The characterisation of *Theileria annulata* sporozoite surface antigens

Pamela Anne Knight

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Wellcome Unit of Molecular Parasitology
Department of Veterinary Parasitology
Glasgow University
Bearsden Road
Glasgow

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

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Abbreviations

BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
cpm	counts per minute
CTVM	Centre for Tropical Veterinary Medicine
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbant assay
FACS	fluorescence activated cell sorter
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GST	glutathione-S-transferase
GUTS	ground up tick supernatant
GUTSGS	ground up tick salivary gland supernatant
hr-IL2	human recombinant interleukin 2
IFA	immuno-fluorescence assay
ILRAD	International Laboratory for Research on Animal Diseases
IPTG	isopropylthio-β-D-galactoside
kb	kilobases
kDa	kilodaltons
McAb	monoclonal antibody
MDP	muramyl dipeptide
MEM	minimal essential medium
MLC	mixed lymphocyte culture medium
NBT	nitro blue tetrazolium
OD	optical density
p67	*T. parva* 67kDa sporozoite antigen
PBM	peripheral blood mononuclear cells
PBM^R	irradiated peripheral blood mononuclear cells
PBS	phosphate buffered saline
RPMI	Roswell Park Memorial Institute medium
SDS	sodium dodecyl sulphate
SPAG1	sporozoite antigen 1 (of *T. annulata*)
SSC	sodium citrate
te	tick equivalent
TE	Tris-EDTA
TLCK	tosyllysine chloromethyl ketone
NK	natural killer (cells)
BoLA	bovine leucocyte antigen
IAA	indol acetic acid

Abstract

Theileria annulata is a major protozoan parasite of cattle in tropical and subtropical countries, transmitted by the tick *Hyalomma anatolicum*. The infection in the host is initiated by the sporozoite, which rapidly attaches to and invades host cells. A humoral response to sporozoites has been identified in immune cattle (Preston and Brown 1985). Two McAbs which block sporozoite invasion of lymphocytes "in vitro", McAb 1A7 and 4B11, have been identified in previous work (Williamson 1988). These McAbs recognise different sets of polypeptides on Western blots. *T. annulata* sporozoite antigen 1 (SPAG1), which contains the epitope recognised by McAb 1A7, has been cloned and sequenced (Hall et al 1992).

This work describes research to analyse the bovine T and B cell response to the *T. annulata* SPAG1. The SPAG1 polypeptide, and a range of subclones covering different regions of the antigen, were expressed using pGEX vectors in *E. coli* and purified in large quantities for the purpose of B and T cell epitope mapping. The recombinant SPAG1 polypeptides were used to analyse humoral responses of immunised cattle by Western blotting and enzyme linked immuno-sorbant assay. A number of regions of SPAG1 containing B cell epitopes recognised by bovine antisera raised against *T. annulata* sporozoites were also identified, and the relevance of this data to SPAG1 neutralising determinants discussed. T cell responses to SPAG1 were analysed in proliferation assays using peripheral blood mononuclear cells and antigen specific T cell lines. A bovine T cell response was found to be induced to SPAG1 in cattle immunised with the recombinant antigen. In addition, the SPAG1 molecule was found to be highly polymorphic according to restriction fragment analysis on Southern blots. Work was also undertaken to prove the surface location of the SPAG1 molecule on sporozoites using immuno-electron microscopy techniques.

The final part of this work was to identify, express and characterise the *T. annulata* sporozoite antigen gene containing the McAb 4B11 epitope. McAb 4B11 was used to screen a λgt11 *T. annulata* genomic expression library, and two positive recombinants were subcloned into the expression vector pGEX1λT to give pGEX1λT-KP6 and pGEX1λT-KP8.

Southern and Northern blot analysis of the KP6 and KP8 inserts confirmed they were parasite sequences expressed at high levels in sporozoites as a 3.1kb mRNA. However, KP6 and KP8 did not cross-hybridise, indicating that they contained different sequences coding for the 4B11 epitope. The pGEX1λT-KP8 fusion protein was expressed in *E. coli* and purified. The fusion protein was found to affinity bind antibodies from bovine antisera raised against *T. annulata* sporozoites, and evoked antibodies capable of blocking sporozoite infectivity of leukocytes "in vitro" and recognising sporozoite antigens in immunofluorescence assays or on Western blots when used to immunise a rabbit.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The Parasite

The protozoan parasite *Theileria annulata*, first described by Dschunkowsky and Luhs in 1904, is the causative agent of the disease tropical or Medditerranean theileriosis, which threatens about 250 million cattle worldwide. The constraints on livestock production and improvement imposed by the disease mean this parasite is of major economic importance. Much of the pathology of the disease is a result of the parasite's ability to infect cells of the immune system; a property also shared by the protozoan species *Trypanosoma cruzi*, *Toxoplasma* and *Leishmania*.

Theileria parasites are a group of tick transmitted apicomplexan parasites infecting wild and domestic animals throughout the world. The genus contains a number of species that are infective to cattle. The characteristics of different *Theileria* species have been reviewed by Uilenberg (1981), Morzaria and Nene (1990) and Dolan (1989a), on which the following account is based. As well as *T. annulata*, *T. parva* is also important as a disease problem, which gives rise to East Coast Fever, January disease and Corridor disease. Other cattle infective species which can also be pathogenic are *T. mutans*, *T. taurotragi* and *T. sergenti*. *T. velifera* is non-pathogenic but may confuse diagnosis of pathogenic species. *T. mutans*, *T. velifera*, *T. annulata* and *T. parva* also infect buffalo. There are also numerous other *Theileria* species infecting various domestic animals. The most important of these is *T. hirci*, which is pathogenic in sheep and goats but is poorly studied. *T. camelensis* has been identified as a parasite in camels and *T. ovis*, *T. recodita* and *T. separata* infect small ruminants.

The genus *Theileria* is included in the phylum Apicomplexa which contains other important genera such as *Plasmodium*, *Eimeria*, *Babesia*, *Sarcocystis* and *Toxoplasma*. These parasites all possess an apical complex at some stage in their life cycle, with intracellular forms occuring in erythrocytes and or other cells (such as leucocytes) in the mammalian host. The genus *Theileria* is distinguished from the closely related genus *Babesia*

by the morphology of the erythrocytic stages and the ability to infect lymphocytes as well as erythrocytes; *Babesia* species infect erythrocytes only. The currently accepted classification of the genus according to Levine (1988), is as follows;

Phylum	Apicomplexa
Subphylum	Sporozoa
Class	Aconoidasida
Order	Piroplasmorida
Family	Theileriidae

The class Aconoidasida also includes the genera *Babesia, Dactylosoma* and *Cytauxzoon.* This classification is based on morphological and biological characteristics of this group, principally the lack of certain elements of the apical complex, namely the conoid, and is still a matter of some debate. The taxanomic position will probably be more fully resolved by molecular and biochemical techniques, such as the small subunit ribosomal RNA sequence comparisons between *Theileria* and other apicomplexan genera carried out by Barta et al (1991) and Gajadhar et al (1991).

The various *Theileria* species of cattle are distinguished by means of morphology of the piroplasms and schizonts, serological differences detected by indirect fluorescent antibody tests (Kimber et al 1973), and differences in their geographical distribution, vector species and pathogenicity. *T. parva* is transmitted by *Rhipicephalus* species of ticks and is only found in Eastern, Central and Southern Africa, where it is a major disease problem (Dolan 1989a). *T. taurotragi* has a similar ditribution and is transmitted by the same vector, but usually the infection is subclinical although occasional mortality has been recorded. *T. mutans* is found in Southern parts of Africa and possibly the Caribbean, being transmitted by *Amblyomma* species. The disease is also usually benign but occasionally pathogenic. *T. velifera,* which has only been associated with benign infections, has a similar distribution. *T. sergenti* is an important pathogen in East Asian countries, transmitted by *Haemaphysalis* species. "*T. orientalis* " has also been described in East Asia but the validity of this species is in doubt. In addition, a number of non-pathogenic *Theileria* species which are not fully described are known from Australia, Asia, Africa, Europe and rarely in the Americas (Uilenberg 1981).

T. annulata has a widespread distribution, being identified in Portugal, Spain, Italy, the Balkan countries especially Bulgaria and Greece, Turkey, Southern Russia and Central Asia, the Near and Middle East, Pakistan, India the Northern and North-Eastern parts of Africa; Morocco, Algeria, Tunisia, Libya, Egypt, Sudan and possibly Eritrea (Dolan 1989a). The worldwide distribution is summarised in Figure 1, and is associated with the distribution of the *Hyalomma* species tick vectors.

It is worth mentioning the nomenclature used in the study of *Theileria*, which will be used in this thesis according to the definitions given by Dolan (1989b). An "isolate" describes viable organisms isolated on a single occasion from a field sample. A "stock" describes all the heterogenous populations of a parasite derived from a single isolate, including derived cell lines and tick stabilates. A stock is identified by the location of the laboratory in the endemic area where it was isolated, such as *T. annulata* Hissar and *T. annulata* Ankara. Considerable heterogeneity has been demonstrated both between and within stocks of *T. annulata* and *T. parva*, for example in their glucose phosphate isomerase patterns (Melrose et al 1984) and their antigenicity (Shiels et al 1989). A "line" is a laboratory derivative of a stock, such as a culture of parasite infected lymphoblastoid cells, while a "clone" is derived from a single cell and therefore consists of genetically identical organisms A "stabilate" describes a cryopreserved sample of organisms from a cell line, clone or isolate.

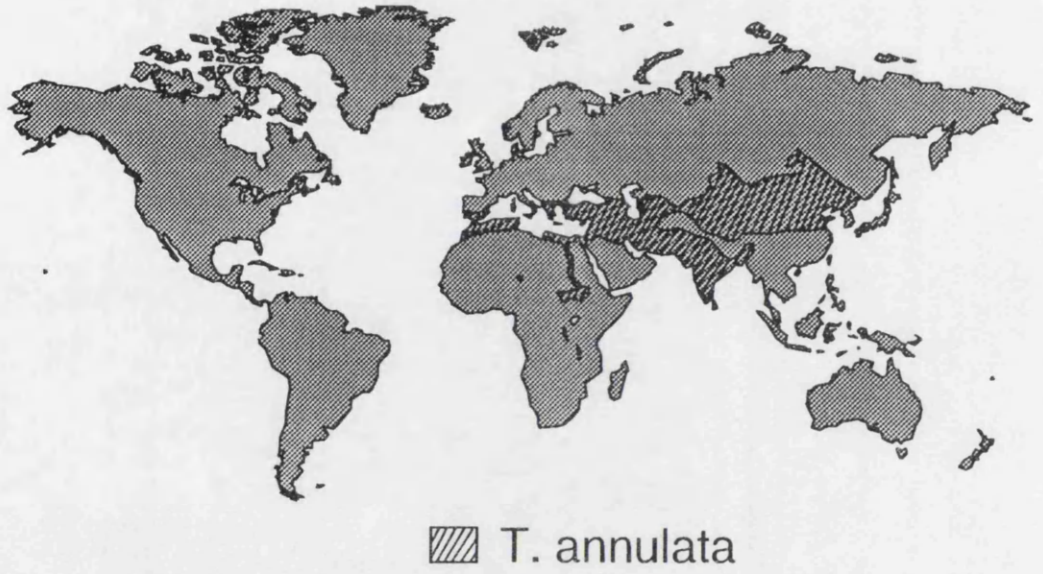
1.1.1 The life cycle

The life cycle of *T. annulata* is typical of that of all *Theileria* species, with three main phases of multiplication; sexual reproduction followed by sporogony in the vector and asexual reproduction by schizogony and merogony in the vertebrate host. A detailed description of the *Theileria* life cycle based on observations from light and electron microscopy is given by Melhorn and Schein (1984), and summarised for *T. annulata* in reviews by Uilenberg (1981), Tait and Hall (1990) and Morzaria and Nene (1990), on which this account is based.

The vectors for *T. annulata* are ticks of the genus *Hyalomma*, with vertebrate hosts being cattle such as *Bos taurus* or *Bos indicus*, or domestic

Figure 1

Worldwide distribution of *T. annulata* (adapted from Dyer and Tait 1987). Parts of the world where the disease is documented are shaded in black.



buffalo. Ticks can only be infected by feeding on the host, and cattle can only be infected by feeding ticks or by artificial inoculation. No transovarial transmission has been detected in the vector. Most *Hyalomma* species, such as *H. detritum* which is capable of transmitting theileriosis, are two host ticks, in which the larva and nymph feed on the same animal, each taking a blood meal, while the adult feeds on a different animal. However, some *T. annulata* vectors are three host ticks where the larva, nymph and adult feed on separate animals, such as *H. anatolicum anatolicum.* In these ticks, one blood meal is taken on each host. After each blood meal the tick moults into it's next stage. If the parasite is acquired in the nymph stage, it can be transmitted to a new host by the adult tick.

The life cycle is summarised in Figure 2. Sporozoites, the infective stage of the parasite, are injected into the host with saliva from a feeding tick. *T. annulata* invades a specific population of lymphocytes; those bearing MHC Class II molecules such as B cells and macrophages (Glass et al 1989). This contrasts with *T. parva* sporozoites which infect the T cell subset. Invasion is an active, parasite induced process, inhibited "in vitro" by low temperature and heat-inactivation of sporozoites (Jura 1984). The ultrastructure of the process has been described (Jura et al 1983). Sporozoites were found to make contact with and invade lymphocytes "in vitro" in less than five minutes, and multiple infections of single lymphocytes were common. The membrane of the host and parasite were found to come into close apposition, with the area of contact spreading laterally as the sporozoite becomes enclosed in a deepening recess until the host cell membrane closes and fuses behind the parasite. The sporozoites were consistently found to invade lymphocytes via their basal end. This contrasts with the situation in other Apicomplexa such as *Plasmodium*, where the sporozoites invade target cells following attatchment of the apical complex (Sinden 1985).

Differentiation of the sporozoite to the trophozoite stage was found to occur within 30 minutes "in vitro". The host cell membrane surrounding the parasite breaks down and is not retained as a parasitophorous vacuole as is the case with *Eimeria* and *Plasmodium.* The trophozoite enlarges to about 2μm apparently by ingesting host cell cytoplasm and undergoes repeated nuclear division, to give rise to the multinucleate shizont or "macroschizont" observed under light microscopy. This stage can be observed about three

Figure 2 The life cycle of *Theileria annulata*

1. Sporozoites are inoculated into the bloodstream of a cow by a feeding tick, and rapidly infect leucocytes.

2. Differentiation of an internalised sporozoite into a trophozoite.

3. Multiplication by schizogony; macroschizont followed by microschizont stages.

4. Release of merozoites and infection of erythrocytes to form piroplasms.

5. Piroplasms may possibly undergo further division, producing merozoites capable of infecting other erythrocytes.

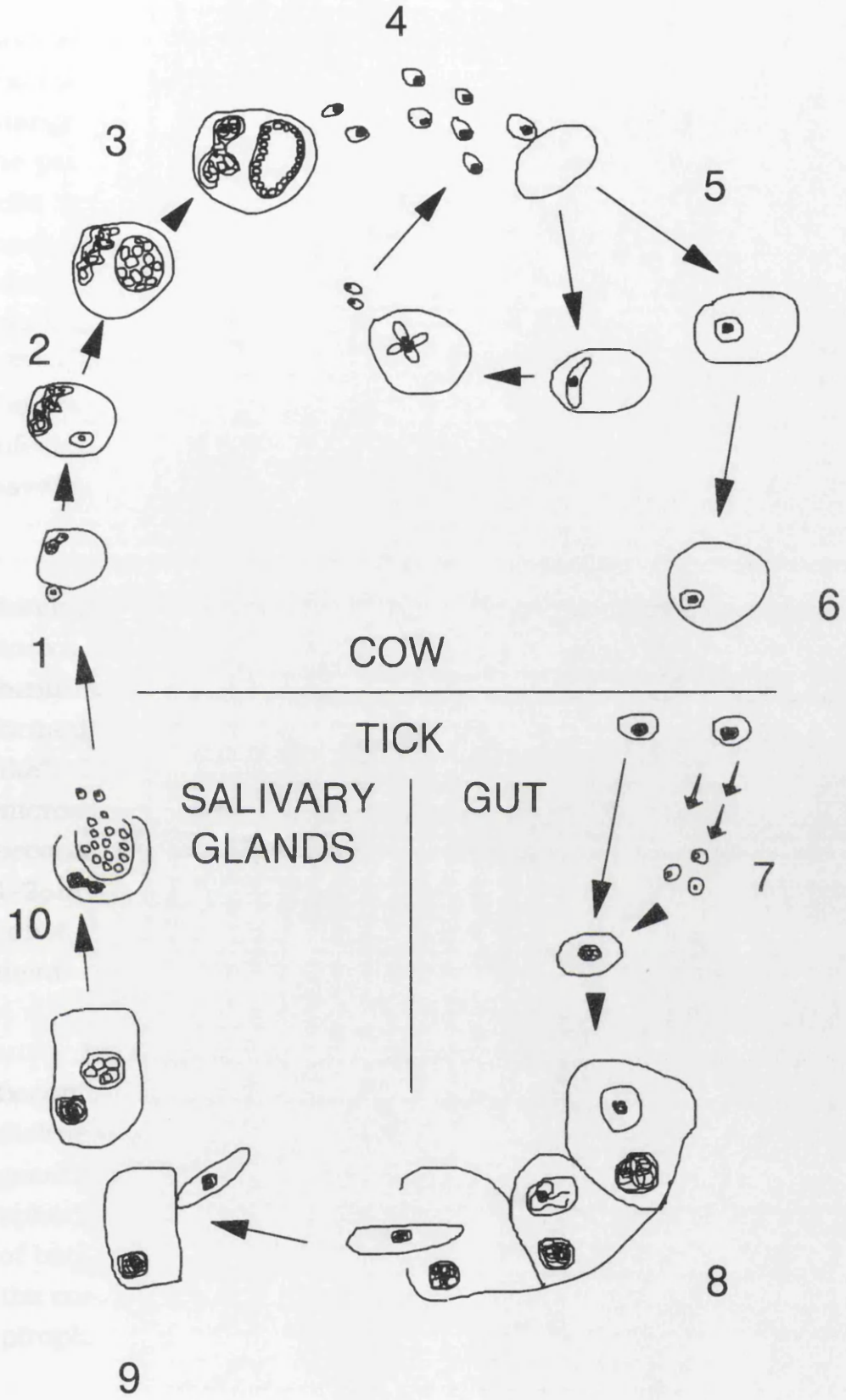
6. Piroplasms are taken up by a feeding tick.

7. Piroplasms undergo further differentiation in the tick gut to produce ray-bodies, which divide to produce microgametes. Macrogametes are also postulated to exist. Syngamy produces an ovoid zygote.

8. The ovoid zygote differentiates into a motile kinete inside an intestinal cell.

9. Kinetes migrate through the haemolymph and infect cells of the salivary glands.

10. The kinete undergoes extensive sporogony, to produce large numbers of sporozoites capable of infecting a new host.


4
3
5
2
6
1
COW
TICK
SALIVARY GLANDS
GUT
7
10
8
9

days after infection "in vivo" (Melhorn and Schein 1984). The macroschizonts replicate by binary fission. This process brings about changes in the host cell, which is also stimulated to undergo division, with the parasite becoming associated with and being distributed to the daughter cells by the host cell spindle apparatus (Melhorn and Schein 1984). The mechanism by which this rapid proliferation of infected lymphocytes occurs, which phenotypically resembles transformation, is poorly understood, but it has been postulated to involve sequences homologous to oncogenes (reviewed by Dyer and Tait 1987) Alterations in the phosphoprotein and protein kinase activity profiles of *T. annulata* infected leukocytes, which could potentially be produced by such sequences, have been identified (Dyer et al 1992).

In *T. annulata*, merogony is an important feature of the parasite's development. This contrasts with the situation in other species such as *T. parva*, where piroplasm replication is limited (Morzaria and Nene 1990). Schizonts start forming merozoites 8-10 days after the initial infection. The merozoites are formed from nuclei at the periphery of the schizont, which appear "rosette-like". This stage is termed the "microschizont" which is observed under light microscopy. The merozoites acquire an apical polar ring and rhoptries, and become free almost simultaneously from the residual body. Merozoites are 1-2μm long, with an apical complex, micronemes and an outer surface consisting of a cell membrane, with two closely apportioned inner membranes sometimes seen (Melhorn and Schein 1984).

Merozoites invade erythrocytes, with up to 90% of erythrocytes becoming infected. The erythrocyte membrane surrounding the merozoite disintegrates, and the parasites differentiate to form piroplasms. Two general types of piroplasms are observed; slender, comma shaped forms and spherical or ovoid forms (Melhorn and Schein 1984). The relative abundance of both forms vary according to the species; about 80% of piroplasms are of the comma shaped type in *T. parva*, while in *T. annulata* both types of piroplasms occur in approximately equal proportions.

It is thought that a second cycle of division occurs at this stage, with the comma shaped piroplasms dividing by binary fission. Division of the nucleus is associated with cellular division of the parasite; no multinucleate schizont-like stage has been identified. Division of piroplasms "in vitro" to

form a tetrad stage has been described by Conrad et al (1985), and these workers also observed similar forms in infected cattle. The merozoites formed were identical to those produced by intralymphatic schizogony, and could presumably re-infect erythrocytes. The relative importance of the two forms in maintaining the infection is not known.

The following account of the development and fate of the spherical forms is based on morphological studies and "in vitro" observations of infected erythrocytes in culture, described by Melhorn and Schein (1984). The spherical piroplasms undergo further development when taken into the gut of feeding ticks, or when cultured "in vitro". These gamete-like forms, known as microgamonts or "ray-bodies" on account of their morphology, appear to be formed from piroplasms 2-4 days after completion of feeding by the tick. Division of these stages has been observed beginning 5 days after tick feeding, and is thought to result in uninucleate gamete-like stages. Larger spherical forms have also been identified in the gut of infected ticks, which are tentatively considered to be macro gametes . Syngamy of gametes occurs 6-13 days after incubation of the intraerythrocytic stages in culture. An ovoid zygote is formed which can also be detected "in vivo". This differentiates to form a kinete, a club-like uninucleate motile stage surrounded by a pellicle. Kinetes can be observed in the gut of infected ticks 12-30 days after feeding. Kinetes penetrate the gut wall and migrate to the salivary glands via the haemolymph. When a nymph is infected, this process is associated with moulting of the nymph to the adult tick in *T. parva* and other species of *Theileria*, but in *T. annulata* kinetes are detected in the haemolymph shortly before moulting.

Development in the salivary glands is only possible after the tick commences feeding. This is because the salivary glands of ixodid ticks become reduced after each feed and only enlarge once the tick attaches to and feeds from a new host. It is thought that the parasite remains dormant until this happens. Kinetes are found inside E cells of the type III acinus of the salivarian alveoli gland (Melhorn and Schein 1984). Kinetes are found directly in the cytoplasm of the gland cell, where they undergo a series of differentiation steps including loss of the pellicle and become amorphous. The nucleus increases in size and undergoes extensive division. The volume of the cytoplasm increases rapidly, and numerous invaginations or "cytomeres" form and surround the nuclei. Formation of ovoid sporozoites

from the cytomeres takes place 3-5 days after the tick commences feeding. The sporozoites are capable of transmission to a new bovine host. In the region of 50,000 sporozoites are produced from a single infected salivary gland cell, which results in an enormous inoculum from a single feeding tick.

1.1.2 The disease

250 million cattle are believed to be at risk from the effects of tropical theileriosis. Indiginous cattle living in areas where the disease is endemic are often infected as calves, with minimal clinical reaction followed by recovery, resulting in a persistant immune carrier state (Brown 1990). However, in "improved" cross-bred or exotic cattle, the disease is highly pathogenic, causing 40-60% mortality. Many countries such as India are attempting to increase dairy production by improving their cattle stock, through cross-breeding indiginous cattle with more productive European breeds such as Friesians (Brown 1990). Tropical theileriosis has become an increasing problem in recent years through the constraints it imposes on such schemes.

The epidemiology of the disease has been reviewed by Uilenberg (1981). Tropical theileriosis has a pronounced seasonal character in subtropical countries, with the majority of cases occurring in the summer when adult ticks are more active. Seasonality is less pronounced in tropical countries where ticks are active all year round. The epidemiology of tropical theileriosis varies according to the climate and vector species. Recovered cattle tend to act as healthy carriers, therefore cattle alone are sufficient as a reservoir for the infection of ticks. However, Asian water buffalo may also act as reservoir hosts in areas where they are common, since they can be infected but usually only undergo a mild form of the disease. Local cattle in endemic areas are generally less susceptible than imported stock.

The pathogenicity of the disease has been described in a number of reviews; such as Melhorn and Schein (1984), Tait and Hall (1990) and Uilenberg (1981). The incubation period between attatchment of an infected tick and fever is about two weeks, with extremes of 8 and 30 days. Mechanically transmitted infection usually results in a longer incubation period. The severity of the disease depends on the susceptability of the

animal, the virulence of the strain and is generally dependent on the dose of sporozoites inoculated.

A typical acute infection starts with a high fever and swelling of the superficial lymph glands, followed by swelling of the regional lymph gland draining the site of infection. Symptoms which are generally apparent are listlessness, accelerated pulse and respiration rate, reduction in milk production, and frequently diarrhoea. Haemolytic anaemia and jaundice are common later features. In fatal infections the animal dies usually 1-2 weeks after the onset of clinical symptoms, although this can be as early as three days in hyperacute cases (Uilenberg 1981). Cattle that recover show symptoms that are less severe and gradually wane over several weeks after the onset of the infection, although a complete recovery may take some time.

Not all aspects of the pathology are well understood. Destruction of lymphocytes by macroschizonts and of erythrocytes by piroplasms both seem to be key factors. The involvement of toxins has also been postulated. Infected lymphocytes displace uninfected lymph node tissue and bring about symptoms identical to leucosis (lymphocyte depletion). The infection also has a mitogenic effect on infected and uninfected lymphocytes which can be detected early on in the infection, even before the appearance of schizonts. The main pathogenic effects which often lead to death occur during the phase of intralymphocytic schizogony. Destruction of erythrocytes by piroplasms leads to the symptoms of haemolytic anaemia frequently associated with later infections, although this is postulated to be due to removal of infected erythrocytes by phagocytosis rather than direct parasite-induced lysis, and it has also been suggested that autoimmune mechanisms contribute to the anaemia (Uilenberg 1981). The pathology of *T. annulata* infections differ in some ways from the effects caused by other *Theileria* species. For example, in the disease caused by *T. mutans* pathogenesis is apparently solely due to the erythrocytic stage. In *T. parva* piroplasm replication is not a prominant feature of the infection (Morazia and Neme 1990), and haemolytic anaemia is uncommon.

Diagnosisis is usually based on observation of clinical symptoms and the use of Giemsa stained blood or tissue smears to detect macroschizonts and piroplasms. Indirect immunofluorescence against fixed piroplasms or macroschizonts has also been used, but this method also identifies immune

animals as being positive. It may be possible to develop ELISA assays for detecting circulating antigens or immune complexes using monoclonal antibodies (McAbs) to various stages, as has been developed for *T. mutans* (Katende et al 1990). DNA hybridisation techniques using cloned parasite genes as probes may also prove useful. *T. parva* isolates have been characterised using specific oligonucleotide probes (Allsopp et al 1989).

1.1.3 The bovine immune response

The bovine immune response to *T. annulata* infection has recently been reviewed by several authors; Hall (1988), Tait and Hall (1990) and Brown (1990). The heterogenous life cycle expose the host immune system to different sets of antigenic determinants at each stage. Indeed, different surface polypeptides have been identified in the sporozoite, macroschizont and piroplasm stages (Shiels et al 1989). This antigenic diversity appears to result in the heterogenous immune response observed in cattle, directed against different stages in the life cycle, and involving both cellular and humoral components. Nevertheless, cattle that recover from an infection are solidly immune to further challenge. This immunity develops irrespective of the source of primary infection, whether through injection of sporozoites from a feeding tick or artificially, with or without drug treatment. Immunity can also be engendered by vaccination with schizont infected lymphocyte cell lines derived from tissue culture (to be discussed futher in section 1.1.4). Immunity in the absence of further challenge usually lasts at least three years. The immune response to each stage of the life cycle will be reviewed separately.

The sporozoite

The fact that cattle can be immunised with schizont infected lymphocytes directly from another animal or from a cell line cultured "in vitro" (as reviewed by Brown 1990) indicates that an immune response to the sporozoite stage is not essential for cattle to be protected against challenge. However, there is a body of evidence indicating that humoral immunity to the sporozoite, the first stage of the parasite to which the cow is naturally exposed, does have an important role. Studies on the immunity to this stage

have been carried out both "in vitro" and "in vivo" using sporozoites obtained from infected ticks.

Evidence that immunity to *T. annulata* involves a humoral response to sporozoites was first presented by Gray and Brown (1981), who showed serum from immune cattle was capable of neutralising sporozoite infectivity of lymphocytes "in vitro". These findings were confirmed and extended by Preston and Brown (1985), who found that sera from cattle immunised against *T. annulata* by the infection and treatment method was capable of both suppressing sporozoite invasion and the initial development of intracellular trophozoites in culture. These two effects appeared to be due to different serum factors; while sera from cattle exposed to single or multiple immunisations with sporozoites was capable of suppressing trophozoite development, only sera from multiply immunised cattle were capable of neutralising sporozoites. They proposed that the trophozoite inhibiting effect was due to the recognition of parasite antigens on the surface of the infected host cell, although the involvement of other serum factors such as tumour necrosis factor (TNF) is also a possibility (Hall 1988). Serum factors, thought to be antibody, which were capable of neutralising sporozoite infectivity were also identified from *T. annulata* immune cattle by Ahmed et al (1988).

Two sporozoite specific surface antigens have been identified using mouse McAbs raised against *T. annulata* sporozoites (Williamson 1988; Williamson et al 1989). Tissue culture supernatant and ascites fluid preparations of the McAbs 1A7 and 4B11 were found to effectively neutralise sporozoite infection of bovine leucocytes "in vitro". Both the McAbs recognised live and formalin or acetone fixed *T. annulata* Ankara sporozoites in indirect fluorescent antibody tests (IFAT) but gave little or no reaction with acetone fixed piroplasm or macroschizont stages. The McAbs also reacted with formalin fixed sporozoites from a geographically distinct stock, *T. annulata* Gharb. However, the McAbs recognised different groups of sporozoite specific antigens on Western blots. While McAb 1A7 reacted with four protein doublets of 85, 70, 63 and 54kDa, plus an additional antigen of 104 kDa in sporoblasts and immature stages, McAb 4B11 was found to recognise antigens of about 20 and 17kDa in molecular weight. Both sets of antigens were also found to be recognised by cattle immunised with viable or irradiated sporozoites, although the combination of bands detected varied from animal to animal. Similar results were obtained from rabbit antiserum

raised against live sporozoites. Sera from a number of the cattle also recognised additional antigens of sizes 126, 100, 48, 36 and 27kDa.

Further work was carried out to characterise the 1A7 antigen (Williamson et al 1989). The McAb was used to probe a *T. annulata* λgt11 genomic expression library, and identified a positive clone (λgt11-SR1), containing a 300bp insert of parasite DNA. The insert hybridised to a 3.1kb band in total RNA from tick salivary glands infected with *T. annulata* sporozoites on a Northern blot, but not to RNA from uninfected tick salivary glands, a macroschizont cell line or piroplasms. The insert also hybridised to bands of 6, 4.8 and 3.4kb in Southern blots of uncloned *T. annulata* Hissar and Ankara DNA, but only single bands in DNA from cloned macroschizont cell lines. This implied that the gene encoding the 1A7 antigen was polymorphic at the DNA level which will be discussed further in Chapter 3. Antisera from cattle immunised with λgt11-SR1 recognised SPAG1 antigens on Western blots of *T. annulata* sporozoite material, *T. annulata* sporozoites in IFA tests and were capable of blocking sporozoite infectivity of bovine lymphocytes "in vitro".

The SR1 insert was used to isolate the entire sequence for the antigen gene from a *T. annulata* sporozoite cDNA library (Hall et al 1992). The DNA and amino acid sequence are shown in Figure 77 in the Appendix. The SR1 sequence containing the 1A7 epitope was located in the C-terminal region of the gene.

It was suggested that the set of antigens recognised by 1A7 could potentially be the proteolytically processed products of a single gene or the products of separate genes sharing a common epitope (Williamson et al 1989). In order to resolve this, rabbit antiserum raised against the λgt11-SR1 fusion protein was used to immunoprecipitate the expressed products from sporozoite mRNA translated in a reticulocyte lysate cell-free system. The primary translation product was found to be a single 115kDa polypeptide, showing that only a single mRNA species was present; therefore the McAb 1A7 only recognised the product of a single gene (Hall et al 1992). Thus it was concluded that the smaller polypeptides recognised by 1A7 are derived from a common precursor by proteolytic processing . Since the products on mature sporozoites all contained the C-terminal 1A7 epitope, it seemed likely that the antigen was being processed from the N-terminal end.

In a further study to characterise sporozoite surface antigens, mouse antiserum raised against *T. annulata* sporozoites was used to immunoprecipitate radiolabelled proteins from cell surface iodinated sporozoites (Shiels et al 1989). Antigens of sizes analogous to SPAG1 were precipitated, as well as a further set of antigens of sizes 190, 127 and 98kDa. This set of antigens were also precipitated by a single McAb, 4E5, and so also appeared to contain a common epitope. Therefore the sporozoite surface appears to consist of several groups of immunologically related antigens.

The immune response to *T. parva* sporozoites was also found to be mediated via humoral factors capable of neutralising sporozoite infectivity of lymphocytes "in vitro" (Musoke et al 1982). A number of McAbs which were raised against *T. parva* sporozoites were also found to block infectivity (Dobbelaere et al 1984, Musoke et al 1984). Three distinct groups of immunologically related antigens recognised by a bovine antiserum were identified on Western blots *T. parva* sporozoite material (Iams et al 1990a). Two of these antigens have been characterised further; namely the p67 sporozoite surface antigen (Nene et al 1992) and the 104kDa microneme-rhoptry protein (Iams et al 1990b). It was suggested that the 104kDa antigen was processed to a number of smaller immunologically related polypeptides of 90, 85 and 35kDa. The genes coding for both these antigens have been identified and sequenced.

The macroschizont

There is good evidence that the macroschizont infected lymphocyte is the main target of the immune response to *T. annulata*, since animals can be immunised with macroschizont infected lymphocyte cell lines attenuated in tissue culture (described in section 1.1.4) or from an infected animal (Hall 1988, Brown 1990). While anti-macroschizont antibodies have been identified in cattle immune to tropical theileriosis, this antibody is not considered to play a major role in protection (Pipano 1977). Immunity produced to macroschizont infected lymphocytes appears to be predominantly cellular, involving a variety of lymphocyte subsets.

It was shown by Preston et al (1983) that cytotoxic cells directed against macroschizont infected lymphocytes appeared in the blood and

lymph nodes of cattle recovering from theileriosis, but not in cattle that succumbed to the disease. Two sequential populations of cytotoxic cells were observed during recovery from a primary infection; the first mediating BoLA restricted lysis in culture, and so were postulated to belong to the cytotoxic T cell subset, while the action of the second population of cells was not BoLA restricted and were postulated to be NK like cells. When these animals were challenged with live sporozoites and underwent a secondary response, one or two peaks of BoLA restricted cytotoxic activity were observed. These workers therefore postulated that cytotoxic T cells directed against antigens on the surface of macroschizont infected lymphocytes were retained in the immunological memory. Interestingly cattle that were immunised with BoLA mismatched cell lines produced a cytotoxic cell population specific for the immunising cell line only, while cattle immunised with BoLA matched cell lines produced only a very transient population of BoLA restricted cytotoxic cells (Preston and Brown 1988).

A third type of cellular response to macroshizont infected lymphocytes was identified by Preston and Brown (1988). Adherent cells, which appeared to be macrophages, from cattle that had recovered from theileriosis or that had been immunised with macroshizont infected lymphocyte cell lines were shown to inhibit proliferation of both BoLA matched and BoLA mismatched cell lines. This type of response, termed macrophage mediated cytostasis, was detected consistantly after both immunisation and challenge. The mechanism by which macroshizont infected lymphocyte proliferation was inhibited is not known but appears to be mediated by soluble factors, such as tumour necrosis factor (TNF), since proliferation could still be inhibited even when contact between the macrophage and target cells was prevented by a 0.45μM filter.

A number of infection associated antigens have been identified on the surface of *T. annulata* transformed cells (Shiels et al 1986b), which could be potential targets for cellular responses. There are several postulated sources for these antigens; they could be entirely derived from the parasite and transported to the host surface, where they could possibly associate with MHC molecules; they could be parasite-altered lymphocyte surface molecules or parasite-induced lymphocyte surface molecules not normally expressed (reviewed by Hall 1988).

A number of McAbs have been raised against macroschizonts, specific for antigens on the surface of the macroschizont or macroschizont infected leucocytes (Shiels et al 1986). One of these antigens, identified by the McAb 4H5, has been further characterised (Shiels et al 1989). 4H5 was found to immunoprecipitate an antigen of 95-120kDa from surface iodinated macroshizont infected lymphocytes. Preston et al (1986) showed that McAb 4H5 specifically mediated complement lysis and suppressed proliferation of macroshizont infected lymphocytes, showing that the antigen could function as a target for immune mechanisms. Whether the 4H5 antigen can act as a target for cytotoxic T cell responses remains to be determined. Although the 120kDa molecule itself is too large to be a processed product expressed in association with Class I molecules, a requisite for it's recognition by cytotoxic T cells, it is possible that processed fragments could contain cytotoxic T cell epitopes.

A number of infection associated antigens on the surface of *T. parva* macroshizont infected lymphocytes have also been identified (reviewed by Morrison et al 1989). The effector mechanisms against *T. parva* macroschizonts also appear to be predominantly cellular, with transient populations of BoLA resticted cytotoxic cells produced in animals undergoing immunisation or challenge. Both cytotoxic and helper T cell clones specific for macroshizont infected lymphocytes have been derived from *T. parva* immunised animals. Two groups of helper T cell clones were derived which recognised distinct antigenic fractions from homogenised *T. parva* infected cells (Brown et al 1990). Furthermore, cytotoxic T cell clones were found to be either parasite strain specific or recognised cross-reactive determinants, indicating that this cell subset was also responding to more than one determinant.

T. parva immune cattle have been found to produce an antibody response to a macroschizont encoded polymorphic immunodominant molecule (PIM) characterised by Toye et al (1991). This antigen also occurs on the surface of sporozoites, and varies in molecular weight in different parasite stocks. It is currently being assessed as a potential target for cytotoxic cells.

The merozoite/ piroplasm

While sera from immune cattle do not appear to recognise the surface of infected erythrocytes, a humoral response to merozoite and piroplasm antigens has been identified. A number of radiolabelled polypeptides have been immunoprecipitated from surface-iodinated *T. annulata* piroplasms extracted from infected erythrocytes using antiserum from an immune cow (Shiels et al 1989). These antigens probably originate from the merozoite stage, since antigens on the surface of piroplasms would be unlikely to be exposed to the bovine immune system. The effects of a bovine immune response to such antigens has not so far been elucidated, but inhibition of merozoite infection of erythrocytes might be expected to reduce the anaemia associated with the disease, and also to reduce piroplasm transmission to the tick.

Merozoites, like sporozoites, are an invasive stage of the parasite in the bovine host. Work is less advanced than to the sporozoite stage, impeded by the difficulty in obtaining sufficient amounts of this stage for molecular analysis and the lack of an "in vitro" assay for erythrocyte invasion. However, merozoites have now been produced "in vitro" by culturing macroschizont infected cell lines at 41^{o}C (Glascodine et al 1990). This differentiation step was associated with a change in antigenic profile of the lymphocytes. A number of McAbs raised to piroplasms were found to recognise heat induced merozoites by indirect immunofluorescent antibody tests. The McAb 5E1 was found to recognise a 30kDa antigen on the surface of merozoites and piroplasms, which was expressed during or following the heat-induced differentiation process. The 30kDa antigen was further characterised by Dickson and Shiels (1993). Two forms of the molecule, of 30 and 32kDa, were detected in heat-induced cell lines. While the peptide sequences of these molecules were related, only one form was detected by the McAb, which was found to recognise a carbohydrate epitope on the 30kDa form.

Molecules with similar characteristics to the 30/32kDa antigen have been identified in other species of *Theileria*. A 32kDa glycoprotein has been identified on the merozoites of *T. mutans* (Katende et al 1990), and a 33-34kDa polypeptide has been identified in *T. sergenti*, *T. buffeli* and *T. orientalis* (Kawazu et al 1992). Passive administration of McAbs raised

against the antigen in *T. sergenti* were found to protect cattle against infection by *T. sergenti* merozoites (Tanaka et al 1990).

The tick

It is important to consider immune responses to the ectoparasitic tick as well as to *T. annulata*, since infective sporozoites entering the host are probably first exposed to the cell types attracted to the point of tick attachment. Ticks remain associated with their host generally for at least a week, which would give adequate time for priming of the immune system. Tick feeding is preceded by production of saliva and a cement like material which holds the tick to the skin. This antigenic material persists in the skin for several days where it can be detected on the surface of Langerhan's cells by indirect immunofluorescence (Wakelin 1984). Both innate and acquired immune responses have been described to ixodid ticks.

Tick bites produce pronounced inflammatory responses even in naive hosts (Wakelin 1984). In immune hosts a rapid reaction occurs which may prevent tick feeding completely and can result in death of the tick. The immune response of guinea pigs to the ixodid tick *Amblyomma americanum* was studied by Brown and Askenase (1983) and compared with immune mechanisms previously identified to other genera of the family Ixodidae. An inflammatory response is provoked around the point of insertion of the mouthparts. On primary exposure, the feeding site is first infiltrated predominantly by neutrophils, and later (after 3-5 days of feeding) by basophils and eosinophils. Basophils and eosinophils are also the predominant cell types to appear on secondary exposure to ticks. These workers found that sensitised T cells and IgG_1 antibody produced to *A. americanum* antigens appeared to recruit basophils and eosinophils to the tick attachment site. Basophil infiltration is also a feature of guinea pig immune responses to a number of other ixodid ticks, such as *Ixodes* and *Rhipicephalus* species, and has also been demonstrated in the immune response of cattle to *Ixodes holocyclus*.

The effect of attraction of eosinophils and basophils to the tick feeding site on immune responses to *T. annulata* is not known. However, this inflammatory response may mean that non-specific immune responses

would already be established at the tick feeding site prior to sporozoite inoculation.

1.1.4 Control measures

The steps which are taken to control tropical theileriosis and East Coast fever have been reviewed by Brown (1990), Tait and Hall (1990), Morzaria and Nene (1990), Musisi (1990), Dolan (1989a) and Uilenberg (1981), on which many of the following descriptions are based. The three main types of control which are also areas of current research are vector control, chemotherapy and vaccination, and these will be reviewed here.

Vector control

Since there are several other tick borne parasitic infections in parts of the world where *T. annulata* and *T. parva* occur, such as heartwater and babesiosis, control measures taken against ticks has the advantage of controlling several of these diseases at the same time. The current method is by spraying or dipping cattle in acaricides, such as butocarb or amitraz. Normally, dipping or spraying cattle once a week is adequate, due to a residual effect of the acaricide for four days, plus the fact that sporozoites are only transmitted 3-5 days after commencement of feeding (Urquhart et al 1987).

This method does have a number of disadvantages, however. Firstly, it is impossible to irradicate the two or three host ticks such as the *Hyalomma* species which transmit *T. annulata* because they have reservoir hosts of various wild and domestic animals which maintain the population. It is recommended to treat cattle twice a week under these conditions. Other drawbacks are the high cost of wide scale use of acaricides, the necessity for a highly organised programme of regular dipping and acaricide resistance in ticks. While dipping programmes have been applied successfully to control *T. parva*, their use has been limited in *T. annulata* control.

Steps have also been taken to avoid contact between infected ticks and susceptible cattle. This includes control of cattle movement between areas where the disease is endemic and areas relatively free of theileriosis, or

housing cattle under conditions of zero grazing. This last method is expensive and only occasionally used for valuable imported breeds. Such cattle would not have any aquired immunity to the disease through lack of previous exposure to ticks. The construction of cattle sheds can be an important factor in controlling exposure to ticks in some subtropical countries such as Morocco, where the vectors often behave as barn ticks, hibernating in cracks in the walls instead of remaining in grazing areas. Improved construction of animal housing and spraying walls with acaricides has helped to reduce the incidences of tick infestation.

Chemotherapy

While chemotherapy has been used fairly sparingly for the prevention and treatment of *T. annulata* infections, it has been used on a fairly wide scale to treat *T. parva* infections and immunise cattle by the infection and treatment method, to be discussed in the section on "Vaccination". Chlorotetracycline was one of the earlier drugs used. There has been considerable advances in the development of new drugs in the past 10-15 years, aided by the development of "in vitro" culture techniques useful for screening for anti-theilerial activity (Brown 1987). The use of such techniques coupled with "in vivo" tests led to the identification of several drugs active against *T. parva* and *T. annulata* infections; the anticoccidial drug halofuginone, a napthaquinone menoctone, and the menoctone analogues parvaquone and buparvaquone. Extensive field trials have shown buparvaquone to be a highly effective therapeutic agent against *T. annulata* and *T. parva* infections of cattle (McHardy 1991). Menoctone and buparvaquone have both been found to be active against *T. parva* and *T. annulata* macroschizonts in culture (McHardy 1978, McHardy et al 1985). Interestingly, many compounds shown to be active against *Babesia*, *Eimeria* and *Plasmodium* infections, such as proguanil, diaveridine and chloroquine, had no effect on *Theileria* infections in cattle or on cultured macroschizonts (McHardy 1978), postulated to be due to lack of penetration of infected lymphocytes by these compounds.

Vaccination

Vaccination by attenuated schizont infected cell lines is the most widely used control method against *T. annulata*. This relies on the fact that

lymphocytes transformed by *T. annulata* schizont infection can be continuously cultured "in vitro" without the need for additional growth factors (Brown 1987). The production and use of schizont tissue culture vaccines has been reviewed by Pipano (1981), Hall (1988) and Brown (1990). Briefly, *T. annulata* schizont infected cells are established in culture either from an infected cow or by infecting healthy bovine lymphocytes with sporozoites "in vitro". The infected cells are cultured in RPMI 1640 medium supplemented with 20% calf serum, and are passaged continuously to make a cell line. Schizonts gradually become attenuated in culture, which means they become less virulent when used to infect a cow, producing milder clinical symptoms and lower parasitaemia with increasing passage. Attenuation of vaccine cell lines is currently tested by periodic inoculations into susceptible cattle. A cell line is considered to be properly attenuated when it no longer produces clinical symptoms or piroplasms when used to inoculate cattle. This requires 20-300 passages in culture to achieve, depending on the *T. annulata* isolate, which can take between several months and three or more years. Once attenuated, the cell line can be cryopreserved for storage and transport and resucitated. The usual vaccine dose is 10^6 -10^7 cells, although much lower doses have been used successfully (Hall 1988). The vaccine protects most breeds of cattle against severe clinical theileriosis, and has been frequently shown to be protective against heterologous as well as homologus challenge. A single vaccination is usually adequate, since the immunity produced is reinforced by subsequent tick challenge in the field. However, in exotic Friesian cattle it has been found necessary to follow up the primary immunisation with a second immunisation of heterologous schizonts from a low passage culture, in order to provide adequate protection against pathogenic effects (Pipano 1981).

The method of attenuation is not known, but a number of other changes have been identified in cultured schizont infected lymphocytes apart from loss of virulence. These include the loss of ability to differentiate into piroplasms (Pipano 1989), and changes in the level of a protease in a *T. annulata* macroschizont infected leucocyte cell line (Baylis et al 1992).

The schizont vaccine has been in use for about 20 years in Israel (Pipano 1981) where it was first developed and where it has reduced incidence of the disease dramatically (Brown 1990). The development of a similar vaccine in India has been reviewed by Singh (1990); effective vaccines

are also in various stages of development in other countries such as Iran, Russia, Morocco and Turkey (Hall 1988). Attempts to produce a schizont vaccine for *T. parva* have met with little success. While *T. parva* schizont infected lymphoblastoid cells can be successfully maintained in culture, they often fail to protect cattle when used for immunisation. The problem appears to be that the schizonts from the vaccine fail to transfer to host cells, which is a crucial step in the generation of *T. annulata* immunity (Dolan 1989). It is thought that BoLA mismatching inhibits the process in *T. parva*, but not in *T. annulata* (Musisi 1990).

Another form of immunisation with live parasites is by the "infection and treatment" method. This method, reviewed by Morzaria and Nene (1990) and Brown (1990), comprises of infecting cattle with live virulent sporozoites, from a cryopreserved stabilate or infected ticks, and treating with chemotherapeutic agents during the incubation period, usually tetracyclines but more recently buparvaquone. Concomitant administration of drugs reduces the parasitaemia and minimises the clinical symptoms, and good protection against homologous and usually heterologous challenge is obtained. The availability of the attenuated schizont vaccine for *T. annulata* means that the infection and treatment method is not widely used for *T. annulata* immunisation. The infection and treatment method is, however, an important means of immunising against *T. parva*; it is currently used in Kenya, Malawi, Zambia, Burundi and Rwanda.

While both these methods are successful in immunising cattle, there are a number of drawbacks of using live parasitic material. The parasites used for the infection and treatment method are fully virulent and vaccination can lead to a carrier status in cattle if piroplasms are produced which can be taken up by ticks and potentially spread to unprotected cattle, particularly dangerous if the vaccine stock is virulent (Musisi 1990, Morzaria and Nene 1990). This is not an expected problem in the case of the schizont vaccine, since fully attenuated cell lines should not be able to produce piroplasms. However, the schizont vaccine does not tend to prevent the formation of a carrier state in cattle once they are exposed to tick challenge, since piroplasms can form even though the cattle are protected against clinical symptoms (Hall and Tait 1991). This could become an increasing problem in the future as the vaccine is used more widely, with potential for reversion to virulence or selection for variation in whichever schizont

antigens the immune responses of cattle are directed. Another potential limitation is the need for a "cold chain" from the point of production to the point of immunisation; while cryopreservation circumvents many of the problems, the schizont vaccine has a limited shelf life once it is thawed. This could be a hindrance of the use of the vaccine in some less accessible parts of the world. A further possible hindrance of using live material is the potential for transfer of other pathogens in stabilates and cell cultures, unless they are effectively screened before use.

Other problems associated with the infection and treatment method but not with the schizont vaccine are the high cost of the drugs used, the difficulty in it's application on a large scale, the fact that it can be only be implemented by specially trained veterinary personnel and the lack of cross-protection between many antigenically diverse strains in *T. parva* (Musisi 1990). While the *T. annulata* schizont vaccine normally protects against heterologous challenge, cross-protection tends to be less common between *T. parva* stocks and the infection and treatment immunisation programmes generally use a "cocktail" of three different geographically isolated stocks. Another drawback is that the margin of error in the titrated stabilates used to inoculate cattle can be large. This is because the infectivity of stabilates can only be titrated by inoculation into susceptible cattle, which is impeded by the cost of the animals (Musisi 1990).

The problems associated with "live parasite" vaccination methods, whether real or potential, have resulted in several programmes of research aimed at defining possible candidate antigens for inclusion in subunit vaccines. For a molecular vaccine to have a significant advantage over the schizont vaccine for *T. annulata*, it should produce effective immunity, be cheap to produce, have a long shelf life and require only a single shot.

A number of antigens have been identified on the different life cycle stages of *T. annulata* as summarised in section 1.1.3, which are potential candidates for inclusion in a molecular vaccine. The potential for developing recombinant antigen vaccines for the control of *T. annulata* and *T. parva* has been reviewed by Musoke and Nene (1990). The recombinant sporozoite antigen p67 has already been used in a small vaccine trial where it successfully protected most of the cattle against homologous challenge (Musoke et al 1992). While these results suggest it is possible to engender

immunity by vaccination with sporozoite antigens alone, a vaccine would perhaps be more effective and reliable if it included recombinant macroschizont antigens as well as sporozoite antigens, due to the high reproductive capacity of the parasite in the cow. Otherwise even if only a small percentage of sporozoites broke through the immune responses of an vaccinated animal, each parasite would still have the capacity to develop into a macroschizont and produce thousands of merozoites. Such a multi-stage vaccine should also include antigens from the piroplasm/merozoite stage, to block transmission to a new host and to perhaps reduce the pathology associated with haemolytic anaemia in *T. annulata.* Inclusion of several different antigens in a molecular vaccine would also theoretically reduce the probability of selecting parasite strains with variation occuring simultaneously in epitopes of all the molecular vaccine components .

The reasons outlined above demonstrate the importance of examining all stages of the parasite to identify the immune mechanisms effective against them and the antigens involved. An effective molecular vaccine should activate antigen presenting cells capable of processing the recombinant antigen, and evoke appropriate T and B cell subsets capable of producing a protective immune response against the parasite. Therefore it is necessary to identify Tc and Th epitopes as well as B cell epitopes which produce effective anti-parasite immune responses. It is also important to investigate the effects of bovine MHC type and to choose an effective delivery system. In general, molecular vaccines should fulfill a number of objectives as follows. Ideally, the vaccine should include Th, Tc or B cell epitopes which enhance anti-parasite immune responses advantageous to the host, while excluding any Ts epitopes which may reduce it's effectiveness. The vaccine itself should have no pathological effects, therefore it should not include any epitopes whose recognition by the immune system abrogates rather than cures the disease. The vaccine should also avoid any hazards associated with the use of live material, such as reversion to virulence or contamination by pathogenic organisims. These and other considerations will be discussed in the next section.

1.2 Molecular vaccines

The following accounts summarise what is known about some of the immune mechanisms which act against parasites, and their effect on molecular vaccine design. Summaries will be given of the development of such vaccines, approaches taken to identify B and T epitopes on parasite and other antigens, and some of the commonly used expression systems and adjuvants available.

1.2.1 The immune response to parasites

Immunity to pathogens comprises of both innate responses ie. those which act non-specifically against any infectious agent and adaptive responses which are specific for each pathogen (described by Roitt 1986). Adaptive immunity involves clonal expansion of the cell population specific for the antigen, and the production of "memory" cells capable of a faster and more efficient response when the antigen is next encountered. Innate immunity comprises exterior defences such as the skin, phagocytic cells such as the monocyte/macrophage line and the polymorphonuclear neutrophil leucocytes (neutrophils, eosinophils, basophils and mast cells), the complement system, natural killer (NK) cells and soluble factors such as interferons, which have a role in NK activation. The action of many of these cells is enhanced or mediated through adaptive responses. For example, macrophages activated by lymphocytes of the adaptive immune system aquire microbicidal/cytocidal properties not seen in "innate" inflamatory reactions, and become capable of other cytocidal functions apart from phagocytosis, such as antibody dependent cellular cytotoxicity. Adaptive immune responses are produced by lymphocytes capable of recognising antigen and found in the blood, spleen, lymph fluid and lymph nodes. These are subdivided into antibody secreting B cells which mediate humoral immunity and T cells which mediate cellular immunity.

Antibodies may act in a combination of different ways against pathogens; through agglutination, lysis or attraction of phagocytic cells (opsonisation), all of which may or may not involve complement activation. Antibodies are classified into different subclasses according to their structure, namely IgA, IgD, IgE, IgG and IgM, which often have different

modes of action. B cell responses to a single antigen usually involves production of a combination of these subclasses. T cells are subdivided according to their mode of action into cytotoxic (Tc), helper (Th) and suppressor (Ts) subsets. Tc recognise and destroy target cells expressing foreign antigens through production of cytolysins, while Th cells control the development and function of effector cells through lymphokine secretion. Ts cells appear to inhibit the functions of effector and/or helper cells, but their action is still poorly characterised (Dorf et al 1992). These subsets can also be differentiated according to their cell surface markers (CD molecules in man), which can be identified by McAbs. In general, Th cells are CD4+ while Tc cells are CD8+. Homologous Th and Tc subsets have been identified in a number of animals, including cattle (Baldwin et al 1988). Two types of Th cell have been identified in mice; the Th1 subset which generally produce IFNγ, mediating macrophage activation and Th2 which tend to be producers of IL-4, IL-5 and IL-6, mediating B cell activation (Bottomly 1989) although these two functions are far from exclusive. Th1 and Th2 cells may have different induction requirements, depending on the cytokines required for their expansion (Hseih et al 1993, Janeway 1989). Similar T cell populations have been identified in man, although whether these are distinct subsets or not is still controversial (Bottomly 1989), and there is no evidence so far that they are present in cattle. Another lymphocyte subset which has also been identified in man are the "null" cells which are of uncertain lineage and function. These are classified as T cells although they lack any of the CD markers, but appear to have the $\gamma\delta$ T cell receptor. "Null" cells have been identified in cattle (Clevers et al 1990).

All of the immune responses described above have been shown to be important in parasite immunity, generally involving both innate and adaptive components. Eosinophils, neutrophils and macrophages have all been demonstrated in antibody mediated destruction of a number of species of filarial nematodes, and eosinophils have a critical role in the destruction of *Trichinella* larvae and schistosomes also mediated through antibody (Wakelin 1984). Macrophages have been shown to be important in the destruction of a number of protozoa, such as *Toxoplasma gondii* and *Leishmania.* The role of macrophages and NK type cells in the destruction of *T. annulata* schizont infected cells has already been described in section 1.1.3. NK cells are thought to also have a role in malaria immunity (Wakelin 1984). Specific immunity involving a combination of humoral, Th and Tc responses has

been demonstrated for the *P. falciparum* and *P.berghei* circumsporozoite proteins (Kumar et al 1988, Romero et al 1989), and the promastigote glycoprotein gp63 of *Leishmania major* (Jardim et al 1990). Th1 and Th2 cell subsets can have different roles in parasite infections; for example, it is thought that stimulation of the Th1 subset mediates protective immune responses to *Leishmania major*, while stimulation of the Th2 subset enhances the disease, possibly through inhibition of Th1 expansion (Locksley et al 1989). These two subsets may be preferentially stimulated by different antigens, according to the work of Yang et al (1991).

The Major Histocompatability Complex

The major histocompatability complex (MHC) products are a set of highly polymorphic cell surface molecules found on most mammalian cells which enable distinction between "self" and "non-self", by guiding T cell recognition of cell surface antigens (Roitt 1986). "Non-self" may be cells from another individual of different MHC type, or pathogen derived antigens carried on an antigen presenting cell. Two classes have been identified, both of which are in the form of heterodimers. MHC Class I molecules comprise of a 45kDa α chain encoded by MHC genes and a 12kDa non-MHC product, β_2 microglobulin. Class I molecules are expressed essentially on all cells of the body to various levels. MHC Class II molecules comprise of an α and β chain of molecular weight 27-35kDa, both encoded by MHC genes. Class II molecules are only found on specialised cells of the body, such as B cells and dendritic cells where they are expressed constituitively, on activated (but not resting) T cells and on macrophages where the level increases during activation.

MHC antigens and the genes coding for them have been best characterised in mice (H-2 antigens) and humans (HLA antigens). Homologous polymorphic MHC molecules have been identified in a number of other animals including cattle (BoLA antigens) where they have been characterised by serotyping, one-dimensional iso-electric focussing techniques and analysis of DNA restriction fragment length polymorphisms (Bernoco et al 1991). Immune response (Ir) genes have been identified in mice which map to part of the H-2 locus coding for Class II molecules. These genes appear to control the magnitude of the immune response to certain antigens. The response to ovalbumin in cattle has also been

correlated with certain BoLA Class II haplotypes, indicating that Ir genes are also important in cattle (Glass et al 1990). There is a similar link between the T cell response to *P. falciparum* circumsporozoite protein and MHC haplotype in humans (Good et al 1988b).

T/B cell co-operation

Adaptive immune responses to the majority of pathogens are T-dependent, which means they are dependent on T cells for both cellular responses and for B cell activation by the T helper subset in humoral responses. However, a small number of antigens are classified as T-independent, which means that antibody can be produced without the action of T helper cells. Such antigens are usually polymeric antigens from prokaryotes, such as polymeric bacterial flagellin, lipopolysaccharide, and antigens from the bacteria *Corynebacterium parvum* and *Brucella arbortus.* Secondary immune responses to such antigens generally resemble primary immune responses, being almost entirely confined to the IgM isotype (Austyn 1989). The degree that T-independent immune responses are really disconnected from T cell activity is a matter of some debate, since the magnitude of the response to several such antigens was shown to be influenced by T helper cells (Elkins et al 1991). In the majority of cases where T cell responses to parasite antigens have been analysed, such as the studies on *Leishmania* and *Plasmodium* antigens mentioned earlier in this section, the immune responses appear to be T cell dependent. The secondary response to T dependent antigens has several features. The response generally has a much shorter lag phase, a higher titre of antibody is produced and remains higher for a longer period. While primary humoral immune responses result in the production of mostly IgM, secondary immune responses involve other antibody subclasses such as IgG and IgE, and evidence suggests this class switching is mediated by T helper cells (Roitt 1986).

T and B cells differ in the way they recognise antigen. While B epitopes may be linear or conformational, most T cell epitopes are based on the linear sequence only. B cells recognise "free" antigen through binding of immunoglobin on their cell surface, while T cells can only recognise antigen presented in association with MHC molecules on the surface of an antigen presenting cell. Antigen recognition by T cells is via the T cell receptors, $\alpha\beta$

or γδ heterodimers. Tc cells recognise antigen in association with MHC Class I molecules and so can be stimulated by any cell in the body, while Th cells can only be presented with antigen in the context of MHC Class II, by specialised cells such as macrophages, dendritic cells and B cells (Austyn 1989). The sequence of events involved in the processing and presentation of antigen are still poorly understood. Two major types of processing appear to occur; the association of intracellular antigens with MHC Class I molecules and transport to the cell surface, or processing exogenous antigens by the endosomal pathway and association with MHC Class II molecules (Ash 1991). The biochemical basis of processing and association with MHC Class II was examined using hen egg lyzozyme (HEL) by Allen et al (1987). These workers found that HEL could only be recognised by T cells in the presence of live antigen presenting cells unless the antigen was already degraded into peptides, demonstrating that this was a prerequisate step for T cell activation, and were also able to demonstrate binding to MHC Class II molecules. Similar observations were made for a *Listeria monocytogenes* antigen (Unanue 1984). The cellular compartments in which these steps take place have not been confirmed, but Van Noort and coworkers (1991) demonstrated that proteases extracted from macrophage endosomes in a cell free processing system determined the regions of an exogenous protein antigen (sperm whale myoglobin) recognised by T cells, implicating the endosomes as an antigen processing site. It is still not entirely clear where association between processed antigen and MHC molecules occurs, but MHC Class I is thought to bind processed antigen in a pre-golgi compartment such as the endoplasmic reticulum, while MHC Class II molecules are thought to transit through the golgi apparatus and bind antigen in the endosomes, or another post-golgi compartment (Braciale and Braciale 1991), where they would intersect with the endocytic pathway.

The features of T/B cell co-operation have been reviewed by Abbas (1989). The help provided by T cells to B cells is directional, which means that B cells specific for the antigen can only be activated by T cells specific for the same antigen, and the interaction is restricted by MHC Class II components. Th cells induce proliferation and differentiation of small B lymphocytes into antibody secreting plasma cells through secretion of lymphokines and possibly through cell-cell contact. A system has been postulated whereby B cells internalise antigen bound to their immunoglobin receptors by pinocytosis, process and present it in the context of MHC Class

II to T cells recognising the antigen and capable of providing specific "help" (Lanzavecchicia 1985, Howard 1985). This model readily explains the specificity and MHC restriction of T cell help, and was demonstrated using human B and T cell clones specific for tetanus toxoid.

The importance of this mode of presentation compared with that of macrophages and dendritic cells is unclear. These cell types appear to be crucial for T cell priming, a process which is crucial before any adaptive immune responses can take place. This involves an immunostimulation step in which small, resting lymphocytes are activated and differentiate into large functional lymphoblasts, capable of responding to antigen presenting cells and undergoing clonal expansion to produce effector cells which can activate B cells and memory cells. This process takes about five days "in vivo" (Janeway et al 1989). It has been postulated, therefore, that dendritic cells and macrophages mediate primary immune responses, while B cells act as antigen presenting cells in secondary immune responses (Abbas 1989). However, while B cells are highly effective in presenting soluble antigen such as toxins, their poor ability to phagocytose particulate material is likely to make them poor presenters of particulate antigens such as bacteria and parasites, or recombinant antigens rendered insoluble by adjuvants such as Freund's incomplete adjuvant or alum (Janeway 1989).

1.2.2 The development of molecular vaccines

A vaccine can be defined as a substance capable, on inoculation, of inducing an immunological "memory" which the appearance of the antigen on a pathogen would be able to recall, possibly years afterwards. Early studies concerning the immunity engendered by molecular vaccines used "hapten-carrier" complexes. These consisted of a small molecule, a peptide or polysaccharide, linked to a larger molecule, usually a protein such as keyhole limpet haemocyanin, bovine serum albumin or ovalbumin (reviewed by Cease 1991). An example is foot-and-mouth disease virus epitopes linked to β-galactosidase as a carrier, which evoked FMDV specific antibodies on immunisation (Cease 1991). Immunisation with a hapten linked to a carrier generally produces an enhanced hapten specific response, compared with immunisation using the hapten alone; this is known as the "carrier effect". Carriers were found to elicite cell mediated immunity; usually being recognised by both T and B cells, while haptens generally are only

recognised by B cells. However, use of a heterologous carrier in vaccines fails to induce specific cellular immunity to the pathogen, and the immunity produced cannot be increased by natural boosting. It is now considered necessary to include both B and T cell epitopes derived from the same pathogen in molecular vaccines.

The current status of "new generation" or molecular vaccines is reviewed by Ada (1988), Ertl and Bona (1988), and Zanetti et al (1987). These include synthetic peptide, recombinant and anti-idiotypic vaccines. Recombinant vaccines are those produced using the DNA sequence for the antigen, or a part of it containing appropriate epitopes, expressed in a host cell; some of the expression systems available will be reviewed in section 2.2.5. Synthetic vaccines consist of short peptide sequences synthesised from amino acids, covering an epitope or epitopes of the primary antigenic sequence. Synthetic vaccines have been produced from a linked B and Th epitope of *P. falciparum* circumsporozoite protein (Good et al 1987) and from *Leishmania major* gp63 antigen using two peptides containing Th epitopes (Yang et al 1991) which were able to produce some protection from challenge with the parasite. The development of the multiple antigen peptide (MAP) system, in which multiple copies of the same or different synthetic peptides are linked to an "immunologically inert" core (Briand et al 1992) may facilate the development of "multiple epitope" synthetic vaccines. Anti-idiotype vaccines are basically antibodies directed against idiotypes; the part of an antibody that binds to an epitope (paratope) on the antigen. If anti-idiotype antibodies are raised against anti-pathogen antibodies, they can effectively immitate the "real" epitope by producing an "internal image" and evoke the same antibodies if used to immunise an animal. Anti-idiotype vaccines were found to induce some degree of protection against the parasites *Trypanosoma rhodesiense* and *Schistosoma mansoni* as well as to a number of viruses and bacteria (Ertl and Bona 1988). These vaccines are aimed at the production of humoral immunity; and while induction of antigen specific cellular immunity has been noted, it was not MHC restricted and so unlikely to be from T cells.

Effective vaccines for a large number of infectious diseases caused by bacteria and viruses such as whooping cough, Hepatitis B, measles, poliomyelitus, rabies, yellow fever and smallpox have been available for some time, made from the attenuated or killed pathogen. There has also been

considerable success in the development of molecular vaccines to bacterial and viral pathogens. Vaccines made from recombinant subunits of toxin from *Bordetella pertussis*, responsible for whooping cough, are already in use and others are under clinical trial (Pizza et al 1990) and a recombinant antigen is also under trial for Hepatitis B (Wang et al 1990). In contrast with the wide range of vaccines available for bacterial and viral diseases, there is a paucity available for diseases caused by parasites. Three that are available in the veterinary field are an attenuated larvae vaccine for *Dictyocaulus* infections in cattle (Wakelin 1984), an infected erythrocyte calf-attenuated vaccine for *Babesia* infections of domestic animals (Timms 1989) and the attenuated *Theileria* tissue culture vaccine described in section 1.1.4.

There are a number of factors that are problematic in the development of antiparasite vaccines. Parasites tend to display a complex series of antigens evoking a wide range of different forms of immune response as summarised in section 1.2.1. Parasites have also evolved varied strategies for avoiding or limiting immune attack, such as antigenic variation, adopting an intracellular environment, imitation of host molecules, immunocompromising the host and stimulation of immune responses to antigens not vital to the parasite's survival, such as shed antigen complexes (reviewed by Hyde 1990). In some cases, such as malaria, complete natural immunity never develops even in endemic areas (Miller and Good 1988, Good et al 1988a). A vaccine would only be effective in these instances if it could engender more effective immunity than that evoked by the parasite itself (Hyde 1990). There has been more success in the development of anti-parasite molecular vaccines in cases where long lasting immunity does develop naturally in animals that recover; this is the case in the three parasite vaccines mentioned above, and in cestodes such as *Taenia* species. A vaccine is now being commercially developed for *T. ovis* in sheep using a recombinant onchosphere antigen (Johnson et al 1989), and promising results have been obtained using a similar antigen from *T. taeniformis* (Ito et al 1991).

In order to develop molecular vaccines, it is necessary to have some knowledge of the immune responses to the parasite, identifying appropriate antigens and the types of immunity they evoke. In the case of T dependent immune responses, it is necessary that both T and B epitopes are present on the immunising polypeptide which are recognised by the two partner cells in

the co-operation (Celada and Sercarz 1989). It is therefore necessary to properly define the humoral and cellular responses that occur to a given antigen, evaluating the roles of both the Th and Tc subsets. The method of delivery and the innate immune responses produced by any adjuvant that is included have a considerable influence on the form and duration of the immunity induced, as will be discussed in sections 2.2.5 and 2.2.6. Therefore it is important to ensure that systems chosen for expression and delivery of the antigen, and the choice of adjuvant, are appropriate for the types of specific immunity required. The approaches taken to identify immunodominant B and T cell epitopes, and the expression systems and adjuvants available, are reviewed in the following sections.

1.2.3 Approaches to mapping B cell epitopes

Antigenic sequences of interest containing B cell epitopes recognised by protective McAbs or immune antisera are usually identified directly or indirectly by screening of a recombinant genomic expression library in a vector such as λgt11, as described for *Mycobacterium tuberculosis* (Young et al 1985), *Taenia taeniformis* (Ito et al 1991) and *T. parva* (Iams et al 1990a).

As mentioned in section 1.2.1, B cells are capable of recognising continuous (linear) determinants of the primary sequence of an antigen, or discontinous (conformational) epitopes brought together by secondary or tertiary folding. Indeed, it has been suggested by some workers (Barlow et al 1986, Laver et al 1990) that in fact all B cell epitopes are discontinuous, and linear determinants that are identified on antigens only represent the primary binding site rather than the complete epitope. The ability of part of an antigen to be recognised as a B cell epitope is influenced by a number of factors such as hydrophilicity, antibody accessibility, mobility and protrusion from the rest of the molecule (Barlow et al 1986, Miles et al 1989). There are a number of computer algorithms available for predicting B cell epitopes from the primary amino acid sequence of an antigen, such as the algorithm described by Hopp and Woods (1981) based on points of highest local hydrophilicity. While these programs have some success their use is limited to predicting linear determinants only, and even then they do not always correspond to epitopes identified experimentally.

There are numerous methods for the experimental identification of B cell epitopes, such as Western blotting, expression library screening and enzyme linked immuno-absorbant assay (ELISA). Epitope mapping has been facilitated in recent years by the introduction of multiple peptide synthesis (the "Pepscan" technique) in which short, overlapping peptides based on the primary sequences of antigens are synthesised on polyethelene rods and used directly in ELISA tests (Geysen 1984). This technique has been used to identify epitopes recognised by antisera or McAbs on a number of antigens, such as the *P. falciparum* 51kDa merozoite surface antigen (Epping et al 1988) and the *P. chabaudi adami* merozoite antigen precursor (Lew et al 1989). Longer recombinant antigenic fragments can also be used in ELISA tests and Western blotting; overlapping recombinant fragments created by exonuclease deletion and expressed in *E. coli* were used to map McAb epitopes of canine parvovirus (López de Turiso et al 1991) and *Mycobacterium bovis* (Thole et al 1988). The use of recombinant antigenic fragments can provide preliminary data on the location of B cell epitopes before undertaking more detailed analysis using peptides. Another approach using recombinant antigens is to construct an expression sublibrary in a vector such as λgt11 with fragments of the antigen gene, and screen the clones with McAbs or antisera. This approach was used to map epitopes recognised by McAbs to *Mycobacterium leprae* (Mehra 1986).

It should be noted that most of these techniques are limited to mapping linear determinants; small peptides used in ELISA tests would have little folding ability due to the absence of surrounding determinants and Western blotting results in the dissociation of secondary and tertiary structure. There is a paucity of methods suitable for identifying conformational B cell epitopes. ELISA tests using recombinant antigens of sufficient length to show some protein folding may identify conformational epitopes, and competition assays between different McAbs or McAbs and antisera can provide information on the number of immunodominant B cell epitopes on a given antigen. This latter approach was used to investigate immunodominant B cell epitopes of *P. falciparum* circumsporozoite protein, using native sporozoite antigen in a competition ELISA (Zavala et al 1983). It has also been suggested that the Pepscan technique could be used to identify conformational B cell epitopes, through analysing the various peptide sequences recognised by a given McAb, starting at the dipeptide level (Miles et al 1989). In this way it is theoretically possible to reconstruct a

"mimotope", by identifying the combination of sequences that could make up the three-dimensional structure of an epitope.

1.2.4 Approaches to mapping T cell epitopes

Antigens of interest selected for T cell epitope mapping are often initially identified using monoclonal or polyclonal antibodies to screen a genomic expression library as described in the previous section, although the products of expression libraries can also be used directly to stimulate T cells from an immune animal in proliferation assays, as described by Lamb et al (1987).

In contrast to B cells, T cells tend to recognise a small number of immunodominant sites on an antigen; this has been demonstrated for myoglobin (Berzofsky et al 1987) and hen egg lyzozyme (Adorini 1988). A number of factors govern which epitopes are immunodominant. These include features that are intrinsic to the amino acid sequence itself; regions containing T cell epitopes often correlate with their ability to form an amphipathic α helix, with hydrophilic and hydrophobic amino acid residues segregated on opposite sides of the secondary structure (Bezofsky et al 1987). It is thought that these structures have a tendency to be recognised by T cells because of their ability to bind MHC molecules; the hydrophobic side probably binds to the MHC on an antigen presenting cell while the hydrophilic side of the structure can associate with the T cell receptor. The MHC haplotype of the host animal also appears to influence which epitopes are recognised. This was demonstrated in the response of mice to hen egg lyzozyme; the epitopes recognised "in vivo" were found to depend on the MHC Class II haplotype of the mouse strain, and binding of different peptides derived from the same antigen by different MHC Class II antigens could be demonstrated "in vitro" (Adorini 1988). These workers were able to demonstrate that peptides processed from the same antigen could compete for binding to an MHC Class II molecule. MHC Class II haplotype has also been shown to determine T cell epitope recognition of *P. falciparum* circumsporozoite protein (Good et al 1988b) and myoglobin (Berkower et al 1984) by different strains of mice. Other factors that influence immunodominance are the T cell subset activated; activation of suppressor T cells could inhibit the action of helper or cytotoxic T cells recognising

different epitopes, and the presence of T cells in the repertoire capable of recognising the peptide/MHC complex (Adorini 1988).

T cell epitopes can potentially be more readily predicted from the primary amino acid sequence of an antigen, since they only recognise the antigen after it has been denatured and processed into peptides. There are a number of computer algorithms available which can predict T cell epitopes from the primary sequence with reasonable accuracy. These include the algorithm to predict amphipathic α helices developed by Berzofsky et al (1987) and an algorithm to predict primary sequences in a structure that are common among a data base of previously defined T cell epitopes (Rothbard and Taylor 1987). The usefulness and limitations of these predictive algorithms will be discussed further in Chapter 6.

The binding affinity between an antigenic peptide and a particular MHC Class I or Class II molecule appears to be determined by only a few residues, known as "anchors" due to their property of fitting into "pockets" inside the groove of MHC molecules. Anchor motifs have been defined for a number of MHC Class I and Class II alleles by sequence comparisons between MHC restricted peptides (Hobohm and Meyerhans 1993). Prediction of anchor motifs can be used as another method to search for epitope containing sites in antigen sequences, and may facilitate the development of synthetic peptides with multiple MHC specificity. The association of H-2 MHC Class I molecules on the surface of cells with synthetic peptides corresponding to anchor motifs predicted from ovalbumin and influenza nucleoprotein has been demonstrated (Rock, Rothstein and Bernacerraf 1992).

The methods for identifying T cell epitopes are reviewed by Sinigaglia et al (1991). T cell epitopes can be defined experimentally using T cells derived directly from the blood, spleen or lymph nodes of an immunised animal, or cultured "in vitro" in the presence of antigen or IL-2 to produce an antigen specific line or clone. Responsiveness to an antigen can be determined in a number of ways. Proliferation assays involve culturing the cells in the presence of the test protein with ^{3}H thymidine, which is incorporated into the DNA of proliferating cells, then harvesting the culture and measuring the amount of radioactivity incorporated using a β-counter. The use of this method for the mapping of *P. falciparum* circumsporozoite

protein Th cell epitopes has been described by Sinigaglia et al (1991). Although it is primarily used for mapping Th cell epitopes, antigen specific Tc cell lines and clones will also proliferate in these assays (Tetzlaff et al 1992). An alternative method for detection of Th cell activity is to assay for IFNγ as described by Whalgren et al (1991), which is produced by proliferating T cells. In mice, where Th cells can be divided into subsets, IFNγ secretion is predominantly by cells of the Th1 subset as described in section 1.2.1. Antigen specific Tc cells can be identified by their cytolytic activity on radiolabelled target cells presenting test peptides, as described by Romero et al (1989) and Kumar et al (1988). These workers used this method to map Tc epitopes of *Plasmodium* circumsporozoite proteins. The T cell subsets involved in any of these assays can be verified by cell surface phenotypic analysis.

T cell epitopes can be determined experimentally by using fragments of the molecule produced by recombinant DNA techniques, proteolytic digestion or peptide synthesis; some examples of these methods are given below. Generally, the first two methods are useful in determining larger epitope containing regions, with more detailed analysis being undertaken using synthesised peptides. Regions of a *Mycobacterium leprae* 65kDa antigen containing T cell epitopes were identified using fragments of the antigen prepared by specific chemical and proteolytic cleavage and used in proliferation assays (Demotz et al 1989), but this method is limited to antigens containing appropriate sites. Recombinant fragments of the antigen of interest can be generated from it's coding sequence by selection of appropriate restriction enzyme sites or exonuclease digestion. Recombinant fragments were generated from the 65kDa antigen of *M. bovis* for use in proliferation assays, by producing and expressing a series of overlapping deletions of the gene using exonuclease (Thole et al 1988). The products of a recombinant DNA expression sub-library of *M. bovis* MPB70 coding sequence in λgt11 were used in proliferation assays to identify the T cell epitopes recognised by an immune cow (Lamb et al 1987).

The Pepscan technique described for mapping B cell epitopes in section 1.2.4 can also be applied to T epitope mapping, by releasing the synthesised peptides from the polypropylene pins using formic acid (Miles et al 1989). This has been applied successfully to identify Th epitopes of *M. bovis* 65kDa antigen (Van der Zee et al 1989), the gp63 glycoprotein of

Leishmania major (Yang et al 1991) as well as Tc epitopes on the circumsporozoite proteins of P. berghei (Romero et al 1989) and *P. falciparum* (Kumar et al 1988). Another approach is to identify regions of an antigen most likely to contain T cell epitopes using predictive computer algorithms, then to synthesise peptides covering these regions for use in experimental analysis. This method was used to map Th epitopes of *L. major* gp63 antigen (Jardim et al 1990) and *P. falciparum* clustered-asparagine-rich protein (Whalgren et al 1991).

1.2.5 Expression and delivery systems

Expression in E. coli

While intracellular expression of recombinant antigens in *E. coli* is often a cheap and effective means of producing large quantities of a protein, there are a number of problems associated with expressing a eukaryotic coding sequence in a prokaryotic host, reviewed by Marston (1986) and Kelley and Winkler (1990). These include lack of glycosylation, incorrect disulphide bond formation, degradation, accumulation as insoluble inclusion bodies and lethality of the expressed product to the host cell. Prokaryotic cells cannot carry out post-translational modifications such as proteolytic processing or recognition of sequences specifying carbohydrate attatchment, which can pose problems since many parasite surface proteins are glycoproteins. It is thought that poor formation of disulphide bridges is a result of the more reducing environment that exists in *E. coli* cells compared with eukaryotic cells. Both of these factors can effect the tertiary structure of the expressed protein. The problems of degradation or insolubility may be caused by recognition of the expressed protein as "foreign" and potentially toxic, although inclusion body formation is also likely to result from incorrect tertiary folding of the protein for the reasons given above. Inclusion body formation can be used as a means of purifying the recombinant protein, provided it can be denatured and refolded correctly.

The vectors available for expression in *E. coli* either enable direct expression of a eukaryotic coding sequence using bacterial promotors and terminators, or expression as a fusion protein with a bacterial polypeptide such as β-galactosidase. While the latter choice is thought to render the protein less "foreign" and toxic to the host cell, it can effect the

immunological properties of the product unless the prokaryotic product can be cleaved off. The problem of incorrect folding in the prokaryotic environment can sometimes be circumvented by secretion into the periplasmic space of the bacterium. A recently developed set of vectors which can be used for this purpose is the pTO-N system (Deng et al 1990). These vectors allow production of the eukaryotic protein fused to a prokaryotic signal sequence (omp A), which codes for secretion through the inner membrane where the signal sequence is cleaved off.

Salmonella typhimurium

Attenuated strains of *S. typhimurium* have also been used to express recombinant coding sequences inserted into the bacterial genome, and are expressed constituitively by the bacterium. The host is immunised with the whole live cells, which are attenuated so they are only capable of limited growth "in vivo". The advantages of using *S. typhimurium* as an expression and delivery system are that it is not necessary to purify the antigen, the ability of the bacteria to induce cellular immunity and the fact that inoculation is by the oral route. Following oral administration in humans, attenuated *S. typhimurium* strains are found in the lymphatic system where they are ingested by macrophages; the ability to engender cell mediated immunity appears to rely on survival within the macrophage for a short time (Sadoff et al 1988).

This expression system, therefore, is useful for the production of parasite antigens which are the targets for cell mediated immune responses. Oral administration of *S. typhimurium* transformed with the circumsporozoite protein of *P. berghei* was found to produce cell-mediated but not humoral immune responses and partial protection from challenge in mice (Sadoff et al 1988). A similar approach using the *L. major* gp63 antigen evoked both T helper cell mediated and humoral immunity in mice, some of which were subsequently protected from challenge (Yang et al 1990).

Eukaryotic cells

There are a number of vectors available for the expression of parasite antigens in yeast and higher eukaryotic cells, such as the SV40 based pCYM vectors, which have been developed for heterologous expression in cultures

of *Saccharomyces cerevisiae* and mouse cell lines (Camonis et al 1990). Additionally, vaccinia virus has been used to express a number of antigens in mammalian cells from a range of species both "in vivo" and "in vitro" (reviewed by Moss et al 1988). Expression in eukaryotic cells avoids the problems caused by lack of post-translational modification in prokaryotic hosts.

The vaccinia virus can harbour up to 20-25 kb foreign DNA in it's genome without effecting it's ability to infect host cells. The vector is particularly effective for expressing antigens capable of inducing cell mediated immunity. Mouse L cells transformed with recombinant vaccinia virus expressing the circumsporozoite protein of *P. falciparum* have been used to identify both Tc responses (Kumar et al 1988) and Th activity (Good et al 1987) in immunised mice.

An alternative vector system which has been used extensively to express foreign proteins in eukaryotic cells are the baculoviruses. These viruses are infective to invertebrate cells only, and have been used extensively for high level expression of recombinant proteins in insect cell cultures. The expression of a number of proteins, including glycoproteins in this system has been described by Matsuura et al (1987).

1.2.6 Adjuvants

Adjuvants are substances included in a vaccine which potentiate non-specific immune responses. They may be entirely of bacterial origin such as *Bordetella pertussis* or *Corynebacterium parvum*, simply an oil emulsion such as Freund's incomplete adjuvant (FIA), aluminium hydroxide based (alum), a plant extract such as saponin or a bacterial extract in an oil emulsion base; for example Freund's complete adjuvant (FCA) and muramyl dipeptide (MDP). Freund's complete adjuvant consists of mycobacterial extract in a mineral oil base, while the recently developed MDP is the smallest subunit of mycobacterial antigen that can still potentiate the immune response. The effects of these different adjuvants on the cell mediated and humoral responses of cattle immunised with *Trypanosoma brucei* is described by Wells et al (1982). These workers showed there were marked differences in the strength of the immune responses to this antigen in the presence of different adjuvants, with FIA, CFA, MDP and saponin proving to be the most

effective. Since many of the more effective adjuvants, such as CFA, produce undesirable reactions in the animal, there is a considerable research effort into developing new effective formulations without this drawback, such as the Syntex Adjuvant Formulation reviewed by Byars et al (1991). Another group of potentiating agents are liposomes; small lipid membrane vesicles in which the antigen can be incorporated. Liposomes prepared from the capsular polypeptide of *Streptococcus pneumoniae* induced a protective immune response in mice against the bacterium (Snippe, Verheul and van Dam 1989) and trials have been carried out with a liposomal malaria vaccine incorporating peptides from the *P. falciparum* circumsporozoite protein (Alving et al 1989).

The mode of action of adjuvants are poorly understood. In general they appear to activate antigen presenting cells and stimulate lymphocyte proliferation (Hyde 1990) and are important in priming "in vivo" (Janeway 1989). Adjuvants such as alum and oil emulsions render the antigen particulate, enabling it to be phagocytosed by macrophages (Janeway 1989). These types of adjuvants also slow down the rate of antigen release producing a "depot effect", enabling the antigen to be exposed to the immune system over a longer period of time. Liposomes have also been shown to be avidly taken up by macrophages (Alving et al 1989). Different adjuvants can influence the types of humoral or cell mediated immune responses evoked, as reviewed by Bomford et al 1991. They found that while FIA stimulated a strong IgG_1 antibody response in guinea pigs, FCA and saponin evoked IgG_2 and cellular reactions. These workers suggested that these differences could be accounted for by selective induction of different Th subsets. Furthermore, the type of adjuvant included in immunisations of mice with *P. falciparum* major merozoite antigen was found to influence the epitope specificity of the antibodies produced (Hui et al 1991). It is therefore important that an adjuvant chosen for immunisation with a molecular vaccine is appropriate for the type of immunity required, and this in turn depends on a knowledge of the immune response to the pathogen.

1.3 Objectives of this work

The *T. annulata* sporozoite antigen SPAG1 includes the epitope for McAb 1A7 in the C-terminal SR1 region and is recognised by sera from

sporozoite immunised cattle, as already described in section 1.1.3. The sporozoite neutralising activity of McAb 1A7 and bovine λgt11-SR1 antisera indicates that SPAG1 has a role in the recognition and/or invasion of host cells, and is a possible candidate for inclusion in a molecular vaccine. So far, only humoral responses to native sporozoites (Preston and Brown 1985) or λgt11-SR1 recombinant protein (Williamson et al 1989) have been identified. However, it is also likely that a T-helper cell response is elicited to sporozoites, provided the antibody response is T-cell dependent as already discussed in section 2.2.1.

The primary objectives of this project were to verify whether a bovine T cell response was induced to SPAG1, and to attempt to define regions of the antigen containing T and B cell epitopes. The importance of the identification of T and B cell epitopes in molecular vaccine design has already been discussed in section 2.2.2. It was first necessary to express and purify the SPAG1 antigen, for the purpose of immunising cattle. An expression system in *E. coli* using the pGEX vectors (Smith and Johnson 1988) was chosen for this, which will be described in Chapter 4. Despite the drawbacks of expression in a prokaryotic host already summarised in section 1.2.5, this is an economical method of producing large quantities of the antigen provided expression is successful. Secondly, it was necessary to produce a range of subclones of different regions of the SPAG1 molecule for use in B and T cell epitope mapping. Work on the generation and expression of SPAG1 subclones and B cell epitope mapping was a collaborative effort with Dr. R. Hall, Dr. E. Hennessey, and Ms. N. Boulter (Department of Biology, University of York). The B cell response was analysed by Western blotting and ELISA, and T cell responses assessed by producing T cell lines and clones from animals immunised with recombinant SPAG1. These approaches will be discussed further in Chapter 5 and Chapter 6 respectively. Since the immunisation of cattle with recombinant SPAG1 was the first vaccination trial carried out using the full length molecule, the humoral response to native sporozoite antigen and the response of the animals to challenge with *T. annulata* sporozoites were also assessed.

In addition, polymorphism of the SPAG1 molecule was investigated, as this has a bearing on the immune response to the antigen if it occurs within a B or T cell epitopes. Work was also undertaken to ascertain the exact location of the SPAG1 molecule on sporozoites and it's potential role in

recognition or invasion of host cells using immuno-electron microscopy techniques. It was considered important to take into account the biological role of this antigen in the sporozoite when investigating the bovine immune responses to it, since the most effective immune responses may well be directed against those parts of the antigen essential to the parasites survival.

As described in section 1.1.3, a second *T. annulata* sporozoite McAb has been identified, McAb 4B11, directed against a different set of sporozoite antigens according to Western blot analysis (Williamson 1988). The final part of this project was work towards the identification, characterisation and expression of part of the sporozoite antigen containing the 4B11 epitope. Like SPAG1, this antigen may also be involved in host cell recognition and/or invasion, and could be another potential vaccine candidate, since McAb 4B11 is effective in neutralising sporozoite infectivity "in vitro". It was anticipated that by the end of the project, a more complete picture of the biological role and immune response to these two major sporozoite antigens would be obtained.

CHAPTER 1

MATERIALS AND METHODS

2.1 Materials

The following were used and referred to in the text by the abbreviations given.

2.1.1 Buffers

10x PBS

1.5 M NaCl
160mM $Na_2HPO_4.2H_2O$
40mM $NaH_2PO_4.2H_2O$
made up in H_2O, pH adjusted to 7.3 using HCl.

10x TE

100mM Tris base
10mM EDTA, diluted from 0.5M EDTA pH 8.0 stock solution
made up in H_2O, pH adjusted to 7.5 using HCl.

0.5M EDTA pH 8.0

0.5M disodium ethylenediaminetetra-acetate.$2H_2O$ made up in H_2O and pH adjusted to 8.0 with NaOH pellets (approximately 2g/100ml)

1M Tris-HCl pH 8.0

1M solution of Tris base made up in H_2O with 42ml HCl per litre, and the pH adjusted to 8.0.

1M Tris-HCl pH 7.5

1M solution of Tris base made up in H_2O with 50ml HCl per litre, and the pH adjusted to 8.0.

2.1.2 Tissue culture media

TBL medium

0.07% sodium bicarbinate
0.75μg ml^{-1} Fungizone (amphotericin B)
10u ml^{-1} penicillin/streptomycin
10μM 2-mercaptoethanol
20% heat inactivated foetal calf serum
made up in RPMI 1640 25mM HEPES (Gibco) and filter sterilized

RPMI 16% FCS

100iu ml^{-1} benzyl penicillin
100μg ml^{-1} streptomycin sulphate
2mM L-glutamine
16% heat inactivated foetal calf serum
made up in RPMI 1640 25mM HEPES (Gibco) and filter sterilized.

Eagles MEM 3.5% BSA

3.5% bovine serum albumin
200iu ml^{-1} benzyl penicillin
200μg ml^{-1} nystatin
made up in Eagles MEM medium (Gibco) with Hanks salts and filter sterilized.

Eagles MEM without BSA

made up as described above, but BSA not included.

MLC medium

10% foetal calf serum
2mM L-glutamine
5μg ml^{-1} gentamicin
10μM 2-mercaptoethanol
made up in RPMI 1640 25mM HEPES (Gibco) and filter sterilised.

FACS medium

5% horse serum
0.02% sodium azide
made up in RPMI 1640 25mM HEPES (Gibco).

2.1.3 Bacterial culture media

Luria-Bertani (LB) medium

10gms Bacto-tryptone
5gms Bacto yeast extract
10gms NaCl
per litre H_2O and sterilized by autoclaving (20minutes at 15lb/ square inch, 120°C). $MgSO_4$ and glucose was added to 10mM and 0.2% respectively before use.

LB Agar

1.5 gms Difco Agar was added per 100ml LB medium prior to autoclaving. $MgSO_4$ was added to10mM before pouring the plates.

2xTY medium

16gms Bacto-tryptone
10gms yeast extract
5gms NaCl
per litre H_2O and sterilized by autoclaving.
Glucose added to 0.2% before use.

2xTY agar

1.5gms Difco agar added per 100ml 2xTY medium prior to autoclaving.

Ampicillin stock

100mg ml^{-1} ampicillin (sodium salt) made up in H_2O, sterilized by filtration and stored at -20°C.

5-Bromo-4-chloro-3-indolyl-β-D-galactoside.(X-gal) stock

20mg ml^{-1} made up in dimethylformamide. Aliquots stored at -20°C wrapped in aluminium foil to exclude light.

1M Isopropylthio-β-D-galactoside (IPTG) stock

238mg ml^{-1} made up in H_2O and filter sterilized. Aliquots stored at -20°C.

2.1.4 Solutions for extracting eukaryotic DNA

Sodium acetate/SDS solution with Proteinase K

0.3M sodium acetate

10mM Tris -HCl pH 7.9
1mM EDTA
1% SDS
200μgml^{-1} Proteinase K
made up in H_2O

Proteinase K

Stock solution made up fresh at 10mg ml^{-1} in H_2O

RNAase A

Stock solution made up at 20mg ml^{-1} in H_2O, boiled for 15minutes to destroy DNAases and stored in aliquots at -20°C.

Dialysis tubing

Dialysis tubing 1.5cm in diameter was cut into 20cm strips, autoclaved (20minutes at 2.2psi,120°C) in TE buffer and stored at 4°C.

2.1.5 Solutions for extracting plasmid DNA

20% Sucrose solution

100mM Tris-HCl pH 8.0
50mM EDTA pH 8.0
20% sucrose
made up in H_2O and autoclaved.

Chloroform/Isoamyl alcohol (IAA)

1part IAA equilibriated with 24 parts chloroform.

Phenol

Phenol was equilibriated wiith TE buffer and stored at 4°C until use.

Phenol/Chloroform/Isoamyl Alcohol (IAA)

50% phenol and 50% chloroform/IAA was equilibriated wiith TE buffer and stored at 4°C until use.

2.1.6 Solutions for working with bacteriophage λ

SM phage dilution buffer

0.1M NaCl
8mM $MgSO_4.7H_2O$
50mM Tris.HCl (diluted from 1M Tris HCl stock solution)
made up in H_2O and sterilised by autoclaving. Gelatin added to 0.07% prior to use.

NZCYM medium

10gms NZ amine
5gms NaCl
5gms bacto-yeast extract
1gm casamino acids
2gms $MgSO_4.7H_2O$
dissolved per litre H_2O. pH adjusted to 7.0. Sterilised by autoclaving.

2.1.7. Solutions for agarose gel electrophoresis

10x Tris-borate (TBE)

0.9M Tris base
0.9M boric acid
20mM EDTA (diluted from 0.5M stock solution pH 8.0)
made up in H_2O

50x Tris-acetate (TAE)

2M Tris base
20mM EDTA (diluted from 0.5M stock solution pH 8.0)
57.1ml per litre glacial acetic acid
made up in H_2O

Low melting point and high melting point electrophoresis agarose were purchased from Sigma.

Ethidium bromide stock solution

Made up in H_2O at 10mg ml^{-1}.

Molecular weight markers

1kb ladder (BRL), fragment sizes 75bp-12.2 Kb
Made up according to the manufacturer's instructions.

2.1.8. Solutions for Southern blotting

20x SSC

3M NaCl
0.3M sodium citrate
made up in H_2O and pH adjusted to 6.8

Denaturation solution

0.5M NaOH
1M NaCl
made up in H_2O.

Neutralisation solution

0.5M Tris-HCl
3M NaCl
made up in H_2O

20% Sodium dodecyl sulfate (SDS)

20% electrophoresis grade SDS dissolved in H_2O, heating to 68°C to assist dissolving. pH adjusted to 7.0 with NaOH.

SDS hybridisation solution

0.5M Na_2HPO_4 pH 7.2
7% SDS
1mM EDTA
in H_2O

5% SDS phosphate buffer

40mM Na_2HPO_4 pH 7.2
1mM EDTA
5% SDS
in H_2O

1% SDS phosphate buffer

40mM Na_2HPO_4 pH 7.2
1mM EDTA
1% SDS
in H_2O

1M sodium hydrogen phosphate

1M Na_2HPO_4

0.34% orthophosphoric acid
in H_2O

2.1.9. Solutions for SDS polyacrylamide electrophoresis

Solutions for 10%, 12% and 15% SDS-polyacrylamide gels were made up as described by Sambrook, Fritsch and Maniatis (1989). 30:0.8 acrylamide:bisacylamide mix was purchased from BRL.

4x sample buffer

200mM Tris HCl (diluted from 0.5M Tris HCl pH 6.8 stock solution)
40% glycerol
8mM EDTA
8% SDS
4% 2-mercaptoethanol
few grains Bromophenol Blue
in H_2O

5x gel running buffer

7.7M glycine
1M Tris base
2% SDS
40mM EDTA
in H_2O

Coomassie blue R250 stain

0.5mg ml^{-1} Coomassie blue R250 powder dissolved in 30% methanol, 10% acetic acid in H_2O.

Destaining solution

30% methanol and 10% acetic acid in H_2O.

Molecular weight markers

High and low molecular weight markers (SDS-6H and SDS-7, Sigma) were made up according to the manufacturer's instructions.

2.1.10. Solutions for Western blotting

Transfer buffer

200mM glycine
27mM Tris base
5% methanol
made up fresh in H_2O, pH adjusted to 8.3.

10x Ponceau S

2% Ponceau S powder
1.8M trichloroacetic acid
1.18M sulfosalicyclic acid
made up in H_2O.

10x Tris saline

100mM Tris
1.5M NaCl
made up in H_2O, pH adjusted to 7.4.

Blocking buffer

5% non-fat skimmed milk powder
10% horse serum
0.02% sodium azide
in 1x Tris-saline.

Tris Tween

0.1% Tween 20 in 1x Tris saline

Diethanolamine developing solution

5mM magnesium chloride hexahydrate
1.05% diethanolamine
made up in dH_2O, pH 9.5
0.033% nitro blue tetrazolium (prepared in 70% dimethyl formamide) 0.016% bromochloroindolyl phosphate (prepared in 100% dimethyl formamide) added per 10ml developing solution just before use.

2.1.11. Solutions for use in enzyme linked immunosorbant assay (ELISA)

PBS 0.05% Tween

0.05% Tween 20 in PBS (2.1.1)

PBS-Tween-BSA

As for PBS Tween but containing 0.1% normal bovine serum.

BSA blocking buffer

1% BSA in PBS

Peroxidase substrate solution

1mg ml^{-1} o-phenylenediamine

0.1M sodium citrate pH 4.5

Hydrogen peroxide is added to a final concentration of 0.01% just before use.

2.2 Methods

2.2.1 Preparation of parasite material

All the tissue culture techniques described were performed under sterile conditions.

Culture of macroshizont cell lines from cryopreserved stocks

TBL medium (2.1.2) was warmed to 37°C and dispensed into 10ml centrifuge tubes in aliquots of 5ml. Vials containing cryopreserved cells were thawed at 37°C, and the contents added to a 5ml aliquot of medium before centrifuging at 1100g for 5 minutes at 22°C. The cells were washed a second time and the medium discarded. 5ml fresh medium was added and the cells resuspended by gentle shaking before transfering to a $50cm^3$ tissue culture flask. The appearance of the cells was checked under a bifocal optical microscope and the flask incubated at 37°C in 5% CO_2.

An additional 5ml medium was added to the flask after 24 hours, and the culture maintained by diluting 1:10 with fresh medium every second day. For DNA preparations, the culture was diluted up to 100ml in a $150cm^3$ flask and cultured for 2-3 days until the cells had grown to high density before harvesting (10 minutes at 500g). The cells were washed twice in PBS before DNA extraction.

Preparation of piroplasms from infected blood

Piroplasm infected bovine blood was first depleted of leucocytes by centrifugation at 1300g for 5 minutes and removing the buffy coat, and washed 4-5 times in PBS. The erythrocyte fraction was passed through CF11 columns and piroplasms released from infected erythrocytes by the ammonium chloride lysis method (Martin et al 1971).

Preparation of sporozoites from infected ticks

Sporozoites were prepared as ground up tick supernatant (GUTS) for the purposes of Western blotting or "in vitro" sporozoite invasion assays, ground up tick salivary gland supernatant (GUTSGS) or purified on Percoll for Western blots for which purer preparations of sporozoites were required. In all cases, the concentration of sporozoites in the final preparation was assessed by light microscope observation of Giemsa stained cytospin smears (described in section 2.2.12).

Sporozoites were prepared in the form of sterile GUTS from *T. annulata* infected adult *Hyalomma anatolicum* ticks fed for 3-4 days on a rabbit to allow maturation of the parasites. The ticks were detached manually, rinsed in water, surface sterilised and the GUTS prepared according to methods described (Brown 1987). The ticks were ground on Eagles MEM 3.5% BSA (2.1.2) for use in invasion assays, or in Eagles MEM without BSA for use in Western blots. The GUTS was passed through a 8μ filter to remove particulate tick debris and either used directly or further purified on a Percoll gradient according to the protocol given by S. Williamson (1989).

Sporozoites were also prepared from ground up tick salivary gland supernatant (GUTSGS) from *T. annulata* infected adult *Hyalomma anatolicum* ticks according to the following method. The ticks were fed on rabbits for 3-4 days to allow maturation, washed and surface sterilised as described above. The ticks were embedded dorsal side uppermost in sterile wax in a petri dish, covered with sterile PBS and dissected aseptically under the x20 objective of a light microscope. The dorsal integument was removed by cutting round the midline, the paired salivary glands freed from the surrounding midgut and tracheal tissues, the main salivary ducts were severed just behind the mouthparts, and the two glands were transferred to 0.5ml PBS in a small petri dish. After dissection of all the ticks, the pooled salivary glands were transferred to 1ml PBS in a tissue grinder (a 1ml Griffiths tube) and ground for 50 strokes. The ground up tick salivary glands (GUTSG) were transferred to a 10ml conical centrifuge tube and a further 1ml PBS was used to rinse out the tissue grinder and added to the GUTSG. The suspension was

centrifuged at 100g for 5 minutes at 22°C, and the supernatant (GUTSGS) collected.

2.2.2 Preparation of DNA

From piroplasms or macroshizont cell lines

The cells were harvested at 540g for 10minutes (22°C) and resuspended in a small quantiy of TE buffer before adding 10ml sodium acetate/SDS solution with proteinase K (2.1.4) for approximately 0.5ml packed cell volume. The cells were incubated overnight at 37°C. RNAase A was added to a final concentration of 100μg ml^{-1}, and the suspension incubated for 2 hours before addition of Proteinase K to 100μg ml^{-1}. After a further 2 hours incubation the lysate was extracted once with phenol, once with phenol/chloroform/IAA and finally with chloroform alone in polypropylene tubes, to remove proteins and other contaminants. In each case, the tubes were gently agitated for 10 minutes, and the phases were separated by centrifugation (1100g for 5 minutes, 22°C).

The aqueous phase was dialysed at 4°C against TE buffer (3litres, two changes) and the DNA concentration estimated before ethanol precipitation. 2.5 volumes of ethanol were added and the DNA left to precipitate for 2 hours at 4°C. The DNA was collected by centrifugation (10 minutes at 11,000g, 4°C), and the pellet washed carefully in 70% ethanol and allowed to drain thoroughly. A sufficient volume of 0.1x TE buffer was added to give a final DNA concentration of 0.5-1 mg ml^{-1}. The pellet was allowed to dissolve at 4°C overnight and the final DNA concentration estimated by measuring the optical density (OD) at 260nm. If the DNA was heavily contaminated with protein the phenol extractions and/or DNA precipitation steps were repeated.

Small preparations of plasmid DNA

A) Alkali lysis method

Colonies were picked from a fresh L-agar plate and grown in 3ml LB medium (2.1.3) with 100μg ml^{-1} ampicillin overnight at 37°C with

shaking. DNA extraction by the alkali lysis method was carried out as described by Sambrook, Fritsch and Maniatis (1989).

B) Using "Magic Minipreps" purification kit (Promega)

A 3ml culture of each transformant was grown overnight at 37°C with shaking in LB medium with 100μg ml^{-1} ampicillin. Production of a cleared lysate and plasmid purification using a disposble 5ml syringe to pass the supernatant through the column was carried out according to the protocol given by the manufacturer.

Large preparations of plasmid DNA

A 200ml culture was grown from a single colony of the transformant to high density overnight at 37°C with shaking in LB medium with 100μg ml^{-1} ampicillin. The cells were harvested by centrifugation for 5 minutes at 5000g, 4°C and resuspended in 3.6ml 20% sucrose solution (2.1.5). The suspension was transferred to a large conical flask and 3.6ml of 4mg ml^{-1} lyzosyme in 20% sucrose solution was added. The mixture was swirled for 1-2 minutes before addition of 6ml 10% Triton-X 100. The suspension was boiled over an open flame with constant swirling for a few minutes, until the cells lysed and the mixture became viscous. The flask was incubated briefly in a boiling water bath then cooled in iced water for 5-10 minutes.

The lysed cells were poured into a 50ml Oakridge tube and centrifuged for 30minutes at 44,000g, 4°C to remove chromosomal DNA and cell debris. The supernatant was recovered and 0.5 volume 7.5M Ammonium acetate pH 7.5 added. The mixture was incubated on ice for 20 minutes to precipitate out remaining protein, and centrifuged at 24,700g 4°C for 10minutes. 0.7 volumes of isopropanol was added to the supernatant and the DNA precipitated for 20minutes at -20°C. The DNA was pelleted at 24,700g for 10minutes at 4°C, and the pellet drained carefully.

The DNA was further purified on a Caesium chloride density gradient. The pellet was resuspended in 4ml TE buffer and 4.3gms caesium chloride added, together with ethidium bromide to a final

concentration of 0.5mg ml^{-1} and the mixture transferred to a 2" x 0.5" Beckman Quick-seal tube. The tube was topped up with mineral oil, sealed and spun at 200,000g for 16 hours at 20°C (no brake). The band of plasmid DNA was visualised with UV light (260nm) and recovered. Ethidium bromide was extracted 3 times with butan-1-ol saturated with sodium chloride, and 3 volumes H_2O added to dilute the caesium chloride. The DNA was precipitated by adding 0.5 volumes of 7.5M ammonium acetate and 2 total volumes ethanol. Following a 30 minute incubation on ice, the DNA was pelleted at 15,800g for 20 minutes at 4°C. The supernatant was poured off carefully, and the pellet washed with 70% ethanol. The pellet was allowed to dry, then resuspended in 200µl TE buffer.

Preparation of bacteriophage λ DNA

Bacteriophage λ was prepared from a 1litre culture, the phage purified on a caesium chloride density gradient and DNA extracted using described methods (Sambrook, Fritsch and Maniatis 1989). The recipes for the NZCYM medium and SM phage dilution buffer used are given in section 2.1.6.

Spectrophotometric determination of DNA concentration

The optical densities of DNA samples diluted in TE buffer were taken at wavelengths 260 and 280nm. A reading of 1 OD at 260nm was taken to correspond to a concentration of 50µg ml^{-1} of DNA, and the ratio of OD 260nm / OD 280nm was used to assess purity of the sample (Sambrook, Fritsch and Maniatis 1989).

2.2.3 Electrophoresis and Southern blotting of DNA

Restriction endonuclease digestion of the DNA was carried out as described in section 2.2.5. Agarose gel electrophorisis in TBE buffer (2.1.7) of the DNA samples was carried out at low voltage overnight as described (Sambrook, Fritsch and Maniatis 1989). 8-10µg macroschizont cell line DNA, 2µg piroplasm DNA or 1µg phage DNA was loaded per track. 1µg ml^{-1} ethidium bromide was included in the gel.

Samples were diluted in Type III gel loading buffer (Sambrook, Fritsch and Maniatis 1989) and molecular weight markers (2.1.7) were loaded in the outermost tracks.

Once the run was completed the agarose gel was photographed under UV light to record the positions of the DNA bands and the markers. Prior to transfer, the gel was depurinated in 0.25M HCl (two changes, 15 minutes each with shaking), washed in H_2O, soaked in denaturation solution (2.1.8) for 2 x 15 minutes with shaking, washed again and then soaked in neutralisation solution for 1 hour with shaking. After a brief wash in H_2O, Southern transfer onto a nylon membrane (Hybond N; Amersham) was carried out in 20x SSC as described (Southern 1975). The DNA was fixed onto the nylon membrane by exposing to UV light for 7 minutes.

The probes used for hybridisation were restriction enzyme digested inserts from appropriate recombinant plasmids, excised from low melting point agarose gels (method described in section 2.2.5). The quantity of DNA in the agarose was estimated by calculation from the ratio of insert to vector size, and TE buffer added to give a DNA concentration of 5ng/μl. Just before labelling, the DNA was denatured and the agarose melted by boiling for 10 minutes, then cooling the tube to 37°C. Approximately 25ng DNA was labelled to high specific activity with 50μCi α^{32}P dCTP by random priming (Random Priming DNA Labelling Kit, Boehringer-Mannheim Pharmaceuticals) according to the manufacturer's instructions. The reaction was stopped by addition of 3 volumes TE buffer, and the DNA again denatured by boiling just before hybridisation.

The filter was prehybridised at 65°C in SDS phosphate hybridisation solution (2.1.8) for 1 hour. Hybridisation was carried out using cylinders in a Techne HB1 hybridiser. The prehybidisation solution was discarded from the filter and 20ml fresh hybridisation solution was added. The labelled DNA was added and hybridisation carried out at 65°C overnight with constant agitation. The filter was washed at high stringency in 5% SDS phosphate buffer (2.1.8) twice for 15 minutes each then in 1% phosphate buffer twice for 15 minutes each at 65°C with constant agitation. The filter was sealed in a bag and exposed for several days to Kodak X-OMAT S film in a film cassette

containing an intensifying screeen. If it was desired to reprobe a hybridised Southern blot, the original probe was removed by incubating the blot in two changes of approximately 500ml H_2O at 100°C, not allowing it to dry out at any stage. The blot was checked for removal of the probe by overnight exposure to film as described above. Hybridisation with a new probe carried out as described previously in this section.

Southern blotting of Hind III partial digests

Partial digests were carried out of DNA from a cloned macroschizont infected cell line using 1 unit of Hind III restriction enzyme per μg DNA. Eight tubes were set up, each containing 10μg DNA and 10u Hind III enzyme in medium salt buffer supplied by the manufacturer (BRL). The tubes were incubated in a 37°C waterbath and removed at time intervals of 1, 3, 5, 10, 20, 30, 60 and 120 minutes, halting the reaction by adding EDTA to a final concentration of 10mM and heating at 65°C for 5 minutes. The samples were run on an agarose gel and Southern blotted as described above. Partial digestion was indicated by the presence of DNA fragments larger in size than those produced in complete Hind III digests.

2.2.4 Molecular cloning techniques

Restriction enzyme digests

All restriction enzyme digests were carried out at 37°C for 2 hours (for plasmid DNA) or overnight (for phage or eukaryotic DNA) using buffers supplied by the manufacturer (BRL). Restriction enzyme digested DNA fragments were separated on low melting point agarose gels in TAE buffer (2.2.7) by electrophoresis as described (Sambrook, Fritsch and Maniatis 1989), and using a "Geneclean II" kit (Bio 101). If the DNA sample was to be digested by more than one enzyme which could not be carried out simultaneously (if different buffers were required by each enzyme) DNA from the first digest was purified by phenol extraction and ethanol precipitation as follows. The DNA was mixed with an equal volume phenol/chloroform/IAA and the phases separated by

centrifugation (10,000g for 5 minutes). The DNA was precipitated from the aqueous phase with 0.5 volumes of 7.5M ammonium acetate pH 7.5 and 2 total volumes of ethanol for 20 minutes at $^{-}20^{o}C$, pelleted at 10,000g for 10 minutes, washed in 70% ethanol and allowed to dry. Digestion was then carried out by the second restriction enzyme as described above.

Partial digests

It was necessary to carry out partial digests of recombinant plasmids with restriction enzymes where they contained more than a single recognition site, and the cutting of one specific site was desired. Partial digestion of a recombinant plasmid with Bam HI restriction enzyme were carried out in a similar fashion to partial digestion of macroschizont DNA (described in section 2.2.3), identifying an appropriate enzyme concentration and incubation time by carrying out trial digestions and assessing the sizes of bands produced on an ethidium bromide stained 1% agarose gel. 0.1 unit of the restriction enzyme was used per μg plasmid DNA to produce partial digest products, and the appropriate insert identified by it's size on the gel.

Treatment with alkaline phosphatase

This technique was used to prevent religation of restriction enzyme digested plasmid vectors. 1 unit of phosphatase enzyme was added to 1-5μg DNA in 9μl 50mM Tris-HCl, 0.1mM EDTA pH 8.0. After incubating for 30 minutes at 37°C the enzyme was heat inactivated at 65°C for 10 minutes. After cooling, the DNA was extracted with phenol/chloroform/IAA as described in the previous section.

Generation of blunt ends with S1 nuclease and Klenow enzyme

4 units of S1 nuclease enzyme were added to 1-5 μg DNA dissolved in 50μl 0.25M NaCl, 1mM $ZnSO_4$ and 30mM CH_3COOK. The DNA was incubated for 30 minutes at 22°C, and the enzyme was heat inactivated by adding 6μl of 0.125M EDTA, 0.5M Tris-HCl pH 8.0. The DNA was extracted with phenol/chloroform/IAA and ethanol precipitated as described earlier.

For Klenow filling-in, the pellet was dissolved in 10µl of 20mM Tris-HCl, 7mM $MgCl_2$ and 2 units of Klenow enzyme added. The mixture was incubated for 2 minutes at 37°C before addition of 1µl of a solution containing 0.125M each of dGTP, dATP, dCTP and dTTP, then incubated for a further 2 minutes. The DNA was extracted with phenol/chloroform/IAA and ethanol precipitated.

Ligation of DNA fragments with blunt ends

300-500µg DNA with insert:vector in the ratio of 3:1 was dissolved in 10µl H_2O, 6.33µl 2:5 freshly made ligase mix and 1µl (1 unit) Bacteriophage T_4 ligase. 2:5 ligase mix consisted of the following; 5µl 10x ligase buffer with ATP (BRL), 5µl PEG, 5 µl DTT and 22µl H_2O. The ligation mixture was incubated overnight at 22°C and diluted x5 before being used to transform cells.

Ligation of DNA fragments with staggered ends

The ligation mixture consisted of approximately 100ng total insert and vector DNA in the ratio 3:1 dissolved in 12.5µl H_2O, 1.5µl 10x T_4 ligase buffer and 1µl (1 unit) T_4 DNA ligase. The ligation mixture was incubated at room temperature for 1 hour, then overnight at 4°C. The mixture was diluted 3x in TE buffer prior to transformation of cells.

Making and transforming competent cells by the $CaCl_2$ method

100ml LB medium was inoculated with 1ml of a fresh overnight culture from a single colony of an appropriate untransformed strain of *E. coli* (Table B, Appendix), and grown to exponential phase with good aeration (OD at 450nm = 0.5 for JM101 and XL-1 Blue cells). The cells were cooled on ice before centrifugation for 3 minutes at 540g, 4°C. The cells were washed once in ice cold 100mM $MgCl_2$, then resuspended in 20ml ice cold 100mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were again harvested, resuspended in 6ml 100mM $CaCl_2$ and dispensed into precooled eppendorf tubes in 200µl aliquots. The cells were used fresh or stored for up to 24 hours at 4°C.

5-40ng DNA in a maximum volume of 10μl was used to transform 200μl competent cells. The cells were vortexed briefly and stored on ice for 30 minutes. DNA uptake was induced by incubating the cells for 90 seconds at 42°C, then cooling them on ice. 1ml LB medium was addded to each tube and the cells were transferred to 37°C for 45 minutes. The cells were briefly centrifuged and the medium removed, resuspending them in 200μl fresh medium. Dilutions were plated out on selective LB agar plates containing 100μg ml^{-1} ampicillin (2.1.3) and incubated overnight at 37°C.

In addition to transforming competent cells with ligated vector and insert, control aliquots were also transformed with 100ng of each of the following; uncut vector only to check the efficientcy of the transformation (Control A), restriction enzyme digested and phosphatased vector DNA to check that self-religation of the vector had been prevented by phosphatasing (Control B) and vector which had been restriction enzyme digested only to check the efficiency of the ligation (Control C). Normally colonies from the recombinant plate would only be used for DNA minipreps if they occured in sufficiently higher numbers than the colonies on Control B, ie were likely contain an insert, rather than have been transformed by religated vector alone.

Testing bacteria for α -complementation

Where vectors containing the N-terminal part of the β-galactosidase gene (such as pGEM7ZF or pBluescript) were used in a host cell coding for the C-terminal part of the β-galactosidase gene (such as JM109 and XL-1 Blue) it was possible to identify recombinant clones by α-complementation. This can only occur in colonies that lack an insert of foreign DNA; complementation results in the production of active β-galactosidase which can be detected on indicator plates containing X-gal. This chromogenic substrate imparts a blue colour to colonies in which α-complementation occurs.

Transformed competent cells were plated out and incubated as described in the previous section except that 20μg ml^{-1} X-gal and 0.1mM IPTG (2.1.3) were included in the agar plates. After overnight incubation, plates with colonies were incubated at 4°C for several hours.

Colonies lacking an insert, ie containing active β-galactosidase, could be identified by their blue colouration.

2.2.5 DNA sequencing

Sequencing was carried out using the dideoxy chain termination method of Sanger et al (1977). Double stranded plasmid DNA was denatured in 200mM NaOH from a freshly made solution for 5 minutes at 22°C. 0.4 volumes of 5M ammonium acetate pH 7.5 was added, immediately followed by 4 total volumes of ethanol. The DNA was allowed to precipitate at -20°C for 2 hours. The single stranded DNA was pelleted, washed in 100% ethanol, dried and resuspended at a concentration of 300-500ng ml^{-1} and 7μl was used for each set of sequencing reactions.

Sequencing was carried out using "Sequenase Version 2.0" (United States Biochemical Corporation). Annealing of the DNA to the primer was performed at 65°C with slow cooling. The DNA was labelled using ^{35}S dATP before termination with ddGTP, ddATP, ddTTP or ddCTP. Reactions were stopped in 95% formamide, 20mM EDTA plus loading dye and the samples stored at -20°C for up to a week.

40 x 30cm buffer gradient sequencing gels were set up using methods described (Sambrook, Fritsch and Maniatis 1989). 6% acrylamide 0.5x TBE non-gradient gels were also used. Samples were heated to 75°C for 2 minutes prior to loading, and gels were run at 2500V for 2-6 hours. The gels were fixed in 40% methanol/10% acetic acid for 15 minutes before being carefully transferred to 3mm paper. Gels were dried for 30-60 minutes at 80°C in a Biorad gel dryer, before exposure to Kodak X-OMAT-S film in a film cassette at room temperature for 24hours or more.

2.2.6 Antibody techniques

SDS polyacrylamide gel electrophoresis of proteins

SDS polyacrylamide electrophoresis was carried out on 10%, 12% or 15% SDS-polyacrylamide gels (2.1.9) as appropriate for the molecular weights of the protein samples, following described methods (Sambrook, Fritsch and Maniatis 1989) on Biorad gel running equipment. Protein samples were boiled in 1x sample buffer (diluted from 4x stock, 2.1.9) for 5 minutes and cooled prior to loading. High or low molecular weight markers were run in the outer tracks (2.1.9). Minigels (8 x11cm) were run for 2-3 hours at 100v, while large gels (18 x 20cm) were run overnight at 50-60v. On completion of the gel run, the proteins were either blotted onto 0.45μm nitrocellulose or visualised by staining in Coomassie Blue R250 (2.1.9) for 30 minutes at room temperature, then incubating overnight in destaining solution (2.1.9).

Western blotting

Transfer of protein from an SDS polyacrylamide gel onto nitrocellulose was carried out following the technique of Western blotting described by Sambrook, Fritsch and Maniatis (1989), using a "Protean II" blotter (Biorad). Blots were stained in 1x Ponceau S protein stain (2.1.10) to check transfer, then incubated overnight in blocking buffer (2.1.10). The blots were incubated with primary antibody for 1 hour with constant agitation. Primary antibodies were McAb culture supernatants used undiluted, McAb ascites fluid diluted 1:100 in blocking buffer or polyclonal antisera diluted 1:100-1:10,000 in blocking buffer. The blots were washed once in 1x Tris saline (2.1.10), once in Tris Tween (2.1.10) then finally in Tris saline, for 15 minutes each at 22°C with shaking. This was followed by incubation with alkaline phosphatase conjugated second antibody diluted in blocking solution for 1 hour at 22°C. Alkaline phosphatase conjugates used were rabbit anti mouse IgG, goat anti-rabbit IgG or rabbit anti-bovine IgG (Sigma) diluted 1:300, as appropriate for the primary antibody. The washing steps were repeated as described above. The bands were visualised by incubating the filter in NBT and BCIP in diethanolamine developing solution (2.1.10) for 5-10 minutes, monitoring the reaction carefully by eye until bands appeared. The

reaction was halted by washing the filters in water and allowing them to dry.

Slot blotting of proteins directly onto nitrocellulose

A series of dilutions of the protein samples to be blotted were made in Tris saline. Nitrocellulose strips were cut to size and soaked in Tris saline for a few minutes before placing them in a "HibriSlot" manifold (BRL). 100µl of protein solution was loaded into each slot and drawn through the nitrocellulose paper under a vacuum. Each slot was washed through twice with 200µl Tris saline. The nitrocellulose strips were stained in 1x Ponceau S to check protein binding. Blocking and antibody reactions were carried out as described for Western blots.

Affinity purification of antibodies using antigen immobilised on a column

Protein antigens were cross linked onto agarose beads using "Amino Link" columns and reagents (Pierce). Coupling of protein to the gel and blocking of uncoupled sites were carried out according to the manufacturer's instructions. 1mg protein was coupled to 1ml "Amino-Link" beads. Columns were stored at 4°C in 0.2% sodium azide.

Columns were washed with 10-20 bed volumes of Tris saline prior to use. A maximum of 0.5ml of serum was run into the agarose and incubated at room temperature for 30 minutes. The column was washed with 5-7ml Tris saline and fractions containing non-binding antibody were combined. Antibody bound to the column was eluted using "Immunopure Gentle Ab/Ag Elution Buffer" (Pierce) pH 6.9 and elution monitored by measuring OD at 280nm. Eluted antibody was dialysed against 3litres of Tris saline for 3-4 hours at 4°C (two changes) prior to use in immunoassays. The agarose beads were regenerated by washing in 0.1M Glycine pH 2.8 and measuring OD at 280nm to ensure any bound antibody had been eluted. The columns were then washed in 10 volumes of PBS and 5 volumes of PBS 0.2% sodium azide before storing at 4°C.

Removal of antibodies using antigens immobilised on a column

This method was used to remove unwanted reactivities to antigens such as to normal *E. coli* proteins from antisera. Coupling of the protein to an "Amino Link" column and incubation with the serum were carried out as described above. Antibodies which failed to bind to the column were washed off with 5-7ml Tris saline, collecting and retaining the fractions. Fractions containing antibody were identified by measuring OD at 280nm, combined and passed through the column twice more as described above, noting the final dilution of the original serum sample. The reactivities of the antisera with the test antigen were compared on slot blots.

Immunofluorescence assays on formaldehyde fixed cells

Formaldehyde fixation of *Theileria annulata* sporozoites (Williamson 1988), macroshizonts (Minami et al 1983) and piroplasms (Glascodine et al 1990) onto 12 or 15 well IFA slides were carried out using the methods described. The slides were stored at -20°C until use.

The IFA slides were thawed in a dessicator for 20 minutes at room temperature prior to use. Serial dilutions of the serum to be tested were prepared in PBS and pipetted onto the wells. 10µl were used per well on a 15 well slide or 20µl per well on a 12 well slide. The slides were incubated for 30 minutes in a humidified box at room temperature, then washed once in PBS by carefully pipetting fluid from each well, avoiding cross contamination. A further two washes of 3 minutes each were carried out in a staining dish. Excess fluid was blotted away from the wells and the slides air dried before an appropriate FITC conjugated second antibody diluted in PBS was added to each well. FITC conjugates used were rabbit anti-mouse IgG diluted 1:80, goat anti-rabbit IgG diluted 1:100 or rabbit anti-bovine IgG diluted 1:32 (Sigma). After a 30 minute incubation the slides were washed in PBS as described before and counter stained in 0.1% Evans blue for 10 minutes. The slides were washed three times in PBS in a staining dish and dried. A drop of 80% glycerol was added to each slide, and a coverslip placed over the wells. The slides were examined under a Leitz fluorescence microscope and the degree of fluorescence observed in each well recorded.

Enzyme-linked immuno-sorbant assays (ELISA)

This method was used to titre antibody levels in bovine sera by antigen capture. All incubations were performed at room temperature in a humidified box. The concentration of the recombinant antigen was adjusted to 20μg ml^{-1}, and 50μl added to each well of a microtitre plate which was incubated overnight to coat the plates with antigen. The wells were washed three times in PBS. 0.2ml of BSA blocking solution (2.1.11) was added to each well and incubated for 30 minutes, and the washing step repeated. Serial dilutions were made of the serum sample in PBS/Tween/BSA (2.1.11), 50μl of each dilution was added per well and the plates incubated for 2 hours. The wells were washed three times in PBS 0.05% Tween (2.1.11), and incubated with peroxidase conjugated rabbit anti-bovine IgG at a working dilution of 1:8000 in PBS/Tween/BSA for 2 hours. The washing step was repeated. 200μl peroxidase substrate (2.1.11) was added to each well, the plates incubated for 5 minutes and the reactions halted by addition of 12.5% sulphuric acid. The absorbance of each well was read at 490 nm in a microplate reader.

2.2.7 Preparation of GST fusion proteins

The methods descrbed here are modified from Smith and Johnson (1988). Glutathione sepharose 4B beads (Pharmacia) were washed in 20 volumes PBS and equilibriated in PBS/1% Triton-X 100 before use. After each purification, the glutathione beads were regenerated with 3M NaCl in PBS (5 volumes), washed in PBS (10 volumes) then 20% ethanol (10 volumes) and stored at 4°C. Beads were discarded after being used for 6 purifications.

Small scale preparations for screening pGEX recombinants

5-25ml aliquots of 2xTY media (2.1.2) with 100μg ml^{-1} ampicillin were inoculated with each recombinant to be tested and grown overnight in a 30°C shaking incubator. The cultures were diluted 1:10 with fresh media with ampicillin and grown for 2 hours before induction with 0.1mM IPTG. The cultures were grown for a further 3 hours before

harvesting at 540g, 4°C for 5 minutes. The cell pellets were drained thoroughly, resuspended in 400µl ice cold PBS/1% Triton-X 100 and transferred into eppendorf tubes. Cells were lysed by sonnicating on ice at 6µ in three 30 second bursts (MSE Soniprep 150, 3mm probe). Cell debris was removed by centrifugation for 5 minutes at 10,000g, 4°C, retaining the supernatant.

The sonicates containing fusion protein were either used at this stage, boiling the samples in 1xSDS sample buffer (2.1.9) before loading onto an SDS-polyacrylamide gel, or the fusion proteins were further purified as follows. Each sonicate was transferred into a fresh eppendorf and 100µl of a 50:50 suspension of glutathione sepharose 4B beads in PBS/1% Triton-X 100 were added to each tube. The tubes were incubated for 15 minutes at 4°C with constant agitation to allow binding of the fusion proteins, and the beads spun down at 10,000g for 10 seconds. Unbound proteins were removed by washing the beads three times in 1ml PBS. Fusion protein was eluted by boiling each sample in 50µl 1x SDS sample buffer. The samples were run on SDS polyacrylamide gels which were either stained in Coomassie Blue R250 or transferred onto nitrocellulose by Western blotting and visualised with an appropriate antibody, as described in section 2.2.6.

Large scale preparations from 1 litre cultures

A 2litre flask containing 450 or 900ml 2xTY medium with 100µg ml^{-1} ampicillin was inoculated with 1/20 volume from an overnight culture and incubated at 30°C with good aeration until the cells had grown to late exponential phase (O.D. 450nm = 1-2 for JM101 or XL Blue cells). The culture was induced with 0.1mM IPTG and incubated for a further 2-3hours. The culture was cooled in iced water for 20 minutes prior to centrifugation at 470g, 4°C for 4 minutes. The cells were resuspended without delay in an ice cold mixture of PBS/1% Triton-X 100 and protease inhibitors (2mM 1,10 phenanthroline, 5µM antipain, 7µM aprotinin, 1mM benzamidine, 5µM leupeptin, 5mM pepstatin, and 10mM TLCK (Nα-p-Tosyl-L-lysine Chloromethyl Ketone)). Formulae for stock solutions of these protease inhibitors are given in section 2.1.12. The cells could be stored at this stage at -70°C.

Lysis was carried out by sonnicating 6-7ml of cell suspension in a 1x10cm polypropylene tube on ice for 5 bursts of 30 seconds each at 6μ (MSE Soniprep 150, 8mm probe). The suspensions were centrifuged at 100,000g for 60 minutes to remove insoluble matter and unlysed cells. The supernatant was passed through 1-2ml bed volume of Glutathione Sepharose beads in a 10ml disposable polypropylene column. The column was washed thoroughly with PBS, measuring the OD at 280nm until it dropped to 0. The fusion protein was eluted using 5mM glutathione in 50mM Tris-HCl pH 8.0. The eluate was collected in 1ml fractions and protein concentration was estimated by measuring OD at 280nm. Fractions containing reasonable quantities of fusion protein (over 0.1 OD at 280nm) were combined and dialysed against 3 litres PBS at 4°C for 4-5 hours (two changes) to remove the glutathione. If necessary, proteins were concentrated using a "Centricon" microconcentrator, and the amount of fusion protein quantified.

Bulk preparations from 4-16 litre cultures

This method is identical to the one given previously except for certain modifications. 4-16 2litre flasks each containing 900ml 2xTY medium with 100μg ml^{-1} ampicillin were inoculated, grown to late exponential phase and induced with 0.1mM IPTG, using a shaking platform in a 30°C incubation room. Cells were cooled and harvested as described but were resuspended in a much smaller volume of PBS/protease inhibitors, using 2-3ml for every litre of original culture volume. The cells were lysed in a French press at 950pi. The lysate was centrifuged at 100,000g for 60 minutes, and passed through a 1.6x20cm chromatography column containing 6ml bed volume of glutathione sepharose beads at a rate of 1ml/minute using a peristaltic pump. The column was washed in PBS overnight at 0.3ml/minute until the OD at 280nm of the eluates dropped to 0. The fusion protein was eluted at 0.3ml/min and dialysed as described above.

Factor Xa proteolytic cleavage of fusion proteins expressed in pGEX3X

PBS dialysed fusion proteins were incubated with 15 units/ mg protein Factor Xa protease (New England Biolabs) at 23°C overnight in 100mM NaCl and 20mM Tris pH 8.0. Uncleaved fusion protein and free

glutathione-S-transferase were removed by passing the reaction mixture through a 10ml disposable polypropylene column containing 2-3ml bed volume glutathione sepharose beads. The column was incubated for 1 hour at 4°C. PBS was passed through the column and 1ml fractions collected. The ODs at 280nm were measured and fractions containing protein were combined and concentrated.

Thrombin proteolytic cleavage of fusion proteins expressed in pGEX2T

Thrombin cleavage of PBS dialysed fusion proteins was carried out in PBS containing 2.5mM $CaCl_2$ and 2μg thrombin enzyme (Sigma) for every mg fusion protein. The reaction was carried out at 25°C for 2 hours. Uncleaved fusion protein and free GST were removed by passing the reaction mixture through a column as described above.

Concentration of fusion proteins

The samples were filtered at 5000g through "Centricon 10" microconcentrator tubes (Amicon) for1-2 hours according to the protocol given by the manufacturer.

Protein quantification

Protein concentration was estimated by direct measurement of OD 280nm as described by Harlow and Lane (1988), taking one absorbance unit to indicate approximately 1mg ml^{-1} protein. More accurate measurements were obtained by using Coomasie Plus Protein Assay Reagent (Pierce). The microassay procedure was followed according to the manufacturer's instructions. A series of bovine serum albumin standards from 2-40μg ml^{-1} were prepared and mixed with reagent. The OD at 595nm was measured from each sample, and used to plot a standard curve of OD 595nm against protein concentration (Figure 3). Test samples were diluted 1:10 or 1:100 as appropriate, mixed with the reagent and the ODs read at 595nm. The quantity of protein in each sample was then estimated from the standard curve.

2.2.8 Immunisations with recombinant protein

Immunisations of cattle with GST-2.7 fusion protein

Five Ayrshire or Ayrshire cross male calves aged between 2 and $3^1/_2$ months were immunised with GST-2.7 fusion protein for analysis of serum antibody and response to challenge with *Theileria annulata* sporozoites. Cattle 26x and 32x were immunised with saponin (Quil A; Quillaria) as an adjuvant while 23x, 758 and 759 were immunised using Freund's Complete Adjuvant (Gibco). Details of the immunisations are given in Table 2A.

Four Freisian male cattle aged 6-9 months were also immunised with GST-2.7 for T cell work. Cattle 11659 and 11663 were immunised with Freund's incomplete adjuvant (Gibco) and saponin, while cattle 12045 and 12056 were immunised with Freund's complete adjuvant and boosted with Freund's incomplete adjuvant. Details of the immunisations are given in Tables 2B and 2C. Sera was collected from cattle 11659 and 11663 prior to immunisation (day 0) and after the third immunisation (day 142). Sera was collected from cattle 12045 and 12056 prior to immunisation (day 0), 14 days after the initial immunisation (day 14), 7 days after boosting (day 35) and seven months after the last immunisation (day 253).

For all the immunisations the antigen in PBS was emulsified in an equal quantity of the adjuvant, to give a total volume of 0.5-1ml for each immunisation. All the immunisations were intramuscular. For sera, blood was collected from the jugular vein into tubes without anticoagulant, allowed to clot for 30-60 minutes at 37°C, then stored overnight at 4°C to allow the clot to contract. The serum was removed from the clot and centrifuged at 10,000g for 10 minutes to remove insoluble debris, and the supernatant removed.

Immunisation of a rabbit with GST-KP8 fusion protein

A rabbit was immunised intramuscularly with GST-KP8 recombinant fusion protein using alum as an adjuvant. The adjuvant was prepared as described by Harlow and Lane (1988). Details of the

immunisations are given in Table 3. Blood was collected from the marginal ear vein and serum prepared as described above. Serum samples were taken prior to immunisation (day 0), 8 days after the first boost (day 56), and 8 and 18 days after the second boost (days 87 and 97).

2.2.9 Techniques used in culturing T cells

The various stages of setting up and testing cell lines and clones are summarised in Figure 3, and will be referred to in the text.

Preparation of peripheral blood mononuclear cells (PBM)

Cattle were immunised with recombinant GST-2.7 as described in section 2.2.8 (Figure 3(i)). PBM were prepared from bovine blood for setting up helper T cell lines and for testing in helper T cell proliferation assays. Blood was collected from the jugular vein in acid-citrate dextrose (3 parts blood:1 part anticoagulant) in siliconised 20ml tubes. PBM were separated on Ficoll-Hypaque and washed in PBS and RPMI according to the method described by Teale et al (1985).

Generation of antigen specific bovine helper T cell lines

To culture bovine helper T cell lines, PBM were prepared from an immunised cow which responded well to the antigen in helper T cell proliferation assays. The cells were then cultured for alternate weeks in a selection of antigen concentrations found to induce proliferation in PBM proliferation assays (described later in this section) and in human recombinant IL-2. After the first expansion in antigen it was necessary to include antigen presenting cells in the culture in the form of irradiated PBM from the donor animal. The methods used were based on those described by Glass and Spooner (1990a).

PBM were prepared and diluted to $4x10^6$ in MLC medium with 10% Serumax (2.1.2). The PBM were pipetted into $2cm^3$ wells of a 24 well Costar plate to give $4x10^6$ cells per well in each chosen concentration of antigen (Figure 3 (ii)). Several lines were set up in

different antigen concentrations. Two or three plates were set up for each line, and incubated at 37°C in 5% CO_2 for 5-7 days.

After the first expansion the cells were harvested by centrifuging at 800g at room temperature and the number of viable blasts assessed by staining with 0.25% Trypan blue in PBS and counting in a haemocytometer chamber. Blasting cells were separated from small and non viable cells on a 20-100% Percoll step-wise gradient (Figure 3 (iii)), according to the method given by Kurnick et al (1979). The gradients were centrifuged for 20 minutes at 3000g without a brake, and the layer of blasting cells carefully removed from the 40%/50% interface. The blasts were washed twice in medium and resuspended at $4x10^5 ml^{-1}$ in MLC medium with 10% FCS. $4x10^5$ cells were aliquoted into each $2cm^3$ well of a 24 well plate, with 20u ml^{-1}hrIL-2 (BRL) in each well (Figure 3 (iv)). The plates were incubated in 5% CO_2 at 37°C, adding additional IL-2 at 20u ml^{-1} after 3 days.

After 5-7 days the cells were harvested, washed twice in medium and assessed for the proportion of viable blasts. The cells were either cloned at this stage or further expanded in antigen as follows. PBM was prepared from the donor animal for antigen presenting cells and irradiated at 3000 rads. The cells were washed three times in RPMI and resuspended in MLC medium with 10% FCS to give $2x10^6$cells ml^{-1}. 1ml was aliquoted into each well of a 24 well plate, with $2x10^5$blasts and antigen at the same concentration used when the line was set up. The plates were incubated at 37°C for 7 days (Figure 3 (v)).

The lines were then given a second expansion in IL-2. The contents of the wells were harvested and dead cells removed by separation on Ficoll Hypaque as previously described (Teale et al 1985). The blasts were washed, counted and resuspended in MLC 10% FCS at $4x10^5$/ml. Wells were set up each containing $2x10^5$ blasts and 20u ml^{-1} IL-2, and incubated at 37°C. After a further 7 days, the blasts were again expanded in antigen as described earlier. The lines were then tested and cryopreserved as described later in this section.

Cloning antigen specific bovine helper T cell lines

The methods given here are based on those of Fathman and Fitch (1982). Cloning by limiting dilution was carried out after expansion of the line in IL-2. The clones were set up in round bottomed 96 well plates each containing a volume of 200μl. PBM was prepared from the donor animal, irradiated at 3000rads and washed in RPMI. Blasts were diluted in MLC medium with 10% FCS to give an average of 0.3 blasts per well. To each well was also added 10^5 irradiated PBM (PBM^r), 20u ml^{-1} hr IL-2 and antigen at the same concentration used in the original line.

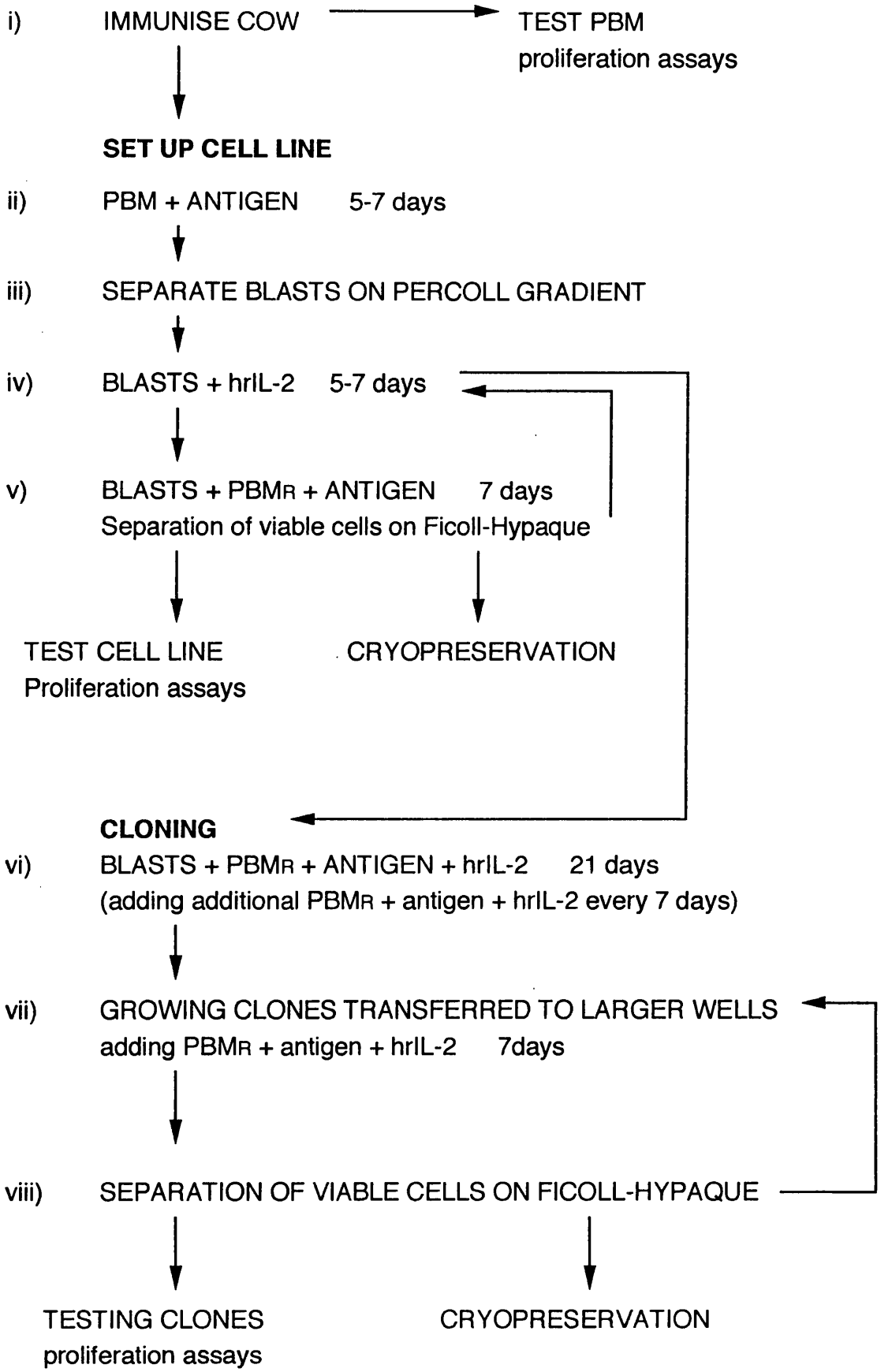
The plates were cultured in 5% CO_2 at 37°C for 21 days, adding additional PBM^r, antigen and IL-2 every 7 days (Figure 3 (vi)). This was done by carefully removing 150μl aliquots from each well and adding back 150μl of a mixture containing PBM^r, hrIL-2 and antigen at 1.3x the original concentrations. After 14-21 days, the plates were examined for growing clones. Clones were first split into four wells of a 96 well plate by resuspending the cells and aliquoting 50μl per well. Fresh PBM^r, hr IL-2 and antigen were added as described above. After cultivating for a further 7 days the clones were transferred to a 24 well plate, by carefully removing 150μl medium from each of the wells, resuspending the cells and combining them in one $2cm^3$ well. PBM^r was added to give 10^6 cells per well, with 20u ml^{-1} hr IL-2 and the correct antigen concentration (Figure 3 (vii)). Any expanding clones were centrifuged in 3ml Ficoll Hypaque at the end of an antigen cycle to remove dead cells, washed and counted (Figure 3 (viii)). They were then aliquoted into $2cm^3$ or $10cm^3$ wells. Each well contained the following; $2.5x10^5$ blasts ml^{-1} of the clone, 10^6 cells ml^{-1} of PBM^r, 20u ml^{-1} hr IL-2 and the correct concentration of antigen. This procedure was repeated at 7 day intervals until the clone had expanded sufficiently to be tested in proliferation assays and cryopreserved.

Cryopreservation of lymphocytes

Cells were harvested from a T cell line or clone were cooled on ice and resupended in ice cold RPMI with 50% FCS at $1-2x10^7$ blasts ml^{-1}. An equal quantity of 20% DMSO in FCS was added, the contents mixed

Figure 3

Flow diagram to summarise the steps in the production and testing of T cell lines and clones. See text for explanation of the different stages.


i)
IMMUNISE COW
TEST PBM
proliferation assays
SET UP CELL LINE
ii)
PBM + ANTIGEN 5-7 days
iii)
SEPARATE BLASTS ON PERCOLL GRADIENT
iv)
BLASTS + hrIL-2 5-7 days
v)
BLASTS + PBMR + ANTIGEN 7 days
Separation of viable cells on Ficoll-Hypaque
TEST CELL LINE
Proliferation assays
CRYOPRESERVATION
CLONING
vi)
BLASTS + PBMR + ANTIGEN + hrIL-2 21 days
(adding additional PBMR + antigen + hrIL-2 every 7 days)
vii)
GROWING CLONES TRANSFERRED TO LARGER WELLS
adding PBMR + antigen + hrIL-2 7days
viii)
SEPARATION OF VIABLE CELLS ON FICOLL-HYPAQUE
TESTING CLONES
proliferation assays
CRYOPRESERVATION

and dispensed into precooled vials. The vials were stored overnight in a polystyrene box at -70°C, then transferred to liquid nitrogen.

To resuscitate cryopreserved cells, the vials were thawed rapidly and 10ml ice cold MLC medium was added dropwise slowly with mixing. The cells were spun down at 800g for 10 minutes and washed in medium again, resuspending them gently. The concentration was adjusted to 2-5x10^5 viable blasts ml^{-1} and PBM^r were added at 2x$10^6 ml^{-1}$, plus 20u ml^{-1} hr IL-2 and the original antigen concentration used. The blasts were harvested after 5-7 days and either cultured for 1 week in hrIL-2 alone (for a line) or in IL-2 plus antigen (for a clone) before testing.

Proliferation assays for T helper activity

A) Testing PBM

Proliferation assays were carried out using methods previously described (Glass, Oliver and Spooner 1990a). Briefly, tests were set up in quadruplicate in 200µl flat bottomed wells of a 96 well plate. The wells were set up with PBM at 1x10^6cells ml^{-1} plus medium only, control antigen, Concanavilin A mitogen or the test antigen at various concentrations.

Plates were incubated at 37°C in 5% CO_2 for 5 days before addition of ^3H thymidine (0.2µCi per well). The plates were incubated for a further 6 hours to allow incorporation of the label into proliferating cells. The contents of the wells were transferred onto filter paper using a cell harvester. The filter paper was dried by incubating at 65°C overnight, then the discs were removed and transferred to individual scintillation vials with 3ml scintillation fluid ("OptiSafe"). Vials were labelled and the levels of ^3H thymidine determined by scintillation counting (MR 300 Kontron Ltd).

B) Testing cell lines

Wells of a 96 well plate were set up containing 5x10^5 blasts ml^{-1}, plus PBM^r at 3x$10^6 ml^{-1}$ with the required concentration of antigen. Tests were also set up in quadruplicate and controls were included of

blasts without PBM^r in the presence or absence of antigen, PBM^r alone in the presence or absence of antigen and blasts without PBM^r but with antigen. The plates were incubated for 3 days, then labelled and harvested as described above.

C) Testing clones

The same procedure was followed as for testing lines except that 20u ml^{-1} hrIL-2 was included in the medium when setting up the test.

Analysis with a fluorescence activated cell sorter

Cells from a PBM preparation, a T cell line or a clone were labelled by indirect immunofluorescent staining using McAbs to a variety of cell markers, listed in Table C in the appendix.

The harvested cells were cooled on ice and resuspended at $2x10^7ml^{-1}$ in FACS medium (2.1.2). The McAbs were diluted in FACS medium to 2x their working concentrations. 50µl of the cell suspension plus 50µl of each McAb were dispensed into round bottomed wells of a 96 well plate. Controls were included containing medium only instead of McAb. The plates were incubated on ice for 30 minutes with occasional agitation, then centrifuged at 4°C for 3 minutes at 300g and the supernatant removed. The cells were washed twice in medium and resuspended in 25µl per well of rabbit anti-mouse IgG FITC (Sigma) diluted1:200 in medium. The plates were covered in foil and incubated on ice for 30 minutes, then washed in medium as before. The cells were either tested immediately or fixed in 0.2% formalin in FACS medium. Fixed cells were stored at 4°C in the dark for up to 48 hours.

Flow cytometric analysis was performed using a FACS IV (Becton Dickinson) as described by Spooner et al (1988). The parameters of forward and side scatter were set before running each group of samples using unlabelled cells from the control wells. Normally 10,000 cells were analysed from each sample.

2.2.10. Sporozoite invasion of lymphocytes "in vitro"

Invasion assays

A sporozoite suspension was prepared as sterile GUTS as previously described (2.2.1) and diluted to 0.5 te (tick equivalents) ml^{-1} in RPMI 16% FCS (2.1.2). Peripheral blood mononuclear cells were prepared from bovine blood according to the method of Glass and Spooner (1990a) and diluted to 2 x $10^6 ml^{-1}$ in RPMI 16% FCS.

Infection of lymphocytes was carried out as described (Brown 1987). GUTS and PBM were pre-incubated at each temperature; 4°C, 22°C or 37°C, prior to mixing. 0.5ml GUTS and 0.5ml PBM were mixed in each $1cm^3$ well of a Costar 48 well plate. The final contents in each well was therefore 0.25 te of sporozoites and $1x10^6$ PBM. The plates were incubated at 4°C, 22°C or 37°C in 5% CO_2. 50µl samples were taken at specific timepoints and used to prepare Giemsa stained cytospin smears. The percentage of parasite infected cells was assessed.

Antibody inhibition assays

Inhibition assays were carried out using a method similar to that described by Williamson (1989). Invasion assays were set up as described above except that 0.25ml of serum or McAb at appropriate dilutions was incubated with 0.25ml sporozoite suspension at 1 te ml^{-1} for 1 hour at 37°C prior to the assay. 0.5ml PBM at $2x10^6 ml^{-1}$ was then added to each well, and incubation was continued at 37°C in 5% CO_2 for four days. Lower concentrations of sporozoites were used in assays when the ticks contained high levels of infection; in these assays, the sporozoite suspension was diluted to 0.25 te ml^{-1} to give a final concentration of 0.0625 te per well. Each test was set up in quadruplicate. The proportions of infected cells were assessed from Geimsa stained cytospin smears and used to calculate percentage inhibition.

Fusion protein inhibition assays

Inhibition by fusion protein was tested in a similar way except that 0.25ml PBL at $4x10^6ml^{-1}$ were incubated with dilutions of fusion protein (0.5ml volumes) for 1 hour at 37°C prior to the assay. 0.25ml sporozoite suspension at 0.25 te ml^{-1} was added to each well, and the cultures incubated and assessed for inhibition as described for the antibody inhibition assays.

Preparation of Giemsa stained cytospin smears

Approximately 50µl of each cell suspension to be tested was spun onto a slide for 5 minutes at 5000g in a Cytospin centrifuge, air dried and fixed in methanol for 1 minute. The slides were then stained in 4% Merck's Giemsa stain in Giemsa buffer (BDH) for 40 minutes. The excess stain was removed by washing in Giemsa buffer and the slides were air dried.

2.2.11 Preparation of sporozoite infected lymphocytes for immuno electron microscopy

Fixation in 0.5% glutaraldehyde/2% formaldehyde

PBM were infected "in vitro" using the method described for invasion assays (2.2.10). Assays were set up in 8ml culture volumes in each of six $10cm^3$ wells of 6 well plates. Each well contained 2 te of sporozoites and $8x10^6$ PBM. The cultures were incubated at 22°C in 5% CO_2 and wells were harvested at timepoints of 5, 30 and 60 minutes and 3 and 6 hours. The additional well was also harvested at 5 minutes in order to provide material for incubation with a control McAb. A third of the contents of each well (2.7ml) was transfered to a 10ml centrifuge tube and centrifuged at 400g for 5 minutes, the medium removed and the pellet resuspended in 1ml PBS. An equal quantity of 4% formaldehyde in PBS was added and the suspension was mixed gently and incubated at room temperature for 30 minutes before pelleting at 400g. The cell pellet was resuspended in 0.5% glutaraldehyde/ 2% formaldehyde in PBS, incubated again for 30 minutes and harvested at 400g. After removing

the fixative, the pellet was stored overnight at 4°C in 8% sucrose in PBS with a few drops of 2% formaldehyde.

Fixation in 4% formaldehyde

2.7ml from each culture was harvested and resuspended in PBS as described above, and an equal quantity of 4% formaldehyde in PBS was added. After a 30 minute incubation at room temperature the cells were pelleted, resuspended gently in 4% fomaldehyde and incubated for a further 30 minutes. The cells were again pelleted at 400g, the fixative removed and replaced with 8% sucrose in PBS with a few drops of formaldehyde.

Fixation in 1.5% glutaraldehyde

2.7ml of each culture was harvested at 400g, resuspended directly in 1.5% glutaraldehyde in PBS, and incubated for 30 minutes at room temperature. The fixative was carefully removed and replaced with 8% sucrose in PBS with a few drops of 25% glutaraldehyde.

Antibody incubations

The pellets were first incubated for 15 minutes in 0.2M glycine in PBS to block aldehyde residues and washed twice in a solution of 20mM Tris HCl pH 7.5 (2.1.1) including 1% BSA. Incubation with primary antibody diluted in 20mM Tris/1% BSA was carried out for 1 hour at room temperature. Primary antibodies used were mouse McAbs 1A7 (IgM) undiluted supernatant or a control McAb (anti-trypanosome (IgG); kindly provided by Dr. L. Tetley, Dept. of Chemistry, University of Glasgow) diluted 1:50. The pellets were washed 3 times in 20mM Tris/ 1% BSA (5 minutes each change), before incubation with secondary antibody for 1 hour at room temperature. Second antibody was goat anti-mouse IgG or goat anti-mouse IgM (as appropriate for the primary McAb) conjugated with 10nm colloidal gold particles, diluted 1:40 in 20mM Tris/1% BSA. The pellets were again washed 3 times in the buffer as described above.

Dehydration and embedding

The pellets were embedded in 2% high melting point agarose at 60°C, washed in H_2O and stained in 0.5% uranyl acetate for 30 minutes at room temperature in the dark. The pellets were completely dehyrated by incubating them in a series of increasing concentrations of ethanol (50%, 70%, 90%, 100% and dehydrated 100%) for 10 minutes each. The pellets were finally washed in three changes of propylene oxide before embedding for 36 hours at 60°C in a 50:50 mixture of propylene oxide and araldyte.

Sectioning and staining

The resin blocks were trimmed to size with a razor blade and ultrathin sections cut using an LKB ultramicrotome. The sections were collected on uncoated copper mesh grids. Sections were stained in uranyl acetate and Reynolds lead citrate prior to examination.

Grids were immersed in uranyl acetate in the dark for 5 minutes at room temperature, before thorough washing in H_2O. Grids were then washed in 0.02M NaOH. Staining in lead citrate was carried out in a petri dish containing NaOH pellets to remove CO_2, which tends to react with lead citrate to produce artefacts. After 5 minutes the grids were washed thoroughly in 0.02M NaOH then H_2O. The grids were allowed to air dry completely before examination under a Zeiss 902 transmission electron microscope. Images were recorded on Kodak SO281 70mm roll film.

TABLE 1. STOCK SOLUTIONS OF PROTEASE INHIBITORS

The following table gives the concentrations (concn) and solvents used to make up stock solutions of protease inhibitors, together with the final concentrations at which they were used.

PROTEASE INHIBITOR	SOLVENT	STOCK CONCN	FINAL CONCN
PMSF	Acetone	1M	1mM
1,10 PHENANTHROLINE	Methanol	2M	2mM
LEUPEPTIN	Water	5mM	5μM
ANTIPAIN	Water	5mM	5μM
TLCK	Methanol	25M	10mM
BENZAMIDINE	Water	100mM	1mM
APROTININ	Water	1mM	10μM

TLCK = Nα-p-Tosyl-L-lysine Chloromethyl Ketone

The following tables summarise the immunisations of cattle with recombinant GST-2.7 fusion protein.

TABLE 2A. IMMUNISATIONS OF CATTLE 23X, 26X, 32X, 758 AND 759.

DAY	NUMBER	ADJUVANT	GST-2.7
0	23x 758 759	Saponin	500μg
	26x 32x	FCA	
36	23x 758 759	Saponin	100μg
	26x 32x	FIA	
56	23x 758 759	Saponin	100μg
	26x 32x	FIA	
78	23x 758 759	Saponin	100μg
	26x 32x	FIA	
92	23x 758 759	Challenged	
	26x 32x		

FCA = Freund's complete adjuvant

FIA = Freund's incomplete adjuvant

GST-2.7 = GST-2.7 purified recombinant protein

TABLE 2B. IMMUNISATION OF CATTLE 11659 AND 11663

DAY	ADJUVANT	GST-2.7
0	FIA	500μg
34	FIA	100μg
95	Saponin	100μg
179*	Saponin	100μg
253*	Saponin	100μg

*11663 only

TABLE 2C. IMMUNISATION OF CATTLE 12045 AND 12056

DAY	ADJUVANT	GST-2.7
0	FCA	500μg
28	FIA	100μg

TABLE 3. IMMUNISATIONS OF RABBIT WITH GST-KP8 FUSION PROTEIN

DAY	ADJUVANT	GST-KP8
0	ALUM	500μg
48	ALUM	100μg
79	ALUM	100μg

CHAPTER 3

SPAG1 POLYMORPHISM

3.1 Introduction

The structure of a parasite surface antigen would be expected to reflect both it's functional requirements and the need to evade any immune mechanisms directed against it. Antigenic variation and antigenic diversity are exhibited by many parasitic protozoa in order to avoid host immune responses. Antigenic polymorphism or antigenic diversity is defined as the existence of genetically stable and alternative forms of an antigen transmitted through successive infections. Polymorphism can also be defined as allelic variation at a single gene locus; a locus is said to be polymorphic when the frequency of the most common allele is equal or less than 0.99 (Arnot 1989). Antigenic polymorphism is distinct from antigenic variation; the successive expression of alternative forms of an antigen throughout an infection, in which the alternative forms are non-allelic.

Evidence that the coding region that includes the SPAG1 gene shows restriction fragment length polymorphism (RFLP) was first provided by S. Williamson (Williamson et al 1989, Williamson 1988). The SR1 insert (described in section 1.1.3) was found to hybridise to three bands of different molecular weights on a Southern blot of restriction endonuclease Eco RI digested *T. annulata* piroplasm or macroschizont DNA from uncloned Hissar and Ankara stocks. Since there are no internal Eco RI sites in the SR1 insert, these bands were thought to represent either three gene copies in each stock, or two gene copies if polymorphism produced an EcoR1 site in the SR1 region of the second copy. This data was interpreted as indicating the presence of multiple copies of the SPAG1 gene per haploid genome, or of multiple alleles for a single polymorphic locus (Williamson et al 1989). However, when the SR1 insert was used to probe Southern blots of Eco RI digested DNA from cloned *T. annulata* Ankara macroschizont infected cell lines, only single bands were detected for each clone (Williamson 1988). This separation of the Eco RI restriction fragments according to clone types implied that the

SPAG1 gene was present as a single copy in the genome, and that the different sized bands arose through the presence of alleles on different length Eco RI restriction fragments. The different Eco RI fragments could result directly through polymorphism of the SPAG1 gene itself, although they could also be produced by polymorphism in the sequences outside the coding region of the SPAG1 gene.

In this chapter work is described to confirm and extend these studies. The extent of restriction fragment polymorphism in the SPAG1 coding region was assessed on Southern blots of DNA from a range of geographically separated *T. annulata* parasite stocks digested with a number of restriction endonucleases, using SPAG1 specific probes. These results are compared with sequence data from polymerase chain reaction (PCR) analysis of the SPAG1 locus, kindly communicated by Dr. R. Hall (Department of Biology, University of York). Work is also described to confirm that the SPAG1 gene exists as a single copy, using Southern blots of restriction endonuclease digested DNA from cloned *T. annulata* macroschizont infected cell lines.

3.2 Results

Analysis of restriction fragment length polymorphism of the SPAG1 gene was carried out using the Southern blotting technique, as described in section 2.2.3. *T. annulata* piroplasm DNA was kindly provided for this work by Dr. B. Shiels and S. McKellar, Wellcome Unit of Molecular Parasitology, University of Glasgow. Genomic DNA was prepared from cloned and uncloned macroschizont infected cell lines as described in section 2.2.2. The *T. annulata* stocks used in this study were kindly provided by C. G. D. Brown and T. R. Melrose, Centre for Tropical Veterinary Medicine, Edinburgh. Digests with restriction endonucleases were carried out as described in section 2.2.4.

Two probes were derived from the SPAG1 sequence; the 300bp SR1 insert (from Eco RI digested primary clone λgt11-SR1 or the subclone pGEX1λT-SR1; described later in section 4.2.1) corresponding to nucleotides 2403-2724 of the C-terminal end of the molecule, or a 1.6kb fragment corresponding to nucleotides 1197-2825 (from BamH1

restriction endonuclease digestion of subclone pGEX2T-2.4; described in section 4.2.2). The latter insert corresponded to the central and C-terminal regions of the SPAG1 molecule, covering a more extensive region than SR1. The nucleotides refered to above correspond to those of the published SPAG1 sequence (Hall et al 1992 and Figure 77, Appendix).

3.2.1 Analysis of uncloned stocks

In order to verify whether the banding patterns seen in the Ankara, Hissar and Gharb stocks were common to isolates from other regions, DNA was prepared from macroschizont infected cell lines of ten different stocks derived from several geographically separated locations; Ankara in Turkey, Razi in Iran and Soba, Sagadi, Um Banein and Shambat in Northern Sudan. The DNA was digested completely with the restriction endonuclease Eco RI, Southern blotted and probed with the SR1 insert derived from Eco RI digestion of λgt11-SR1. The resulting autoradiograph is shown in Figure 4, and the sizes of the bands to which SR1 hybridised are summarised in Table 4. Despite the poor quality of some of the tracks it was still possible to distinguish a total of ten different sized restriction fragments, and there were obvious variations in the number and sizes of bands detected between the different stocks. Many of these size differences were quite small, such as the differences between the bands of 6.2 and 6.0kb in the Sagadi DNA, and 5.8kb in Soba 1biii. Restriction fragments could be identified which were common to different stocks from the same location, such as the 3.1kb band in both the Razi S3 and Razi S15 stocks, the 6kb band in the Shambat stocks and the 5.1kb band in Um Banein 21/29 and 24. However, the patterns of restriction fragments observed did not always correlate to the geographical origin of the isolate, since considerable heterogeneity was exhibited between the various stocks from Northern Sudan.

In order to identify other restriction endonucleases apart from Eco RI which reveal restriction fragment polymorphisms in the *T. annulata* genome, DNA was prepared from macroschizont infected cells of the uncloned Ankara 46 stock and digested with a number of restriction enzymes chosen at random. Many of these had recognition sites predicted within the SPAG1 sequence, as shown by the cSPAG1

Figure 4

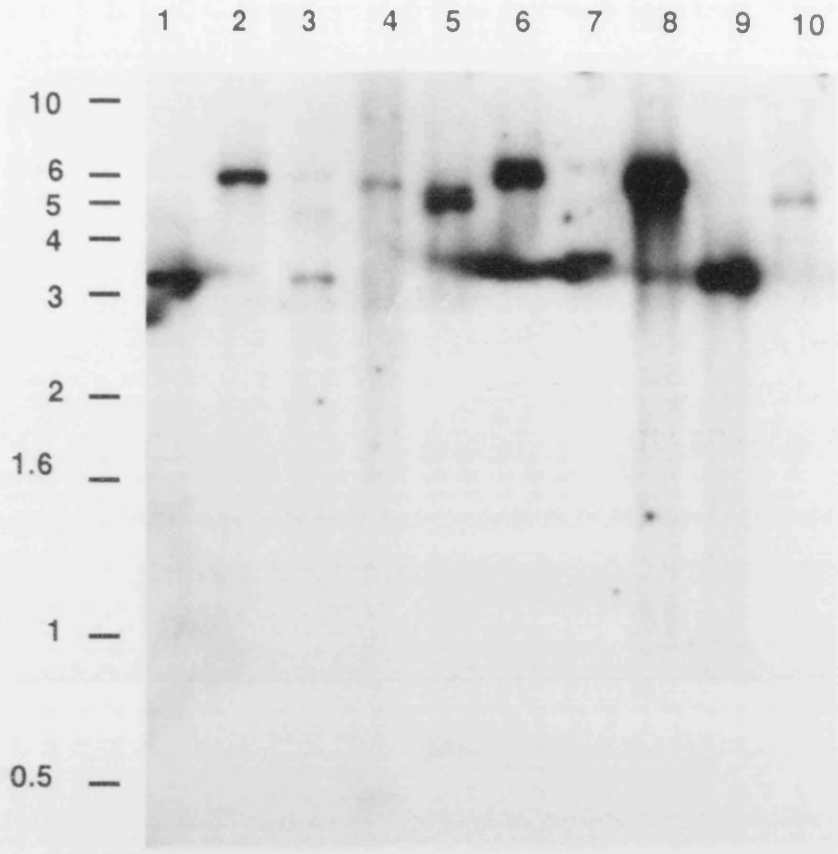
Autoradiograph (exposed for 3 days) of a Southern blot of Eco RI digested DNA from uncloned *T. annulata* macroschizont cell lines hybridised with the SR1 insert from λgt11-SR1. The markers are shown on the left hand side (sizes in kb). Tracks contained DNA from the following stocks:

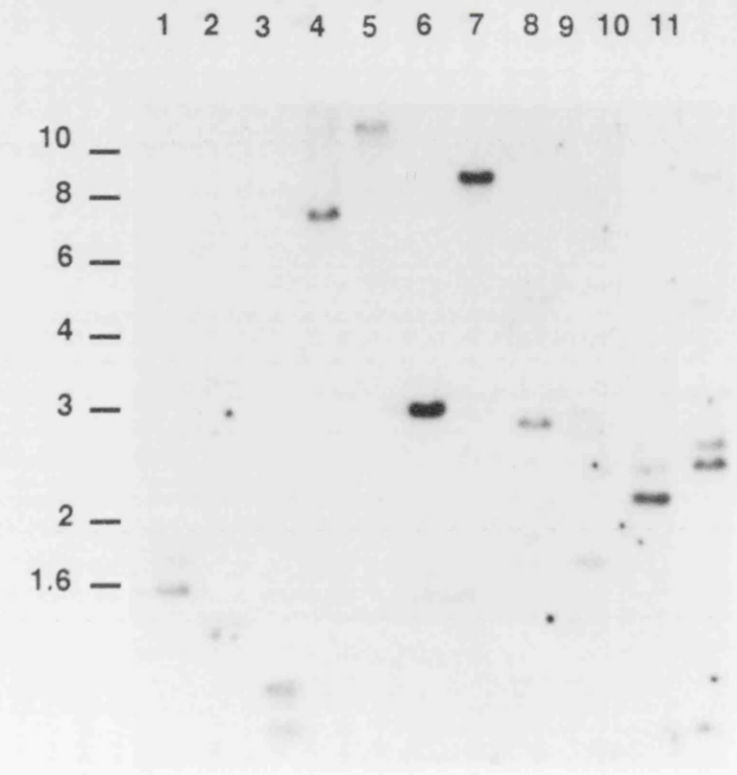
Track 1	Razi S15	Track 6	Sagadi
Track 2	Shambat 32	Track 7	Soba 46
Track 3	Ankara 46	Track 8	Shambat 33
Track 4	Soba 1biii	Track 9	Razi S3
Track 5	Um Banein 21/29	Track 10	Um Banein 24

Figure 5

Autoradiograph (exposed for 3 days) of a Southern blot of DNA from uncloned Ankara 46 macroschizont infected cell line digested with a variety of restriction endonucleases, following hybridisation with the 1.6kb Bam H1 insert from pGEX2T-2.4. The markers are shown on the left hand side (sizes in kb). Tracks contained DNA digested with the following enzymes:

Track 1	Cfo I (Hha I)	Track 7	Pst I
Track 2	Acc I	Track 8	Eco RI
Track 3	Ava II	Track 9	Hinc II
Track 4	Ava I	Track 10	Xba I
Track 5	Sst I	Track 11	Hpa I
Track 6	Hind III		


1 2 3 4 5 6 7 8 9 10
10
6
5
4
3
2
1.6
1
0.5


1 2 3 4 5 6 7 8 9 10 11
10
8
6
4
3
2
1.6

restriction map (Figure 78A, Appendix). The Southern blot of these digests was probed firstly with the SR1 insert, then the blot was stripped as described in section 2.2.3 and reprobed with the 1.6kb insert. The autoradiograph following hybridisation with the 1.6kb probe is shown in Figure 5, and the restriction fragments produced by digestion with each enzyme seen after hybridisation with each probe are summarised in Table 5. The differences observed in the banding pattern obtained in the Hinc II and Hpa I digest following hybridisation with either probe were probably due to recognition of restriction sites covered by the 1.6kb but not the SR1 insert, ie within the region 1197-2403 of the SPAG1 sequence, with the probe being able to hybridise with both the fragments. Both the restriction enzymes do contain a single recognition site within this region (starting at nucleotide 1949), as predicted from the cSPAG1 sequence (Figure 78A, Appendix). Four of the enzymes, Cfo I (Hha I), Pst I, Hinc II and Hpa I, which all hybridised to multiple bands, were chosen to be used in further analysis of SPAG1 restriction fragment polymorphism. It was decided to use the SR1 probe in further hybridisations since it contained no internal restriction sites for any of the enzymes mentioned above, while the 1.6kb probe contained internal Hpa I, Hinc II and Cfo I sites which could make the resulting hybridisation patterns more difficult to interpret.

The enzymes Pst I, Hinc II and Eco RI were used to digest *T. annulata* Ankara, Gharb and Hissar piroplasm DNA, for a Southern blot which was probed with the SR1 insert. These stocks were originally isolated in Turkey, Morocco and India respectively. The autoradiograph is shown in Figure 6. The SR1 probe hybridised to multiple bands from each digest. The majority of fragments produced by each restriction endonuclease were common to all three stocks, although their intensity varied considerably between the stocks: this is particularly apparent in the Eco RI digested DNA (tracks 6, 7 and 8). A few of the restriction fragments, however, are absent in some of the stocks; such as the 3.3kb Hinc II fragment, which is present in the Ankara and Hissar stocks and absent in Gharb. The implications of all these results will be discussed further in section 3.4.

Figure 6

Autoradiographs (exposed for 3 days) of Southern blot of *T. annulata* Ankara, Gharb and Hissar piroplasm DNA digested with restriction enzymes Pst I, Hinc II or Eco RI and hybridised with the SR1 insert from pGEX1λT-SR1. The sizes of the bands seen (kb) are indicated by arrows. Tracks contained DNA from the following stocks:

Track 1	Gharb, digested with Pst I
Track 2	Hissar, " "
Track 3	Ankara, digested with Hinc II
Track 4	Gharb, " "
Track 5	Hissar, " "
Track 6	Ankara, digested with Eco RI
Track 7	Gharb, " "
Track 8	Hissar, " "

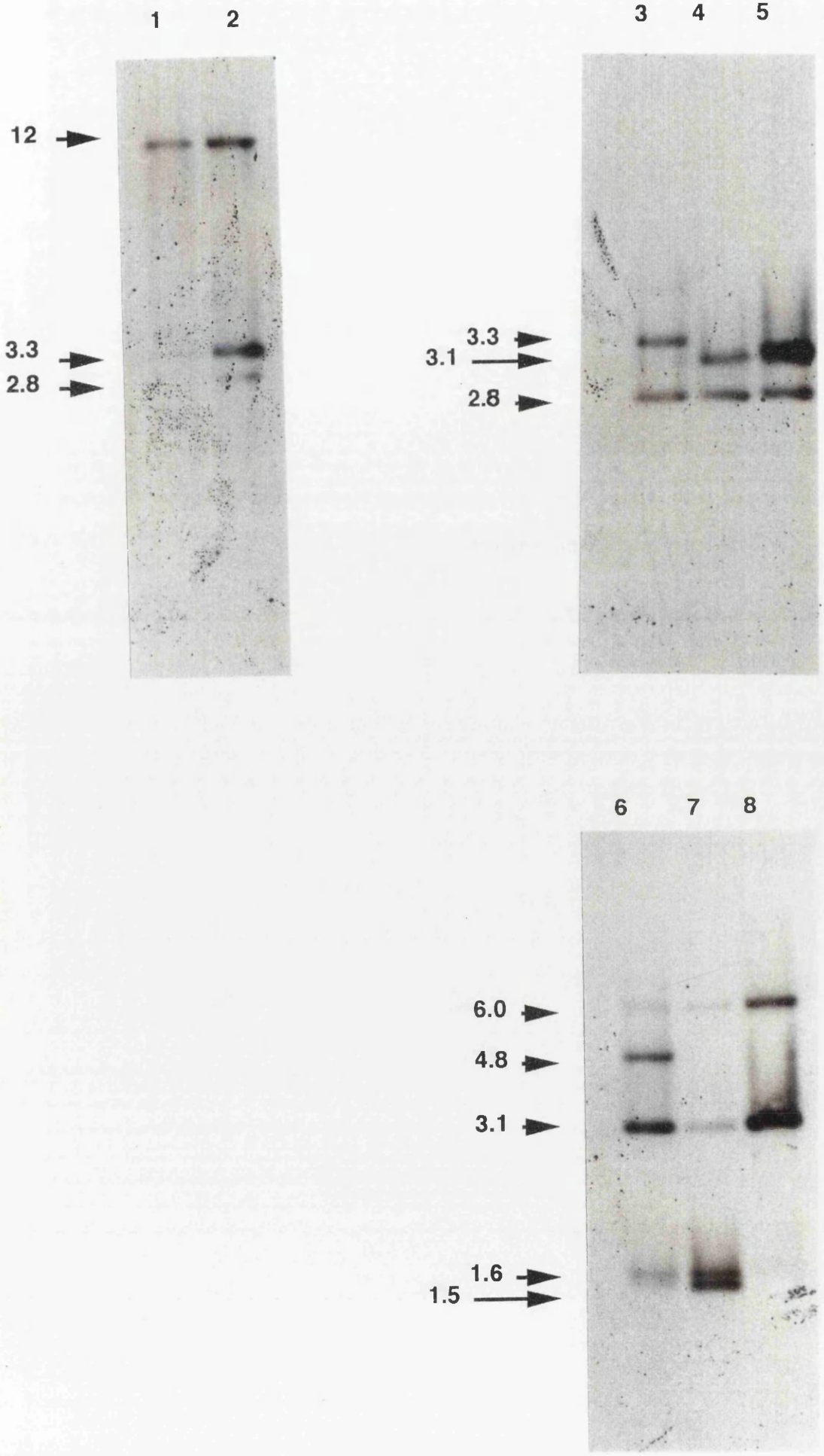
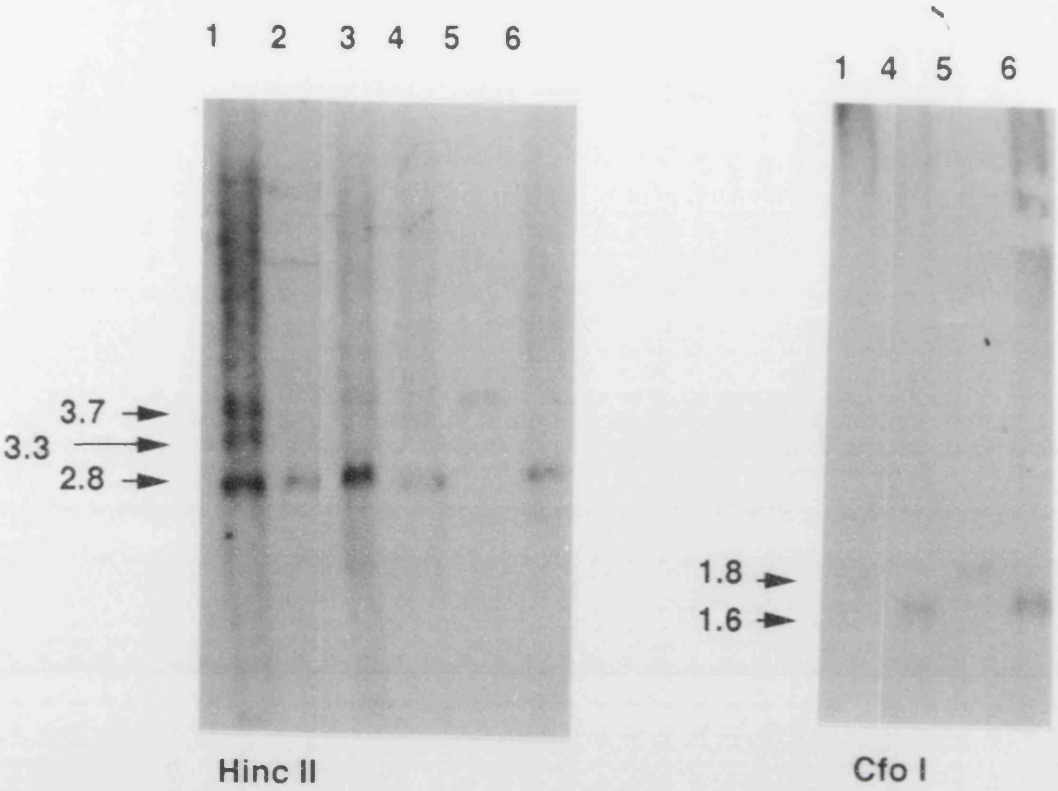

1
2
12
3.3
2.8
3
4
5
3.3
3.1
2.8
6
7
8
6.0
4.8
3.1
1.6
1.5

Figure 7

Autoradiographs (exposed for 7 days) of DNA from uncloned Ankara 46 and cloned Ankara macroschizont infected cell lines digested with Hinc II, Cfo I or Hpa I restriction enzymes, following hybridisation with the SR1 insert from pGEX1λT-SR1. The sizes of the bands seen (kb) are indicated by arrows. Tracks contained DNA from the following:

Track 1	uncloned Ankara 46
Track 2	clone A1-A3
Track 3	clone 46-2
Track 4	clone 139-D4
Track 5	clone 139-D6
Track 6	clone 139-E5


1 2 3 4 5 6
3.7
3.3
2.8
Hinc II
1 4 5 6
1.8
1.6
Cfo I
1 4 5 6
8
5.5
Xba I

3.2.2 Analysis of cloned stocks

DNA was extracted from a number of cloned *T. annulata* Ankara macroschizont infected cell lines, A1-A3, A46-3, A139-D4, A139-D6 and A139-E5, and uncloned A46. These clones were established by J.M. Wathanga and G. Wilkie as described by Williamson (1988). Digests were set up using the restriction endonucleases Hinc II, Cfo I and Hpa I, and used in Southern blots which were probed with SR1. The resulting autoradiograph is shown in Figure 7, and the bands seen are summarised in Table 6. These clones were also known to exhibit polymorphism in parasite glucose phosphate isomerase isoenzyme patterns according to analysis by starch gel electrophoresis (Wilkie et al 1986) and the isoenzyme type of each clone are included in Table 6 for comparison. While two or three bands were detected in the endonuclease digests of uncloned material (8A, B and C, track 1) these bands appeared to segregate in the digests of cloned material in the case of all three restriction enzymes. The faint 3.7kb band detected in the Hinc II digest of uncloned Ankara 46 which was not seen previously (Table 5) was probably due to the longer exposure of this autoradiograph. The clone 139-D6 (track 5) consistantly produced a larger restriction fragment in digests with all three enzymes than the other clones examined, which appeared to be identical in their restriction fragment patterns. The clones appeared to segregate into two types according to their restriction fragment sizes.

The SR1 insert was also used to probe a Southern blot of DNA from four Soba 46 cloned macroschizont infected cell lines digested with Eco RI (Figure 8). Although the bands were weak, probably due to poor hybridisation of the probe, two bands could be distinguished in the track containing DNA from uncloned Soba 46, of 6 and 3.3kb (track 1). In digests of DNA from the clones the 6kb band detected in the uncloned stock appears to resolve into two bands of slightly different sizes, 6.2 and 6kb. The 6.2kb restriction fragment was detected in Soba 46 clones 1E7 and 2D4 (tracks 2 and 3), while the 6kb band was detected in clones 2G5 and 2G7 (tracks 4 and 5). Again only single bands could be detected in the clones. None of the clones analysed contained the 3.3kb restriction fragment apparent in the uncloned stock.

Figure 8

Autoradiograph of Southern blot of Eco RI digested DNA from *T. annulata* Soba 46 cloned and uncloned macroschizont infected cell lines, following hybridisation with the SR1 insert from pGEX1λT-SR1 (after a 10 day exposure). The sizes of the bands detected (kb) are indicated by arrows. Tracks contained DNA from the following cell lines:

Track 1	uncloned Soba 46
Track 2	Soba 46 clone 1E7
Track 3	" " 2D4
Track 4	" " 2G5
Track 5	" " 2G7

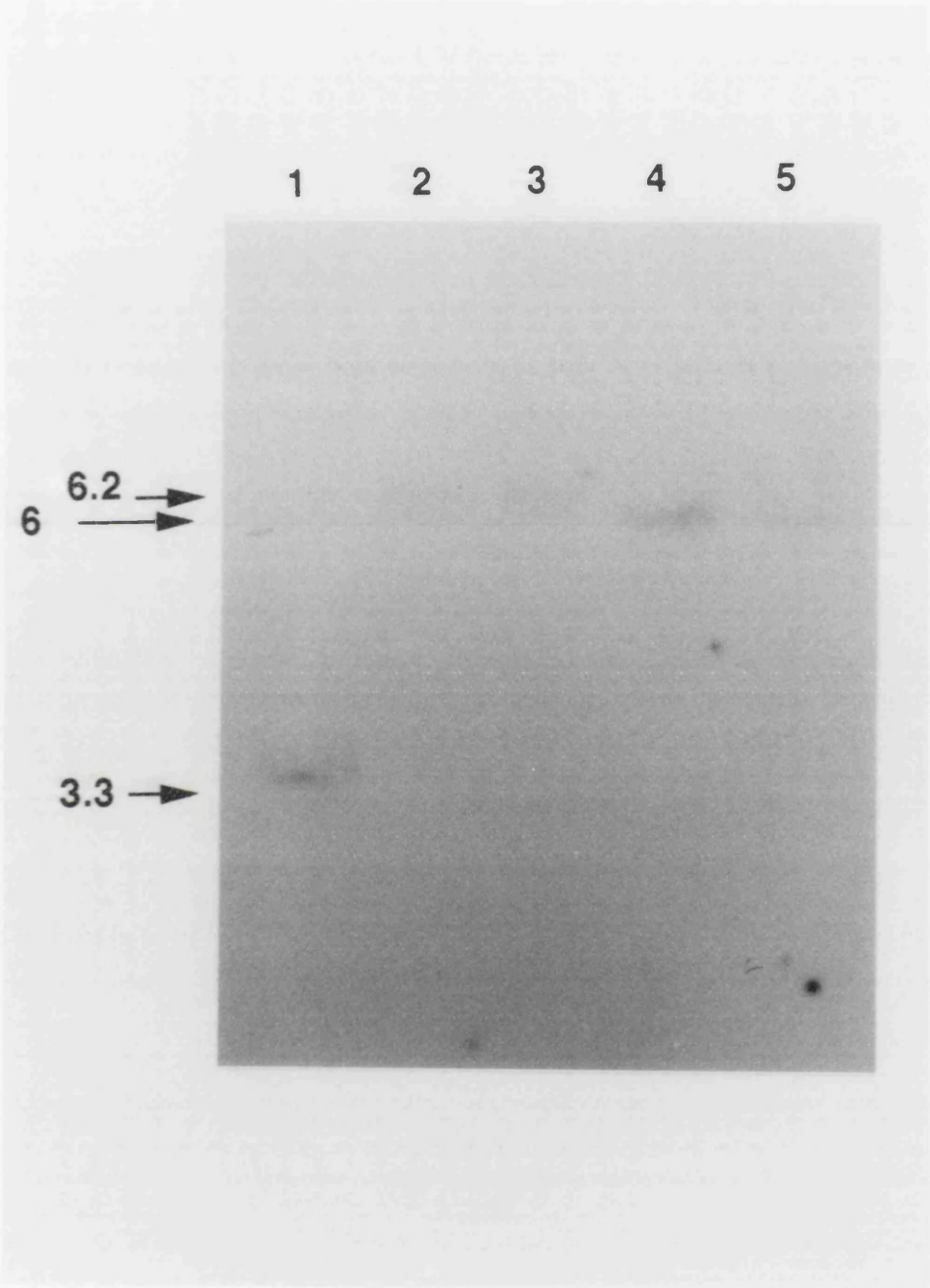

1
2
3
4
5
6.2
6
3.3

Figure 9

Autoradiograph (exposed for 4 days) of a timecourse of Hind III digested DNA from *T. annulata* Ankara cloned macroschizont infected cell line, 139-E5, probed with the SR1 insert from pGEX1λT-SR1. The total incubation time in minutes following enzyme addition is shown above each track, and the sizes of the bands detected (kb) are indicated by arrows.

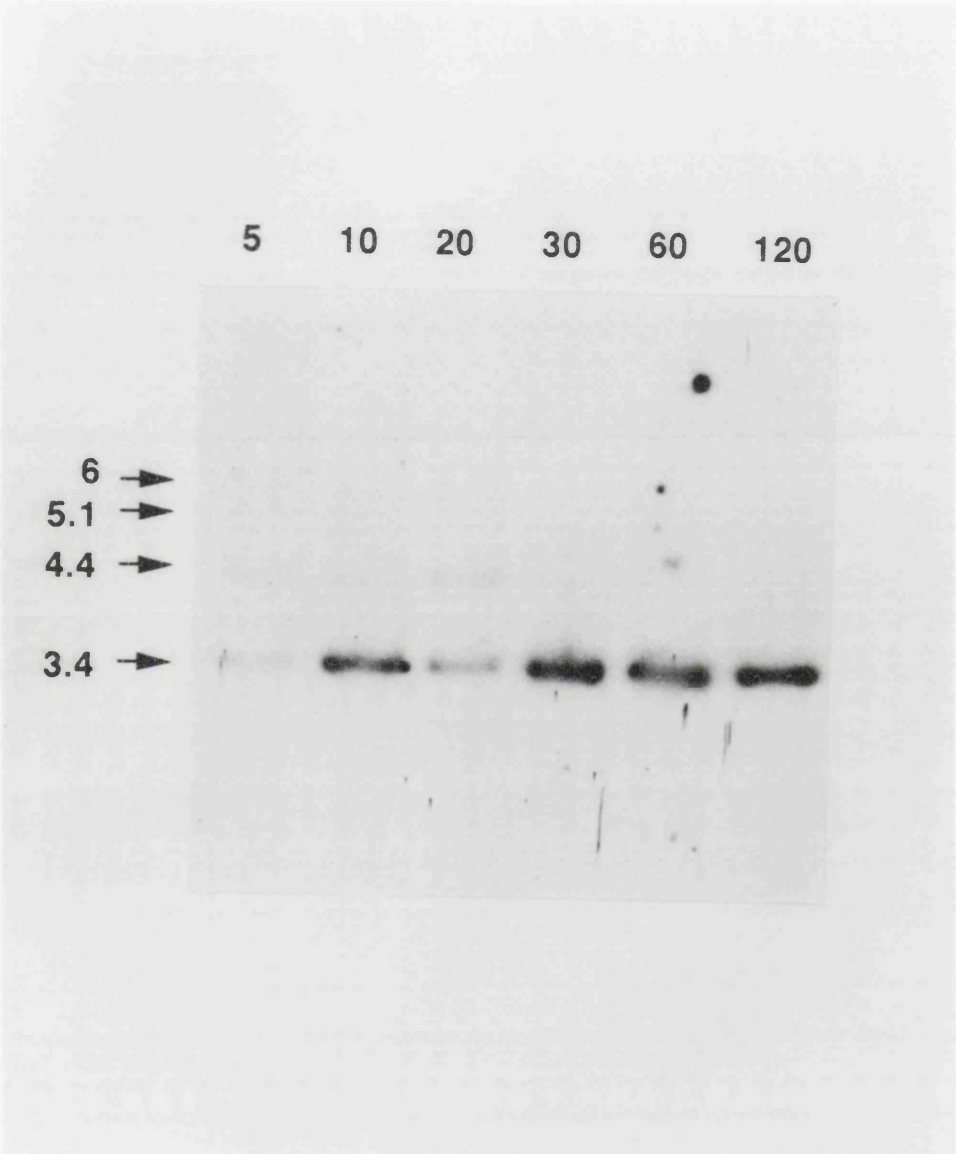
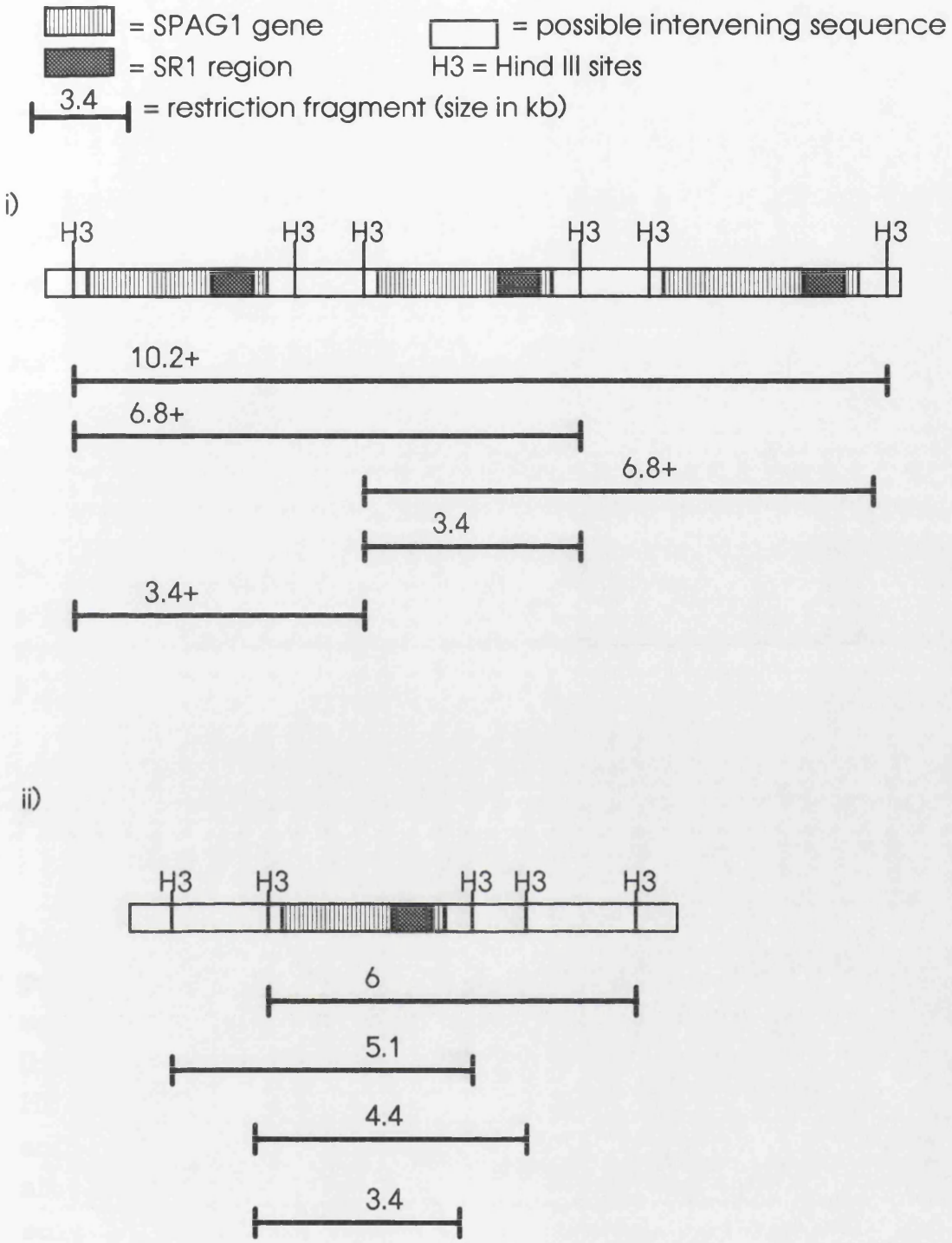

5
10
20
30
60
120
6
5.1
4.4
3.4

Figure 10

Predicted restriction fragments from Hind III endonuclease partial digestion of *T. annulata* Ankara 139-E5 DNA probed with SR1 (i) and postulated arrangement of restriction fragments obtained (ii).

Complete digestion of Ankara 139 DNA with Hind III produced a single band of size 3.4kb (Figure 10A, track 120'). It is possible that the SPAG1 gene occurs as tandemly arranged copies, each capable of producing identical 3.4kb restriction fragments with Hind III, leading to detection of a single band. However if this was the case, partial digestion of the DNA with Hind III would be expected to produce restriction fragments of sizes in multiples of 3.4kb (or larger if intervening sequences were present between the genes) as shown in (i). Since this pattern was not obtained, with Hind III sites occuring closer together than 3.4kb (ii), it was concluded that the SPAG1 gene is present as a single copy and not as a series of tandemly arranged repeats.


= SPAG1 gene
= possible intervening sequence
= SR1 region
H3 = Hind III sites
3.4
= restriction fragment (size in kb)
i)
H3
H3
H3
H3
H3
H3
10.2+
6.8+
6.8+
3.4
3.4+
ii)
H3
H3
H3
H3
H3
6
5.1
4.4
3.4

In order to provide further evidence that SPAG1 is present as a single copy in the *T. annulata* genome, partial digests were carried out of DNA prepared from the cloned macroschizont cell line A139-E5 using the restriction enzyme Hind III, as described in section 2.2.3. This enzyme was not predicted to recognise sites in the SPAG1 sequence (Figure 78A, Appendix) and does not appear to show restriction fragment polymorphism in uncloned Ankara 46 DNA (Figure 5). The reaction was halted at time intervals ranging from 5 to 120 minutes. Southern blotting was carried out and the DNA hybridised with the SR1 insert; the resulting autoradiograph is shown in Figure 9. Complete digestion with Hind III produced a single band of 3.4kb (at 60 and 120 minutes), while shorter digestion times produced larger restriction fragments of 6, 5.1 and 4.4kb. These results were consistant with SPAG1 gene existing as a single copy and not as tandem repeats. If there were tandem repeats of SPAG1 in the genome, with or without intervening sequences, the SR1 probe would be expected to hybridise to restriction fragments of sizes that were multiples of 3.4 or larger in partial digests (as explained in Figure 10).

3.2.3 Sequence data

The sequence data in this section was provided by R. Hall, Department of Biology, University of York and are included here with his permission. In addition to the published SPAG1 sequence obtained using a recombinant from a *T. annulata* Hissar sporozoite cDNA library (Hall et al 1992), a second variant of this gene derived from a *T. annulata* Hissar piroplasm genomic library constructed in λgt11 was also sequenced (gSPAG1). Sequence comparisons were made between the two alleles at both the nucleotide level and between the predicted amino acid sequences, using the GCG computer package. The amino acid sequence comparison is shown in Figure 11. While both sequences were clearly homologous, they contain obvious regions of divergence. There was 78.5% identity between the cSPAG1 and gSPAG1 nucleotide sequences, and 87.2% identity (94.7% similarity) between the predicted amino acid sequences. Restriction enzyme maps of both sequences are shown in Figure 78A and B, Appendix.

Figure 11

The result of a comparison between the two amino acid sequences gSPAG1 (g) and cSPAG1 (c), using the computer program GCG WordSearch. Amino acids are indicated in single letter code. The vertical dashed lines indicate identity between amino acids, the dotted lines indicate conservative substitutions. The horizontal dahed lines indicate gaps in the sequence. There was 87.2% identity (exact homology) and 94.7% similarity (which includes conservative substitutions) between the two sequences.

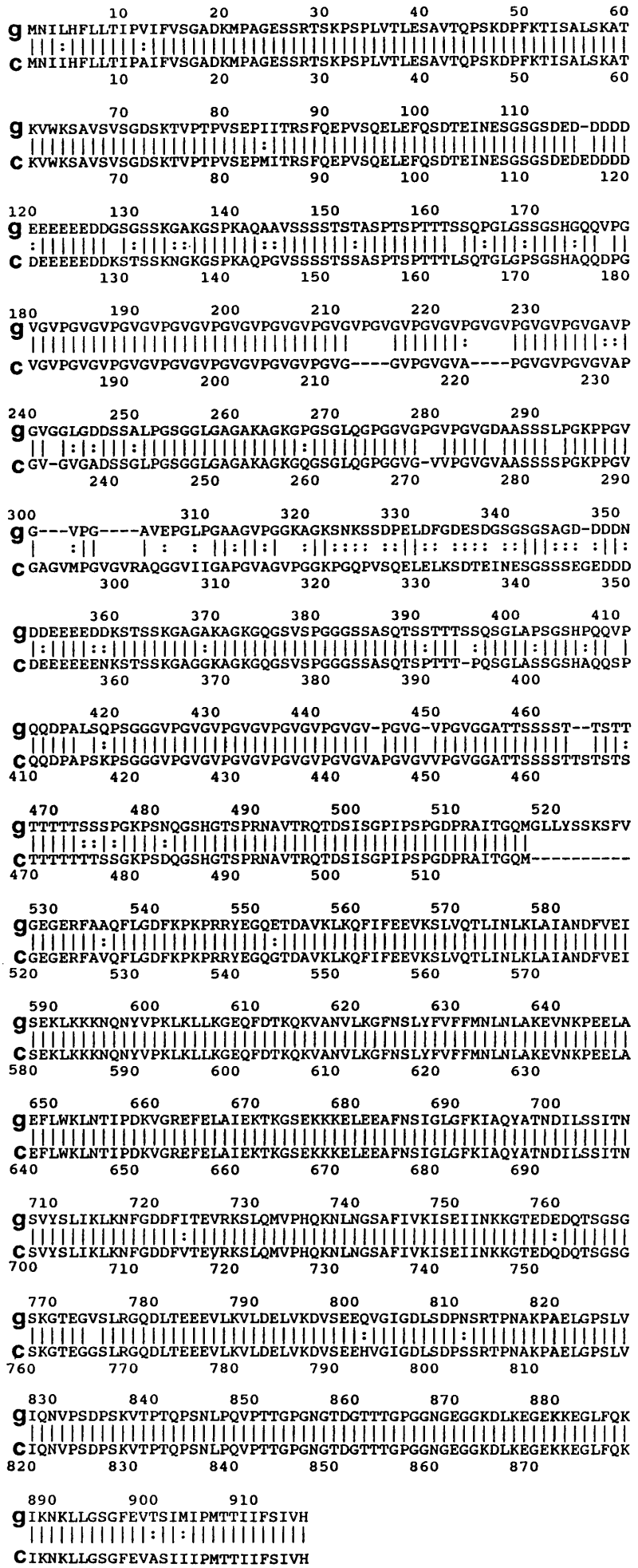


Figure 12

Histogram to summarise the extent of variation between the nucleotide sequences of cSPAG1 and gSPAG1 according to a comparison made using the computer program GCG WordSearch. The cSPAG1 sequence was divided into blocks of 150 bases, and the total number of bases that were either changed or absent in the corresponding gSPAG1 sequence was calculated. The x-axis denotes nucleotides of the cSPAG1 sequence (given in Figure 77, Appendix), while the y-axis gives the total number of these nucleotides that were substituted or were missing in the gSPAG1 sequence per block of 150 bases.

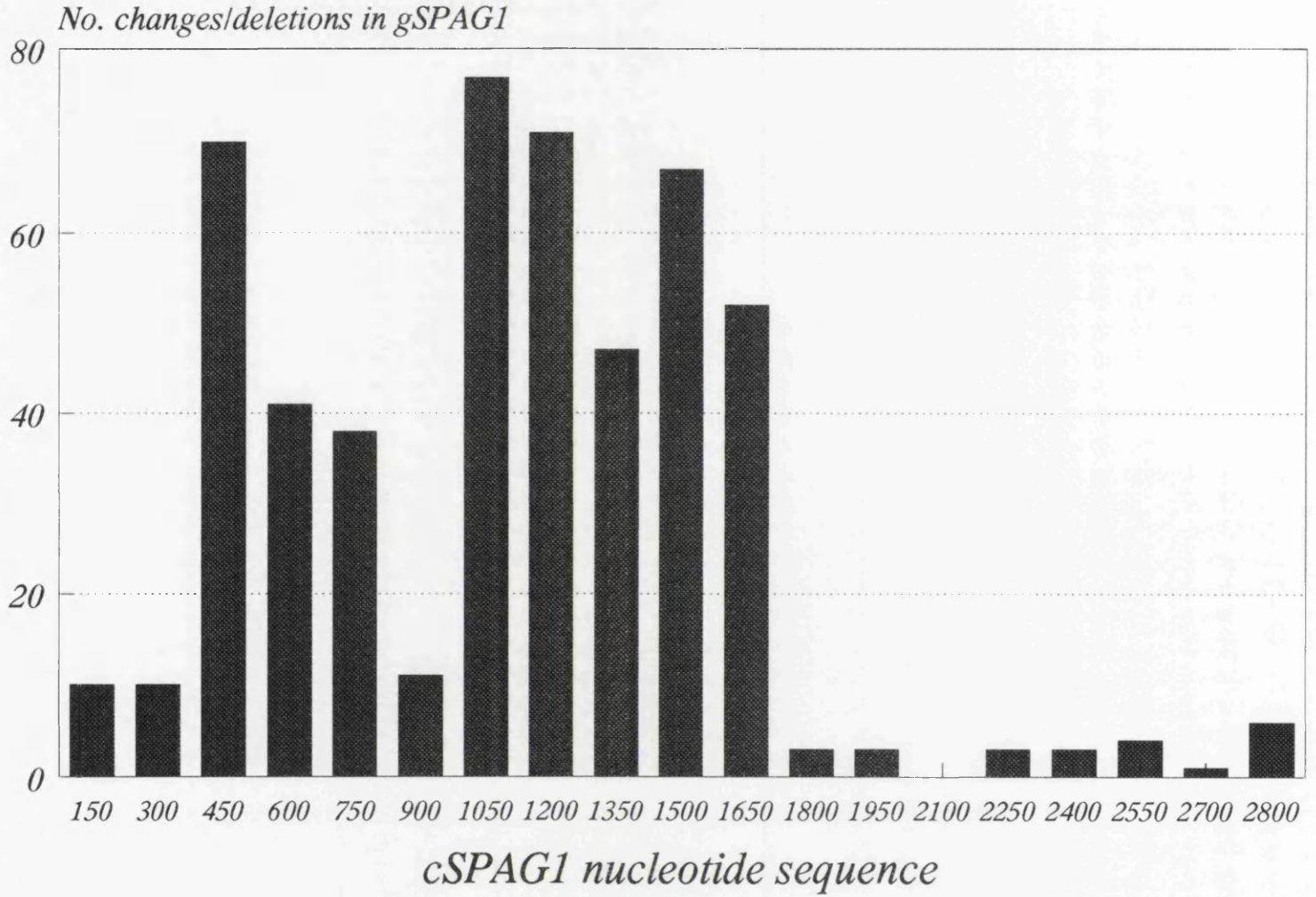
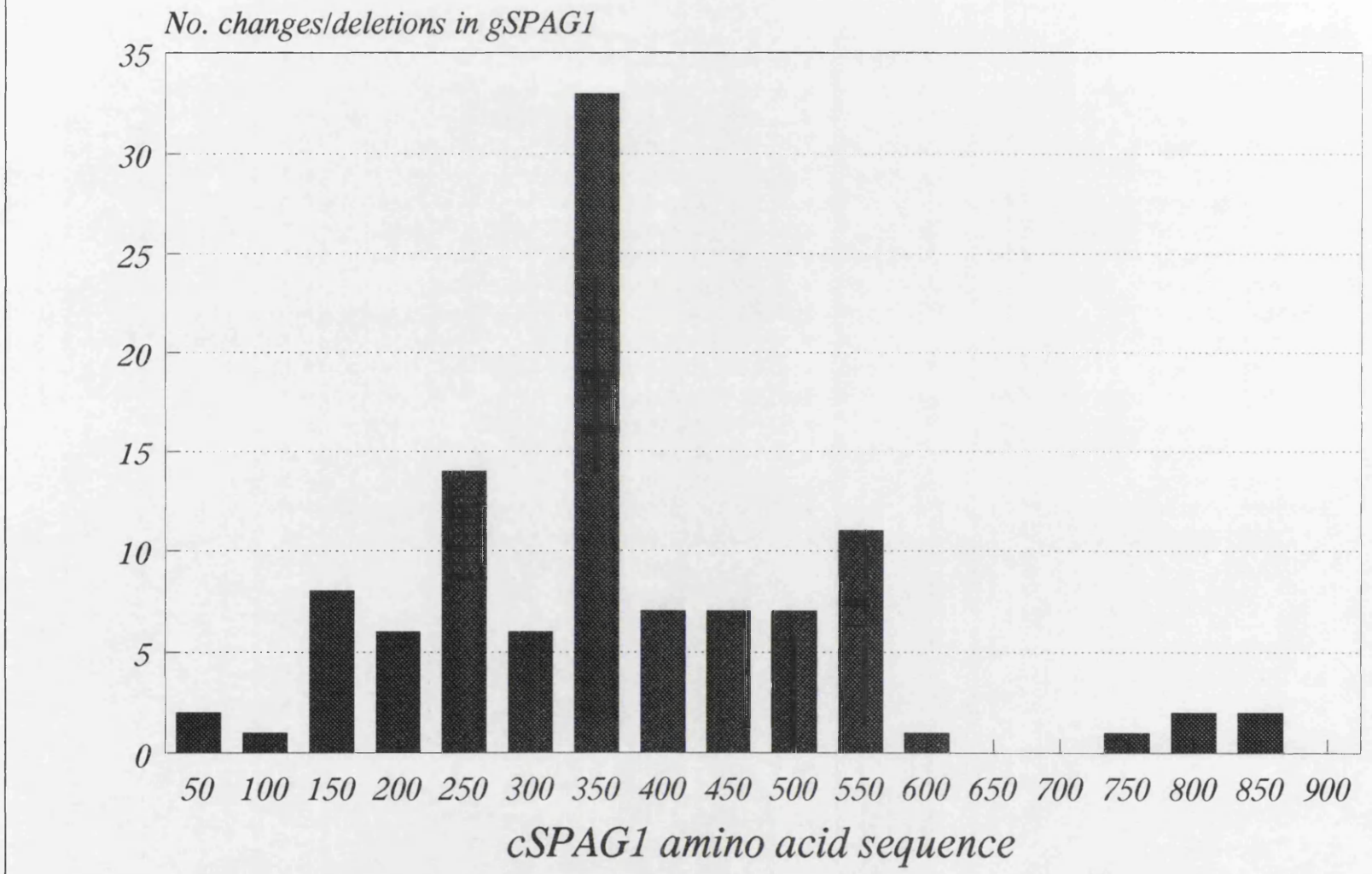

No. changes/deletions in gSPAG1
80
60
40
20
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
1950
2100
2250
2400
2550
2700
2800
cSPAG1 nucleotide sequence

Figure 13

Histogram to summarise the extent of variation between the predicted amino acid sequences of cSPAG1 and gSPAG1 according to a comparison made using the computer program GCG WordSearch. The cSPAG1 sequence was divided into blocks of 50 amino acids and the total number that were either changed or absent in the corresponding gSPAG1 sequence was calculated. The x-axis denotes amino acids of the cSPAG1 sequence, while the y-axis gives the total number of these that were substituted or were missing in the gSPAG1 sequence per block of 50 amino acid residues.


No. changes/deletions in gSPAG1
35
30
25
20
15
10
5
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
cSPAG1 amino acid sequence

In order to show the areas of greatest divergence more clearly, the result of the comparison at the nucleotide level is summarised in Figure 12, and at the amino acid level in Figure 13. Differences between the two sequences were scored as follows; the number of "deletions" was the number of amino acids or nucleotide bases present in the cSPAG1 sequence but absent in gSPAG1, while the number of "substitutions" refered to amino acids or nucleotide bases in the cSPAG1 sequence which were substituted with different amino acids in the gSPAG1 sequence. The areas of the highest divergence between the amino acid sequences of gSPAG1 and cSPAG1 correlated closely with the greatest differences in the nucleotide sequences. The most variant region between the two alleles appeared to cover amino acids 100-550 of the cSPAG1 sequence (78% identity), particularly between amino acids 300-350 (66% identity) between the two blocks of elastin homologous repeats, although this region did contain a high level of conservative sustitutions (Figure 11). The corresponding region of the coding sequences, between nucleotides 353-1703 of the cSPAG1 sequence, also showed low homology (76% identity). Conversely, the the region corresponding to SR1 (nucleotides 2403-2825, amino acids 725-893) appears to be highly conserved between gSPAG1 and cSPAG1, with 96% identity between the coding sequences and 95% identity at the amino acid level. It was not possible to tell from the restriction maps of the cSPAG1 or gSPAG1 nucleotide sequences (Figure 78A and B, Appendix) whether they corresponded to any of the SPAG1 alleles identified by restriction fragment analysis on the Southern blots. This was due either to the enzymes having a single cutting site within the SPAG1 sequence, or the cutting sites being too close together for the internal fragment to be visualised as a band on the Southern blots.

3.3 Discussion

The Southern blot analysis of uncloned parasite material showed the SPAG1 coding region to display extensive restriction fragment polymorphism. This confirmed previous observations (Williamson et al 1989) and showed that the polymorphism was more extensive than the two or three alleles originally described. Ten different sized bands were detected altogether on the Southern blot of macroschizont DNA (Figure 4,

Table 4) and five on the blot of piroplasm DNA (Figure 6). The SR1 probe used for these blots contained no internal restriction sites for any of the endonucleases used; Eco RI, Hinc II and Pst I, although polymorphisms in other copies of the SPAG1 sequence could give rise to restriction sites and result in the detection of two bands derived from the same allele. However, even taking this into account, a minimum of six restriction fragment polymorphisms would be required to produce the ten different sized bands seen in Figure 4. These could arise from polymorphism within the SPAG1 gene itself, although polymorphism in adjacent sequences could also have produced some of the restriction fragments observed.

It is notable that in the Southern blot of Ankara 46 piroplasm DNA, SR1 hybridises to two faint bands of 1.5 and 1.6kb (Figure 6, track 6) which are not detected in Ankara 46 macroschizont DNA (Figure 4, track 3). This could be purely a result of the lower and more variable proportion of parasite DNA in total DNA extracted from macroschizont infected lymphocytes rather than purified piroplasms, or through depletion of specific genotypes during "in vitro" culture. Changes can occur in the biological properties of macroschizonts during tissue culture, as discussed in section 1.1.4. It is interesting that the SR1 probe hybridised to several bands of identical size in restriction enzyme digested *T. annulata* piroplasm DNA from uncloned Hissar, Gharb and Ankara stocks, since this implies that at least some of the restriction sites, which may be within the SPAG1 gene or in adjacent DNA sequences, are shared between geographically separated parasite populations. Some of the restriction fragments from the uncloned macroschizont DNA were also shared between stocks isolated in different countries, such as the 6kb band seen in Ankara 46, Sagadi, Soba 46 and the two Shambat stocks, and the 3.1kb band common to Ankara 46 and the two Razi stocks. The heterogeneity seen in the stocks derived from Northern Sudan may be due simply to the greater number of stocks examined, or imply that *T. annulata* parasite populations in this area are particularly polymorphic in the region of DNA containing SPAG1. These same stocks have also been found to exhibit considerable heterogeneity in their glucose phosphate isomerase isoenzyme patterns (Melrose et al 1984).

The single bands observed in endonuclease digests of DNA from the Ankara and Soba 46 macroschizont clones (Figures 7 and 8), provided further evidence for SPAG1 existing as a single copy in the *T. annulata* genome. The hybridisation of SR1 to larger restriction fragments in Hpa I, Cfo I and Hinc II digests of Ankara clone 139-D6 implies that this clone carries a different allele of SPAG1 from the other clones. This correlates with earlier observations that SR1 hybridised to a larger restriction fragment (4.8kb) in Eco R1 digest of 139-D6 compared with Eco RI digested DNA from clones 139-D4, 139-E5 and 46-2, which produced bands of 3.1kb. The fact that the SPAG1 restriction fragment patterns of the clones did not correlate with differences already identifed in their glucose phosphate isoenzyme patterns (Wilkie et al 1986; Table 4) indicates that the SPAG1 gene and the gene coding for the isoenzyme are not closely associated in the *T. annulata* genome. Furthermore, while three alleles were identified from the glucose phosphate isoenzyme types, only two SPAG1 alleles were identified from the restriction enzyme patterns. The two Eco RI fragments identified in the Soba 46 clones, of 6.0 and 6.2 kb (Figure 8) indicated that the two Soba 46 clonal types were different from those identified on Eco R1 digestion of the Ankara clones (Williamson 1988: described above).

The hybridisation of SR1 to single bands in the clones cannot discount the presence of tandemly arranged repeats of SPAG1 in the *T. annulata* genome, which could result in generation of restriction fragments of identical size from each copy of the gene. However, the results from the partial digest with Hind III provides evidence to discount the presence of multiple tandemly arranged copies of the gene, as summarised in Figure 10. This observation confirms that the multiple bands identified in the uncloned stocks described previously in this section are restriction fragment polymorphisms rather than originating from multiple copies of the gene.

The differences in the cSPAG1 and gSPAG1 nucleotide and amino acid sequences (Figures 11-13) confirm that the gene itself is polymorphic. A proportion of the nucleotide changes appear to be synonymous, ie not reflected at the amino acid level, since the two sequences are more homologous at the amino acid level (87.2% identity) than at the nucleotide level (78.5% identity, 94.7% similarity).

Furthermore, some of the "peaks" of divergence between the DNA sequences (such as between nucleotides 300-450 and 1050-1650, Figure 12) are not entirely reflected at the amino acid level (amino acids 100-150 and 350-550, Figure 13). Indeed, these regions contained a number of conservative amino acid substitutions (Figure 11). However, it is interesting that the overall % identity between the nucleotide sequences is not much higher than between the amino acid sequences. This indicates that a proportion of the variants in the nucleotide sequence were non-synonymous; ie. they resulted in amino acid changes. Such changes could potentially manifest themselves as alterations in T or B cell epitopes, as will be discussed later in this section. Sequence data from regions of SPAG1 amplified by the polymerase chain reaction using DNA from a number of different *T. annulata* Ankara and Soba 46 cloned and uncloned macroschizonts indicated that other variants of the gene existed apart from the alleles corresponding to gSPAG1 and cSPAG1 (personal communication; P. Hunt and R. Hall, Department of Biology, University of York). Two areas of the SPAG1 gene were amplified using primers based on conserved regions of the cSPAG1 sequence; the N-terminal region including the first set of elastin homologous repeats and the C-terminal SR1 region. Variants were identified in both these regions and the sequence data indicated that three other polymorphic versions of SPAG1 existed in the parasite population apart from the alleles corresponding to gSPAG1 and cSPAG1. These polymorphisms occured in both the DNA and predicted amino acid sequences. Interestingly, the SR1 region which was conserved between cSPAG1 and gSPAG1 sequences was considerably more variable in some of the other alleles sequenced. SPAG1 sequences from two of the Ankara clones, A1-A3 and 139-D6, were not identical to each other, confirming the Southern blot analysis indicating that these clones contained different alleles of SPAG1. Neither of these sequences were identical to cSPAG1 or gSPAG1, although a sequence identical to cSPAG1 was amplified by the polymerase chain reaction from uncloned Soba 46 DNA.

There are a number of other examples of polymorphism described in *T. annulata*. These include glucose phosphate isoenzyme patterns (Melrose et al 1984) and diversity of macroschizont antigens (Shiels et al 1986) among different stocks of macroschizont infected cell lines. In both cases, variability could be identified between stocks from identical and

different geographical locations. The 30-32kDa *T. annulata* merozoite surface antigen has been shown to be polymorphic in Ankara, Gharb and Hissar parasite stocks with respect to it's molecular weight and recognition by a McAb (Dickson and Shiels 1993). Extensive restriction fragment polymorphism between and within stocks has been identified in *T. annulata* isolates from Tunisia using a number of *T. annulata* sequence specific probes (Ben Miled et al 1993). Genotypic heterogeneity between and within stocks has also been observed for *T. parva* (Allsopp et al 1989, Conrad et al 1989). Polymorphism in *Theileria* species may have immunological implicatons where it results in antigenic diversity. Immunisation of cattle with *T. annulata* macroschizont infected lymphocytes or viable sporozoites usually results in complete protection from homologous challenge, as already discussed in section 1.1.4. However, cattle immunised with *T. parva* sometimes fail to be protected against heterologous challenge (Musisi 1990) and a case of this in *T. annulata* immunisation has been reported (Gill et al 1980). It can be speculated that instances of decreased protection to heterologous parasite stocks are the result of antigenic diversity.

Polymorphism has also been identified in antigens of other apicomplexan parasites, such as the surface membrane proteins of *Babesia rodhaini* (Snary and Smith 1988) and in a number of antigens of *P. falciparum* such as the circumsporozoite protein, S- (soluble) antigens, several antigens expressed on the erythrocyte membrane and the merozoite surface antigens MSA1 and MSA2 (Anders et al 1989). Evidence that polymorpism of such antigens contribute to immune evasion through variation in their T and B cell epitopes is reviewed by Mendis et al (1991). Variation in the coding sequences of *P. falciparum* circumsporozoite proteins has been studied particularly extensively. The gene is highly polymorphic, with many of the same polymorphic forms existing in geographically widely separated populations according to sequence analysis (McCuthchan et al 1992). These workers suggested that these common forms resulted from homoplasy; similarity not directly attributable to common ancestry, but possibly resulting from similar biological pressures such as that from the immune system. The circumsporozoite protein shows a high level of non-synonymous mutations compared to synonymous ones, ie. a high proportion of the genetic changes are phenotypically expressed at the amino acid level.

Since the cytotoxic and helper epitopes identified in the circumsporozoite protein tend to centralise on the most polymorphic regions of the antigen sequence, it has been suggested that polymorphism of the antigen evolved as a direct result of immune pressure from T cells (Good et al 1988b, Good et al 1988c and Lockyer et al 1989). However, this has been questioned by other workers who suggest that polymorphism is a more general feature of large, rapidly evolving sequences and not necessarily through selection by immune pressure (Arnot 1989).

The results described in this chapter indicate that the SPAG1 gene contains polymorphic regions, which are reflected in part at the amino acid level, and at least some of the polymorphic forms could be shared by geographically isolated stocks. Since these features are also salient in the circumsporozoite protein of *P. falciparum*, it is interesting to speculate that allelic variation in *T. annulata* SPAG1 is also a direct result of immune pressure. The next few chapters describes work towards identifying T and B cell epitopes of SPAG1, and the location of any epitopes identified with respect to the polymorphic regions of the molecule will be discussed.

TABLE 4. RESTRICTION FRAGMENT POLYMORPHISM OF SPAG1.

The following table summarises the sizes of restriction fragments that were observed in EcoRI digested DNA from *T. annulata* macroshizont stocks probed with SR1, as shown in Figure 4. The appropriate tracks on Figure 4 are referred to.

COUNTRY OF ISOLATION	STOCK	SIZE OF BANDS DETECTED (kb)	TRACK
Turkey	Ankara 46	6 4.8 3.1	3
Iran	Razi S3	3.1	9
	Razi S15	3.1	1
Sudan	Sagadi	6.2 6	6
	Shambat 32	6	2
	Shambat 33	6	8
	Soba 1biii	5.8 0.4	4
	Soba 46	6 3.3	7
	Um Banein 21/29	5.4 5.1 0.6	5
	Um Banein 24	5.1	10

TABLE 5. RESTRICTION FRAGMENTS PRODUCED BY ENDONUCLEASE DIGESTION OF ANKARA 46.

The following table summarises the restriction fragments generated by digestion of DNA from uncloned Ankara 46 macroschizont infected cell line digested with a variety of restriction endonucleases, on hybridisation with the SR1 or 1.6kb probe described in the text. The size of restriction fragments for the SR1 probe are only noted where they were different from the 1.6kb probe. The tracks refer to Figure 5, which shows the autoradiograph after hybridisation with the 1.6kb probe.

ENZYME	RESTRICTION FRAGMENTS (KB)		TRACK
	1.6KB PROBE	SR1 PROBE	
Cfo I	1.8 1.6	Identical to 1.6	1
Acc I	4.4 1.4	" " "	2
Ava II	1 0.8	" " "	3
Ava I	9	" " "	4
Sst I	20+	" " "	5
Hind III	3.4	" " "	6
Pst I	12 3.3	" " "	7
Eco RI	6 3.1	" " "	8
Hinc II	3.3 2.8 1.9	3.3 2.8	9
Xba I	2.8 2.4	Identical to 1.6	10
Hpa I	9 5.5 3 2.8	9 5.5 2.8	11

TABLE 6. RESTRICTION FRAGMENTS IDENTIFIED IN ANKARA 46 CLONES

The following table summarises the sizes of restriction fragments seen in Figure 7, generated by digestion of DNA from cloned and uncloned Ankara macroschizont infected cell lines by restriction endonucleases Hinc II, Cfo I and Hpa I, following hybridisation with SR1. The tracks on Figure 7 are referred to. The glucose phosphate isomerase (GPI) isoenzyme types (Wilkie et al 1986) are also included for comparison; these are arbitarily classified as 1, 2 or 3.

ENZYME	CELL LINE	TRACK	RESTRICTION FRAGMENTS (KB)	GPI TYPE
Hinc II	Uncloned A46	1	3.7 3.3 2.8	**1/ 2/ 3**
	A1-A3	2	2.8	**1**
	A46-2	3	2.8	**1**
	A139-D4	4	2.8	**3**
	A139-D6	5	3.7	**2**
	A139-E5	6	2.8	**1**
Cfo I	Uncloned A46	1	1.8 1.6	**1/ 2/ 3**
	A139-D4	4	1.6	**3**
	A139-D6	5	1.8	**2**
	A139-E5	6	1.6	**1**
Hpa I	Uncloned A46	1	9 5.5	**1/2/3**
	A139-D4	4	5.5	**3**
	A139-D6	5	9	**2**
	A139-E5	6	5.5	**1**

CHAPTER 4

THE CLONING, EXPRESSION AND PURIFICATION OF SPAG1 POLYPEPTIDES

4.1 Introduction

To enable the bovine immune response to SPAG1 to be investigated, it was of primary importance to express the recombinant protein on a large scale, using a suitable expression vector. It was also necessary to express a series of constructs covering defined regions of SPAG1 in order to dissect B and T cell responses more closely.

For these purposes, it was decided to use the pGEX vectors (Smith and Johnson 1988) for intracellular expression in *E. coli*. These plasmids are constructed to give a fusion protein with the carboxyl terminus of the *Schistosoma japonicum* Glutathione-S-transferase (GST) protein. The fusion proteins can be isolated from bacterial lysates by affinity purification on Glutathione Sepharose 4B gel, which consists of reduced glutathione coupled to agarose beads. The proteins can then be eluted under mild conditions by competitive binding from free reduced glutathione.

The cloning procedures which were used to generate these vectors are described by Smith and Johnson (1988). Since the coding sequence for both GST and the protein of interest are eukaryotic, the vector includes prokaryotic initiation and termination sequences. Therefore, the ATG codon for methionine, which initiates translation in bacterial systems, precedes the GST gene. This results in an unnatural methionine residue at the N-terminus of the expressed GST, but this was found not to effect it's enzymic activity. The normal termination sequence of the GST gene is absent, being replaced by a polylinker containing BamH1, Sma1 and EcoR1 restriction enzyme recognition sites, followed by TGA termination codons in all three reading frames. The vectors also include coding sequences for the IPTG inducible *tac* promotor (Amann et al 1983), the Lac I^q allele, which is highly efficient in

repressing transcription from the *tac* promotor and the Ap^R gene for ampicillin resistance. The plasmids also contain coding sequences for specific protease recognition sites immediately upstream from the multiple cloning region, enabling the required polypeptide to be cleaved from the GST portion of the fusion protein.

It was decided to express the SPAG1 proteins intracellularly in *E. coli* as a means of producing large quantities economically in a system that can be readily scaled up to fermentation of several litres of culture. The problems of protein folding in *E. coli* have been discussed in Chapter 1 (1.2.5). One of the main factors which influences folding of foreign proteins in the prokaryotic environment is the incorrect formation of disulphide bridges. Since the SPAG1 and GST amino acid sequences reveal a paucity of cysteine residues, this problem would not be anticipated.

Another problem associated with expression of eukaryotic proteins in bacterial hosts is the inability of the host to add appropriate carbohydrate moities to the product, which can also effect the tertiary structure of the resultant protein (Kelley and Winkler 1990). The SPAG1 amino acid sequence contains five potential asparagine linked glycosylation sites (Hall et al 1992), which could possibly effect the way in which it is expressed in this system. However, lack of glycosylation has been found in many instances to have no obvious effect on the tertiary structure and biological activity of a number of recombinant proteins, such as IFN_γ, IFN_β and IL-2 (Smith and Johnson 1988). Potential limitations in mapping T and B cell epitopes that could occur due to lack of glycosylation will be discussed in the relevant chapters.

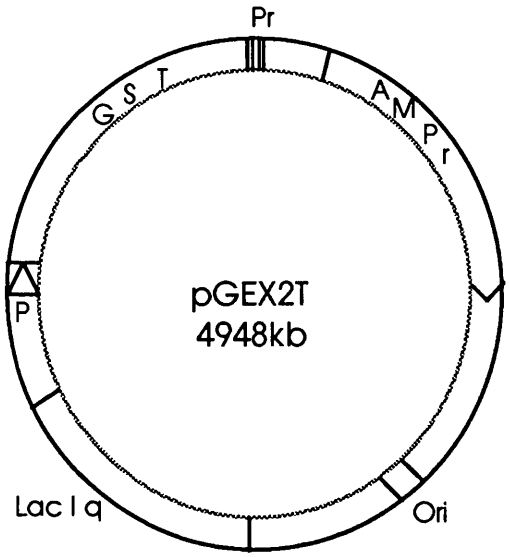
The pGEX system was chosen because it enables the recombinant protein to be purified without resorting to harsh conditions which could potentially destroy important epitopes. This system has also been used successfully in a number of similar applications; to express fourteen different *Plasmodium falciparum* antigens (Smith and Johnson 1988), to express a vaccine candidate antigen of *Taenia ovis* (Johnson et al 1989) and to ascertain T and B epitope location using a series of subfragments constructed from a *Mycobacterium bovis* protein antigen (Billman et al 1990).

In this chapter the cloning and expression of a number of SPAG1 subfragments in pGEX vectors are described. Details are also given of the results from a series of trial experiments set up to verify the conditions which minimised degradation and maximised expression of these fusion proteins. A number of variables were tested, such as induction time, IPTG concentration, degree of sonication and the effects of a reduced temperature and inclusion of protease inhibitors during the purification steps. The effect of using *E. coli* strains deficient in the *lon* gene was examined. This gene codes for the Lon A protease, which has an important role in the degradation of recombinant proteins. The effect of using *lon-* strains to express recombinant proteins can vary from a 150 fold increase in yield to no effect at all (Marston 1986).

Once a set of optimum conditions had been chosen, large scale fusion protein preparations were produced from all the SPAG1 derived constructs. Finally, trial experiments were set up for the proteolytic cleavage of the GST carrier from the fusion proteins by bovine thrombin or the blood coagulation factor Xa.

4.2 Cloning

Cloning was carried out using specific restriction enzyme sites chosen from the cSPAG1 DNA sequence (the restriction enzyme map is shown in Figure 78A, Appendix) and the multiple cloning sites of the vectors. The features of the vectors utilised, pGEX1λT, pGEX2T and pGEX3X, are summarised in Figure 14. Procedures for preparation of plasmid and phage λ DNA, restriction enzyme digestion, purification of DNA fragments on agarose gels, ligations and transformation of competent cells were carried out as described in Chapter 2 (sections 2.2.2 and 2.2.4). Large DNA preparations were made from each recombinant using the lysis by boiling method (section 2.2.2). The method used for making small scale fusion protein preparations is described in section 2.2.7, and SDS polyacrylamide gel electrophoresis was performed following the methods given in section 2.2.6.



Polylinker regions (Pr);

pGEX1λT

			Thrombin										
Leu	Val	Pro↓	Arg	Gly	Ser	Pro	Glu	Phe	Ile	Val	Thr	Asp	
CTG	GTT	CCG	GGA TCC		CCG	GAA TTC		ATC	GTG	ACT	GAC	TGA	
			Bam H1			EcoR1				Stop codons			

pGEX2T

			Thrombin		Sma1			
Leu	Val	Pro↓	Arg	Gly	Ser			
CTG	GTT	CCG	CGT	GGA TCC	CCG	GGA ATT	CAT	
				BamH1		EcoR1		

pGEX3X

			Factor Xa		Sma1			
Ile	Glu	Gly	Arg↓					
ATC	GAA	GGT	CGT	GGGATC	CCC	GGGAAT	TCA	
				Bam H1		EcoR1		

Figure 14; Features of pGEX expression vectors

P = Ptac promotor; GST = gene for glutathione-S-transferase; Pr = polycloning region; Amp^R = gene for ampicillin resistance; P = *tac* promotor; O = *ori* from pBR322; Lac I^q = lac I^q gene controling transcription.

Nucleotide sequences of the polylinker regions are given for pGEX1λT, pGEX2T and pGEX3X.

4.2.1 pGEX1λT-SR1

It was decided to subclone the insert from λgt11-SR1 into the pGEX system to facilate it's expression and purification. The subcloning procedure is summarised in Figure 15.

A large preparation of bacteriophage DNA was made from the λ gt11-SR1 recombinant (Williamson et al 1989) and digested completely with Eco RI. The 300bp insert was separated from the 20 and 24kb arms of the bacteriophage vector on an agarose gel, from which it was excised and purified. The vector pGEX1λT, which contains an Eco RI site in the same reading frame as bacteriophage λ, was also digested completely with Eco R1. A staggered end ligation of vector and insert DNA was carried out and the resultant plasmids used to transform *E. coli* JM101 competent cells (Table A, Appendix), with appropriate controls as described in section 2.2.4.

Twenty-four transformants were picked at random and screened for presence of the insert by Eco RI digestion of alkali lysis DNA minipreps, and the orientation of the insert checked by making small scale fusion protein preparations which were run on a 12% SDS polyacrylamide gel and stained with Coomassie Blue. Six of the colonies screened were found to contain an insert, and one of these expressed a fusion protein of the expected size (Figure 21A). The size of the 45kDa fusion protein correlated with the combined molecular weights of the GST carrier (26kDa) and the SR1 polypeptide, which was expected to have a molecular weight of 19kDa (Williamson et al 1989). Western blotting of the fusion protein preparations using rabbit antiserum raised against λ gt11-SR1 fusion protein (provided by Dr.S. Williamson, CTVM, Edinburgh University) showed a reaction with this clone only (Figure 22).

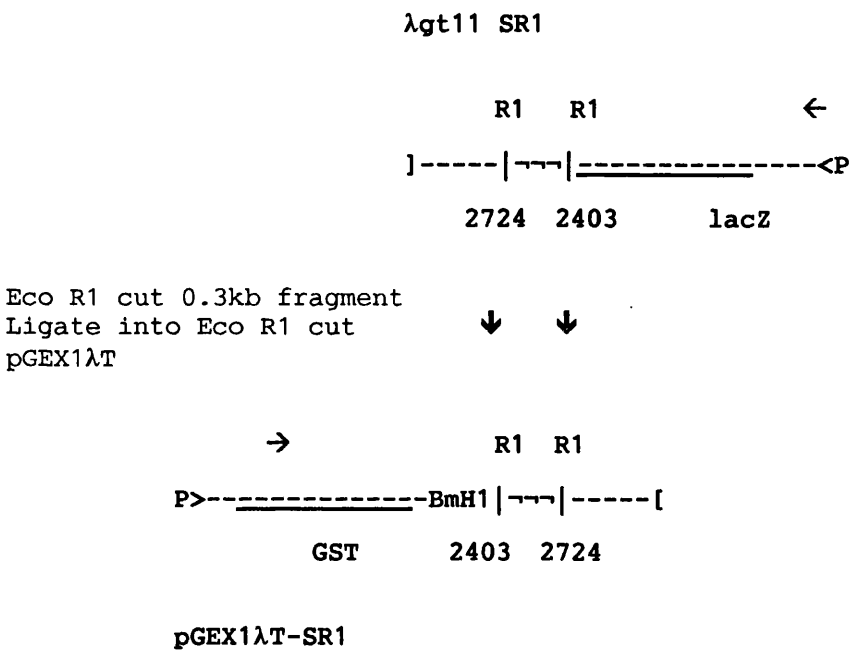
4.2.2 pGEX2T-2.4

A *T. annulata* Hissar sporozoite cDNA library in the vector pCDM8 was screened with the Eco RI insert from λgt11-SR1 (Hall et al 1992). The positive clone pTS-SPAG7, which was found by sequence analysis to contain the entire SPAG1 gene, was used for subcloning a 2.7kb

Figure 15. Cloning of pGEX1λT-SR1

The following diagram summarises the cloning procedure used. The numbers refer to nucleotides of the SPAG1 sequence (Figure 77, Appendix)

R1 = Eco R1 recognition site
BmH1 = BamH1 recognition site
---- = vector
¬¬¬¬ = insert
→ = direction of transcription
P = promotor



fragment into the Hind III and Pst I sites of pUC18 to generate the clone pUC18 SPAG7 (Figure 16A). These cloning steps were carried out by Dr. R. Hall, Dept. of Biology, University of York. This recombinant was used to generate subfragments of SPAG1 in pGEX2T.

The procedures used to subclone pGEX2T-2.4 are summarised in Figure 16B. It was decided to attempt to cut the pUC18-SPAG7 insert at the polylinker Eco R1 site and at Bam HI site 378, thus generating a fragment that would ligate into EcoR1/Bam HI digested pGEX2T to give the 2.4kb insert in the correct orientation. Bam HI site 378 is in an identical reading frame to the Bam HI site in the pGEX2T polylinker. The pUC18-SPAG7 recombinant was first digested completely with Eco RI and the 2.5kb fragment gel purified. Since the insert contained three Bam HI sites, as indicated in Figure 16B, it was necessary to carry out a partial digest as described in section 2.2.4 in order to cut site 378 only. The fragments produced by the partial digest were separated on a 1% agarose gel; the band sizes are summarised in Figure 16C. The Eco RI/ Bam HI (site 378) fragment could be identified by it's size of 2.4kb as indicated. The BamH1/EcoR1 2.4kb band was excised from the gel, purified and ligated into the polycloning site of pGEX2T, which would be expected to give the insert in the correct orientation. The ligation mixture was used to transform *E. coli* XL-1 Blue competent cells (Table A, Appendix).

Bam HI digestion of DNA minipreps, prepared from thirty randomly chosen recombinants by alkali lysis, revealed a single clone to contain the insert. Small scale fusion protein preparations run on a 12% polyacrylamide gel and stained with Coomassie Blue showed this clone to express a fusion protein of 130kDa. This was larger than the expected size of 106.3kDa, predicted from the coding capacity of the inserted DNA sequence combined with the 26kDa GST polypeptide. The fusion protein was completely soluble in PBS 1% Triton X-100, but quite heavily degraded (Figure 21B). Western blot analysis with McAb 1A7 detected a fusion protein in the preparation from pGEX2T-2.4, but no protein was detected in a clone lacking an insert (Figure 23).

Figure 16 Cloning pGEX2T-2.4

Figure 16a and b summarise the cloning of pGEX2T-2.4.

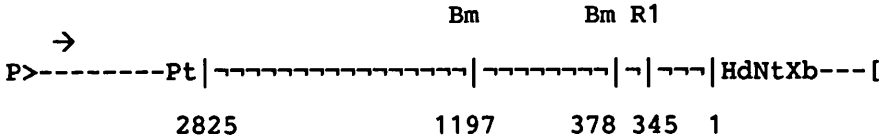
The following restriction enzyme sites are represented;
Pt = Pst1; Bm = BamH1; Hd = Hind III; Nt = NotI; Xb = Xba 1; R1 = EcoR1; Sc = Sac1; Kp = Kpn1; Sm = Sma1; Sa = Sal1.

Pr = polylinker region of plasmid containing a variety of restriction enzyme sites. See legend for Figure 15 for explanation of other symbols.

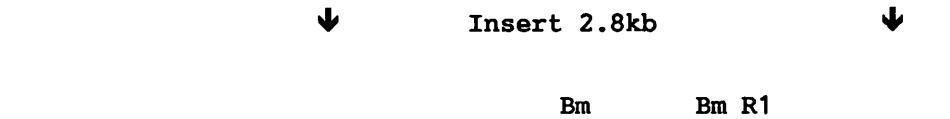
The numbers represent nucleotides of the SPAG1 DNA sequence (Figure 77, Appendix).

Figure 16c summarises the sizes of the restriction fragments generated from partial Bam H1 digestion of Eco RI linearised pGEX-2.4. The 2.4kb Bam HI/Eco RI fragment, which would have been generated by cutting of the Bam HI site 378, was excised from the gel and ligated into the Eco R1/Bam HI digested vector.

a) pTS-SPAG7

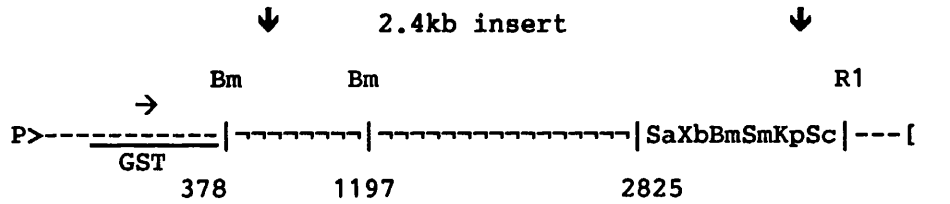


HindIII/Pst1 cut 2.8kb
Ligate into HindIII/Pst1
sites of pUC18



pUC18-SPAG7

b) EcoR1 cut 2.5kb fragment
Partial BamH1 digest to cut site
378 only
Ligate into BamH1/Eco R1
cut pGEX2T



pGEX2T -2.4

c) Fragments generated from Bam H1 partial digest of Eco RI insert

	Size (bp)	Bam HI sites cut
_____	2480	None
_____	2447 ⇔	378 (or 378 + Pr BamHI)
_____	1628	1197 (or 1197 + 378)
_____	852	1197 (or 1197 + Pr Bam HI)
_____	817	1197 + 378

⇔ excised from gel. The Bam HI/Bam HI fragment of identical size would not ligate into the Bam HI/Eco RI cut vector

4.2.3 pGEX2T-0.8

The subcloning of pGEX2T-0.8 from pGEX2T-2.4 is summarised in Figure 17. The plasmid pGEX2T -2.4 was digested completely with Bam HI to give fragments of 5, 1.6 and 0.8 kb. The 0.8kb fragment was excised from the gel, purified and ligated into Bam HI digested pGEX 2T, and the ligation mixture used to transform *E. coli* XL-1 Blue competent cells. Bam HI site 378 was in frame with the Bam HI site in the polycloning region of pGEX2T. Bam HI digestion of minipreps prepared by alkali lysis from twelve transformants demonstrated eight of them to contain the insert. The orientation of the inserts was checked by making small scale fusion protein preparations and running the samples on an SDS polyacrylamide gel which was stained in Coomassie Blue. Three of the clones were found to express a soluble fusion protein of 63kDa with little degradation (Figure 21C), which was slightly larger than the expected size of 50.9kDa.

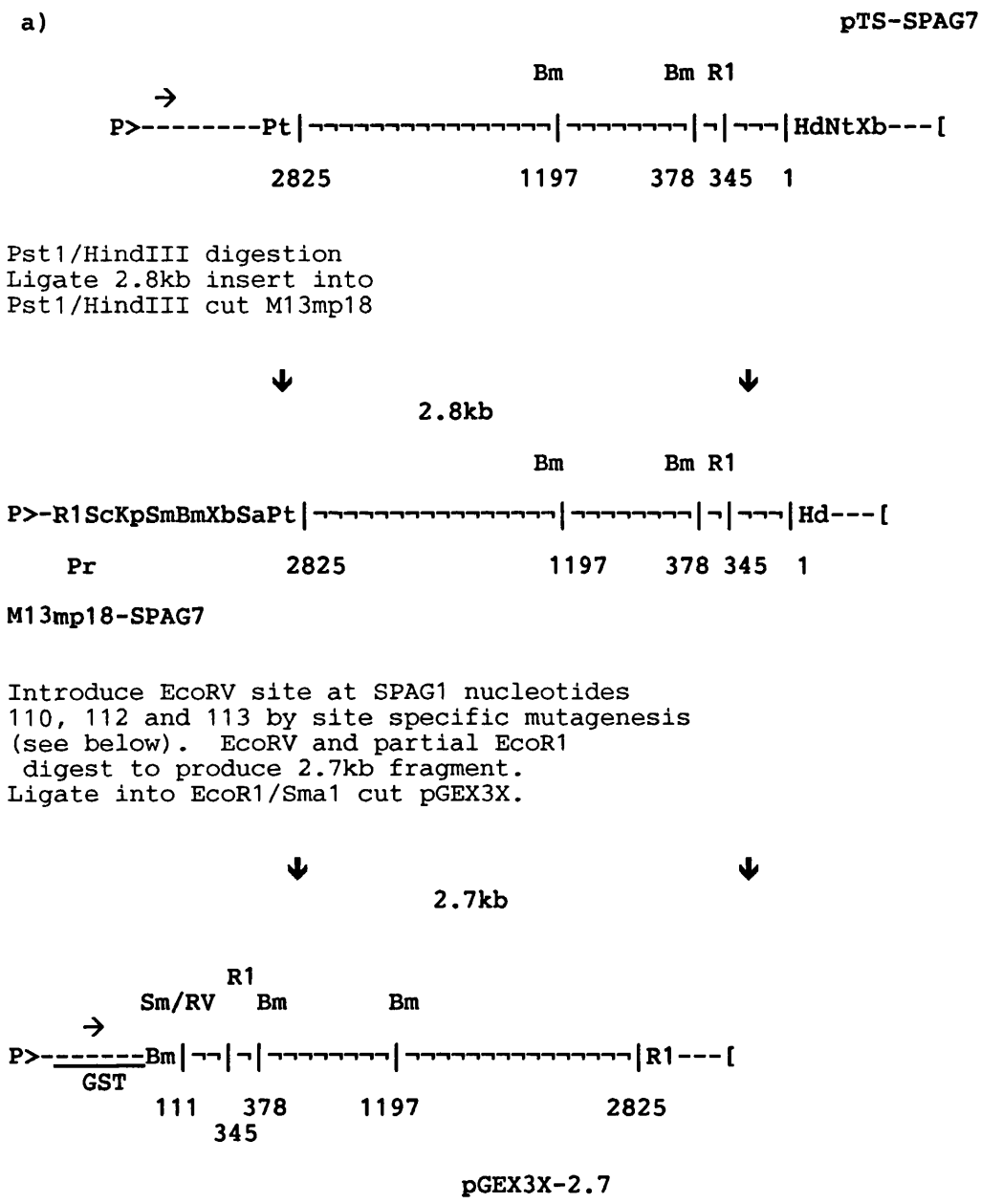
4.2.4 pGEX2T-2.1

The subcloning of pGEX2T-2.1 from pGEX2T-2.4 is summarised in Figure 18A. pGEX2T-2.4 was cut at the Spe1 site 2504 by partial digestion (as described in section 2.2.4) and at the Xba1 site in a separate digest, due to the different buffering conditions required by the enzymes. The size of the DNA fragments generated from the partial Spe I digest, 7.1 and 0.3kb, indicated that Spe I site 2504 had cut rather than 2405, which would have generated fragments of 7.0 and 0.4kb. The staggered ends of the Spe I site in the insert and the Xba I site of the vector could be religated since they share a common central four base pairs in their sequences (Figure 18B). This subcloning removing 300 nucleotides at the C-terminal end of the original 2.4kb insert. *E. coli* XL-1 Blue competent cells were transformed with the ligation products.

Bam HI digestion of minipreps prepared by alkali lysis from twelve recombinants identified five with the correct insert size. These were screened for expression as described above, and all of them were found to produce a soluble protein of 110kDa, larger than the predicted size of 96kDa. Reactivity of the fusion protein with McAb 1A7 and sequence

Figure 19. Cloning of pGEX3X-2.7

The cloning of pGEX3X-2.7 is summarised in 19a.
See Figures 15 ad 16 for explanations of symbols.The EcoRV site was introduced into the M13mp18-SPAG7 insert by site specific mutagenesis (at nucleotides 110,112 and 113) using the oligonucleotide shown in19b.



b)

```
                    110 112/113
          100         |  ||          124
SPAG1       CTGGAGCGGACAAGATGCCTGCGGG

OLIGO       CTGGAGCGGATATCATGCCTGCGGG
                    EcoRV
```

analysis of the C terminal coding region (Dr. R. Hall; personal communication) confirmed that the Spe1 site 2505 rather than 2405 had been ligated into the polycloning site.

4.2.5 pGEX3X-2.7

The cloning of almost the entire SPAG1 sequence into pGEX3X was carried out by Dr. R. Hall and Dr. P.Hunt (Dept. of Biology, University of York). The recombinant pTS-SPAG7 was used for subcloning into pGEX3X (Figure 19A). In order to be able to subclone the insert into one of the pGEX polycloning sites, an EcoRV site was introduced into the N terminal end of the SPAG1 sequence at nucleotides 108-113 (at amino acid 20), using site specific mutagenesis with an oligonucleotide containing the required coding sequence (Figure 19B) Eco RV and partial EcoR1 digestion of the SPAG1 insert enabled it to be ligated into the Sma1 and EcoR1 sites of pGEX3X in the correct reading frame and orientation. The pGEX3X recombinant, containing most of the SPAG1 sequence apart from the N-terminal 20 amino acid residues, was used to transform *E. coli* JM101. A Coomassie Blue stained gel of the fusion protein from a large preparation is shown in Figure 24A, and Western blots developed with McAb 1A7 and McAb 5E1 (anti-merozoite control kindly provided by Dr. B. Shiels, WUMP) are shown in Figure 24B) The recombinant plasmid expressed a soluble 145kDa fusion protein recognised by McAb 1A7, but not by 5E1. The size of this fusion protein was of a higher molecular weight than the expected size of 115.8kDa.

4.2.6 Additional constructs

Seven other SPAG1 derived constructs were cloned from pTS-SPAG7 into pGEX expression vectors by Dr. R. Hall and Dr. P. Hunt, using appropriate restriction enzyme sites. The procedures used are summarised in Table 7, and the relationship between all the SPAG1 constructs is illustrated in Figure 20. All of these constructs expressed fusion proteins completely soluble in PBS 1% Triton X-100. The orientation of the inserts in all of the recombinant clones described in this section was confirmed by sequencing (Dr. R.Hall and Dr. N. Boulter,

Figure 20

Diagram to summarise the relationships between the different SPAG1 sub-constructs which were cloned and expressed in the pGEX vectors. The numbers correspond to amino acid residues of the SPAG1 sequence (Hall et al 1992; Figure 77, Appendix).

¬¬¬¬ = elastin homologous regions

------ = inserts

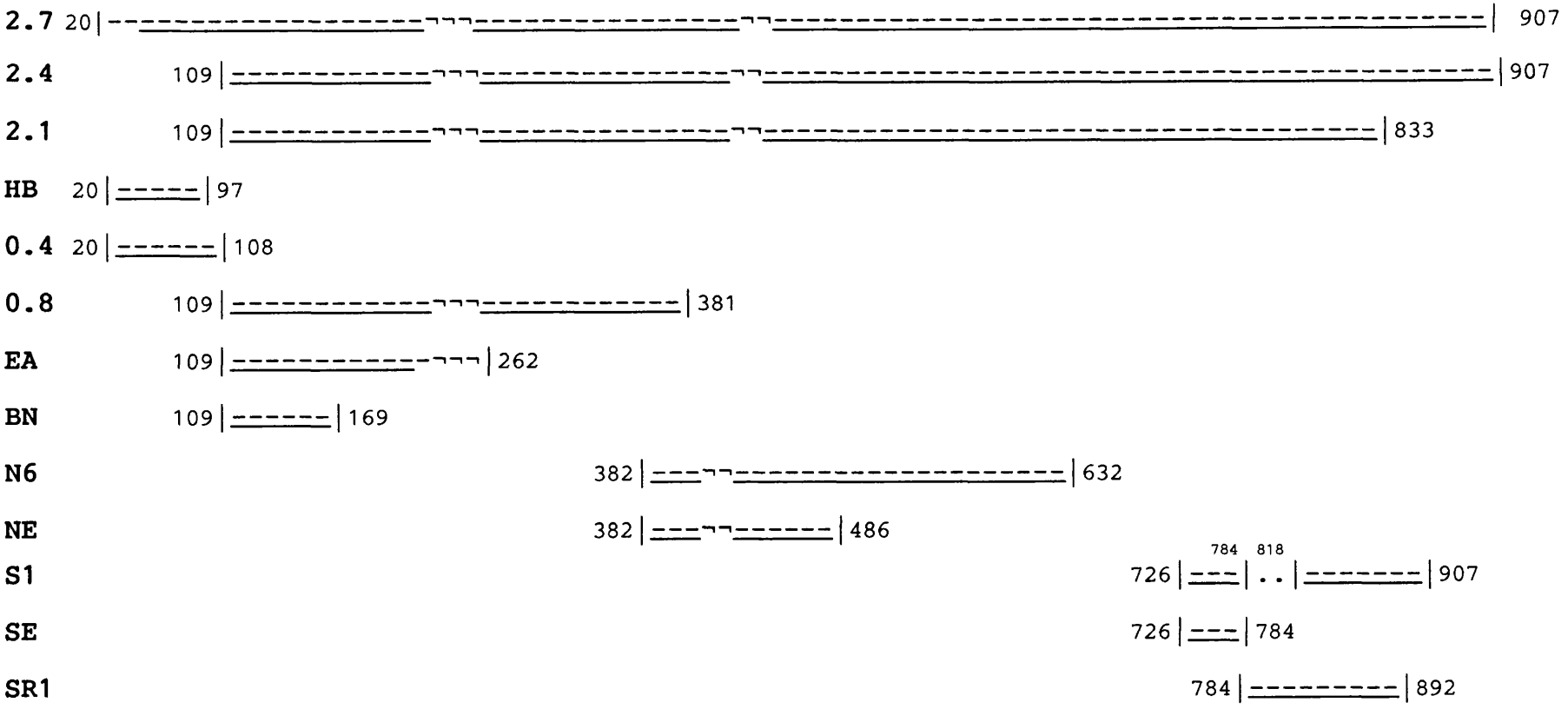

2.7 20 907
2.4 109 907
2.1 109 833
HB 20 97
0.4 20 108
0.8 109 381
EA 109 262
BN 109 169
N6 382 632
NE 382 486
S1 726 784 818 907
SE 726 784
SR1 784 892

Figure 21

Coomassie Blue stained 12% SDS-polyacrylamide gels, to show the screening of recombinants for expression of GST fusion proteins. The gels were loaded with small fusion protein preparations from induced 5ml cultures of each transformant purified using glutathione sepharose 4B beads. The molecular weights (kDa) of the fusion proteins are indicated by arrows.

A) Screening pGEX1λT recombinants in *E. coli* JM101 for expression of the SR1 insert. The numbers at the top of the gel refer to the clones screened. Clones 3, 5, 8, 10, 11 and 12 all contained the 300bp insert. Clone 13 was untransformed .

B) Screening pGEX2T recombinants in *E. coli* XL-1 Blue for expression of the 2.4kb insert. Tracks contained the following;

1 Clone 21, without an insert

2 Clone 23, with 2.4kb insert

3 Insoluble cell pellet from clone 23, obtained after sonication.

C) Screening pGEX2T-0.8 recombinants in *E. coli* XL-1 Blue for expression of 0.8kb insert. The numbers 1-9 refer to the clones screened. Clones 3, 4, 6, 7, 8 and 9 all contained the 0.8kb insert. Clone P was transformed with pGEX2T only.

D) Screening pGEX2T-2.1 recombinants in *E. coli* XL-1 Blue for expression of 2.1kb insert. The numbers 1-6 refer to the clones screened. Of these, clones 2-6 all contained an insert of the correct size. Clone P was transformed with pGEX2T only.

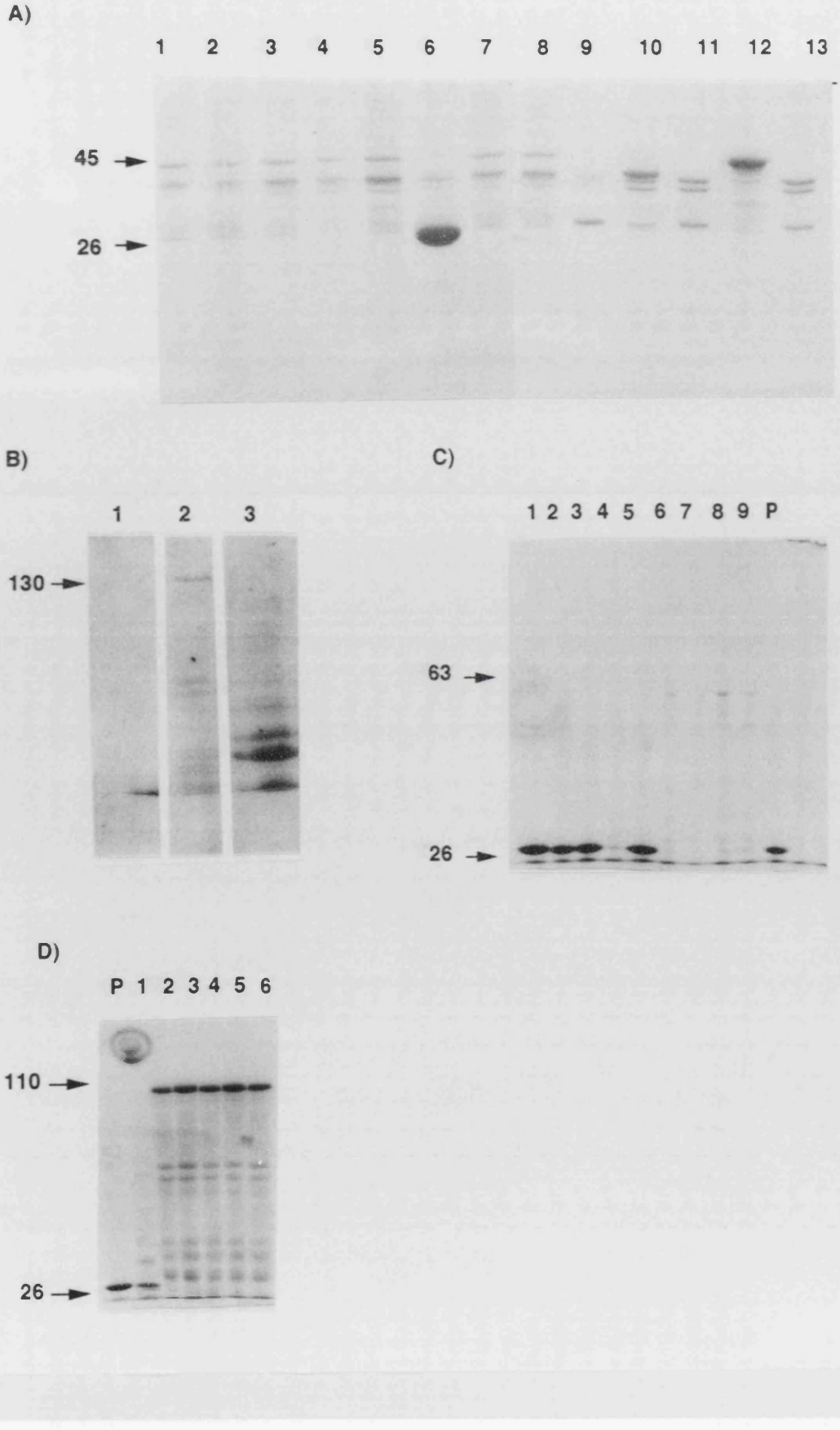

A)
1 2 3 4 5 6 7 8 9 10 11 12 13
45
26
B)
1 2 3
130
C)
1 2 3 4 5 6 7 8 9 P
63
26
D)
P 1 2 3 4 5 6
110
26

Figure 22

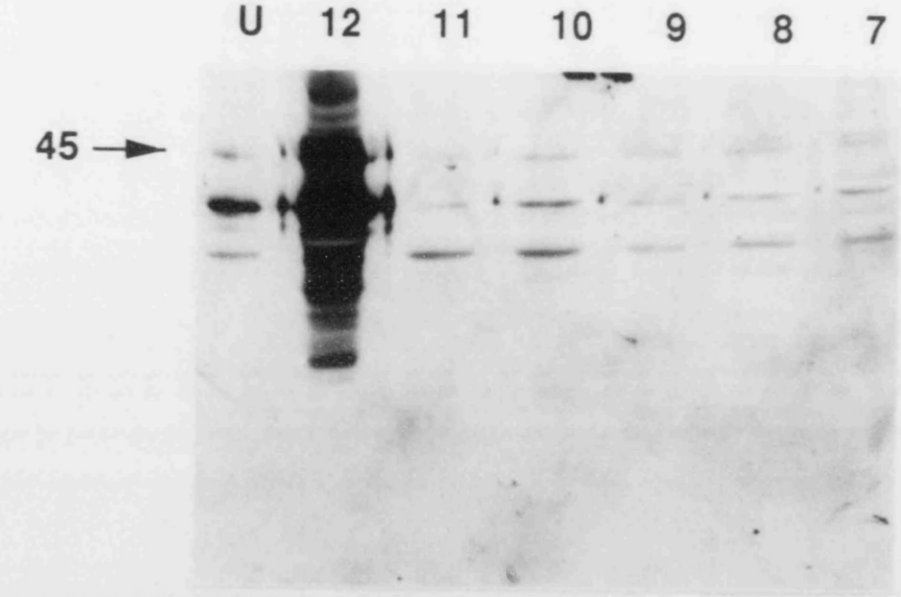
Western blot to show the screening of pGEX1λT-SR1 recombinants with λgt11-SR1 rabbit antiserum, diluted 1:100. The second antibody was alkaline-phosphatase conjugated goat anti-rabbit IgG (1:300). The numbers refer to the clones screened, as described for Figure 21A. The size (kd) of the GST-SR1 fusion protein is indicated by an arrow.

Figure 23

Western blot to show the screening of pGEX2T-2.4 recombinants with McAb 1A7 undiluted supernatant. The second antibody was alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300). The tracks are as follows;

1 clone 23, with 2.4kb insert
2 clone 21, without an insert
3 insoluble cell pellet from clone 23, obtained after sonnication

The size (kD) of the GST-2.4 fusion protein is indicated by an arrow.


U
12
11
10
9
8
7
45

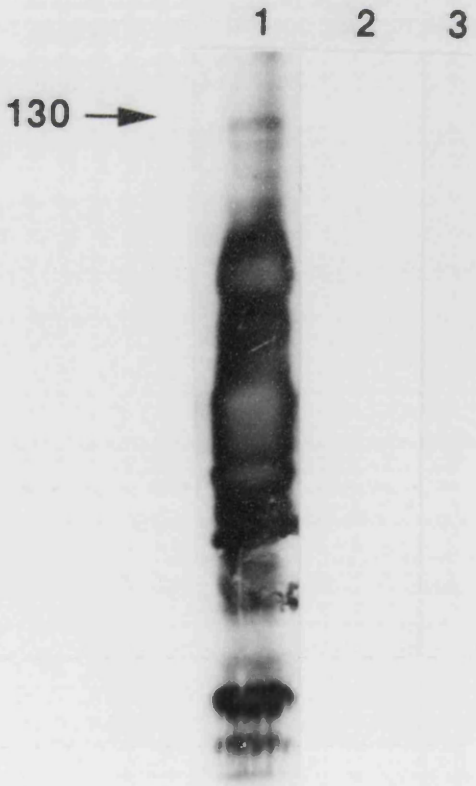

1
2
3
130

Figure 24A

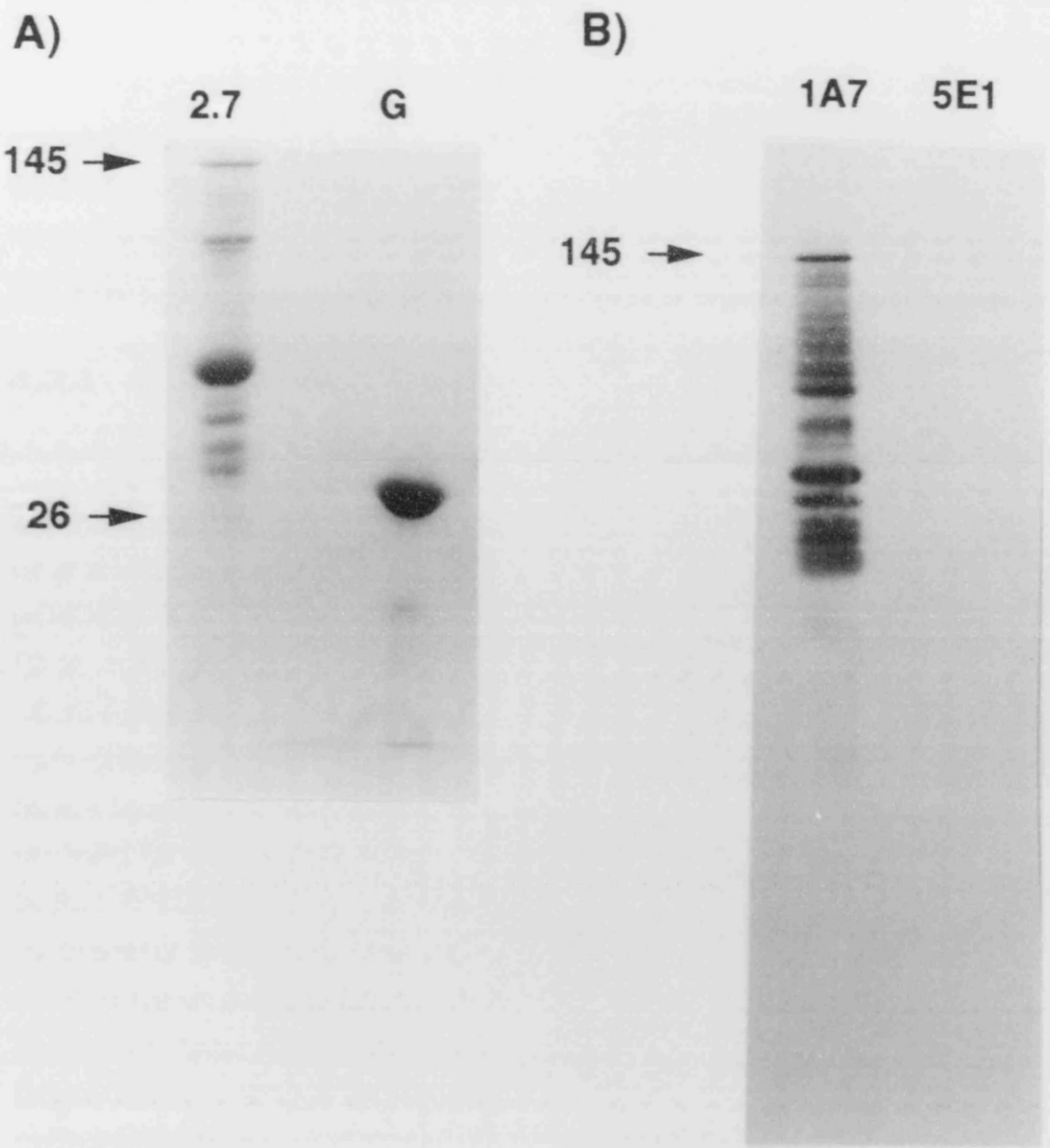
Coomassie blue stained 12% SDS-polyacrylamide gel to show expression of pGEX3X-2.7. The sizes of the fusion proteins are indicated by arrows. Tracks contained the following;

2.7 20μg GST-2.7 fusion protein from pGEX3X-2.7 transformed *E. coli* XL-1 Blue, purified on a glutathione sepharose 4B column.

G 20μg GST protein from pGEX3X transformed XL-1 Blue, purified on a glutathione sepharose 4B column.

Figure 24B

Western blot of GST-2.7 fusion protein (1μg per track) developed with McAb 1A7 or 5E1 undiluted supernatant as indicated. The second antibody was alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300)


A)
2.7
G
145
26
B)
1A7
5E1
145

personal communication) using a primer to the GST coding sequence, given in the paper by Smith et al (1986). The primer sequence was as follows: CCT TTG CAG GGC TGG CAA GC. The oligonucleotide starts 70 nucleotides 5' from the pGEX polylinker region.

4.3 Large scale expression

Most of the work undertaken to optimise conditions for large scale expression was carried out using pGEX2T-2.4, since this was the first SPAG1 coding sequence covering most of the protein to be cloned and expressed in pGEX. The conditions were then applied to production of the other fusion proteins.

4.3.1 Timecourse of induction

Since the length of time between IPTG induction and harvesting of the cells can be critical to the yield of fusion protein and the level of degradation, a timecourse was set up to analyse the expression of pGEX2T-2.4. A 200ml culture of pGEX2T-2.4 transformed *E. coli* XL-1 Blue was grown up and induced at 30°C as described (2.2.7), removing 40ml aliquots at time intervals of 0, 30, 60, 120 and 240 minutes after induction in 0.1mM IPTG. Small preparations of the fusion protein were made from each timepoint, run on a 12% SDS polyacrylamide gel and stained in Coomassie Blue. There was no apparent expression at time 0, before IPTG addition, but the total yield of fusion protein rose with increasing induction time up to 120 minutes after the addition of IPTG, according to estimation made from the gel by eye (Figure 25). A longer induction time of 240 minutes appeared to result in increasing degradation without increasing the yield of fusion protein in relation to culture volume.

A similar timecourse ws set up but growing and inducing the cells at 37°C instead of 30°C, and assessed in the same way (data not shown). Much lower levels of expression were detected at all the timepoints, and the proportion of degradation products was noticably higher with the 130kDa band being barely visible.

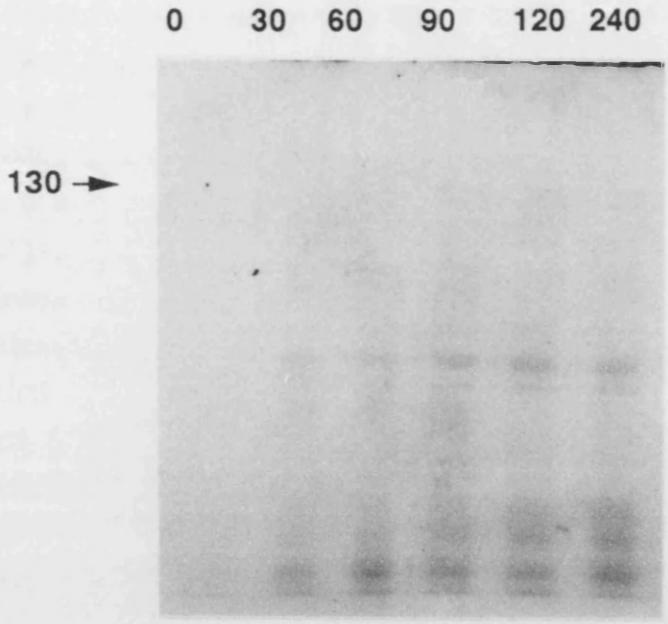
Figure 25

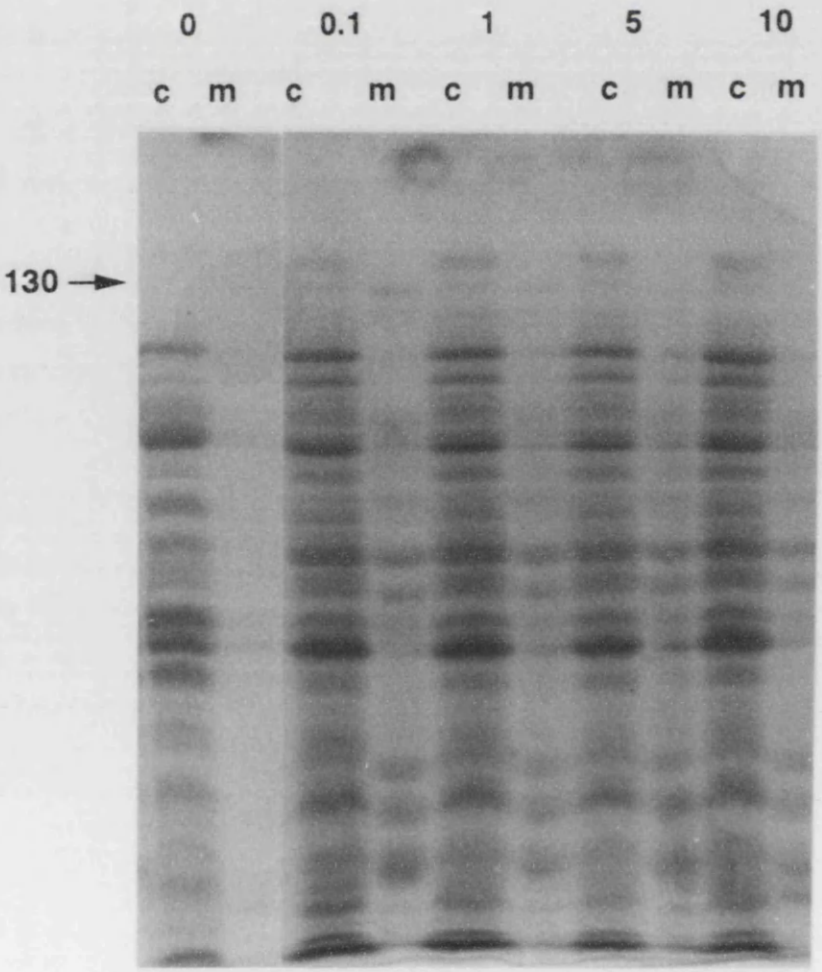
Coomassie blue stained 12% polyacrylamide gel, to show a timecourse of expression of pGEX2T-2.4 in *E. coli* XL-1 Blue. The tracks were loaded with small preparations of fusion protein from 5ml cultures, purified with glutathione sepharose beads. The time between IPTG addition and harvesting of each culture (0-240) is shown in minutes. The position of the 130 kDa GST-2.4 fusion protein is indicated.

Figure 26

Coomassie Blue stained 12% SDS-polyacrylamide gel to show the effect of IPTG concentration on the level of fusion protein expression by pGEX2T-2.4 in *E. coli* XL-1 Blue. All the cultures were induced for 3 hours at 30°C. Tracks are indicated as follows;

m	small preparations of fusion protein from 5ml cultures, purified with glutathione sepharose beads
c	whole cell lysates from induced 5ml cultures
0, 0.1, 1, 5, 10	Final concentration of IPTG added to each culture in mM


0
30
60
90
120
240
130


0
0.1
1
5
10
c m c m c m c m c m
130

4.3.2 IPTG concentration

A second trial was set up using pGEX2T-2.4 transformed *E. coli* XL-1 Blue to analyse the effect of an increase in the concentration of IPTG used to induce the cells. Ten 5ml cultures were set up and duplicate cultures induced after 2 hours as described (2.2.7), using 0, 0.1, 1, 5 and 10mM final concentrations of IPTG. A 12% SDS polyacrylamide gel was loaded with a whole cell lysate and glutathione sepharose purified fusion protein from each IPTG concentration, and the gel stained with Coomassie Blue. The results are shown in Figure 26. The faint 130kDa fusion protein was absent in the samples prepared without IPTG addition (Track 0), but present in equal quantities in all the preparations to which IPTG was added. There was no visible increase in yield of the 130kDa protein produced by increasing the IPTG concentration. Purification on glutathione sepharose appeared to reduce the presence of other proteins seen in the crude preparations which were not apparently IPTG induced; the effects of glutathione sepharose purification are further investigated later in this chapter.

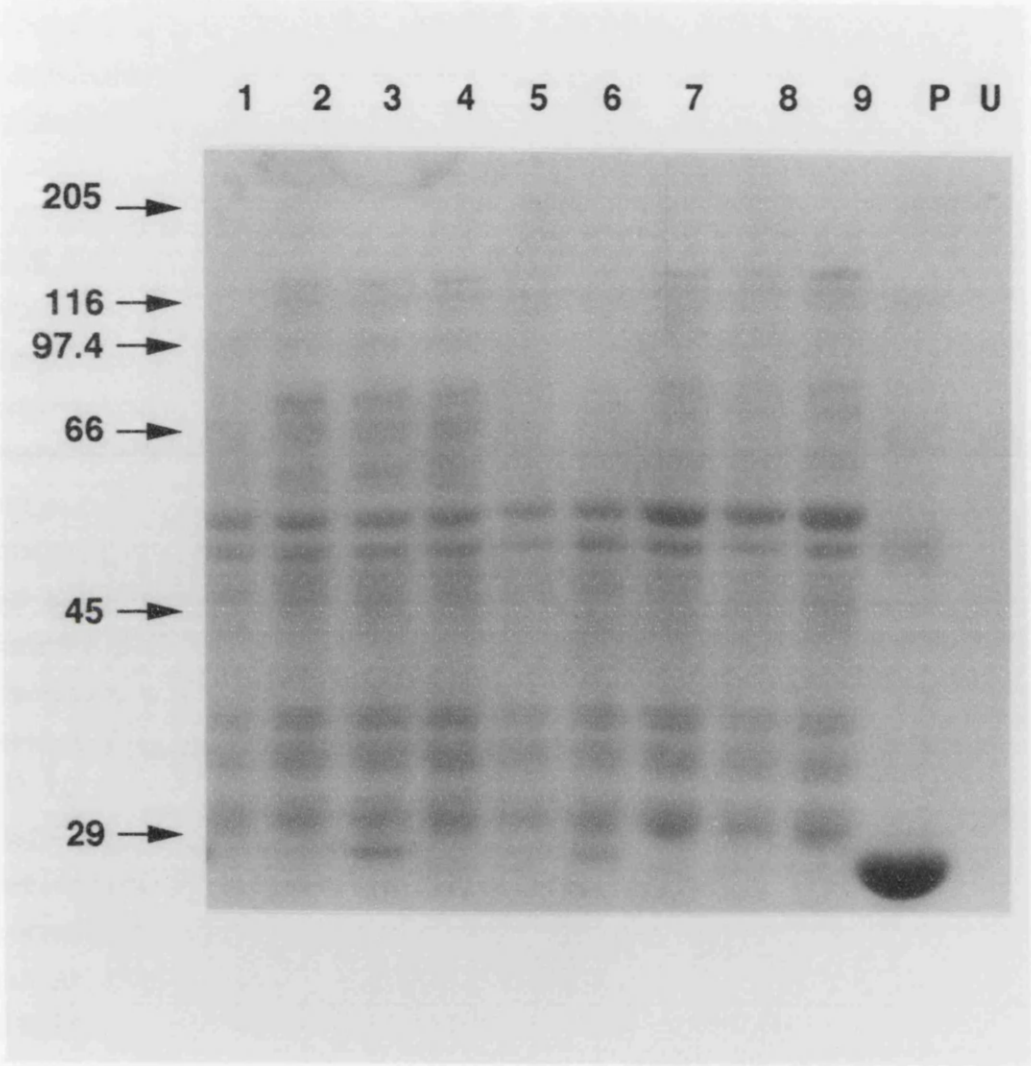
4.3.3 Using protease deficient host strains

The *E. coli* strain XL-1 Blue is not deficient in the Lon A protease coded for by the gene *lon-*, which may have exacerbated degradation of the expressed fusion proteins. Therefore, three *lon-* strains of *E. coli* were transformed with the recombinant pGEX2T-2.4 to ascertain whether degradation would be reduced. The genotypes of the strains used are given in the Appendix (Table A). *E. coli* DS884, DS885 and DS890 competent cells were transformed with pGEX2T-2.4 and a number of recombinants checked for the presence of inserts by BamHI digestion of DNA minipreps prepared by alkali lysis. Small scale fusion protein preparations were made from three recombinants in each strain, run on a 12% SDS polyacrylamide gel and Coomassie Blue stained (Figure 27). There was no apparent reduction in the level of degradation of the 130kDa fusion protein expressed in any of these hosts compared with XL-1 Blue.

Figure 27

Coomassie Blue stained SDS-polyacrylamide gel to show the effect of using a *lon-* host on the level of expression of pGEX2T-2.4. Small scale fusion protein preparations were made from 5ml cultures of each transformant, purifying the fusion protein on glutathione sepharose 4B. The positions of the molecular weight markers are indicated in kDa. Tracks were loaded with fusion protein prepared from the following clones;

1,2,3 *E. coli* DS884 pGEX2T-2.4 transformants
4,5,6 *E. coli* DS884 pGEX2T-2.4 transformants
7,8,9 *E. coli* DS890 pGEX2T-2.4 transformants
P *E. coli* DS890 pGEX2T transformants
U *E. coli* DS890 untransformed cells


1
2
3
4
5
6
7
8
9
P
U
205
116
97.4
66
45
29

4.3.4 Purification conditions

Since switching to a *lon-* host did not appear to influence the quality of the fusion protein produced, it was decided to optimise conditions under which cell harvesting, lysis and column purification of the fusion protein were carried out. The first step was to carry out a preparation at a low temperature to decrease the activity of proteases. A second fusion protein preparation was carried out using protease inhibitors specific for different protease groups. The effect of the sonication time used to lyse the cells was also investigated.

A large scale preparation of fusion protein from a 1 litre culture of pGEX2T-2.4 transformed *E. coli* XL-1 Blue was carried out at temperatures kept as close to 4°C as possible. GST-2.4 was also prepared from an identical 1 litre culture in the same way except that fusion protein binding and elution from the column was carried out at room temperature. Protease inhibitors were not included in either preparation. The eluate from both columns was collected in 1ml fractions and 60µl from each sample run on a 10% SDS polyacrylamide gel which was stained in Coomassie Blue (Figure 28A and B). In both cases spectrophotometric estimation of protein concentration in the fractions indicated elution was occuring primarily in fractions 4-8, decreasing in subsequent fractions and reaching a minimum in fractions 10-12. The presence of the fusion protein was confirmed by the appearance of the samples on the gel. There was a noticable difference in the degree to which the fusion protein was degraded in each preparation. According to estimations by eye from the gel, in the preparation carried out at 4°C the fractions contained approximately 20% of undegraded 130kDa fusion protein, while in the purification conducted at room temperature this was only about 5%. The total yields of fusion protein from the preparation carried out at 4°C and room temperature were 2.2mg and 1.7mg respectively, as determined by Coomassie Plus microassay (section 2.2.7).

Fusion protein was prepared from a 4 litre culture of the pGEX2T-2.4 XL-1 Blue transformant as described in section 2.2.7, carrying out the purification steps at 4°C and including a selection of protease inhibitors active against various protease groups (section

2.1.13). Collection of the eluate and visualisation of the fusion protein on an SDS polyacrylamide gel was carried out as described above; the results are shown in Figure 28C. The elution profile was similar to that described for the other preparations, but addition of protease inhibitors did have a small effect on the degree of degradation, with approximately 30% of the 120kDa fusion protein remaining intact. The total yield was 2.3mg per litre of culture.

Since it was possible that the degradation products could be produced during sonication, a small trial experiment was also carried out to verify the optimum sonication time. A 100ml culture of pGEX2T-2.4 transformed *E. coli* XL-1 Blue was grown up and induced as described in section 2.2.7. 10ml aliquots were taken, spun down and resuspended in 100µl PBS/ 1% Triton X-100. Aliquots were sonicated for between 1 and 6 x 30 second bursts at 6µ. Small fusion protein preparations were made from each sample and run on a 10% SDS polyacrylamide gel, which was stained in Coomassie Blue and used to estimate the amount of degradation in each sample (data not shown). An increase in sonication time had no effect on the percentage of degradation products present in the preparations. Higher yields of fusion protein were obtained with sonications of 3-6 x 30 second bursts than with lower sonication times, as estimated by the intensity of the Coomassie Blue staining.

4.3.5 Bulk preparations of GST-2.7

In order to generate sufficient quantities of GST-2.7 fusion protein for immunisations and assessment of immune responses, it was necessary to express and purify the recombinant protein on a large scale. Two bulk preparations of fusion protein from pGEX3X-2.7 transformed JM101 were carried out from 16 litres culture each, purifying the fusion protein at 4°C in the presence of protease inhibitors exactly as described in section 2.2.7. Lysis was performed using a French press rather than by sonication since this is a more efficient method for breaking open a high density cell suspension. Column eluates were collected in 1ml fractions, and 30µl samples from each fraction were run on a 10% SDS polyacrylamide gel and stained with Coomassie Blue.

Figure 28 A,B,C

Coomassie Blue stained 10% SDS-polyacrylamide gels to show the effect of addition of protease inhibitors and a reduced temperature during the purification process on large scale expression of fusion protein from pGEX2T-2.4 in *E. coli* XL-1 Blue. The numbers labelling each track refer to the sequential 1ml fractions of eluted protein collected from each column. 60µl was loaded from each sample. The positions of the molecular weight markers are indicated in kDa for each gel.

28A (upper photograph)

Fractions 1-11 from a large scale fusion protein preparation carried out at room temperature without addition of protease inhibitors.

28B (lower photograph)

Fractions 1-11 from a large scale fusion protein preparation carried out at 4°C, without the addition of protease inhibitors.

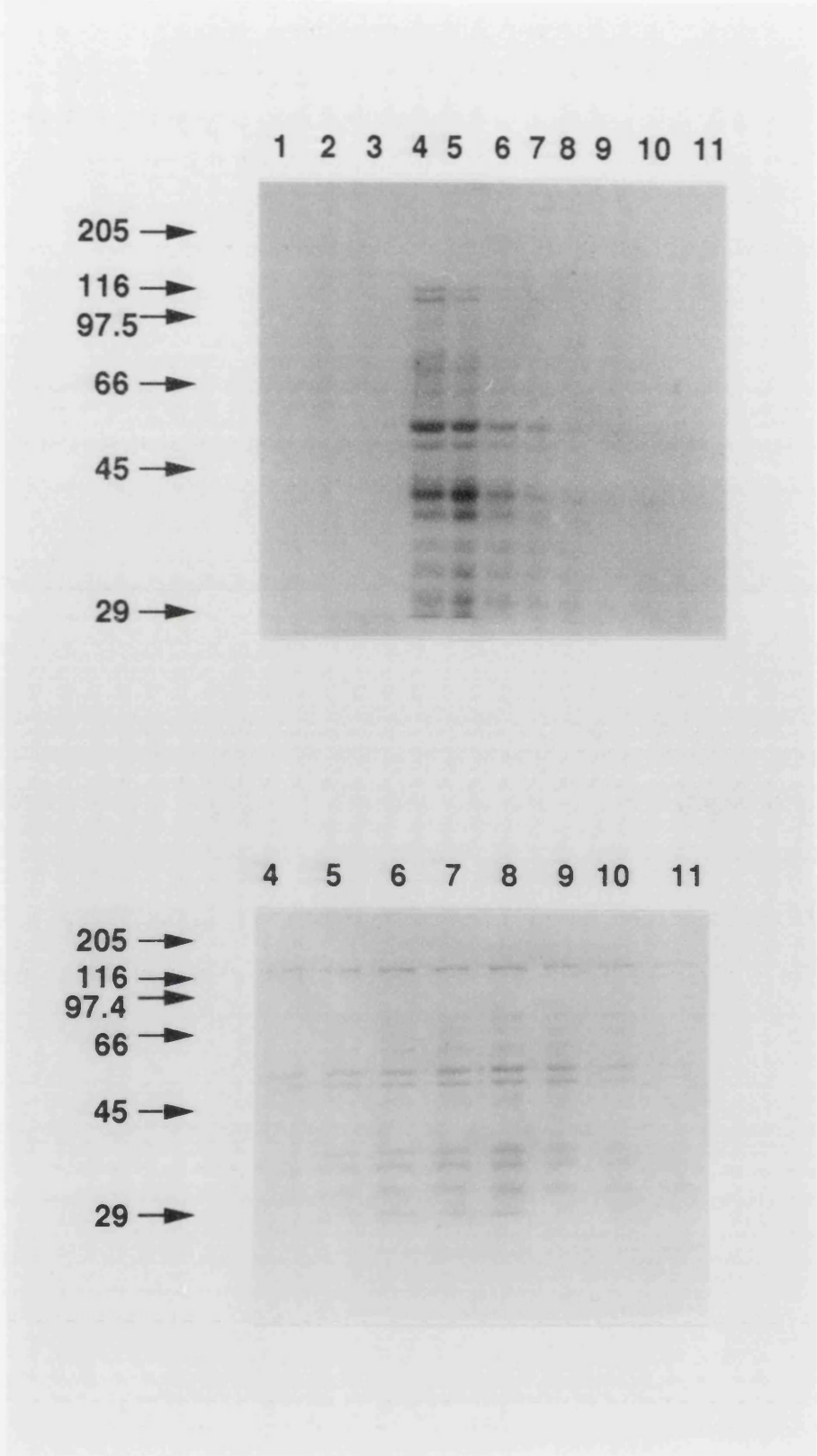
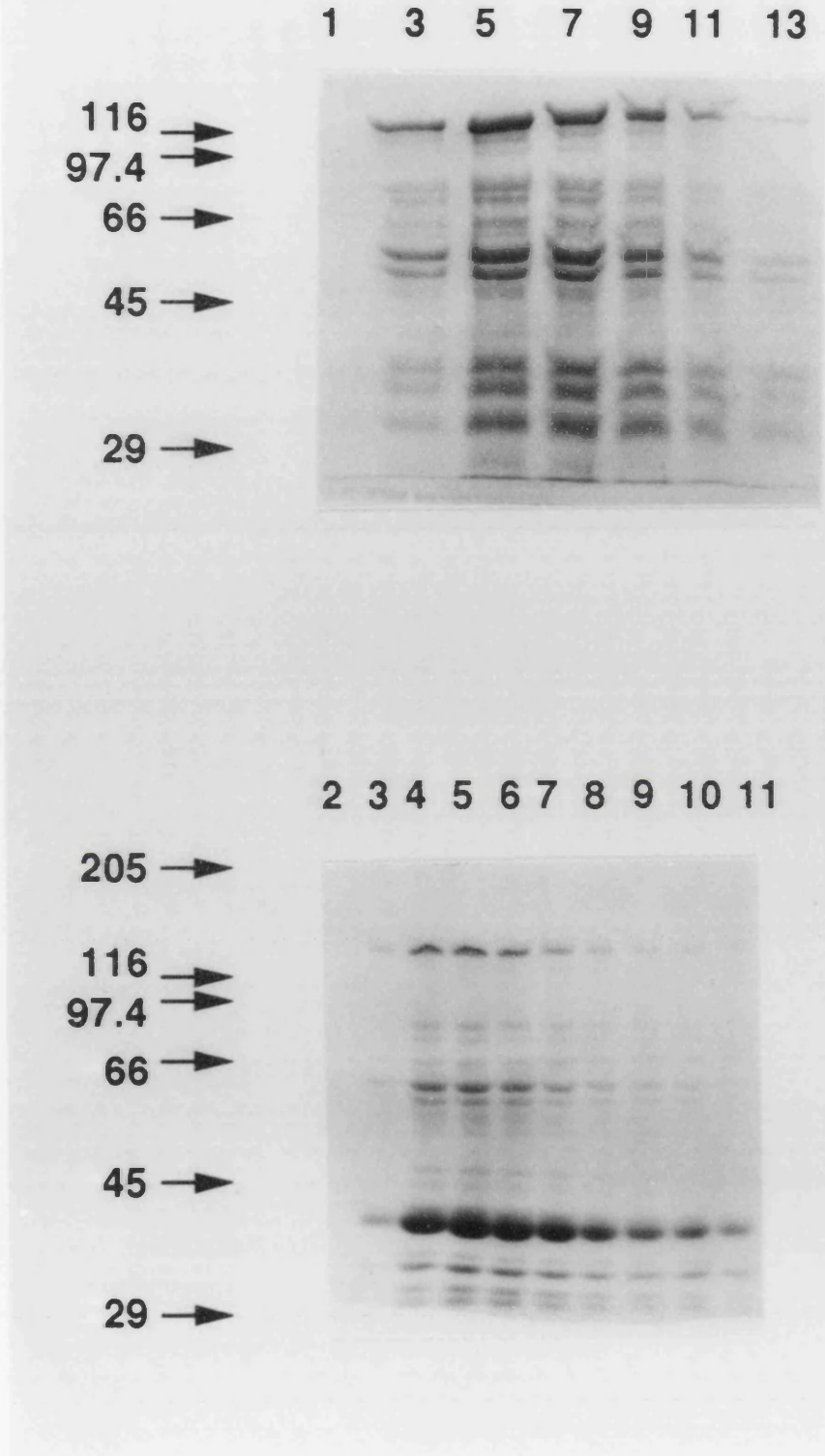

1 2 3 4 5 6 7 8 9 10 11
205
116
97.5
66
45
29
4 5 6 7 8 9 10 11
205
116
97.4
66
45
29

Figure 28C (continued from previous page)

Fractions 1, 3, 5, 7, 9, 11 and 13 from a large scale fusion protein preparation, carried out at 4°C with the addition of protease inhibitors.

Figure 29

Coomassie blue stained 10% SDS-polyacrylamide gel to show the products of a large scale fusion protein preparation from pGEX3X-2.7 transformed *E. coli* JM101, carried out at a temperature of 4°C with the addition of protease inhibitors. The numbers labelling each track refer to the sequential 1ml fractions of eluted protein collected from each column. 60µl was loaded from each sample. The positions of the molecular weight markers are indicated in kDa for each gel.


1 3 5 7 9 11 13
116
97.4
66
45
29
2 3 4 5 6 7 8 9 10 11
205
116
97.4
66
45
29

The samples from the first bulk preparation are shown in Figure 29. Spectrophotometric estimations of the protein content in the fractions indicated protein was first eluted in fractions 3-4, then decreased steadily in subsequent fractions. The appearance of the samples on the gel confirmed this elution profile. Undegraded 145kDa fusion protein comprised approximately15% of the total protein eluted, according to estimates made from the gel. The mean yield of fusion protein from the bulk preparations was 1.3mg/litre of culture; the total yield from each of the two preparations were 22.95mg and 18.76mg as determined by Coomassie Plus microassay (2.2.7).

4.3.6 Large preparations of other SPAG1 derived fusion proteins

Fusion protein preparations of all the SPAG1 derived GST fusion proteins, and of GST alone, were carried out from 1 litre cultures as described in section 2.2.7, purifying the proteins at 4°C in the presence of protease inhibitors. Spectrophotometric estimation of protein content of the 1ml fractions collected in each case showed an elution profile similar to that of GST-2.7, with maximum elution occuring in fractions 4-7 and then decreasing. Samples of 20-30μg from each purified and dialysed fusion protein were run on an SDS polyacrylamide gel; the resultant Coomassie Blue stained gel is shown in Figure 30. The actual sizes of all the fusion proteins produced according to estimations from the gel, and that predicted from the DNA sequences are summarised in Table 8, together with the yields per litre of culture and the proportions of undegraded fusion protein according to estimations made from the gel by eye.

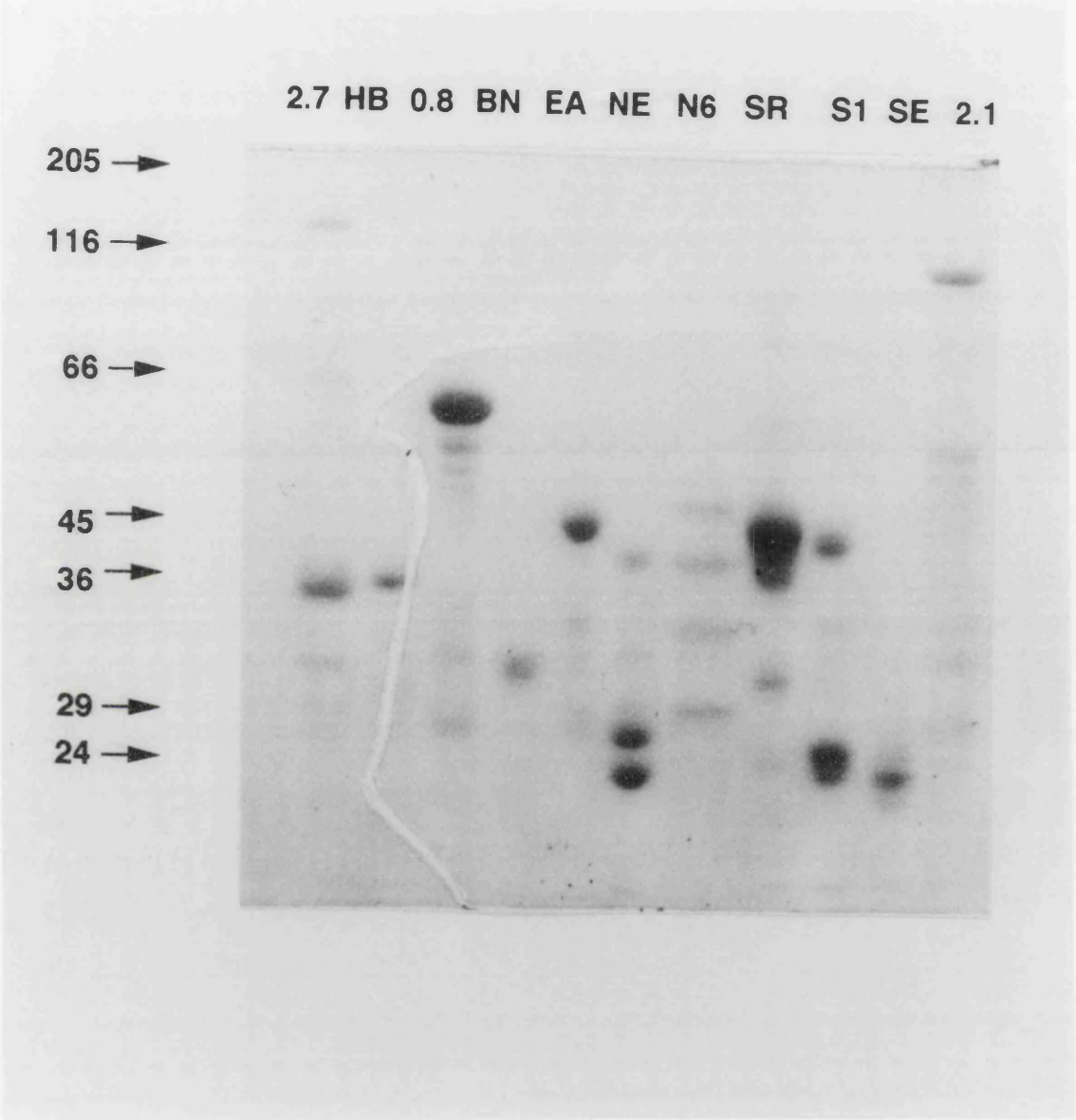
4.3.7 Thrombin cleavage of fusion proteins

Since the vectors pGEX2T and pGEX1λT contain a recognition site for thrombin protease in their polycloning regions, it was intended to cleave these fusion proteins and remove the GST portion by affinity purification on a glutathione sepharose 4B column. The recognition site for bovine thrombin protease consists of the amino acid sequence Pro-Arg-Gly-Ser, which is absent in the SPAG1 sequence according to

Figure 30

Coomassie Blue stained 12% SDS-polyacrylamide gel to show the expression of eleven SPAG1 fusion proteins. The positions of the molecular weight markers are indicated in kDa. 10-20μg of each fusion protein, purified from large scale preparations using glutathione sepharose columns, was loaded per track. Tracks contained the following proteins;

2.7	GST-2.7
HB	GST-HB5
0.8	GST-0.8
BN	GST-BNI
EA	GST-EA
NE	GST-NE
N6	GST-N6
SR	GST-SR1
S1	GST-S1
SE	GST-SE
2.1	GST-2.1


2.7 HB 0.8 BN EA NE N6 SR S1 SE 2.1
205
116
66
45
36
29
24

analysis using the computer program GCG WordSearch. However, thrombin protease can cleave any peptide bond N-terminal to an arginine residue (Sambrook et al 1989), of which there are 11 in the SPAG1 sequence (Figure 77, Appendix). It was therefore necessary to carry out experiments to verify whether these sites internal to SPAG1 were cleaved when the fusion proteins were treated with thrombin.

An experiment was first carried out using the SR1 fusion protein, to verify the incubation time required for effective thrombin cleavage. The SR1 sequence contains a single arginine residue near the N terminal end (Figure 77). 1mg of pGEX1λT-SR1 was cleaved with bovine thrombin in a total volume of 1ml according to the method given in section 2.2.7. Aliquots were removed at time intervals of 0, 30 minutes, 1 and 2 hours after thrombin addition, and GST and any uncleaved fusion protein removed from the remaining sample using Glutathione Sepharose 4B beads. A sample of protein eluted from the beads by boiling in SDS sample buffer was also taken. The samples were run on a 12% SDS polyacrylamide gel which was stained with Coomassie Blue; the gel is shown in Figure 31A. The 45kDa polypeptide representing the intact fusion protein is clearly visible at time 0, steadily decreasing with increasing incubation time and becoming very faint after 2 hours incubation (tracks 0, 30, 1 and 2). A 26kDa polypeptide, corresponding to the size of GST, simultaneously increased in intensity during the incubation, and a band at 20kDa, probably the cleaved SR1 polypeptide, was faintly visible on the gel in the sample incubated for 2 hours. The appearance of these two lower molecular weight bands indicated that cleavage was taking place, although the 20kDa band was poorly visible, probably due to the acrylamide concentration in the gel being to low to clearly show bands of this size. Most of the 45kDa fusion protein appeared to have been cleaved in the sample taken after a 2 hour incubation time. Incubation with Glutathione Sepharose 4B reduced the strength of the 26kDa band (track B). Elution of bound protein from the beads resulted in a 26kDa band corresponding to GST and a 45kDa band of uncleaved fusion protein (track E). The band at 70kDa is likely to be the thrombin protease.

While this experiment showed that an incubation time of two hours was sufficient for thrombin proteolysis of the fusion protein to take place,

Figure 31A

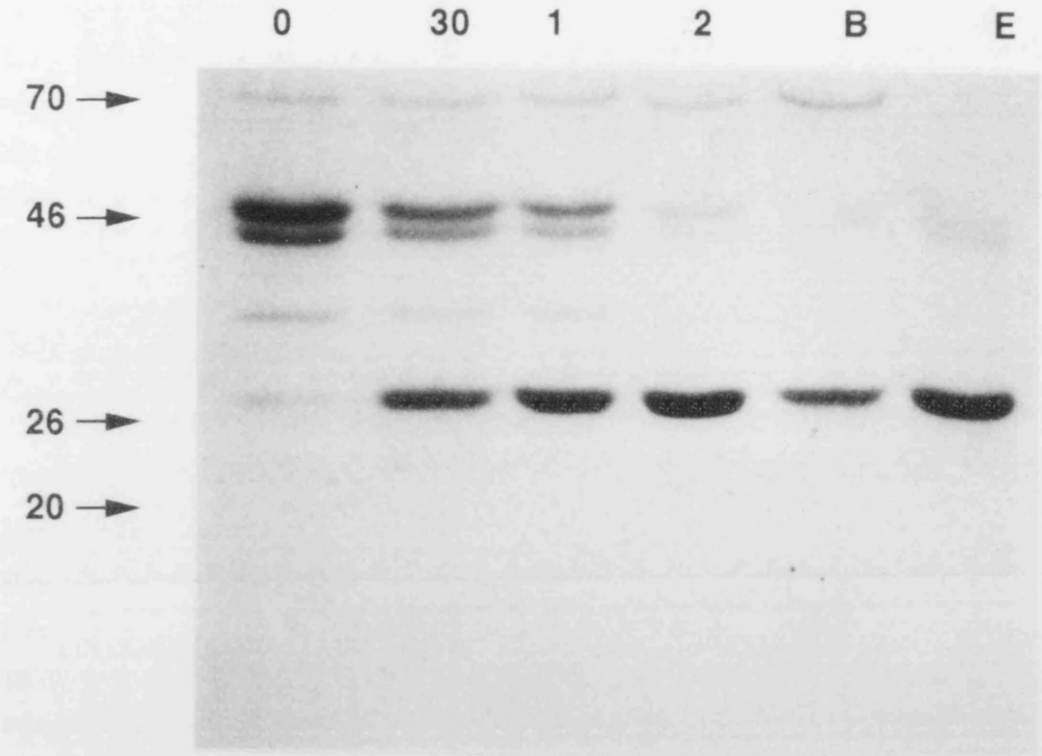
Coomassie blue stained 12% SDS-polyacrylamide gel to show a timecourse of proteolytic cleavage of GST-SR1 fusion protein by bovine thrombin. 1mg GST-SR1 was cleaved with 2μg bovine thrombin over a period of two hours. The sizes of the bands detected (kDa) are indicated by arrows. Tracks are labelled as follows;

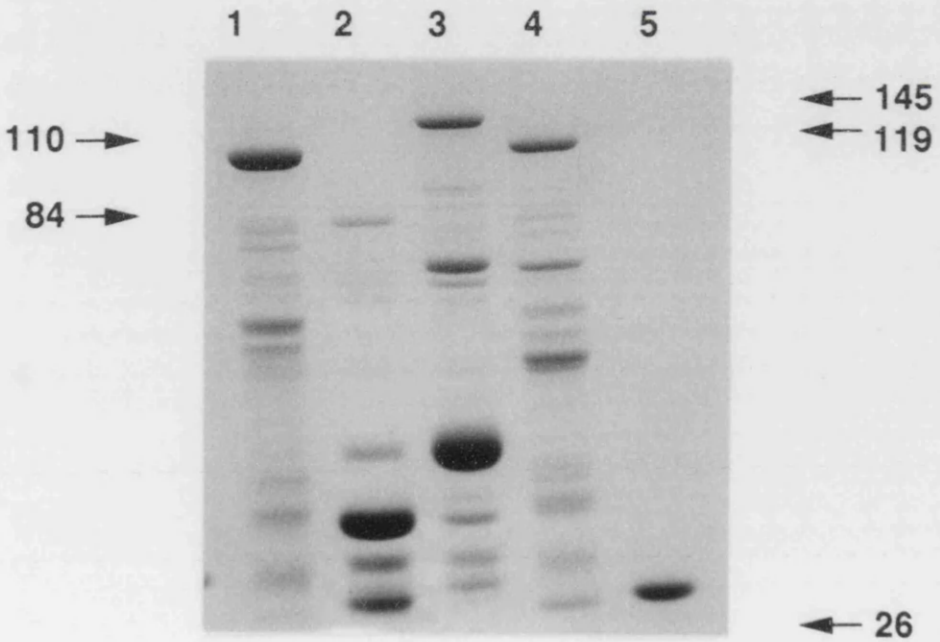
0 45μg sample taken immediately following thrombin addition (time 0).
30 45μg sample taken after 30 minutes incubation
1 45μg sample taken after 1 hour
2 45μg sample taken after 2hours
B Remaining protein after 800 μg GST-SR1 incubated with glutathione sepharose 4B beads
E Eluate from the glutathione sepharose 4B beads boiled in SDS-sample buffer

Figure 31B

Coomassie blue stained 12% SDS-polyacrylamide gel to show the products of thrombin proteolytic cleavage of GST-2.7 and GST-2.1 fusion proteins. The molecular weights (kDa) of the main cleavage products are indicated by arrows. Samples were taken prior to and following a 2 hour incubation of 2mg each fusion protein with 4μg bovine thrombin protease. Tracks contained the following;

1 50μg GST-2.1 prior to thrombin addition.
2 5 μg GST-2.1 following incubation with thrombin
3 50μg GST-2.7 prior to thrombin addition
4 50μg GST-2.7 following incubation with thrombin
5 50μg GST


0
30
1
2
B
E
70
46
26
20


1
2
3
4
5
110
84
145
119
26

it did not confirm whether the arginine site within the SR1 sequence was being cleaved since the peptide produced would have been too small to visualise on the gel. To demonstrate proteolysis of a larger SPAG1 fusion protein and to check whether or not any sites could be recognised within SPAG1, simultaneous cleavage of fusion protein prepared from pGEX2T-2.1 and pGEX3X-2.7 by thrombin protease were set up as described in section 2.2.7. The vector pGEX3X does not contain the complete thrombin recognition site Pro-Arg-Gly-Ser in it's polylinker region, instead it has the Factor Xa recognition site Ile-Glu-Gly-Arg. 2mg of each fusion protein was cleaved in a total volume of 1ml. Samples were removed prior to and 2 hours following thrombin addition, run on a 12% SDS polyacrylamide gel including purified GST in one of the tracks. The Coomassie Blue stained gel is shown in Figure 31B.

Both the 110 and the 145kDa fusion proteins disappeared after 2 hours incubation with thrombin (tracks 2 and 4). In both cases a number of smaller bands were detected, including a 26kDa band corresponding to GST (track 5). This implied that both the fusion proteins were being cleaved by thrombin, despite the lack of the complete recognition sequence in pGEX3X. The highest molecular weight bands in the cleaved preparations were approximately 75kDa for GST-2.1 and 110kDa for GST-2.7, which were slightly less than the sizes expected from removal of the GST fragment alone. Furthermore, some of the bands in the cleaved preparations could not be accounted for by removal of the GST fragment alone from the intact fusion proteins or their degradation products, such as the heavily stained 35kDa band in the cleaved GST-2.1 preparation. This data indicates that thrombin proteolytic cleavage was not confined to the Pro-Arg-Gly-Ser sequence coded for by the pGEX2T polylinker.

4.3.8 Factor Xa proteolytic cleavage of pGEX3X fusion proteins

The SPAG1 amino acid sequence was screened for the blood coagulation Factor Xa protease cleavage site using the computer program GCG WordSearch. Factor Xa protease is specific for the recognition site comprising the four amino acids Ile-Glu-Gly-Arg (Nagai and Thøgersen 1984). No groups of three or four amino acids identical to the above

Figure 32A

Coomassie Blue stained 12% SDS-polyacrylamide gel to show a timecourse of proteolytic cleavage of GST-2.7 by Factor Xa. The positions of the molecular weight markers are shown in kDa. 250μg GST-2.7 fusion protein was incubated with 5 units bovine Factor Xa protease and 15μg samples taken at the following time intervals;

Track

1 taken prior to protease addition
2 after 1 hour
3 after 2 hours
4 after 4 hours
5 after 16 hours

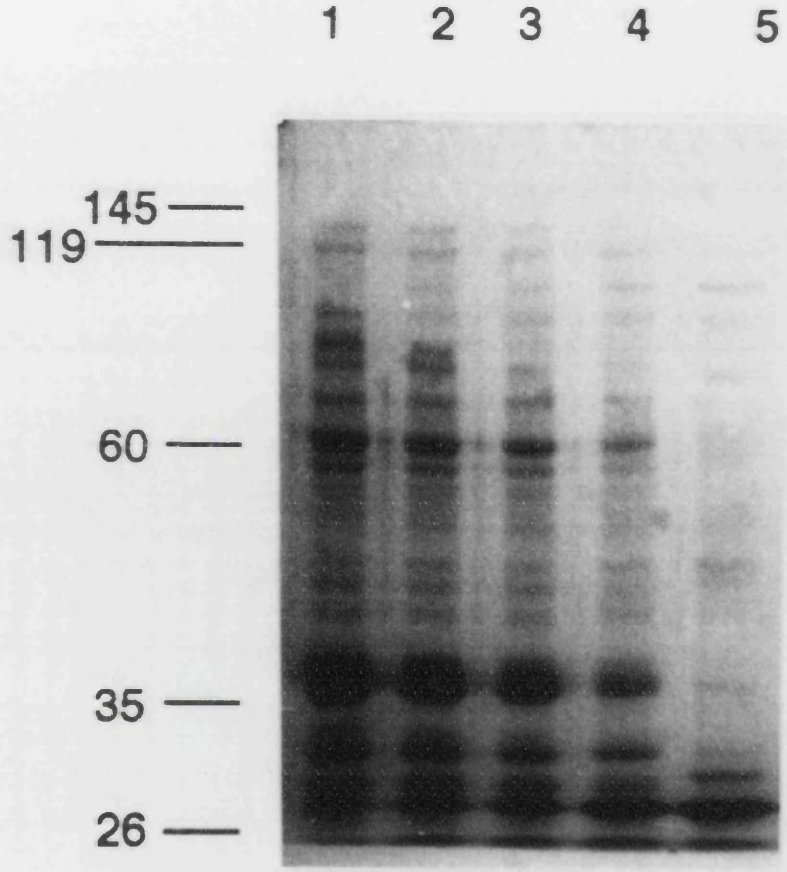
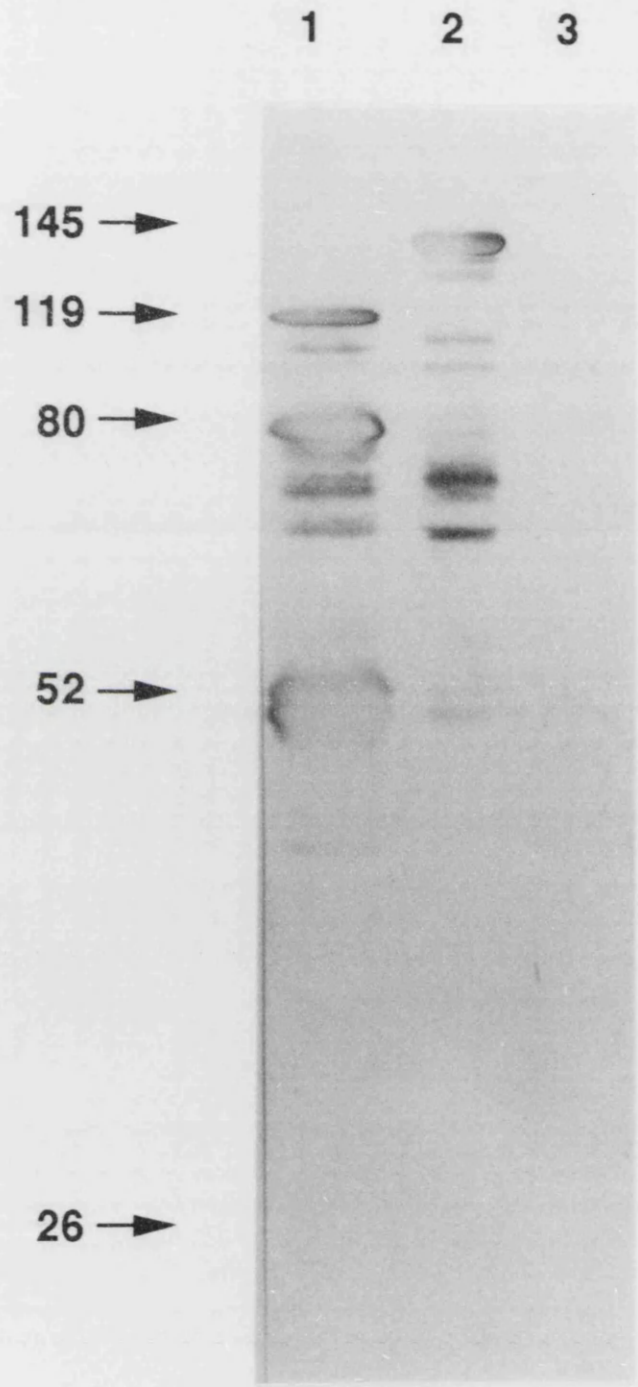

1
2
3
4
5
145
119
60
35
26

Figure 32B

Western blot to show the proteolytic cleavage products of GST-2.7 by Factor Xa protease (16 hour incubation), developed with McAb 1A7 and alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300) as a second antibody. The positions of the molecular weight markers are shown in kDa. Tracks contained the following;

1 2μg Factor Xa cleaved GST-2.7
2 2μg uncleaved GST-2.7 (no protease)
3 2μg GST


1
2
3
145
119
80
52
26

sequence could be detected in the SPAG1 sequence. Since the vector pGEX3X contains the Factor Xa recognition site in the polycloning region, it was feasable to remove the GST carrier from the fusion protein GST-2.7 to generate isolated SPAG1 protein, without cleaving sites within the SPAG1 sequence itself.

A trial experiment was set up in which 250μg of GST-2.7 fusion protein was cleaved with bovine Factor Xa protease as described in section 2.2.7. Samples were removed prior to and 1, 2, 4 and 16 hours following addition of the protease. The samples were visualised on a Coomassie Blue stained 12% SDS polyacrylamide gel, as shown in Figure 32A. Cleavage of the fusion protein was indicated by decreasing intensity of the principal 145, 60 and 40kDa bands in the original preparation (track 1) with longer incubation time and the increasing intensity of bands at 120 and 26kDa (tracks 2-5). Maximum cleavage had occured in the overnight incubation (track 5), in which the 145 kDa band had almost disappeared. The 26kDa band corresponded to the molecular weight of GST, while the 120kDa band corresponded to the expected size of the SPAG1 polypeptide (119kDa) with the 26kDa GST removed from the 145kDa fusion protein. There was also a noticable increase in density of a band at the dye front of the gel with longer incubation time, indicating the accumulation of small peptides, possible degradation products.

Having chosen an incubation time of 16 hours, which gave the maximum cleavage of the fusion protein, Factor Xa protease was used to cleave 8mg GST-2.7 in an overnight incubation, passing the digest through a Glutathione Sepharose 4B column as described in section 2.2.7. The protein concentration in the end product was quantified by Coomassie Plus microassay. 2μg samples of GST, uncleaved GST-2.7 and cleaved GST-2.7 which had been passed through the glutathione sepharose column were run on an SDS polyacrylamide gel and Western blotted. The blot was developed using McAb 1A7, as shown in Figure 32B. A total of 1.5mg SPAG1 protein was obtained after removal of GST and uncleaved fusion protein by the Glutathione Sepharose, which represents only 20% recovery of the original protein. On the Western blot, 1A7 reacted with the cleaved and uncleaved fusion protein but not GST. The 145kDa band was present in the uncleaved preparation only,

while in the cleaved preparation the highest band was about 120kDa. As in the smaller scale preparation, the 120kDa band corresponded to the expected size of the SPAG1 polypeptide with the 26kDa GST fragment removed.

4.4 Discussion

This chapter described the successful cloning and expression of SPAG1 and eleven subfragments derived from it in pGEX vectors. The fusion proteins were all capable of binding to glutathione, implying that the tertiary structure of the GST active site had not been disrupted during the purification process. Furthermore, the reactivity of McAb 1A7 with GST-2.4 and GST-2.7 (Figures 23 and 24B) on Western blots indicates that the McAb epitope was correctly expressed. Further examples demonstrating the antigenicity of the fusion proteins will be discussed in Chapter 5. The expression of the intact GST binding site and of a SPAG1 epitope capable of being recognised by McAb 1A7, raised to the native sporozoite antigen indicate that the primary structure of the expressed fusion proteins was correct.

Trial experiments using GST-2.4 showed the optimum yield of the fusion protein was obtained by inducing the culture at 30°C for 2 hours in 0.1mM IPTG, followed by purification at 4°C in the presence of protease inhibitors. Expression at 30°C was found to be preferable to expression at 37°C. 30°C has frequently been found to be an optimal temperature for expression of recombinant proteins in *E. coli*, and has been shown to enhance solubility of pGEX fusion proteins (manufacturer's information) There was no identifiable expression of fusion protein in the absence of IPTG, indicating that control of expression by the Lac I^q gene in the vector was very efficient. This is advantageous as it allows the host cells to reach a high density prior to induction, without any problems of toxicity resulting from premature fusion protein expression. Induction for longer than 2 hours resulted in increasing degradation of the fusion protein. A likely explanation for this is the increased transcription of proteases by *E. coli* which has been found to occur with increased synthesis of recombinant proteins (Marston 1986).

Expressing GST-2.4 in *E. coli* strains deficient in the Lon A protease, which is primarily responsible for proteolysis of recombinant proteins, had no apparent effect on the level of expression or the degree of degradation. This does not preclude the involvement of other protease groups in degradation of the fusion protein, however. Conversely, the conditions under which the fusion protein was purified had a significant effect on the yield and level of degradation of the final product. Conducting the purification steps at a low temperature in the presence of protease inhibitors increased the yield of GST-2.4 from 1.7mg to 2.3mg per litre and almost tripled the proportion of undegraded fusion protein in the preparation. These observations imply that much of the fusion protein degradation was occuring during lysis of the cells and/or isolation of the fusion protein, rather than during it's expression in the cell.

A common characteristic associated with intracellular expression of eukaryotic proteins in *E. coli* is degradation of the product or it's accumulation as insoluble "inclusion bodies", refractile granules which can be observed in the cytoplasm. The reasons for inclusion body formation are not clear; they are thought to arise through the incorrect folding of the recombinant fusion protein causing it to lose solubility and/or a reaction of the prokaryotic host to the accumulating "foreign" and potentially toxic protein when it's degradation systems become saturated (Kelley and Winkler 1990). As a result of the frequency of this problem, most protocols for purification of recombinant proteins involve isolation of inclusion bodies and refolding and solubilising the protein. The refolding step can be problematic since the protein can lose it's biological activity.

The pGEX system is unusual in that enzymically active GST can be expressed in the cytoplasm of *E. coli* without forming insoluble inclusion bodies or undergoing detectable degradation (Smith and Johnson 1988). These workers found these properties could be extended to other eukaryotic proteins expressed as fusions with GST; of twenty-one different *Plasmodium falciparum* antigens they attempted to express, fourteen were completely or partially soluble. It is possible that the GST fragment can convey solubility on the rest of the fusion protein, or change the way in which it is recognised by the bacterial host. In cases

where insolubility was a problem, they found this was associated with high proportions of strongly hydrophobic or charged residues within the sequences, or with fusion proteins greater than 100kDa in molecular weight. As discussed in section 4.1, insolubility of fusion proteins can also be a result of incorrect disulphide bond formation.

All of the SPAG1 derived fusion proteins were completely soluble in PBS/1% Triton X-100, despite the high molecular weight of many of the fusion proteins. Regions of hydrophobic and charged residues are rare in the SPAG1 sequence, primarily restricted to stretches of hydrophobic residues at the N and C termini, two short regions of high negative charge (glutamic and aspartic acid residues, amino acids 113-128 and 341-351) and a block of 20 polar amino acids (amino acids 451-472) (Hall et al 1992). The effects of these regions were probably counteracted by the more hydrophilic regions of SPAG1 and the GST carrier. There is also a lack of cysteine residues implying that disulphide bonds were not involved in stabilising the structure, as already discussed in section 4.1. The fact that the SPAG1 proteins were soluble provides evidence that they posessed correct tertiary structure, since poor solubility is frequently associated with the inability of recombinant proteins to fold correctly in the prokaryotic environment (Kelley and Winkler 1990).

While expression of the SPAG1 constructs in pGEX was not hindered by insolubility, apparent degradation of the fusion proteins was problematic. In preparations of the fusion proteins from most of the SPAG1 constructs, additional bands could be seen smaller in size than the intact fusion protein (Figure 30). The positions of the bands were not identical in different recombinants, but were identical, however, in different preparations made from the same recombinant (Figures 28B and 28C). Furthermore, McAbs recognising the intact fusion protein also recognised the smaller bands (Figures 23 and 24B). These bands appeared, therefore, to have derived from the expressed fusion protein rather than the bacterial host. Since these products all bound to glutathione sepharose it can be assumed that they all contain the GST portion of the fusion protein and so any degradation would be from the C terminus. The yield of fusion protein and the extent of degradation varied considerably between the different recombinants, even though they were all derived from the SPAG1 sequence (Table 8). These factors

did not seem to be influenced by the size of the fusion protein. For example, preparations of the 110kDa fusion protein GST-2.1 produced a higher yield and was less degraded then in preparations of the 50kDa recombinant GST-N6.

There are three potential sources for these fragments. One possibility is that they represent the products of premature termination of translation. This was reported by Marston (1986) to result in shorter versions of recombinant proteins lacking amino acid residues at the C terminal end. If this was the case, however, it would be hard to explain the effect of a reduced temperature during the preparation. Perhaps a more likely explanation is that the recombinant protein is degraded by proteases at specific sites. Native SPAG1 is extensively processed in the parasite and is assumed to contain sites for *T. annulata* proteases (Hall et al 1992). However, it seems unlikely that identical sites would be recognised by bacterial proteases, and the sizes of the degradation products do not correspond to those of the native processed antigen. As expression in *lon*$^-$ strains did not reduce degradation, any proteases responsible would be likely to be non-Lon A, or originate from external sources during the purification. While protease inhibitors were added during the purification steps, degradation could still have occured during the induction process, during which they would have been unlikely to have penetrated the cells sufficiently to prevent degradation during harvesting prior to lysis.

It was decided not to attempt to separate the degradation products from the intact fusion proteins, since the proportion of intact protein in each preparation was thought to be sufficient for use in immunological assays. Since the smaller fragments were derived from the same source there was no reason to suppose they would produce any adverse effects. Furthermore, cleavage of the protein into fragments would not be expected to effect it's recognition by antibody on Western blots or by T cells in proliferation assays, unless the cleavage site actually passed through an epitope or an adjacent site necessary to it's structure. The amount of undegraded fusion protein in the preparations would probably be sufficient to compensate for this.

Most of the SPAG1 recombinant proteins were of a larger size than expected from the coding capacity of their DNA sequences, according to their migration on SDS-polyacrylamide gels (Table 8). This phenomenon was also apparent in the size of the λgt11-SR1 fusion protein, with the expressed product of the SR1 insert having an apparent molecular weight of 19kDa compared with the expected size of 12kDa estimated from the coding capacity of the insert (Williamson et al 1989). In this case the reason was thought to be that the high proline content reduced the migration of the protein in SDS-polyacrylamide gels, resulting in a higher size estimation. This is a likely explanation for anomalous sizes of most of the fusion proteins, since the entire SPAG1 amino acid sequence is rich in proline residues. It is notable that the fusion protein GST-SE, which has a low proline content, does not give an anomalous size.

The protease thrombin was found to recognise sites within SPAG1, precluding it's use as a means of removing the GST carrier from the fusion proteins (Figure 31B). This protease appears not to be specific for the sequence Pro-Arg-Gly-Ser, but probably only specifically recognises arginine as described in section 4.3.7, which appears in the SPAG1 sequence. The Factor Xa protease recognition site Ile-Glu-Gly-Arg is longer and so less likely to appear in the coding sequences of recombinant proteins. Data from the GCG WordSeach analysis and from the trial experiments indicated that the Factor Xa cleavage site did not occur in the SPAG1 sequence, so removal of GST from the GST-2.7 fusion protein was feasible. The drawback is that Factor Xa cleavage is much less efficient than thrombin cleavage, requiring a longer incubation time (Figure 32A).

After removal of GST and uncleaved fusion protein the total loss of protein during the preparation was about 80%. Removal of the 26kDa GST polypeptide from the 145kDa fusion protein would only account for about 15% of this. The remaining loss could be due to a combination of incomplete cleavage of the fusion protein, degradation during the preparation or loss of solubility of SPAG1 due to removal of the GST polypeptide. There is some evidence for degradation occuring during Factor Xa cleavage, in that peptides appear to accumulate at the dye front during the incubation (Figure 32A). This could be due to contamination of the Factor Xa by other proteases, as well as decreased

stability of the SPAG1 polypeptide resulting from removal of the GST fragment. Recognition of the cleaved product by McAb 1A7 showed the epitope to be intact, implying that antigenicity of the protein was unaffected by the cleavage process (Figure 32B).

As a result of the problems encountered in proteolytic cleavage of the fusion proteins, it was decided to use them in an uncleaved state when assessing T and B responses, but including purified GST as a control in all tests. Isolated SPAG1 derived from Factor Xa cleaved GST-2.7 was used to confirm anti-SPAG1 responses.

TABLE 7. CLONING SPAG1 CONSTRUCTS

The following table summarises the cloning procedures used to subclone the SPAG1 fragments HB, EA, BN, N6, NE, S1 and SE into pGEX expression vectors. (supplied by Dr. R. Hall). The nucleotide numbers refer to the original SPAG1 sequence (Figure 77, appendix). The positions of the recognition sites in the cSPAG1 sequence for the enzymes used are given in the cSPAG1 restriction map (Figure 78A). The predicted molecular weight (kDa) of the fusion protein with 26kDa GST predicted from each construct is also given.

Construct	Nucleotides	Cloning	Predicted size (kDa)
HB	110-378	N-terminal BamH1 fragment from 2.7 ligated into BamH1 cut pGEX3X	35.6
EA	378-839	Acc1/EcoR1 fragment removed from 0.8*, end-filled and religated	39.8
BN	378-559	BamH1-NlaIV fragment from 0.8 (partial digest), end-filled and religated	32.1
N6	1197-1949	BamH1-Hpa1 fragment cut from 2.7, ligated into BamH1-Sma1 cut pGEX2T	51.9
NE	1197-1512	Nco-EcoR1 cut N6, end-filled and religated	35.5
S1	2227-2405 2504-2777	Spe1 cut N2 (NlaIV/EcoR1 fragment from 2.7) end-filled and religated after removal of fragment	38.7
SE	2227-2405	Spe1/EcoR1 cut S1, endfilled and religated after removal of fragment	32.3

* This subclone was derived from a pGEX2T-0.8 subclone with the insert in the opposite orientation.

TABLE 8. SIZE,YIELD AND %DEGRADATION OF EXPRESSED GST-SPAG1 CONSTRUCTS

The following table summarises the results from large scale preparations of the SPAG1 constructs expressed as GST fusion proteins in pGEX expression vectors. The final yeild of fusion protein per litre induced culture is shown, plus the proportion of undegraded protein according to estimation from Coomassie Blue stained SDS polyacrylamide gels. The amino acid residues of the SPAG1 sequence are also given, the molecular weight of each fusion protein estimated from it's position on SDS -polyacrylamide gels (actual size) and the expected molecular weight calculated from the sequence (expected size).

CONSTRUCT	AMINO ACIDS	EXPECTED SIZE (kDa)	ACTUAL SIZE (kDa)	% INTACT	YIELD (MG/LITRE)
GST	-	26	26	>90%	22.5
GST-2.7	20-907	115.8	145	15-20%	1.3*
GST-2.4	109-907	106.3	130	20%	2.3◊
GST-2.1	109-833	96.0	110	70%	4.96
GST-HB	20-108	35.6	37.5	80%	8.56
GST-0.8	109-381	50.9	63	70%	3.39
GST-EA	109-262	39.8	49	80%	#
GST-BN	109-169	32.1	36	80%	#
GST-N6	382-632	51.9	50	25%	1.4
GST-NE	382-486	35.5	44	20-30%	#
GST-S1	726-784 818-907	38.7	46	30%	#
GST-SE	726-784	32.3	36	20%	2.18
GST-SR1	784-892	34.1	45	70%	5.42

* mean from two 16L preparations
◊ mean from two 4L preparations
fusion protein provided by Dr.P.Hunt/Dr. R. Hall, Dept. Biology, University of York

CHAPTER 5

THE B CELL RESPONSE TO SPAG1

5.1 Introduction

As discussed in section 1.2.2, in order for a molecular vaccine to be effective it needs to contain relevant T and B epitopes, which immitate the epitopes on the native parasite molecule sufficiently to trigger an immune response to the native parasite antigen. Analysis of the B cell responses to SPAG1 will be described here; work to identify the T cell responses to the antigen will be dealt with in the next chapter.

It is important to identify relevant B cell epitopes for inclusion in a subunit vaccine ie. those whose recognition contributes to destruction of the parasite, and preferably are not subject to a high degree of antigenic diversity. In extracellular stages of parasites such epitopes tend to be located in proximity to ligands necessary for invasion of host cells. An example of this is the MSP1 antigen of *Plasmodium falciparum* merozoites, which is extensively processed during maturation to produce fragments of various molecular weights. Antibodies which inhibited infection of erythrocytes "in vitro" were found to recognise a single 19kDa fragment, the only processed product to be retained on the surface of invading merozoites (Blackman et al 1990).

In many cases, humoral responses are stimulated to parasite epitopes which are ineffective at preventing or terminating the infection, and this is a potential evasion mechanism to prevent recognition of antigens more vital to the parasite's survival. Such may be the role of many of the highly immunogenic repetitive amino acid sequences unusually common in antigens of *Plasmodium* species. It has been suggested (Hyde 1990) that some of these repetitive epitopes may produce a "smoke screen" effect, diverting the humoral immune response away from more vital epitopes.

This chapter describes work towards identifying B cell epitopes of SPAG1 recognised by bovine antisera and McAbs, and the results of a

vaccination trial using the recombinant fusion protein. In an experiment conducted previously (Williamson 1988), two cattle were immunised with SR1 expressed in λgt11 as a fusion protein with β-galactosidase, prepared by electoelution from SDS-PAGE gels. The post-immune sera gave a strong reaction to formalin fixed sporozoite antigen in IFA tests and recognised sporozoite antigens on Western blots to give a banding pattern identical to that produced by McAb 1A7. The post-immune sera also neutralised sporozoite infectivity of lymphocytes in "in vitro" assays. However, both animals remained susceptible to challenge when injected with an estimated LD50 of *T. annulata* Hissar sporozoites. The results of this experiment could be interpreted as an indication that stimulation of humoral responses to SPAG1 is not sufficient alone to induce protective immunity to *T. annulata*. Nevertheless, there are a number of other possible reasons why protective immunity was not induced. Primarily, it may have been inappropriate to present the SR1 peptide as a fusion protein with β-galactosidase. While no humoral response was recorded to β-galactosidase alone from the cattle serum, it is likely that the much larger prokaryotic protein acted as a carrier for the SR1 peptide hapten, and so probably evoked a T helper cell response. The "carrier effect" of fusing a small hapten to a larger molecule has already been discussed in section 1.2.2. However, fusion to β-galactosidase has been found to result in poor immunity to some eukaryotic antigens. For example, in studies on the response to vaccination of sheep with a *Taenia ovis* onchosphere antigen, recombinant antigen was found to induce a high level of protective immunity when expressed as a fusion protein with GST, but not with β-galactosidase (Johnson et al 1989). The lack of protection from the β-galactosidase recombinant was unlikely to be due to it's purification on SDS polyacrylamide gels as the native onchosphere antigen prepared in the same way was protective. A similar result was obtained in investigations carried out to ascertain whether *Taenia taeniformis* onchosphere antigens could induce protective immunity in rats (Ito et al 1991).

Fusion to β-galactosidase may, therefore, result in inefficient recognition of some polypeptides, perhaps by stimulation of inappropriate T cell subsets unable to give "help" to hapten-specific B cells, or by stimulation of supressor T cells. Recognition of T cell epitopes on the eukaryotic peptide may also have been inhibited by the influence of

β-galactosidase on the antigen processing pathways. Denaturation of any conformational epitopes resulting from the extraction of the fusion protein from SDS polyacrylamide gels could have also been a reason for the lack of protection in the λgt11-SR1 immunised cattle. The potential problems of incorrect glycosylation and folding associated with expression of a eukaryotic polypeptide in a prokaryotic host, which could also interfere with immune recognition of the antigen, have already been discussed in Chapter 4.

In the work done here, the bovine humoral responses to the entire SPAG1 antigen are examined, using polypeptides expressed in the pGEX system as described in Chapter 4. Five cattle were immunised with GST-2.7 fusion protein and their immune responses to sporozoite antigen assessed using Western blots,.IFA tests and in "in vitro" sporozoite neutralisation assays. The sera was also tested for reactivity against the different SPAG1 constructs in order to map regions of the SPAG1 sequence containing B cell epitopes recognised by the cattle, and the patterns of reactivity compared to that given by serum from cattle immunised with sporozoite extracts. The GST-2.7 immunised cattle and four unimmunised controls were challenged with an estimated LD50 dose of *T. annulata* Hissar sporozoites and the levels of parasitaemia assessed.

The advantages of using the pGEX system to express SPAG1 rather than the λgt11 expression system have already been discussed in the previous chapter. Immunising the cattle with GST-2.7 rather than the cleaved product would not be expected to greatly influence the immune responses to the SPAG1 part of the fusion protein, since the GST would probably be cleaved from the fusion protein by bovine Factor Xa protease in the blood. Furthermore, GST is a much smaller protein than SPAG1, and so anti-GST responses would not be expected to predominate in the recognition of the fusion protein by B and T cells.

The cattle were immunised with GST-2.7 in conjunction with Freund's complete adjuvant (FCA) or saponin. In a comparative study of various adjuvants, both FCA and saponin were found to be highly effective at triggering bovine immune responses to a *Trypanosoma brucei* antigen (Wells et al 1982). Furthermore, saponin was the adjuvant used

in a successful immunisation trial with a *T. parva* recombinant sporozoite antigen, p67 (Musoke et al 1992). Two different immunomodulators were used since the choice of adjuvant can have a considerable influence on the the antibody response induced to a specific antigen, probably by influencing the way in which it is processed and presented to T helper cells (Hui et al 1991).

As well as mapping the regions of SPAG1 containing B cell epitopes recognised by cattle, the epitopes recognised by mouse McAbs generated to *T. annulata* sporozoites were also mapped. The generation of the sporozoite neutralising McAbs 1A7 and 4B11 from a mouse immunised with *T. annulata* Ankara sporozoites has been described previously (Williamson 1988, Williamson et al 1989). The reactivity of these McAbs with sporozoite material has already been described in Chapter 1 (1.1.3). A number of other McAbs were produced at the same time which also recognised formalin fixed *T. annulata* Ankara and Gharb sporozoites, but not macroschizonts or piroplasms (Williamson 1988). However, these McAbs were considerably less effective at inhibiting sporozoite infectivity of lymphocytes when assayed "in vitro".

These McAbs were screened for reactivity with SPAG1 antigens on a Western blot of *T. annulata* sporozoite material. Three McAbs which were reactive with bands of an identical size to those seen by McAb 1A7 were used to probe Western blots of GST-2.7 and smaller GST SPAG1 constructs, in order to identify regions of SPAG1 containing the epitopes recognised.

The final part of this work was to examine sequence homology and serum cross reactivity between *T. annulata* SPAG1 and the *T. parva* sporozoite surface antigen p67, since there is some evidence of structural similarity between the two antigens, according to the work of Nene et al (1992). The p67 gene, derived from a *T. parva* Muguga cDNA library, has been expressed in the vector pMG1 to give a fusion protein with NS1, derived from the influenza A non-structural gene. On Southern blots of EcoR1 digested *T. annulata* DNA the p67 insert was found to hybridise to a 3.4kb fragment at low stringency, but not to *T. mutans* or *T. taurotragi* DNA. The 3.4 kb band is identical in size to the restriction fragment which the SR1 probe hybridises to on *T. annulata* genomic Southern

blots, sugesting that the two probes hybridise to the same gene. Futhermore, amino acid sequence comparison between SR1 and p67 using the computer package GCG WordSearch showed 54% similarity. Therefore the p67 gene appeared to show some sequence homology with the C-terminal end of SPAG1, covered by the SR1 region, and this region could potentially contain cross-reactive epitopes.

In this chapter the amino acid sequences of p67 and the entire SPAG1 sequence are compared, in order to identify any other regions of sequence homology existing outside the SR1 region of SPAG1. Experiments were also performed to verify the existence of any cross reactive epitopes between the two gene products. Reactivity of serum from cattle immunised with *T. parva* sporozoites or NS1-p67 fusion protein with *T. annulata* sporozoite material and recombinant SPAG1 was assessed using Western blots and IFA tests on formalin fixed sporozoites. Reactivity of this serum with the GST-2.7 sub-constructs was also examined in order to map regions of SPAG1 containing epitopes homologous to p67. Reciprocal Western blots were also performed on *T. parva* sporozoite material and on NS1-p67 fusion protein using serum from cattle which had been immunised with *T. annulata* sporozoites and GST-2.7 fusion protein.

In summary, the questions being addressed here are as follows. Firstly, to identify regions of SPAG1 containing B cell epitopes recognised by bovine antisera raised against recombinant SPAG1, and to compare them with B cell epitopes recognised by bovine antisera raised against *T. annulata* sporozoites. Secondly, to ascertain whether any SPAG1 B cell epitopes are also shared by the *T. parva* sporozoite antigen p67.

5.2 RESULTS

5.2.1. GST-2.7 immunised cattle

Five male Ayrshire cattle were immunised with GST-2.7 fusion protein prepared as described in Chapter 4. Details of the immunisations are given in section 2.2.8. Two animals, 26x and 32x, were immunised with saponin as an adjuvant, while animals 23x, 758

and 759 were immunised with Freund's complete adjuvant in the first immunisation, and Freund's incomplete adjuvant with subsequent immunisations. After an initial immunisation, all five animals were boosted at intervals of 5, 4 and 3 weeks, and then challenged with an estimated LD 50 of *T. annulata* Hissar stabilate (0.2 tick equivalents) 14 days following the final boost, on day 92 after the first immunisation. Sera for Western blots, sporozoite inhibition assays and IFA tests was collected 14 and 20 days following each immunisation, and immediately prior to challenge. After challenge, blood smears were taken every 2-4 days and used to assess the level of infection by counting the percentage of piroplasms. The immunisations, serum collection, sporozoite inhibition assays, IFA titres and assessment of blood smears was carried out by C.G.D. Brown, G. Wilkie and L. Bell-Sakyi at CTVM, Edinburgh. The results are included here with their permission.

The results of the IFA tests on acetone fixed *T. annulata* Hissar sporozoite antigen using pre-immune sera and post-immune sera collected on day 14 after the second, third and fourth immunisations are summarised in Table 9. The tests were carried out as described in section 2.2.6. All the post-immune sera were found to recognise sporozoite antigen. Sera collected after the second boost gave a titre of 1/640, compared with 1/10 or 1/40 for the pre-immune serum. In three of the five animals, 23x, 758 and 759, the titre increased to 1/2560 in serum collected after further boosting.

Western blots of *T. annulata* Hissar sporozoite material were developed with Day 92 antiserum from cattle 26x, 32x, 758, and 759, pooled Day 0 sera from all five animals and McAb 1A7, as shown in Figure 35. The day 92 sera from 32x, 758 and 759 all reacted with groups of bands identical in size to those recognised by McAb 1A7, of approximately 110, 80-85, 70, 63 and 50-54kDa. Interestingly, day 92 sera from 26x reacted with the three higher molecular weight groups of bands only, at 110, 80-85 and 70kDa. There was no reaction from the pooled day 0 serum with the sporozoite material.

ELISA titres were carried out against GST-2.7 fusion protein using serum collected 35 days after the initial immunisation, and 14 days after each subsequent boost. The assay was carried out as described in

section 2.2.6. In all 5 animals the optical densities recorded for the post-immune sera were substantially higher than those recorded with the pre-immune serum, indicating the presence of antibodies to the fusion protein (Table 10). The antibody level in all the day 56 sera apart from 26x was higher than that recorded in the day 35 sera, but was not found to increase in sera collected after subsequent immunisations.

The effect of day 0 and day 92 serum from animals 26x, 32x, 758 and 759 on the infectivity of *T. annulata* (Hissar) sporozoites "in vitro" was examined by carrying out antibody inhibition assays as described in section 2.2.10. Two assays were carried out using different dilutions of the sera. Percentage inhibition was calculated as shown in Table 11. All the post-immune sera blocked sporozoite infection of lymphocytes almost completely when used neat or diluted 1/4, but this effect was reduced or eliminated when the serum was used at dilutions of 1/16.

The results of challenge of the five cattle immunised with recombinant GST-2.7 and the four unimmunised controls with an estimated LD50 of *T. annulata* Hissar sporozoites are summarised in Figures 33 and 34. Figure 33 and Table 12 summarises the percentages of piroplasm infected erythrocytes as assessed from the blood smears taken from all nine cattle over a period of 21 days following challenge. In all the cattle, piroplasms were seen by day 12 post-challenge, and generally increased subsequently. However, there was considerable variation between the animals in the levels of parasitaemia recorded. All four control cattle; 739, 740, 741 and 740, and two of the immunised cattle; 23x, which received FCA, and 32x, which received saponin as adjuvants, suffered high levels of infection, reaching a plateau between days 14 and 19 in most cases. Three of the GST-2.7 immunised animals; 26x, which had saponin as an adjuvant, and 758 and 759, which received FCA, showed much lower increases in their parasitaemia after day 12. Figure 34 summarises the mean percentage piroplasms recorded in the control and immunised groups of animals. The mean parasitaemia for the immunised cattle was significantly lower than for the control cattle on day 12 (Student's T test; $P > 0.2$). The mean for the immunised group was also considerably lower than the controls on days 14 and 16, although this was not of statistical significance due to the

Figure 33

Graph to show the parasitaemias (% piroplasms in blood smears) of the five cattle immunised with GST-2.7 and four unimmunised controls over 21 days following challenge with an estimated LD50 of *T. annulata* Hissar sporozoite stabilate. Cattle 26x and 32x were immunised with GST-2.7 and saponin, cattle 23x, 758 and 759 were immunised with GST-2.7 and Freund's complete adjuvant while cattle 739, 740, 741 and 742 were unimmunised controls.

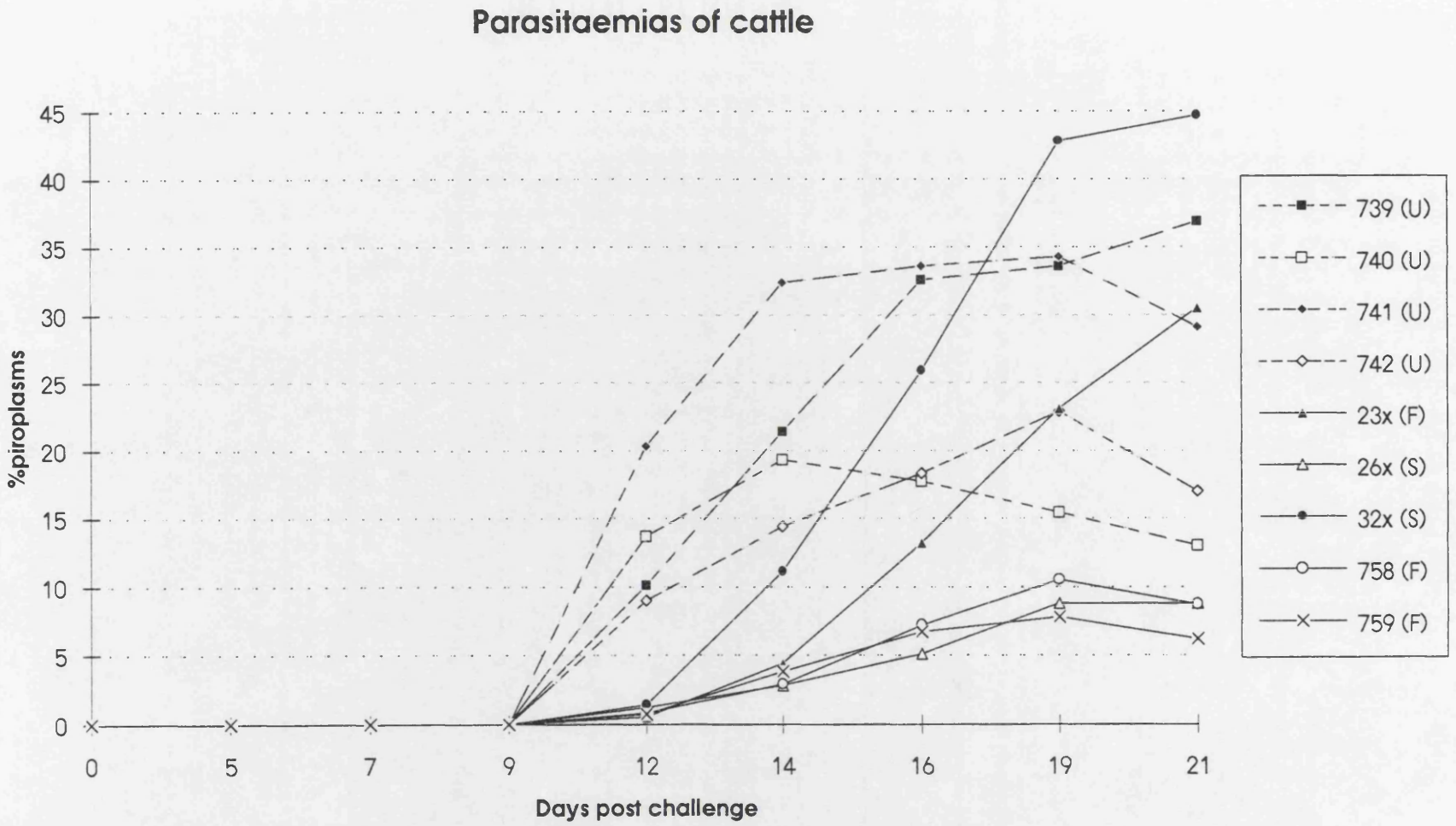

Parasitaemias of cattle
%piroplasms
45
40
35
30
25
20
15
10
5
0
0
5
7
9
12
14
16
19
21
Days post challenge
739 (U)
740 (U)
741 (U)
742 (U)
23x (F)
26x (S)
32x (S)
758 (F)
759 (F)

Figure 34

Histogram to show the mean parasitaemia (% piroplasms) for the control and GST-2.7 immunised cattle over 21 days following challenge with an LD50 *T. annulata* Hissar sporozoites.

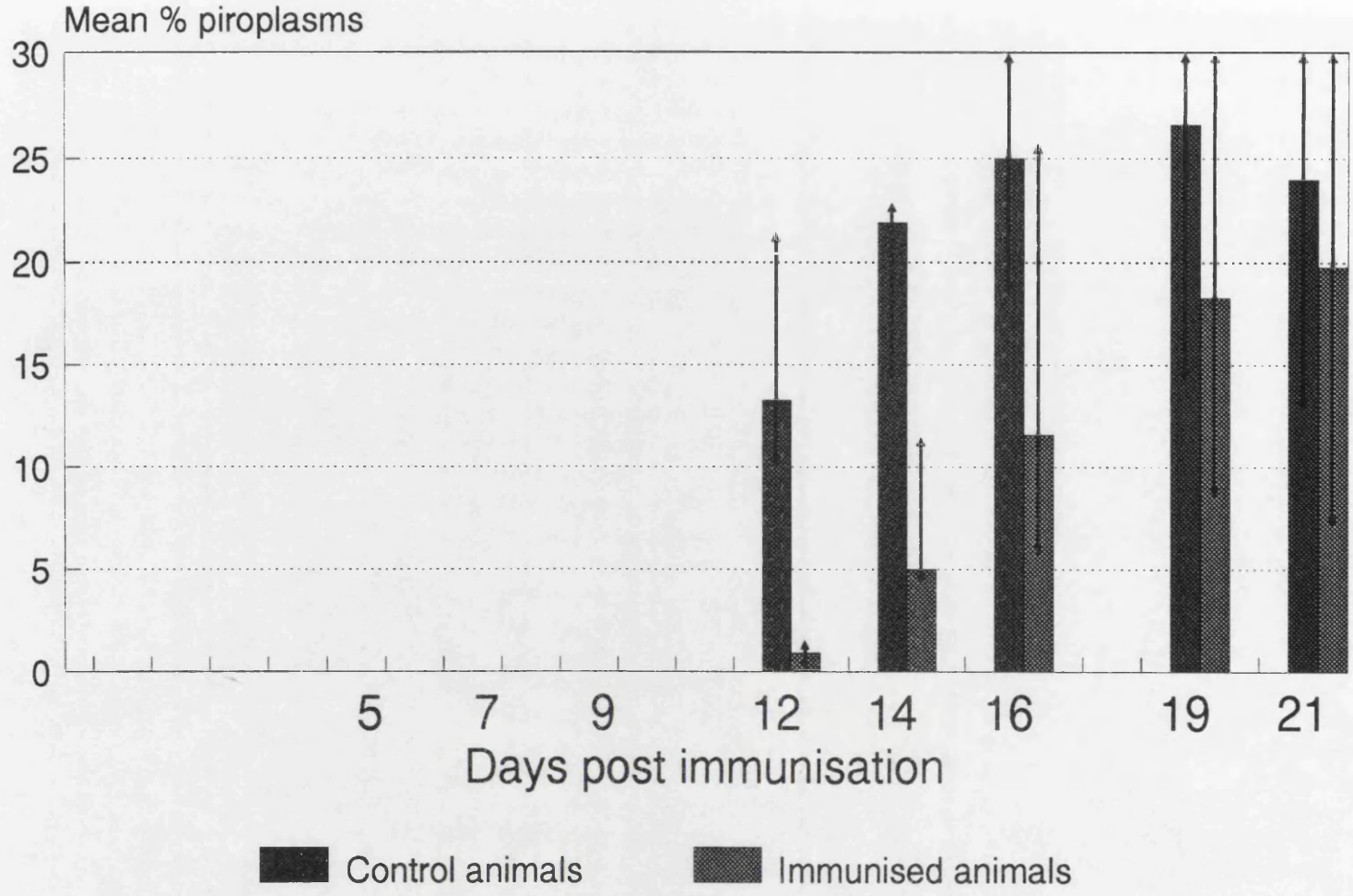

Mean % piroplasms
30
25
20
15
10
5
0
5
7
9
12
14
16
19
21
Days post immunisation
Control animals
Immunised animals

Figure 35

Western blot of a 10% SDS-polyacrylamide gel loaded with *T. annulata* Hissar sporozoites (tick salivary gland extract; 0.2 tick equvalents per track) developed with day 0 and day 92 antisera from the cattle immunised with GST-2.7. The positions of the molecular weight markers are shown in kDa. Tracks were incubated with the following antibodies;

1A7 McAb 1A7 undiluted supernatant, with alkaline-phosphatase conjugated rabbit anti-mouse IgG as a second antibody (1:300)

26x, 32x Day 92 antisera from cattle immunised with GST-2.7 and saponin, using alkaline-phosphatase conjugated rabbit anti-bovine IgG as a second antibody.

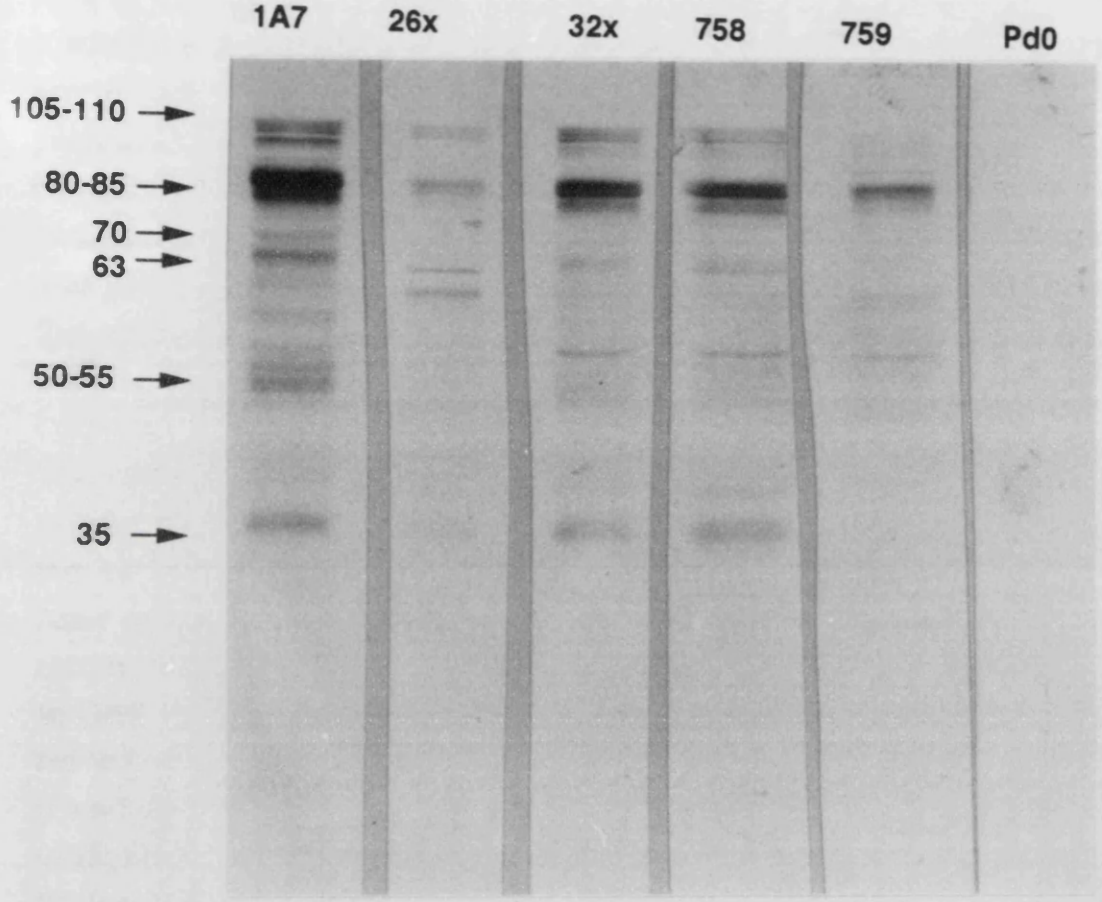
758, 759 Day 92 sera from cattle immunised with GST-2.7 and Freund's complete adjuvant. Second antibody as above.

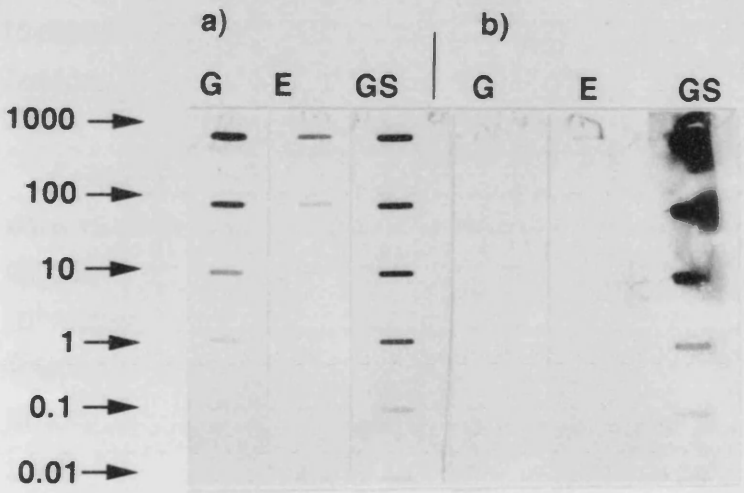
Pd0 Pooled Day 0 antiserum from all five cattle. Second antibody as above.

Figure 36

Slot-blot of GST, GST-2.7 fusion protein and normal *E. coli* proteins incubated with 23x day 92 GST-2.7 antiserum before (a) and after (b) adsorbtion with GST and *E. coli* proteins on an AminoLink column. The antisera was used at a final dilution of 1:100 (taking into account the dilution produced by washing the antiserum from the column). The second antibody was alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300). The amount of protein loaded per slot is shown in μg. Tracks contained the following proteins;

G GST recombinant protein
E Proteins extracted from untransformed *E. coli* XLBlue
GS GST-2.7 recombinant protein


1A7
26x
32x
758
759
Pd0
105-110
80-85
70
63
50-55
35


a)
b)
G
E
GS
G
E
GS
1000
100
10
1
0.1
0.01

variability within each group. The experiment was terminated on day 23 after challenge.

In order to assess the antibody response of the GST-2.7 immune sera to fragments of SPAG1 expressed in *E. coli* as GST fusion proteins, it was first necessary to remove those antibodies reactive to GST and to any *E. coli* antigens which may also be present in the fusion protein preparations. This was done by passing the sera through a column containing "AminoLink" agarose beads coupled to GST and *E. coli* proteins as described in section 2.2.6. Day 92 sera from each animal and pooled day 0 sera consisting of combined pre-immune sera from the five cattle were adsorbed in this way. The reactivites of adsorbed and unadsorbed sera were then compared on slot blots loaded with a range of GST and *E. coli* protein concentrations, as described in section 2.2.6. The resulting blots are shown for 23x day 92 serum only (Figure 36). The unadsorbed serum reacted with GST and *E. coli* proteins at quantities as low as 1ng and 100ng respectively, and with GST-2.7 down to 0.01ng. After adsorbtion there was no detectable reaction to GST and only a minor reaction to *E. coli* at 1μg. There was no detectable loss of reactivity to GST-2.7. Adsorbtion of the other sera produced similar results when tested on identical slot blots (data not shown). GST/ *E. coli* adsorbed day 0 and day 92 serum from animal 23x was used in an ELISA test against GST, GST-2.7 and eight of the SPAG1 subfragments expressed as GST fusion proteins. The results are summarised in Table 13. The binding ratios were calculated by comparing the OD 490nm given by the post-immune serum with the day 0 sera. A "binding ratio" of 5 or more, indicating a positive humoral response, was obtained with most of the fusion proteins except for BN, SE and GST alone.

More detailed analysis of SPAG1 epitopes recognised by the cattle was carried out on Western blots. To identify an appropriate working dilution for the sera, day 92 GST adsorbed serum from 23x was used at dilutions of 1/100, 1/1000, 1/5000 and 1/10,000 to develop strips of a Western blot with 1μg GST-2.7 loaded per track. The optimum serum dilution was found to be 1/5000, which gave clear resolution of bands (data not shown). Sera were used at this dilution in all subsequent blots. Western blots were undertaken using the ten SPAG1 fusion protein constructs listed in Table 15A. The cloning and expression of all these

Figure 37 A-C

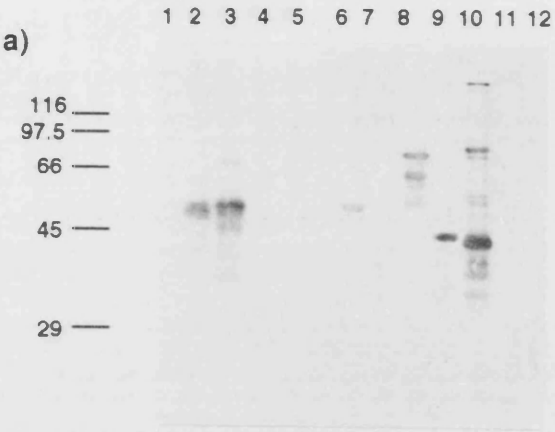
Triplicate Western blots of 12% SDS-polyacrylamide gels each loaded with eleven SPAG1 subclones expressed and purified as GST fusion proteins, recombinant GST and unpurified *E. coli* lysate, incubated with antisera from the GST-2.7 immunised cattle. 1μg protein was loaded per track. The positions of the molecular weight markers are shown in kDa. The second antibody was alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300). The blots were developed with the following primary antibodies;

A day 92 antisera from animal 759
B day 92 antisera from animal 32x
C Pooled day 0 sera from all five GST-2.7 animals

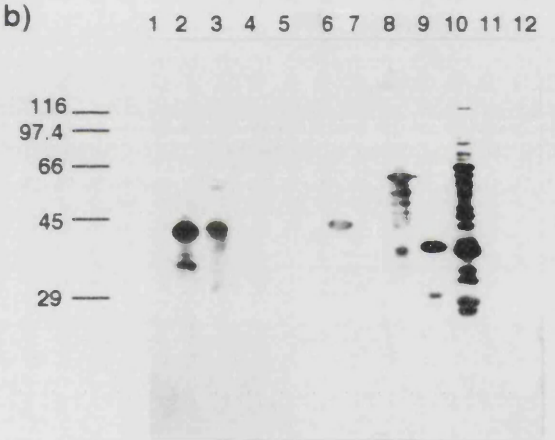
Tracks contained the following recombinant proteins;

1 GST-SE
2 GST-S1
3 GST-0.3
4 GST-N6
5 GST-NE
6 GST-EA
7 GST-BN
8 GST-0.8
9 GST-HB
10 GST-2.7
11 GST
12 *E. coli* extract

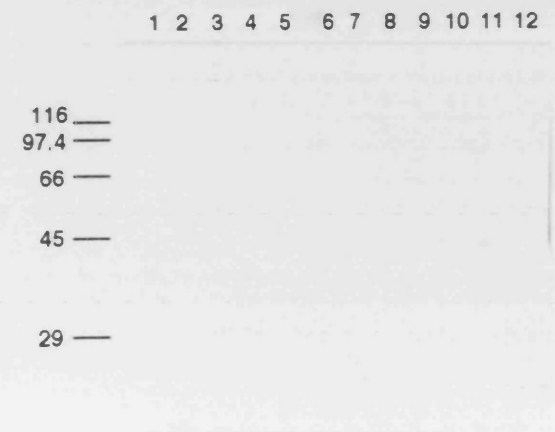
a)


1 2 3 4 5 6 7 8 9 10 11 12
116
97.5
66
45
29

b)


1 2 3 4 5 6 7 8 9 10 11 12
116
97.4
66
45
29

c)


1 2 3 4 5 6 7 8 9 10 11 12
116
97.4
66
45
29

fusion proteins have been described in Chapter 4. Recombinant GST and proteins from untransformed *E. coli* cells were included in two of the tracks as controls. Identical blots were incubated with GST/ *E. coli* adsorbed pooled day 0 serum and day 92 sera from each animal. The reactivities are summarised in Table 15A. Figure 37 A, B and C shows representative blots incubated with 759 day 92 serum, 32x day 92 serum and pooled day 0 serum are shown in respectively. All five post-immune sera gave similar results, reacting with GST fusion proteins 2.7, HB, 0.8, EA, S1 and SR1, but failing to react with BN, SE, GST or *E. coli* proteins. There was some variability in the reactivites to N6 and NE, which were recognised by 23x, 32x and 758 day 92 sera only, although the bands seen were faint. Recognition of fusion proteins by the day 0 serum was negligable.

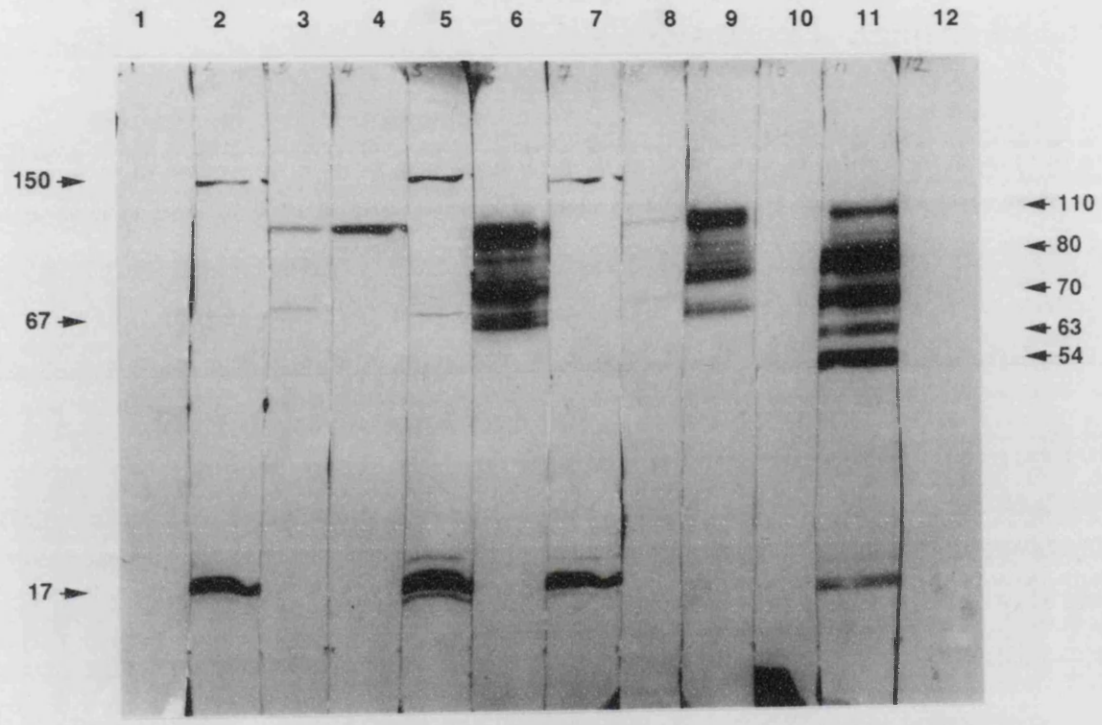
5.2.2 Mouse anti-sporozoite McAbs

In order to identify anti-sporozoite McAbs reacting with SPAG1 antigens, tracks from a Western blot of *T. annulata* Hissar sporozoite material were incubated with McAbs 4B11, 4D3, 5D1, 1B4, 4A7, 4G5 and 5D1 and the banding pattern obtained compared with 1A7. The results are shown in Figure 38 and summarised in table 14. McAbs 4D3, 5D1. 5F1 and 4A7 all reacted with antigens identical in molecular weight to at least some of those recognised by 1A7. McAbs 4D3, 5D1 and 5F1 reacted predominantly with the 110 kDa antigen, while McAb 4A7 recognised a complex of polypeptides with molecular weights identical to those recognised by 1A7 except for the two lower molecular weight bands of 63 and 54 kDa. Conversly, McAbs 4B11 and 1B4 reacted with a different group of antigens, of 150, 67 and 17kDa. No polypeptides were recognised by McAb 4G5, despite it's previously observed reactivity with *T. annulata* formalin fixed sporozoites (Williamson 1988). No bands were recognised by the control McAb 1C7, or when blots were incubated with second antibody only. The detection of a 17kDa antigen by 1A7 was unexpected; this was not obtained on repetition of the blot, and so presumably was a result of non specific binding. Repetition of the blot also confirmed the sizes of antigens recognised by 4A7, 4D3 and 5D1 (data not shown). All of these McAbs also reacted with *T. annulata* Gharb

Figure 38

Western blot of a 7-20% gradient SDS-polyacrylamide gel loaded with one tick equivalent per track *T. annulata* Hissar sporozoite material (GUTS), incubated with McAbs raised against *T. annulata* sporozoites. The molecular weights (kDa) of the bands detected are indicated by arrows. The second antibody used in each case was alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300). All the primary antibodies were ascites diluted 1:100 except for the control McAb 1C7, which was undiluted supernatant. Tracks were incubated with the following McAbs;

1 1C7 (anti-schizont control)
2 4B11/C12
3 4D3/E8
4 5D1/D11
5 1B4/B12
6 4A7/E12
7 4B11/E9
8 5F1/E11
9 4A7/A8
10 4G5/C10
11 1A7/G8
12 None (second antibody only)


1
2
3
4
5
6
7
8
9
10
11
12
150
67
17
110
80
70
63
54

and Ankara formalin fixed sporozoites to a titre of 1/160 (S. Williamson, personal communication).

Another available McAb, known to react with *T. annulata* sporozoite material, was BA4 (supplied by Dr. B. Mecham, Washington University Medical Center, St. Louis). McAb BA4 detects the epitope VGVAPG in bovine elastin (Wrenn et al 1986), which is also present in the elastin homologous repeats of *T. annulata* SPAG1. BA4 has been shown to react with the surface of formalin fixed sporozoites (Hall et al, 1992) indicating that it recognises these repeats in SPAG1.

When McAbs 4A7/A8, 4D3/E8 and 5D1/D11 were incubated with Western blots of GST-2.7 fusion protein, they all recognised the intact antigen and it's degradation products (Figure 39 C, D and E, track 11). Epitope mapping was carried out on Western blots of GST SPAG1 fusion proteins as described for the antisera. The reactivities of 1A7, 4A7, 4D3, 5D1 and anti-elastin McAb BA4 are shown in Figure 39, and the results are summarised in Table 15B. The anti-*T. annulata* merozoite McAb 5E1 (supplied by Dr. B. Shiels) which does not recognise the sporozoite stage, was included on the blots to act as a negative control. The weak bands observed in the BN and HB tracks in Figure 39C are likely to be due to leakage of the GST-0.8 fusion protein; a second blot confirmed this.

Because 4A7, 4D3 and 5D1 gave similar reactivities to BA4, it was possible that they recognised the elastin homologous repeats. Therefore they were screened for reactivity to α elastin using slot blots (Figure 40). Both 4A7 and BA4 gave a positive reaction, recognising quantities of α elastin as low as 0.1ng, while the tracks incubated with 5D1, 4D3 and second antibody only produced little detectable reaction. This indicated that only McAb 4A7 recognised an epitope within the elastin homologous repeats, the implications of which will be discussed later in this chapter.

5.2.3 Comparison with *T. parva* p67 sporozoite antigen

The result of the amino acid sequence comparison between *T. parva* p67 and *T. annulata* SPAG1 using GCG WordSearch is shown in Figure 41. Overall there is 47% identity and 64% similarity between the

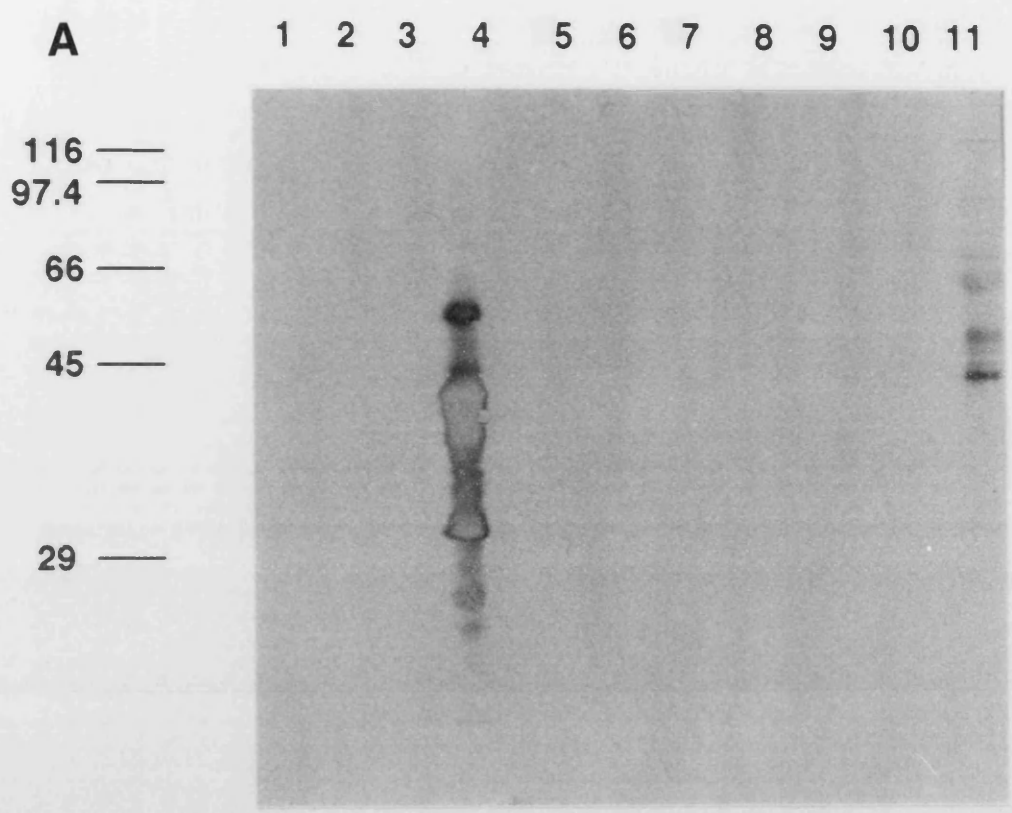
Figure 39 A-F (opposite and subsequent pages)

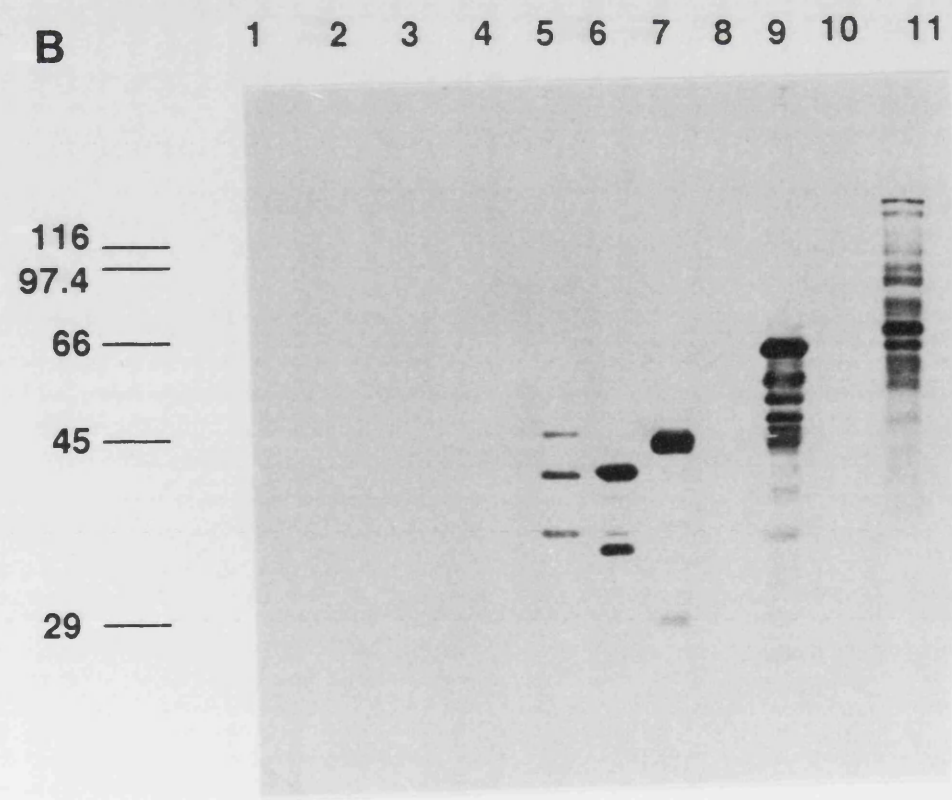
Western blots of 12% SDS-polyacrylamide gels each loaded with eleven SPAG1 constructs expressed and purified as GST fusion proteins and recombinant GST in an unpurified *E. coli* lysate, incubated with anti-sporozoite McAbs. 1μg protein was loaded per track. The positions of the molecular weight markers are shown in kDa. Tracks were loaded with the following recombinant proteins;

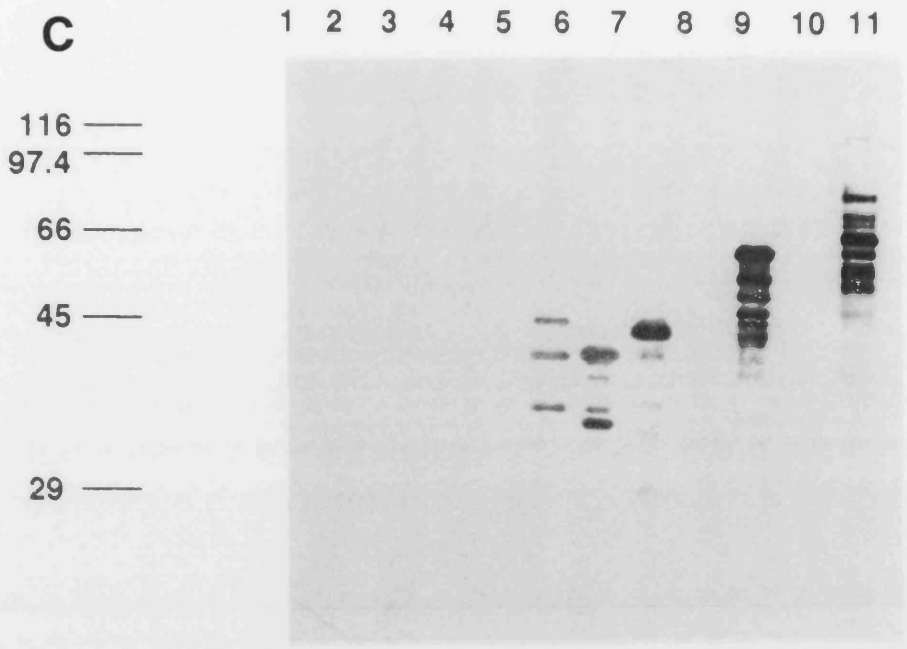
1 GST in an unpurified *E. coli* lysate
2 GST-SE
3 GST-S1
4 GST-0.3
5 GST-N6
6 GST-NE
7 GST-EA
8 GST-BN
9 GST-0.8
10 GST-HB
11 GST-2.7

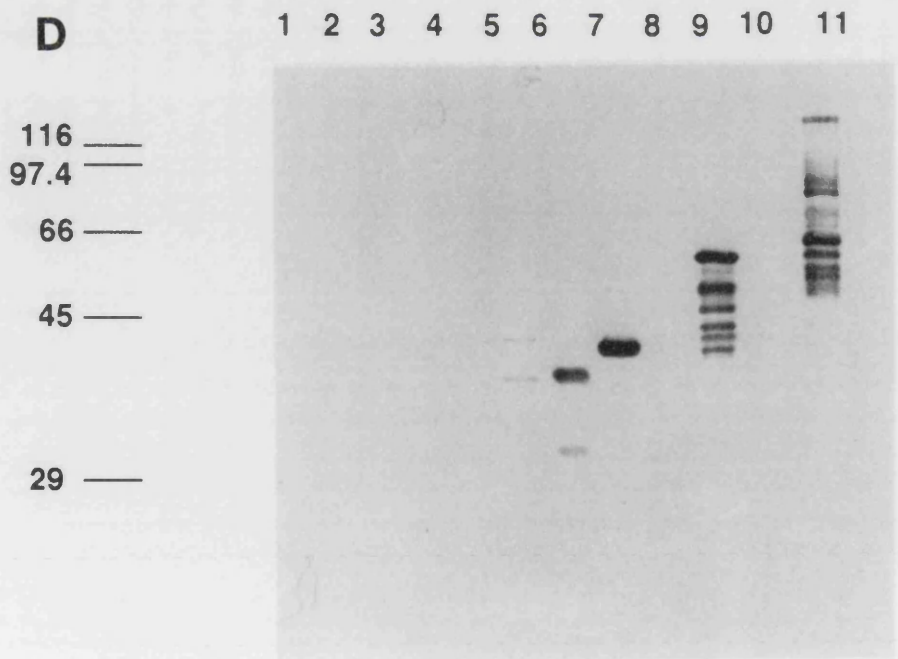
The second antibody used in each case was alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300). Blots were incubated with the following antibodies, with alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300) as a second antibody;

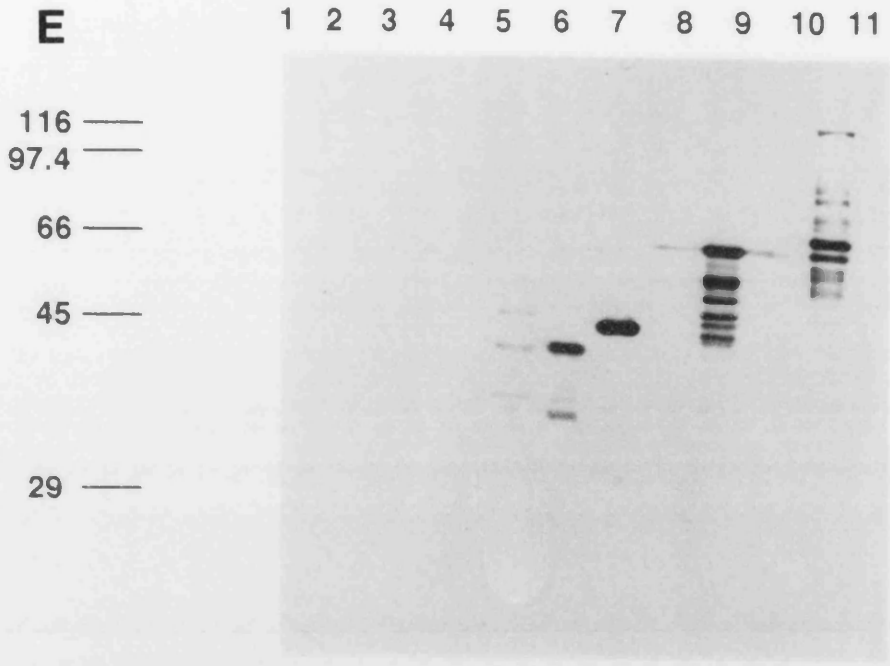
A 1A7 undiluted supernatant
B BA4 ascites 1:100
C 4A7 ascites 1:100
D 5D1 ascites 1:100
E 4D3 ascites 1:100
F 5E1 anti-merozoite control, undiluted supernatant


A
1 2 3 4 5 6 7 8 9 10 11
116
97.4
66
45
29


B
1 2 3 4 5 6 7 8 9 10 11
116
97.4
66
45
29


C
1 2 3 4 5 6 7 8 9 10 11
116
97.4
66
45
29


D
1 2 3 4 5 6 7 8 9 10 11
116
97.4
66
45
29


E
1 2 3 4 5 6 7 8 9 10 11
116
97.4
66
45
29

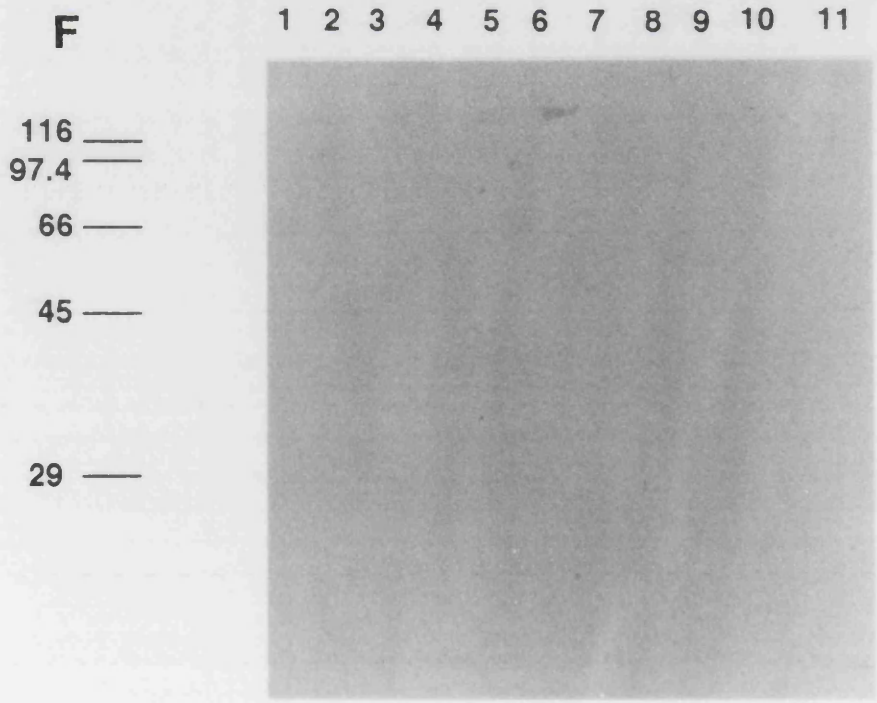

F
1 2 3 4 5 6 7 8 9 10 11
116
97.4
66
45
29

Figure 40

Slot blots of bovine α elastin incubated with the *T. annulata* sporozoite McAbs. The second antibody used in each case was alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300). The amount of protein (μg) loaded in each slot is indicated by arrows. Tracks were incubated with the following primary antibodies;

1 None (second antibody only)
2 McAb 4A7 ascites 1:100
3 McAb BA4 ascites 1:100
4 McAb 5D1 ascites 1:100
5 McAb 4D3 ascites 1:100
6 McAb 1A7 undiluted supernatant
7 None, stained in Ponceau S protein stain

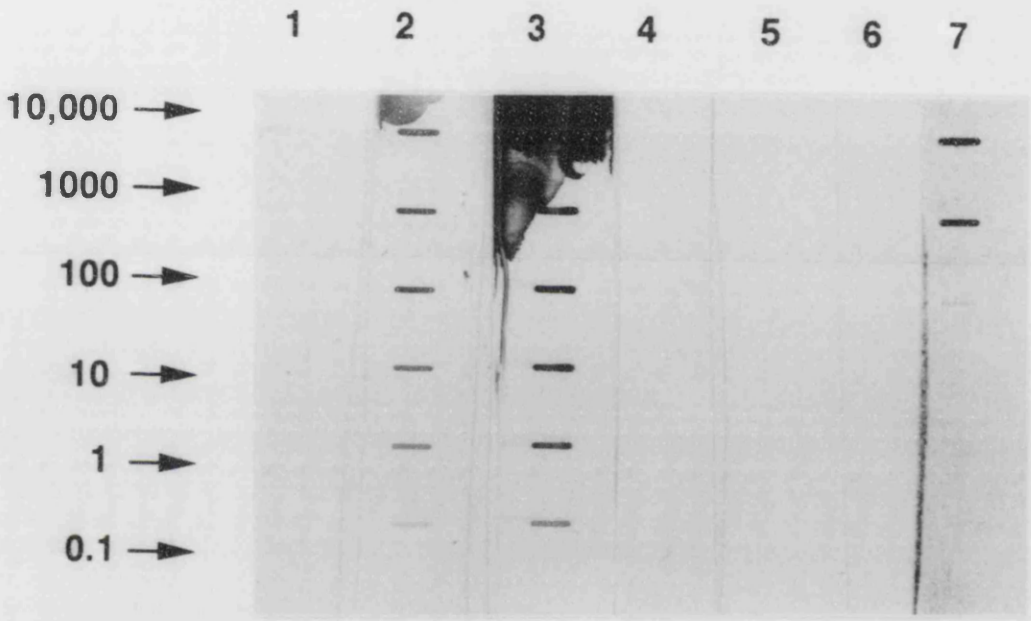

1
2
3
4
5
6
7
10,000
1000
100
10
1
0.1

Figure 41

Result of a sequence comparison between the predicted *T. annulata* SPAG1 and *T. parva* p67 amino acid sequences using the GCG computer package. The amino acids are shown in single letter code. The dashed vertical lines indicate identity between amino acids, while the dotted lines indicate similarity. There was 47% identity and 64% similarity between the two sequences.

sequences. The homologous regions appeared to be predominantly in the C-terminal sections of both sequences, but there is also a high similarity in the first 80 amino acid residues stretching from the N-termini. The elastin homologous repeats in SPAG1 are absent in p67, except for a single PGVGV sequence (residues 274-279). The regions of glutamic and aspartic acid (residues 114-128 and 346-358) and of alternating threonine and serine (residues 459-478), which are both features of the SPAG1 sequence, are completely absent in p67.

Bovine sera from animals 151, 155 and E99, *T. parva* sporozoite material from tick salivary gland extracts and NS1-p67 fusion protein in a lysate of *E. coli* were kindly donated for the work examining cross-reactivity between SPAG1 and p67 by Dr. T. Musoke, ILRAD, as part of a collaborative project. Cattle 151 and 155 had both been immunised with NS1-p67 partially purified from an *E. coli* lysate (Musoke et al 1992). Post-immunisation sera from these animals recognised native p67 in ELISA tests and on Western blots, and showed *T. parva* sporozoite neutralising activity "in vitro" (Dr. T. Musoke, personal communication). Animal E99 was immunised by infection with *T. parva* sporozoites then treatment with oxytetracycline; the antiserum recognised several *T. parva* antigens including p67 on Western blots (Toye et al 1991). All three animals were immune to challenge from a potentially lethal dose of *T. parva* sporozoites. Post-immune sera collected before challenge was provided from all three animals, in addition to pre-immune sera from 151 and 155 for use as controls. The reactivities of these sera were compared to that of post-immune sera from animals 23x, 758 and the anti- *T. annulata* sporozoite McAbs described earlier in this chapter, and from animals 38T and 45T, which were both immunised with freeze-thawed *T. annulata* sporozoites and were immune to subsequent challenge (kindly provided by C.G.D. Brown,CTVM). The day 0 and day 92 sera from 23x and 758 had been adsorbed with GST and *E. coli* proteins as described earlier in this chapter.

The reactivities of anti-SPAG1 bovine sera and McAbs with *T. parva* Muguga sporozoite lysate are shown in Figure 42. As expected, post-immune sera from cattle 155, 151 and E99 recognised bands of 110, 80 and 67kDa (Figure 42A, tracks B, C and H). Post-immune sera from the two cattle immunised with GST-2.7 (23x and 758) also

Figure 42A

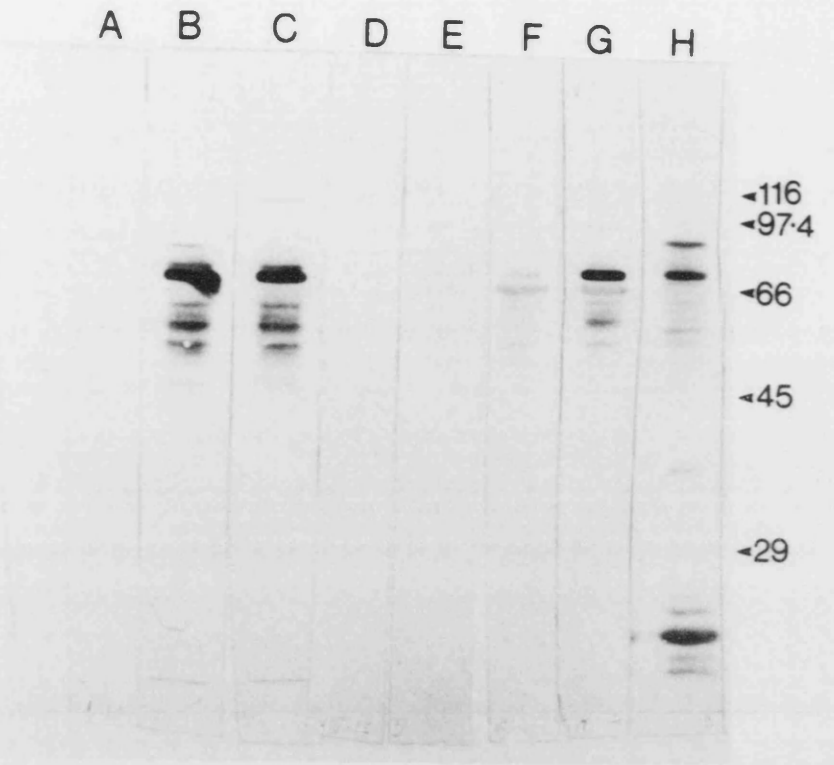
Western blot of a 10% SDS-polyacrylamide gel of *T. parva* sporozoite lysate (approximately one tick equivalent per track) incubated with antisera raised against *T. annulata* recombinant GST-2.7, *T. parva* recombinant NS1-p67 or *T. parva* sporozoites. The positions of the molecular weight markers are shown in kDa. Alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300) was used as the second antibody. Tracks were incubated with the following antisera at a dilution of 1:100;

A 151 pre-immune serum
B 151 antiserum raised against recombinant NS1-P67
C 155 " " " " "
D 155 pre-immune serum
E pooled day 0 serum from 23x and 758
F 758 day 92 antiserum against recombinant GST-2.7
G 23x " " " " "
H E99 antiserum raised against freeze-thawed *T. parva* sporozoites

Figure 42B

Western blot of *T. parva* sporozoite lysate as described above, incubated with anti-sporozoite McAbs and alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300) as a second antibody. Tracks were incubated with the following McAbs (specificity in brackets);

I None, second antibody only
J 1A7 undiluted supernatant (*T. annulata* sporozoites)
K 4B11 undiluted supernatant " "
L BA4 ascites 1:100 (bovine elastin)
M 4A7 ascites 1:100 (*T. annulata* sporozoites)
N 4D3 " " " "
O 5D1 " " " "
P 23F ascites 1:25 (*T. parva* sporozoites)


A
B
C
D
E
F
G
H
116
97·4
66
45
29

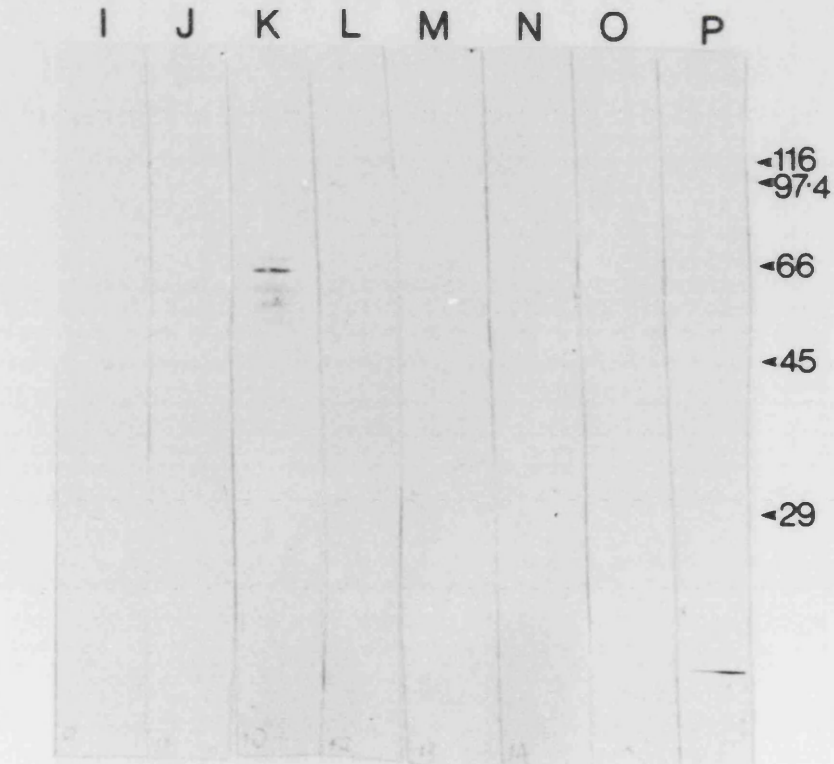
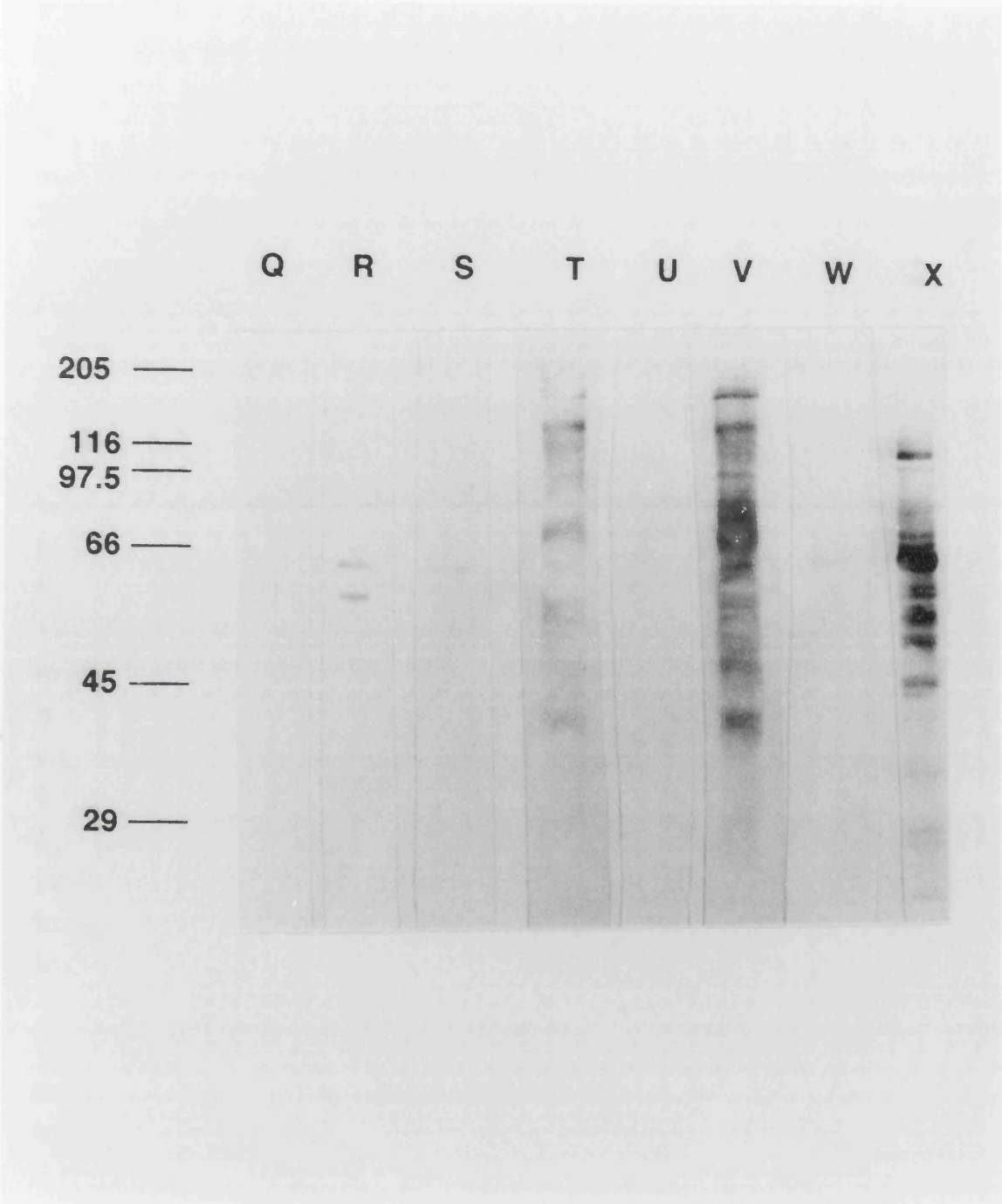

I
J
K
L
M
N
O
P
116
97·4
66
45
29

Figure 42C

Western blot of a 10% SDS-polyacrylamide gel of *T. parva* sporozoite lysate incubated with antisera raised against *T. annulata* sporozoites, and appropriate control antisera. The positions of the molecular weight markers are shown in kDa. The second antibodies used were alkaline-phosphatase conjugated rabbit anti-mouse IgG (tracks Q, R) or alkaline-phosphatase conjugated rabbit anti-bovine IgG (tracks S-X) diluted 1:300. Tracks were incubated with the following as primary antibodies;

Q	McAb 1A7 undiluted supernatant
R	McAb 4B11 " "
S	Day 0 serum from 38T
T	Day 63 antiserum from 38T, against *T. annulata* sporozoites
U	Day 0 serum from 45T
V	Day 63 antiserum from 45T, " " "
W	Day 0 serum from 155
X	Post-immune antiserum from 155


Q
R
S
T
U
V
W
X
205
116
97.5
66
45
29

recognised the 67kDa band (tracks F and G), although the reaction produced by 758 was noticably much weaker than 23x. Sera from cattle 38T and 45T, which were immunised with freeze-thawed *T. annulata* sporozoite material and known to be reactive to SPAG1 antigens (S. Williamson, personal communication), showed very weak reactivity to the 67kDa antigen (Figure 42C, tracks T and V). However, they appeared to show quite strong reactivity to a number of other *T. parva* antigens, of molecular weights approximately 190, 150, 90, 80 and a group of smaller bands of 40-60kDa. 45T gave a noticably stronger reaction than 38T. Pre-immune sera from the animals failed to recognise any bands. There were no strong reactions from any of the anti-SPAG1 McAbs to *T. parva* sporozoite material (Figure 42B). However, McAb 4B11 gave a weak reaction with bands of 65-75kDa (track K). A repeat of the blot confirmed this result (Figure 46C track R).

A Western blot of *T. annulata* sporozoites was developed with post-immune sera from animals E99, 155, 45T and 758 (Figure 43). The McAb 1A7 was also included to highlight the SPAG1 antigens. 155 post-immune sera (track C) produced a pattern of reactivity similar to McAb 1A7, or sera 45T and 758 (tracks B, E and G), although the bands were much fainter. The reactions were predominantly to groups of bands of molecular weights around 104, 85, 70, 63, and 54kDa, plus a number of smaller bands. The sera E99 only appeared to recognise bands of about 75 and 85kDa (track A); the band near the lower edge of the blot is at the dye-front. Only 45T serum reacted with uninfected tick material, probably present in the sporozoite preparation with which it was immunised. Little or no reaction was obtained from 155, 45T or 758 pre-immune sera.

The reactivities of 155 and E99 *T. parva* sporozoite antisera to *T. annulata* antigens were also investigated by carrying out IFA titres against formalin fixed sporozoites, as described in section 2.2.6, using 23x day 92 serum as a positive control (Figure 44). The results are summarised in Table 16. Both 155 and E99 post-immune sera reacted to a titre of 1:100 and 1:1000 respectively. A titre of 1:1000 was also recorded using 23x day 92 sera. Titres of 23x and 155 pre-immune sera were 1:25 and 1:10 respectively.

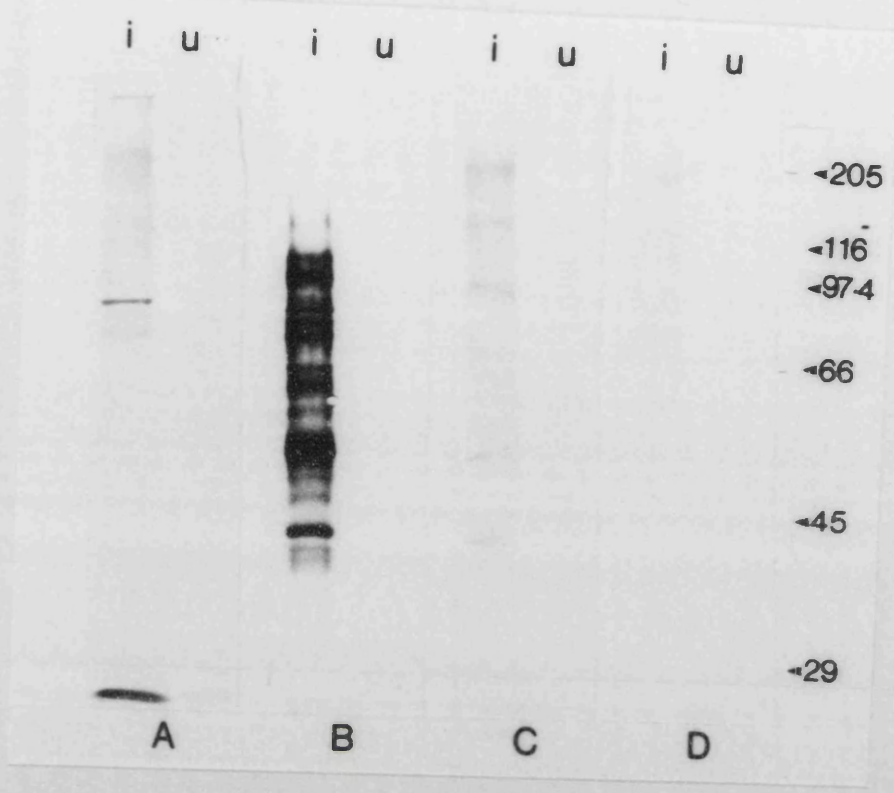
Figure 43

Western blots of 10% SDS-polyacrylamide gels loaded with one te /track *T. annulata* Gharb sporozoite material (tick salivary gland extract) incubated with *T. annulata* and *T. parva* antisera against sporozoites and recombinant antigens. The positions of the molecular weight markers are shown in kDa. The second antibodies were alkaline-phosphatase conjugated rabbit anti-mouse IgG (track B) alkaline-phosphatase conjugated rabbit anti-bovine IgG (all remaining tracks). Tracks were loaded with;

i salivary gland extract from *T. annulata* sporozoite infected ticks
u GUTS from uninfected ticks

Tracks were incubated with the following as primary antibodies (specificities in brackets);

A E99 antiserum 1:100 (*T. parva* sporozoites)
B McAb 1A7 undiluted supernatant (*T. annulata* sporozoites)
C 155 post-immune antiserum 1:100 (*T. parva* recombinant NS1-p67)
D 155 pre-immune serum 1:100
E 45T day 63 antiserum 1:100 (*T. annulata* sporozoites)
F 45T day 0 serum 1:100
G 758 day 92 antiserum 1:100 (*T. annulata* recombinant GST-2.7)
H 758 day 0 serum (1:100)


i u i u i u i u
205
116
97·4
66
45
29
A B C D

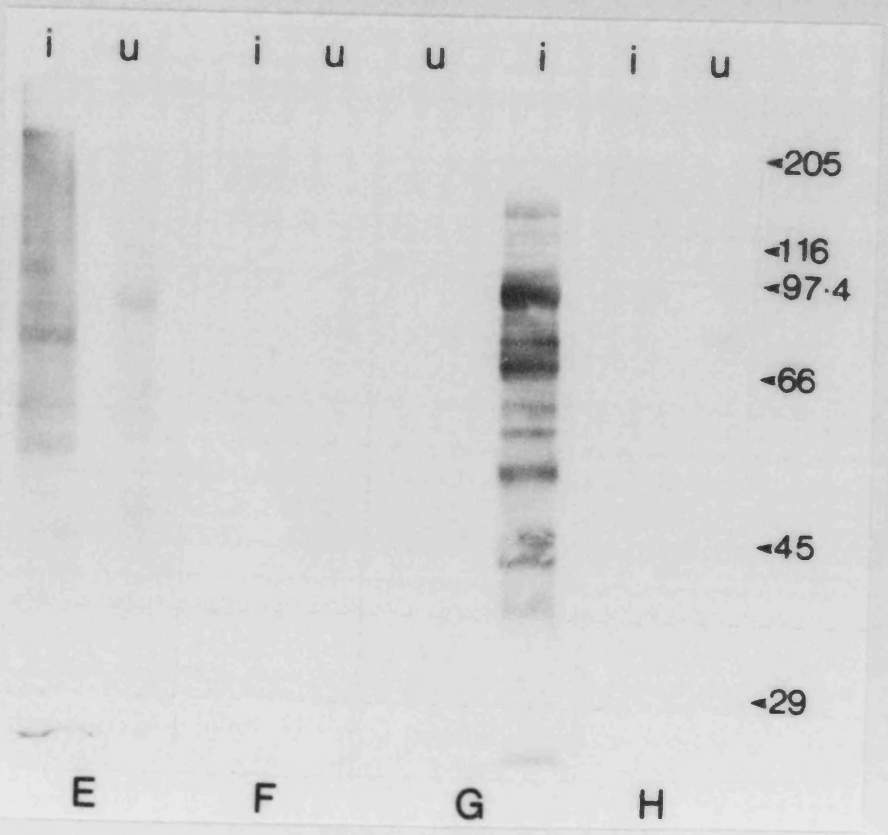
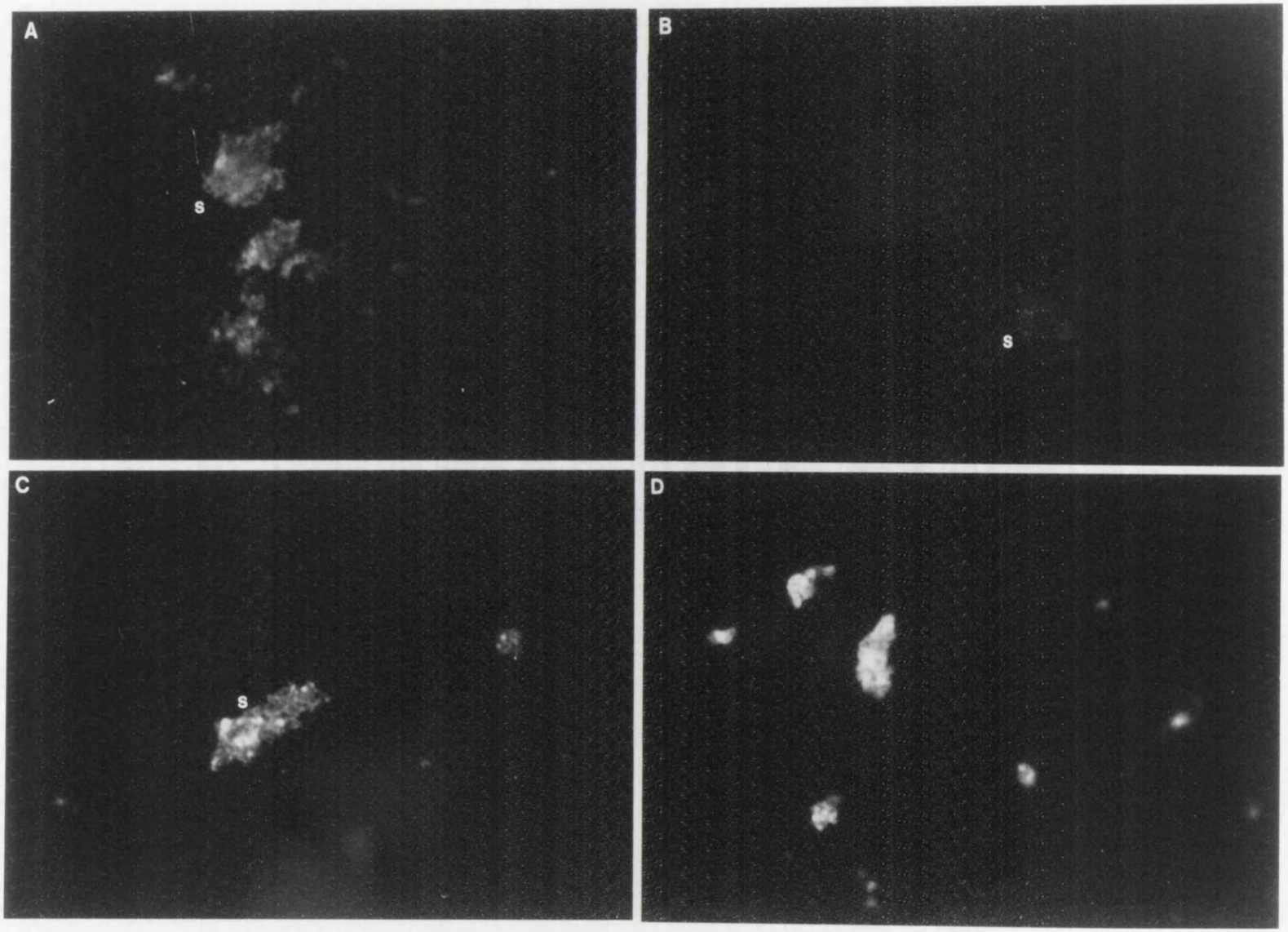

i u i u u i i u
205
116
97·4
66
45
29
E F G H

Figure 44

Results from IFA tests against *T. annulata* Gharb formalin fixed sporozoites using antisera against *T. parva* sporozoites or recombinant NS1-p67 antigen. The secondary antibody was FITC conjugated rabbit anti-bovine IgG (1:32). The material shown was incubated in the following antisera at a dilution of 1:25;

A 155 antisera to recombinant *T. parva* NS1-p67
B 155 pre-immune serum
C E99 antisera to *T. parva* sporozoites
D 23x day 92 antiserum to recombinant *T. annulata* GST-2.7

s = sporozoites


A
S
B
S
C
S
D

The reactivities of *T. annulata* and *T. parva* bovine antisera and McAbs with Western blots of purified GST-2.7 fusion protein (molecular weight 145kDa) and a lysate of *E. coli* cells expressing p67-NS1 fusion protein (molecular weight 110kDa) are shown in Figures 45 A and B. Tracks containing purified GST and a lysate of untransformed *E. coli* cells were included as controls. As expected, the 110kDa band was recognised by post-immune sera from animals 151 and 155 (45A tracks 1 and 2), which were immunised with the p67 fusion protein, and by sera from E99 (45A track 4), which had been immunised with *T. parva* sporozoites. There were also reactions to *E. coli* antigens from these sera, but the sizes of these bands do not correspond to the 110kDa antigen. Day 92 sera from 23x immunised with GST-2.7 also recognised the 110kDa band, but not *E. coli* proteins (track 5). While the 110kDa band was recognised by 38T anti- *T. annulata* sporozoite post-immune serum, the pre-immune serum also reacted (45B tracks 9 and 10). McAbs 1A7 and 4B11 both gave reactions to the 110kDa fusion protein (tracks 8 and 12), but not the control McAb 5E1 (track 7). Both the SPAG1 antisera and the McAbs failed to recognise any antigens in the *E. coli* track.

Post-immune sera from 23x recognised GST-2.7 fusion protein as expected, but not GST alone (Figure 45A, tracks 5). E99, 155 and 151 post-immune sera showed weak reactivity to the 145kDa GST-2.7 and it's degradation products, but not to GST (Figure 45A, tracks 1,2 and 4). The reaction of 151 serum was noticably weaker than that of 155. There was no significant reaction from any of the pre-immune sera tested.

Since the post-immune sera from E99 and 155, raised against *T. parva* sporozoites and p67 fusion protein respectively, clearly reacted with SPAG1, it was decided to use these sera to map the regions of SPAG1 containing the cross reacting epitopes using Western blotting as described previously in this chapter. The sera was incubated with duplicate Western blots of nine GST-2.7 fusion proteins, using day 92 serum from 758 as a positive control (Figure 46, A-E). The SPAG1 fragments recognised are summarised in Table 17. The patterns of reactivity produced by the post-immune sera from animals 155 and E99 were identical to that of 758 GST-2.7 antiserum, apart from the reaction with the C-terminal fusion protein GST-SE, which was not detected by 758 antisera. Pre-immune 155 serum failed to react with any of the

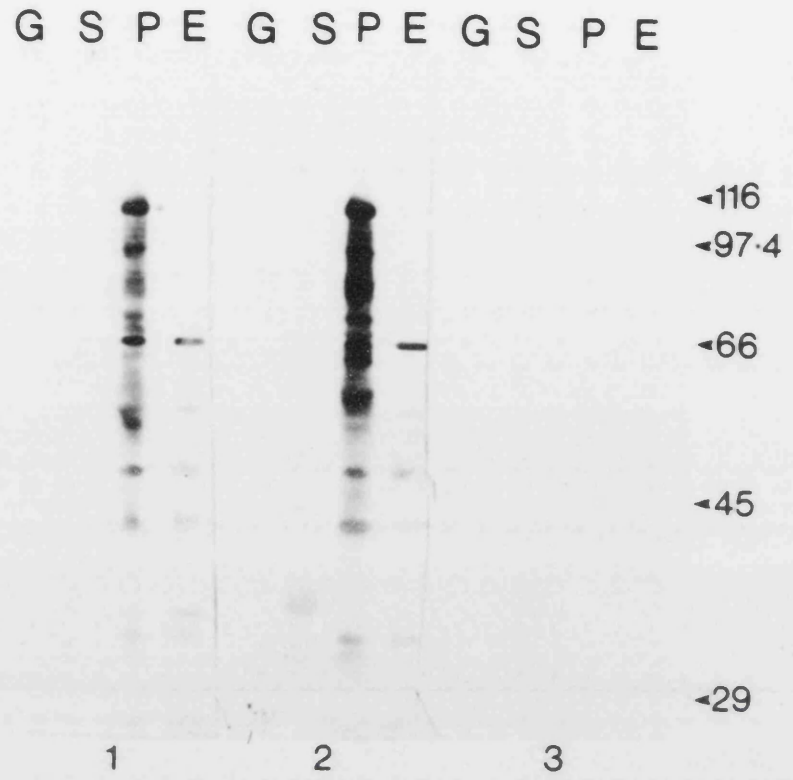
Figure 45A

Western blots of 12% SDS-polyacrylamide gels loaded with 1μg per track recombinant GST, GST-2.7 and NS1-p67 fusion proteins and normal *E. coli* proteins incubated with antisera against *T. annulata* and *T. parva* sporozoites and recombinant antigen. The positions of the molecular weight markers (kDa) are indicated by arrows. Tracks contained the following proteins;

G purified recombinant GST
S " " GST-2.7
P recombinant NS1-p67 in an *E. coli* lysate
E lysate from untransformed *E. coli*

The blots were incubated with the following bovine antisera (specificitie in brackets), with alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300) as a second antibody;

1 155 post-immune antiserum 1:100 (NS1-p67)
2 151 " " " " "
3 151/155 pooled pre-immune serum 1:100
4 E99 antiserum 1:100 (*T. parva* sporozoites)
5 23x day 92 antiserum (GST-2.7)
6 23x day 0 serum


G S P E G S P E G S P E
116
97·4
66
45
29
1
2
3

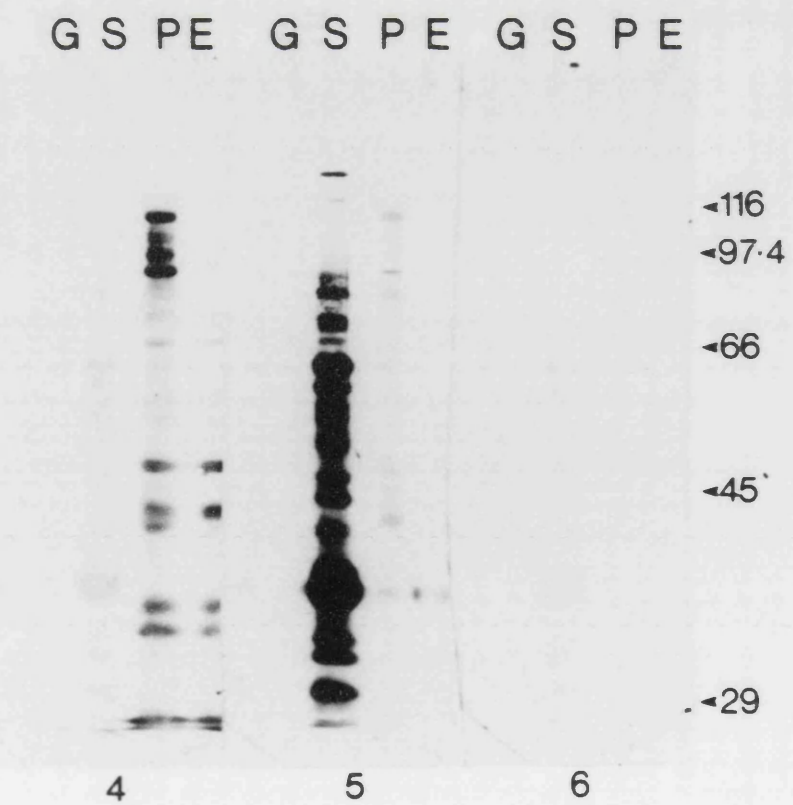

G S PE G S P E G S P E
116
97·4
66
45
29
4
5
6

Figure 45B

See legend for Figure 45A. Tracks were loaded with the following proteins (1μg per track);

67 recombinant NS1-p67 in an *E. coli* lysate
E lysate from untransformed *E. coli*

The blots were incubated with the following bovine antisera (specificities in brackets), with alkaline-phosphatase conjugated rabbit anti-mouse IgG (tracks 7, 8 and 12) or alkaline-phosphatase conjugated rabbit anti-bovine IgG (tracks 9-11) 1:300 as a second antibody;

7 McAb 5E1 undiluted supernatant (*T. annulata* merozoites)
8 McAb 4B11 " " (*T. annulata* sporozoites)
9 38T day 0 serum 1:100
10 38T day 63 antiserum 1:100 (*T. annulata* sporozoites)
11 E99 antiserum 1:100 (*T. parva* sporozoites)
12 McAb 4B11 undiluted supernatant (*T. annulata* sporozoites)

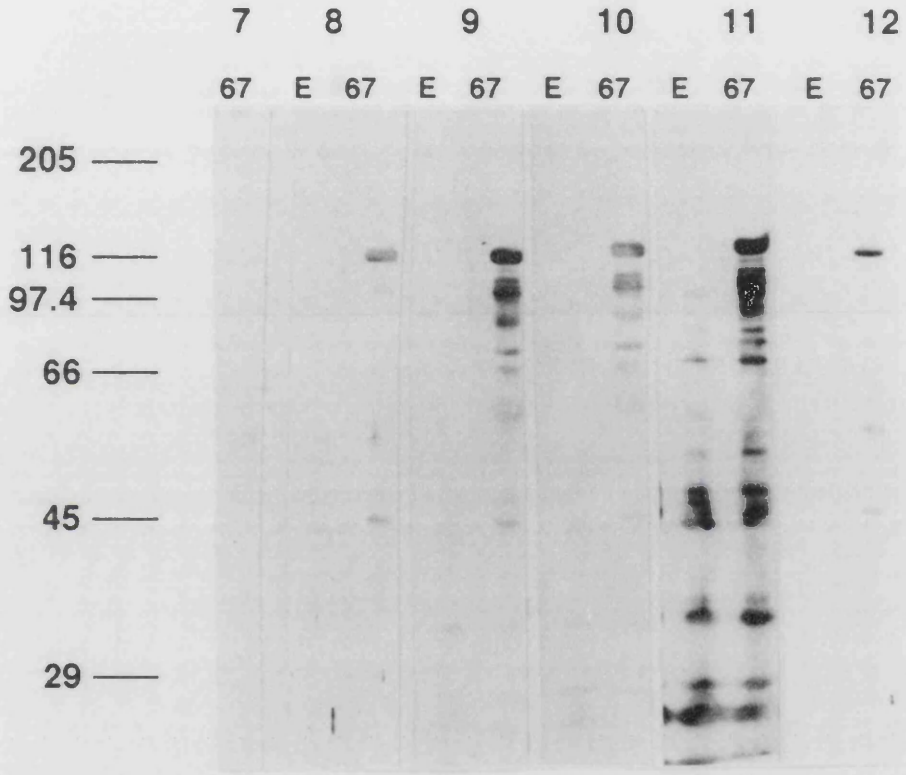

7
8
9
10
11
12
67
E
67
E
67
E
67
E
67
E
67
205
116
97.4
66
45
29

Figure 46A

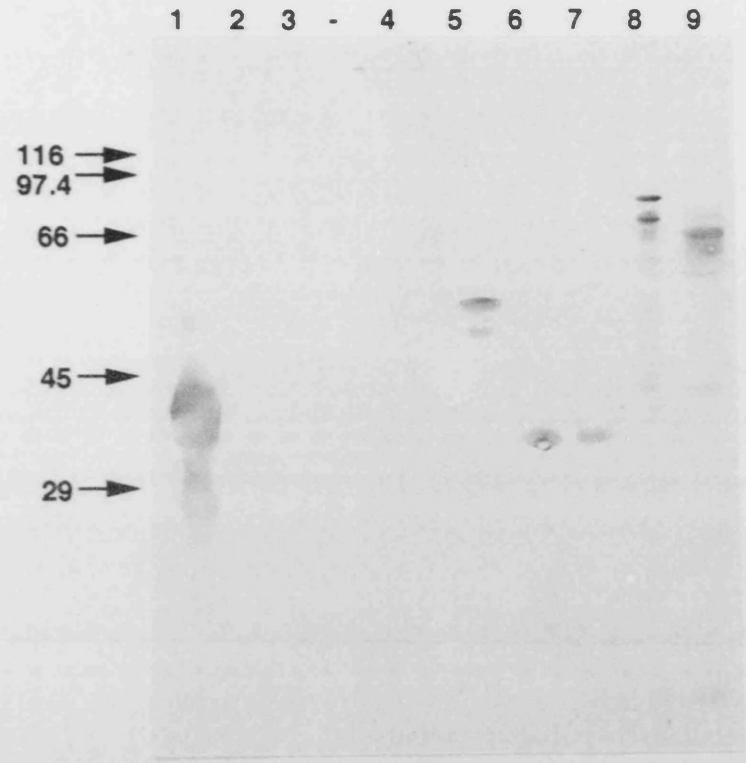
Western blot of a 12% SDS-polyacrylamide gel loaded with eight SPAG1 subclones expressed and purified as GST fusion proteins, NS1-p67 recombinant protein and unpurified *E. coli* lysate, incubated with *T. parva* antisera. 1μg protein was loaded per track. The positions of the molecular weight markers are shown in kDa. Tracks were loaded with the following proteins;

1 GST-0.3 purified fusion protein
2 GST-SE " "
3 GST-NE " "
4 GST-N6 " "
5 GST-0.8 " "
6 GST-HB " "
7 GST-2.7 " "
8 NS1-p67 in *E. coli* lysate
9 untransformed *E. coli* lysate

The blot was incubated with day 92 GST-2.7 antiserum from animal 758, with alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300) as a second antibody.

Figure 46B

See legend for Figure 46A. The first antibody was 155 *T. parva* NS1-p67 immune serum (1:100).


1 2 3 - 4 5 6 7 8 9
116
97.4
66
45
29

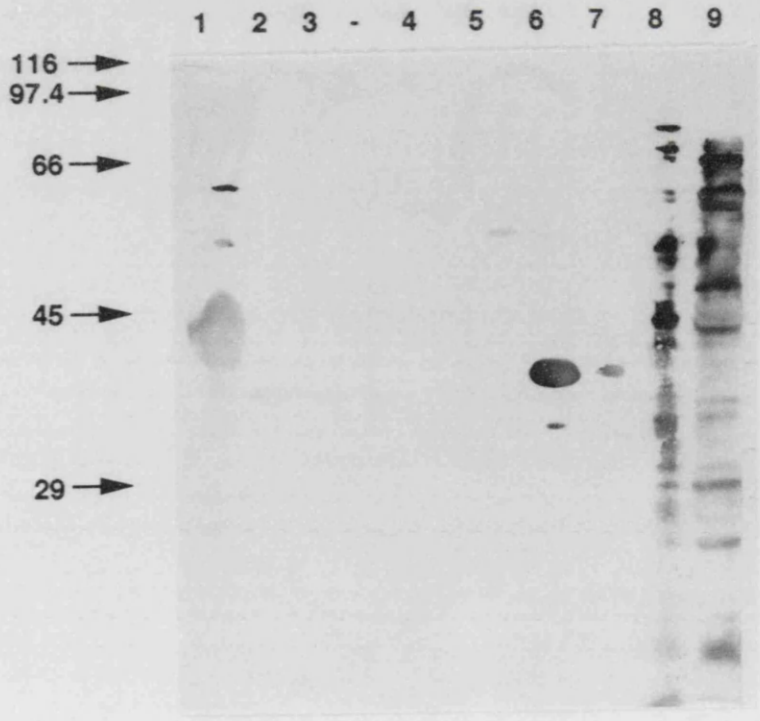
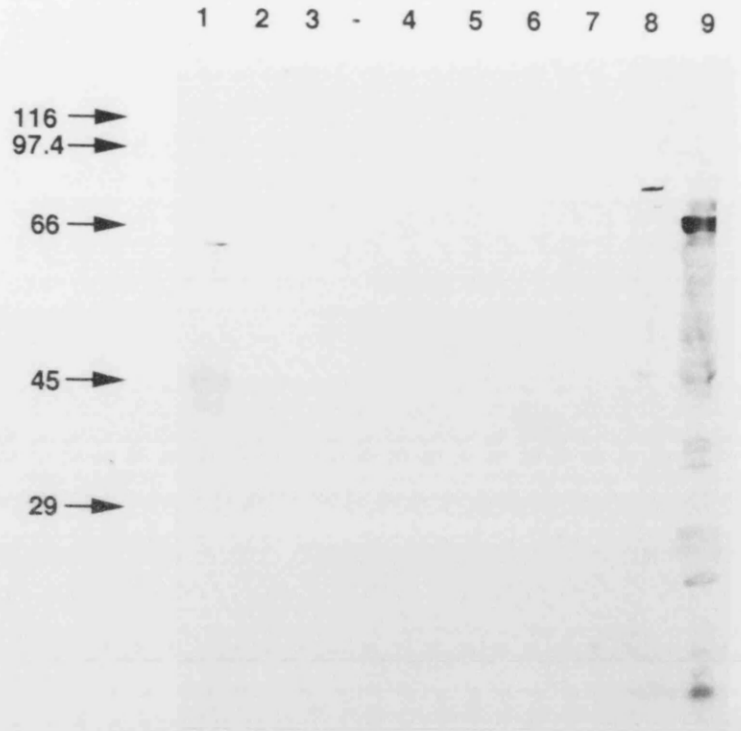

1 2 3 - 4 5 6 7 8 9
116
97.4
66
45
29

Figure 46C

See legend for Figure 46A. The first antibody was E99 *T. parva* sporozoite immune serum (1:100).

Figure 46D

See legend for Figure 46A. The first antibody was day 0 serum from cow 155 (1:100).


1 2 3 - 4 5 6 7 8 9
116
97.4
66
45
29

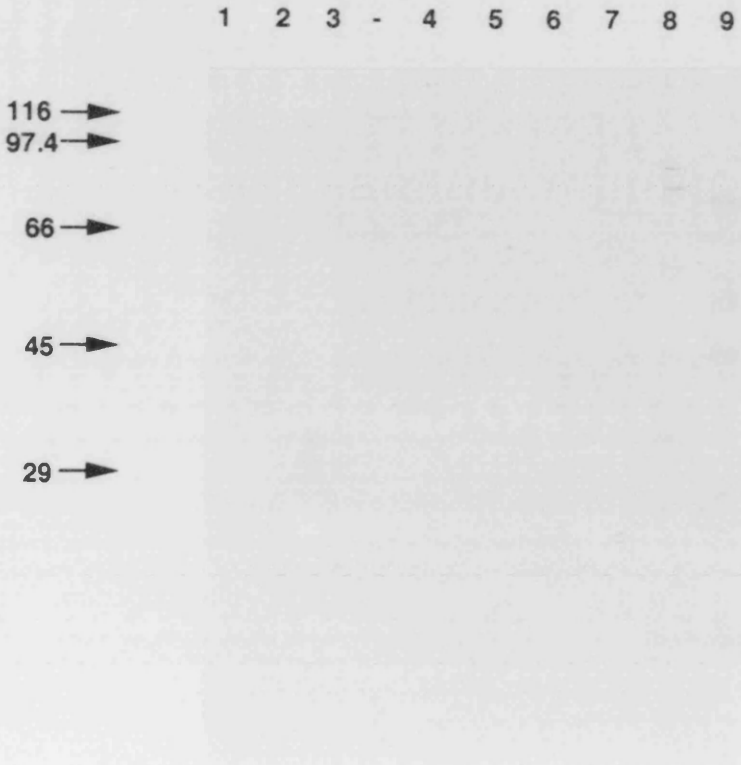

1 2 3 - 4 5 6 7 8 9
116
97.4
66
45
29

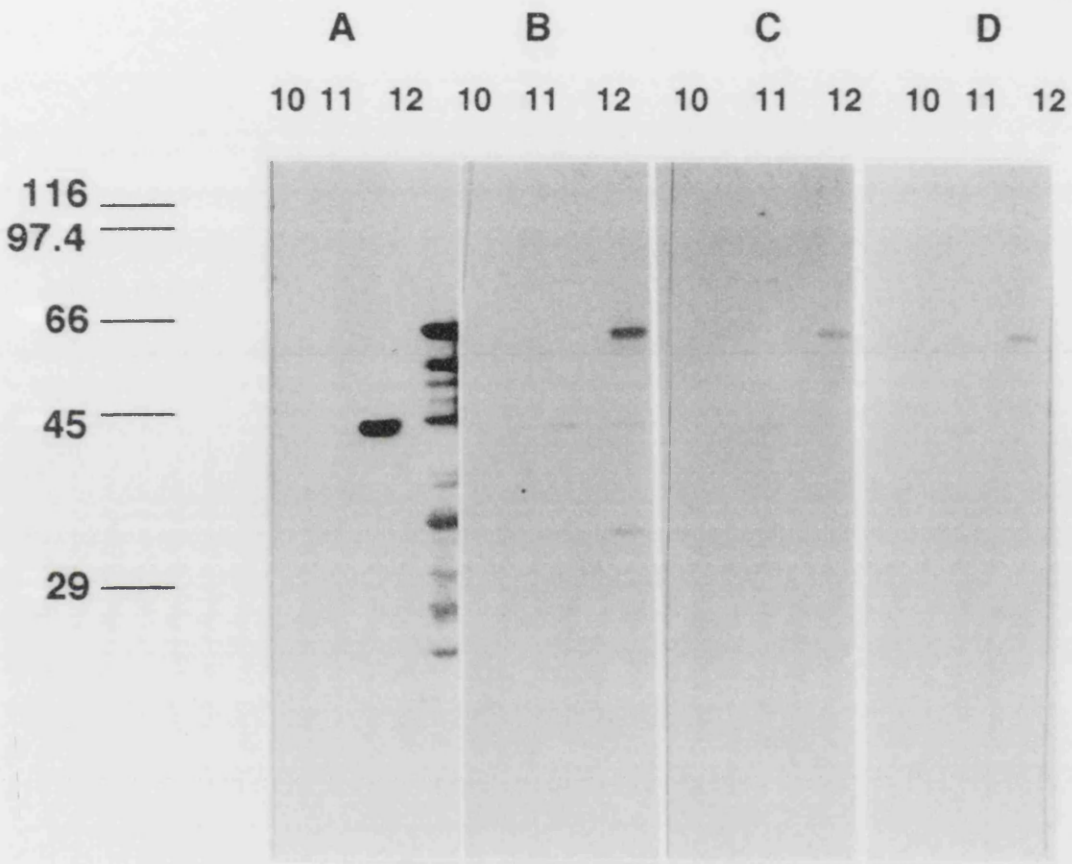
Figure 46E

Western blot of a 12% SDS-polyacrylamide gel loaded with the SPAG1 subclones BN, EA and 0.8, expressed and purified as GST fusion proteins, incubated with *T. parva* antisera. 1μg protein was loaded per track. The positions of the molecular weight markers are shown in kDa. Tracks were loaded with the following proteins;

10 GST-BN
11 GST-EA
12 GST-0.8

The primary antibodies (1:100) were as follows, with alkaline-phosphatase conjugated rabbit anti-bovine IgG as a second antibody;

A day 92 GST-2.7 immune serum from cow 758
B NS1-p67 immune serum from cow 155
C *T. parva* sporozoite immune serum from cow E99
D day 0 serum from cow 155


A
B
C
D
10 11 12 10 11 12 10 11 12 10 11 12
116
97.4
66
45
29

fusion proteins. The implications of these patterns of reactivity will be discussed in the next section.

5.3 DISCUSSION

Sera from all five of the cattle immunised with recombinant GST-2.7 recognised the fusion protein in ELISA tests (Table 10) and on Western blots (Figure 37). A positive ELISA reading was obtained using serum collected after each boost, compared with readings around zero for the pre-immune sera. The increase in absorbance at 490nm between sera collected after the first and second immunisations indicates an increase in antibody production on second exposure to the antigen. This rise was noticable in all the cattle except 26x. There was no noticable increase in the ELISA readings produced by subsequent immunisations.

All the cattle antisera reacted to the surface of formalin fixed *T. annulata* sporozoites in IFA tests (Table 9). The antisera also neutralised the ability of viable sporozoites to infect bovine lymphocytes "in vitro" (Table 11), indicating they were reacting to native SPAG1 on the sporozoite surface. This blocking activity was 100% when the serum was used either undiluted or at 1/4. Post-immune sera from four of the animals tested reacted with sporozoite extract on Western blots (Figure 33). The molecular weights of the sporozoite antigens recognised corresponded to those detected by McAb 1A7, indicating they were reacting to the native SPAG1 antigens. Interestingly, 26x did not appear to recognise the two lower bands of 54 and 63kDa detected by McAb 1A7 and the other cattle.

There was noticable variation between the cattle both in the intensity of their humoral responses and in the degree of parasitaemia suffered by them after challenge. Although good humoral responses were recorded in the animals, on the Western blots of SPAG1 fusion proteins (shown for 32x and 759 only in Figure 37) and in the ELISA test against GST-2.7 (Table 9), 26x antiserum showed noticably weaker reactivity to GST-2.7 than antiserum from the other four cattle. 26x antiserum also gave poorer recognition of sporozoite derived SPAG1 on Western blots (Figure 35), and 26x and 32x antisera, the two cows which received saponin, produced slightly lower IFA titres against acetone fixed

sporozoites (Table 9) than sera from 23x and 758. The different levels of antibody recorded for each of these animals did not seem to be associated with the differences in the levels of parasitaemia observed, with 23x and 32x suffering the highest percentage of piroplasms (Figure 33). These different levels of parasitaemia also did not correlate with the adjuvants received by each cow; 23x, 758 and 759 had Freund's complete adjuvant in the initial immunisation with Freund's incomplete adjuvant for the boosts, while 26x and 32x had saponin. Both adjuvants appeared to be highly effective in potentiating anti GST-2.7 immune responses, although the results were variable between the animals used.

Despite the strong anti-sporozoite humoral responses induced in all of these animals, none were protected against homologous challenge with *T. annulata* Hissar sporozoites. However, there was some apparant overall effect on the levels of parasitaemia (Figure 34). Although piroplasm infected erythrocytes were first detected on day 12 in both the immunised and control groups, the mean percentage of infected cells in the immunised animals was considerably lower and remained so for 3-5 days subsequently. Nevertheless, the high level of variation in each group is typical of a small sample size, and probably precludes too much significance being attached to these differences.

The most likely effect that was expected from immunisation with recombinant SPAG1 was a delay in the onset of parasitaemia, due to a reduction in the challenge dose. This was found to be the case when cattle were immunised with sporozoites inactivated by irradiation or freeze-thawing (Williamson 1993). It was found that the onset of macroshizont infection in these cattle was significantly delayed when compared with control animals on challenge. This effect was considered to be due to immune responses evoked to the sporozoite stage only, since no schizont infection could be identified in the animals following immunisation. Furthermore, we might expect recombinant SPAG1 to induce some protection since six out of nine cattle immunised with the homologous antigen p67 as a recombinant were resistant to challenge with *T. parva* (Musoke et al 1992). In this vaccine trial, the antibody titres in the immunised cattle, as assessed by ELISA tests and sporozoite neutralisation assays, also varied considerably between the animals and did correlate with their disease reactions.

There are numerous possible reasons for the recombinant SPAG1 to fail in protecting cattle fully against challenge. One plausible explanation lies in the polymorphism exhibited by SPAG1, described in Chapter 3, which needs to be taken into account when assessing it as a candidate antigen for a molecular vaccine. Comparison between the cSPAG1 and gSPAG1 amino acid sequences showed that polymorphic regions existed at the protein level as well as in the genomic sequence. The Hissar stock was an appropriate isolate to choose for challenging the cattle since the SPAG1 insert was derived from a *T. annulata* Hissar cDNA library (Hall et al 1992). However, the Hissar challenge stock could have contained as many as five allelic forms of the gene according to restriction fragment analysis of DNA from uncloned stocks (Chapter 3, Table 4). Since GST-2.7 represented only one SPAG1 variant, the recombinant antigen may not have elicited immune responses sufficient to block sporozoites "in vivo" carrying other SPAG1 variants. The nature of the disease means that very few sporozoites need to survive the host's immune responses in order to initiate the next stages of development, due to the high level of asexual reproduction that occurs in macroschizonts.

Another possibility is that the expression system was inappropriate for production of SPAG1 with all the correct epitopes. If there were some differences between folding of SPAG1 on sporozoites and in the recombinant antigen, this could have meant that important discontinuous (conformational) epitopes were not recognised. Possible interference in the immune response by the GST part of the fusion protein, such as the induction of T supressor cells, will be discussed in Chapter 6. The importance of trying out different vector systems for a given antigen was highlighted in a review of cestode vaccines by M.D. Rikard (1989). While nothing is known about the glycosylation of SPAG1 at present, the amino acid sequence does contain five potential glycosylation sites (Hall et al 1992, Figure 77, Appendix). Therefore, it may have been inappropriate to use *E. coli* as an expression system since carbohydrate moities would not have been added to the expressed product, as discussed in section 1.2.5. As well as being important in maintaining protein tertiary structure, glycosylated sequences can also form some epitopes of an antigen. An example of this in *T. annulata* is the

anti-merozoite antibody 5E1 (Dickson and Shiels 1993) which recognises carbohydrate moities on a 30kDa merozoite surface antigen recognised by *T. annulata* immune cattle. It may be necessary, therefore, to use a vector system based on mammalian cells to produce SPAG1 in a form akin to it's native structure, such as the vaccinia virus system described in section 1.2.5.

The possibility that the dose of antigen was just not sufficient to induce effective immunity cannot be discounted. In the immunisation trial with *T. parva* recombinant p67, the animals were immunised with 5mg of antigen in total during five inoculations (Musoke et al 1992), while the GST-2.7 immunised animals received no more than 1mg over four inoculations. Both groups of cattle were challenged by subcutaneous injection with a lethal dose of sporozoites. The adjuvants used for GST-2.7 immunisation both appeared to be adequate in potentiating anti-SPAG1 humoral responses; saponin was also found to be successful in inducing anti-p67 responses as described in the aforementioned vaccine trial. Overall, there are a considerable number of variables that should be taken into account before rejecting SPAG1 as a possible vaccine candidate.

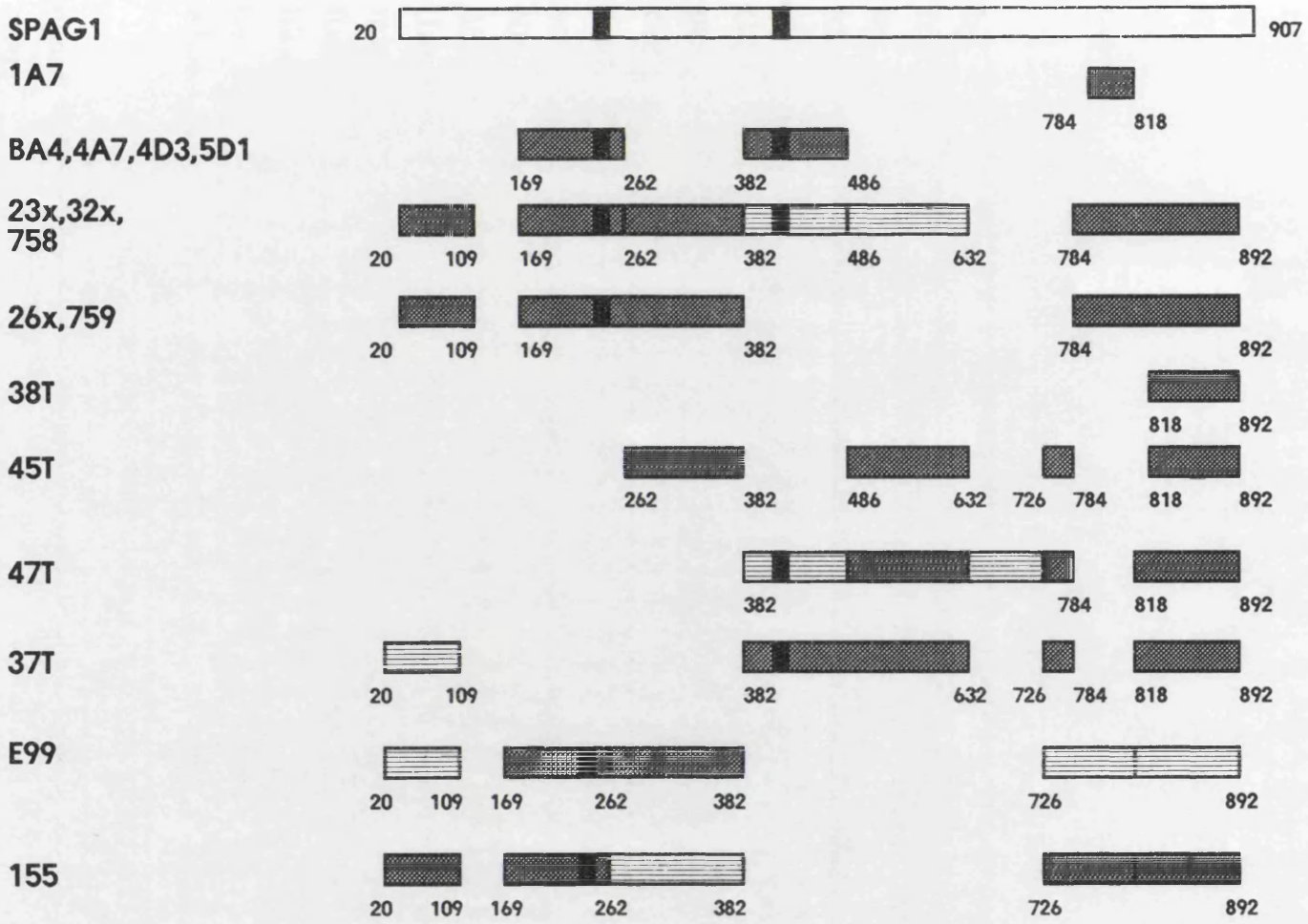
Analysis of humoral activity by Western blotting is limited in that only continuous (linear) determinants can be detected, not dicontinuous (conformational) sites which are brought together by protein folding, since SDS binding to charged residues disrupts all secondary and tertiary structure. The reactions of the bovine sera and the majority of mouse McAbs to sporozoite derived and recombinant SPAG1 on Western blots indicated that continuous determinants were being detected. Therefore it was considered appropriate to use the detection of recombinant polypeptides on Western blots as a means of locating the SPAG1 epitopes recognised. The lack of reactivity of McAb 4G5 with sporozoite antigen on a Western blot, despite it's (weak) reaction with formalin fixed sporozoites (Dr. S. Williamson, personal communication), could indicate that this McAb was reacting with a conformational epitope.

The majority of examples in the literature concerning B epitope mapping identify continuous epitopes through the use of techniques

Figure 47

Summary of regions of SPAG1 containing B cell epitiopes ascertained from Western blots of recombinant SPAG1 subclones. B cell epitopes are indicated which were recognised by the following antibodies;

1A7, 4A7, 4D3, 5D1	*T. annulata* sporozoite McAbs
BA4	Bovine elastin McAb
23x, 32x, 758, 26x, 759	Bovine GST-2.7 immune serum (grouped according to their reactivities)
38T, 45T, 47T, 37T	Bovine *T. annulata* sporozoite serum
E99	Bovine *T. parva* sporozoite immune serum
155	Bovine NS1-p67 immune serum



EPITOPE MAP SUMMARY

ND = NOT DONE = EPITOPE = WEAK EPITOPE = ELASTIN HOMOLOGOUS REPEATS

which disrupt folding such as Western blotting (López de Turiso et al 1991) or ELISA tests using small peptides which would have little or no intrinsic folding ability (Geysen et al 1984). However, recent evidence suggests that many B cell epitopes may be discontinuous, and that reactions to peptides may only represent binding to the primary interaction site (Barlow et al 1986, Laver et al 1990). It is therefore necessary to bear in mind when interpreting the Western blotting data that reactivities to denatured polypeptides may in fact represent part of the epitope only, since the linear epitopes of SPAG1 detected by bovine antisera and McAbs could potentially represent part of more extensive conformational epitopes.

The predominant SPAG1 epitope containing regions as determined by Western blotting are summarised in Figure 47. The epitopes recognised by the GST-2.7 antisera are compared with those recognised by four cattle immunised with *T. annulata* freeze-thawed sporozoites (data from R. Hall) and showing strong sporozoite neutralising activity "in vitro" (Williamson 1988, S.Williamson, personal communication). The GST-2.7 antisera reacted with a number of constructs, and all showed particularly strong recognition of epitopes within the N and C-terminal regions of SPAG1. Sera from 23x, 32x and 758, but not sera from 26x and 759, also recognised weak epitopes in the central part of the SPAG1 amino acid sequence, as indicated by their reactivities to recombinants NE and N6. However, the level of antibody to the GST-2.7 was lower in 26x and 759 than in the other three antisera according to the intensity of the bands, so reactivity to NE and N6 may not have been detected for this reason alone. The ELISA titres of 23x antisera against the GST-2.7 fusion proteins appeared to confirm the pattern of reactivity on Western blots, with high readings in the presence of most of the constructs except for SE (amino acids 726-784) and BN (amino acids 109-169) (Table 13), which were also not recognised on the blots.

The SPAG1 fragments detected by the sporozoite immunised animals was highly variable between the four animals, and showed some major differences from the GST-2.7 antisera. In general, there were no strong epitopes recognised by the sporozoite antisera in the N-terminal region of SPAG1, although serum 37T reacted weakly with the fusion protein containing amino acid residues 20-109 (HB). Sera from 37T, 47T

and 45T also gave strong reactions SPAG1 regions 486-632 and 726-784, which were recognised poorly or not at all by the GST-2.7 antisera. These differences may relate to the way SPAG1 is folded when attatched to the sporozoite surface or as a free antigen in it's recombinant form; thus exposing cattle to slightly different sets of epitopes.

The reactivities of the mouse McAbs showed some key differences from those of the bovine antisera. According to previous work McAb 1A7 recognises a continuous epitope within the C-terminal SR1 region of SPAG1, since it recognised the λgt11-SR1 recombinant on Western blots (Williamson et al 1989). The lack of reactivity of the antibody to fusion proteins S1 and SE (Figure 39A) indicated that the epitope was in the region covered by SPAG1 amino acid residues 784-818. The epitope has now been located more precisely to a stretch of 16 amino acid residues 807-822 by testing with synthetic peptides (Boulter et al 1993)

The VGVAPG epitope for which BA4 is specific is predicted to occur twice in the SPAG1 sequence, among the two blocks of elastin homologous repeats (Hall et al 1992). The constructs that were positive for BA4; 2.7, 0.8, EA, N6 and NE were those predicted to contain the VGVAPG epitope. This served to validate the primary structure of these constructs. Interestingly, the SPAG1 McAbs 4A7, 4D3 and 5D1 recognised the same non overlapping polypeptides as BA4 (Figure 47). This indicated that they also reacted with epitopes that occur more than once in the SPAG1 sequence. Only 4A7 reacted with α elastin on slot blots (Figure 40), indicating that it recognised epitopes within the elastin homologous repeats. The McAbs which failed to react with elastin presumably recognised an epitope outside the elastin homologous regions which was also repeated in the SPAG1 sequence covered by constructs NE (382-486) and EA, but not BN (169-262). THese two regions do share small areas of identity, such as HAQQ (174-178, and 404-408) or GSH (172-174, and 475-477) but these are probably too small to form an epitope. An alternative explanation is that the McAbs actually recognise a conformational epitope on the sporozoite surface, and the reactivity to two regions of the SPAG1 primary sequence on Western blots actually represents binding to two parts of the complete recognition site.

In studies on epitope recognition in the *Plasmodium falciparum* circumsporozoite protein, it was found that similar groups of epitopes were recognised by mouse McAbs and human antisera (Zavala et al 1983). Therefore it is plausible that the 4D3 and 5D1 epitopes are also recognised by 23x, 32x and 758 antisera, which react with EA and NE. Cattle would not be expected to react with epitopes within the repeated regions homologous to bovine elastin, to which their immune system would be tolerant. A possible function of these repeats in the parasite would be to reduce humoral recognition of certain epitopes (Hall et al 1992). While BA4 shows limited cross-reactivity with elastin from other species such as humans and sheep, rodent elastin is sufficiently different for it to fail to react (Wrenn and Mecham 1987) This would explain why a humoral response to the *T. annulata* elastin homologous repeats could be mounted in the mouse, resulting in the production of McAb 4A7. It is not surprising that repeated epitopes in SPAG1 proved to be immunogenic. Repeated regions in a number of antigens of *Plasmodium* species have been found to evoke high levels of antibody in individuals exposed to the antigen (Hyde 1990).

It is notable that while the bovine antisera to both recombinant and sporozoite derived SPAG1 have a wide range of reactivities, they all recognise epitopes in the C-terminus, which is also the only region to be recognised by sporozoite antiserum 38T. A C-terminal epitope was also recognised by McAb 1A7. This may correlate with the ability both 1A7 and SPAG1 antisera to block sporozoite infection of lymphocytes "in vitro" to a high level. Furthermore, the McAbs BA4, 4A7, 4D3 and 5D1, which fail to see C-terminal epitopes, did not significantly neutralise sporozoite infectivity (Williamson 1988, Boulter et al 1993). These data imply that sequences concerned with sporozoite entry or recognition of lymphocytes and immunological neutralising activity are located in the C-terminus of SPAG1, as described in a recent paper (Boulter et al 1993). It is interesting that the GST-2.7 antisera recognised epitopes in the S1 polypeptide which were not recognised by 1A7. Bovine antisera from animals 10T and 34A, which were immunised with λgt11-SR1 recombinant fusion protein and show a high level of neutralising activity (Williamson 1988), also react with epitopes in the C-terminal region not covered by the 16aa peptide recognised by 1A7 (Boulter et al 1993). It appears, therefore, that bovine antisera reacts with different epitopes

from those recognised by 1A7, so the C-terminal region must contain several potentially neutralising antibody recognition sites.

It is notable that McAbs 4A7, 4D3, and 5D1 only react with the higher molecular weight SPAG1 polypeptides recognised by 1A7 on Western blots. The identical patterns of reactivity produced by 4A7 and BA4 were to be expected since they both recognise epitopes in the elastin homologous regions. It is thought that the group of antigens recognised by 1A7 represent proteolytic processing products from the SPAG1 N-terminus, since 1A7 recognises the C-terminal end of SPAG1 (Williamson et al 1989, Hall et al 1992). It has been proposed that this results in the exposure of C-terminal ligands, similar to the mechanism thought to operate in *P. falciparum*. The 190kDa merozoite surface antigen MSP1 is extensively processed from the N-terminal end, to give a 19kDa antigen found on the surface of invading merozoites (Blackman et al 1991). It seems likely that McAbs 4A7, 4D3 and 5D1 are reacting with SPAG1 in various stages of processing. It would be expected that some of the proteolytic cleavage sites would lie between the epitope recognised by 1A7 and those recognised by the other three SPAG1 McAbs, since the lower molecular weight bands of 63 and 54kDa carry the 1A7 epitope only. It is impossible to predict where the other cleavage sites are located, since the McAbs 4A7, 4D3 and 5D1 all recognise an epitope repeated in the SPAG1 sequence.

Amino acid sequence comparison between SPAG1 and p67, the *T. parva* homologue of SPAG1, revealed regions of homology which were not purely confined to the C-terminus, although the latter appeared to be the most highly conserved region between the two sequences. The polypeptides clearly contain common epitopes based on the cross-reactivities of sera raised to either fusion protein. NS1-p67 bovine antisera recognised SPAG1 antigen both in recombinant form and in *T. annulata* sporozoite preparations, as well as reacting with formalin fixed *T. annulata* Gharb sporozoites in IFA tests. Moreover, GST-2.7 antisera 23x and 758 reacted with NS1-p67 and with p67 in *T. parva* sporozoite material on Western blots.

While E99 antisera raised against *T. parva* clearly recognised *T. annulata* SPAG1 recombinant and sporozoite derived antigen, *T. annulata*

sporozoite antisera from 38T and 45T gave negligable reactions with p67, both as a recombinant and in *T. parva* sporozoite material. It is notable that a number of bands of molecular weights not corresponding to the p67 antigen were recognised by 45T antiserum on the Western blot of *T. parva* material. Since 38T and 45T would have received a combination of sporozoite antigens when they were immunised with freeze-thawed sporozoites, this result perhaps indicates that homology could exist between *T. annulata* antigens and other *T. parva* antigens apart from p67. The bands of 90, 80, and 60-40kDa could correspond to three of the *T. parva* microneme/rhoptry protein antigens, of molecular weights 104, 90, 85 and 35kDa (Iams et al 1990b). Another possible candidate is the polymorphic immunodominant molecule (PIM), which is 86/90kDa in *T. parva* Muguga, and is also recognised by E99 antiserum (Toye et al 1991). Since uninfected *Rhipicephalus* sp. tick material was unavailable, the possibility that some of these bands derived from tick material cannot be excluded. However, this seems unlikely for several reasons; the tick material in salivary gland extract is minimal, tick antigens would only be recognised if they cross reacted with proteins from *Hyalomma* sp. and no equivalent antigens were recognised by E99 antisera, which was immunised with *T. parva* sporozoites prepared from infected ticks, when used to probe Western blots of uninfected *Hyalomma* extracts (Figure 43 track A (u)).

The lack of reactivity of McAb 1A7 with p67 in *T. parva* sporozoite material did not correlate with it's recognition of NS1-p67. This was probably due simply to the lower concentration of p67 antigen in sporozoite extract than in preparations of the recombinant fusion protein. A weak reaction was in fact obtained against p67 in *T. parva* sporozoite lysate when carried out at ILRAD (Personal communication; T. Musoke), confirming that the 1A7 epitope was cross-reactive.

Within the region of SPAG1 identified as containing the C-terminal cross reactive epitope (807-822), seven continuous amino acid residues are shared by p67. It is unlikely that p67 contains the complete epitope, however, as the reaction of 1A7 with p67 on Western blots is considerably weaker than with SPAG1. The failure of the other three McAbs to react with p67 probably reflects the fact that their epitopes lie in part of the SPAG1 sequence with poor homology to p67. The weak

reaction of McAb 4B11 with NS1-p67 and *T. parva* sporozoites will be discussed in Chapter 8.

Since the antisera raised against *T. parva* sporozoites or recombinant p67 recognised *T. annulata* sporozoite material and recombinant SPAG1 on Western blots, at least some of the common epitopes must be continuous. Therefore it was plausible to attempt to map these regions of homology using Western blots of a number of the SPAG1 GST constructs, as described for the SPAG1 antisera and McAbs. A summary of the regions containing common epitopes recognised by E99 and 155 antisera is included on Figure 47. Both sera recognised epitopes in four main regions of the SPAG1 amino acid sequence; residues 20-109, 169-381, 726-784 and 814-907. The strength of reaction varied between the two antisera, with 155 giving a stronger reaction to constructs covering the C-terminus. These regions are also all recognised by the SPAG1 antisera, with the exception of 726-784 (SE), although this construct is recognised by some of the *T. annulata* sporozoite antisera. The N-terminal region 20-109, and the C-terminal regions 726-784 and 814-907, corresponded to regions of high predicted homology between p67 and SPAG1. While homology is low in the SPAG1 sequence between amino acid residues 169-381, there is one near identical stretch of 12 amino acids (354-368) which would be sufficient to form a continuous common epitope. This sequence also lies within an area corresponding to part of the p67 sequence predicted to contain B cell epitopes, according to analysis by McAbs (Nene and Musoke 1990).

Interestingly, the only other part of the p67 sequence identified by these workers as containing B cell epitopes was in the C-terminal region, corresponding to SPAG1 amino acid residues 726-848. This region is particularly homologous to p67 and contains epitopes recognised by 155 and E99 antisera. The importance of this region in neutralisation of *T. annulata* has been discussed previously in this chapter. Therefore it seems plausible that some of the epitopes capable of neutralising *T. annulata* sporozoite infectivity are recognised by antibodies raised against *T. parva* sporozoites. It would be worth testing this theory by carrying out "in vitro" sporozoite inhibition assays, to see if *T. parva* antisera were capable of reducing *T. annulata* sporozoite infectivity and vice versa. If neutralising epitopes are recognised, this could have important

implications for vaccine design. However, it should be borne in mind that there are likely to be some key differences in the neutralising determinants of SPAG1 and p67, since *T. annulata* and *T. parva* sporozoites infect different lymphocyte subsets. *T. annulata* sporozoites infect Class II bearing cells, particularly B cells and macrophages, while *T. parva* sporozoites primarily infects T cells (Spooner et al 1989). Therefore, if p67 and SPAG1 are involved in host cell recognition, which has not so far been elucidated, they would be expected to contain non-identical recognition sequences for these different cell subsets. This is comparable with two species of malaria parasites, *P. falciparum* and *P. knowlesi*, where the merozoites both carry similar MSP1 molecules but recognise different receptors on the erythrocyte surface (Miller et al 1977).

It is likely that several determinants are involved in lymphocyte recognition and invasion. There is evidence that *T. parva* sporozoite entry is mediated by binding to MHC Class I molecules on the lymphocyte surface (Shaw et al 1991). Such a mechanism alone could not convey target cell specificity as MHC Class I is ubiquitous on bovine lymphocytes, although it may convey host species specificity if binding takes place to bovine MHC Class I only. Therefore, *T. parva* sporozoites would be expected to have other recognition sites. Since both sporozoites have been demonstrated to carry a variety of antigens, it cannot be ruled out that the sites conveying target cell specificity might reside on molecules other than p67 or SPAG1. However, specific binding of SPAG1 to MHC Class II bearing cells has been demonstrated (Personal communication; F. Katzer, Dept. of Biology, University of York). If the recognition sites for lymphocyte subsets are carried on SPAG1 and p67, these would be likely to occur in one of the non-identical regions of the molecules.

Structural similarity and cross-reactivity between the p67 and SPAG1 antigens is not unexpected. Similarities between antigens within a number of Apicomplexa genera have been identified on numerous occasions, such as the cross reactivity of epitopes in the circumsporozoite proteins of several *Plamodium* species (Nussenzweig and Nussenzweig, 1985) and between the sporozoite microneme antigens of three *Eimeria* species (Tomley et al 1991). Such similarities probably demonstrate

evolutionary relationships; it is likely that certain molecules necessary to the parasite's survival would be conserved across species.

As discussed in Chapter 3, SPAG1 is polymorphic at the DNA level, and this is reflected at the amino acid level to an extent. Comparison between the two SPAG1 sequences gSPAG1 and cSPAG1 (Figure 13) revealed polymorphic regions. However, 100 amino acid residues at the N-termini and a long stretch of the C-termini were highly conserved between the sequences. The fact that McAb 1A7 reacts with *T. annulata* Hissar, Gharb and Ankara sporozoite material on Western blots and in IFA tests also indicates that the C-terminus is conserved between different geographical isolates. The data presented in this chapter demonstrates the SPAG1 C-terminus to be consistantly recognised by antisera raised against both recombinant SPAG1 and *T. annulata* sporozoites, and to contain common epitopes with *T. parva* p67. This is consistant with evidence for the presence of determinants in the SPAG1 C-terminus necessary for host cell invasion, which is a likely reason for it's conservation among different isolates and species, although it does imply that this part of the molecule might not be responsible for host cell specificity.

In this chapter Western blotting techniques were used successfully to map a number of regions of SPAG1 containing B cell epitopes recognised by McAbs and antisera, and to examine cross reactivity with a homologous molecule on *T. parva* sporozoites. However, this technique is confined to the detection of linear epitopes only, as discussed previously in this chapter. There is a paucity of techniques available for the mapping of conformational epitopes, since they can be detected only by using the molecule in it's native conformation. ELISA tests using the SPAG1 constructs may detect some conformational epitopes since the fragments were probably of sufficient length to show some secondary and tertiary folding. Another approach that would verify whether the bovine antisera to GST-2.7 or *T. annulata* sporozoites reacted to identical epitopes to those recognised by the SPAG1 McAbs would be to carry out competetive binding assays, using recombinant SPAG1 or the native antigen isolated from sporozoites. This method was used to identify the number of immunodominant regions in the *P. falciparum* circumsporozoite protein (Zavala et al 1983).

TABLE 9. RESULTS OF IFA TESTS OF SERA FROM GST-2.7 IMMUNISED COWS AGAINST *T. ANNULATA* SPOROZOITES.

The following table shows the maximum serum dilutions giving bright fluorescence with *T. annulata* Hissar acetone fixed sporozoite antigen. Day 0 sera was tested at dilutions of 1/10 to 1/640, while sera from days 50, 70 and 92, collected 14 days after the second, third and fourth immunisations respectively, was tested at dilutions of 1/10 to 1/2560. F = immunised with Freund's complete adjuvant, S = immunised with saponin as an adjuvant. No fluorescence was observed in wells incubated with second antibody alone (PBS instead of first antibody).

	DAYS POST IMMUNISATION			
ANIMAL NO.	0	50	70	92
23x (F)	1/10	1/640	1/640	1/2560
26x (S)	1/10	1/640	1/640	1/640
32x (S)	1/40	1/640	1/640	1/640
758 (F)	1/10	1/640	1/640	1/2560
759 (F)	1/10	1/640	1/640	1/2560

TABLE 10. ELISA TITRES AGAINST GST-2.7

The following table shows the optical densities at 490nm obtained from ELISA tests of sera from the five cattle immunised with GST-2.7 fusion protein against the antigen on a microtitre plate. F = immunised with Freund's complete adjuvant, S = immunised with saponin as an adjuvant. The tests included pre-immune sera and sera collected 35 days after the first immunisation, and 14 days after each subsequent immunisation. The sera was used at a final concentration of 1:50, and the second antibody was rabbit anti-bovine IgG peroxidase conjugate used at 1:8000. Each well contained 1μg GST-2.7.

	DAYS POST IMMUNISATION				
ANIMAL NO.	0	35	56	70	92
23x (F)	0.003	0.221	0.589	0.479	0.563
26x (S)	0.002	0.333	0.233	0.194	0.281
32x (S)	0.003	0.103	0.387	0.232	0.322
758 (F)	0.001	0.093	0.329	0.265	0.406
759 (F)	0.003	0.342	0.573	0.373	0.509

TABLE 11. SPOROZOITE INHIBITION ASSAYS

The following table shows the results for two assays for inhibition of *T. annulata* Hissar sporozoite infection of PBM "in vitro", using day 0 and day 92 serum from animals 26x, 32x, 758 and 759 at initial concentrations of x1, 1:4 and 1:16. F = immunised with Freund's complete adjuvant, S = immunised with saponin as an adjuvant. The figures show the percentage inhibition produced by the post immune serum, calculated according to the following formula:

$$\% \text{ inhibition} = \frac{x-y}{x} \times 100$$

where x = % infection recorded in pre-immunisation serum
and y = % infection recorded in post-immunisation serum

Assay 91/9

	SERUM DILUTION	
ANIMAL NO.	X1	1/4
26x (S)	100	100
32x (S)	100	100
758 (F)	100	100
759 (F)	100	100

Assay 91/9A

	SERUM DILUTION	
ANIMAL NO.	1/4	1/16
26x (S)	91	77
32x (S)	100	27
758 (F)	100	7
759 (F)	100	0

TABLE 12. PARASITAEMIA OF GST-2.7 IMMUNISED ANIMALS

The following table gives the percentage of piroplasms as assessed from Giemsa stained blood smears taken from the five GST-2.7 immunised cattle and the four unimmunised controls. Samples were taken over a period of 21 days following challenge. CON = unimmunised control animal, F = animal immunised with GST-2.7 and Freund's Complete adjuvant, S = animal immunised with GST-2.7 and saponin as an adjuvant.

	DAYS POST CHALLENGE								
ANIMAL NO.	**0**	**5**	**7**	**9**	**12**	**14**	**16**	**19**	**20**
739 (CON)	0	0	0	0	10.1	21.9	32.6	33.6	36.9
740 (CON)	0	0	0	0	13.7	19.3	17.7	15.4	13.0
741 (CON)	0	0	0	0	20.3	32.4	33.6	34.3	29.1
742 (CON)	0	0	0	0.1	9.0	14.4	18.3	22.9	17.0
23X (F)	0	0	0	0	0.5	4.4	13.2	23.1	30.5
26X (S)	0	0	0	0.1	1.2	2.8	5.1	8.8	8.8
32X (S)	0	0	0	0	1.4	11.1	25.9	42.8	44.7
758 (F)	0	0	0	0	0.8	2.9	7.2	10.5	8.7
759 (F)	0	0	0	0	0.7	3.8	6.7	7.8	6.2

TABLE 13. ELISA TITRES AGAINST GST-SPAG1 CONSTRUCTS

The following table shows optical densities recorded at 490nm in an ELISA against different regions of SPAG1 expressed as GST fusion proteins using GST adsorbed day 0 and day 92 serum from animal 23x. Sera dilutions were identical to those given above. All the fusion proteins were used at 1μg/well. The amino acid residues of the SPAG1 sequence to which each construct corresponds are shown. Also included are the "binding ratios", given by the following formula;

$$\frac{\text{OD 490nm Day 0 serum}}{\text{OD 490nm Day 92 serum}} = 5 \text{ or more for a positive reaction (denoted by *)}$$

GST FUSION PROTEIN	DAY 0 SERUM	DAY 92 SERUM	BINDING RATIOS
2.7 20-907	0.010	0.627	62.7*
HB 20-108	0.115	0.833	7.243*
0.8 109-381	0.065	0.671	10.32*
EA 109-262	0.090	0.661	7.344*
BN 109-169	0.178	0.688	3.865
N6 382-632	0.111	0.660	5.946*
NE 382-486	0.086	0.581	6.756*
S1 726-784/ 818-907	0.138	0.689	4.99*
SE 726-784	0.398	0.592	1.487
GST	0.105	0.130	1.238

TABLE 14. SUMMARY OF REACTIVITIES OF ANTI-SPOROZOITE MCABS WITH *T. ANNULATA* SPOROZOITE MATERIAL

The following table summarises the predominant molecular weights of antigens recognised by a number of anti-sporozoite McAbs on a Western blot of salivary gland extract of *T. annulata* Hissar infected tick material, as shown in Figure 38.
McAb 1C7 supernatent was used undiluted, while the other McAbs were used in the form of ascites fluid diluted 1:100.

TRACK	MCAB	REACTION	MW AGS RECOGNISED
1	1C7*	-	-
2	4B11/C12	+	140, 67, 17
3	4D3/E8	+	110, 70
4	5D1/D11	+	110
5	1B4/B12	+	140, 67, 17
6	4A7/E12	+	110, 80, 70
7	4B11/E9	+	140, 67, 17
8	5F1/E11	+	110, 70
9	4A7/A8	+	110, 80, 70
10	4G5/C10	-	-
11	1A7/G8	+	110, 80-85, 70, 63, 54, (17)
12	None #	-	-

* 1C7 is a control anti-schizont McAb (supplied by Dr. B. Sheils, WUMP)
# Incubated with second antibody only

The following tables summarise the results from Western blots of SPAG1 fusion proteins using sera from cattle immunised with GST-2.7 fusion protein and McAbs directed against *T. annulata* sporozoites. 1μg fusion protein was loaded per track, plus GST and protein from untransformed *E. coli* lysate. The amino acid residues of the SPAG1 sequence covered by each construct are shown.
Reactivities were scored as "-" = no detectable reaction, "+/-" = negligable reaction,"+" = weak reaction and "++" = strong reaction .

TABLE 15A REACTIVITIES OF GST-2.7 ANTISERA

The reactivities of sera from the GST-2.7 immunised cattle are summarised. The sera were used at a dilution of 1:5000. The second antibody was alkaline phosphatase conjugated rabbit anti-bovine IgG used at 1:300.

	DAY 0	DAY 92				
FUSION PROTEIN	(Pooled)	23x	26x	32x	758	759
2.7 20-907	+/-	++	++	++	++	++
HB 20-108	+/-	++	++	++	++	++
0.8 109-381	+/-	++	++	++	++	++
EA 109-262	-	++	++	++	++	++
BN 109-169	-	-	-	-	-	-
N6 382-632	-	+	-	+	+	-
NE 382-486	-	+	-	+	+	-
S1 726-784/ 818-907	-	++	++	++	++	++
SE 726-784	-	-	-	-	-	-
SR1 784-892	-	++	++	++	++	++
GST	-	-	-	-	-	-
E. coli proteins	-	-	-	-	-	-

TABLE 15B. REACTIVITIES OF MONOCLONAL ANTIBODIES

1A7 and 5E1 supernatants were used undiluted. 4A7, 4D3, 5D1 and BA4 ascites fluid were used at a dilution of 1:100. Second antibody was alkaline conjugated rabbit anti-mouse IgG, used at 1:300.

	MONOCLONAL ANTIBODIES					
FUSION PROTEIN	5E1	1A7	BA4	4A7	4D3	5D1
2.7 20-907	-	++	++	++	++	++
HB 20-108	-	-	-	-	-	
0.8 109-381	-	-	++	++	++	++
EA 109-262	-	-	++	++	++	++
BN 109-169	-	-	-	-	-	-
N6 382-632	-	-	++	++	++	++
NE 382-486	-	-	++	++	++	++
S1 726-784/ 818-907	-	-	-	-	-	-
SE 726-784	-	-	-	-	-	-
SR1 784-892	-	++	-	-	-	-
GST	-	-	-	-	-	-
E. coli proteins	-	-	-	-	-	-

TABLE 16. RESULTS OF IFA TESTS USING *T. PARVA* IMMUNE SERUM

The results of the I.F.A. titres of 155, E99 and 23X bovine sera against *T.annulata* sporozoites are shown.

Fluorescence in each well was scored as very bright (+++), moderate (++), weak (+) or absent (-).

D.0 = Day 0
I.M. = post immunisation sera

SERUM DILUTION	23X D.0	23X I.M.	155 D.0	155 I.M.	E99 I.M.
1:10	++	+++	+	+++	+++
1:25	+	+++	+/-	++	+++
1:100	+/-	++	-	+	++
1:1000	-	+	-	+/-	+
1:10,000	-	-	-	+/-	+/-

TABLE 17. REACTIVITIES OF *T. PARVA* IMMUNE SERUM

Summary of reactivities of bovine sera and McAbs to GST fusion proteins covering different regions of SPAG1. 1μg fusion protein was loaded per track, plus GST and protein from untransformed *E. coli* lysate. The amino acid residues of the SPAG1 sequence covered by each construct is shown. The sera were used at a dilution of 1:100. The second antibody was alkaline phosphatase conjugated rabbit anti-bovine IgG used at 1:300.

Reactivities were scored as "-" = no detectable reaction, "+/-" = negligable reaction,"+" = weak reaction, "++" = moderate reaction and "+++" = strong reaction .

FUSION PROTEIN	758	155 d. 0	155d.92	E99
2.7 20-907	+++	-	++	++
HB 20-108	++	-	++	+
0.8 109-381	++	-	+	++
BN 109-169	-	-	-	-
EA 109-262	+	-	++	++
N6 382-632	-	-	-	-
NE 382-486	-	-	-	-
SE 726-784	-	-	+	+
SR1 784-892	+++	-	+++	+

CHAPTER 6

THE T CELL RESPONSE TO SPAG1

6.1 Introduction

The differences in T and B cell immune responses and their interactions have been described in section 1.2.1. Helper T cells (Th) have a central role in the immune response to pathogens, since they aid the activation of small B cells into antibody secreting plasma cells, enable macrophages to acquire more effective microbicidal and cytocidal capabilities and co-operate in the maturation of cytotoxic T cells (Wakelin 1984, Roitt et al 1986). While there is evidence that humoral responses to some polysaccharide antigens are T cell independant, antibody recognition of the majority of protein antigens is T cell dependant (Roitt et al 1986). Usually the helper function is "directional", ie. it occurs as a specific response to the antigen on the surface of an antigen presenting cell, and is MHC restricted. It was found that specific T cell subsets help specific populations of B cells produce antibody, and where more than one antibody isotype is produced to a given antigen this help may even be isotype specific. The problem of poor stimulation of cellular responses has been a major factor in attempts to develop molecular vaccines to parasites (Hyde 1990), so there has been a lot of work in recent years to identify Th cell epitopes on parasite antigens. The various approaches taken to map T cell epitopes have been summarised in section 1.2.4.

In Chapter 5 it was shown that a number of B cell epitopes, recognised by antiserum to recombinant SPAG1 and to sporozoite material, could be defined in the SPAG1 amino acid sequence. When cattle were experimentally immunised with recombinant SPAG1, a clear secondary humoral response was observed, demonstrating immunological memory. This has also been demonstrated in the case of sporozoite immunised cattle (Preston and Brown 1985). Secondary

responses tend to consist predominantly of IgG, as opposed to the primary response which is generally of the IgM isotype (Roitt et al 1986). This sequence of events has been demonstrated in the case of cattle immunised with *T. parva* sporozoites (Musoke et al 1992). While the isotypes of bovine antibodies to *T. annulata* sporozoites has not been determined, the predominant isotype in rabbits immunised with λgt11-SR1 recombinant was found to be IgG (Williamson 1988). Most evidence suggests T helper cells are a requirement for such class switching to take place, and are important in the formation of B cell memory (Roitt et al 1986). Therefore it is likely that T cell epitopes are contained within the SPAG1 sequence, although the possibility of T cell help being derived from a separate sporozoite antigen cannot be excluded. Such a relationship has been identified between two separate antigens of Hepatitis B virus (Milich 1987).

The objectives of this part of the work were twofold; to verify whether or not cattle recognised any Th cell epitopes on SPAG1, and to try and identify which regions of the molecule contained such epitopes. To resolve whether or not a Th response was induced to SPAG1, PBM taken from cattle immunised with *T. annulata* sporozoites were used in Th cell proliferation assays against GST-2.7 fusion protein (2.2.9). Two cattle which had had no previous exposure to sporozoites or SPAG1 fusion proteins, 11659 and 11663, were immunised with GST-2.7. PBM proliferative responses were then analysed both to GST-2.7 and to SPAG1 alone, derived from the intact fusion protein by Factor Xa cleavage. Cattle were immunised with uncleaved GST-2.7 due to the difficulties in producing the cleaved protein in sufficient quantities. All the GST fusion proteins used in these experiments were produced as described in Chapter 4.

Several approaches were taken to try and identify the regions of SPAG1 containing Th cell epitopes. Firstly, the computer algorithm TSites, reviewed by Feller and Cruz (1991), was used to predict Th epitopes within the SPAG1 amino acid sequence conforming to Rothbard and Taylor motifs or amphipathic α helices. These algorithms are among several available that predict T cell epitopes based on the primary structure of the protein sequence, and are capable of predicting both cytotoxic and helper T cell epitopes. The amphipathicity algorithm is

based on periodic variations in hydrophilicity/ hydrophobicity and identifies regions likely to form an amphipathic α helix, with hydrophobic and hydrophilic residues segregated on opposite sides of the structure (Berzofsky et al 1987). T cell epitopes of this nature were first identified in sperm whale myoglobin, and it was found subsequently that a number of other experimentally identified epitopes could produce this conformation. These workers postulated that while the hydrophobic residues were capable of associating with the MHC on the antigen presenting cell, the hydrophillic residues associated with the T cell receptor. This algorithm has been used to sucessfully predict regions containing T cell epitopes in a number of antigens, such as the TH2R epitope in the *Plasmodium falciparum* circumsporozoite protein (Good et al 1987) and the pf155 merozoite surface antigen (Perlmann et al 1988).

The Rothbard and Taylor algorithm is based directly on the amino acid sequence of the antigen (Rothbard and Taylor 1988). It was produced by analysing primary sequences found to be common in a database of T cell epitopes which had already been identified experimentally. These two methods overlap in that many Rothbard and Taylor motifs can form amphipathic α helices, but many can also form other types of secondary structures such as β pleated sheets. For this reason the algorithm is capable of identifying a broader range of T cell epitopes. It has been used to predict both cytotoxic and helper T cell epitopes in a 65kDa mycobacterial antigen (Brett et al 1989) and in the GP63 antigen of *Leishmania major* (Jardim et al 1990).

The second approach was to attempt to identify T helper cell epitopes experimentally. PBM from an animal immunised with GST-2.7 (11663) was used to produce T cell lines by culturing in the presence of GST-2.7 fusion protein. These cell lines were tested for proliferation against SPAG1 constructs, and the nature of the responding cell types assessed by FACS analysis, using a range of bovine lymphocyte markers. Cloning of some of the cell lines was carried out by limiting dilution, based on methods described by Sinigaglia et al (1991). Cloning is necessary for Th epitope mapping since Th cell lines normally recognise a number of different epitopes on a single protein, due to the mixture of cell populations they contain. Cloned Th cells also tend to grow more successfully in culture than lines (Dr. E. Glass; personal

communication). A further advantage of cloning was that since each clone would be expected to recognise a single epitope, clones responding to GST could be rejected prior to testing with GST-2.7 constructs.

In a second experiment carried out by Mrs. P.Millar and Dr. E. Glass (AFRC Roslin Institute, Edinburgh) two more cattle, 12045 and 12056, were immunised with GST-2.7 fusion protein. These were also used to produce Th cell lines to GST-2.7, which were tested as described above. The data obtained is included and analysed as part of this chapter with their permission as their results are relevant to the previous experiments.

6.2 Results

Th cell proliferation assays on PBM, lines and clones, the setting up of Th cell lines and clones "in vitro" and the FACS analysis of lymphocyte subsets were carried out as described in section 2.2.9.

6.2.1 Use of a predictive algorithm

The results obtained using the computer program TSites run on an Apple Mackintosh computer to predict Th cell epitopes within the SPAG1 amino acid sequence are shown in Figure 48. A large number of potential epitopes were predicted, both Rothbard and Taylor motifs and amphipathic α helices. Both forms were detected throughout the molecule but were at a noticably lower density between amino acid residues 234 and 434, increasing in density in the C-terminal portion of the molecule.

Sequences where Rothbard and Taylor motifs overlap with an amphipathic block 15-20 amino acids long have a high probability of containing T cell epitopes recognised by one or more animals in a group of outbred individuals (Personal communication; T. Collen, Institute for Animal Health, Pirbright). Eighteen such regions in the SPAG1 amino acid sequence were identified, and these are summarised in Table 18. The algorithm was also used to predict any potential T cell epitopes in GST. Sixteen Rothbard and Taylor motifs and eleven blocks of

amphipathic α helices were predicted throughout the molecule, but there was only one region where the epitopes overlapped as described above (data not shown).

6.2.2 Cattle immunised with sporozoites

The results from the Th cell proliferation assays to GST-2.7 using PBM from sporozoite immunised cattle are shown in Table 19. Cattle 11052,11055 and 11434 were immunised with *T. annulata* Hissar sporozoites by infection with live stabilates and treatment with buparvaquone. These cattle were part of an experiment being performed by Dr. E. Glass et al, AFRC Roslin Institute, Edinburgh. Serum and PBM was collected for assays 10 days following a third challenge of 11055 and 11052, and a second challenge of 11434 with *T. annulata* Hissar sporozoites. 11435, a genetically identical twin of 11434, was an unimmunised control. Serum from cattle 11052, 11055 and 11434 all reacted with acetone fixed *T. annulata* Hissar, Ankara and Gharb sporozoites, macroschizonts and piroplasms in IFA tests to a titre of at least 1/160, while serum from 11435 did not (Mrs. L. Bell-Sakyi, CTVM). PBM from animal 5692 was included as a positive control for the tests. This animal had been immunised with ovalbumin as part of a different experiment being carried out by Dr. E. Glass, and was known to respond well to the immunogen in PBM proliferation assays. The MHC Class I and Class II types of the four cattle are summarised in Table 20A.

Sera from the three immunised animals collected after they were challenged and taken at the same time from 11435 were used at a dilution of 1:100 on Western blots of GST-2.7, SPAG1 and a lysate of *E. coli* XL-1 Blue expressing GST (Figure 49). McAb 1A7 was included as a control. 11052 and 11055 only gave a weak reaction with a 145kDa band in the GST-2.7 track, while serum from 11434 gave a stronger reaction. 11434 alone also reacted weakly with bands of 119kDa and 80kDa in the track containing SPAG1 purified from cleaved GST-2.7. These bands correspond to the sizes of the cleaved fusion protein visualised by McAb 1A7, and their sizes do not correspond to any bands in the *E. coli* /GST lysate. Serum from 11435 reacted with *E. coli* proteins only.

Figure 48

Analysis of SPAG1 amino acid sequence using the computer algorithm TSITES.

BOLD = amphipathic α helices, midpoint of a 12 residue block. Where several residues are shown, this indicates the midpoints of several overlapping 12 residue blocks.

UNDERLINED = Rothbard/Taylor motifs, 4, 5 or 8 amino acid residues. Longer stretches are produced by overlapping motifs.

The amino acid sequence is shown in single letter code. The numbers correspond to the amino acid residues of the SPAG1 sequence (given in the Appendix, Figure 77)

1 M N I I H F L L T I P A I F V S G A D K M P A G E S

27 S R T S K P S P L V T L E S A V T Q P S K D P F K T

53 I S A L S K A T K V W K S A V S V S G D S K T V P T

79 P V S E P M I T R S F Q E P V S Q E L E F Q S D T E

105 I N E S G S G S D E D E D D D D D E E E E E D D K S

131 T S S K N G K G S P K A Q P G V S S S S T S S A S P

157 T S P T T T L S Q T G L G P S G S H A Q Q D P G V G

183 V P G V G V P G V G V P G V G V P G V G V P G V G V

210 P G V G G V P G V G V A P G V G V P G V G V A P G V

236 G V G A D S S G L P G S G G L G A G A K A G K G Q G

262 S G L Q G P G G V G V V P G V G V A A S S S S P G K

288 P P G V G A G V M P G V G V R A Q G G V I I G A P G

314 V A G V P G G K P G Q P V S Q E L E L K S D T E I N

340 E S G S S S S E G E D D D D E E E E E E N K S T S S K

366 G A G G K A G K G Q G S V S P G G G S S A S Q T S P

392 T T T P Q S G L A S S G S H A Q Q S P Q Q D P A P S

418 K P S G G G V P G V G V P G V G V P G V G V P G V G

444 V A G V G V V P G V G A T T S S S S T T S T S T S

470 T T T T T T T S S G K P S D Q G S H G T S P R N A V

496 T R Q T D S I S G P I P S P G D P R A I T G Q M G E

522 G E R F A V Q F L G D F K P K P R R Y E G Q G T D A

548 V K L K Q F I F E E V K S L V Q T L I N L K L A I A

574 N D F V E I S E K L K K K N Q N Y V P K L K L L K G

600 E Q F D T K Q K V A N V L K G F N S L Y F V F F M N

626 L N L A K E V N K P E E L A E F L W K L N T I P D K

652 V G R E F E L A I E K T K G S E K K K E L E E A F N

678 S I G L G F K I A Q Y A T N D I L S S I T N S V Y S

704 L I K L K N F G D D F V T E V R K S L Q M V P H Q K

730 N L N G S A F I V K I S E I I N K K G T E D Q D Q T

756 S G S G S K G T E G G S L R G Q D L T E E E V L K V

782 L D E L V K D V S E E H V G I G D L S D P S S R T P

808 N A K P A E L G P S L V I Q N V P S D P S K V T P T

834 Q P S N L P Q V P T T G P G N G T D G T T T G P G G

860 N G E G G K D L K E G E K K E G L F Q K I K N K L L

886 G S G F E V A S I I I P M T T I I F S I V H

The data from the proliferation assay is summarised in Table 19. PBM from the animal 11434 showed a significant proliferative response to GST-2.7 ($P > 0.005$ at $10\mu g\ ml^{-1}$) when compared with proliferation in medium alone or ovalbumin. No significant response was obtained to GST-2.7 using PBM from any of the other animals. A second proliferation assay carried out using PBM from 11434, 11435 and 5692 seven days later produced a similar result.

6.2.3 GST-2.7 immunised cattle; 11659 and 11663

Proliferation assays to GST-2.7 were set up using PBM obtained from five unimmunised cattle of various MHC Class I and II haplotypes. The two animals which gave the lowest non specific proliferative response in the test, 11663 and 11659, were selected for immunisation. The MHC Class I and Class II haplotypes of the cattle are shown in Table 20B. Details of the immunisations are given in Chapter 2 (2.2.8). Cattle 11659 and 11663 were boosted on days 34 and 95 following the initial immunisation, with 11663 only boosted on day 179 and 253.

Sera taken from both cattle on days 0 and 142 after initial immunisation were used to develop a Western blot of GST-2.7, isolated SPAG1 polypeptide derived from Factor Xa cleaved GST-2.7 and GST recombinant proteins. Day 142, but not Day 0 serum from both cattle gave strong reactions with all three antigens (data not shown). The pattern of reactivity was identical to that of cattle 12045 and 12056, the second pair of cattle immunised with GST-2.7 (Figure 53 A and B). 11663 serum gave a stronger reaction than 11659.

Table 21 gives the results from Th proliferation assays carried out using PBM taken from cattle 11659 and 11663 on days 33, 73, 142 and 184 after the initial immunisation. PBM from 5692 was included in assays set up on days 73 and 142 to act as a positive control. The lectin Concavalin A was also included to act as a positive control for the proliferation assays, since it is mitogenic for lymphocytes. Counts of Trypan Blue stained cells made when the assay was set up showed over 90% of the cells were viable.

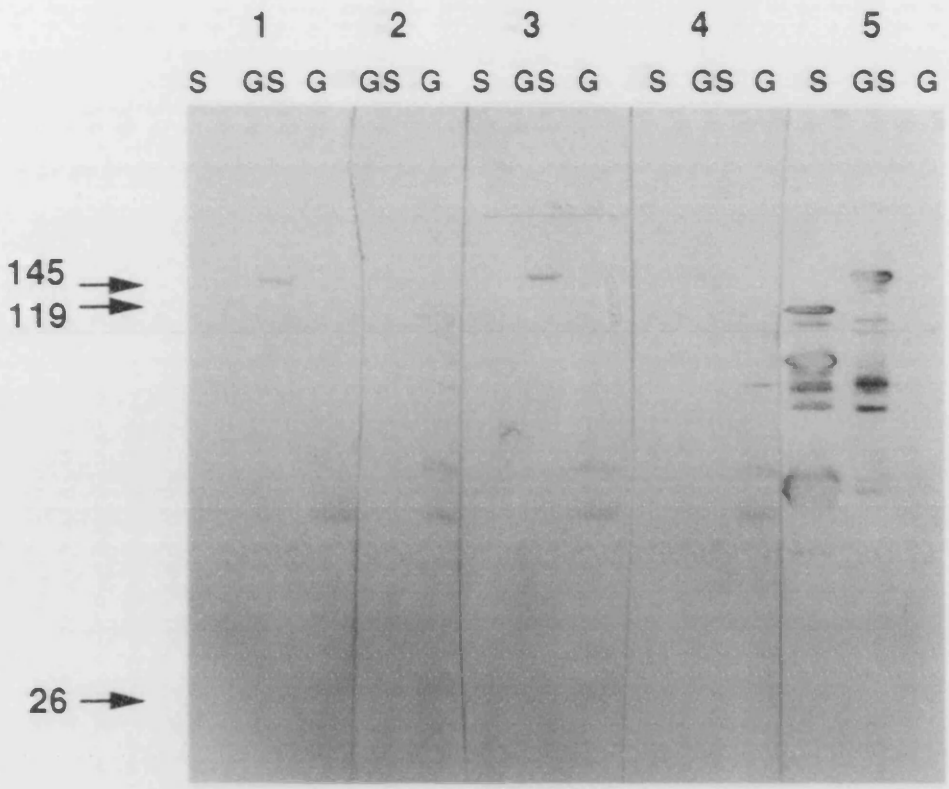
Figure 49

Western blot of a 12% SDS-polyacrylamide gel of GST-2.7, SPAG1 from cleaved fusion protein and recombinant GST in an unpurified *E. coli* lysate, developed with bovine *T. annulata* sporozoite immune sera. The sizes of the principal bands (kDa) are indicated by arrows. Tracks were loaded with the following fusion proteins (1μg per track);

S SPAG1 from cleaved GST-2.7
GS GST-2.7
G GST in unpurified *E. coli* lysate

Western blots were incubated with the following antibodies, with alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300) as a second antibody;

1 11055 bovine immune serum (1:100)
2 11435 " " "
3 11434 " " "
4 11052 " " "
5 McAb 1A7 undiluted supernantant


1
2
3
4
5
S GS G GS G S GS G S GS G S GS G
145
119
26

Significant proliferation to GST-2.7 at 0.1, 1 and 10μg ml^{-1} (Student's T test; P>0.05 compared with proliferation in medium) was obtained from 11663 at day 142, while 11659 gave a less significant response. It was decided to continue boosting 11663 and use this animal as a source of PBM for setting up cell lines. A much greater dose dependant proliferative response was obtained on day 184 from 11663 PBM, 5 days after the third boost. The highest mean cpm was recorded at 5μg ml^{-1} GST-2.7. Significant proliferation was also observed in the presence of SPAG1 purified from cleaved GST-2.7 (P>0.005 at 10μg ml^{-1}), although this was at a lower level than with the complete fusion protein. No significant proliferation was observed in the presence of ovalbumin except from the positive control, 5692 PBM (P>0.005). Significant proliferation was observed in all the tests containing Concavalin A.

6.2.4 11663 cell lines and clones

11663 PBM from day 189, 10 days after the third boost, were used to set up cell lines in 1, 2 and 5μg ml^{-1} GST-2.7 . Blasts could be observed in the wells at the end of the week 1 and week 2 in culture, but after further culture the cell numbers decreased and the cell lines died. Cloning of the cell lines by limiting dilution was carried out at the end of Week 2 but the cloned cells failed to grow.

One week following a further boost on day 253 cell lines were again set up in GST-2.7 at final concentrations of 0.1μg ml^{-1} (line L1), 1μg ml^{-1} (line L2) and 5μg ml^{-1} (line L3). Extensive cell proliferation and blasting could be observed in all three lines until the end of week 3, after which cell numbers begun to decrease. The lines died out by the end of week 6. Tests on line L2 were set up in the form of Th proliferation assays and FACS analysis of cell types at the end of weeks 1 , 3 and 5. FACS analysis was also carried out on day 260 PBM when the lines were set up. The cell lines L1 and L3 were also tested in proliferation assays and analysed with a FACS at the end of week 3.

Data from the proliferation assays on the line L2 are shown in Table 22. There was no significant proliferation to any of the test

proteins at weeks 1 or 3. The results from the FACS analysis are summarised in Table 23. The plots obtained using the LYSYS program are given for normal PBM and L2 at week 1 (Figures 50 and 51 respectively). When the FACS analysis of L2 at week 1 is compared to PBM a marked increase in Class II expressing cells from 40 to 90% of the population is apparent. The plot of forward versus side scatter obtained from the analysis indicates a high proportion of large, blasting cells in the line compared to that of PBM (Figures 50(i) and 51(i), demarked regions). There was also a drop in the proportion of B cells in the line, but only a slight increase in BoT2+ and BoT4+ cells. Further analysis of the line after three and five weeks in culture shows an increase in the BoT8+ population, and a decrease in the percentage of cells expressing Class II. The proportion of cells with markers for B cells, the γδ T cell receptor and macrophages dropped to a minimum in the lines, while the pecentage of Class I positive cells remained consistently high. The tests on L1 and L3 gave similar results.

Cloning by limiting dilution was carried out in 0.1, 1 and 5μg ml^{-1} of GST-2.7 from lines L1, L2 and L3 respectively, at the end of Week 2 in culture. Three weeks after the cloning step, rapidly growing and blasting cells could be observed in some of the wells, and these were harvested and expanded further. Many more clones were obtained in 1μg ml^{-1} GST-2.7 than in 5μg ml^{-1}. Only the clones 2C3, 4E5, 5F6, 5F8 and 6E4, which were all established at 1μg ml^{-1}, expanded to sufficient numbers for testing. These clones all expanded rapidly during the first 4-5 weeks of culture and large, blasting cells could be observed in the wells. The rate of expansion did slow down considerably, however, in subsequent weeks. The clones were tested in proliferation assays and the cell types analysed by FACS after 9 weeks in culture.

The results from the proliferation assays are summarised in Table 22. Counts in Trypan Blue made of each clone when the tests were set up showed 80-90% of the cells were viable. The clones 2C3, 4E5, 5F8 and 6E4 showed significant proliferation in the presence of Concavalin A, but none showed any significant proliferative response to GST-2.7, GST or SPAG1 alone. The data from FACS analysis of the clones are summarised in Table 23, with the plots from the LYSYS program shown for clones 5F6 and 6E4 only in Figures 52A and 52B respectively. The

BoT4 marker was surprisingly absent from all the cloned cell populations, although some expressed the BoT8 marker. Clones 2C3, 6E4 and 4E5 consisted of cell populations which were BoT2-, BoT4- , BoT8+, while the clones 5F6 and 5F8 appeared to be BoT2+, BoT4-, BoT8-. For example, clone 6E4 consisted of 98% cells labelled BoT8+, but produced no positive peak for the BoT4 or BoT2 markers (Figure 52B). Conversely, the cells analysed for clone 5F6 were 71% positive for BoT2, but failed to react significantly with the other two bovine lymphocyte markers (Figure 52A). The plots of forward versus side scatter demonstrated that the clones consisted almost exclusively of large cells (Figures 52A (i) and 52B (i)).

6.2.5 GST-2.7 immunised cattle 12045 and 12056

Cattle 12045 and 12056 were also immunised with GST-2.7 but Freund's complete adjuvant was used in the initial immunisation instead of Freund's incomplete adjuvant, which was used in the previous experiment. Details of the immunisations are given in section 2.2.8. The MHC Class II haplotypes of the two cattle are shown in Table 20B. They were boosted on Day 28 after the initial immunisation using Freund's incomplete adjuvant, and were not boosted further. Serum taken on days 0, 14, 35 and 253 was used in Western blots at a dilution of 1:100 against GST-2.7, GST and SPAG1 from cleaved GST-2.7 (Figure 53A and B).

Pre-immune serum from both cattle showed a weak reaction to GST-2.7 only. Day 35 sera collected 10 days after the boost gave a strong reaction to both GST-2.7 and cleaved SPAG1. An identical but weaker pattern of reactivity was still apparent in day 253 sera. The higher molecular weight bands recognised in the tracks containing GST-2.7 and SPAG1 correspond to those normally recognised by McAb 1A7 (Figure 49 track 5). The serological reactions of animal 12045 to GST-2.7 and SPAG1 generally appeared to be stronger than those of 12056.

Data from 12045 and 12056 PBM proliferation assays, carried out at regular intervals after the initial immunisations, are summarised in Tables 24 and 25 respectively. A proliferative response was shown by

Figure 50

Plots from LYSYS program of 11663 PBM (taken on day 260) analysed by FACS as described in the text. The shaded peak is that obtained from each labelled cell population, while the unshaded peak represents the unlabelled cell population (negative peak) obtained in medium alone.

a Plot of forward (FSC) vs. side scatter (SSC) to show the sizes of the cell populations analysed

b-l Plots to show the degree of fluorescence obtained with each cell marker specific McAb, and the percentage of cells positive for each marker is given. The cell populations labelled were as follows:

b None (fluorescein-conjugated second McAb only)
c MHC Class I positive
d CD4+
e CD8+
f CD2+
g Gamma-delta T cell receptor positive
h Macrophages
i B cells
l MHC Class II positive

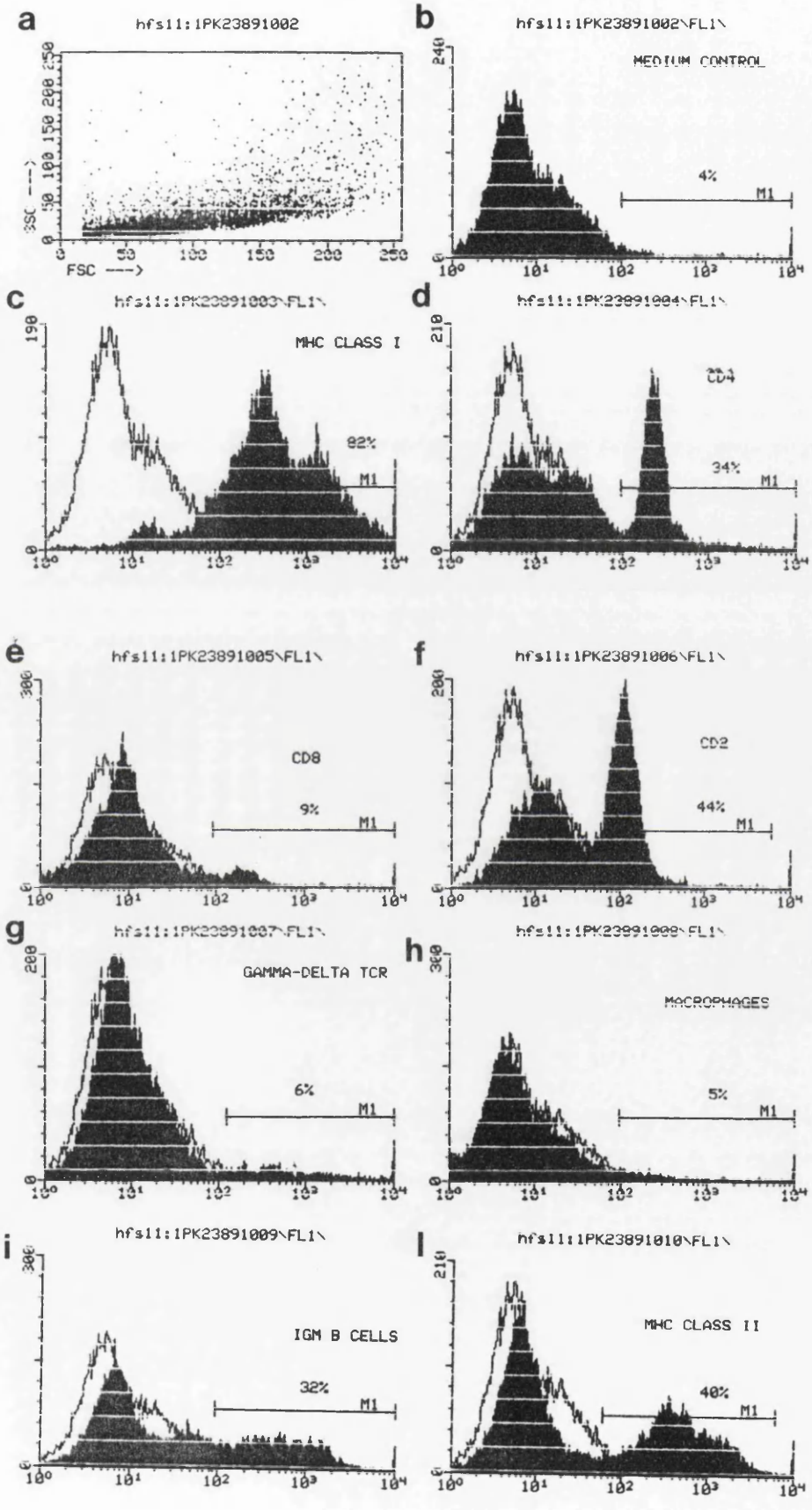

a
hfs11:1PK23891002
SSC ---->
FSC ---->
b
hfs11:1PK23891002\FL1\
MEDIUM CONTROL
4%
M1
c
hfs11:1PK23891003\FL1\
MHC CLASS I
82%
M1
d
hfs11:1PK23891004\FL1\
CD4
34%
M1
e
hfs11:1PK23891005\FL1\
CD8
9%
M1
f
hfs11:1PK23891006\FL1\
CD2
44%
M1
g
hfs11:1PK23891007\FL1\
GAMMA-DELTA TCR
6%
M1
h
hfs11:1PK23891008\FL1\
MACROPHAGES
5%
M1
i
hfs11:1PK23891009\FL1\
IGM B CELLS
32%
M1
l
hfs11:1PK23891010\FL1\
MHC CLASS II
40%
M1

Figure 52A

Plots from LYSYS program of 11663 clone 5F6, analysed by FACS after 9 weeks in culture as described in the text. See legend for Figure 50 for further explanation.

a Plot of forward (FSC) vs. side scatter (SSC)

b-f Plots to show the degree of fluorescence obtained with each cell marker specific McAb, and the percentage of cells positive for each marker is given. The cell populations labelled were as follows:

b None (fluorescein-conjugated second McAb only)
c CD2+
d CD4+
e MHC Class I positive
f CD8+

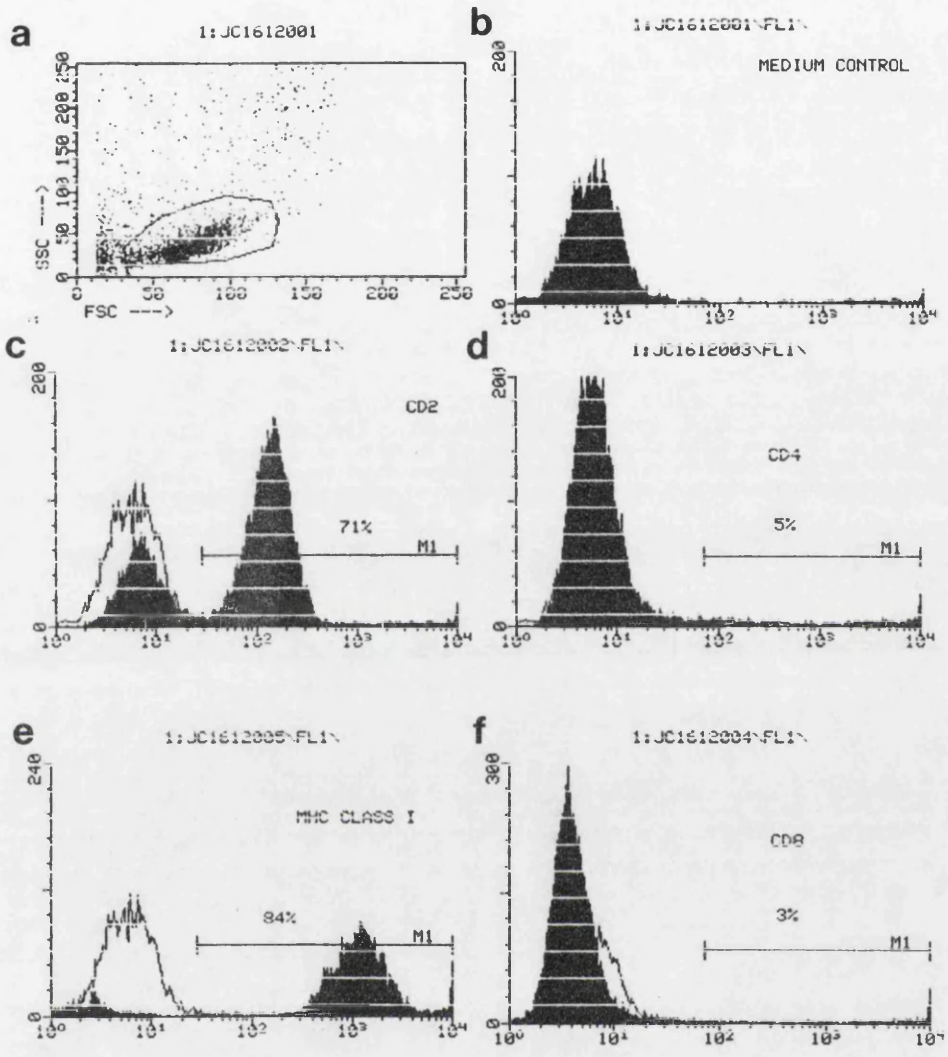

a
1:JC1612001
SSC --->
FSC --->
b
MEDIUM CONTROL
c
CD2
71%
M1
d
CD4
5%
M1
e
MHC CLASS I
84%
M1
f
CD8
3%
M1

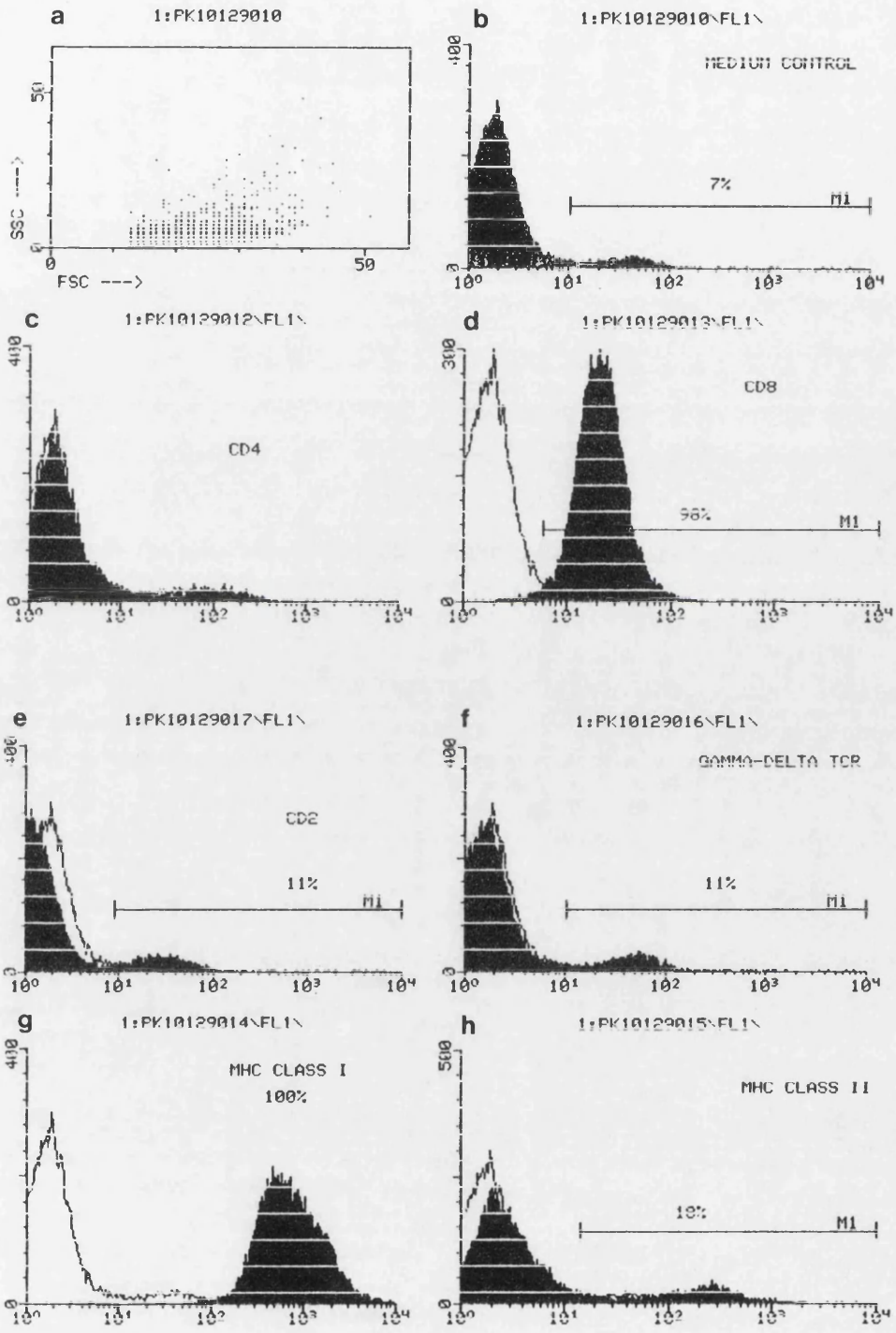
Figure 52B

Plots from LYSYS program of 11663 clone 6E4, analysed by FACS after 9 weeks in culture as described in the text. See legend for Figure 50 for further explanation.

a Plot of forward (FSC) vs. side scatter (SSC)

b-h Plots to show the degree of fluorescence obtained with each cell marker specific McAb, and the percentage of cells positive for each marker is given. The cell populations labelled were as follows:

b None (fluorescein-conjugated second McAb only)
c CD4+
d CD8+
e CD2+
f Gamma-delta T cell receptor positive
g MHC Class I positive
h MHC Class II positive


a
1:PK10129010
SSC --->
FSC --->
b
1:PK10129010\FL1\
MEDIUM CONTROL
7%
M1
c
1:PK10129012\FL1\
CD4
d
1:PK10129013\FL1\
CD8
98%
M1
e
1:PK10129017\FL1\
CD2
11%
M1
f
1:PK10129016\FL1\
GAMMA-DELTA TCR
11%
M1
g
1:PK10129014\FL1\
MHC CLASS I
100%
h
1:PK10129015\FL1\
MHC CLASS II
18%
M1

PBM from both cattle on day 14 after immunisation and remained high in all subsequent assays, even 224 days after the primary immunisation. High levels of proliferation were recorded in all the concentrations of GST-2.7 tested. An obvious response to boosting was only seen in animal 12056, since the proliferative response in all concentrations of the antigen increased by day 42, fourteen days after the cattle were boosted.

The effect of GST-2.7 concentrations on proliferation of PBM from both animals was investigated more thoroughly using PBM taken on Day 98 after immunisation (Figure 54). A significant proliferative response was obtained in concentrations as low as 10ng ml^{-1} compared against proliferation in medium alone (Student's T test; $P>0.005$). Antigen dose dependant proliferation was observed at concentrations between 0.01 and 0.1μg ml^{-1} for 12045 PBM and between 0.01 and 1μg ml^{-1} for 11056 PBM, after which there was little increase in proliferation with increasing antigen concentration.

12045 PBM taken on Day 112 were tested in a proliferation assay against proteins from an untransformed *E. coli* lysate, GST alone and a number of expressed GST-SPAG1 subfragments (Table 26). There was significant proliferation (Student's T test; $P>0.005$) in the presence of both GST alone and all the fusion proteins tested when compared with proliferation to normal *E. coli* proteins. This indicated that T cell epitopes were being recognised on the GST polypeptide, in addition to or instead of the SPAG1 polypeptide. It was therefore not possible to identify SPAG1 T cell epitopes by assessing PBM proliferative responses, since all the SPAG1 constructs were expressed as fusions with GST.

6.2.6 Cell lines from cattle 12045 and 12056

Cell lines were set up on day 28 after immunisation from 12045 PBM in 1μg ml^{-1} (L4) and 2.5μg ml^{-1} (L5) of GST-2.7 fusion protein. These were stimulated for 7 days in antigen, passed through a Percoll gradient and then depleted of any BoT8+ cells using a FACS as described by Baldwin et al (1986) using anti BoT8+ antibody CC63 (Howard et al 1991). The lines were then expanded for a further 4 weeks alternately in IL-2 and GST-2.7 before testing in proliferation assays.

Table 27 summarises the FACS profiles of both lines from tests set up after 1 week in antigen just before BoT8+ depletion, and after 4 weeks in culture, following BoT8+ depletion. The lines tested at week 1 contained about 20% BoT8+ cells, while at week 4 BoT8+ cells were almost absent. Conversely, the BoT4$^+$ and Pan T markers became predominant in the population, while the population of B and $\gamma\delta$ cells became negligable. The line L4, set up at 1μg ml^{-1}, displayed a significant proliferative response to GST-2.7 at all concentrations tested, when compared to proliferation in the presence of PBM without antigen (Table 28). Proliferation to GST and SPAG1 alone was significantly lower than proliferation to the complete fusion protein at identical concentrations (Student's T-test, $P > 0.005$). Line L5 was also tested at the end of week 4 in a proliferation assay, but failed to respond to any of the antigens tested.

Cloning by limiting dilution was carried out at the end of Week 2 in culture. Clones were set up from L4 in 1μg ml^{-1} GST-2.7 and from L5 in 2.5μg ml^{-1}. A number of growing clones were expanded for six weeks and tested in proliferation assays, but none of them responded to GST-2.7, GST or isolated SPAG1 (data not shown).

Additional 12045 and 12056 lines were set up on Day 156 after the cattle were immunised, in 0.1 (L6), 0.25 (L7), 0.5 (L8) and 1μg ml^{-1} (L9) of GST-2.7 fusion protein. The lines were tested at the end of Week 1 in culture, and those that were still blasting were also tested at Week 4. The results for the 12045 lines L6 to L9 are summarised in Table 28. Of the lines tested at Week 1, only L6 set up at 0.1μg ml^{-1} showed a significant response to GST-2.7 when compared to proliferation in PBM alone. The lines L7 to L9, set up at 0.25, 0.5 and 1μg ml^{-1} showed a high response to PBM in the absence of antigen. When L7 was retested at week 4, however, the response to PBM had dropped but a proliferative response to GST-2.7 could still be observed. Significant proliferation was recorded independantly in the presence of isolated GST and SPAG1.

Table 29 summarises similar data from the 12056 cell lines, L10 to L13. All these lines showed significant proliferative responses to GST-2.7 and GST alone when tested at Week 1. Significant proliferation was also found in the presence of SPAG1 and GST independantly when L12 and

Figure 53A

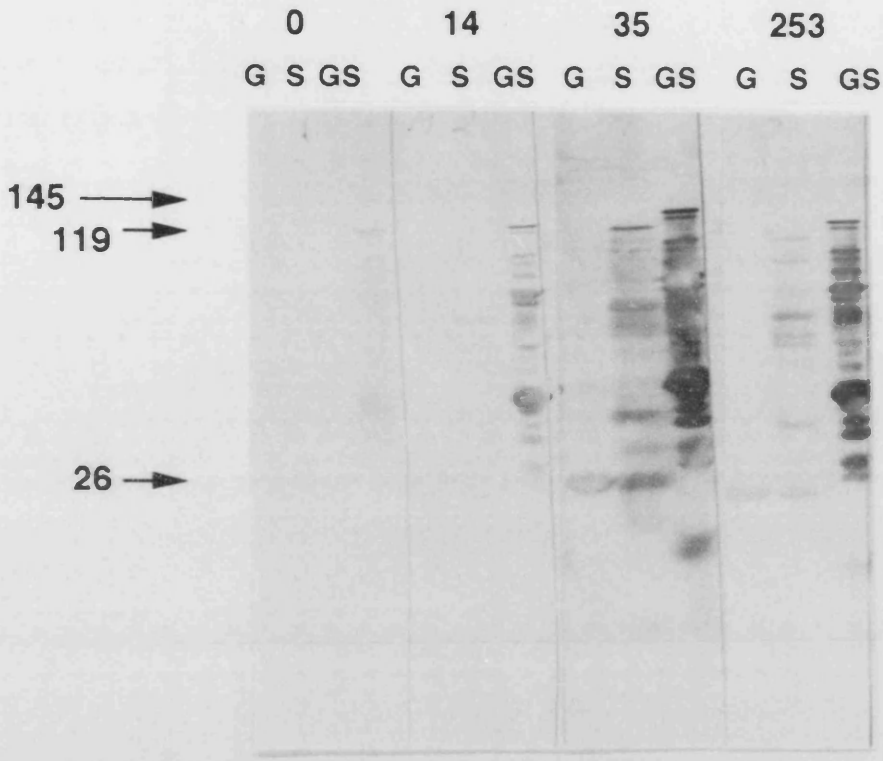
Western blot of a 12% SDS-polyacrylamide gel of GST-2.7, SPAG1 from cleaved fusion protein and recombinant GST in an unpurified *E. coli* lysate, developed with bovine *T. annulata* sporozoite immune sera. The sizes of the principal bands (kDa) are indicated by arrows. Tracks were loaded with the following fusion proteins (1μg per track);

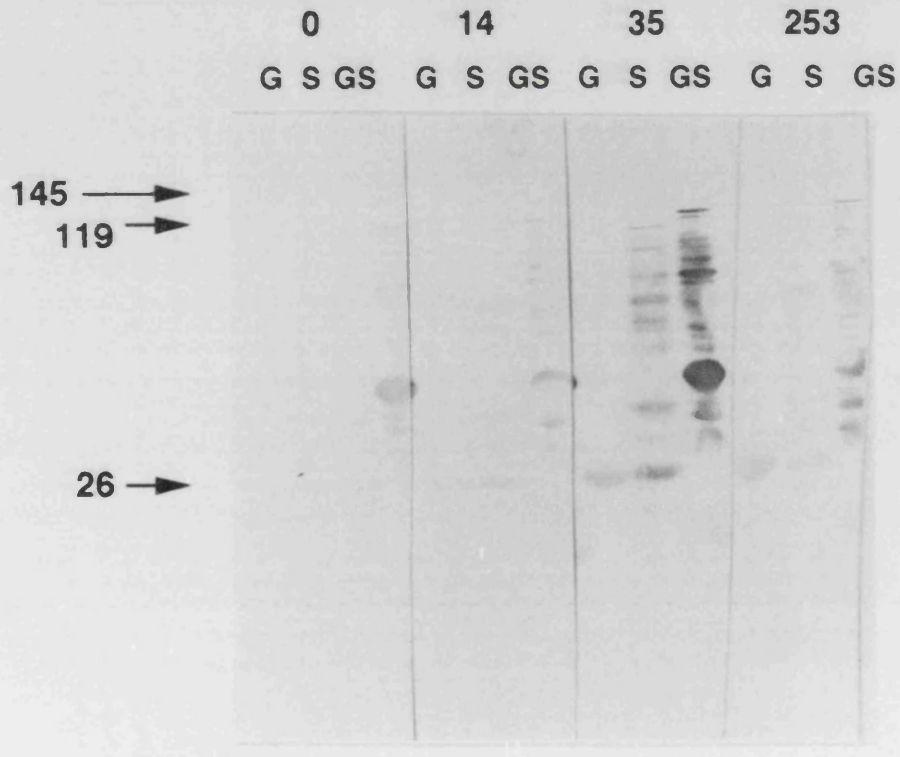
S SPAG1 from cleaved GST-2.7
GS GST-2.7
G GST in unpurified *E. coli* lysate

The primary antibody was GST-2.7 immune serum from cow 12045, collected on days 0, 14, 35 and 253 after immunisation, as indicated (1:100 dilution). The second antibody was alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300).

Figure 53B

See legend for Figure 54A for contents of tracks. The primary antibody was GST-2.7 immune serum from cow 12056, collected on days 0, 14, 35 and 253 after immunisation, as indicated (1:100 dilution).


0
14
35
253
G S GS G S GS G S GS G S GS
145
119
26


0
14
35
253
G S GS G S GS G S GS G S GS
145
119
26

L13 were tested at Week 4. In both cases the response to an identical concentration of the intact fusion protein was significantly higher (Student's T test; P>0.005). It is notable that when L9 was tested, the proliferative response recorded to the intact GST-2.7 was four times greater than that which occured to GST or SPAG1 alone. However, again the proliferation of these lines in the presence of GST as well as SPAG1 precluded the use of the GST fusion proteins in testing them.

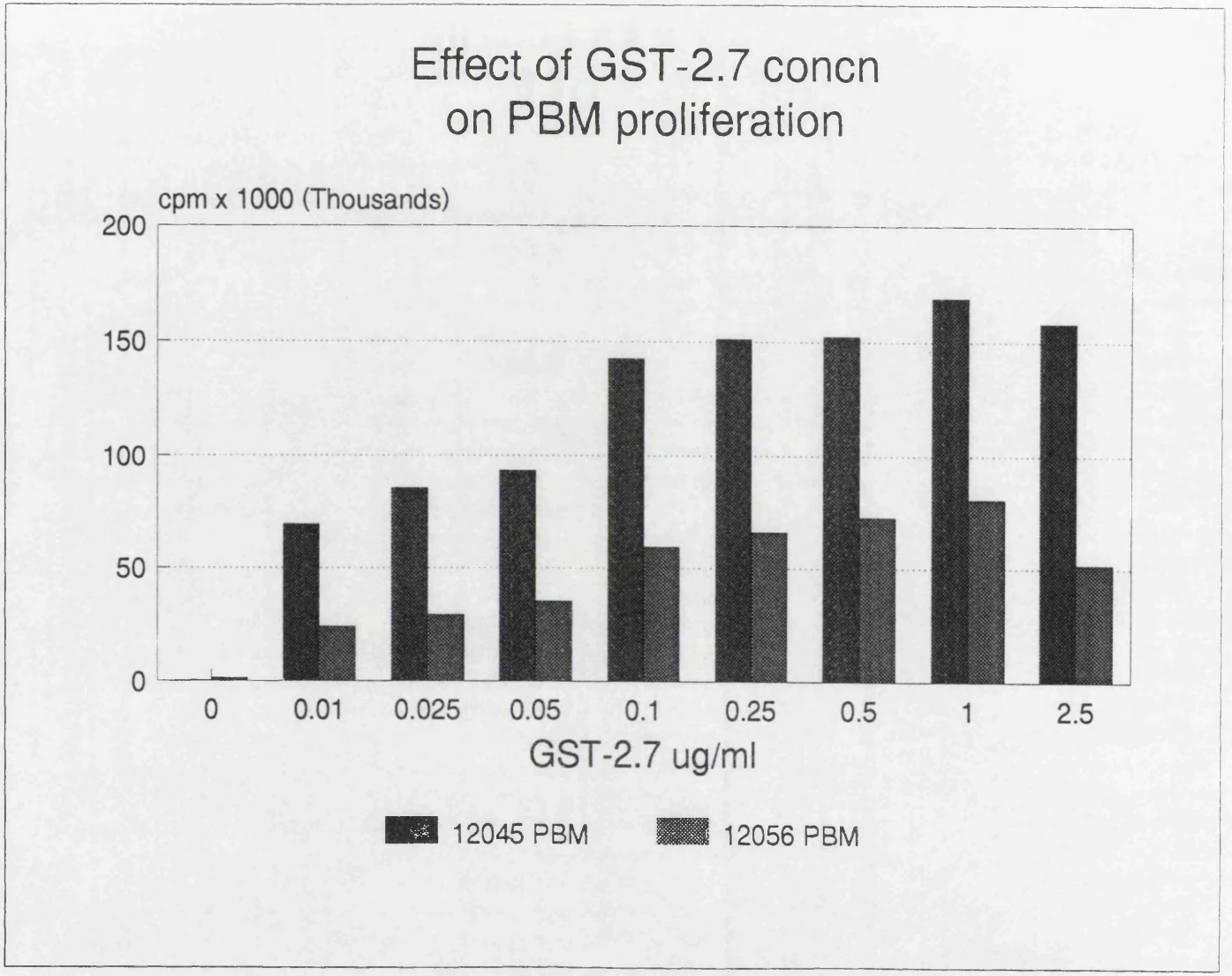
6.3 Discussion

The program TSites predicted a large number of amphipathic α helices and Rothbard and Taylor motifs throughout the length of the SPAG1 sequence (Figure 48). The large number of predicted epitopes probably relates to the size of the antigen, giving a wide potential T cell repertoire that could be directed against the recombinant protein. There were eighteen regions, however, where Rothbard and Taylor motifs overlapped with 15-20 residue amphipathic α helices, and so were perhaps more likely to be recognised by the immunised animals (Table 18). This pattern occurs at the N-terminus of SPAG1 (regions A-D), in each block of elastin homologous repeats (regions E and H), between the blocks of elastin repeats (regions F and G) in the C-terminal-to-central part of the molecule (regions I-Q) and in the C-terminal SR1 sequence (region R). The two regions in the blocks of amino acid residues homologous to bovine elastin would be unlikely to be recognised due to their identity to host antigens, resulting in the animal being tolerised to these parts of the molecule. A single overlapping region was also detected in GST, which could potentially be recognised by the animals immunised with the fusion protein.

In a number of cases where T cell epitopes have been mapped on parasite antigens, they were found to lie within polymorphic regions of the molecule. This has been found to be the case in the *P. falciparum* circumsporozoite protein, where the two main T helper epitopes map to regions of the molecule which are highly polymorphic in isolates even from the same geographical regions, and the polymorphism is limited to these areas only (Lockyer et al 1989). This was interpreted as indicating that polymorphic epitopes were being selected for by immune pressure,

Figure 54

Histogram to show the effect of GST-2.7 concentration on the degree of proliferation (cpm) of PBM taken from cattle 12045 and 12056, taken on day 98 after initial immunisation with GST-2.7 recombiannt protein.


Effect of GST-2.7 concn
on PBM proliferation
cpm x 1000 (Thousands)
200
150
100
50
0
0
0.01
0.025
0.05
0.1
0.25
0.5
1
2.5
GST-2.7 ug/ml
12045 PBM
12056 PBM

as discussed in section 3.3. This contrasts with the area of the molecule immunodominant for antibody recognition, which is highly conserved (Nussenzweig and Nussenzweig 1985). Extensive variation in the T cell epitopes of *P. falciparum* is thought to be a means by which the parasite avoids detection by human T cells, and is considered to be the key factor in the failure of the circumsporozoite protein as a vaccine so far (Good et al 1988).

Studies on the polymorphism of SPAG1 are described in Chapter 3. If the amino acid sequence comparison between the cSPAG1 and gSPAG1 alleles is taken to indicate polymorphic areas of the molecule (Figure 13), the most variant region lies between amino acid residues 100-550. Six of the SPAG1 regions predicted to have a good possibility of containing T cell epitopes given in Table 18, regions E-J, map to this polymorphic section of the antigen. Whether these epitopes can be detected experimentally remains to be determined. It is interesting that regions A and G contain a high proportion of amino acids identical to the homologous sequences in *T. parva* P67 sporozoite antigen (section 5.2.3) indicating that the two antigens could potentially cross-react at the T cell level.

When PBM from the sporozoite immunised cattle were tested in proliferation assays to GST-2.7, only animal 11434 gave a significant response. Serum from this animal also recognised the antigen on a Western blot, while sera from 11052 and 11055 produced a barely detectable reaction. The lack of reactivity of the11434 sera with GST and of 11435 serum with GST-2.7, and the fact that 11434 PBM did not proliferate in the presence of the unrelated antigen ovalbumin, indicate that 11434 produced a specific T and B cell response to SPAG1.

Data from the IFA titres against sporozoites, macroshizonts and merozoites indicate that the immunity of animals 11434, 11052 and 11055 was directed against all three stages of the parasite, which is presumably a result of the viable immunising sporozoites infecting lymphocytes and developing into subsequent stages. The anti-sporozoite immunity of 11052 and 11055 demonstrated by reactivity with acetone fixed sporozoites could well have been directed against sporozoite antigens other than SPAG1. Variation in the specificity and magnitude of

B and T cell responses to antigens is a common feature of outbred animal populations. For example, variation in the responses of cattle to the antigen ovalbumin has been correlated to their MHC Class II haplotype (Glass et al 1990). All three of the sporozoite immunised cattle had different MHC Class II haplotypes (Table 20), which could be a reason for their variable response to SPAG1.

PBM from cattle 11663 and 11659 taken on day 142 following initial immunisation with GST-2.7 gave a significant and apparently dose dependant responses to the fusion protein, although in the case of 11659 PBM the response was only significant at a final concentration of 10μg ml^{-1}. (Table 21). No proliferative responses were observed following the first immunisation and they still remained low even after the first boost. The proliferation assay set up using day 184 PBM from animal 11663 after a further boost showed significant dose dependant T cell responses to GST-2.7 and SPAG1. This indicates that the animal was reacting to T cell epitopes in the SPAG1 portion of the fusion protein. T cell epitopes also appeared to be recognised in the GST carrier, since the proliferative response to the complete fusion protein was considerably greater than proliferative responses to SPAG1 alone. Assessment of the serological response indicated that antibodies in the 11663 serum also recognised epitopes on both sections of the fusion protein.

When 11663 cell lines and clones were set up in varying concentrations of GST-2.7 and tested in T cell proliferation assays however, none of them responded to the uncleaved fusion protein, GST or SPAG1 (Table 22). The cell lines failed to respond to Concavalin A, indicating they may have been losing viability by the time they were tested, although four of the five clones did proliferate in response to Concavalin A. It is difficult to explain why the clones failed to proliferate in the assays when they had been expanding in culture in the presence of antigen, although the rate expansion and blasting of the cells which was initially very high, had decreased considerably after several weeks in culture when the tests were carried out.

FACS anaysis of the cell line L2 showed an increase in the proportion of BoT8+ cells and a decrease in the proportion of BoT4+ cells in subsequent weeks of culture (Table 23). The percentage of BoT2+ cells

also decreased, which was unexpected since this is a marker for most mature and immature T cells. The proportions of Class I and Class II positive cells remained fairly high, while the cells bearing macrophage, γδ or B cell markers became negligable. FACS analysis of the 5 clones set up from line L2 showed the BoT4+ marker to be almost absent, indicating they were not of the T cell inducer/ helper phenotype. Clones 2C3, 4E5 and 6E4 consisted almost entirely of cells bearing the BoT8+ marker, but the BoT2+ marker was almost absent in the populations. This is unexpected since the BoT2 molecule is found on most mature and immature T cells, of which cells positive for the BoT8+ antigen are a subset (Baldwin et al 1988). The other two clones 5F6 and 5F8 contained cells positive for the BoT2 marker, but a negligable proportion were positive for BoT4 or BoT8 antigens. The clones therefore appeared to possess an unusual combination of markers. Interestingly, there is a precedent for T cell populations with the unusual combination of markers Bo T2-, BoT4-, BoT8+; such cells have been identified in *T. parva* infections in cattle (Innes et al 1992).

As discussed in section 1.2.1, the BoT4+ and BoT8+ bovine leucocyte populations correspond in antigenicity and functional capabilities to the CD4+ and CD8+ cells in humans, the BoT4 marker tending to occur on T cells of the helper/inducer phenotype and the BoT8+ marker being identified primarily on cytotoxic or suppressor T cells. However, it should be borne in mind that the functional capabilities of BoT4+ and BoT8+ cells may not be as well defined as in analogous T cell populations in humans or mice. For example, there are documented cases for CD4+ cytotoxic cells in mice (Shinohara et al 1988) and cytotoxic BoT4+ cells have been identified in cattle (Baldwin et al 1988).

The fact that the 11663 lines only survived for a few weeks in culture appears to be associated with the increase in the proportion of BoT8+ cells during this period. While BoT4+ and BoT8+ cells are both capable of proliferation in culture with antigen, BoT8+ cells have been found to proliferate poorly to mitogens and antigen in the absence of IL-2 (TCGF), and generally at a lower rate than BoT4+ cells in the presence of IL-2 (Baldwin et al 1988). This is probably because BoT4+ cells and their analogues are capable of producing endogonous IL-2 wheras BoT8+ cells

are not, although the ability to produce IL-2 is likely to vary between different BoT4+ subsets. During culture of the 11663 lines IL-2 was added on alternate weeks, which appears to have been sufficient to allow some expansion of the BoT8+ subset. A high proportion of BoT8+ cells in the line could therefore have reduced the ability of the cell population as a whole to proliferate in long term culture. This would also explain the lack of response in the proliferation assays to soluble antigen, when IL-2 was not included. Another possible explanation for the failure of the proliferation assays is that the BoT8+ population included supressor cells able to inhibit BoT4+ responses.

When T cells are cloned, it has been found necessary to include IL-2 throughout the culture period to allow sufficient growth of the cells to maintain the culture (Sinigaglia et al 1991). This could explain why BoT8+ clones expanded so rapidly in the initial stages of culture. It has been noted that activated T cells can grow in IL-2 alone, but such cells usually cease growth after about two months (Fitch 1986). There is no satisfactory explanation for the results of the proliferation assays performed on the 11663 clones. IL-2 was also present in the proliferation assays, yet the clones failed to respond to antigen, even though most responded to Concavalin A. If their proliferation in culture was due to IL-2 alone, proliferation would be expected to occur in all the wells of the assay.

There are several possible origins for the BoT8+ cells in the clones and lines. They could have been cytotoxic or supressor cells produced specifically to the antigen but lacking the normal BoT2+ marker, or possibly have been cells proliferating non-specifically due to a mitogenic effect of SPAG1. Cytotoxic activity has been identified on numerous occasions in parasitic infections and examples will be discussed below. T supressor lymphocytes may be found within the CD4+ or CD8+ subsets in mice and humans, although their role in immune responses has been poorly characterised (Dorf et al 1992). T supressor responses have been identified which involve recognition of specific antigenic sequences, such as the response of mice to hen egg lyzozyme (Wicker 1984). Supressor activity has been identified in cattle, although this was not necessarily antigen specific (Smith et al 1981).

CD8+ type cells are stimulated when presented with the antigen in the context of MHC Class I. For this reason they are frequently induced in response to intracellular pathogens. For example, both BoT4+ and BoT8+ cells have been found to be involved in the cellular response to schizont infected lymphocytes of *T. parva* (Morrison 1987) and the cytotoxic response was found to be confined to the BoT8+ subset (Goddeeris 1986). Similarly, cytotoxic T lymphocyte responses have been identified to *T. annulata* schizont infected lymphocytes (Preston, Brown and Spooner 1983). The possibility that the BoT8+ lymphocyte induction was purely a feature of the recombinant antigen and did not reflect the T cell response to *T. annulata* sporozoites cannot be excluded.
For example, BoT4+ and BoT8+ T cell subsets were apparently both induced by a recombinant *Babesia bovis* merozoite antigen in cattle, but cells of the BoT4+ subset were only induced to the crude antigen extracted from merozoites (Tetzlaff et al 1992). It can be speculated that such anomalies could arise through differing antigen presentation and processing pathways, perhaps through interaction and competetion with other parasite antigens within the antigen presenting cell.

However, cytotoxic T cells have also been identified in response to a number of exogenous parasite stages. T lymphocytes of both the CD4+ and CD8+ subsets have been found to recognise epitopes on the circumsporozoite protein of *Plasmodium falciparum* (Kumar et al 1988) and of *P. berghei* (Romero et al 1989). There is also evidence for the induction of T lymphocytes in response to *Leishmania major* promastigotes (Müller et al 1989). However, in both the latter cases the CD8+ lymphocytes were thought to be induced by parasite antigens shared by both the exogenous and intracellular stages of the parasite. For example, the circumsporozoite protein has been identified on the surface of *P. falciparum* and *P. berghei* infected hepatocytes (Good et al 1988a, Romero et al 1989). The antigen may be shed during sporozoite entry (Good et al 1988a), where it could become associated with MHC Class I molecules. An alternative explanation was that the source of the antigen was the intracellular parasite (Romero et al 1989). For this to occur, the antigen would have to be transported into the cytoplasm of the infected cell from the parasitophorous vacuole before becoming associated with antigen processing pathways.

There are several possible means whereby sporozoite derived antigen could also be presented to cytotoxic cells in the context of MHC Class I. There is evidence that the *T. parva* sporozoite surface antigen P67 is shed during infection of lymphocytes, and persists for a time on the surface of the infected cell (Webster et al 1985). While the McAb 1A7, which is specific for SPAG1, fails to recognise the antigen on the surface of schizont infected lymphocytes, this cannot exclude the possibility of the antigen's persistance on the surface of the host cell for a short time following infection (to be discussed further in Chapter 7). Another possibility is that SPAG1 could occur on the surface of infected cells in a processed form not recognisable by the monoclonal antibody. SPAG1 could also be processed and presented with MHC Class I molecules on the surface of macrophages which had engulfed antibody opsonised sporozoites, and so be able to stimulate BoT8+ and/or BoT4+ lymphocytes. It is thought that association with Class I molecules occurs in the cytoplasm of macrophages, wheras association with Class II takes place in another cell compartment such as the Golgi apparatus (reviewed by Ash 1991). It has been shown in the case of *Toxoplasma gondii*, which like *T. annulata* infects macrophages, that parasites coated in antibody lose their resistance to lysosomal attack, rendering their antigens capable of being processed (Ash 1991).

The recombinant SPAG1 could have been able to induce a CD8+ response by being processed in a similar way. While the antigen was soluble in PBS, it would have been rendered insoluble by the adjuvants used when immunising the cattle. Adjuvants such as alum and oil based emulsions (eg. FIA, FCA) render the antigen particulate, enabling it to be phagocytosed by macrophages (Janeway 1989) as discussed in section 1.2.6. If the recombinant antigen was phagocytosed by macrophages in this way, it could have been presented with MHC Class I as described above.

Another possible origin of the BoT2-BoT8+ cells is through non-specific cell proliferation induced by binding of SPAG1 to the cell surface. The binding of recombinant SPAG1 to the surface of cells bearing the MHC Class II marker, the target cell population for *T. annulata* sporozoites, has been demonstrated (Personal communication; F. Katzer, Department of Biology, University of York), although this binding was not

to the MHC Class II antigen itself. Certain mitogens, such as Staphylococcal enterotoxins, have been shown to induce lymphocyte proliferation through "bridging" MHC Class II molecules (Ash 1991). It is conceivable that SPAG1 could also have a mitogenic effect on lymphocytes through cross linking of receptor molecules on the cell surface. While the receptor molecule for SPAG1 has not been verified at present, infection of lymphocytes by *T. parva* sporozoites has been shown to involve binding to MHC Class I molecules, presumably through interaction with an antigen on the sporozoite surface (Shaw et al 1991).

If SPAG1 did cross-link molecules on the surface of the host cell, this might alter the surface markers and so explain the unusual FACS profiles of the 11663 clones. Indeed, it is notable that during infection of cattle with *T. parva*, a predominant population of proliferating but uninfected cells which were BoT2-BoT4-BoT8+ have been identified (Innes et al 1992). Proliferating uninfected cells are also a common feature in cultures of *T. annulata* infected lymphocytes (Glass and Spooner 1990b). The origin of these cells is not known at present; their proliferation may be induced by interaction of shed parasite antigens with their surface receptors, although a more likely explanation is that this is the result of growth-inducing factors secreted by infected lymphocytes. Secretion of soluble factors postulated to be IFNγ by *T. annulata* schizont infected lymphocytes has been demonstrated (Preston, Brown and Spooner 1983).

When cattle 12045 and 12056 were immunised with GST-2.7, a PBM proliferative response to the antigen was observed by day 14 after the initial immunisation (Tables 24 and 25), at a much earlier stage than with the animals 11663 and 11659. This would normally be sufficient time for priming of peripheral blood T cells to occur, an event which normally takes about five days following immunisation "in vivo" (Janeway 1989). In the case of 11663 and 11659, PBM proliferative responses to the antigen were not observed until after the first boost. It appears that priming of the T cells occured much more effectively in 12045 and 12056, probably as a result of using Freund's complete adjuvant as opposed to Freund's incomplete adjuvant. The use of different adjuvants can have a major influence on both the T and B cell responses to antigens, possibly by effecting the way in which they are processed (Hui et al 1991).

Higher levels of proliferation to GST-2.7 were recorded in both cattle after boosting on day 28, and were maintained in all subsequent assays even several months later, without the animals being boosted further. PBM proliferation could be detected at GST-2.7 concentrations as low as 10ng ml^{-1} (Figure 54), indicating that the antigen was highly effective in provoking a cellular response in these animals. Antibodies were also produced to GST-2.7 and SPAG1 polypeptide according to Western blot analysis (Figures 53A and 53B), with a considerable increase in reactivity to GST-2.7 and isolated SPAG1 following the day 28 boost. Since both GST and SPAG1 were recognised as well as the intact fusion protein, this indicates that the sera contained antibodies reactive to B cell epitopes on both sections of the fusion protein. These antibodies were still present in the sera on day 253 after the initial immunisation.

Overall, the animal 12045 appeared to show a more effective immunological response to GST-2.7 than 12056. PBM proliferation was at a higher level in all the antigen concentrations tested and the bands recognised by the sera on the Western blot were much stronger. A possible explanation for these differences is the effect of the MHC Class II haplotypes of the cattle on their immune responsiveness to this antigen (Table 20B), as discussed previously in this section.

When 12045 PBM was tested against a variety of different GST-SPAG1 constructs (Table 26) the proliferative response to all of them was found to be indistinguishable from the response to GST alone, indicating that T cell epitopes were being recognised on the GST carrier instead of or in addition to epitopes on the SPAG1 sequence. This made it impossible to map epitope containing regions based on PBM reactivity.

The L4 and L5 cell lines from 12045, like those of 11663, were found to contain about 20% BoT8+ cells when they were first set up (Table 27). Four weeks after removal of this subset by the anti-BoT8 McAb CC63, the cell lines were found to consist almost exclusively of BoT4+ BoT2+ cells, so it seemed that removal of the BoT8+ cells had facilated expansion of the T helper/inducer subset.

All of the cell lines tested at the end of week 1 in culture proliferated in the presence of GST-2.7 at all the concentrations tested. However, the 12045 cell lines L7, L8 and L9 also responded to "self " PBM in the absence of antigen, indicating non-specific proliferation was taking place. This seemed to be confirmed by the fact that when L7 was tested again 4 weeks later, the cells proliferated in the presence of antigen. Probably the assays at 4 weeks in culture are a better indication of cell line reactivity, as by this stage the lines would probably be more uniform in cell type.

The proliferative responses of cell lines L4 and L12 tested after 4 weeks in culture to GST and SPAG1 as well as the intact fusion protein indicated that T helper cell epitopes were being recognised in both parts of the fusion protein (Tables 28 and 29). It is interesting that the cell line L13 gave a much higher response to the intact fusion protein than to GST or SPAG1 independantly. This suggests that cleavage of the fusion protein had an effect on the T cell epitopes being recognised, perhaps due to location of epitopes near the cleavage site. The group of epitopes predicted near the N-terminal region of SPAG1 by the TSites algorithm would be in this region. The line L7 tested at 4 weeks showed a higher proliferative response to GST than to identical concentrations of GST-2.7 and SPAG1, indicating that T cell epitopes recognised in this line were located primarily on the GST section of the fusion protein. The variability in the cell lines of their proliferative responses to the three antigens indicates that a number of different T cell epitopes were capable of being recognised on the GST-2.7 fusion protein. The response of all of the cell lines to GST precluded their use in mapping regions of specificity by testing with other GST-SPAG1 fusion proteins.

To summarise, the data presented in this chapter demonstrates that a strong cellular proliferative response can be evoked to GST-2.7 in cattle immunised with the recombinant protein. The recombinant antigen also appears to induce production of memory T cells, since proliferation in the assays set up after boosting was noticably increased (Tables 21, 24 and 25), indicative of a secondary response. Data from the 12045 and 12056 lines tested in proliferation assays indicated that at least a proportion of the epitopes recognised are located on the SPAG1 molecule. Reactivity of the sporozoite immunised animal 11434 with

GST-2.7 indicated that epitopes were also recognised on the native SPAG1 antigen. Since the cell line L4, which was almost entirely BoT4+, responded to SPAG1 in proliferation assays, it appears that T helper/inducer epitopes are among those recognised on SPAG1.

The high proportion of BoT8+ cells in lines set up in the presence of GST-2.7 indicates that epitopes could also recognised on the fusion protein by the supressor or cytotoxic T cell subsets, unless these cells originate from non-specific interactions. Whether or not this is also a feature of the true parasite infection remains to be determined.

The induction of high proliferative responses to the GST portion of the fusion protein indicates that it was inappropriate to immunise cattle with SPAG1 expressed as a GST recombinant. It would be preferable to immunise cattle with SPAG1 produced in a vector capable of expressing the fusion protein directly, or to cleave large quantities of the GST fusion protein. The possibility of using different vector systems will be further discussed in Chapter 9. The SPAG1 proliferative responses of cattle immunised with sporozoites should also be further examined. Since the native sporozoite antigen could be processed differently from recombinant SPAG1, this could profoundly effect the T cell epitopes recognised by the host animal, and the T cell subsets that are induced.

TABLE 18. REGIONS OF SPAG1 PREDICTED TO HAVE A HIGH PROBABILITY OF CONTAINING EPITOPES

The following table lists eight regions of the SPAG1 amino acid sequence where one or more 15-20 residue amphipathic α helices overlap with a Rothbard and Taylor motif according to analysis with the computer algorithm "TSites", shown in Figure 48.

REGION	AMINO ACIDS	REGION	AMINO ACIDS
A	5-9	J	520-530
B	51-54	K	575-578
C	58-64	L	605-612
D	74-78	M	637-644
E	217-223	N	674-678
F	278-281	O	690-694
G	313-316	P	709-716
H	449-452	Q	740-747
I	513-516	R	852-856

TABLE 19. PBM PROLIFERATION ASSAYS; CATTLE 11052,11055,11434 AND 11435

The following table shows the results obtained in proliferation assays using PBM taken from cattle 11052, 11055 and 11434, which were immunised with *Theileria annulata* (Hissar) sporozoites, 11435, which was not immunised and 5692, immunised with ovalbumin. The mean CPM from each group of four wells are shown, with the sample standard deviations in brackets. Final concentrations of test proteins are given in $\mu g\ ml^{-1}$.

MED= medium only; OVB=ovalbumin ; G-2.7= GST-2.7 fusion protein
Proliferation significantly higher than in medium alone is indicated by;
* $P > 0.05$, ** $P > 0.005$.

		ANIMAL NO.				
ANTIGEN	CONCN.	5692	11052	11055	11434	11435
MED	0	13255 (5162.8)	1679 (1316.8)	3670 (907)	6165 (3025.6)	17357 (6821.7)
OVB	1	151349** (47843)	1391 (1661.6)	3508 (1350.5)	2639 (68.5)	13017 (8496)
G-2.7	0.1	12169 (5065)	1798 (1105.3)	3408 (1430.6)	24294 (18177)	13837 (10332)
	1	9350 (5153)	2862 (1481.4)	3010 (1362.6)	52879* (25843)	13622 (4039)
	10	10344.8 (4139.9)	2305 (1387)	5026 (973.9)	59623** (16291.4)	14956 (4038)

TABLE 20A. MHC HAPLOTYPES OF SPOROZOITE IMMUNISED CATTLE

The following table gives the MHC Class I and II haplotypes of the cattle 11052, 11055, 11434 and 11435, as determined by serotyping and one-dimensional iso-electric focussing (Personal communication: Dr. E. Glass, AFRC Roslin Institute, Edinburgh)

ANIMAL NO.	CLASS I	CLASS II
11052	17 18	2 6
11055	11 eu27	8 ?
11434	19 20	3 7
11435	"	"

TABLE 20B. MHC HAPLOTYPES OF GST-2.7 IMMUNISED CATTLE

The following table shows the MHC Class I and Class II haplotypes of the four cattle immunised with GST-2.7, determined as described above.

ANIMAL NO.	CLASS I	CLASS II
11659	6,18	2,13
11663	10	13
12045	11,14	7,8
12056	14,20	6,13

TABLE 21. PBM PROLIFERATION ASSAYS; CATTLE 11663 AND 11659

The following table shows the results obtained in proliferation assays using PBM taken at 33, 73, 142 and 184 days post GST-SPAG1 immunisation from cattle 11663 and 11659, and from 5692, a control immunised with ovalbumin. The mean CPM obtained from each group of four wells is shown, with the sample standard deviations in brackets. Final concentrations of test proteins are given in $\mu g\ ml^{-1}$. " - " = not tested

CON A = Concanavalin A; OV = ovalbumin; G-2.7 = GST-2.7 fusion protein; SPAG1 = SPAG1 alone; MED = medium only.
Proliferation significantly higher than in medium alone according to Student's T test is indicated by ; ** = P > 0.005 and * = P > 0.05.

		MED	CON A	OVB		G-2.7				SPAG1		
			1	1	10	.1	1	5	10	0.1	1	10
11663	d.33	1457 (476.1)	-	2858 (2668.2)	2232 (1347.5)	-	718 (437.1)	811 (282.1)	1380 (980)	-	-	-
	d.73	2758 (1281)	-	4485 (1256.2)	10715* (1374.7)	2899 (1096.1)	5172 (1842.1)	-	5816* (1621.9)	-	-	-
	d.142	337 (142.1)	112442** (13709.9)	257 (108.7)	738 (286.8)	758* (180)	1170* (557.2)	-	12332** (2594.2)	-	-	-
	d.184	417 (131)	43099** (16564)	748 (355)	741 (265.4)	8807* (4816)	34211** (14247)	71242** (12356)	40720** (13033)	782 (346)	3624 (1876.4)	16495** (6192.3)
11659	d.33	3369 (1537.4)	-	1663 (1135)	2570 (703)	-	167 (102.8)	-	2158 (556.2)	-	-	-
	d.73	4516.7 (992)	-	6082.2 (2228)	7472 (2324)	7099 (2634)	14241* (7490)	-	18101* (7317)	-	-	-
	d.142	1128 (133.6)	203818** (5924)	1128 (134)	951 (64)	1407 (672)	2406 (1980)	-	21216** (5206)	-	-	-
5692	d.73	13572 (4419)	-	80944** (9403)	84750** (4438.6)	9699 (5513)	17568 (1399.8)	-	12932 (5168.6)	-	-	-
	d.142	1558 (1216.8)	109056** (32079.6)	101370** (26033.5)	117274** (10388.8)	4401 (857.5)	-	-	5803 (3551.6)	-	-	-

TABLE 22. PROLIFERATION ASSAYS; 11663 CLONES

The following table shows mean CPM from each group of four wells in proliferation assays using clones and a cell line cultured from 11663 PBM (L2) in the presence of GST-2.7 fusion protein. All the test proteins were used at a final concentration of $1\mu g\ ml^{-1}$. - = not tested.

MED = medium only; CONA = Concanavalin A; PBM = irradiated PBM only; G-2.7 = GST-2.7 fusion protein; SPAG1 = SPAG1 from cleaved fusion protein, OVB = ovalbumin.
Proliferation significantly higher than in medium alone is indicated by; *; P > 0.05; ** P > 0.005. The standard deviations are shown in brackets.

	LINE L2		2C3	4E5	5F6	5F8	6E4
	WK1	WK3					
MED	153 (42)	417 (96)	563 (364)	6338 (1989)	687 (307)	1611 (823)	1522 (633)
CONA	113 (56)	209 (117)	39094** (2524)	43123** (1723)	4679 (689)	14250** (2634)	4663* (42)
PBM	150 (33)	-	344 (516)	3455 (2804)	572 (61)	2053 (100)	686 (97)
GST	187 (39)	215 (56)	2214 (1483)	11795 (7284)	1148 (588)	5280 (5153)	2067 (1402)
G-2.7	253 (41)	312 (22)	3967 (569)	1489 (1225)	1642 (1841)	8808 (1216)	666 (552)
SPAG1	155 (26)	176 (44)	6860 (1029)	1088 (1376)	899 (265)	3003 (322)	704 (301)
OVB	130 (32)	162 (46)	952 (799)	1367 (1417)	2867 (252)	2596 (92)	-

TABLE 23. DATA FROM FACS ANALYSIS OF 11663 CLONES

The following table shows the percentage of the cell population sampled carrying each marker. Details of the McAbs and the markers they recognise are given in the Appendix (Table C). - = not tested. The 4H5 control McAb was specific for *T. annulata* infected cells.

		PBM	LINE L2			2C3	4E5	5F6	5F8	6E4
McAb	MARKER		WK1	WK3	WK5					
None	-	<4	<4	<4	<4	<4	<4	<4	<4	7
ILA-19	MHC Cl I	82	74	99	85	96	77	84	94	100
J11	MHC Cl II	40	90	63	54	8	15	-	<4	18
CC42	BoT2+	44	49	34	32	<4	7	71	44	11
ILA-12	BoT4+	34	37	38	12	<4	10	5	<4	10
ILA-51	BoT8+	9	9	17	44	90	92	3	<4	98
ILA-29	γδ TCR	6	8	8	5	<4	-	-	-	11
ILA-30	IgM Bcells	32	11	9	5	7	-	-	-	-
ILA-24	Macrophage	5	9	<4	<4	5	-	-	-	-
4H5	Control	<4	<4	<4	-	-	-	-	-	-

TABLE 24. PBM PROLIFERATION ASSAYS; COW 12045

The following table shows the results obtained in proliferation assays (mean cpm from quadruplicate wells) against GST-2.7 using PBM taken at intervals (days post immunisation) from animal 12045. Standard deviations are shown in brackets. Final concentrations of GST-2.7 are given in $\mu g\ ml^{-1}$. - = not tested

Proliferative responses to GST-2.7 significantly higher than in medium alone according to Student's T-test are indicated by ; *; $P > 0.05$, **; $P > 0.005$.

	GST-2.7 FINAL CONCENTRATIONS						
	0	0.1	0.5	1	2.5	5	10
DAY 0	29.8 (3.8)	-	-	30.8 (2.2)	-	30 (5.4)	115 (13.6)
14	2070 (937)	-	-	28230** (4684)	-	19790** (2028)	18270** (4846)
21	1676 (1120)	-	13816** (2626)	16677** (3292)	25555** (5542)	20993** (3166)	32007** (6769)
28	804 (127)	-	5986** (1635)	11325** (3773)	11561** (5089)	13963** (7363)	16568** (8786)
35	72 (47)	-	13353** (1722)	11747** (3467)	17884** (1283)	16969** (1610)	-
42	1013 (471)	-	-	24356** (3873)	21652** (6295)	24429** (6269)	92353** (1868)
56	2985 (3390)	-	-	46334** (8335)	62819** (2094)	62663** (6269)	-
70	9936 (5624)	-	72807** (5925)	71989** (7792)	62818** (2094)	-	-
98	237 (145)	142483** (8224)	152419** (4390)	168782** (8294)	157924** (13699)	-	-
105	7315 (1087)	157309** (17712)	176908** (6316)	152761** (9228)	-	-	-
126	483 (101)	173817** (9988)	149150** (6651)	130275** (12830)	-	-	-

TABLE 25. PBM PROLIFERATION ASSAYS; 12056

The following table shows the results from proliferation assays (mean cpm from quadruplicate wells) against GST-2.7 using PBM taken at intervals (days post immunisation) from animal 12056. Sample standard deviations are shown in brackets. Final concentrations of GST-2.7 are given in $\mu g\ ml^{-1}$. - = not tested

Proliferative responses to GST-2.7 significantly higher than in medium only according to Student's T-test are indicated by ; *; $P > 0.05$, **; $P > 0.005$.

	GST-2.7 FINAL CONCENTRATIONS						
	0	0.1	0.5	1	2.5	5	10
DAY 0	30.3 (5.5)	-	-	68.8 (30)	-	32.8 (5.9)	27.8 (4.7)
14	250 (130)	-	-	8598** (2329)	-	11050* (3778)	41284* (4986)
21	2448 (990)	-	30960 (9164)	39958* (9164)	41635** (4453)	30080 (11466)	39664** (2608)
28	3748 (1169)	-	5088 (2660)	22253 (4416)	13865 (923)	31929* (9292)	34223* (6767)
35	1115 (669)	-	20239** (3707)	14410** (3337)	17913** (6686)	23024** (7911)	-
42	10304 (2409)	-	-	104138** (11805)	86506** (24210)	137429** (12929)	137611** (11234)
56	592 (488)	-	-	15131** (4909)	-	12956** (2883)	-
70	2925 (1208)	69755** (4324)	62542** (4324)	62542** (9400)	70659** (2055)	-	-
98	1398 (620)	59444* (6104)	72714** (12833)	80628** (10309)	51716** (21734)	-	-
126	178 (39)	59353** (15553)	77894** (16815)	74305** (23870)	-	-	-
175	5610 (4375)	-	71221** (31070)	85996** (35254)	-	-	-
224	354 (132)	-	43526** (21939)	60853** (14362)	76004** (5015)	68851** (15584)	-

TABLE 26. EFFECT OF SPAG1 CONSTRUCTS ON PBM PROLIFERATION

The following table shows the results from a proliferation assay (mean cpm from quaruplicate wells) using 12045 PBM taken on Day 112 against a variety of GST fusion proteins covering different regions of SPAG1. Sample standard deviations are shown in brackets. Concentrations are in $\mu g\ ml^{-1}$. - = not tested.
G-2.7 = GST-2.7, G-2.1= GST-2.1, G-0.3= GST-0.3; G-HB5=GST-HB5, G-0.8= GST-0.8 and *E. coli*= lysate from untransformed *E.coli* XL Blue.

Mean proliferation in medium with no antigen added was 397 cpm (standard deviation 88).

	FINAL CONCENTRATION			
ANTIGEN	0.25	0.5	1	2.5
G-2.7	17603 (21483)	183956 (24271)	191642 (81926)	126996 (20365)
GST	-	-	178306 (18117)	189055 (12273)
G-2.1	-	-	190122 (7924)	191475 (5768)
G-0.3	-	-	188071 (9738)	159298 (36467)
G-HB5	-	-	168564 (33004)	183664 (3260)
G-0.8	-	-	18714 (8160)	187594 (18380)
E.coli	-	-	2682 (3539)	2961 (2867)

TABLE 27. FACS ANALYSIS OF 12045 LINES L4 AND L5

The following table shows the percentage of the cell population sampled carrying each marker according to FACS analysis before (Week 1 in culture) and after (Week 5 in culture) BoT8+ depletion. GST-2.7 concentrations in which lines were cultured are given in $\mu g\ ml^{-1}$. - = not tested

		WEEK 1 (+BoT8)		WEEK 5 (-BoT8)	
McAb	MARKER	L4 - 1.0	L5 - 2.5	L4 - 1.0	L5 - 2.5
IL-A19	CLASS I	99	99	98	97
IL-A12	BoT4	47	48	89	95
IL-A29	γδ TCR	13	12	<4	<4
IL-A51	BoT8	19	17	<4	<4
VPM 30	B CELLS	10	10	<4	<4
J11	CLASS II	-	-	94	93
IL-A21	"	-	-	91	96
IL-A26	PAN T	-	-	94	91
J4	CD18	-	-	94	98

TABLE 28. PROLIFERATION ASSAYS; 12045 LINES

The following table shows mean cpm obtained in the presence of test proteins, "self" PBM and medium alone using GST-2.7 cell lines tested after 1 or 4 weeks in culture. Sample standard deviations are shown in brackets. Final concentrations of the test proteins, and the concentrations of GST-2.7 in which the cell lines were cultured, are shown in $\mu g\ ml^{-1}$. All the lines were set up on day 156 after the initial immunisation, except L4 which was set up on day 28. - = not tested

The mean cpm obtained was tested for significance against proliferation with PBM alone: * $P > 0.05$, ** $P > 0.005$ according to Student's T-test..

MED = no antigen, medium only, PBM = "self" PBM only, G-2.7 = GST-2.7 fusion protein, SPAG1 = SPAG1 purified from cleaved GST-2.7

	L4 - 1.0	L6 - 0.1	L7 - 0.25		L8 - 0.5	L9 - 1.0
ANTIGEN	WK4	WK1	WK1	WK4	WK1	WK1
MED	750 (149)	145 (26)	240 (34)	6120 (914)	6869 (1158)	1026 (94)
PBM	1636 (79)	16112 (9291)	57601 (3063)	9600 (302)	117765 (11345)	32953 (5053)
G-2.7 0.1	-	55550** (8648)	76478 (7841)	-	102361 (7895)	44306 (12077)
0.25	14783** (2452)	55444** (8648)	84806 (6744)	14273** (2702)	96115 (11042)	45610 (14983)
0.5	23365** (1990)	53008** (1523)	81220 (75641)	15587** (1610)	95509 (6622)	46032 (10606)
1	21425** (2149)	51916** (5487)	75641 (5432)	13642** (2499)	81813 (5517)	43253 (6703)
GST 1	13578** (1955)	38685* (8034)	65492 (8133)	18281** (960)	91238 (6670)	46755** (434)
SPAG1 0.1	11824** (2388)	-	-	11798** (1046)	-	-
0.25	15067** (1212)	-	-	19276** (1933)	-	-
0.5	16828** (2924)	-	-	14434* (5666)	-	-
1	13018** (2186)	-	-	9002* (1230)	-	-

TABLE 29. PROLIFERATION ASSAYS; 12056 CELL LINES

Legend as for Table 28. All the lines were established on day 156 after initial immunisation.

	L10 - 0.1	L11 - 0.25	L12 - 0.5		L13 - 1.0	
ANTIGEN	WK1	WK1	WK1	WK4	WK1	WK4
MED	179 (52)	145 (49)	206 (35)	1248 (404)	288 (78)	2495 (459)
PBM	647 (337)	13176 (1883)	7195 (4257)	12469 (1660)	2100 (763)	3272 (170)
G-2.7 0.1	12283** (4567)	39989** (2867)	53062** (3631)	-	23752** (5322)	-
0.25	26608** (4364)	52712** (7894)	62801** (11729)	94465** (7099)	40795** (11750)	26378** (6931)
0.5	19131** (2240)	46935** (4201)	34959** (6351)	65415** (4014)	20315** (7430)	15864** (799)
1	32378** (3560)	65146** (6075)	77341** (11171)	87685** (19726)	51157** (7841)	19863** (2309)
GST 1	9283** (434)	29631** (3441)	29925** (2037)	25927** (6350)	222538** (825)	5217** (406)
SPAG1 0.1	-	-	-	12873** (4999)	-	4759** (768)
0.25	-	-	-	26041** (3421)	-	6552** (872)
0.5	-	-	-	-	-	6824** (820)
1	-	-	-	-	-	4877** (376)

CHAPTER 7

SPAG1 AND LYMPHOCYTE INVASION

7.1 Introduction

The neutralisation of *T. annulata* sporozoite infectivity for bovine lymphocytes "in vitro" by both McAb 1A7 (Williamson et al 1989) and bovine GST-2.7 antisera (described in section 5.2.2) suggest that the SPAG1 molecule has a role in host cell recognition and/or invasion. The neutralising effects of anti-SPAG1 antibodies, together with the reactivity of McAb 1A7 with viable sporozoites in indirect IFA tests (Williamson et al 1989) also imply that the antigen has a surface location. It was decided to further characterise the functional role of SPAG1 by examining the effect of SPAG1 fusion protein on invasion of lymphocytes "in vitro", and investigating the location and fate of the antigen during host cell invasion using immuno-electron microscopy techniques.

Immuno-electron microscopy has been used to identify the location of numerous protozoan parasite antigens by a variety of techniques. In general, the parasite material is preserved under conditions which cause minimal damage to epitopes of the antigen being investigated, before incubation with primary antibody, which may be a McAb or antisera, followed by incubation with secondary antibody conjugated to electron dense colloidal gold particles. Embedding and sectioning may be carried out before or after the antibody incubations, depending on how effectively the material can be preserved prior to antibody labelling without losing it's immunogenicity. The technique of cryo-ultramicrotomy followed by antibody labelling of sections has been used to identify the location of a *T. annulata* merozoite surface antigen (Glascodine et al 1990) and a *T. parva* sporozoite surface antigen (Webster et al 1985). Expression of a *P. falciparum* antigen (RESA) on the surface of infected erythrocytes has been studied by labelling sections of embedded, glutaraldehyde-fixed material (Culvenor et al 1991). This method was only possible because

the epitopes recognised by the primary antibody were not damaged by glutaraldehyde fixation.

The material for the immuno-electron microscopy work described here was produced by infecting bovine lymphocytes with *T. annulata* sporozoites "in vitro" and taking samples at specific timepoints. The location of SPAG1 was assessed by immuno-labelling with McAb 1A7. It was decided to use a "whole cell" surface labelling technique rather than embedding the material and taking sections. Briefly, this involved fixation of the pelleted sporozoites and lymphocytes in minimal concentrations of formaldehyde or glutaraldehyde, incubation of the whole cells with McAb 1A7 followed by colloidal gold conjugated second antibody, then post-fixation in glutaraldehyde, dehydration, embedding and sectioning. While this method can only provide information about the location of the antigen on the surface of the cells rather than on internal structures, it obviates the need for cryo-ultramicrotomy which requires a considerable amount of expertise to perform, or harsh fixation techniques with the capacity to damage epitopes prior to immunolabelling. The initial fixation of whole cells was carried out in three different concentrations of formaldehyde and glutaraldehyde since their effect on antibody binding by 1A7 was not known, although the reaction of the antibody with formalin fixed sporozoites in IFA tests indicated this fixative did not damage epitopes excessively.

The invasion of lymphocytes by *T. annulata* sporozoites has been shown to be an active process, inhibited "in vitro" at a temperature of 0°C (Jura 1984). Since the process occurs within a few minutes at 37°C (Jura et al 1983), it was decided to carry out invasion at the lower temperature of 22°C in an attempt to slow down the rate of sporozoite entry, thus providing a higher yield of sporozoites undergoing the entry process at the time of fixation for immuno-electron microscopy. A small-scale trial experiment was performed initially to examine the effect of carrying out sporozoite invasion at 22°C, assessing the rate of infection from Giemsa stained cytospin smears.

7.2 Results

7.2.1 Effect of SPAG1 fusion protein on sporozoite infectivity

Fusion protein inhibition assays were set up as described in section 2.2.10, assessing the percentage of PBM that became infected with sporozoites by examination of Giemsa stained cytospin smears after four days in culture. Recombinant GST-2.7, GST alone or SPAG1 polypeptide generated from Factor Xa cleavage of the GST fusion protein were used in the assay at final concentrations ranging from 0.1 to 1mg ml^{-1}. An assay was also set up using the McAb 1A7 as a positive control. The percentage inhibition by the fusion proteins was calculated by comparison with cultures which received no fusion protein or were incubated with GST; the results are summarised in Table 30. Sporozoite infectivity in both the control cultures was high, between 29-36% compared with the more commonly observed 10-20% (Jura et al 1983), and all of the 4 day old cultures contained a number of dead cells. The McAb 1A7 produced the expected high level of inhibition. The fusion proteins GST-2.7 and SPAG1 produced some inhibitory effect when compared to infection in medium alone or in the presence of an identical concentration of GST, although this was no more than 50%. There was no obvious concentration effect of GST-2.7 on the level of inhibition, perhaps indicating that the SPAG1 receptors of the leucocytes were saturated.

7.2.2. Effect of temperature on sporozoite invasion "in vitro"

A trial experiment was carried out to assess the appropriate temperature and timepoints at which to take samples for a larger scale invasion assay, in order to provide material for the immuno-electron microscopy work. Sporozoite invasion assays were set up at 4°C, 22°C and 37°C as described in section 2.2.10, using *T. annulata* Ankara sporozoites prepared from 3 day fed ticks. Samples were taken at time intervals ranging from 5 minutes to 18 hours for preparation of Giemsa stained cytospin smears. Counts were made of the percentage of PBM containing internalised sporozoites or trophozoites, and the percentage of

cells which appeared to have sporozoites adhering to their surface. The results are summarised in Table 31.

Infection appeared to be almost absent in the assay carried out at 4°C, while infected lymphocytes containing internalised sporozoites or trophozoites could be observed in the cultures set up at 22°C and 37°C. While there was not a great difference in the percentage of cells containing internalised sporozoites at 22°C and 37°C, trophozoites were noted earlier in the 37°C culture. This indicated that the lower temperature of 22°C was slowing down the infection process at some point. Sporozoites adhering to the surface of lymphocytes were observed in all the cultures and occured in highest numbers at 37°C, but whether they were actually undergoing invasion or only adhering to the lymphocytes as a result of the cytospin process was impossible to verify at the light microscope level. Since invasion obviously was still able to take place at 22°C and the late appearance of trophozoites indicated that the process was being slowed down, it was decided to use this temperature for preparation of the material for electron microscopy.

7.2.3 Immuno-electron microscopy

Bovine PBM were infected "in vitro" with *T. annulata* Ankara sporozoites from 3 day fed ticks and processed for immuno-electron microscopy as described in section 2.2.11. The assay was carried out at 22°C and samples prepared at time intervals of 5, 30 and 60 minutes, 3 hours, 6 hours and 24 hours after sporozoite addition. These time intervals were judged to give a wide range of stages of sporozoite invasion from the results described in section 7.2.2. Samples were also taken for Giemsa stained cytospin smears, which were examined under light microscopy in order to assess the degree of infectivity. The results of these counts are shown in Table 32. The percentage of lymphocytes infected with sporozoites was found to be between 6-9%, with a high percentage of cells with sporozoites attatched to their surface being observed in the cultures incubated for between 30 minutes and 6 hours.

The material for electron microscopy was fixed in 4% formaldehyde, 1.5% glutaraldehyde or 2% formaldehyde/ 0.7% glutaraldehyde, and

antibody incubations, dehydration, embedding, sectioning and examination were carried out as described in section 2.2.11. The pelleted cells were incubated with McAb 1A7 neat supernatant as a primary antibody, except for duplicate samples prepared from the 30 minute incubation which were incubated with a control IgG anti-trypanosome McAb (kindly provided by Dr. L. Tetley, Electron Microscopy Unit, University of Glasgow). The second antibodies used were goat anti-mouse IgG or IgM conjugated to 10nm colloidal gold particles.

The sporozoites were easily distinguished from the PBM by their ultrastructure, which has been described by Jura et al (1983). The sporozoites were of a fairly uniform round or ovoid shape, had a denser cytoplasm and were of a smaller size than the lymphocytes, generally 0.8-1μM in length. Structures resembling a nucleus or rhoptries could also be distinguished in some sections. Sample electron-micrographs are shown in Figures 55-58. No labelling could be detected on sporozoites or lymphocytes incubated with the control primary antibody (Figure 57). In samples incubated with McAb 1A7, binding of colloidal gold could be observed on the surface of sporozoites, but not to bovine lymphocytes (Figure 55 and 56). No colloidal gold labelling could be detected on the surface of lymphocytes that appeared to contain internalised sporozoites (data not shown). The colloidal gold labelling appeared to be bound directly to the outer pellicle of the sporozoites (Figure 56). Labelling was absent in the central area of some of the sections; this was thought to be due to lack of penetration of the antibody or fixative into the central part of the cell pellets. Labelled sporozoites could be observed in the material prepared using all three fixation methods, although the label was noticably much more diffuse in the samples fixed in 1.5% glutaraldehyde than in the other fixatives.

Figure 58A and B shows a labelled sporozoite attatched to the surface of a lymphocyte. The sporozoite and lymphocyte membranes are closely appositioned and thickened indicating that the sporozoite was bound to the lymphocyte and /or undergoing the early stages of the invasion process. The colloidal gold was confined entirely to the sporozoite surface, none had transferred to the surface of the lymphocyte.

Figure 55

Electron-micrograph to show sporozoites and lymphocytes fixed in 2% formaldehyde/0.5% gluteraldehyde 60 minutes following sporozoite addition at 22°C, incubated with McAb 1A7, and goat anti-mouse IgM conjugated to 10nm colloidal gold particles as a second antibody. The line in the bottom right-hand corner on this and subsequent electron-micrographs indicates a scale of 1μM.

In this and subsequent figures;

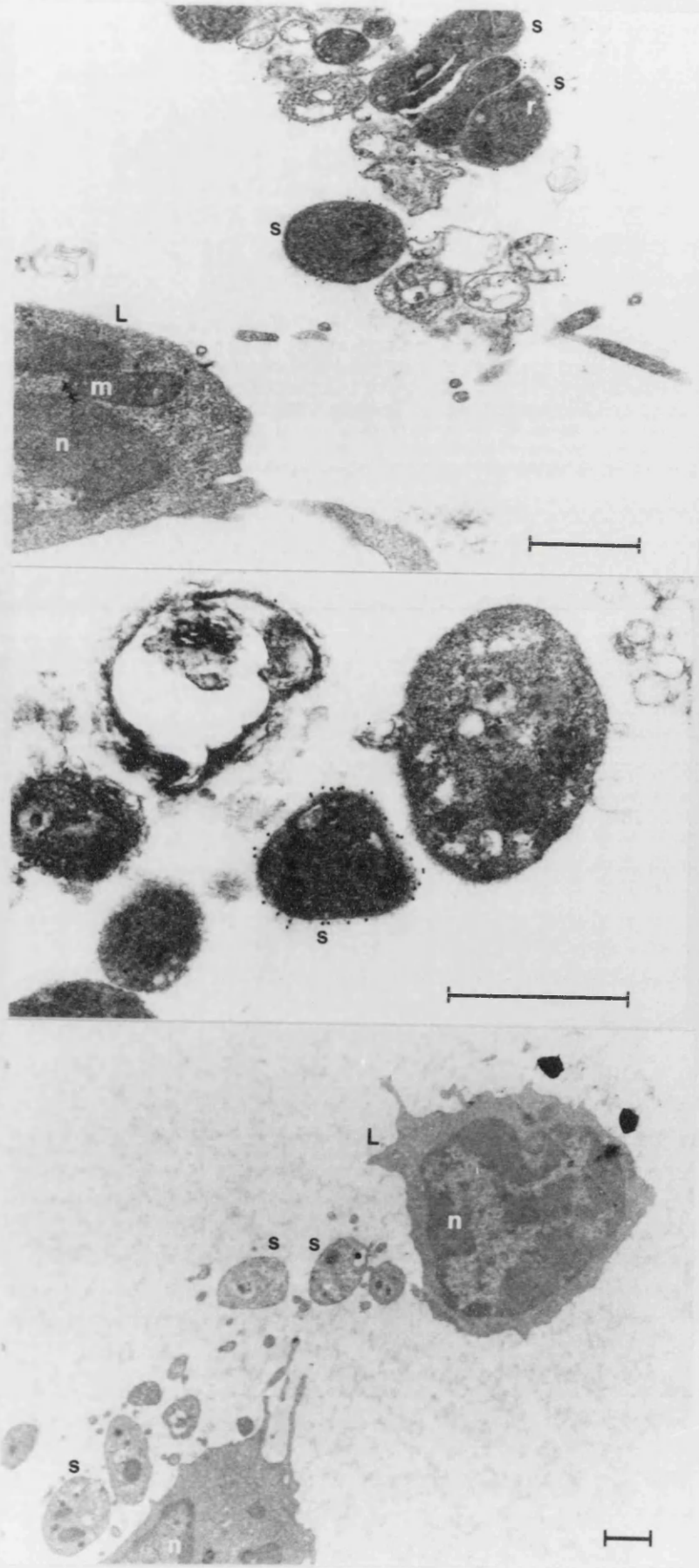
L = lympocyte
s = sporozoite
n = nucleus
m = mitochondrion

Figure 56

Electron-micrograph to show sporozoite and lymphocyte material fixed in 2% formaldehyde/0.5% gluteraldehyde 60 minutes after sporozoite addition, and incubated with antibody as described above.

Figure 57

Sporozoites and lymphocytes fixed in 2% formaldehyde/0.5% gluteraldehyde 60 minutes after sporozoite addition, then incubated with a control McAb (anti-trypanosome IgG), with goat anti-mouse IgG conjugated to 10nm colloidal gold particles as a second antibody.


s
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r
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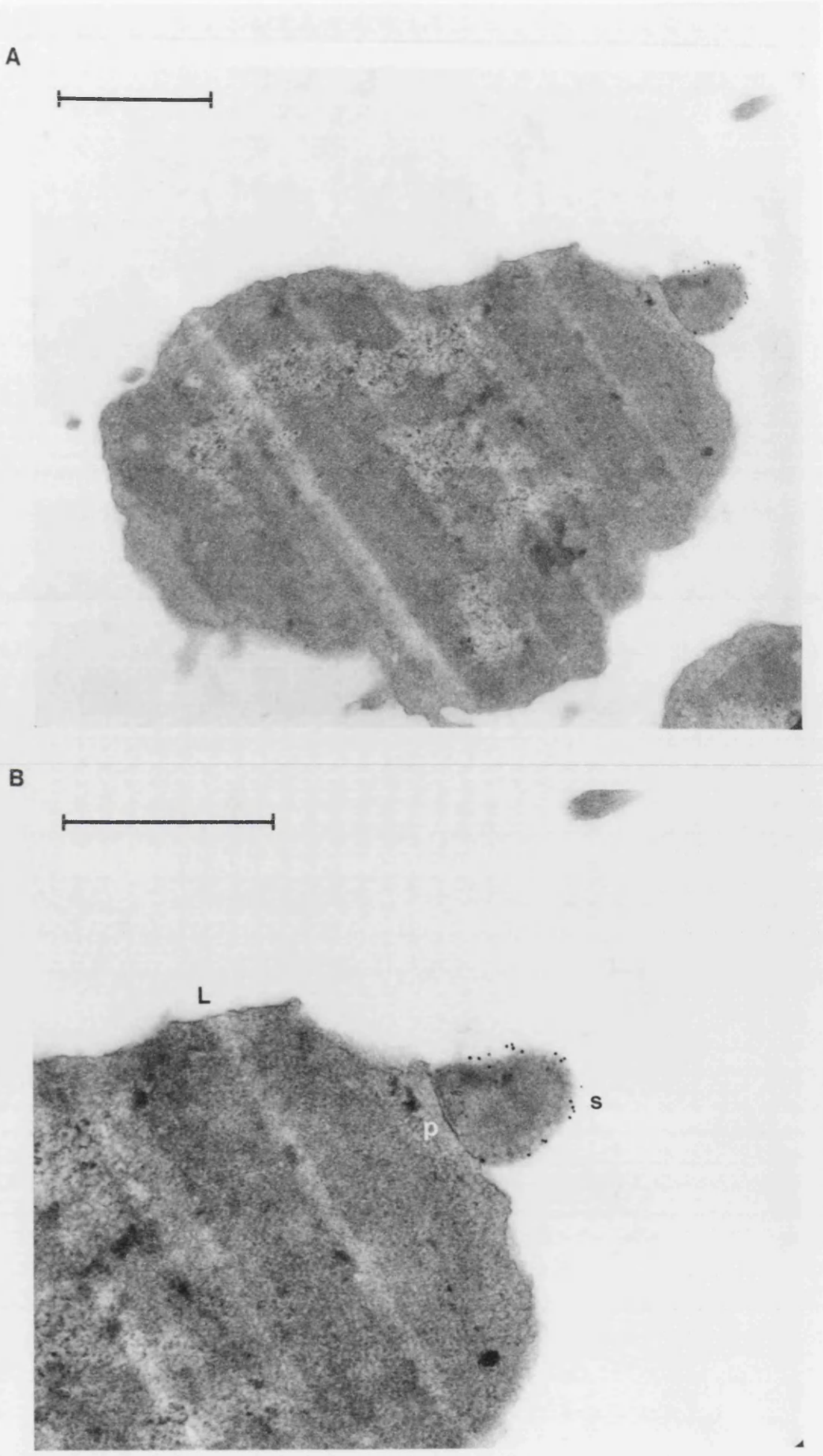
L
m
n
s
L
n
s
s
s
n

Figure 58A and B

Electron-micrographs to show a sporozoite attatched to the surface of a lymphocyte fixed in 2% formaldehyde/0.5% gluteraldehyde 30 minutes following sporozoite addition at 22°C, incubated with McAb 1A7, and goat anti-mouse IgM conjugated to 10nm colloidal gold particles as a second antibody. Figure 57B was taken at a higher magnification to 57A. The line in the bottom right-hand corner electron-micrographs indicates a scale of 1μM.


A
B
L
p
s

7.3 Discussion

In the experiment described in section 7.2.1, some inhibitory effect appeared to be produced by the SPAG1 fusion proteins compared with cultures incubated with GST alone, although this was much lower than that produced by the McAb 1A7 (Table 30). However, it needs to be borne in mind that the antibody and the fusion proteins would be expected to inhibit the process by different mechanisms. While the antibody would produce inhibition by binding to the sporozoite surface, the fusion proteins would expect to manifest their effects by binding to putative sporozoite receptors on the lymphocyte surface. As mentioned previously in section 6.3, recombinant SPAG1 generated from GST-2.7 fusion protein has been shown to bind to the surface of MHC Class II bearing cells (Personal communication; F. Katzer, Dept of Biology, University of York).

The failure of the SPAG1 fusion proteins to produce high levels of inhibition could be due to incomplete blocking of all the SPAG1 receptors on the host cell surface, particularly if the receptors are numerous. It might be that blocking could be obtained using higher fusion protein concentrations than those used in the experiment (up to 1mg ml^{-1}). However, the lack of a concentration effect of GST-2.7 fusion protein on the levels of inhibition does suggest any receptors were saturated. In a similar experiment using synthetic peptides derived from the *P. falciparum* circumsporozoite protein, binding of sporozoites to hepatocytes was completely inhibited by inclusion of 250μg ml^{-1} of peptides identical to the circumsporozoite protein ligand. Probably the concentration of synthetic or recombinant antigen required in such assays is dependant on the abundance of the receptors on the host cell surface. The receptor for SPAG1 is not known. It has been suggested that the N-terminal elastin homologous repeats of SPAG1 could act as a ligand for the elastin receptor on bovine lymphocytes (Hall et al 1992), but lack of inhibition of sporozoite invasion by the anti-elastin McAb BA4 (personal communication; Dr. R. Hall, University of York) and the data presented in Chapter 5 which suggested that neutralising epitopes resided in the C-terminus, did not support this theory. Alternative explanations for the incomplete inhibition produced by the fusion proteins are that the GST polypeptide interfered with binding to the

lymphocyte surface, or that expression of the fusion in *E. coli* resulted in incorrect folding of some determinants, as discussed in section 1.2.5. Nevertheless, the fact that apparent specific inhibition was observed suggests that determinants on the SPAG1 molecule are involved in sporozoite binding to the host cell.

The sporozoite invasion assay carried out at 4°C, 22°C and 37°C confirmed the observations that a reduction in temperature inhibits the infection of bovine lymphocytes with sporozoites "in vitro" (Jura 1984). While infection was inhibited almost completely at 4°C, sporozoites could still be observed attatched to the surface of lymphocytes, but since this could be purely a result of the cytospin process it is hard to conclude anything from this. The later appearance of trophozoites in cultures incubated at 22°C than at 37°C indicated that the lower temperature had some inhibitory effect on the invasion process.

The immuno-electron microscopy studies provide direct proof that SPAG1 was located on the sporozoite surface. The antigen appeared to be bound to the thin pellicle described on *T. annulata* sporozoites by Jura et al (1983). There was no evidence of binding to an outer surface coat, which was found to be the location of the *T. parva* sporozoite p67 antigen according to immuno-electron microscopy studies (Webster et al 1985). These workers also observed binding of the p67 antibody to bovine lymphocytes, and suggested that the antigen is shed and transferred to the lymphocyte surface when the sporozoite enters the cell. This did not appear to be the situation in *T. annulata*, since no label was observed on the surface of bovine lymphocytes and no transfer of label was apparent in the electron micrograph of the sporozoite actually undergoing invasion, although more sections of invading sporozoites would need to be obtained in order to confirm the latter observation. It needs to be borne in mind that the McAb 1A7 used in these studies recognises an epitope in the C-terminal region of SPAG1, thought to be retained on the sporozoite surface during proteolytic processing, as discussed in section 1.1.4. If the proteolytic processing of SPAG1 actually occurs during the invasion process, as is thought to be the case with the *P. falciparum* merozoite antigen MSP1 (Blackman et al 1991), the C-terminal region of SPAG1 may be retained on the surface of the invading sporozoite while N-terminal fragments are shed and persist on the lymphocyte surface.

It was notable that in all of the labelled sporozoite sections observed in the electron micrographs (20-25 in total) the labelling appeared to be uniformly around the surface. In the ultrastructural studies of sporozoite invasion by Jura et al (1983) sporozoites were observed to bind to lymphocytes consistantly by their basal end, ie. that containing the nucleus. This contrasts with the situation in other apicomplexan protozoa such as *Plasmodium*, *Babesia*, *Eimeria* and *Toxoplasma* where sporozoites and merozoites enter host cells via the apical complex, usually involving discharge of rhoptry contents (Aikawa et al 1978). It might be expected that receptors involved in sporozoite recognition and initial attatchment to host cells would be confined to the basal end, with molecules involved in the entry process itself having a more even distribution; this data could implicate the latter role for SPAG1.

The existence of two types of ligands on the surface of sporozoites and merozoites of Apicomplexan protozoa has been suggested by a number of authors. In a review by Sinden (1985) the existence of two sets of ligands on malaria parasites is postulated; primary ligands mediating initial attachment, and secondary ligands mediating close binding. This theory was based on ultrastructural observations, in which long filaments were detected between the surfaces of merozoites and erythrocytes during the early stages of invasion. Initial sporozoite attachment and subsequent invasion were also postulated to be separate processes in *T. parva* sporozoite entry of host cells (Shaw et al 1991). These workers found that initial attatchment appeared to be a passive process that could occur at 0-2^{o}C and did not require the sporozoites to be viable or intact. All later stages, however, required the sporozoites to be viable and were temperature dependant. Since the process was blocked by addition of protease inhibitors, it was also suggested that surface proteases were involved in the entry process.

Since at least one other antigen has been identified on the surface of *T. annulata* sporozoites containing neutralising epitopes (described in section 1.1.3), host cell recognition and invasion may involve other antigens apart from SPAG1. The next section describes work to characterise a second antigen found on the surface of *T. annulata* sporozoites, which may also play a role in attachment or invasion of host

cells. The possible involvement of several antigens in the invasion process will be further discussed in Chapter 9.

TABLE 30. RESULTS OF FUSION PROTEIN INHIBITION ASSAY

The following table summarises the results of a fusion protein inhibition assay carried out to assess the effects of recombinant GST-2.7, SPAG1 alone and GST alone on the infectivity of *T. annulata* Ankara sporozoites for bovine PBM "in vitro". Tests were carried out using the fusion proteins at the final concentrations indicated, and each test well duplicated. The percentage of macroschizont infected cells observed in the culture after four days was assessed from 400 cells in total from each test, and the percentage inhibition calculated according to the following formula;

$$\% \text{ inhibition} = \frac{x - y}{x}$$

where x = % infection in control wells
and y = % infection in test wells

The control wells refered to in the calculation were either the cultures set up in medium alone ("medium control"), or the cultures set up in the presence of GST at the same concentration as the test fusion protein ("GST control") as indicted on the table. Cultures were also set up in the presence of McAb 1A7 ascites fluid (diluted 1:100) to act as a positive control.

FUSION PROTEIN (CONCN IN MG/ML)		% INFECTION	% INHIBITION	
			MEDIUM CONTROL	GST CONTROL
None (medium only)		36	-	-
GST	0.1	29.8	17.2	-
	0.5	29.5	18.1	-
	1.0	33.5	6.9	-
GST-2.7	1.0	15.3	57.5	47
	0.5	15.8	56.1	46.4
	1.0	17	52.8	49.3
SPAG1	0.1	24	33.3	19.5
McAb 1A7		12	3	91.7

TABLE 31. EFFECT OF TEMPERATURE ON SPOROZOITE INVASION

The following table summarises the results of an assay carried out to examine the effects of temperature on sporozoite invasion of bovine PBM "in vitro". Cultures were set up at temperatures of $4^{o}C$, $22^{o}C$ and $37^{o}C$. Samples were taken for Giemsa stained cytospin smears at various timepoints after sporozoite addition as indicated on the table, and the percentage of macroschizont infected cells calculated (% infected). 100 cells were counted from each timepoint. The percentage of total lymphocytes counted containing trophozoites (5 minutes to 18 hours), internalised sporozoites, or sporozoites in contact with their surface (irrespective of whether or not the host cell were infected) was also calculated.

TEMP.	TIMEPOINT	% INFECTED	% TROPHOZOITES	% INTERNALISED SPOROZOITES	% ATTATCHED SPOROZOITES
$4^{o}C$	6 hrs	0	0	0	1
	18 hrs	1	0	1	4
$22^{o}C$	5 mins	4	0	4	2
	10 mins	2	0	2	3
	20 mins	3	0	3	6
	30 mins	1	0	1	6
	1 hr	4	2	2	2
	2 hrs	4	0	4	12
	3 hrs	7	1	6	8
	6 hrs	7	6	2	3
	18 hrs	8	8	2	4
$37^{o}C$	5 mins	3	0	3	10
	10 mins	4	2	3	13
	20 mins	3	2	1	9
	30 mins	4	2	2	7
	1 hr	5	4	1	10
	2 hrs	4	0	4	12
	3 hrs	9	5	4	18
	6 hrs	7	7	5	4
	18hrs	14	14	3	1

TABLE 32. MATERIAL USED FOR ELECTRON MICRSCOPY

The following table summarises the results of the sporozoite invasion assay which was carried out at 22^{O}C to provide material for the immuno-electron microscopy work. The % lymphocytes infected with sporozoites and the % with sporozoites adhering to the cell surface were calculated from Giemsa stained cytospin smears. 400 cells were counted from each timepoint (30 minutes to 18 hours).

TIMEPOINT	% INFECTED	% WITH SPOROZOITES ATTATCHED
30 mins	7.8	22.6
1 hr	6.9	17.7
3 hrs	9.4	21.9
6hrs	6.5	18.1
18 hrs	9.1	3

CHAPTER 8

CHARACTERISATION OF THE 4B11 ANTIGEN

8.1 Introduction

As described in Chapter 1 (1.1.3) a second sporozoite specific antigen apart from SPAG1 has been identified using the mouse McAb 4B11 raised against *T. annulata* sporozoites. Like the anti-SPAG1 McAb 1A7, this McAb specifically reacted with formalin or acetone fixed *T. annulata* Ankara sporozoites in IFA tests (Williamson 1988). The McAb was also found to neutralise *T. annulata* sporozoite infection of bovine lymphocytes "in vitro". The neutralising activity of 4B11 ascites fluid diluted 1:100 was found to be 100% compared with a control McAb, compared with 57% for McAb 1A7 ascites, while the equivalent figures for undiluted supernatant are 75% for 4B11 and 58% for 1A7 (Williamson 1988), thus indicating a more effective invasion blocking activity.

However, McAb 4B11 was found to recognise antigens of a different molecular weight to those recognised by 1A7 on Western blots of *T. annulata* sporozoite extracts. 4B11 reacted with antigens of approximately 150, 67 and 17-20kDa (Figure 37, tracks 2 and 7), distinct from the group of antigens of 54-104 kDa of recognised by McAb 1A7 (Figure 42A track B). Like SPAG1 the antigens detected by 4B11 were also recognised on Western blots of Percoll purified *T. annulata* sporozoites by antisera from a number of immunised cattle (Williamson 1988). Antisera from two cattle immunised with live *T. annulata* sporozoites and treated with buparvaquone (134x, N61), two cattle immunised with sporozoites inactivated by freeze-thawing (37T and 47T) and an animal immunised with irradiated sporozoites (L21) reacted with antigens of 17kDa and a high molecular weight molecule (120-130kDa), as well as SPAG1. Antisera from two other cattle immunised with irradiated sporozoites, L37 and 102, recognised the 17kDa antigen only on Western blots. Sera from all of these cattle showed high blocking activity of *T. annulata* sporozoite infection of lymphocytes "in vitro", and with the exception of animal 102 the cattle were protected from severe

clinical reaction on challenge with viable sporozoites, although a mild clinical reaction was detected in animal L37 (data from Williamson 1988 and personal communication (37T and 47T)). The recognition of the 17kDa antigen by sporozoite immunised cattle, and the high molecular weight antigen which may correspond to the 150kDa antigen recognised by 4B11, indicates that the 4B11 antigen has a role in sporozoite immunity. It is particularly interesting that the only antigen detected by antisera from two of the cattle was a polypeptide carrying the 4B11 epitope.

The McAb 4B11 reacted with the surface of live *T. annulata* Ankara and acetone and formalin fixed Ankara and Gharb sporozoites in IFA tests. A "halo effect" could be observed in these tests, suggesting that the 4B11 antigen was located at or near the sporozoite surface. 4B11 reacted very weakly with acetone fixed macroschizonts, with fluorescence confined to a speckled cytoplasm, and failed to react with acetone fixed piroplasms. A surface location for the 4B11 antigen is also implied by the ability of the McAb to block infectivity of viable sporozoites and by the immunoprecipitation of a 17-20 kDa antigen by McAb 4B11 from surface iodinated sporozoites (Williamson 1988).

This chapter describes preliminary work undertaken to further characterise the 4B11 antigen. The McAb 4B11 was used to screen a λ gt11 genomic expression library. Inserts from two positive recombinants were used to probe Southern blots of sporozoite and bovine DNA and a Northern blot of RNA from *T. annulata* sporozoites, macroschizonts and uninfected ticks, in order to verify that the inserts were of parasite origin and to verify whether their expression was stage specific. The relationship between the inserts was analysed on Southern blots. Nucleotide sequence analysis was also carried out. Both the inserts were subcloned into the expression vector pGEX1λT and trial expression of both the recombinants was undertaken. The role of antibodies recognising one of the expressed recombinants was investigated. The fusion protein was used to immunopurify antibodies from antiserum of a sporozoite immunised cow (47T), and the reactivity of these antibodies to various stages of the parasite assessed in IFA tests. The fusion protein was also used to immunise a rabbit, and the reactivity of the antisera to parasite material examined on Western blots and in IFA tests. The ability

of the antisera to block *T. annulata* sporozoite infectivity of lymphocytes "in vitro" was also investigated.

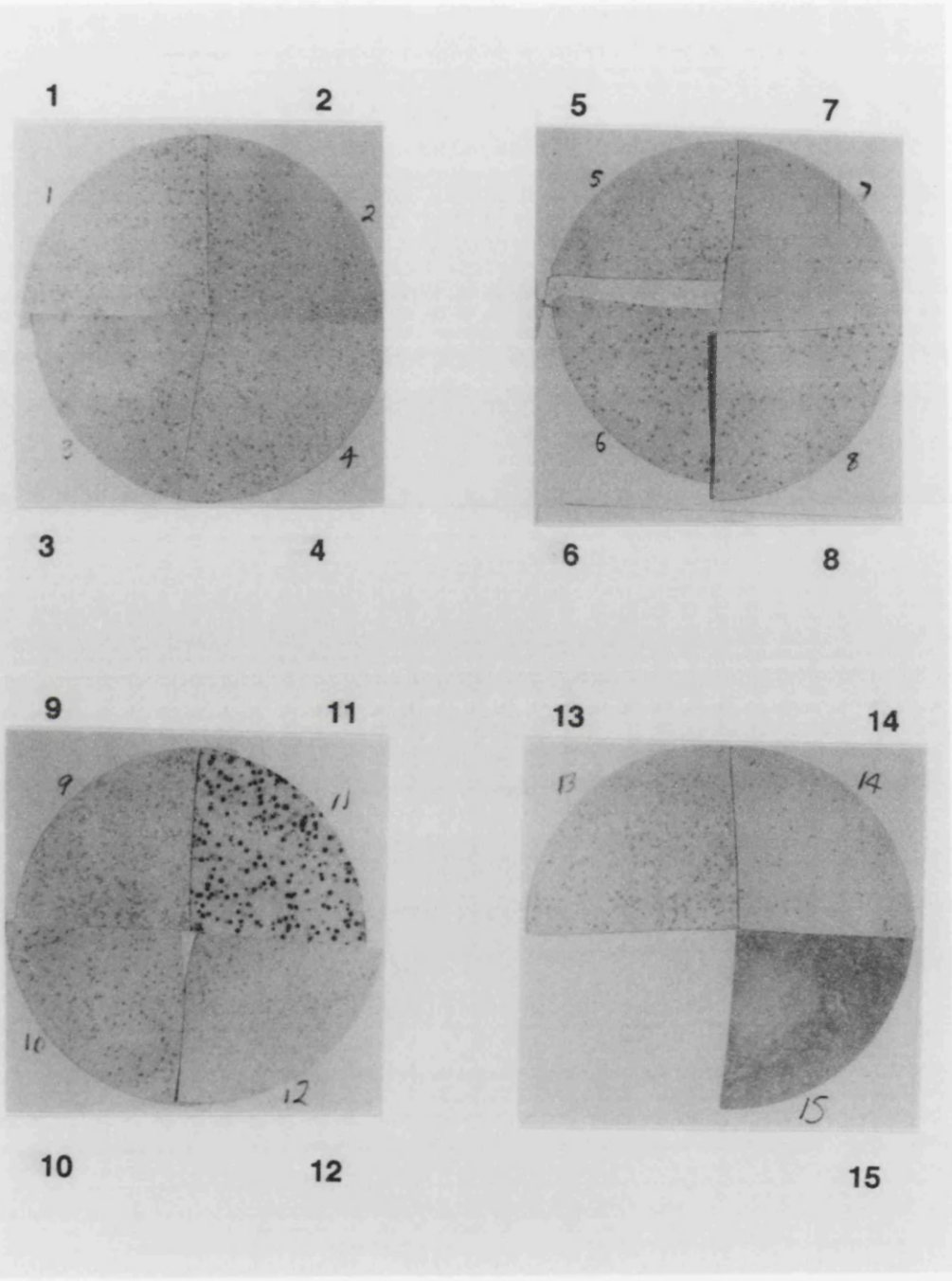
8.2 Results

8.2.1 Library screening

The library screening was carried out by Dr. K. Hussain, Wellcome Unit for Molecular Parasitology, University of Glasgow; details are included here with his permission. The McAb 4B11/C12 was used to screen a λgt11 genomic expression library in *E. coli* Y1090, made by Dr. J. Kinnaird, Wellcome Unit for Molecular Parasitology, University of Glasgow. The library was constructed using DNA made from *T. annulata* Ankara D7 merozoites, randomly sheared by sonication to produce fragments of 300bp-2kb, and ligated into the Eco RI site of λgt11 using Eco RI adaptors. A total of approximately 1.2×10^7 plaques were plated out from the unamplified library and screened according to the method of Young et al (1985). A total of 15 recombinants, numbered λgt11-KP1 to λgt11-KP15, were originally identified which reacted with McAb 4B11. The 15 plaques were picked, plated out and secondarily screened with McAb 4B11; the reactivity with all 15 of the recombinants is shown in Figure 59. The recombinants were further screened with day 42 post-immunisation serum from cow 102 (recognising the 17kDa sporozoite antigen), McAb 1A7 culture supernatant, which recognises SPAG1, and alkaline phosphatase conjugated rabbit anti-bovine IgG and rabbit anti-mouse IgG second antibodies. The 102 bovine antiserum reacted with all 15 of the clones (data not shown), while the McAb 1A7 reacted with the recombinant λgt11-KP11 only (Figure 60 B), indicating that it contained an insert derived from the SPAG1 sequence. The anti-mouse and anti-bovine second antibodies failed to react with any of the recombinants screened (Figure 60C and D). The result of a tertiary screen of five of the recombinants which gave the strongest reactions with the McAb 4B11, λ gt11-KP1, λgt11-KP4, λgt11-KP6, λgt11-KP8 and λgt11-KP11, and of λ gt11 without an insert, is shown in Figure 61. While the McAb 4B11 reacted with the recombinants, no reaction was obtained to λgt11 alone.

Figure 59

Secondary screening of 15 recombinants from the *T. annulata* Hissar D7 merozoite genomic expression library in λgt11 with McAb 4B11 undiluted supernatant. The second antibody used was alkaline phosphatase conjugated rabbit anti-mouse IgG, used at a working dilution of 1:300. The filters are shown labelled 1-15, corresponding to the recombinants λ gt11-KP1 to λgt11-KP15.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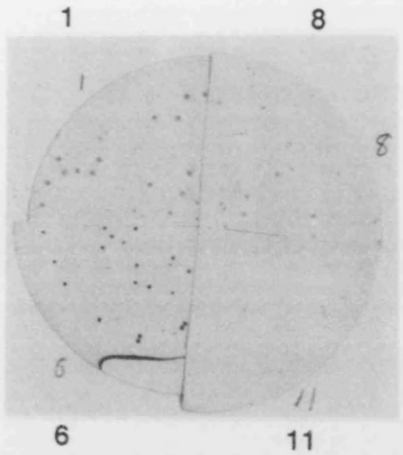
15

Figure 60

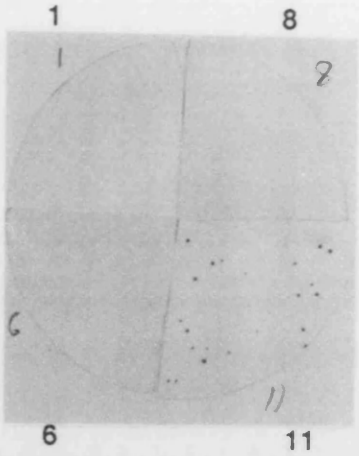
Secondary screening of recombinants λgt11-KP1 (1), λgt11-KP6 (6), λgt11-KP8 (8) and λgt11-KP11 (11) with;

A McAb 4B11 undiluted supernatant
B McAb 1A7 undiluted supernatant
C Alkaline conjugated rabbit anti-mouse IgG only (1:300)
D Alkaline conjugated rabbit anti-bovine IgG only (1:300)

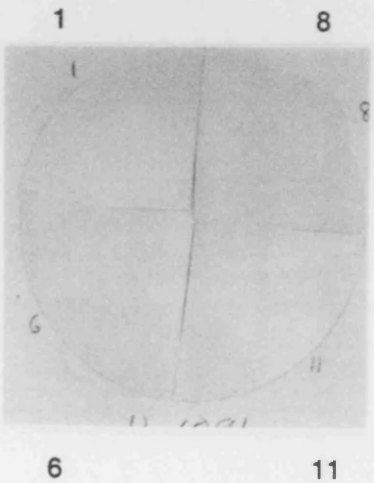
A



B



C



D

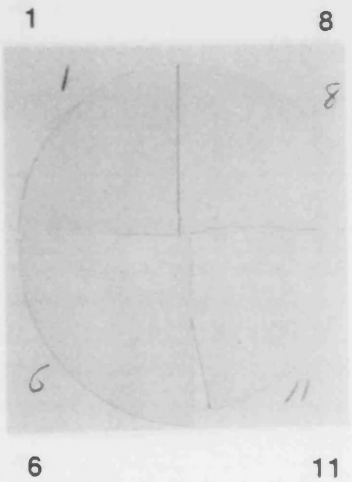


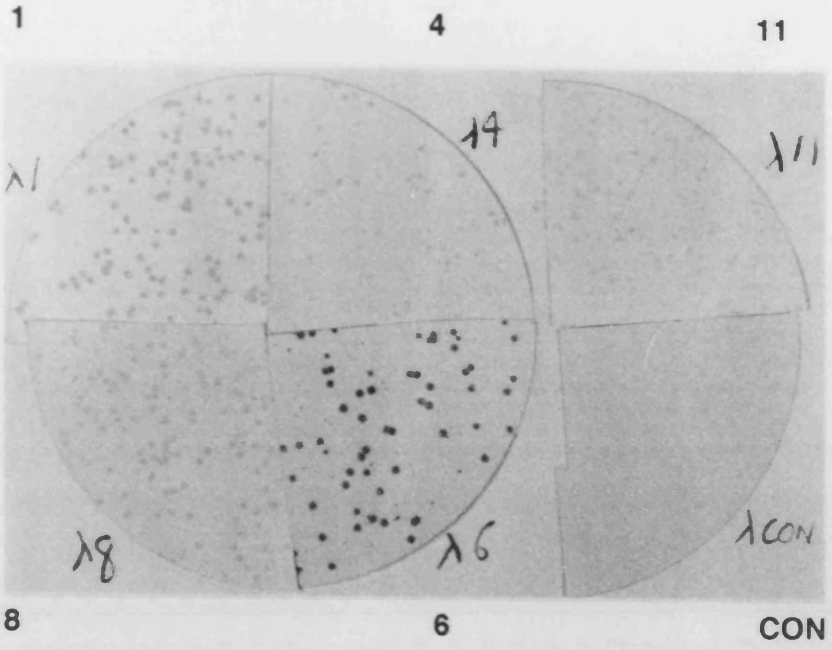
Figure 61

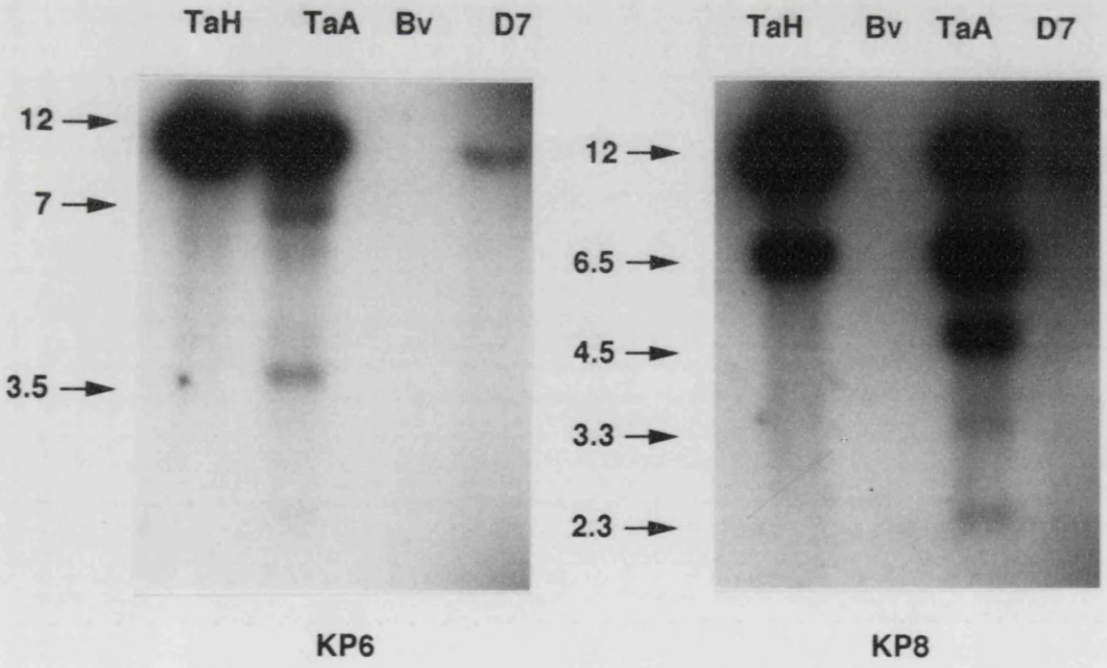
Tertiary screening of the recombinants λgt11-KP1 (1), λgt11-KP4 (4), λ gt11-KP6 (6), λgt11-KP8 (8), λgt11-KP11 (11) and a λgt11 transformant without an insert (CON) with McAb 4B11 undiluted supernatant.

Figure 62

Autoradiographs (exposed for 3 days) of Southern blots of Eco RI digested *T. annulata* and bovine DNA run on a 0.8% agarose gel, and probed with the KP6 insert from λgt11-KP6 (left hand photograph) or the KP8 insert from λgt11-KP8 (right hand photograph). The sizes (kb) of the bands are indicated by arrows. Tracks contained the following;

TaH *T. annulata* Hissar piroplasm DNA
TaA *T. annulata* Ankara piroplasm DNA
Bv Bovine fibroblast DNA
D7 *T. annulata* Ankara clone D7 merozoite DNA


1
4
11
λ1
λ4
λ11
λ8
λ6
λCON
8
6
CON


TaH
TaA
Bv
D7
12
7
3.5
KP6
TaH
Bv
TaA
D7
12
6.5
4.5
3.3
2.3
KP8

Two of the recombinants, λgt11-KP6 and λgt11-KP8, to which 4B11 reacted strongly, were selected for further analysis. DNA was prepared from both the λgt11 recombinants as described in section 2.2.2. Eco RI digestion and agarose gel electrophoresis (as described in section 2.2.4) revealed λgt11-KP6 to contain an insert of 800bp and λgt11-KP8 to contain an insert of 900bp. Both the KP6 and KP8 inserts were subcloned into the Eco RI site of the expression vector pGEX1λT as indicated for KP8 in Figure 66(i), and used to transform *E. coli* XL-1 Blue using methods described in section 2.2.4. Recombinants containing the insert were identified by Eco RI digestion of small scale DNA preparations (prepared by the "Magic Minipreps" method) and electrophoresis on a 1% agarose gel stained with ethidium bromide to visualise the fragments. Six of the seven recombinants screened from the KP6 ligation contained the insert, as did three of the four recombinants screened from the KP8 ligation. The orientation of both inserts was checked by making small scale fusion protein preparations from all of the recombinants, running the whole cell lysates on an SDS-polyacrylamide gel for Western blotting as described in section 2.2.7. The Western blot was developed with McAb 4B11, identifying a single pGEX1λT-KP6 recombinant and a single pGEX1λT-KP8 recombinant which appeared to express fusion protein (data not shown). The expression of KP6 and KP8 will be further discussed in section 8.2.6.

8.2.2 Genomic Southern blot analysis

Both the inserts from the Eco RI digested recombinants λgt11-KP6 and λgt11-KP8 were used to probe Southern blots of Eco RI digested DNA from a bovine fibroblast cell line, *T. annulata* Hissar and Ankara piroplasms and *T. annulata* Ankara clone D7 merozoites. The DNA was kindly provided by Dr. B. Shiels and Ms S. McKellar, Wellcome Unit for Molecular Parasitology, University of Glasgow. The resulting autoradiograph is shown in Figure 62. Neither insert hybridised to bovine DNA, while both inserts hybridised to a single band of 12kb in the track containing DNA from D7 merozoites. The KP6 and KP8 inserts hybridised to a number of bands in *T. annulata* uncloned Hissar and Ankara piroplasm DNA. While KP6 hybridised to bands of sizes 12, 7 and 3.5 kb, KP8 hybridised to five bands in total of sizes 12, 6.5, 4.5, 3.3

and 2.3kb. Identical banding patterns were obtained in both the Hissar and Ankara stocks, though the relative proportions of each band were different for the two stocks.

8.2.3 Northern blot analysis

A Northern blot of total RNA prepared from the salivary glands of uninfected ticks, *T. annulata* Ankara sporozoite infected ticks and Ankara clone C9 macroschizont RNA was probed with the KP6 and KP8 inserts from the λgt11 recombinants. This work was carried out by Dr. S. Williamson, CTVM, University of Edinburgh, and the autoradiograph is included here with her permission. The C9 macroschizont RNA was provided by Dr. J. Kinnaird. The autoradiograph is shown in Figure 63. The KP6 and KP8 inserts hybridised predominantly to a band of 3kb plus a weak band of 6kb. There was no hybridisation to RNA from uninfected tick salivary glands but weak hybridisation to 3kb and 6kb bands could be detected in the track containing macroschizont RNA.

8.2.4 Analysis of KP8 and KP6 inserts on Southern blots

Although both the KP6 and KP8 inserts hybridised to a 12kb band in cloned and uncloned DNA on Southern blots, the smaller bands to which they hybridised in the piroplasm DNA were of different sizes (Figure 62). The relationship between the two inserts was investigated further by examining their cross-hybridisation on Southern blots. Both inserts from the Eco R1 digested λgt11 recombinants were used to probe duplicate Southern blots of the Eco R1 digested recombinants λgt11-KP1, λgt11-KP6, λgt11-KP8 and λgt11-KP11. Prior to Southern blotting, inserts were observed on the ethidium bromide stained agarose gel of 800bp from λgt11-KP6, 900bp from λgt11-KP8 and 600bp from λgt11-KP11, while no insert was apparent in the track containing λgt11-KP1 DNA. However if the KP1 insert was smaller than about 200bp, it probably would not have been visualised on the gel. The resulting autoradiograph is shown in Figure 64. The two inserts did not cross-hybridise at high stringency on the Southern blot, neither did they hybridise to the KP11 insert, thought to be derived from SPAG1 on the

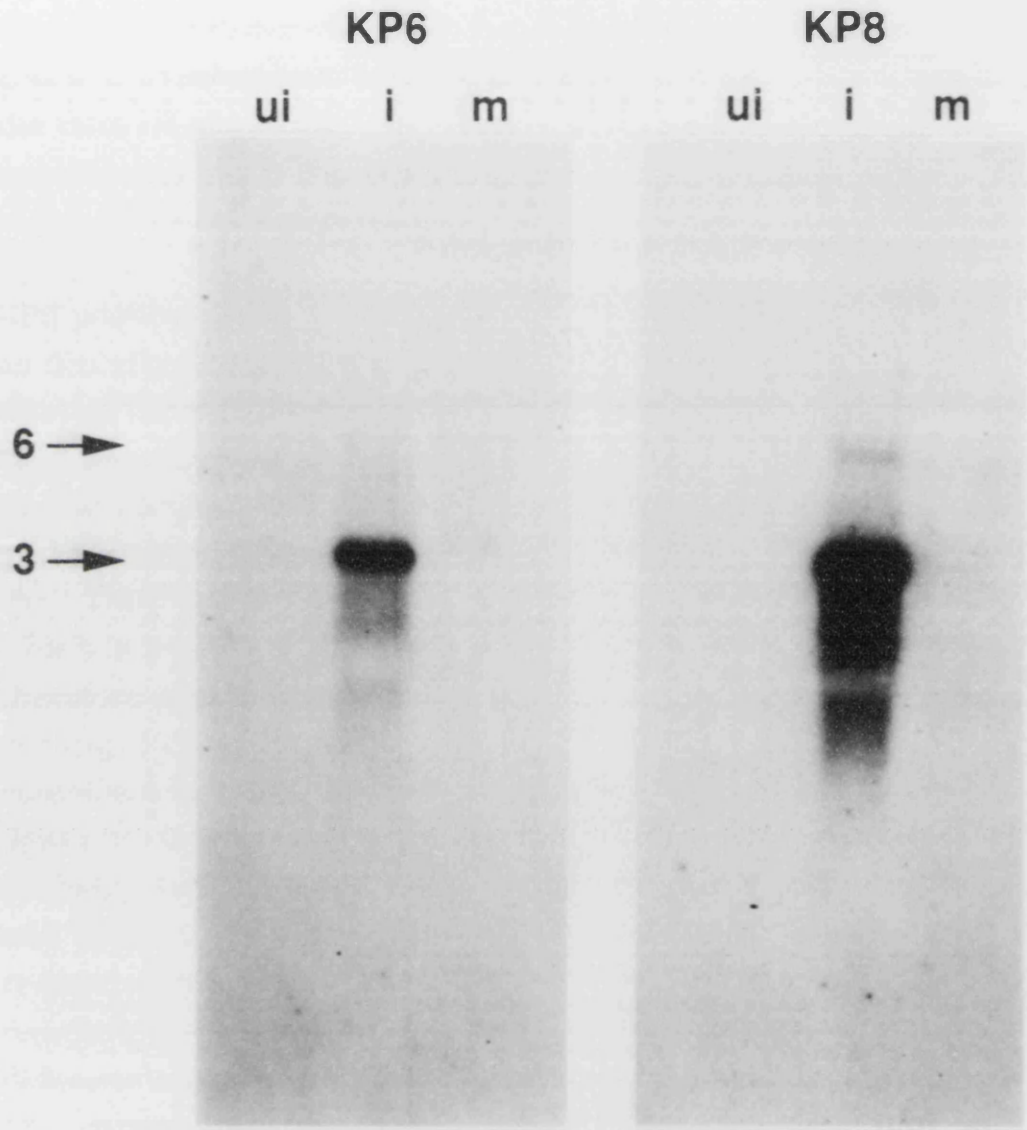
Figure 63

Autoradiograph of Northern blots of *T. annulata* sporozoite, macroschizont and tick salivary gland RNA probed with the KP6 insert from λgt11-KP6 (left hand photograph) or the KP8 insert from λgt11-KP8 (right hand photograph). The sizes of the bands are indicated by arrows. Tracks contained the following:

ui RNA from salivary glands of uninfected ticks

i RNA from salivary glands of ticks infected with *T. annulata* Ankara sporozoites

m RNA from *T. annulata* Ankara clone C9 macroschizonts


KP6
KP8
ui
i
m
ui
i
m
6
3

basis of it's reactivity with the anti-SPAG1 McAb 1A7. The hybridisation to the λ arms detected in this blot was probably due to contamination by λgt11 DNA of the KP6 and KP8 inserts.

Further cross-hybridisation analysis was carried out using recombinant clones from a λ DASH II genomic library in *E. coli* PLK17, containing larger inserts than the λgt11 library. The library was constructed by Dr. J. Kinnaird from Sau 3A partial digests of DNA from *T. annulata* Ankara clone D7 merozoites, size fractionated on a sucrose gradient to select restriction fragments of sizes 12-20kb and ligated into the Bam HI site of the λ DASH II vectors. The library was screened by hybridisation with the KP6 and KP8 inserts derived from Eco R1 digestion of the pGEX1λT recombinants by Dr. K. Hussain, using methods described by Sambrook et al (1989). DNA preparations were made from a KP8 positive clone, λDASH-KP8+, and a KP6 positive clone, λDASH-KP6+, as described in section 2.2.2. Sal I digestion of the two recombinants showed them to contain inserts of 13.5 and 15.6kb respectively. The DNA was digested with a variety of restriction enzymes; Sal I, Xba I, Eco RI, Sst I and an Eco RI/Sal I double digest, run on a 0.8% agarose gel and Southern blotted. All of these enzymes had recognition sites within the λ DASH polylinker sequence of the recombinants, except for Sst I, which is located within the Bam HI sites of the λDASH polylinker and therefore absent in the recombinants. None of these enzymes had cutting sites within the λ arms. The sizes of the restriction fragments visualised by observation of the ethidium bromide stained DNA under UV light are summarised in Table 33. The enzyme Xba I failed to cut, probably due to loss of activity, while Eco RI and Sal I produced the 20kb and 9.2kb λ arms, produced from the sites in the polylinker region. The remaining fragments produced from digests with Eco RI, Sal I and Sst I, resulting from the presence of restriction sites within the inserts, were different between the λ DASH-KP8+ and λ DASH-KP6+ recombinants. The enzyme Sst I, which recognised sites within the KP6+ insert, apparently failed to cut the KP8+ insert, suggesting there were no Sst sites within the KP8 clone. The 6.5kb band seen in the digests with Sal I and Eco RI was probably due to close proximity of the two sites.

The Southern blot was hybridised with the KP8 insert from λgt11-KP8, and the autoradiograph is shown in Figure 65. The insert only

Figure 64

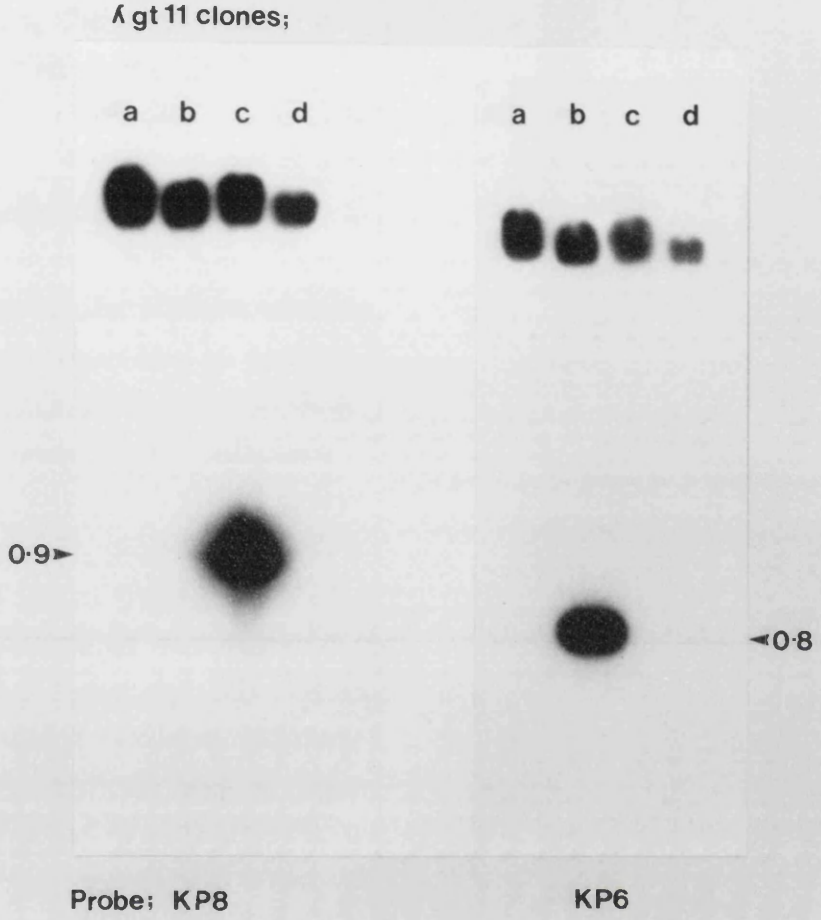
Autoradiograph (24 hour exposure) of a Southern blot of Eco RI digested DNA from four λgt11 recombinants run on a 1% agarose gel and probed with the KP8 insert from λgt11-KP8 (left hand photograph) or the KP6 insert from λgt11-KP6 (right hand photograph). The sizes (kb) of the bands are indicated by arrows. Tracks contained the following;

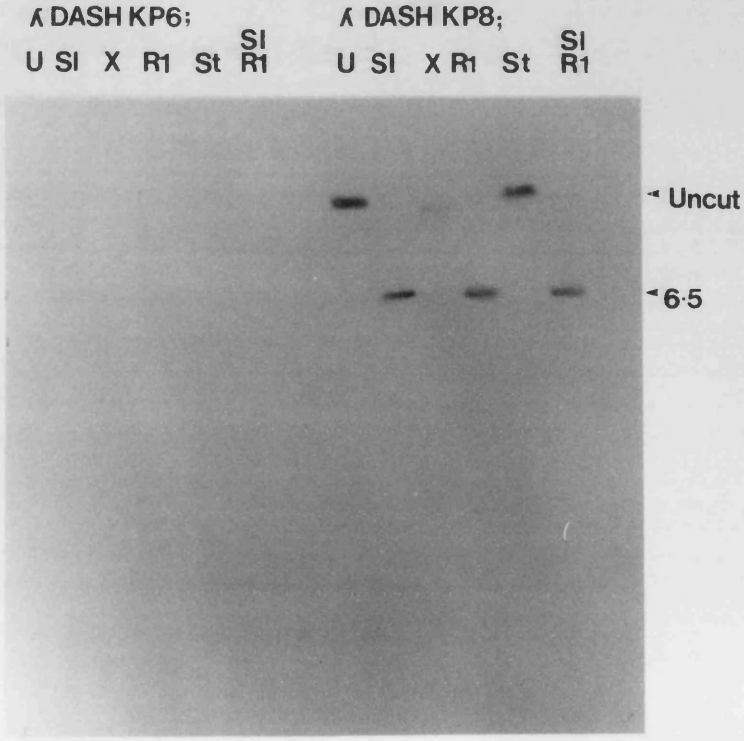
a λgt11-KP1 DNA
b λgt11-KP6 DNA
c λgt11-KP8 DNA
d λgt11-KP11 DNA

Figure 65

Southern blot of restriction enzyme digested DNA from the recombinants λDASH-KP6+ (left hand side) and λDASH-KP8+ (right hand side) run on a 0.8% agarose gel and probed with the KP8 insert from pGEX1λT-KP8. The sizes (kb) of the bands seen are indicated by arrows. Tracks contained DNA digested with the following restriction enzymes;

U Uncut; no enzyme
Sl Sal I
X Xba I
R1 Eco RI
St Sst I
Sl R1 Sal I / Eco RI double digest


λ gt 11 clones;
a b c d
a b c d
0·9
0·8
Probe; KP8
KP6


λ DASH KP6;
U SI X R1 St SI R1
λ DASH KP8;
U SI X R1 St SI R1
Uncut
6·5

hybridised to DNA from λ DASH-KP8+, detecting a 6.5kb fragment in the digests with Sal I and Eco RI, and to uncut DNA in the tracks containing the Sst I and Xba I digested DNA. The significance of these results concerning the relationship between the KP6 and KP8 sequences will be discussed in section 8.3.

8.2.5 Subcloning and sequence analysis

Two of the KP8 recombinants in pGEX1λT which failed to express the insert, described in section 2.2.1, were partially sequenced by the chain termination method described in section 2.2.7 using the primer for the GST coding sequence described in section 4.2.6. Both of these were found to contain the KP8 insert in the opposite orientation to pGEX1λT-KP8, and one, pGEX1λT-KP8(-), was used in further sequence analysis. The KP8 insert from pGEX1λT-KP8 was further subcloned for sequence determination as summarised in Figure 66. The vectors used were pGEM 7ZF and pBluescript SK+, which contain nucleotide sequences for the commercially available primers T3, T7 and SP6 running in opposite directions, as indicated in Figure 66. Restriction enzyme digests, purification of inserts on low melting point agarose, ligations and transformation of competent cells was carried out as described in section 2.2.4. Small DNA preparations were made from the recombinants using the "magic minipreps" method, given in section 2.2.2.

The KP8 insert from pGEX1λT-KP8 was subcloned into the Eco RI site of the vector pGEM7ZF (Figure 66 (ii)) to give pGEM-KP8, and used to transform *E. coli* JM101 competent cells. Recombinants were identified by α-complementation as described in section 2.2.4, and were confirmed to have the insert by Eco RI digestion of small DNA preparations, visualising the products on an ethidium bromide stained 1% agarose gel. All four recombinants tested were found to contain the 900bp insert. In order to identify appropriate restriction sites within the insert sequence to use for further subcloning, DNA from pGEX1λT-KP8 was digested with a selection of restriction enzymes which had no cutting sites predicted in the vector sequence, the products run on an ethidium bromide stained 1% agarose gel and the sizes of the bands noted. Digestion with the enzyme Hinc II produced a band of 550bp, showing this enzyme

contained two recognition sites within the KP8 insert. These Hinc II sites were used in further subcloning.

DNA from pGEM7ZF-KP8 was digested completely with Hinc II, the products run on a 1% agarose gel and the 550bp insert excised and purified. The insert was ligated into the Sma I site of the vector pBluescript SK+ (Figure 66 (iii)) to give pBluscript-KP27, since both Hinc II and Sma I leave blunt ends. The recombinants were used to transform *E. coli* XL-1 Blue competent cells, the positive recombinants identified by α-complementation and the plasmid DNA linearised by Eco RI digestion as described above, noting the band sizes. One of the four recombinants screened contained the 550bp insert. The Hinc II digested DNA from pGEM7ZF, from which the 550bp insert had been removed, was self-ligated to produce the recombinant plasmid pGEM-KP33 (Figure 66 (iv)), which was used to transform *E. coli* JM109 competent cells. Positive recombinants were identified by α-complementation and Eco RI digestion of small scale DNA preparations. Both the recombinants tested contained the correct deletion.

Small scale DNA preparations from pGEX1λT-KP8, pGEX1λT-KP8(-), pGEM-KP8, pGEM-KP33 and pBluescript-KP27 were used for sequencing by the chain termination method, as described in section 2.2.7. The GST sequence primer was used for unidirectional sequencing of pGEX1λT-KP8 and pGEX1λT-KP8(-). Sequencing of the inserts of the pGEM and pBluescript SK+ recombinants was carried out in both directions. The primers T7 and SP6 were used for the pGEM recombinants, while the primers T7 and T3 were used for pBluescript-KP27. A DNA sequence was obtained for the entire KP8 insert using the GCG computer package, but this was incomplete in that it contained mismatches between the sequences from each clone and was not completed in both directions. Sequence comparison with other nucleotide sequences in the database, including published sequences for SPAG1, *T. parva* p67 and the *T. parva* rhoptry antigen, showed no obvious regions of homology. A partial sequence which was identical between all three recombinants covering this region was obtained for the 252 N-terminal base pairs of the KP8 insert, which was in the correct reading frame with the Eco RI site of pGEX1λT. This nucleotide sequence, with the predicted amino acid sequence, is shown in Figure 67.

Figure 66

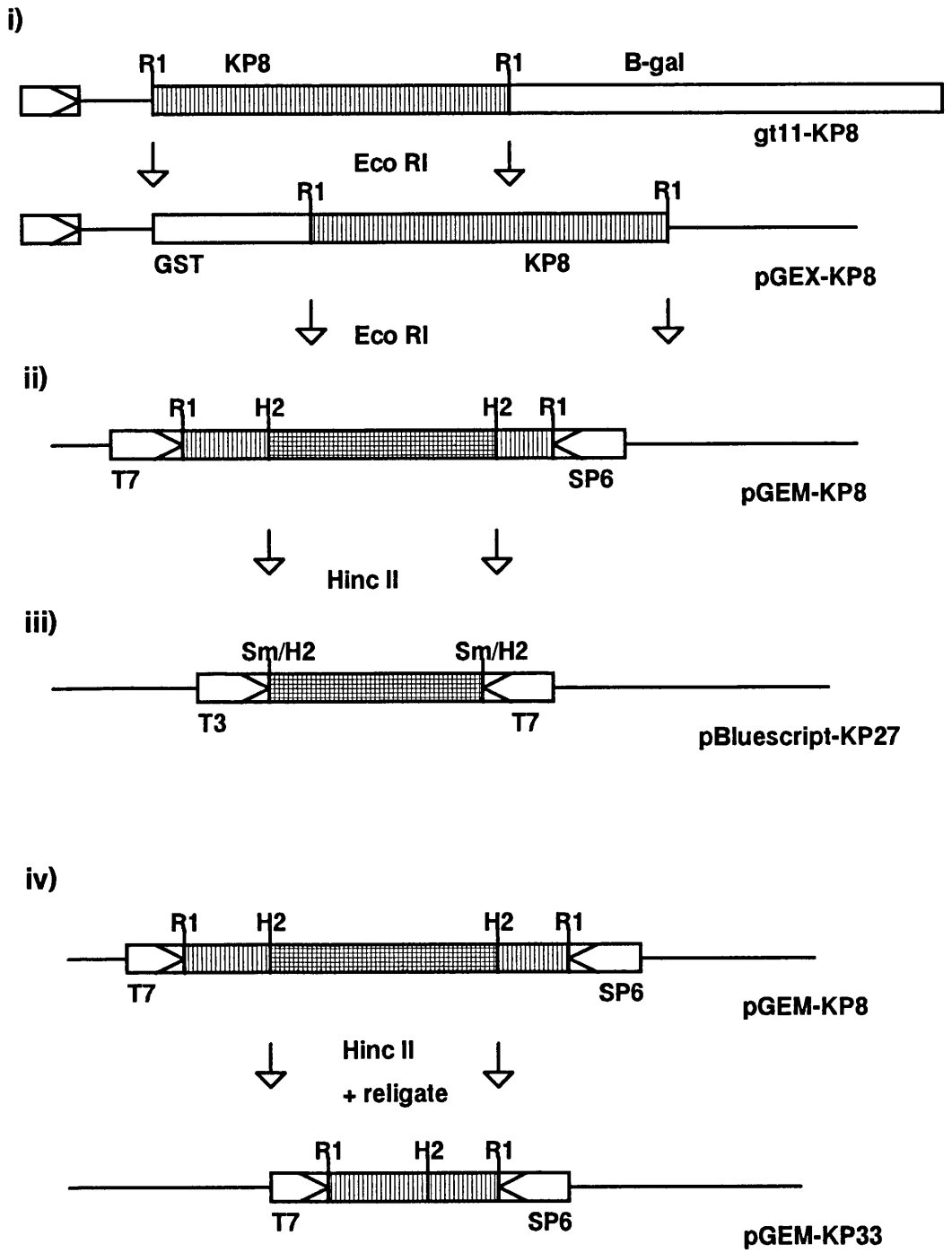
Subcloning the KP8 insert

i) Subcloning the KP8 insert from λgt11-KP8 into pGEX1λT. Both the vector and λgt11-KP8 were digested with Eco RI, and the vector was phosphatased. The 900bp insert was gel purified on a 1% low melting point agarose gel and ligated into the Eco RI site of pGEX1λT to give pGEX1λT-KP8.

ii) Subcloning of the KP8 insert into pGEM7ZF. Both the vector and the recombinant pGEX1λT-KP8 were digested completely with Eco RI, phosphatasing the vector. The 900bp insert was gel purified and ligated into the Eco RI site of pGEM7ZF to give pGEM-KP8.

iii) Subcloning the Hinc II fragment from the KP8 insert into pBluescript SK+. The vector was digested completely with Sma I restriction enzyme and phosphatased. The recombinant pGEX1λT-KP8 was digested completely with Hinc II and the 550bp insert purified on a 1.2% low melting point agarose gel and blunt-end ligated into the Sma I site of pBluescript SK+ to give pBluescript-KP27.

iv) Deletion of the Hinc II insert from KP8. The recombinant pGEM-KP8 was digested completely with Hinc II and purified away from the insert on a 1.2% agarose gel. The two cut Hinc II sites were religated to give pGEM-KP33.



= Promoter (eg. T7, T3, SP6)

= KP8 insert

R1 = Eco RI restriction site

H2 = Hinc II restriction site

Sm = Sma I restriction site

Figure 67

Nucleotide sequence for the 252 N-terminal nucleotides of the KP8 insert, with the predicted amino acid sequence shown in three letter code underneath. The Eco RI site is shown underlined. The consensus sequence was derived from sequences from the inserts of pGEX1λT-KP8, pGEM-KP8 and pGEM-KP33, using the GCG computer package.

1 GAA TTC GGC TCG AGA GAT AGC GCC AAG AAA
Glu Phe Gly Ser Arg Asp Ser Ala Lys Lys

31 ACA AGT GAC CAT GAG ACA AAA GAA AGT AAA
Thr Ser Asp His Glu Thr Lys Glu Ser Lys

61 GAC CAT AGA GAA AGA GAG TAC AAA TAC AAT
Asp His Arg Glu Arg Glu Tyr Lys Tyr Asn

91 AAC AAA GAT GAT AAT TCC AAA GAT TAC GAA
Asn Lys Asp Asp Asn Ser Lys Asp Tyr Glu

121 TGC ATC GAC TCT GAA GCA ATC AAG GCA GTA
Cys Ile Asp Ser Glu Ala Ile Lys Ala Val

151 GTG GAA AAG GCA GTT ATA GAA GCA TTT GAC
Val Glu Lys Ala Val Ile Glu Ala Phe Asp

181 AAG TGC CTG TCA GAA AAA ATT AAG GGT GAC
Lys Cys Leu Ser Glu Lys Ile Lys Gly Glu

211 GAA ACT AGT CTC AAG ACT ACT AGT AAA CCG
Glu Thr Ser Leu Lys Thr Thr Ser Lys Pro

241 AGA GTC ACA TTT
Arg Val Thr Phe

The predicted amino acid sequence showed little homology with any other published sequence. The sequence is rich in AT bases, a feature typical of the *Theileria* genome.

8.2.6 Expression of KP6 and KP8

Small scale preparations of GST fusion proteins were carried out using 5ml cultures of *E. coli* XLBlue transformed with pGEX1λT-KP6, pGEX1λT-KP8, pGEX1λT without an insert and untransformed *E. coli* XL-1 Blue, as described in section 2.2.7. As well as extracting PBS soluble fusion protein using glutathione sepharose beads from the pGEX1λT-KP6 and pGEX1λT-KP8 mini-preparations, the pellet of insoluble cell debris obtained after sonication was washed three times in PBS and retained. A 12% SDS-polyacrylamide gel was loaded with both the soluble and insoluble fractions from the pGEX1λT-KP6 and pGEX1λT-KP8 transformants, plus lysates of whole cells boiled in SDS-sample buffer from the pGEX1λT transformant and untransformed *E. coli* XL-1 Blue. Western blotting was carried out according to the method in section 2.2.6, and the blot developed with McAb 4B11. The resulting blot is shown in Figure 68A. Both pGEX1λT-KP6 and pGEX1λT-KP8 expressed products recognised by 4B11. However, while a band of apparent size 58kDa was recognised in the track containing the soluble fraction from the pGEX1λT-KP8 transformant, the McAb reacted predominantly with a band of 66kDa in the insoluble fraction only from the pGEX1λT-KP6 recombinant.

Similar preparations were carried out from 25ml cultures of the pGEX1λT-KP6 and pGEX1λT-KP8 transformants, as described in section 2.2.7. The soluble and insoluble fractions from the pGEX1λT-KP6 transformant, together with the soluble GST-KP8 fusion protein, were run on a 12% SDS-polyacrylamide gel as described in section 2.2.6. The Coomassie Blue stained gel is shown in Figure 68B. While the 58kDa GST-KP8 PBS soluble fusion protein was clearly visible, there was no obvious fusion protein in the track loaded with the pGEX1λT-KP6 soluble fraction. However, a band corresponding to 66kDa was visible in the insoluble cell pellet.

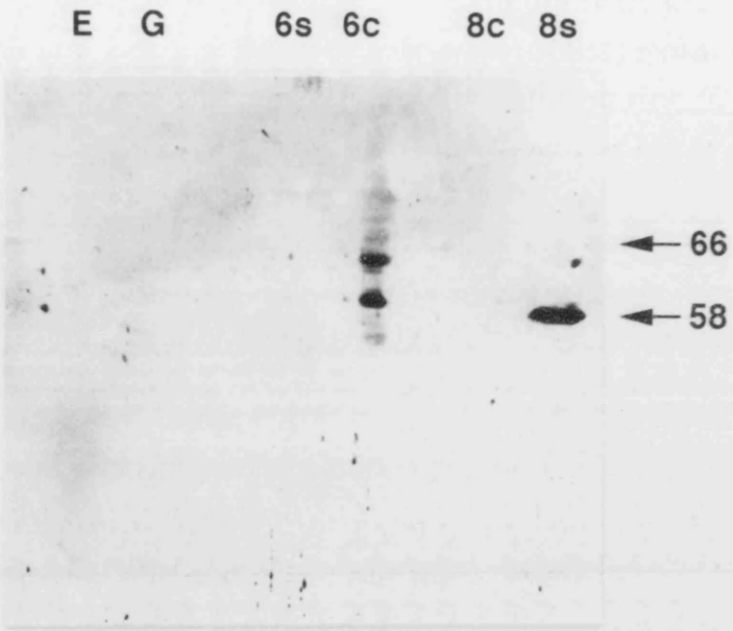
Figure 68A

Western blot of the expressed products from KP6 and KP8 small fusion protein preparations run on a 12% SDS-polyacrylamide gel, developed with McAb 4B11 neat supernatant and alkaline-phosphatase conjugated rabbit anti-mouse (1:300) as a second antibody. The molecular weights (kDa) of the fusion proteins visualised are indicated by arrows. Tracks were loaded with the following;

E Untransformed *E. coli* XL-1 Blue whole cell lysate
G pGEX1λT transformed *E. coli* XL-1 Blue whole cell lysate
6s Eluate from glutathione sepharose 4B beads incubated with the soluble fraction of sonicated pGEX1λT-KP6 transfomed cells
6c Insoluble fraction of sonicated pGEX1λT-KP6 transformed cells
8s Eluate from glutathione sepharose 4B beads incubated with the soluble fraction of sonicated pGEX1λT-KP8 transfomed cells
8c Insoluble fraction of sonicated pGEX1λT-KP8 transformed cells

Figure 68B

Soluble and insoluble fractions from induced pGEX1λT-KP6 transformed cells, run on a 12% SDS polyacrylamide gel and stained in Coomassie Blue. Refer to legend above for explanation of tracks


E
G
6s
6c
8c
8s
66
58

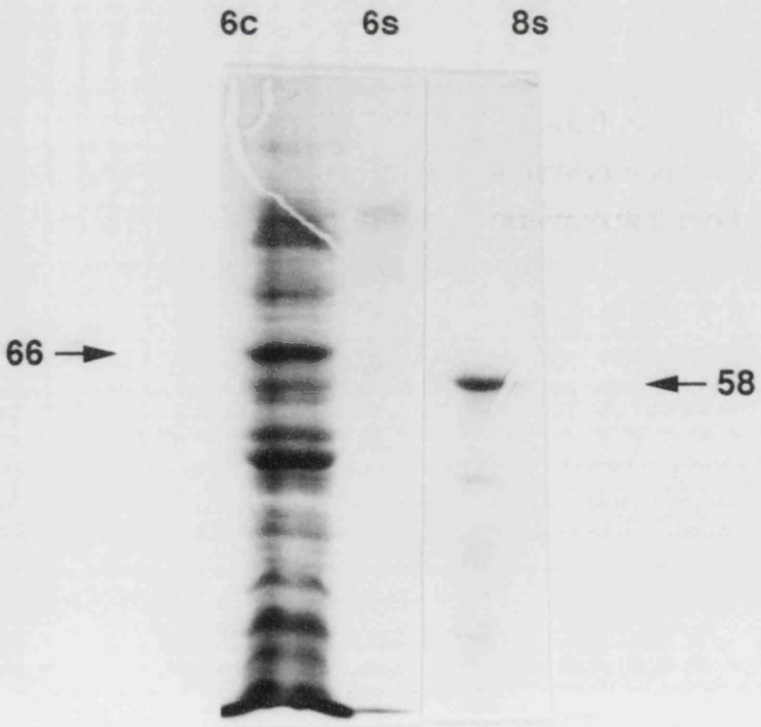

6c
6s
8s
66
58

Figure 69A

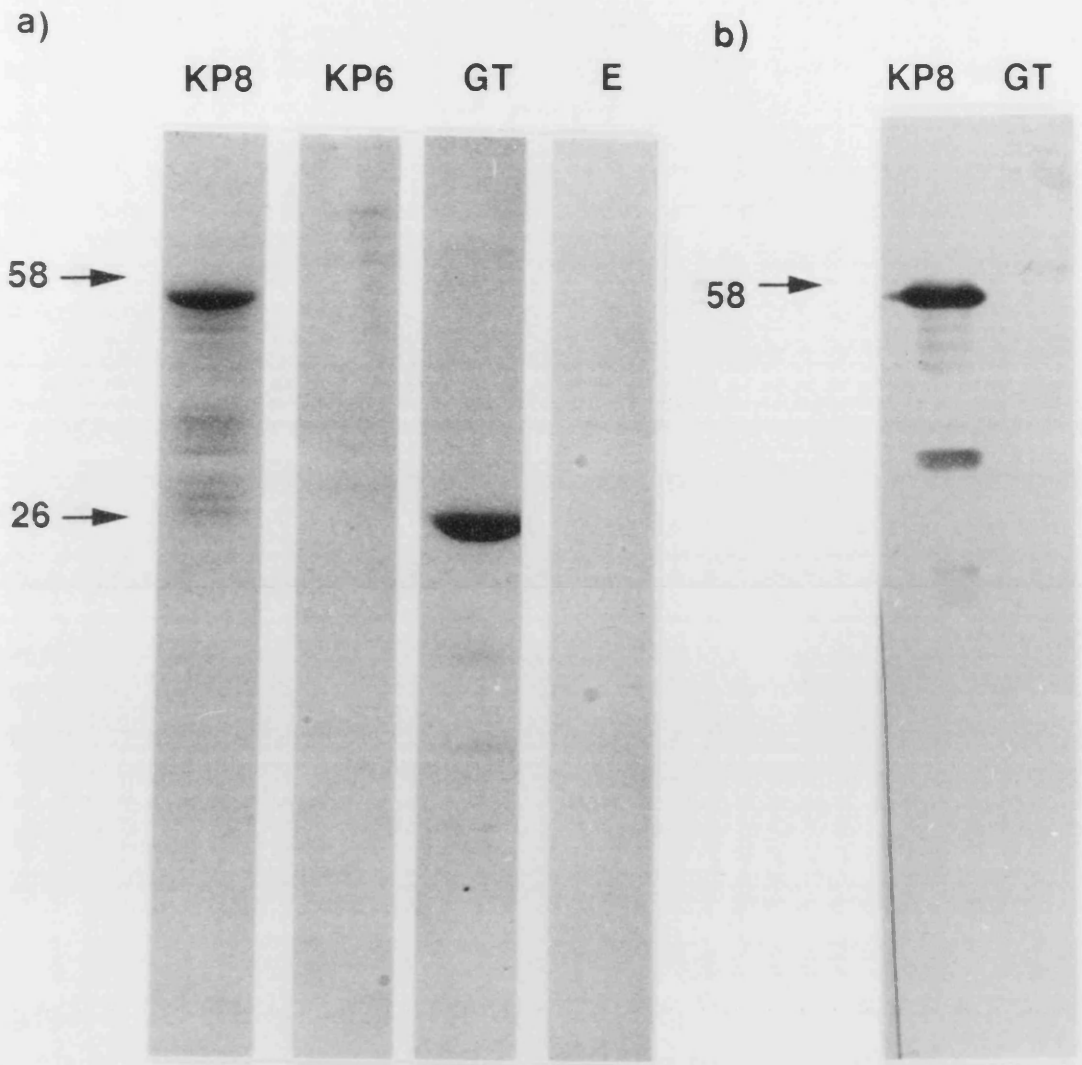
Large scale expression of KP6 and KP8 fusion proteins. Coomassie Blue stained SDS-polyacrylamide gel loaded with 75μl eluate (fraction 4) from glutathione sepharose 4B columns used to affinity purify sonicates of the following IPTG induced cultures;

KP8 pGEX1λT-KP8 transformed *E. coli*
KP6 pGEX1λT-KP6 transformed *E. coli*
GT pGEX1λT transformed *E. coli*
E Untransformed *E. coli*

The molecular weights (kDa) of the bands detected are indicated by arrows.

Figure 69B

Western blot of GST-KP8 and GST fusion proteins run on a 12% SDS-polyacrylamide gel, developed with undiluted McAb 4B11 supernatant and alkaline-phosphatase conjugated rabbit anti-mouse second antibody (1:300).
10μl of each eluate (fraction 4) was loaded per track. See explanation above.


a)
KP8
KP6
GT
E
58
26
b)
KP8
GT
58

It was decided to attempt large scale GST-fusion protein preparations from the KP6 and KP8 recombinants, in case small amounts of soluble GST-KP6 were being produced that were too low to be detected by small scale screening methods. Large scale preparations were made from 500ml cultures of pGEX1λT-KP6, pGEX1λT-KP8 and pGEX1λT transformants, as well as untransformed *E. coli* XL-1 Blue, purifying the proteins on Glutathione Sepharose 4B columns and collecting the eluate in 1ml fractions as described in section 2.2.7. 600μg protein was eluted from the pGEX1λT-KP8 column in fractions 4 and 5 (2ml total), while 7mg was eluted from the pGEX1λT column in fractions 3-8 (5ml total) according to estimation from the O.D. at 280nm. No protein was eluted from the pGEX1λT-KP6 and untransformed *E. coli* columns according to measurement of O.D. at 280nm. 60μl of fraction 4 from each eluate were run on a 15% SDS-polyacrylamide gel which was stained with Coomassie Blue (shown in Figure 69A), while 10μl samples from the same fractions were run on an identical gel and Western blotted. The Western blot was developed with McAb 4B11; the tracks containing GST-KP8 and GST alone are shown in Figure 69B. The 58kDa GST-KP8 fusion protein is clearly visible in the Coomassie stained gel (Figure 69A) and on the Western blot (Figure 69B), with no obvious fusion protein being eluted from the KP6 or *E. coli* columns (Figure 69A). The 26kDa GST protein is visible on the Coomassie stained gel but was not recognised by 4B11 (Figure 69B).

It was decided to carry out further investigations using the GST-KP8 fusion protein, since this was readily expressed in the pGEX system and was completely soluble in PBS. A large scale preparation of the fusion protein was carried out as described above, from a 3 litre culture. 3mg of GST-KP8 fusion protein was eluted in a total volume of 6ml, in fractions 3-8. This and the previous GST-KP8 preparation were used in all further experiments.

The cross-reactivity between KP8 and SPAG1 was investigated on Western blots. Replicate blots with tracks containing protein from untransformed *E. coli* XL-1 Blue and the fusion proteins GST, GST-2.7 and GST-KP8 were incubated with McAbs 1A7, 5E1, 4B11 and the GST pre-adsorbed day 92 anti-SPAG1 bovine serum 32x described in section 5.2.1. The McAb 5E1 was a control anti-merozoite antibody kindly

provided by Dr. B.Shiels (Wellcome Unit for Molecular Parasitology). The four blots are shown in Figure 70. McAb 1A7 and the 32x antiserum reacted with the 145kDa GST-2.7 fusion protein as expected. McAb 1A7 also reacted very weakly with the 58kDa fusion protein in the GST-KP8 track, not obvious in the Western blot incubated with 32x antiserum. McAb 4B11 produced a strong reaction with the 58kDa GST-KP8 and also appeared to react poorly with a single band of 90-100kDa in the GST-2.7 track. McAb 5E1 failed to react with any of the fusion proteins, and none of the antibodies reacted with GST or *E. coli* proteins. The implications of these observations will be discussed in section 8.3.

8.2.7 Affinity purification of KP8 specific antibodies from bovine serum

1mg GST-KP8 fusion protein was conjugated to 1ml AminoLink agarose beads as described in section 2.2.6, and used to affinity purify KP8 specific antibodies from the antiserum from animal 47T, which had been immunised with *T. annulata* freeze-thawed sporozoites and recognised both SPAG1 and the 4B11 antigens on Western blots (S. Williamson, personal communication). The antiserum comprised of pooled serum samples collected on days 7, 11 and 14 following the second immunisation of the animal with freeze-thawed *T. annulata* Ankara sporozoites.

Western blots with tracks containing GST-KP8 or GST fusion proteins were developed with 47T pre-immune serum, pooled post-immune serum or the antibody eluted from the column. The blots are shown in Figure 71. The 47T post-immune serum and the column-purified antibody reacted with the 58kDa GST-KP8 fusion protein but not with GST alone. No reaction was seen from the pre-immune serum. The reactivity of the purified antibody, 47T pre-immune and 47T post-immune serum with formalin fixed *T. annulata* Gharb sporozoites and *T. annulata* differentiating macroschizonts (Ankara D7 clone) were examined in IFA tests as described in section 2.2.6. The D7 IFA slides were provided courtesy of S. McKellar, Wellcome Unit of Molecular Parasitology. The IFA titres against the sporozoite material are summarised in Table 34. The eluted antibody gave an end-point titre of

Figure 70

Western blots of GST-2.7 and GST-KP8 run on a 12% SDS-polyacrylamide gel, developed with McAb 1A7, 5E1, and 4B11, or 32x day 92 GST-2.7 antiserum as indicated. All the McAbs were used undiluted, and the antiserum was used at a dilution of 1:100. The second antibodies were alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:300) for the McAbs, with alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300) for the antiserum. Tracks were loaded with 1μg of each of the following proteins;

SPAG1	GST-2.7 fusion protein
KP8	GST-KP8 fusion protein
GST	GST fusion protein
E. coli	Sonicate from untransformed *E. coli* cells

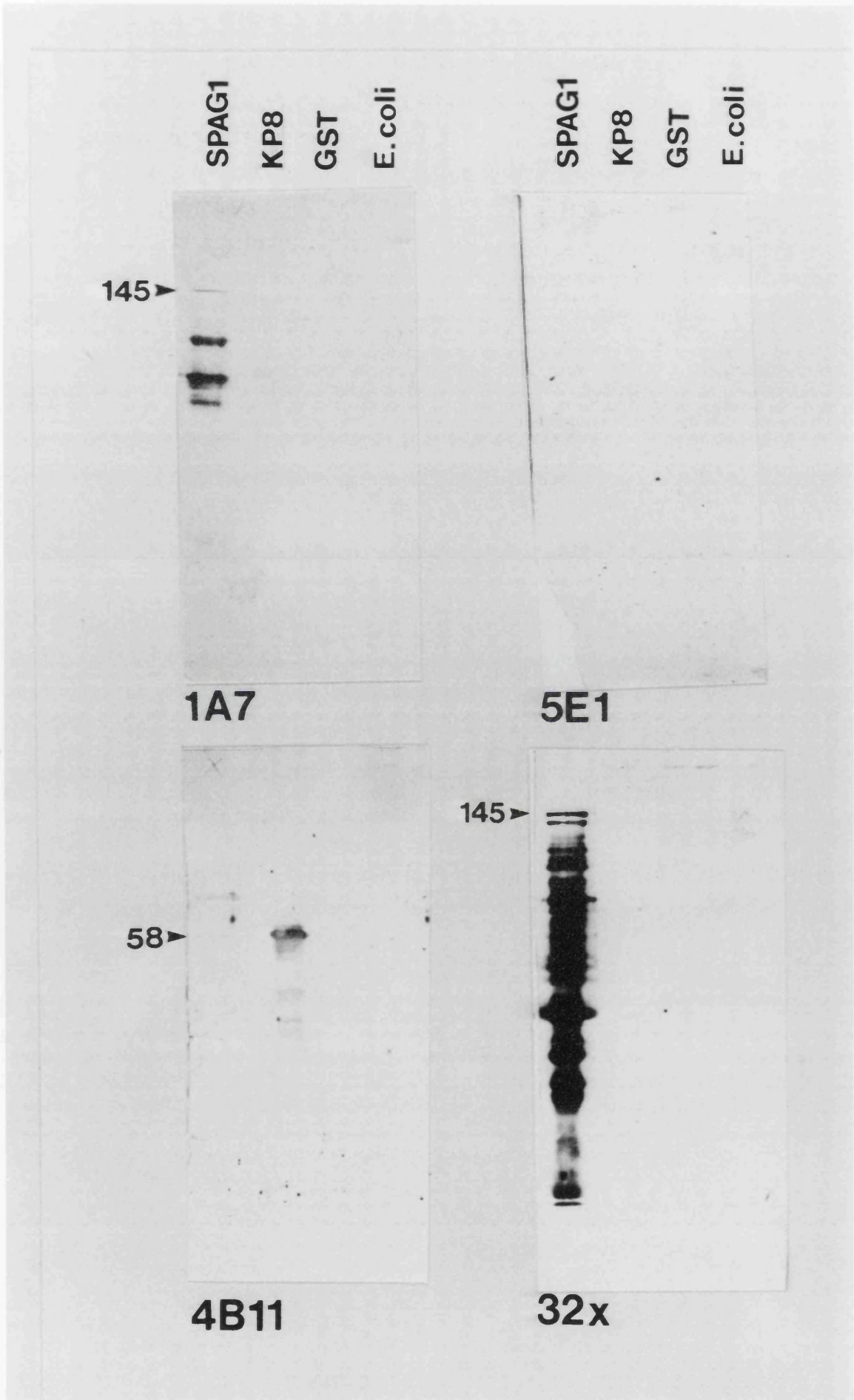

SPAG1
KP8
GST
E.coli
145
1A7
SPAG1
KP8
GST
E.coli
5E1
58
4B11
145
32x

Figure 71

Western blots of a 12% SDS-polyacrylamide gel loaded with 1μg of GST-KP8 fusion protein (K8) and GST (GT) developed with;

a 47T day 0 antiserum (1:100)
b 47T pooled post-immune antiserum (1:100)
c Antibodies eluted from the GST-KP8 column (undiluted)

The second antibody was alkaline-phosphatase conjugated rabbit anti-bovine IgG (1:300)

The 58kDa fusion protein is indicated.

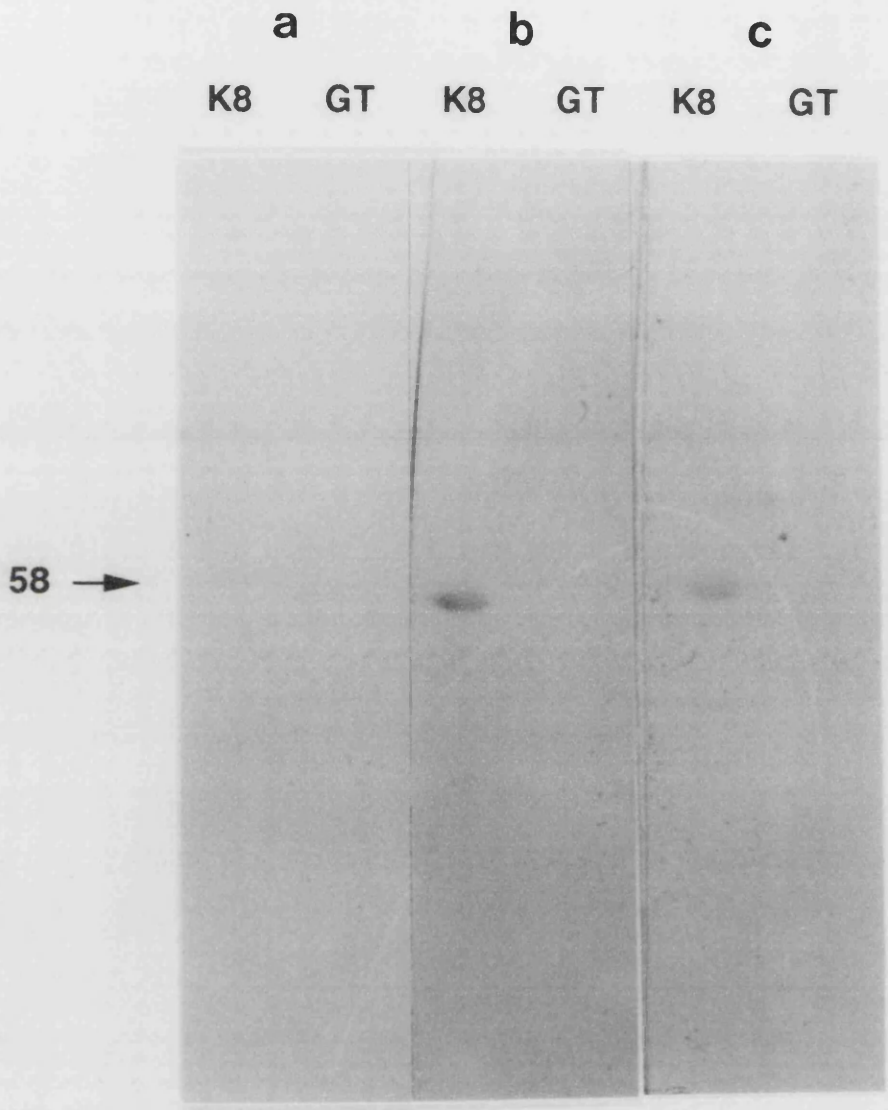
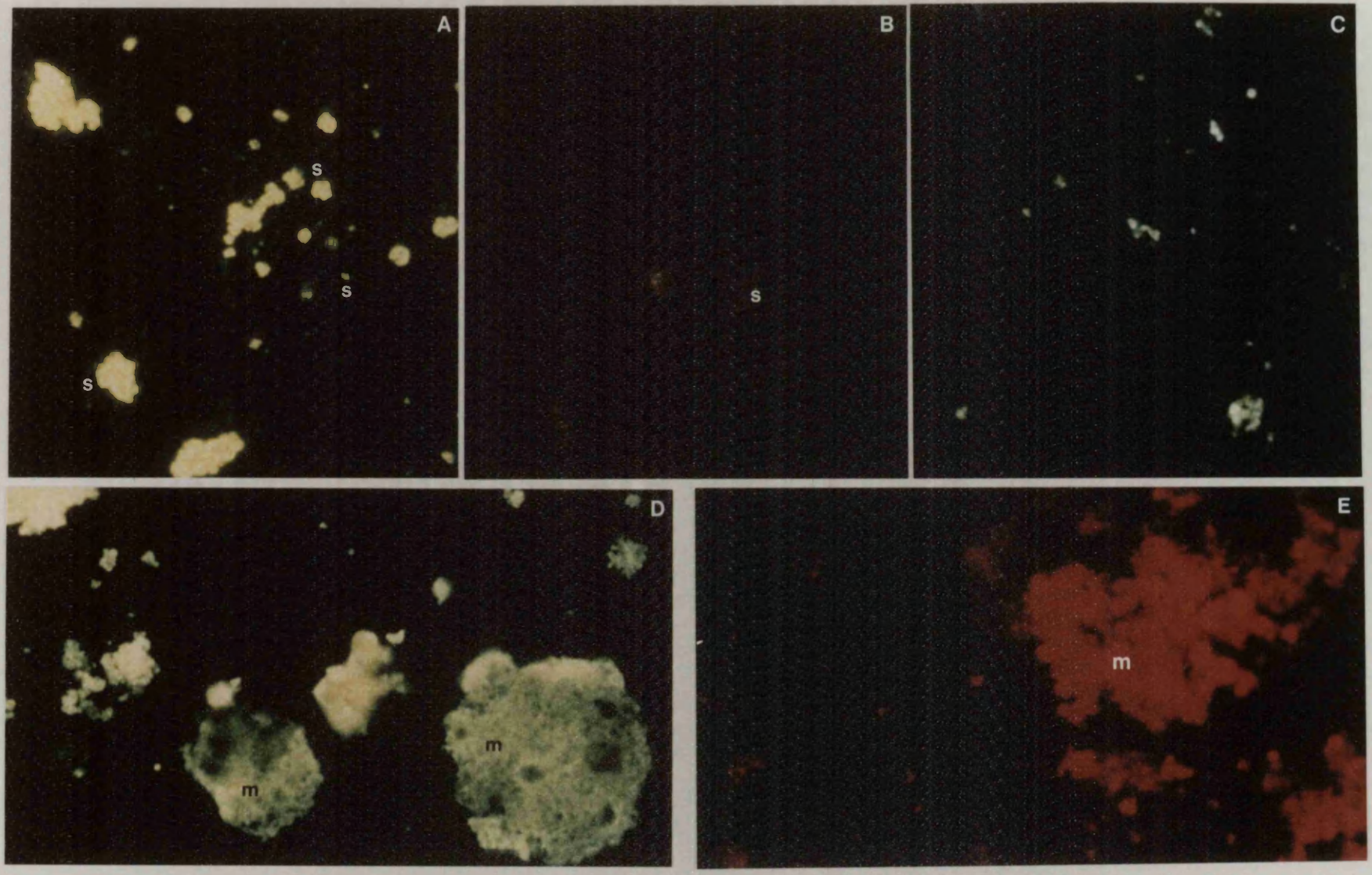

a
b
c
K8
GT
K8
GT
K8
GT
58

Figure 72

Results from IFA tests against *T. annulata* Gharb formalin fixed sporozoites (A,B,C) and *T. annulata* Ankara clone D7 differentiating macroschizonts (D, E). Primary antibodies (at a dilution of 1:10) were as follows;

A, D 47T post immune serum
B 47T day 0 serum
C, E Antibody eluted from GST-KP8 column

The second antibody was fluorescein-conjugated rabbit anti-bovine IgG (1:32).


A
s
s
s
B
s
C
D
m
m
E
m

1:100, compared with 1:10,000 for the 47T post-immune serum and the minimal reaction from the pre-immune serum. No fluorescence was detected in wells incubated with second antibody alone. The IFA reactions at a dilution of 1:10 are shown in Figure 72. While the 47T post-immune serum gave a strong reaction with both sporozoites and differentiating macroschizonts (72A and D), the antibody eluted from the GST-KP8 column reacted with sporozoites only, with no apparent reaction with the macroschizont (72C and E).

8.2.8 Generation of rabbit antiserum to GST-KP8 fusion protein

A rabbit was immunised three times with GST-KP8 fusion protein with alum as an adjuvant as summarised in section 2.2.8. Serum was collected on day 0 prior to immunisation, on day 56 (8 days after the second immunisation), day 87 and day 97 (8 and 18 days following the third immunisation). Reactivity of the serum was examined on a Western blot with tracks containing purified GST-KP8 or a lysate containing both GST and *E. coli* proteins; the results are shown in Figure 73. The blots were also incubated with McAbs 4B11 and 5E1 as positive and negative controls respectively. The day 56, 87 and 97 antiserum produced strong reactions with a 58kDa protein corresponding to the size of GST-KP8, a 27kDa protein probably corresponding to GST and a number of higher molecular weight *E. coli* proteins. The smaller bands in the GST-KP8 track were likely to be degradation products.

The reactivity of the sera with sporozoite and macroschizont /merozoite material was examined on Western blots and in IFA tests as described in section 2.2.6. Western blots of *T. annulata* Gharb sporozoite material (tick salivary gland extract from 4 day fed ticks), *T. parva* Muguga sporozoite lysate (courtesy Dr. T. Musoke, International Laboratory for Research on Animal Diseases), *T. annulata* Ankara D7 macroschizont and *T. annulata* Hissar piroplasm material (Dr. B. Shiels, Wellcome Unit for Molecular Parasitology) were incubated with McAb 4B11 neat supernatant, day 0 and day 87 GST-KP8 rabbit antisera. The blots are shown in Figure 74. The reaction of the antisera with piroplasm material appeared to be non-specific, since both day 0 and day 87 sera gave identical bands. Day 87 serum and 4B11, but not day 0 sera,

reacted with antigens of 150 and 67kDa in the *T. annulata* sporozoite material and showed weak reactivity to similar bands in the macroschizont material. Interestingly day 87 sera and 4B11 also reacted with an antigen in the *T. parva* sporozoite lysate, estimated to be of approximately 75kDa.

IFA titres were carried out using formalin fixed *T. annulata* Gharb sporozoites, and *T. annulata* Ankara D7 differentiating macroschizonts. The titres of day 0, day 56, day 87 and day 97 GST-KP8 rabbit antisera against the sporozoites are summarised in Table 35. Figures 75 and 76 show the appearance of formalin fixed sporozoites and macroschizonts in the IFA tests, using GST-KP8 antisera at a dilution of 1:25. Antisera from a rabbit immunised with merozoite material ("Eve", courtesy Dr. B. Shiels) as well as the McAbs 5E1 and 4B11 were included as controls. Day 56, 87 and 97 antisera gave end point titres of between 1/100 and 1/400, compared with the day 0 serum which only gave a positive non-specific reaction when used undiluted (Table 35). It can be seen from the photographs that while the day 0 sera produced little detectable reaction with sporozoites at a dilution of 1:25 (Figure 75B), the day 56 and 87 sera and 4B11 reacted strongly to produce a "halo" effect (Figure 75A, C and D). McAb 4B11 and day 87 sera also produced a weak reaction with differentiating macroschizonts (Figure 76A and D), while strong reactions were obtained with McAb 5E1 (76B) and merozoite antiserum (75E). The day 0 sera produced no detectable reaction with macroschizonts (76C) and fluorescence was also absent in sporozoite and macroschizont material incubated with second antibody alone (data not shown).

The ability of the GST-KP8 rabbit antiserum to block the infectivity of viable sporozoites for bovine lymphocytes was also examined in two antibody inhibition assays, carried out as described in section 2.2.10. *T. annulata* Ankara sporozoites prepared as GUTS from 3 day fed ticks were used in the assays, and the results are summarised in Tables 36 and 37. The first assay was carried out using day 0 and day 56 antisera, while sera from days 0, 56 and 97 was used in the second assay. λgt11-SR1 antisera from rabbit 77, known to have sporozoite neutralising activity (courtesy Dr. S. Williamson, Centre for Tropical Veterinary Medicine) was included as a positive control. The level of infection obtained in both assays was high, being 24.9% in the absence of antibody in the first

Figure 73

Western blots of a 12% SDS-polyacrylamide gel with tracks containing 1μg GST-KP8 fusion protein (8) or GST (G) developed with McAbs 5E1, 4B11 and day 0, day 56, day 87 and day 97 GST-KP8 rabbit antiserum as indicated. The second antibodies were alkaline-phosphatase conjugated goat anti-mouse IgG (1:300) for the McAbs, and alkaline-phosphatase conjugated goat anti-rabbit IgG (1:300) for the antisera. The molecular weights of the fusion proteins are indicated in kDa.

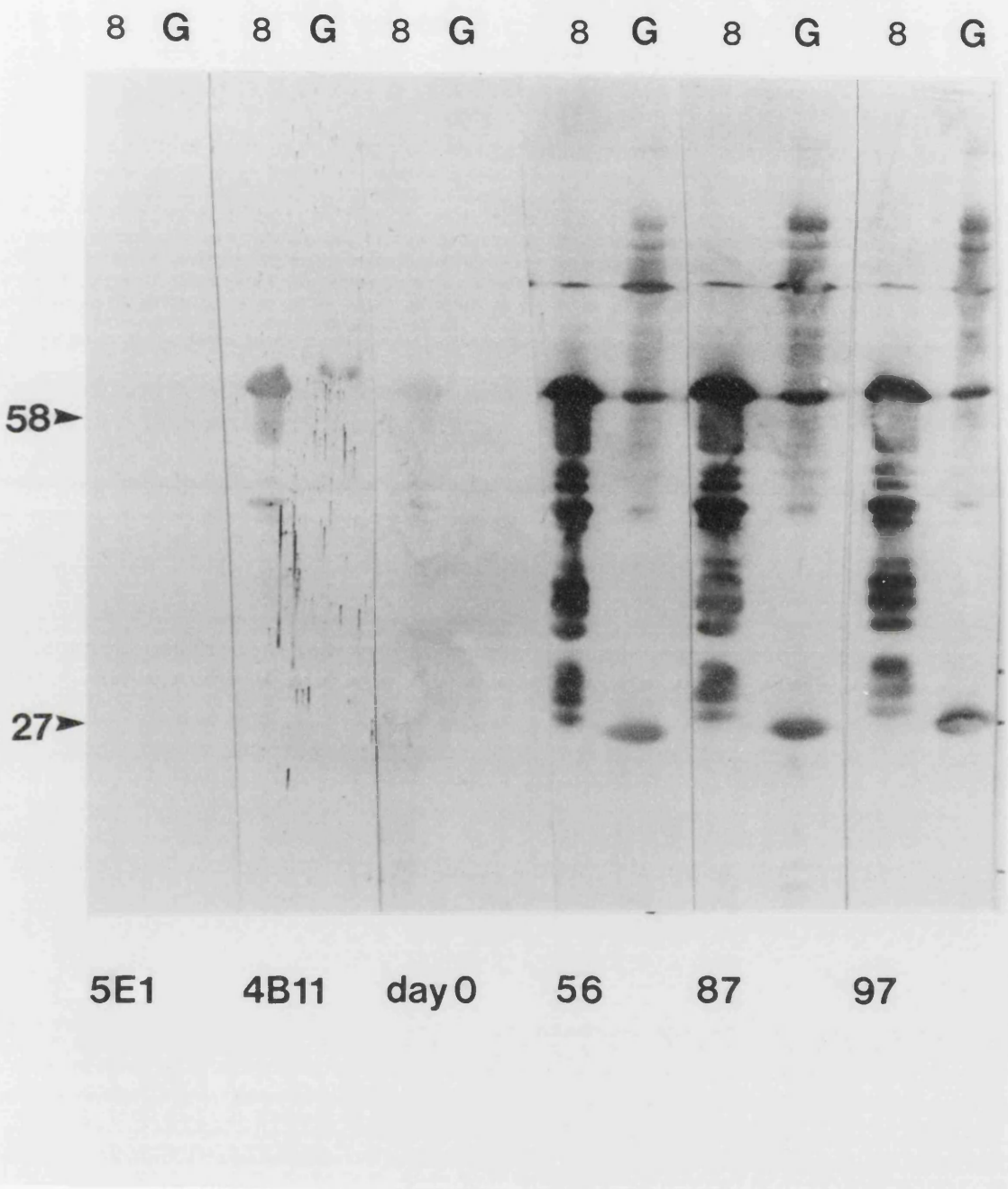
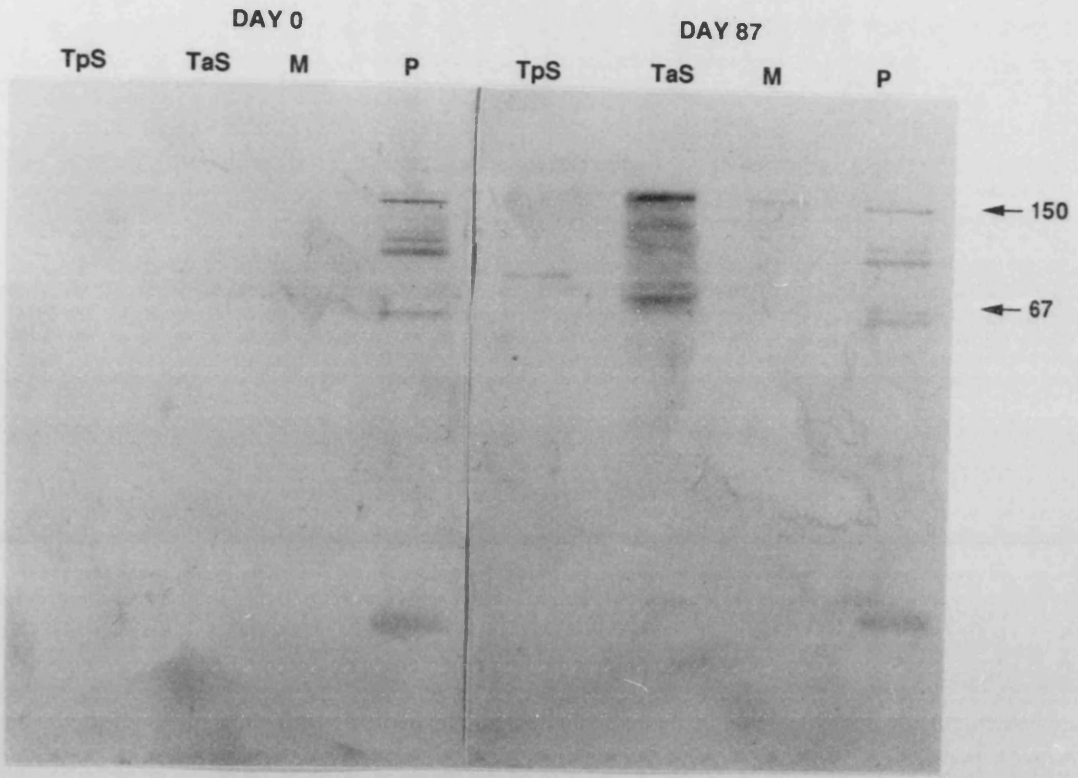

8 G 8 G 8 G 8 G 8 G 8 G
58
27
5E1
4B11
day 0
56
87
97

Figure 74

Western blot of a 15% SDS-polyacrylamide gel loaded with tracks containing the following;

TpS *T. parva* sporozoite lysate (1 tick equivalent)
TaS *T. annulata* Gharb sporozoites infected tick salivary gland extract (1 tick equivalent)
M *T. annulata* Ankara D7 macroschizont/merozoite material (approx. 20μg)
P *T. annulata* piroplasm material (approx. 20 μg)

The primary antibodies were McAb 4B11, day 0 and day 87 KP8 rabbit antisera as indicated. The second antibodies were alkaline-phosphatase conjugated goat anti-mouse IgG (1:300) for the McAb, and alkaline-phosphatase conjugated goat anti-rabbit IgG (1:300) for the antisera.


DAY 0
DAY 87
TpS
TaS
M
P
TpS
TaS
M
P
150
67

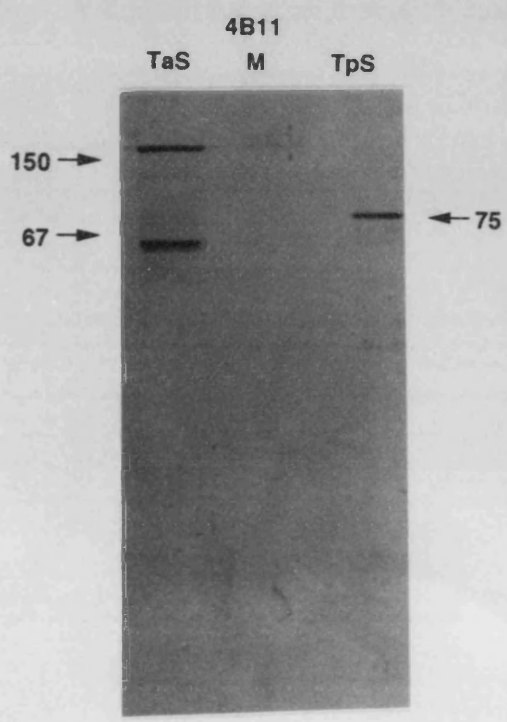

4B11
TaS
M
TpS
150
67
75

Figure 75

Results from IFA tests against *T. annulata* Gharb formalin fixed sporozoites. The primary antibodies were as follows;

A McAb 4B11 undiluted supernatant
B GST-KP8 day 0 rabbit antiserum (1:25)
C GST-KP8 day 56 antiserum (1:25)
D GST-KP8 day 87 antiserum (1:25)

The second antibodies were fluorescein-conjugated rabbit anti-mouse IgG (1:80) (A), or goat anti-rabbit fluorescein conjugated IgG (1:100) (B, C, D)

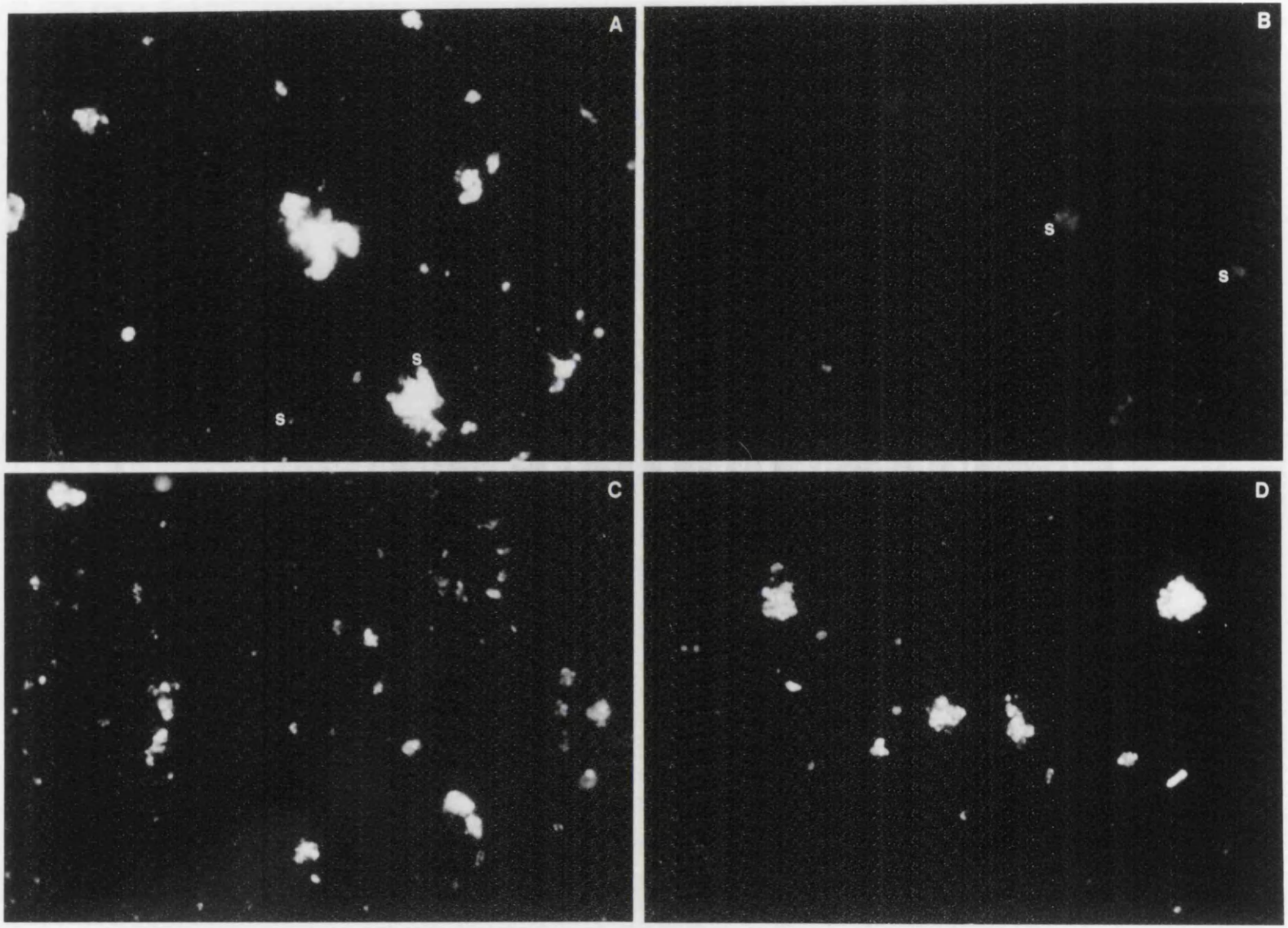
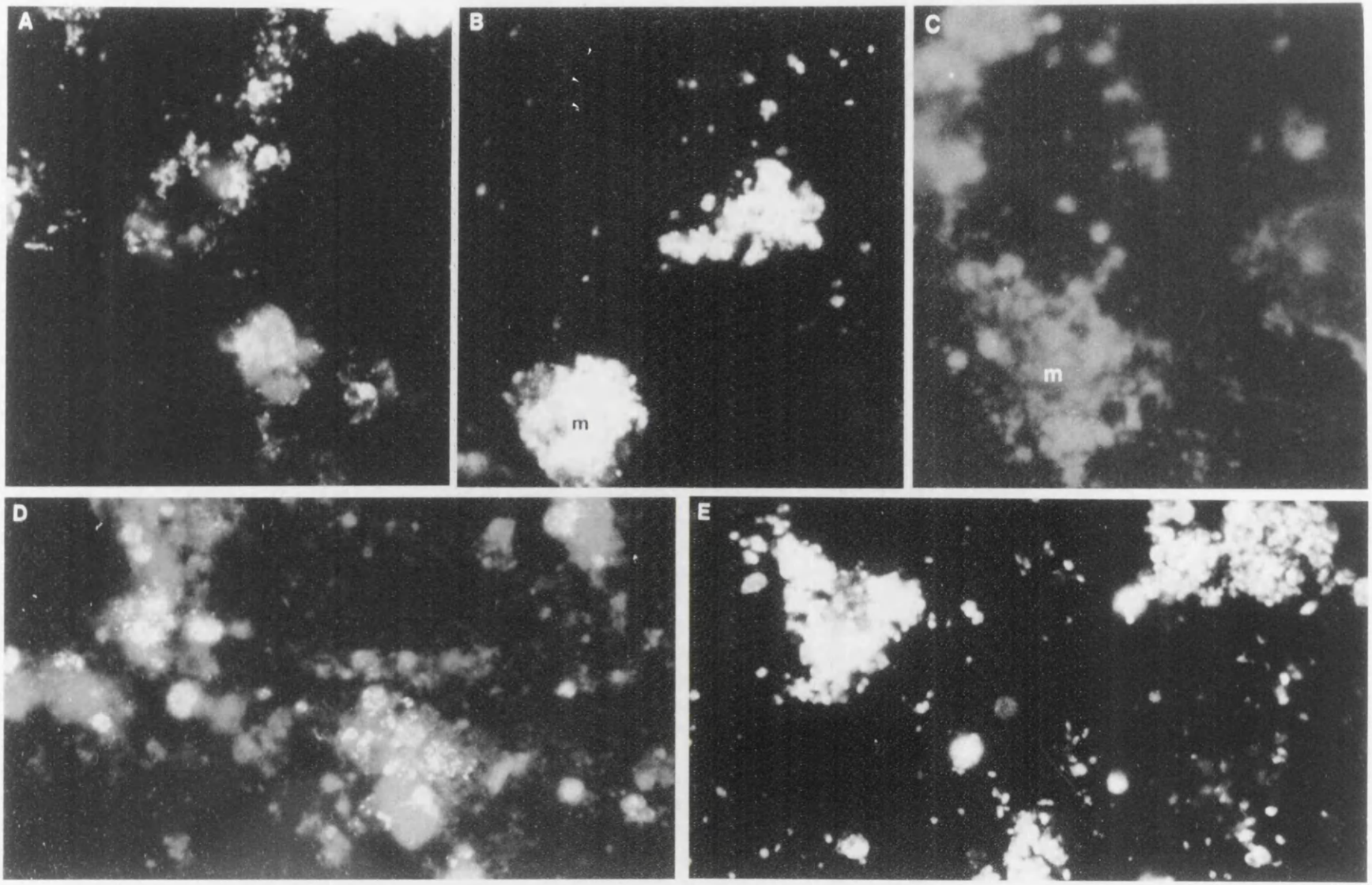

A
S
S
B
S
S
C
D

Figure 76

Results from IFA tests using *T. annulata* Ankara clone D7 differentiating macroschizonts. Primary antibodies were as follows;

A McAb 4B11 neat supernatant
B McAb 5E1 neat supernatant
C Day 0 GST-KP8 rabbit antiserum (1:25)
D Day 87 GST-KP8 rabbit antiserum (1:25)
E "Eve" anti-D7 merozoite rabbit antiserum (1:25)

The second antibodies were fluorescein conjugated rabbit anti-mouse IgG (1:80) (A, B), or goat anti-rabbit fluorescein-conjugated IgG (1:100) (C, D, E)


A
B
m
C
m
D
E

assay, and 33.6% in the second assay. A high level of infection was still obtained in day 0 serum from the GST-KP8 immunised rabbit. High levels of inhibition (up to 94.7%) were produced by the GST-KP8 antiserum when used undiluted in both assays. Inhibition produced by diluted sera was much lower. The λgt11-SR1 antiserum produced high levels of inhibition as expected.

8.3 Discussion

In this chapter work was described on the identification, cloning and expression of two sequences, KP6 and KP8, containing epitopes recognised by McAb 4B11. The hybridisation of the KP6 and KP8 inserts to *T. annulata* DNA only and not to bovine DNA on a Southern blot (Figure 62) and to RNA from sporozoite infected ticks, not from uninfected ticks (Figure 63) showed them to be derived from *T. annulata* sequences. Since hybridisation to macroschizont RNA was weak (Figure 63), the KP6 and KP8 sequences appeared to be expressed primarily in the sporozoite stage. However, the hybridisation to macroschizont RNA and some degree of reactivity of McAb 4B11 and GST-KP8 antiserum with differentiating macroschizont material on Western blots (Figure 74) and in IFA tests (Figure 76) indicates that the 4B11 antigen may also be present at low levels in the macroschizont or merozoite stage.

The lack of cross-hybridisation between the KP6 and KP8 inserts on Southern blots (Figures 64 and 65) indicated they were different sequences. Furthermore, the lack of hybridisation of KP8 to restriction enzyme digests of λ DASH-KP6+ implied that the sequences were not in close proximity, since the large 15.6kb insert of λ DASH-KP6+ would be expected to include some of the KP8 sequence if they were adjacent. However, this would depend on the position of the KP6 insert within the 15.6kb insert, which was not known.

The inserts KP6 and KP8 have been used as part of a *T. annulata* genome mapping project carried out using pulse-field gel electrophoresis, which provided more information about their linkage (Prof. A. Tait and Ms N. Buchannan, personal communication). The inserts were found to be located on the same chromasome of *T. annulata* (chromosome 2), and on the same Sfi I fragment (1.25Mb) and the same Not I fragment

(1.4Mb). However, they were on different Eag I fragments; KP6 was identified on a 60kb fragment with KP8 on a 500kb fragment. There is no evidence for an Eag I site in the KP8 sequence, but this by no means precludes the possibility of the site existing within the rest of the gene. Therefore there are two possibilities for the origin of the KP6 and KP8 sequences; firstly, they are derived from the same gene with an internal EagI site and either overlapping at the 4B11 epitope or containing repeats of the epitope, or secondly, they are from separate genes, both coding for the 4B11 epitope. Sharing of a 4B11 epitope by KP6 and KP8 would not necessarily result in a homologous DNA sequence of sufficient length to allow cross-hybridisation between the inserts; differences in the nucleotide sequences could be generated by different codon usage.

Since the largest expressed product of the 4B11 antigen gene appears to be about 150kDa according to Western blot analysis using McAb 4B11 (Figure 74), this would give the gene a predicted size of at least 3kb without introns, but this is excluding any anomalous migration of the protein. Northern blot analysis indicated that the mRNA was 3kb in size. Such a gene would be of adequate size to accomodate both the KP6 and KP8 sequences. It would be quite possible for the 4B11 epitope to be repeated more than once within the same gene; repeated epitopes have already been identified in *P. falciparum* antigens (Nussenzweig and Nussenzweig 1985). There are also precedents for sharing of small regions of homology between the products of separate genes, such as the cross-reactivity identified between a number of *P. falciparum* antigens (Hyde 1990), and homologies identified between the tandemly arranged genes of four *Babesia rodhaini* surface membrane proteins (Snary and Smith 1988).

The 67kDa and 17kDa antigens identified by McAb 4B11 (Figure 37, tracks 2 and 7) could be proteolytic processing or degradation products of the 150kDa molecule, or could be the products of different genes. The identification of the 150kDa and 67kDa antigen by GST-KP8 antiserum as well as McAb 4B11 in *T. annulata* sporozoite material on Western blots implies they are both the products of a single gene, unless the 4B11 epitope or other cross-reactive epitopes were being recognised by antibodies in the rabbit sera. No 17kDa antigen was detected by McAb 4B11 or the antisera on the Western blots of salivary gland extract

from *T. annulata* infected ticks (Figure 74), while a 17kDa band was present in Western blots of ground up tick supernatant (Figure 37) and was the only antigen detected by 4B11 and bovine sera in Western blots of Percoll purified sporozoites (Williamson 1988). However, while Percoll purified sporozoites consist largely of mature forms and ground up tick supernatant contains both mature and immature sporozoites (Williamson 1988), the salivary gland extracts contained a high proportion of sporoblasts (immature forms) according to light microscope observation of Giemsa stained cytospin smears. Therefore, if the 17kDa antigen is a final processing product found on mature sporozoites only, the inconsistancies in it's detection could be explained by variation in the relative proportions of mature and immature sporozoites. The 4B11 antigen could be processed in a similar fashion to the *P. falciparum* merozoite protein MSP1, in which a high molecular weight precursor is extensively processed to a 19kDa antigen present on the surface of invading merozoites (Blackman et al 1990). Interestingly, antigens of 69 and 140kDa, which could correspond to the 67 and 150kDa bands on the Western blots described here, were immunoprecipitated by mouse anti-sporozoite serum from surface iodinated sporozoites in previous studies (Shiels et al 1989), the differences in molecular size being explained by experimental error.

The KP8 insert was expressed successfully in the vector pGEX1λT as the soluble fusion protein GST-KP8; the size was estimated to be 58kDa estimated according to it's migration on SDS-polyacrylamide gels. By subtraction of the known molecular weight of GST (26kDa) the KP8 polypeptide was estimated to be of size 32kDa, which corresponds to the 30kDa predicted molecular weight from the coding capacity of the 900bp KP8 insert. The GST-KP8 fusion protein was recognised by McAb 4B11 and by serum from a cow immunised with sporozoites (Figure 71). The GST-KP6 fusion protein expressed from pGEX1λT-KP6 was also recoginsed by McAb 4B11, but remained in the insoluble cellular fraction after sonication (Figure 68A and B). The problems of insolubility and inclusion body formation associated with eukaryotic gene expression in *E. coli* have already been discussed in section 1.2.5. The insolubility of GST-KP6 may be due to incorrect disulphide bond formation if the sequence contains cysteine residues, or due to a requirement for post-translational modification not carried out by the prokaryotic host. The

size of the GST-KP6 fusion protein was estimated to be 66kDa according to it's migration on SDS-polyacrylamide gels, which would give a size of 40kDa for the KP6 polypeptide. This is larger than the estimated coding capacity of 26.6kDa predicted from the 800bp KP6 sequence. An explanation could be that the GST-KP8 protein migrated anomalously on SDS-polyacrylamide gels, which can be caused by a high proline content (Williamson 1989) or by the protein being very basic (Hames 1988).

The GST-KP8 fusion protein was able to bind antibodies from 47T antiserum. While the antiserum was reactive to both sporozoite and macroschizont/merozoite antigens in an IFA test (Figure 72A and D) the GST-KP8 affinity purified antibody reacted only with sporozoites (Figure 72C and E). This implies that the GST-KP8 fusion protein was binding sporozoite specific antibodies from the cattle serum. Antiserum to GST-KP8 raised in the rabbit also reacted strongly with *T. annulata* sporozoite material in IFA tests (Figures 75 and 76, and Table 35) and on a Western blot (Figure 74). Weak reactivity was also observed to the differentiating macroschizont material used in the IFA tests and in the Western blot. The GST-KP8 antiserum reacted with antigens of 150 and 67kDa, identical to those detected by McAb 4B11. The ability of the GST-KP8 antisera to neutralise infectivity of live *T. annulata* sporozoites "in vitro" (Tables 36 and 37) indicates that the antiserum contained antibodies recognising neutralising determinants on or in contact with the sporozoite surface.

The reactivity of 4B11 and the GST-KP8 antiserum with a band of approximately 75kDa in *T. parva* sporozoite lysate (Figure 74) and the weak reactivity with the NS1-p67 fusion protein described in chapter 5 (Figure 44C track 8) indicates that there is some cross-reactivity between the 4B11 antigen and p67 and/or other *T. parva* sporozoite antigens. Sequence comparison between p67 and the KP8 nucleotide and amino acid sequences did not reveal any stretches of homology, although the KP8 sequence was incomplete. If they are cross-reactive, it would be expected that the KP8 and p67 amino acid sequences would share the 4B11 epitope or a sequence similar enough for the antibody to bind to p67, but the two antigens may not necessarily share close homology at the DNA level. The complete KP8 amino acid sequence would be required

to properly elucidate whether there could be an epitope in common with any *T. parva* antigens which have been sequenced.

There is considerable evidence that SPAG1 and the 4B11 antigen are different proteins; they are of different molecular weights on Western blots, there was no sequence homology between the KP8 and SPAG1 nucleotide sequences and no obvious cross-reactivity between the KP8 and SPAG1 GST fusion proteins (Figure 70). However, there may be small areas of homology between the two antigens at the amino acid level, since 4B11 reacted with a λgt11 recombinant containing the 1A7 epitope (λgt11-KP11) in the genomic expression library screening (Figure 60A and B). Very weak reactivity was detected to GST-2.7 by 4B11, and to GST-KP8 by 1A7 (Figure 70). Possible relationships between the SPAG1 and the 4B11 antigen will be discussed in Chapter 9, but will probably be more fully understood once the complete sequence of KP8 is obtained.

TABLE 33 RESTRICTION FRAGMENTS FROM λDASH RECOMBINANTS

The following table summarises the sizes of the restriction fragments produced by digestion of λDASH-KP6+ and λDASH-KP8+ by the enzymes Sal I, Xba I, Eco RI, Sst I and an Eco RI/Sal I double digest. (N.B. Fragments much smaller than 1kb may not have been visualised on the gel). * denotes the λ vector arms.

RECOMBINANT	ENZYME	RESTRICTION FRAGMENTS (kb)
λDASH-KP6	Sal I	20* / 12 / 9.2* / 2.6 / 1.0
	Xba I	Failed to cut
	Eco RI	20* / 12 / 9.2* / 3.6
	Sst I	22 / 18 / 4.9
	Sal I/Eco RI	20* / 9.2* / 9 / 2.6 / 2.1 / 1.0
λDASH-KP8	Sal I	20* / 9.2* / 6.5 / 5.2 / 1.8
	Xba I	Failed to cut
	Eco RI	20* / 9.2* / 6.5 /3.7 / 3.5
	Sst I	Failed to cut
	Sal I/Eco RI	20* / 9.2* / 6.5 / 3.7 / 1.9 / 1.2

TABLE 34 IFA TEST; GST-KP8 ADSORBED ANTIBODY

The following table shows the IFA titres of cow 47T pre-immune and pooled post-immune serum, and antibody eluted from the GST-KP8 column, against *T. annulata* Gharb formalin fixed sporozoites. The second antibody used was rabbit anti-bovine IgG diluted 1:32. Fluorescence in each well was scored as very bright (+++), moderate (++), weak (+), barely detectable (+/-) or absent (-). The highest dilution giving a "+" was taken to be the end-point titre.

SERUM DILUTION	47T PRE-IMMUNE SERUM	47T POST-IMMUNE SERUM	ELUTED ANTIBODY
x1	+/-	+++	+++
1/10	+/-	+++	+++
1/100	-	+++	+
1/1000	-	++	+/-
1/10,000	-	+	+/-

TABLE 35 IFA TEST; GST-KP8 ANTISERUM

The following table gives the IFA titres against formalin fixed *T. annulata* Gharb sporozoites of sera collected before immunisation (day 0), 8 days after the first boost (day 56), 8 days after the second boost (day 87) and 18 days after the second boost (day 97) with GST-KP8 fusion protein. The second antibody was goat anti-rabbit IgG diluted 1:100. McAbs 4B11 and 5E1 (anti-merozoite, kindly provided by Dr. B. Shiels, Wellcome Unit for Molecular Parasitology) were included as controls, using rabbit anti-mouse IgG diluted 1:80 as a second antibody. The degree of fluorescence in each well was scored as described above.

SERUM DILUTION	DAY 0	DAY 56	DAY 87	DAY 97
x1	+++	+++	+++	+++
1/25	+/-	++	++	++
1/100	+/-	+	+	+
1/200	+/-	+	+	+/-
1/400	-	+/-	+	+/-
1/800	-	-	-	-

McAb 4B11 neat supernatant was scored as " +++ "
McAb 5E1 neat supernatant was scored as " - "

TABLE 36 ANTIBODY INHIBITION ASSAY 1

The following table summarises the results of an antibody inhibition assay carried out using day 0 and day 56 GST-KP8 rabbit antiserum using *T. annulata* Ankara sporozoites. Each test was repeated in quadruplicate. 400 cells were counted from a Giemsa stained cytospin smear from each well after 4 days in culture and the percentage of cells infected with sporozoites, trophozoites or macroschizonts was assessed. The degree of inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = \frac{x - y}{x} \times 100$$

where x = mean % infection for pre-immune serum

and y = mean % infection for post-immune serum

Standard deviations are included in brackets. Significant inhibition compared to day 0 serum according to Student's T-test are denoted as * = P > 0.05, ** = P > 0.005

SERUM	DILUTION	% INFECTED CELLS	% INHIBITION
Medium only	-	24.9 (1.12)	-
Day 0	x1	28.2 (0.96)	-
	1/4	23.3 (1.36)	-
	1/16	25.3 (3.2)	-
Day 56	x1	7.4 (2.59)	73.8**
	1/4	20 (5.22)	14.2
	1/16	22.4 (4.13)	11.5

TABLE 37 ANTIBODY INHIBITION ASSAY 2

The following table summarises the results of an antibody inhibition assay carried out using day 0, day 56, and day 97 GST-KP8 rabbit antiserum, using *T. annulata* Ankara sporozoites. Day 0 and day 66 antiserum from a rabbit (R77), which had been immunised with λgt11-SR1 fusion protein and was known to show inhibition (Williamson 1988), was also included as a positive control. Quadruplicate wells were set up for each test, and 200 cells counted from a Giemsa stained cytospin smear taken from each well and the percentage of cells infected with sporozoites, trophozoites or macroschizonts was assessed. The degree of inhibition was calculated as described above.

SERUM	DILUTION	% INFECTED CELLS	% INHIBITION
Medium only	-	33.6 (4.28)	-
Day 0	x1	35.6 (4.39)	-
	1/4	30.1 (2.5)	-
	1/16	31.4 (3.96)	-
Day 56	x1	4.6 (1.18)	87**
	1/4	26 (5.9)	13.6
	1/16	33 (2.45)	0
Day 97	x1	1.9 (2.02)	94.7**
	1/4	20.5 (6.51)	31.9*
	1/16	15.1 (2.1)	51.9**
R.77 day 0	x1/4	20.6 (2.5)	-
	x1/16	26.25 (5.91)	-
R.77 day 66	x1/4	2 (0.91)	90.3**
	x1/16	10.4 (4.01)	60.38**

CHAPTER 9

GENERAL DISCUSSION

This work has described research towards the partial elucidation of the bovine immune response to the sporozoite antigen SPAG1, the identification of polymorphisms of the antigen and confirmation of it's surface location. In addition, the cloning, expression and partial characterisation of a second sporozoite antigen, carrying the epitope recognised by the anti-sporozoite McAb 4B11, has also been undertaken. The results have been discussed at the end of each chapter; the following account is an overview of the points raised.

The SPAG1 antigen is highly polymorphic according to the data reported in Chapter 3. Restriction fragment analysis indicated that at least six alleles of SPAG1exist (Table 4). The existance of polymorphic alleles has been confirmed by sequencing (Dr. R. Hall, personal communication). SPAG1 diversity could have immunological implications if it occurs in B or T cell epitopes, as discussed in section 3.4.

As described in Chapter 5, cattle immunised with GST-2.7 recombinant fusion protein, containing most of the SPAG1 amino acid sequence, recognised several groups of linear B cell epitopes according to Western blot analysis using expressed SPAG1 sub-fragments. All five of the cattle produced similar patterns of reactivity, only varying in their recognition of some of the centrally located epitopes in the SPAG1 sequence (Figure 47). This contrasts with the reactivities of antisera from cattle immunised with *T. annulata* freeze-thawed sporozoites, which were highly variable in the epitopes they recognised. However, all of the antisera consistantly recognised epitopes in the C-terminal region. A C-terminal epitope was also recognised by the McAb 1A7, although the McAb and the bovine antisera do not recognise identical epitopes. Since the McAb 1A7 and the bovine antisera had been shown to neutralise the infectivity of *T. annulata* sporozoites "in vitro", a property not shared by the McAbs 4A7, 4D3 and 5D1 (Williamson 1988) which recognise N-terminal SPAG1 epitopes, it was concluded that the C-terminal region of SPAG1 contained sporozoite neutralising epitopes. Since McAb 4A7

raised against *T. annulata* sporozoites clearly reacted with α-elastin (Figure 40) this suggested that the McAb was reacting with the elastin homologous repeats. Since McAb 4A7 fails to show any convincing ability to block sporozoite invasion of lymphocytes (Williamson 1988) this indicates that the elastin homologous regions of SPAG1, which have been postulated to act as a ligand (Hall et al 1992), are not an essential requirement for sporozoite recognition and invasion. Furthermore, sequence comparison between the two SPAG1 alleles gSPAG1 and cSPAG1, which had both been sequenced completely (R. Hall and P. Hunt, personal communication) indicated that the C-terminal region was the most conserved part of the molecule.

Evidence was also presented in Chapter 5 that the *T. annulata* SPAG1 and *T. parva* p67 sporozoite antigens are homologous molecules (Figure 41) and clearly cross react according to Western blot and IFA analysis (Figures 42-45). Interestingly, most of the regions of SPAG1 recognised by GST-2.7 antiserum were also recognised by antisera raised against *T. parva* sporozoites and *T. parva* NS1-p67 recombinant antigen (Table 17 and Figure 47), including C-terminal epitopes.

Despite the strong recognition of sporozoite antigens by the bovine GST-2.7 antisera on Western blots and in immunofluorescence assays, and the ability of the antisera to inhibit sporozoite invasion of lymphocytes almost completely "in vitro", the GST-2.7 immunised animals were not protected against challenge with *T. annulata* Hissar sporozoites. Possible reasons for this failure in protection, such as polymorphism of SPAG1 epitopes or that the vector system used for SPAG1 expression was inappropriate, are discussed in Chapter 5. The advantages and disadvantages of using the pGEX vectors for intracellular expression of the fusion protein will be considered later in this chapter.

The immuno-electron microscopy work using McAb 1A7 described in Chapter 7 establishes that the SPAG1 molecule has a surface location on *T. annulata* sporozoites, which had previously only been deduced indirectly. Furthermore, these studies indicated that SPAG1 is not shed during lymphocyte invasion. However, this observation may only be true for the C-terminal part of the molecule, containing the 1A7 epitope. To identify the fate of SPAG1 N-terminal components during invasion, which

may be shed by proteolytic processing as described in section 1.1.3, it would be necessary to carry out inmmuno-electron microscopy using McAbs recognising N-terminal epitopes, such as 4D3 or 5D1. Better insight could also be gained into the fate of SPAG1 during invasion by carrying out post-embedding immuno-labelling for electron microscopy work, as described in the studies on the *P. falciparum* merozoite invasion of erythrocytes (Culvenor et al 1991).

The results of the work towards identifying T cell epitopes of SPAG1 described in Chapter 6 were dissappointing in that it was not possible to produce BoT4+ clones against SPAG1 which could be used in T helper cell epitope mapping. Explanations for the production of short lived clones with the unusual phenotype $BoT2^-$, $BoT4^-$, $BoT8^+$, such as a mitogenic effect of recombinant SPAG1, were put forward in section 6.3. However, the results that were obtained indicated that SPAG1 did contain T cell epitopes, since PBM from a cow which had been immunised with *T. annulata* sporozoites (11434) proliferated in response to recombinant GST-2.7 (Table 19); PBM and some cell lines from cattle immunised with GST-2.7 fusion protein also proliferated in response to recombinant SPAG1 produced from the fusion protein by Factor Xa cleavage (Tables 21, 28 and 29). While the GST-2.7 cell lines produced by L. Glass and P. Millar (AFRC Roslin, Edinburgh) appeared to recognise SPAG1 epitopes, they could not be used to map the T cell epitopes using the SPAG1 fusion proteins since they also proliferated in response to GST alone, which is present in all the constructs (Tables 28 and 29). This problem could be circumvented by either removing cells responsive to GST from T cell lines during an early stage in culture, by treatment with 5'-bromodeoxyuridine in the presence of light as described by Sakane and Green (1979), or by expressing SPAG1 without GST in a different vector system for immunisation of cattle, only using the GST fusion proteins for testing T cell lines or clones.

The expression of intact SPAG1 and smaller subconstructs of the antigen in *E. coli* using the pGEX vectors was reasonably successful in that the fusion proteins were soluble and milligram quantities could be purified (Chapter 4). While all the fusion proteins were degraded to an extent, there was still at least 15-20% undegraded fusion protein even when large constructs (up to 145kDa) were expressed. The main problem

associated with this vector was the difficulty in removing the GST polypeptide from the fusion proteins, which was recognised by T and B cells to the recombinant protein, and could have potentially interfered with the immune response to SPAG1 as discussed in Chapter 5. While antibodies to GST could be easily removed by affinity purification (described in section 5.2.1), removal of GST reactive T cells from lines is not so easily accomplished, which would involve selective killing of GST reactive T cells. Expression in *E. coli* is also problematic in that post-translational modifications such as glycosylation are not carried out, as discussed in section 1.2.5. This could interfere with the folding of the antigen or the structure of conformational epitopes. It is not known at present if SPAG1 is post-translationally modified, but if this is the case it would be advantageous to express the antigen in a eukaryotic system, such as vaccinia virus in mammalian cells (described by Moss et al 1988).

While preliminary characterisation of a *T. annulata* sporozoite antigen carrying the epitope for McAb 4B11 has been described in Chapter 8, this work raises a considerable number of questions that remain to be addressed. Firstly, the relationship between the two sequences containing the 4B11 epitope, KP6 and KP8, needs to be fully elucidated to verify whether or not they are part of the same *T. annulata* gene. Approaches that could be taken are to screen the inserts from the other λgt11 recombinant clones isolated with McAb 4B11 (λgt11-KP1-5, λgt11-KP7 and λgt11-KP9-15) with KP6 and KP8, to identify any containing sequences common to both. The λDASH recombinant clones isolated using the KP6 and KP8 inserts, apart from λDASH-KP6+ and λDASH-KP8+, could also be screened with both inserts on Southern blots to detect any common sequences. Additionally, the λDASH library could also be further screened with the KP6 and KP8 inserts in an attempt to identify a recombinant containing both sequences, and carrying out sequence analysis of the λDASH insert. Another approach would be to analyse the hybridisation patterns of both the KP6 and KP8 inserts to restriction fragments on Southern blots of restriction enzyme digested DNA from *T. annulata* cloned macroschizont cell lines. The Ankara and Soba clones identified in Chapter 3 as carrying different SPAG1 alleles would be ideal for this purpose. If the inserts consistantly hybridised to

bands of identical size in the clones, this would indicate close proximity in the *T. annulata* genome.

While the KP8 insert could be readily expressed as a soluble fusion protein with GST in the pGEX expression system, the KP6 fusion protein was insoluble. This could have been due to incorrect folding, improper formation of disulphide bridges or lack of post-translational modification which can all result in insolubility as described in section 1.2.5. The problems of incorrect folding and/or failure of disulphide bridge formation may be avoided by using an alternative vector for expression in *E. coli*. Candidate vectors could be the pTO-N system for secretion of recombinant protein into the periplasmic space (described in section 1.2.5) or the pQE vectors (Stüber et al 1990). The latter vector system expresses the recombinant protein fused to a six histidine residue affinity tag, which enable the protein to be purified on a nickel-conjugated resin. Purification can be achieved under denaturing conditions, which can be used to solubilise recombinant proteins (Marston 1986). Problems associated with lack of post-translational modification may be circumvented by expression in a eukaryotic vector. It would be advantageous to immunise an animal with the KP6 protein in order to investigate whether the 150 and 67kDa bands recognised by KP8 antiserum on Western blots of sporozoite proteins are also recognised by KP6 antiserum. This would provide further evidence for the two sequences being part of the same gene.

It is interesting to speculate on the possible relationship between the SPAG1 antigen and the antigen recognised by 4B11. The two antigens may share some regions of homology at the amino acid level, since McAb 1A7 reacted with a 4B11-positive λgt11 recombinant (Figure 60, B) and reacted weakly with the GST-KP8 fusion protein on a Western blot (Figure 70). However, comparison between the partial KP8 DNA sequence and SPAG1 showed no obvious regions of homology, confirming they were separate antigens. The ability of antisera and McAbs raised against the SPAG1 and 4B11 antigens to block infection of leucocytes by sporozoites "in vitro" suggests that both the antigens have a role in sporozoite invasion or recognition of host cells. The antigens may mediate alternative forms of entry, or one of the antigens might be involved in initial recognition, while the other could be involved in the

invasion process. As discussed in Chapter 7, there is evidence that target cell recognition and invasion by apicomplexan protozoa could be mediated by separate determinants. Since the antisera raised against *T. parva* sporozoite material showed extensive cross-reactivity with SPAG1 and recognised B cell epitopes in the C-terminus (the region most likely to contain ligands as described earlier in this section) it seems unlikely that SPAG1 is involved in target cell recognition since the two *Theileria* species infect different cell populations as discussed in section 5.3. Perhaps it is therefore more probable that SPAG1 is involved in the invasion mechanism itself, with another antigen on the sporozoite surface mediating initial attatchment and thus providing host cell specificity. The 4B11 antigen is a possible candidate, although the weak cross-reactivity of the McAb and KP8 antibodies with *T. parva* p67 indicates that these two antigens may also share small areas of homology.

The work described here has a number of potential applications. One possible application could be in the diagnosis of *T. annulata* infections by ELISA. An ELISA test for *T. annulata* could potentially be developed using the SPAG1 recombinant fusion proteins. Such a test could be useful in measuring the levels of anti-SPAG1 antibodies in animals immunised with recombinant SPAG1. A recombinant SPAG1 ELISA test could also have potential uses in diagnosis of *T. annulata* infection in cattle thought to be suffering from theileriosis. However, such constructs would need to be selected carefully to include determinants common to the different SPAG1 alleles but which are not present in any other *Theileria* species found in identical regions, such as *T. sergenti* in East Asia and *T. parva* in the Sudan. An ELISA would also be limited in it's use since sera from immune animals would also react as positive, as well as animals suffering the disease. Perhaps a more useful diagnostic test would be an antigen capture ELISA, such as the test developed for the identification of *T. mutans* infections (Katende et al 1990). Such a test could be developed using McAbs directed against SPAG1 or the 4B11 antigen which do not cross-react with antigens of *T. parva* or any other species of *Theileria*. The anti-sporozoite McAbs 5D1 and 4D3 (section 5.2.2) might be candidates for this since they react with parts of the SPAG1 molecule with low homology to *T. parva* p67 and did not appear to react with *T. parva* sporozoite material on a Western blot.

It must be borne in mind that both the potential ELISA tests described above would be limited in diagnosis of *T. annulata* infection in that they are both specific for the sporozoite stage only. Both types of test would rely on sufficient levels of anti-sporozoite antibody or of sporozoite antigen in the blood of infected animals to be detected. Perhaps a more suitable candidate antigen for the development of an ELISA for *T. annulata* diagnosis would be one that is common to all stages of *T. annulata*, such as the antigen identified on Western blots of sporozoite, macroschizont and piroplasm material by Shiels et al (1989). A fusion protein or antibody ELISA test developed for SPAG1 or the 4B11 antigen could have wider applications in the assessment of anti-sporozoite humoral responses in immune cattle, which would be useful in a vaccine development programme.

Another potential application of the work described here is in the development of a molecular vaccine for *T. annulata*. Since the KP8 fusion protein was capable of eliciting antibody in a rabbit which recognised *T. annulata* sporozoite material on Western blots and in IFA tests, and was capable of neutralising sporozoite infectivity "in vitro" as reported in Chapter 8, this antigen is a potential candidate for inclusion in a molecular vaccine. The immunological relationship between this antigen and SPAG1 would need to be further investigated in order to verify whether or not an enhanced immune response to sporozoites could be obtained by immunisation with both antigens. Sporozoite antigens could be included as part of a "cocktail" vaccine with antigens of other stages, as suggested in section 1.1.4. Hybrid vectors have been constructed expressing epitopes from both sporozoite and merozoite antigens of *P. falciparum*, capable of eliciting immune responses to both stages (Holder et al 1988). Similar "chimeric" recombinant antigens could also be constructed to induce immunity to multiple stages in *T. annulata*.

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Appendix

TABLE A. *E. coli* HOST STRAINS USED

The following table summarises the genotypes of the *E. coli* host strains used. *E. coli* host strains DS884, DS885 and DS890 were kindly provided by Dr. D. Sharpe, Department of Genetics, University of Glasgow. Genotypes of the other strains are described in Sambrook et al (1989).

STRAIN	GENOTYPE
DS884	Δlon - genotype not known
DS885	Δlon - genotype not known
DS890	ilv his sup^o strA Pro^c galop : : IS1 λ[Bam H^+ CI^{857} ΔH1] htpr Δlon
JM109	e14⁻(mcrA) recA1 endA1 gyrA96 thi-1 hsdR17 (r_k^-, m_k^+) supE44 relA1 Δ(lac-proAB) [F' traD36 proAB $lacI^q$ ZΔM15]
XL-1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB $lacI^q$ ZΔM15 Tn10(tet^r)]
PLK17	e14⁻(mcrA) mcrB1 lac hsdR2 (r_k^-, m_k^+) supE44 galK2 galT22 metB1

TABLE B. MCABS USED IN FACS ANALYSIS OF PBM, CELL LINES AND CLONES

The following table summarises the antigens recognised (- = not known) and the cell subsets identified (from Baldwin, Morrison and Naessens (1988) and Howard et al 1991).

MCAB	ANTIGEN IDENTIFIED	CELL POPULATION IDENTIFIED
ILA-19	monomorphic MHC Class I	All cells
J11	monomorphic MHC Class II	Some monocytes, macrophages, B cells and blasting T cells
CC42	BoT2	majority of T cells
J4	CD18	majority of T cells
IL-A12	BoT4	Class II restricted T cells
IL-A51	BoT8	Class I restricted T cells
IL-A29	γδ T cell receptor	γδ T cells
IL-A30	IgM	B cells
VPM-30	-	B cells
IL-A24	-	macrophages
4H5	120kDa molecule	*T. annulata* macroschizont infected leucocytes

Figure 77

cDNA sequence of *T. annulata* SPAG1, reproduced from Hall et al (1992). The predicted amino acid sequence is shown below in single letter code. The PGVGV elastin homologous regions are boxed. The C-terminal region corresponding to the λgt11-SR1 insert, encoding the McAb 1A7 epitope, is underlined. Putative N-linked glycosylation sites are marked with arrowheads.

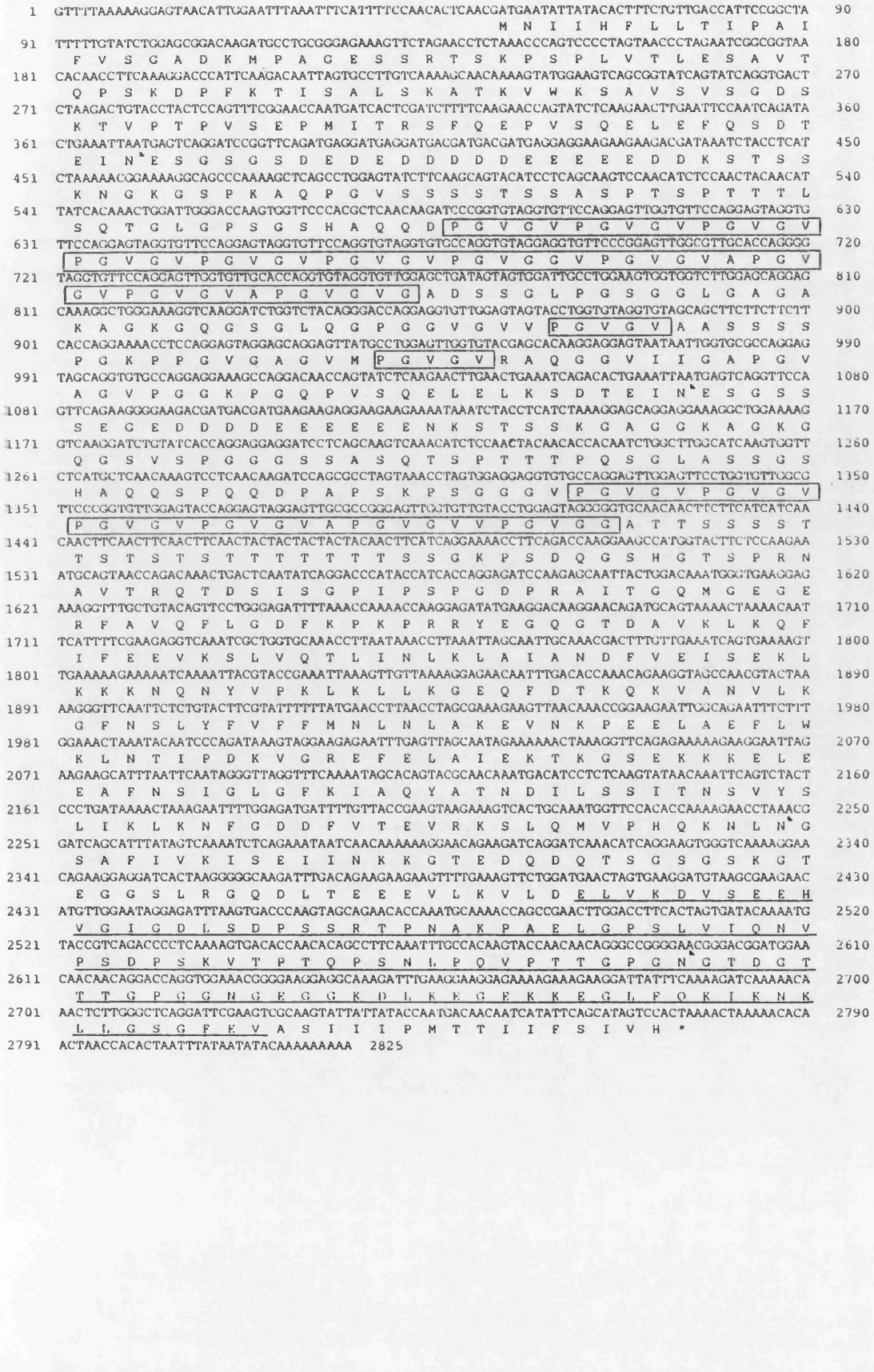

Figure 78A cSPAG1 restriction map

The following diagram summarises the positions of recognition sites for restriction enzymes referred to in this thesis. The numbers refer to nucleotides in the cSPAG1 DNA sequence (Figure 77). Restriction enzyme sites are denoted as follows;

Ac = Acc I	Av = Ava I	B = Bam HI	C = Cfo I	Ec = Eco RI
Hc = Hinc II	Hp = Hpa I	Nc = Nco I	Nl = Nla IV	S = Spe I

The shaded box corresponds to the SR1 region.

cSPAG1 contained no restriction sites for the following enzymes; Ava I, Hind III, Pst I, Sma I or Sst I.

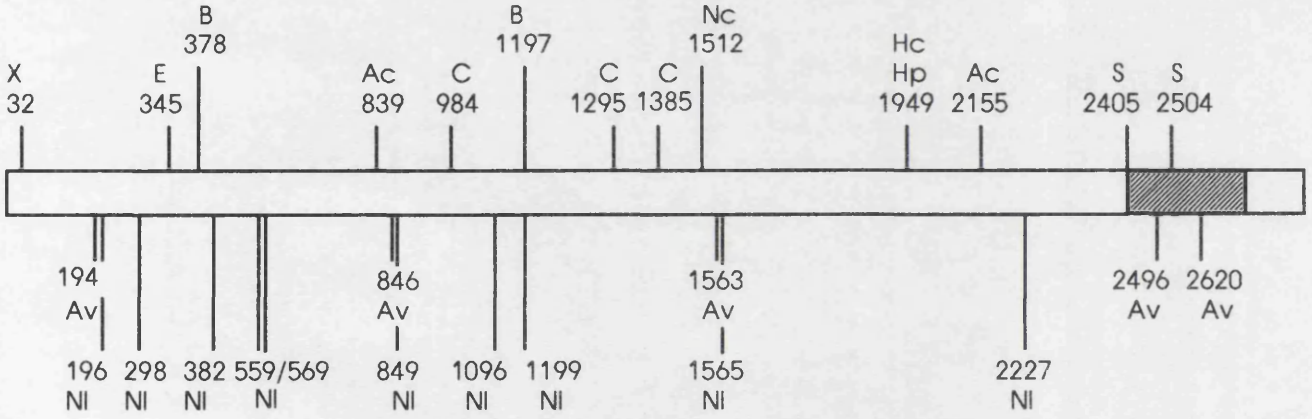

Figure 78B gSPAG1 restriction map

The following diagram summarises the positions of recognition sites for restriction enzymes referred to in this thesis. The numbers refer to nucleotides in the gSPAG1 DNA sequence (Dr. R. Hall; personal communication). Restriction enzyme sites are denoted as follows;

Ac = Acc I	Av = Ava I	B = Bam HI	C = Cfo I	Ec = Eco RI	
Hc = Hinc II	Hp = Hpa I	Nc = Nco I	Nl = Nla IV	P = Pst I	S = Spe I

The shaded box corresponds to the SR1 region.

The gSPAG1 sequence contained no restriction sites for the following enzymes; Ava I, Hind III, Hpa I, Sma I or Sst I.