. 2.3.22.17).

The cDNA sequence of the λSK2 insert has recently been published by others (Knight et al., 1989). These investigators independently isolated the same 147 base pair cDNA from a λgt11 expression library. Moreover, they showed that the fusion protein synthesized by their clone elicited antibodies which precipitated a 25 kDa membrane antigen with an isoelectric point between 4.2 and 4.6. These properties are remarkably similar to those described in Chapter 2 for the 24 kDa membrane antigen. Thus, it seems likely that the 25 kDa antigen characterized by Knight et al. (1989) is identical to the 24 kDa antigen described in Chapter 2.

The size of the λSK2 cDNA and its independent isolation by two different laboratories is interesting. I have been unable to isolate a longer cDNA homologous to the λSK2 cDNA by screening a S. mansoni λgt10 library. This may be related to the method which was used to construct this library. One

step in the construction of S. mansoni cDNA libraries (Chapter 3) which was omitted, was the Eco RI methylation of the cDNA prior to addition of Eco RI linkers. This precaution is usually carried out to minimize cleavage at internal Eco RI sites within the cDNA. Eco RI methylation was not performed on the S. mansoni cDNA preparation, since the cDNA used for cloning into λgt11 was to be released from an amplified cDNA library by Eco RI digestion. There are at least two possible explanations for the failure to isolate full length cDNAs encoding the 24 kDa antigen from these Eco RI libraries. First, it is possible that the full length cDNA encoding the 24 kDa antigen has at least 2 Eco RI sites. This suggestion is supported by the sequence of these sites, ie. both Eco RI sites are missing two nucleotides which should be present if they were linker derived sequences. Secondly, it is also possible that secondary structure in the 24 kDa mRNA precluded the synthesis of full length cDNAs. If the 24 kDa cDNA has internal Eco RI sites, a full length clone might be isolated from cDNA or genomic libraries which were not constructed with Eco RI linkers using the λSK2 cDNA as a probe, eg. the Xba I linker modified λZAP library described in this study. This work is in progress in our laboratory (S. Basalyga, MSc. thesis). Further functional analysis of the 24 kDa protein must await the determination of a near full length sequence.

The nucleotide and deduced amino acid sequence of the cDNA harboured in pSKZ-1 suggests that it encodes a schistosome calpain-like molecule. The

amino acid sequence from this cDNA shows similarities through out its length to calcium-activated neutral proteases from human, chicken and rabbit. In addition, two 28 amino acid stretches within the schistosome sequence contain the structural elements required for E-F hand structure, suggesting that the predicted schistosome protein also binds calcium.

The vertebrate calpain sequences have been divided into 4 functional domains based on the sequence of the first vertebrate calpain to be determined (chicken) (Ohno et al., 1984). Domain I encompasses the amino terminal 70 to 80 residues and may be involved in enzyme activation by calcium. Domain II spans the next approximately 235 amino acids and bears the catalytic activities of calpains. Domain IV is the C-terminal 150 amino acids which comprises the site for calcium binding. Domain III resides between domains II and IV and has no known function. The similarity between the schistosome sequence and vertebrate calpain sequences with respect to conservation of catalytic and calcium-binding sites, suggests that the schistosome sequence might also be divided into 4 domains.

Although a calpain-like molecule has not previously been reported in the schistosome literature, ongoing work in our laboratory (A. Siddiqui, pers. comm.) is emerging which indicates that calcium-dependent neutral protease activity copurifies with a 78 kDa protein. This is consistent with the size of the protein predicted from the sequence and the molecular weight of the translation

product programmed by a pSKZ-1 transcript. The sequence of the schistosome calpain-like molecule should provide a basis for an investigation of the function of this molecule in the schistosome. This is particularly interesting with respect to calcium-mediated signalling systems across the schistosome surface (Podesta et al., 1987).

The possible biological significance of the two polypeptides whose cDNAs have been cloned is discussed in Chapter 5.

Chapter 5- Summary and Future Prospects

The complex and dynamic interactions amongst schistosome membrane constituents play a major role in mediating many physiological functions essential for parasite survival. Individual membrane macromolecules function cooperatively in the transport of ions and nutrients and transduction of signals while simultaneously thwarting potentially lethal immune mechanisms. Although there is currently much emphasis on the characterization of membrane proteins as potential vaccine components, it seems certain that continued study of membrane events will present new and as yet unrealized opportunities for the discovery of therapeutic agents as well as information regarding basic mechanisms of wider biological relevance.

The focus of this thesis was on the characterization of proteins associated with the APM of adult S. mansoni at the molecular level. In this light, two polypeptides emerged as candidates for further study. One polypeptide, a 24 kDa antigen, has been biochemically characterized and a cDNA which encodes antigenic determinants shared with the 24 kDa antigen, has been cloned and sequenced. Another polypeptide, whose complete sequence has been deduced from a cloned cDNA, is a schistosome homologue of vertebrate calpain, a calcium-activated neutral protease. Both of these polypeptides appear to have properties, which have been demonstrated in this study or implied from the sequence, that suggest their involvement in signal transduction mechanisms

operating at the surface of S. mansoni. These two molecules will be discussed separately below.

Three separate research groups are currently focused on a 24-25 kDa antigen to determine the potential of this molecule as a molecular vaccine. It would appear that on the basis of the similar biochemical and antigenic properties described independently by all three groups, that the molecules is the same in all cases (Kelly et al., 1987; Karcz et al., 1988; Wright et al., 1988; Knight et al., 1989; Smithers et al., 1989; Huebert, 1990). Moreover, this molecule has been implicated in resistance to schistosome infection by all three research groups. Wright et al. (1988) and Smithers et al. (1989) have shown that resistance to schistosome infection in mice is correlated with a strong IgG antibody response to the 24 kDa antigen. In addition, Huebert (1990) has demonstrated that protective immunity to challenge infection is correlated with an enhanced IgG2a response to the 24 kDa antigen in mice immunized with the isolated APM of adult S. mansoni. Thus the vaccine candidacy of the 24 kDa antigen is presently being evaluated. Coupled with the demonstration that the 24 kDa antigen is phosphorylated (Chapter 2), it is tempting to speculate that the direction of a potent immune response against a phosphoprotein antigen (a molecule with a potential regulatory role) is a viable strategy for vaccination against this parasite. The use of the 24 kDa antigen in vaccine trials however, must await the determination of the full primary sequence of the molecule and

its expression in a recombinant vector system since it is highly unlikely that sufficient protein can be purified from schistosomes. In this sense, information gained from this thesis will be useful in establishing whether the recombinant product is correctly glycosylated, since this may substantially affect its antigenicity.

The cloning of the schistosome calpain homologue is also significant, since it represents the first invertebrate calpain-like molecule to have been cloned. The levels of sequence homology between the schistosome sequence and either the chicken or human sequence are approximately the same as those reported for other host-like schistosome polypeptides. For example, levels of approximately 40% identity have been demonstrated for schistosome glutathione S-transferase (Smith et al., 1986) superoxide dismutase (Simurda et al., 1989) and tropomyosin (Xu et al., 1989). In the case of glutathione S-transferase, the enzyme has been subsequently purified and shown to possess authentic enzyme activity. Therefore, it is not unreasonable to expect that the schistosome calpain homologue will also possess calcium-activated neutral protease activity. As mentioned previously, calcium-dependent protease activity in S. mansoni has been shown to co-purify with a 78 kDa polypeptide (A. Siddiqui, pers. comm.), a size which is consistent with that predicted from the cDNA sequence.

Calpains in vertebrates appear to have a limited substrate specificity. Most of the substrates which have been characterized to date include enzymes

such as protein kinases, cytoskeletal and structural proteins, and membrane proteins including receptors (reviewed in Wang et al., 1989). In addition, much attention has been focused on calpains for their potential role in cellular processes involving calcium as a second messenger, since their protease activity is absolutely dependent on calcium (Suzuki et al., 1987). Calpains also appear to undergo activation at the cell membrane (Suzuki et al., 1987) which may facilitate their interaction with membrane associated substrates.

The biological function of calpains in cells of vertebrates remains obscure although there is good evidence to indicate an important role for calpain in the cytoskeletal control of exocytosis in both platelets (Verhallen et al., 1987) and neutrophils (Pontremoli et al., 1986). Accordingly, calpain activity is intimately associated with exocytosis of platelet prothrombinase activity and neutrophil granule enzymes. Inherent to both of these events is the fusion of vesicles with the surface of these cells. If the schistosome calpain homologue has a similar function, it is possible that fusion of membrane precursors (such as the MLB or DB) with the parasite surface may also involve calpain activity. This hypothesis is particularly attractive in light of the known effects of C_3 on membrane synthesis and the involvement of calcium in this phenomenon (Young, 1984; Podesta et al., 1987). Therefore, further investigation of calpain activity and its role in calcium-mediated signalling mechanisms in schistosomes may shed light on a major parasite immune evasion strategy; that of rapid membrane

biogenesis and renewal (discussed in Chapter 1).

Thus the approach outlined in thesis has been successful in the sense that two polypeptides which were previously uncharacterized before this study was initiated, have been described. The further study of these molecules is clearly warranted from both an immunological and functional perspective.

REFERENCES

Aoki, K., Imajoh, S., Ohno, S., Emori, Y., Koike, M., Kosaki, G. and Suzuki, K. (1986). Complete amino acid sequence of the large subunit of the low-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease (muCANP) deduced from its cDNA sequence. FEBS Letters 205:313-317.

Balloul, J.M., Sondermeyer, P., Dreyer, D., Capron, M., Grzych, J.M., Pierce, R.J., Carvallo, D., Lecocq, J.P. and Capron, A. (1987). Molecular cloning of a protective antigen of schistosomes. Nature 326:149-153.

Bazzi, M.D. and Nelsestuen, G.L. (1988). Constitutive activity of membrane-inserted protein kinase C. Biochemical and Biophysical Reasearch Communications 152:336-343.

Beall, J.A. and Mitchell, G.F. (1986). Identification of a particular antigen from a parasite cDNA library using antibodies affinity purified from selected portions of Western blots. Journal of Immunological Methods 86:217-223.

Beliveau, R., Fortier, G. and Vachon, V. (1988). Effect of magnesium on ATP labelling by kidney brush border membrane. International Journal of Biochemistry 20:375-380.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7:1513.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. Journal of Biological Chemistry 256:1604-1607.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Bricker, C.S., Depenbusch, J.W., Bennett, J.L. and Thompson. D.P. (1983). The relationship between tegumental disruption and muscle contraction in Schistosoma mansoni exposed to various compounds. Parasitology Research 69:61-71.

Butterworth, A.E. (1988). Control of Schistosomiasis in Man. In "The Biology of Parasitism" Ed. Eglund, P.T. and Sher, A., Alan Liss, New York. pp 43-59.

Butterworth, A.E., Capron, M., Cordingley, J.S., Dalton, P.R., Dunne, D.W., Kariuki, H.C., Kimani, G., Koech, D, Mugambi, M., Ouma, J.H., Prentice, M.A., Richardson, B.A., arap Siongok, T.K., Sturrock, R.F. and Taylor, D.W. (1985). Immunity after treatment of human schistosomiasis mansoni. II. Identification of resistant individuals, and analysis of their immune responses. Transactions of the Royal Society of Tropical Medicine and Hygiene 79:393-408.

Butterworth, A.E., Sturrock, R.F., Houba, V., Mahmoud, A.A.F., Sher, A., and Rees, P.H. (1975). Eosinophils as mediators of antibody-dependent damage to schistosomula. Nature 256:727-729.

Butterworth, A.E., Taylor, D.W., Veith, M.C., Vadas, M.A., Dessein, A., Sturrock, R.F. and Wells, E. (1982). Studies on the mechanisms of immunity in

human schistosomiasis. Immunological Reviews 61:5-39.

Caro, J.F., Shafer, J.A., Taylor, S.I., Raju, S.M., Perrotti, N. and Sinha, M.K. (1987). Insulin stimulated protein phosphorylation in Human Liver plasma membranes: Detection of endogenous or plasma membrane associated substrates for insulin receptor kinase. Biochemical and Biophysical Research Communications 149:1008-1016.

Clegg, J.A., Smithers, S,R., Terry, R.J. (1971). Acquisition of human antigens by Schistosoma mansoni during cultivation in vitro. Nature 232:653-654.

Cohen, P. (1988). Protein phosphorylation and hormone action. Proceedings of the Royal Society of London B234:115-144.

Coles, G.C., Bruce, J.I., Kinoti, G.K., Mutahi, W.T., Dias, E,P, and Katz, N. (1986). Drug resistance in schistosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 80:347.

Colley, D.G. and Colley, M.D. (1989). Protective immunity and vaccines to schistosomiasis. Parasitology Today 5:350-354.

Colley, D.G., Garcia, A.A., Lambertucci, J.R., Parra, J.C., Katz, N., Rocha, R.S. and Gazzinelli, G. (1986). Immune responses during human schistosomiasis. XII. Differential responsiveness in patients with hepatosplenic disease. American Journal of Tropical Medicine and Hygiene 35:793-802.

Cordingley, J.S., Taylor, D.W., Dunne, D.W. and Butterworth, A.E. (1983). Clone banks of cDNA from the parasite Schistosoma mansoni: isolation of clones

containing a potentially immunodiagnostic antigen gene. Gene 26:25-39.

Cousin, C.E., Stirewalt, M.A., and Dorsey, C.H. (1981). Schistosoma mansoni: Ultrastructure of early transformation of skin- and shear-pressure derived schistosomules. Experimental Parasitology 51:341-365.

Dalton, J.P., Tom, T.D. and Strand, M. (1987). Cloning of a cDNA encoding a surface antigen of Schistosoma mansoni schistosomula recognized by sera of vaccinated mice. Proceedings of the National Academy of Science 84:4268-4272.

Damian, R.T. (1964). Molecular mimicry: antigen sharing by parasite and host and its consequences. American Naturalist 98:129-149.

Damian, R.T., Greene, N.D. and Hubbard, W.T. (1973). Occurrence of α2-macroglobulin antigenic determinants on Schistosoma mansoni, with evidence on their nature. Journal of Parasitology 59:64-73.

Davis, A.H., Nanduri, J. and Watson, D.C. (1987). Cloning and gene expression of Schistosoma mansoni. Journal of Biological Chemistry 262:1-5.

Dean, D.A. (1983). Schistosoma and related genera: acquired resistance in mice. Experimental Parasitology 55:1-104.

Dean, D.A. and Mangold, B.L. (1984). Autoradiographic analysis of resistance to reinfection with Schistosoma mansoni in mice. Evidence that the liver is a major site of worm elimination. American Journal of Tropical Medicine and Hygiene 33:97-103.

Dean, D.A., Bukowski, M.A. and Cheever, A.W. (1981). Relationship between acquired resistance, portal hypertension, and lung granulomas in ten strains of mice infected with Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene 30:806-814.

Dean, D.A., Mangold, B.L., Georgi, J.R. and Jacobson, R.H. (1984). Comparison of Schistosoma mansoni migration patterns in normal and irradiated cercaria-immunized mice by means of autoradiographic analysis. Evidence that worm elimination occurs after the skin phase in immunized mice. American Journal of Tropical Medicine and Hygiene 33:89-96.

Dean, L.L. and Podesta, R.B. (1984). Electrophoretic patterns of protein synthesis and turnover in apical plasma membrane and outer bilayer of Schistosoma mansoni. Biochimica et Biophysica Acta 799:106-114.

Dessein, A., Samuelson, J.C., Butterworth, A.E., Hogan, M., and others (1981). Immune evasion by Schistosoma mansoni: loss of susceptibility to antibody or complement-dependent eosinophil attack by schistosomula cultured in medium free of macromolecules. Parasitology 82:357-74.

Dissous, C. and Capron, A. (1989). Schistosoma mansoni and its intermediate host Biomphalaria glabrata express a common 39 kilodalton acidic protein. Molecular and Biochemical Parasitology 32:49-56.

Doehring, E., Reider, F., Schmidt-Ehry, G. and Erich, J.H.H. (1985). Reduction of pathological findings in urine and bladder lesions in infection with

Schistosoma haematobium after treatment with praziquantel. Journal of Infectious Diseases 152:807-810.

Doughty, B.L., Ottesen, E.A., Nash, T.E. and Phillips, S.M. (1984). Delayed hypersensitivity granuloma formation around Schisotsoma mansoni eggs in vitro. III. Granuloma formation and modulation in human Schistosomiasis mansoni. Journal of Immunology 133:993-997.

Edwards, M.E. (1986). Protein Phosphorylation in Schistosoma mansoni. MSc. Thesis, The University of Western Ontario, London, Ontario.

Estey, S.J. and Mansour, T.E. (1987). Nature of serotonin-activated adenylate cyclase during development of Schistosoma mansoni. Molecular and Biochemical Parasitology 26:47-60.

Flanigan, T.P., King, C.H., Lett, R.R., Nanduri, J. and Mahmoud ,A.A. (1989). Induction of resistance to Schistosoma manosoni infection in mice by purified parasite paramyosin. Journal of Clinical Investigation 83:1010-1014.

Fozard, J.R. (1987). 5-HT: The Enigma Variations. Trends in Pharmacological Science 8:501-506.

Gillet, J.D. (1985). The behaviour of Homo sapiens, the forgotten factor in the transmission of tropical disease. Transactions of the Royal Society of Tropical Medicine and Hygiene 79:12-20.

Gitter, B.D., McCormick, S.L. and Damian, R.T. (1982). Murine alloantigen acquisition by Schistosoma mansoni: presence of H-2k determinants on adult

worms and failure of allogenic lymphocytes to recognize acquired MHC gene products on schistosomula. Journal of Parasitology 68:513-518.

Goodman, M., Pechere, J.F., Haiech, J. and Demaille, G. (1979). Evolutionary diversification of structure and function in the family of intracellular calcium-binding proteins. Journal of Molecular Evolution 13:331-352.

Grzych, J.M., Dissous, C., Capron, M., Torres, S., and others (1987). Schistosoma mansoni shares a protective carbohydrate epitope with keyhole limpet hemocyanin. Journal of Experimental Medicine 165:865-878 .

Gubler, U. and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.

Guigemde, T.R. (1989). Education pour la sante, eau et assainissement dans la lutte contre les schistosomiases. Tropical Medicine and Parasitology 40:223-225.

Gutteridge, W.E. (1989). Parasite vaccines versus anti-parasite drugs: rivals or running mates? Parasitology 98:S87-S97.

Hagan, P., Blumenthal, U.J., Chaudri, M., Greenwood, B.M., Hayes, R.J., Hodgson, I., Kelly, C., Knight, M., Simpson, A.J., Smithers, S.R. and Wilkins, H.A. (1987). Resistance to reinfection with Schistosoma haematobium in Gambian children: analysis of their immune responses. Transactions of the Royal Society of Tropical Medicine and Hygiene 81:938-946.

Havercroft, J.C., Huggins, M.C., Nene, V., Dunne, D.W., Richardson, B.A., Taylor, D.W. and Butterworth, A.E. (1988). Cloning of the gene encoding a 50 kilodalton potential surface antigen of Schistosoma mansoni. Molecular and Biochemical Parasitology 30:83-88.

Hazdai, R.T., Levi-Schaffer, F., Brenner, V., Horowitz, S., and others (1985). Protective monoclonal antibody against Schistosoma mansoni: antigen isolation, characterization, and suitability for active immunization. Journal of Immunology 135:2772-2779.

Hedstrom, R., Culpepper, J., Schinski, V., Agabian, N. and Newport, G. (1988). Schistosome heat-shock proteins are immunologically distinct host-like antigens. Molecular and Biochemical Parasitology 29:275-282.

Hirata, Y. and Suzuki, T. (1987). Protein kinase activity associated with the Fcγ2a receptor of a murine macrophage like cell line, P388D1. Biochemistry 26:8189-8195.

Hockley, D.J. (1973). Ultrastructure of the tegument of Schistosoma. Advances in Parasitology 11:233-305.

Hockley, D.J. and McLaren, D.J. (1973). Schistosoma: changes in the outer membrane of the tegument during development from cercariae to adult worm. International Journal of Parasitology 3:13-25.

Huebert, T. (1990). Antibody Responses and Membrane Antigen Recognition in Mice Infected With Schistosoma mansoni. MSc. Thesis, The University of

Western Ontario, London, Canada.

Huganir R.L. and Greengard, P. (1983). cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. Proceedings of the National Academy of Science 80:1130-1134.

Huganir R.L. and Greengard, P. (1987). Regulation of receptor function by protein phosphorylation. Trends in Pharmalogical Science 8:472-477.

Incani, R.N. and McLaren, D.J. (1981). Neutrophil-mediated cytotoxicity to schistosomula of Schistosoma mansoni in vitro: studies on the kinetics of complement and/or antibody-dependent adherence and killing. Parasite Immunology 3:107-126.

James, S.L. (1985). Induction of protective immunity against Schisotsoma mansoni by a nonliving vaccine is dependent on the method of antigen presentation. Journal of Immunology 134:1956-1960.

James, S.L. (1987). Induction of protective immunity against Schistosoma mansoni by a non-living vaccine. V. Effects of varying the immunization and infection schedule and site. Parasite Immunology 9:531-541.

James, S.L., Natovitz, P.C., Farrar, W.L. and Leonard, E.J. (1984). Macrophages as effector cells of protective immunity in murine schistosomiasis: macrophage activation in mice vaccinated with radiation-attenuated cercariae. Infection and Immunity 44:569-575.

Kalopothakis, E., Rumjanek, F. and Evans W.H. (1987). Protein and antigen phosphorylation in the tegument of Schistosoma mansoni. Molecular and Biochemical Parasitology 26:39-46.

Karcz, S.R., Barnard, B.J. and Podesta, R.B. (1988). Biochemical properties of a 24 kilodalton membrane glycoprotein antigen complex from Schistosoma mansoni. Molecular and Biochemical Parasitology 31:163-172.

Kasschau, M.R. and Mansour, T.E. (1982). Serotonin-activated adenylate cyclase during early development of Schistosoma mansoni. Nature 296:66-68.

Kawamoto, F., Shozawa, A., Kumada, N. and Kojima, K. (1989). Possible roles of cAMP and Ca^{2+} in the regulation of miracidial transformation in Schistosoma mansoni. Parasitology Research 75:368-374.

Kayes, S.G. and Colley, D.G. (1979). Immunoregulation in experimental schistosomiasis: in vitro induction and assay of spleen cell suppressor activity. Journal of Immunology 122:2340-2344.

Kelly, C., Knight, M., Simpson, A., Hackett, F., Geisow, M. and Smithers, S.R. (1987). Purification and amino acid sequencing of Schistosoma mansoni surface antigens. UCLA Symposium on Molecular and Cellular Biology 60:257-266.

Kelly, E.A. and Colley, D.G. (1988). In vivo effects of monoclonal anti-L3T4 antibody on immune responsiveness of mice infected with Schistosoma mansoni. Reduction of irradiated cercariae-induced resistance. Journal of

Immunology 140:2737-2745.

Kemp, D.J., Coppel, R.I., Cowman, R.F., Saint, R.B., Brown, G.V. and Anders, R.F (1983). Expression of Plasmodium falciparum blood stage antigens in Escherichia coli: Detection with antibodies from immune humans. Proceedings of the National Academy of Science 80:3787-3791.

Kemp, W.M., Brown, P.R., Merritt, S.C. and Miller, R.E. (1980). Tegument-associated antigen modulation by adult male Schistosoma mansoni. Journal of Immunology 124:806-811.

Kemp, W.M., Damian, R.T. and Greene, N.D. (1976). Immunocytochemical localization of IgG on adult Schistosoma mansoni tegumental surfaces. Journal of Parasitology 62:830-832.

Kenney, J.S., Hughes, B.W., Masada, M.P. and Allison, A.C. (1989). Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. Journal of Immunological Methods 121:157-166.

Khandjian, E.W. (1987). Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. Biotechnology 5:165-167.

King, C.H., Lett, R.R., Nanduri, J., el Ibiary, S., and others (1987). Isolation and characterization of a protective antigen for adjuvant-free immunization against Schistosoma mansoni. Journal of Immunology 139:4218-4224.

Klinkert, M.Q., Felleisen, R., Link, G., Ruppel, A. and Beck, E. (1989). Primary structures of Sm31/32 diagnostic proteins of Schistosoma mansoni and their

identification as proteases. Molecular and Biochemical Parasitology 33:113-122.

Klinkert, M.Q., Ruppel, A., Felleisen, R., Link, G. and Beck, E. (1988). Expression of diagnostic 31/32 kilodalton proteins of Schistosoma mansoni as fusions with bacteriophage MS2 polymerase. Molecular and Biochemical Parasitology 27:233-239.

Knight, M., Kelly, C., Rodrigues, V., Yi, X., and others (1989). A cDNA clone encoding part of the major 25,000-dalton surface membrane antigen of adult Schistosoma mansoni. Parasitology Research 75:280-286.

Koster, B., Dargatz, H., Scroder, J., Hirzmann, J., Haarmann, C., Symmons, P. and Kunz, W. (1988). Identification and localisation of the products of a putative eggshell precursor gene in the vitellarium of Schistosoma mansoni. Molecular and Biochemical Parasitology 31:183-198.

Kresina, T.F. and Olds, G.R. (1989). Antiidiotypic antibody vaccine in murine Schistosomiasis mansoni comprising the internal image of antigen. Journal of Clinical Investigation 83:912-920.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lanar, D.E., Pearce, E.J. and Sher, A. (1985). Expression in Escherichia coli of two Schistosoma mansoni genes that encode major antigens recognized by immune mice. Molecular and Biochemical Parasitology 17:45-60.

Lanar, D.E., Pearce, E.J., James, S.L. and Sher, A. (1986). Identification of paramyosin as the schistosome antigen recognized by intradermally vaccinated mice. Science 234:593-596.

Mangold, B.L. and Dean, D.A. (1986). Passive transfer with serum and IgG antibodies of irradiated cercaria-induced resistance against Schistosoma mansoni in mice. Journal of Immunology 136:2644-2648.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). In: Molecuar Cloning. A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

Mansour, T.E. (1984). Serotonin receptors in parasitic worms. Advances in Parasitology 23:1-36.

Marshall, I. (1987). Experimental Chemotherapy. In "The Biology of Schistosomes from Genes to Latrines". Ed. Simpson, A.J.G. and Rollinson, D. Academic Press, New York. pp.399-430.

McCullough, F. (1986). Snail control in relation to a strategy for reduction of morbidity due to schistosomiasis. Tropical Medicine and Parasitology 37:181-184.

McDiarmid, S.S., Dean, L.L. and Podesta, R.B. (1983). Sequential removal of outer bilayer and apical plasma membrane from the surface epithelial syncytium of Schistosoma mansoni. Molecular and Biochemical Parasitology 7:141-157.

McGuinness, T.B. and Kemp, W.M. (1981). Schistosoma mansoni: a

complement-dependent receptor on adult male parasites. Experimental Parasitology 51:236-242.

McHugh, S.M., Coulson, P.S. and Wilson, R.A. (1987). The relationship between pathology and resistance to reinfection with Schistosoma mansoni in mice: a causal mechanism of resistance in chronic infections. Parasitology 94:81-91.

McLaren, D.J. (1980). Schistosoma mansoni: the parasite surface in relation to host immunity. Research Studies Press, Toronto.. .

McLaren, D.J. (1984). Disguise as an evasive strategem of parasitic organisms. Parasitology 88:597-611.

McLaren, D.J. (1989). Will the real target of immunity to schistosomiasis please stand up. Parasitology Today 5:279-282.

McLaren, D.J. and James, S.L. (1985). Ultrastructural studies of the killing of schistosomula of Schistosoma mansoni by activated macrophages in vitro. Parasite Immunology 7:315-331.

McLaren, D.J. and Smithers, S.R. (1987). The Immune Response to Schistosomes in Experimental Hosts. In "The Biology of Schistosomes from Genes to Latrines". Ed. Simpson, A.J.G. and Rollinson, D. Academic Press, New York. pp.234-263.

Mendlovic, F., Tarrab-Hazdai, R., Puri, J. and Arnon, R. (1989). Genetic control of immune response to a purified Schistosoma mansoni antigen. I. Effect of

MHC class II antigens on the cellular, humoral and protective responses. Parasite Immunology 11:667-682.

Mitchell, G.F. (1989). Problems specific to parasite vaccines. Parasitology 98:S19-S28.

Moore, J.J., Baker, J.V. and Whitsett, J.A. (1983). cAMP-dependent protein kinase and cAMP, cGMP and calcium stimulated phosphorylation in human placenta: comparison of cytosol and membrane fractions. Trophoblast Research 1:185-196.

Mott, K.E. (1987). Epidemiological considerations for development of a schistosome vaccine. Acta Tropica 12:13-20.

Newport, G.R., Harrison, R.A., McKerrow, J., Tarr, P., Kallestad, J. and Agabian, N. (1987). Molecular cloning of Schistosoma mansoni myosin. Molecular and Biochemical Parasitology 26:29-38.

Newport, G.R., McKerrow, J.H., Hedstrom, R., Petitt, M., McGarigle, L., Barr, P.J. and Agabian, N. (1988). Cloning of the proteinase that facilitates infection by schistosome parasites. Journal of Biological Chemistry 263:13179-13184.

Noel, F. and Pardon, R.S. (1989). Vanadate sensitivity of Na+, K+-ATPase from Schistosoma mansoni and its modulation by Na+, K+ and Mg2+. Life Science 44:1677-1683.

Nyame, K., Smith, D.F., Damian, R.T. and Cummings, R.D. (1989). Complex-type asparagine-linked oligosaccharides in glycoproteins synthesized by

Schistosoma mansoni adult males contain terminal beta-linked N-acetylgalactosamine. Journal of Biological Chemistry 264:3235-3243.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. Journal of Biological Chemistry 250:4007-4021.

Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984). Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium binding protein? Nature 312:566-570.

Olmsted, J.B. (1981). Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. Journal of Biological Chemistry 256:11955-11957.

Omer-Ali, P., Smithers, S.R., Bickle, Q., Phillips, S.M., Harn, D. and Simpson, A.J.G. (1988). Analysis of the anti-Schistosoma mansoni surface antibody response during murine infection and its potential contribution to protective immunity. Journal of Immunology 140:3273-3279.

Pang, D.T., Wang, J.K.T., Valtorto, F., Benfenati, F. and Greengard, P. (1988). Protein tyrosine phosphorylation in synaptic vesicles. Proceedings of the National Academy of Science 85:762-766.

Pearce, E.J., Basch, P.F. and Sher, A. (1986). Evidence that the reduced surface antigenicity of developing Schistosoma mansoni schistosomula is due to antigen shedding rather than host molecule acquisition. Parasite Immunology 8:79-94.

Pearce, E.J., James, S.L., Heiny, S., Lanar, D.E. and Sher, A. (1988). Induction of protective immunity against Schistosoma mansoni by vaccination with schistosome paramyosin (Sm97), a nonsurface parasite antigen. Proceedings of the National Academy of Science 85:5678-5682.

Podesta, R.B. (1982). Adaptive features of the surface epithelial syncytium favoring survival in an immunologically hostile environment. In "Parasites- Their World and Ours". Ed. Mettrick, D.F. and Desser, S.S., Elsevier Biomedical Press, Amsterdam. pp. 149-155.

Podesta, R.B., Karcz, S., Ansell, M. and Silva, E. (1987). Schistosoma mansoni: Apical membrane/envelope synthesis, signal transduction and protein phosphorylation. UCLA Symposia on Molecular and Cellular Biology 60:241-255.

Pointier, J.P. and McCullough, F. (1989). Biological control of the snail hosts of Schistosoma mansoni in the Carribbean area using Thiara spp. Acta Tropica 46:147-155.

Pontremoli, S., Melloni, E., Michetti, M., Sacco, O., Salamino, F., Sparatore, B. and Horecker, B.L. (1986). Biochemical responses in activated human neutrophils mediated by protein kinase C and a Ca^{2+}-requiring proteinase. Journal of Biological Chemistry 261:8309-8313.

Pryde, J.G. (1986). Triton X-114: a detergent that has come in from the cold. Trends in Biochemical Science 11:160-165.

Ram, D., Grossman, Z., Markovics, A., Avivi, A., Ziv, E., Lantner, F. and Schechter, I. (1989). Rapid changes in the expression of a gene encoding a calcium-binding protein in Schistosoma mansoni. Molecular and Biochemical Parasitology 34:167-175.

Reis, M.G., Kuhns, J., Blanton, R. and Davis, A.H. (1989). Localization and pattern of expression of a female specific mRNA in Schistosoma mansoni. Molecular and Biochemical Parasitology 32:113-119.

Rumjanek, F.D., Campos, E.G. and Afonso, L.C.C. (1988). Evidence for the occurrence of LDL receptors in extracts of schistosomula of Schistosoma mansoni. Molecular and Biochemical Parasitology 28:145-152.

Ruppel, A. and McLaren, D.J. (1986). Schistosoma mansoni: surface membrane stability in vitro and in vivo. Experimental Parasitology 62:223-236.

Samuelson, J.C. and Caulfield, J.P. (1982). Loss of covalently labeled glycoproteins and glycolipids from the surface of newly transformed schistosomula of Schistosoma mansoni. Journal of Cell Biology 94:363-369.

Samuelson, J.C., Sher, A. and Caulfield, J.P. (1980). Newly transformed schistosomula spontaneously lose surface antigens and C3 acceptor sites during culture. Journal of Immunology 124:2055-2057.

Sanger, F., Micklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science 74:5463-5467.

Shenolikar, S. (1987). Control of cell function by reversible protein phosphorylation. Journal of Cyclic Nucleotide and Protein Phosphorylation Research 11:531-541.

Sher, A. (1988). Vaccination Against Parasites: Special Problems Imposed by the Adaptation of Parasitic Organisms to the Host Immune Response. In "The Biology of Parasitism". Ed. Englund, P.T. and Sher, A. Alan R. Liss, New York. pp 169-182..

Sher, A., Correa-Oliveira, R., Hieny, S. and Hussain, R. (1983). Mechanisms of protective immunity against Schistosoma mansoni infection in mice vaccinated with irradiated cercariae. IV. Analysis of the role of IgE antibodies and mast cells. Journal of Immunology 131:1460-1465.

Sher, A., Hall, B.F. and Vadas, M.A. (1978). Acquisition of muurine major histocompatibility complex gene products by Schistosoma mansoni. Journal of Experimental Medicine 148:46-50.

Sher, A., Hieny, S., James, S.L. and Asofsky, R. (1982). Mechanisms of protective immunity against Schistosoma mansoni infection in mice vaccinated with irradiated cercariae. II. Analysis of immunity in hosts deficient in T lymphocytes, B lymphocytes, or complement. Journal of Immunology 128:1880-1884.

Simpson, A.J., Singer, D., McCutchan, T.F., Sacks, D.L. and Sher, A. (1983). Evidence that schistosome MHC antigens are not synthesized by the parasite

but are acquired from the host as intact glycoproteins. Journal of Immunology 131:962-965.

Simpson, A.J.G. and Smithers, S.R. (1985). Schistosomes: Surface, Egg and Circulating Antigens. Current Topics in Microbiology and Immunology 120:205-239.

Simurda, M.C., van Keulen, H., Rekosh, D.M. and LoVerde, P.T. (1988). Schistosoma mansoni: Identification and analysis of an mTNA and a gene encoding superoxide dismutase (Cu/Zn). Experimental Parasitology 67:73-84.

Smith, D.B., Davern, K.M., Board, P.G., Tiu, W.U., Garcia, E.G. and Mitchell, G.F. (1986). Mr 26,000 antigen of Schistosoma japonicum recognized by resistant WEHI 129/J mice is a parasite glutathione S-transferase. Proceedings of the National Academy of Science 83:8703-8707.

Smith, D.B., Rubira, M.R., Simpson, R.J., Davern, K.M., Tiu, W.U., Board, P.G. and Mitchell, G.F. (1988). Expression of an enzymatically active parasite molecule in Escherichia coli: Schistosoma japonicum glutathione S-transferase. Molecular and Biochemical Parasitology 27:249-256.

Smith, M.A. and Clegg, J.A. (1985). Vaccination against Schistosoma mansoni with purified surface antigens. Science 227:535-538.

Smithers, S.R. and Gammage, K. (1980). Recovery of Schistosoma mansoni from the skin, lungs and hepatic portal system of naive mice and mice previously

exposed to S. mansoni: evidence for two phases of parasite attrition in immune mice. Parasitology 80:289-300.

Smithers, S.R. and Terry, R.J. (1969). Immunity in schistosomiasis. Annals of the New York Academy of Sciences 160:826-840.

Smithers, S.R., Hackett, F., Omer-Ali, P. and Simpson, A.J. (1989). Protective immunization of mice against Schistosoma mansoni with purified adult worm surface membranes. Parasite Immunology 11:301-318.

Smithers, S.R., Terry, R.J. and Hockley, D.J. (1969). Host antigens in schistosomiasis. Proceedings of the Royal Society Series B 171:483-494.

Sogandares-Bernal, F. (1976). Immunoglobulins attached to and in the tegument of adult Schistosoma mansoni, Sambon 1907, from first infection of CF1 mice. Journal of Parasitology 62:222-226.

Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y. and Ohno, S. (1987). Calcium-activated neutral protease and its endogenous inhibitor: Activation at the cell membrane and biological function. FEBS Letters 220:271-277.

Tarleton, R.L. and Kemp, W.M. (1981). Demonstration of IgG-Fc and C3 receptors on adult Schistosoma mansoni. Journal of Immunology 126:379-384.

Tavares, C.A., Cordeiro, M.N., Mota-Santos, T.A. and Gazzinelli, G. (1980). Artificially transformed schistosomula of Schistosoma mansoni: mechanism of acquisition of protection against antibody-mediated killing. Parasitology 80:95-104.

Taylor, J.B., Vidal, A., Torpier, G., Meyer, D.J., Roitsch, C., Balloul, J.M., Southan, C., Sondermeyer, P., Pemble, S., Lecocq, J.P., Capron, A. and Ketterer, B. (1988). The glutathione transferase activity and tissue distribution of a cloned Mr 28K protective antigen of Schistosoma mansoni. EMBO Journal 7:465-472.

Teesdale, C.H. (1986). The role of health education to reduce transmission of schistosomiasis. Tropical Medicine and Parasitology 37:184-185.

Thiel, G. and Soling, H-D. (1988). cAMP-dependent protein phosphorylation of membrane proteins in the parotid gland, platelets and liver. European Journal of Biochemistry 124:604-609.

Timmins J.G., Petrovskis E.A., Marchioli C.C., Post L.E. (1985). A method for efficient gene isolation from phage lambda gt11 libraries: use of antisera to denatured, acetone-precipitated proteins. Gene 39:89-93.

Torpier, G.A., Capron, A., and Ouassi, M.A. (1979). Receptor for IgG (Fc) and human beta 2-microglobulin on S. mansoni schistosomula . Nature 278:447-449.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Science 76:4350-4354.