Towards a *Haemonchus contortus* vaccine

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CONTENTS

Declaration

I have made a significant contribution to the papers presented in this thesis principally through my role as an innovative molecular biologist. Funding for this work and responsibility for the general direction of the research is attributable to Dr. David P. Knox. However, within this broad framework, I have independently taken a full and active role in experimental design, execution of the work, interpretation of results, the overcoming of technical difficulties and in deciding the direction of future work. Permission to include the presented papers has been obtained from all joint authors. With the exception of the first paper presented in this thesis (Knox, Redmond and Jones. 1993. *Parasitology*, **106**: 395-404), which was included in the submission for the Degree of PhD awarded to David P. Knox by Napier University in 1993, this work has not been submitted in full or in part for the award of another degree.

Diane L. Redmond

Acknowledgements

Dedicated to the memory of my brother,
Roland Berkeley Smith

Nothing has happened..... All is well

I would like to extend my gratitude to Dave Knox, with whom I have worked for most of my research career at the Moredun Research Institute. His uncanny knack at securing my continued funding is greatly appreciated. However, my biggest debt to Dave is for encouraging me to think – it has led to some interesting "discussions" between us and we have had a lot of fun over the last 13 years. For your unceasing support, thanks Max!!

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Take a bow everyone!!

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Abstract

Haemonchus contortus is a highly pathogenic nematode parasite of small ruminants, especially sheep. In the ovine host, the blood-feeding habit of the adult parasite can induce symptoms such as anaemia, inappetence, weight loss, poor wool quality and, in severe cases, death. As such, haemonchosis is of considerable worldwide economic and animal welfare importance. Control of infection is largely dependent on the use of anthelmintic drugs. However, the rapid and widespread emergence of anthelmintic resistant strains of *H. contortus* has focused attention on the development of alternative control strategies, such as vaccination.

Parasite enzymes have been postulated to be involved in host tissue penetration and niche establishment, evasion/modulation of the host immune system and, in the case of *H. contortus*, degradation of the host blood meal and, as such, may be considered crucial for parasite survival. Therefore, these enzymes may represent pertinent candidate vaccine antigens.

The first paper presented in this thesis characterises the substrate specificity, inhibitor sensitivity and molecular size of the proteinases present in extracts of adult *H. contortus*. Subsequent work focused on the molecular characterisation of two integral membrane protein antigen complexes isolated from the gut of adult *H. contortus*, namely *Haemonchus* galactose-containing glycoprotein (H-gal-GP) and thiol sepharose-binding proteins (TSBP), which confer a high degree of host protection against homologous challenge. Both complexes possess marked proteinase activity with H-gal-GP exhibiting both aspartyl- and metalloproteinase activity together with a sometimes detectable cysteine proteinase component, whilst the TSBP fraction is greatly enriched for cysteine proteinase activity. Protection tends to correlate with proteinase-containing fractions of these complexes. The isolation and characterisation of full-length complementary DNAs encoding the enzyme components of these antigen complexes is described.

The cDNAs were cloned into appropriate bacterial expression vectors although recombinant proteins were expressed typically as insoluble, enzymatically inactive inclusion bodies which afforded no protection in sheep vaccination trials. Sequential denaturation and reduction of H-gal-GP resulted in loss of its protective

capacity, indicating that conformational epitopes and/or enzyme activity are important for protection. Therefore, expression in a eukaryotic system will probably be required in order to obtain appropriate post-translational modifications of recombinant proteins. Because it is closely related in evolutionary terms to *H. contortus*, the free-living nematode *Caenorhabditis elegans* was evaluated as a potential vector for expression of recombinant *H. contortus* antigens. The pepsinogen component of H-gal-GP was successfully expressed in the gut of transgenic *C. elegans*, albeit at low levels. In addition, isolation of the promoter sequence of the *H. contortus* pepsinogen gene was facilitated by the development of a two-step polymerase chain reaction allowing amplification of unknown flanking DNA sequences. This promoter sequence was shown to be capable of directing correct spatial expression of a reporter gene in transgenic *C. elegans*, further emphasising the degree of evolutionary conservation between these two nematodes.

Detailed analysis of the *H. contortus* pepsinogen gene showed low level *trans*-splicing to a 22 bp SL2 sequence. Typically, *trans*-splicing of an SL2 sequence is reserved for splicing to downstream genes in poly-cistronic pre-mRNAs derived from operons and its presence is, therefore, considered as evidence of an operon. This is the first observed case of SL2 *trans*-splicing in *H. contortus*.

In conclusion, the work presented in this thesis characterises the enzymes present in extracts of adult *H. contortus* with particular attention to the molecular isolation and characterisation of the enzyme components of two protective integral gut membrane protein complexes, together with attempts to produce enzymatically active recombinant proteins for evaluation in sheep protection trials.

ABBREVIATIONS

aa	amino acid
β-gal	β-galactosidase
AChE	acetylcholinesterase
bp	base pairs
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
ES	excretory/secretory
EST	expressed sequence tag
FEC	faecal egg count
GlcNAc	N-acetylglucosamine
GST	glutathione S-transferase
H-gal-GP	*Haemonchus* galactose-containing glycoprotein
Ig	Immunoglobulin
IL	interleukin
IFNγ	interferon gamma
kb	kilobases
kDa	kilo Dalton
L	larval stage
MEP	metallopeptidase
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	pepsinogen
pH	$-\log_{10}$ (hydrogen ion concentration)
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RT-PCR	reverse transcribed polymerase chain reaction
SAGE	serial analysis of gene expression

SDS	sodium dodecyl sulphate
SL	spliced leader
Th	T helper
TSBP	thiol sepharose-binding proteins
µg	microgram

Chapter 1: General introduction

1. Gastrointestinal nematodes

Gastrointestinal nematode infections of grazing ruminants are of considerable economic importance to the farming industry worldwide through their major effects on the efficiency of livestock production (Sykes, 1994). Infection is associated with parasitic gastroenteritis, clinical symptoms of which are inappetence, impaired weight gain, reduced body condition and diarrhoea. If left untreated, these symptoms can cause unacceptable animal welfare problems.

The overall cost of gastrointestinal nematode infections on livestock production is difficult to assess as the impact of subclinical infections and fluctuations in farm animal market values must also be taken into account. However, it has been estimated that somewhere in the region of £71 million is spent on anthelmintic treatment of gastrointestinal nematode infections by the farming community in the UK and that subclinical infections in sheep alone account for a further £65 million (R.L. Coop, personal communication).

In sheep, gastrointestinal nematode infections are caused by worms of the superfamily Trichostrongylidae. In general, these worms are small and slender, with the adult stages being parasitic in the abomasum and small intestine. The most common species in the abomasum are *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus axei* and in the small intestine, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Nematodirus battus* and *Cooperia punctata*. In the UK, the predominant intestinal nematode is *T. vitrinus* whilst *T. circumcincta* is the most prevalent of the abomasal nematodes. *Haemonchus contortus* infections are confined mainly to the southern parts of the country. However, on a worldwide scale, *H. contortus* is prevalent in tropical and subtropical regions and because of its high pathogenicity due to its blood-feeding habits, is economically the most important of the gastrointestinal nematodes.

2. *Haemonchus contortus*

2.1 Life cycle

The life cycle of *H. contortus* is typical of the Trichostrongylidae in general and is direct, involving no intermediate host, as outlined in Figure 1.

Under favourable conditions (i.e. temperature and humidity), eggs passed out in the faeces of an infected host will hatch to release the first larval stage (L1) which, after a period of growth, will moult to become the second larval stage (L2). Both the L1 and L2 feed on bacteria present in the host faeces. On breakdown of the faeces, the L2 moults to the infective third larval stage (L3), which retains the L2 cuticle as a loose protective sheath (Urquhart et al, 1991). This sheath imparts resistance to environmental extremes (Levine, 1978) but also prevents the L3 from feeding, its survival being dependent on nutrients stored in the earlier developmental stages. Development to the L3 stage can occur within 5 days although may be delayed for months in cooler conditions. The L3 migrate up blades of grass where they are ingested by the host grazing on pasture. Once swallowed by the host, exsheathment of the L3 occurs in the forestomach before passing into the abomasum (true stomach) where the parasitic phase of the life cycle occurs. The L3 burrow into the mucosa and moult to become the fourth larval stage (L4). The L4 feed on blood from the mucosal vessels which forms a clot around the anterior end of the worm. One final moult forms the fifth larval stage (L5), or early adult stage, which emerges onto the mucosal surface and matures into the adult worm. In the ovine host, patency is reached 2-3 weeks after ingestion of the infective L3. *H. contortus* is a highly fecund nematode, the female worms producing 5,000 to 10,000 eggs per day, with egg production beginning some 15 days or so after the final moult.

The adult worms are typically 10-30mm in length, the blood that they suck giving the transparent worms a reddish-brown colour. The white ovaries of the female worms wrapped around the red intestine give it the characteristic appearance of its common name, the "barber-pole worm". As an apparent adaptation to the blood feeding habit of the late larval and adult stages, *H. contortus* has a unique buccal tooth which is thought to function in penetration of host blood vessels.

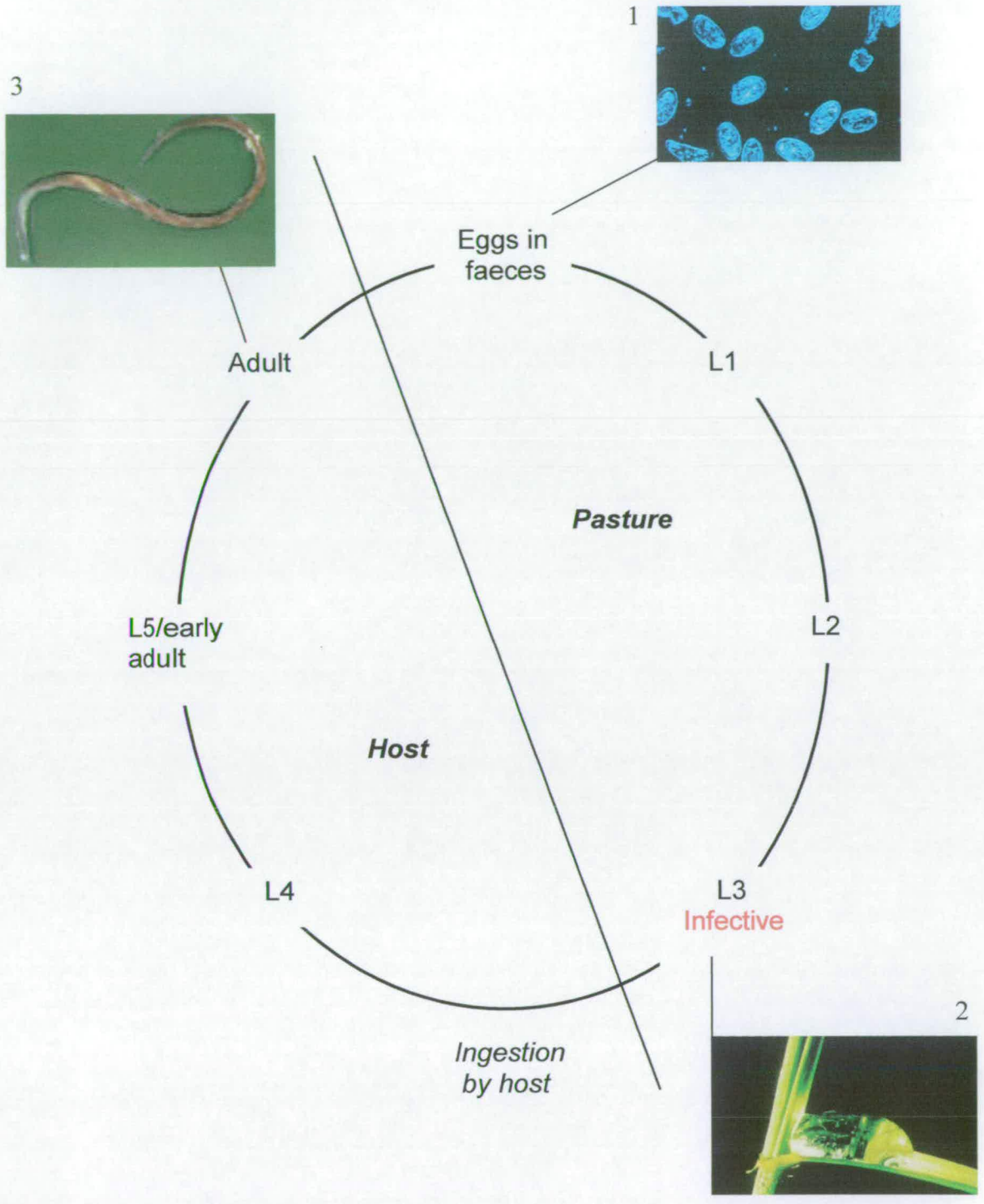

Figure 1. The life cycle of *H. contortus*.

1. *H. contortus* eggs: 2. water droplet containing infective L3 on a blade of grass: 3. adult female worm

(photographs 1 and 2, Moredun Research Institute; photograph 3, EA Munn).

2.2 Epidemiology

Environmental conditions are the principal factors that influence the development and survival of the non-parasitic, free-living stages of *H. contortus*. Larval development occurs optimally at relatively high temperatures although high levels of humidity in the microclimate of the faeces are also essential for development and survival of larvae. Because *H. contortus* eggs are intolerant to desiccation (Waller and Donald, 1970) and to low temperatures (Donald, 1968), very little development will occur below 9^0C. As such, haemonchosis is primarily a disease of sheep in warm climates, with the frequency and severity of outbreaks being largely dependent on the rainfall in any particular area. *H. contortus* is a highly fecund parasite and under optimal climatic conditions, the large numbers of eggs deposited on pasture can lead to the rapid accumulation of infective L3 resulting in the sudden occurrence of acute clinical haemonchosis.

As already mentioned, the sheathed infective L3 are relatively resistant to environmental extremes and in tropical regions, can survive for up to 3 months on pasture depending on the climate and degree of shade (Urquhart et al, 1991). In addition, survival of the parasite in some tropical and subtropical areas is associated with an evolved ability of *H. contortus* larvae to undergo inhibited larval development, or hypobiosis, within the host. Although the exact trigger for hypobiosis is, as yet, unknown, the onset of a prolonged dry season provides an environmental stimulus to the free-living infective larvae. Following ingestion by the host, they survive as arrested L4, before development is resumed just before the onset of seasonal rains. Hypobiosis, which ensures that parasite eggs are not being produced and deposited on dry pasture where they would fail to develop, has been found to occur in counties such as Australia, Brazil, Nigeria and the Middle East (Urquhart et al, 1991). In contrast, hypobiosis is not observed in parts of the tropics which are subject to more frequent rainfall and where free-living larval development is possible all the year round.

The epidemiology of *H. contortus* in temperate regions is different from that observed in tropical and subtropical climates. Temperate regions provide the most unfavourable climatic conditions for survival and most commonly, a single annual cycle is observed. Eggs deposited on pasture by ewes in early spring develop to

infective L3 and are ingested by lambs in late summer. Most of these larvae are arrested as early L4 in the host and do not complete their development until the following spring. The onset of maturation of these hypobiotic larvae may induce clinical signs of acute haemonchosis which often coincides with lambing in the ewes. That clinical haemonchosis is sometimes seen in grazing lambs in late summer may be due to pasture contamination attributable to a proportion of ingested larvae which have not undergone hypobiosis (Urquhart et al, 1991).

2.3 Pathogenesis

The highly pathogenic nature of *H. contortus* is due to the blood-feeding habit of the late larval and adult stages following their emergence from the gastric glands (Holmes, 1985). *H. contortus* primarily affects sheep and goats, causing the typical symptoms associated with parasitic gastroenteritis, although diarrhoea is normally lacking. Animal productivity is impaired through reductions in voluntary food intake and/or reductions in the efficiency of food use (Coop and Kyriazakis, 2001), the magnitude of these effects being influenced by the size of larval challenge and the number and species of worms present (Parkins and Holmes, 1989). Reductions in live weight gain of up to 60% have been recorded in lambs experimentally infected with *H. contortus* (Holmes, 1985).

In acute haemonchosis, where 2,000-20,000 worms may be present on the abomasal mucosa, anaemia due to abomasal blood loss becomes apparent approximately two weeks after infection and is characterised by a progressive and dramatic fall in packed red cell volume which will stabilise at a low level due to a compensatory expansion of erythropoiesis (Urquhart et al, 1991). However, this cannot be sustained, with the continual loss of iron and protein into the gastrointestinal tract and continued inappetence resulting in exhaustion of the bone marrow and a further fall in packed red cell volume before death occurs. In tropical areas, chronic haemonchosis can develop during prolonged dry periods. Although reinfection is negligible, the pasture becomes deficient in nutrients and the continual blood loss from small worm burdens is sufficient to cause weight loss, weakness and inappetence rather than the marked anaemia seen in acute haemonchosis (Urquhart et al, 1991). Lethargy results from the decreased oxygen carrying capacity of blood due

to loss of haemoglobin along with red blood cells and a resulting reduction in oxygen perfusion of tissues such as muscle.

Abomasal parasitism in general compromises abomasal function causing changes in endocrine and enzyme secretion and increasing the pH of its contents. The physical damage to the gastric glands caused by the parasitic stages of *H. contortus* results in leakage of the gastric contents giving a diagnostic rise in host serum pepsinogen levels (Holmes, 1985) and reduction in the efficiency of nutrient absorption. In addition, there is a net loss of endogenous protein into the gastrointestinal tract which is attributable in part to increased leakage of plasma protein, increased sloughing of epithelial cells and increased secretion of mucoproteins (MacRae, 1993) which ultimately impairs animal productivity. Loss of plasma protein will also result in oedema, which is normally seen in live sheep as a gathering of fluid in the submandibular space.

The problems associated with haemonchosis are most marked in naïve animals, lambs that have not yet developed natural immunity and also in ewes where there is a characteristic periparturient rise in worm egg output. This periparturient rise is attributed to a temporary relaxation in immunity related to the high nutritional demands of late pregnancy and early lactation (Coop and Kyriazakis, 2001).

2.4 Immunity to gastrointestinal nematodes

Protective immunity to gastrointestinal nematodes is most marked in animals greater than 6 months of age and is acquired as a result of continuous infection encountered under normal field conditions. The lower resistance to infectious disease of young ruminants appears to be due largely to immunological hyporesponsiveness rather than to lack of exposure to parasites. The stress associated with weaning has been shown to contribute to the delayed development of protective immune responses to *H. contortus* and *T. colubriformis* (Watson and Gill, 1991). Importantly, young sheep, aged 4-8 months old, have significantly lower proportions of $CD4^+$ (T helper) and $CD8^+$ (T cytotoxic) lymphocytes in comparison with mature sheep. In addition, cultured blood lymphocytes from immature sheep produce significantly lower levels of interferon gamma (IFNγ) than those from mature animals (Watson et al, 1994). These relative deficiencies in the immune mechanisms of young sheep are

reflected in the size of the humoral and cell-mediated responses to nematode infection. Vaccination of lambs up to 5 months of age with irradiated *H. contortus* larvae afforded no protection against subsequent challenge and was associated with significantly lower levels of abomasal mucus immunoglobulin (Ig)A and serum IgG antibodies as compared with adult, immune sheep (Duncan et al, 1978). Similarly, Smith et al (1985) reported that, although an increase in basophil and IgA containing cell numbers was detected post infection, 4½ month old lambs infected with *T. circumcincta* were not as efficient at mounting an immune response as 10 month old lambs, and exhibited a lower degree of resistance.

The host immune response to an invading parasite is complex involving cellular, humoral and inflammatory components. The effector responses of the immune system can be directed against:

1. incoming infective larvae which are subject to immune exclusion (Miller et al, 1983) where they are prevented from reaching their niche,
2. emerging L4s and young adult worms which are subject to immune attack and elimination (Emery et al, 1993) or to arrested development (Smith, 1988)
3. an established adult worm population.

The host is capable of mounting both T helper (Th) 1 and Th2-type immune responses against invading parasites. In general, a Th1-polarised response is characterised by the production of the cytokines IFNγ and interleukin (IL) 2 which stimulate IgG2 antibody isotype production and cell-mediated effector responses. The induction of a Th1 response is required to elicit host protection to a range of intracellular parasites and pathogens. In contrast, Th2 cells secrete the cytokines IL-4, -5, -6, -9 and -10 which promote mastocytosis, eosinophilia and the production of IgE and IgG1 antibodies (reviewed by Abbas et al, 1996). An increase in host mast cells, eosinophils, IgA and IgE antibodies are characteristic of nematode infections and strongly suggest a Th2 dominated host immune response. These effector responses are discussed below.

2.4.1 T helper cells and cytokines

Th cells play a central role in eliciting specific antibody responses, in generating cell mediated immune responses and in the recruitment and activation of

macrophages and granulocytes. The overall importance of Th cells in resistance to nematode infection has been demonstrated by treatment of *H. contortus*-primed sheep with monoclonal antibodies to ovine CD4 (expressed on the surface of Th cells). These sheep were rendered susceptible to challenge infection demonstrating that Th-cells play a continuing essential role in the manifestation of resistance even in previously immunised sheep (Gill et al, 1993).

Th-cells can be segregated into functional Th1 and Th2 categories on the basis of the cytokines that they secrete (Miller, 1996). Th2-cells secrete IL-4 and IL-5, which are important in the stimulation of the eosinophil, mucosal mast cell and IgE immune responses typically seen in helminth infections (Finkelman and Urban, 1992). IL-13 has also been shown to be critical for host defence against gastrointestinal nematode parasites (Finkelman et al, 1997 & 1999) increasing gut permeability and mucus secretion, (Shea-Donohue et al, 2001: McKenzie et al, 1998) and exhibits functional overlap with IL-4 (Zurawski et al, 1993). Studies on the role of cytokines in *Trichuris muris* infections showed that IL-2, Il-3 and IFNγ production by Th1-cells was increased in mice susceptible to infection, whereas a Th2 response (IL-4, IL-5, IL-9 and IL-10) was present in resistant mice (Else et al, 1994). In addition, it was found that resistance to *T. muris* infection could be induced in susceptible mice by the administration of IL-4, whereas, conversely, normally resistant mice became persistently infected if treated with IFNγ (Else et al, 1994). *T. muris* expulsion is inhibited in mice deficient in either IL-4 or IL-13 (Finkelman et al, 1999: Urban et al, 2000: Bancroft et al, 2000), demonstrating that both these cytokines are required for the development of protective immunity. However, that IL-13 can mediate IL-4 independent expulsion of *T. muris* in the absence IFNγ (Urban et al, 2000) suggests that other cytokines may also play important regulatory roles. Recently, IL-10 has been shown to be important in protection to *T. muris* through suppression of the counter-regulatory type 1 cytokine response (Schopf et al, 2002). In addition, IL-9 has been shown to enhance intestinal muscle contraction and is associated with accelerated worm clearance in *T. muris* infected mice (Khan et al, 2003). Enhanced levels of IL-9 *in vivo* have also been identified as important in expulsion of *Trichinella spiralis*, increasing parasite-specific IgG1 levels and intestinal mastocytosis (Faulkner et al, 1997).

The immune response to *Nippostrongylus brasiliensis* infection in mice and rats has been extensively studied and is considered a model system for gastrointestinal nematode infections in general. Both mice and rats infected with *N. brasiliensis* develop strong Th2-type responses associated with enhanced production of IL-4 and IL-5 (Urban et al, 1993; Matsuda et al, 1995). Treatment of mice with recombinant IFNγ increased egg production and delayed expulsion of adult *N. brasiliensis* from the intestine (Urban et al, 1993). Repetitive administration of IL-12 to BALB/c mice induced T cell IFNγ secretion, suppressing the Th2-associated intestinal mucosal mast cell hyperplasia, blood eosinophilia and IgE responses and promoting survival of *N. brasiliensis* (Finkelman et al, 1994). This effect could be blocked by administration of anti-IFNγ antibody (Finkelman et al, 1994). The importance of Th2-type responses in resistance to nematode infection was further demonstrated using IL-5 transgenic mice with varying degrees of eosinophilia, all of which displayed enhanced resistance to *N. brasiliensis* relative to non-transgenic hosts as determined by worm numbers, increase in worm size over the course of infection and fecundity (Dent et al, 1999).

Although IL-4 is considered critical in the control of intestinal helminth infections (Else et al, 1994), IL-4 knockout mice are still able to expel *N. brasiliensis* normally (Lawrence et al, 1996). A critical role for IL-13 in mediating resistance, functioning through interaction with an IL-4 receptor, has been demonstrated using IL-13 knockout mice which failed to clear *N. brasiliensis* infections efficiently, despite the production of a robust Th2-like cytokine response (McKenzie et al, 1998). However, mice doubly deficient in IL-4 and IL-13 display more severely impaired host resistance than mice deficient in IL-13 alone (McKenzie et al, 1999), suggesting that IL-4 can play at least a partial role in host protection to *N. brasiliensis* infection.

Very few studies have examined cytokine responses in ruminant gastrointestinal infections. However, in keeping with findings from murine studies indicating a role for Th2-type responses in protection, depletion of IFNγ with monoclonal antibodies has been shown to enhance the acquired immune response to *T. colubriformis* infection in sheep (McClure et al, 1995). More recently, Gill et al (2000) demonstrated increased IL-5 production in sheep in response to *Haemonchus*

infection, this strong Th2-type immune response being greater in genetically resistant as compared to random-bred animals.

2.4.2 Antibodies

Studies to determine the mechanisms of expulsion of gastrointestinal nematodes in ruminants indicate that IgA is the main local immunoglobulin involved. As described earlier, resistance to *H. contortus* infection in 9 month old sheep was associated with elevated levels of IgA antibodies in the abomasal mucosa and increased parasite-specific antibodies in the serum (Duncan et al, 1978). Gill et al (1992) demonstrated that infection of sheep with *H. contortus* resulted in an increase in the numbers of IgA-, IgG1- and IgM-containing cells in the submucosa of the abomasum compared to uninfected controls, of which IgA-containing cells (68-84%) were the most frequent. Interestingly, IgA is generally increased in sheep selectively bred for resistance (Gill et al, 1994).

A positive correlation in human populations has been observed between IgE levels and resistance to schistosome reinfection (reviewed by Hagan, 1993) where it is thought to be involved in antibody-dependent, cell-mediated cytotoxic killing of the parasites (Capron and Capron, 1994). More recently, a pronounced IgE antibody response directed specifically to L3 antigens was detected when previously infected sheep were challenged with *T. circumcincta* (Huntley et al, 1998). During a helminth infection, IgE levels in serum can increase 100-fold (Jarrett and Bazin, 1974) although only a small proportion of this is parasite specific (Turner et al, 1979). However, the results of Huntley et al (1998) suggest that the proportion of total specific IgE may alter during the development of immunity. Since mast cells and IgE have been implicated in the expression of immunity to gastrointestinal parasites (Miller, 1996), the generation of relatively high levels of specific IgE may be of particular relevance in the activation of a mast cell-mediated hypersensitivity response.

2.4.3 Mast cells and globule leucocytes

Mucosal mast cell hyperplasia and the appearance of globule leucocytes, which are derived from mast cells (Huntley et al, 1984), is a well recognised

phenomenon in ruminant gastrointestinal nematode infections (Rothwell, 1989). However, observations that a high degree of resistance in sheep to *H. contortus* can be expressed without any obvious increase in mast cell numbers (Huntley et at, 1992), and that mast cell deficiency has little or no effect on the expulsion of *N. brasiliensis* in rats (Rothwell, 1989), indicate that different effector mechanisms may be important in different infections. Degranulation of mucosal mast cells may have a direct detrimental effect on worm survival (Rothwell, 1989; Emery et al, 1993) although they may also function indirectly, with the release of granule enzymes increasing mucosal permeability thereby facilitating the translocation of plasma proteins, including humoral antibodies, into the gut lumen (Barth et al, 1966). In addition, mast cells are a major source of cytokines and may be able to influence the Th response profile through production of IL-4 and IL-5 and may also upregulate IgA production (Ramsay et al, 1994). The presence of mucosal mast cells and globule leucocytes has been associated with the rapid expulsion of *H. contortus* challenge larvae seen in a proportion of immune sheep (Huntley et al, 1992).

2.4.4 Eosinophils

Eosinophilia is a common feature of helminth infections, occurring both in the blood and at local sites of infection (Butterworth and Thorne, 1993). *In vitro* studies have shown that eosinophils can adhere to and kill a number of helminth parasites, including *Fasciola hepatica* (Duffus and Franks, 1980) and *T. spiralis* (Venturiello et al, 1993). Eosinophils also play a role in antibody-dependent cell-mediated cytotoxic killing of schistosomes through the binding of IgE (Capron and Capron, 1994). More recently, Rainbird et al (1998) showed that eosinophils were able to immobilise and kill *H. contortus* larvae *in vitro* in the presence of antibody to a defined L3 surface antigen. Degranulation of adhering eosinophils onto the surface of the larvae was observed after 6 hours, with many larvae showing signs of damage after 24 hours. However, the *in vivo* role of eosinophils remains uncertain. For example, larval stages of *Ostertagia* spp. and *Teladorsagia* spp. develop in modified gastric pits which exclude eosinophils (Balic et al, 2000) and offer protection from eosinophil-mediated attack.

2.4.5 Mucus

Increased numbers of mucus-secreting goblet cells and qualitative changes in mucus secretion follow infection with a number of nematode parasites. In sheep, increased goblet cell numbers are related to the development of resistance to *T. vitrinus* (Jackson et al, 1983). In primed rats, a substantial proportion of a challenge infection with either *T. spiralis* or *N. brasiliensis* becomes physically entrapped in the intestinal mucus and is rapidly expelled (Miller, 1987).

Studies have suggested that many of the changes which occur in immune rats, such as increased smooth muscle motility and IgE-mediated chloride secretion by epithelial cells, which contributes to intestinal fluidity, also occur in parasitised sheep (Emery et al, 1993; McClure et al, 1992). It therefore seems likely that mucus and gut motility are important components of the rejection response.

In conclusion, the host immune response and development of immunity to gastrointestinal nematode infections is complex with different effector mechanisms working in concert to control parasite infections. However, prototypic type 2 responses composed of peripheral and tissue eosinophilia, elevated IgE levels and intestinal mastocytosis are common responses in all intestinal helminth infections of livestock.

3. Control

Current control of haemonchosis, and of gastrointestinal nematode infections in general, relies principally on prophylactic chemotherapy combined, where feasible, with pasture management.

3.1 Pasture management

The aim of pasture management is to provide "clean grazing" where animals are moved to a rested pasture which will harbour few infective larvae, thereby preventing infection of the most susceptible animals (Boag and Thomas, 1973), i.e. pasture must be kept free of sheep for sufficient time for the ensheathed L3 to die. This strategy is effective in reducing the loss of productivity due to larval challenge

and is particularly successful where animals are moved to fields from which hay or silage have been harvested. A grazing policy where pasture is rotationally grazed with other species such as cattle or horses can also be adopted, although the fact that a number of gastrointestinal nematodes can infect other hosts needs to be taken into consideration (Morley and Donald, 1980). However, pasture management is not compatible with the adoption of intensive farming strategies where there are consequential restrictions on land availability.

3.2 Anthelmintics

Drug treatment is, currently, the control method of choice. Three classes of anthelmintics are currently used for controlling gastrointestinal nematode infections, namely the benzimidazoles, levamisoles and related compounds and the macrocyclic lactones. The biochemical basis of the anthelmintic action of each of these chemicals has been recently reviewed (Köhler, 2001). In brief, the benzimidazole compounds are the most widely used and cause ultrastructural alterations in the intestinal cells of nematodes by binding with high affinity to tubulin, thereby disrupting microtubule structure and function (Köhler and Bachmann, 1981; Lacey, 1988). In *H. contortus*, the effect of fenbendazole has been shown to be associated with the inhibition of microtubule-mediated secretory vesicle transport in the intestine followed by dispersal of the vesicle contents throughout the intestinal cytoplasm resulting in gross disintegration of the anterior intestine (Jasmer et al, 2000).

The levamisole family of drugs are nicotinic agonists which open nicotinic acetylcholine receptors on the surface of somatic muscle resulting in depolarisation and spastic paralysis. The unique properties of the nematode nicotinic acetycholine receptors appear pharmacologically distinct from homologous receptors in higher animals which imparts the selective toxicity of these compounds (Köhler, 2001).

The macrocyclic lactones comprise the avermectins and milbemycins which act by binding to glutamate-gated chloride channels resulting in irreversible hyperpolarisation of the cell membrane and flaccid muscle paralysis. The muscles of the pharynx are particularly sensitive and inhibition of nematode feeding results from inhibition of pharyngeal pumping (Geary et al, 1993; Kotze, 1998).

3.3 Anthelmintic resistance

Although they have been used successfully in controlling gastrointestinal nematode infections over the last 40 years, there are growing concerns over the long term application of anthelmintic drugs resulting from the rapid and widespread appearance of resistant strains of worms. The first case of anthelmintic resistance reported in the UK was in 1982 where a benzimidazole resistant strain of *T. circumcincta* was detected in sheep in Cheshire (Britt, 1982). A recent study (Jackson and Coop, 2000) has reported that anthelmintic resistance is now a global phenomenon. A high prevalence of nematodes exhibiting multiple resistance has been reported in South Africa and South America (Echevarria et at, 1996; Eddi et al, 1996; Nari et al, 1996). In the UK, resistance is predominantly to the benzimidazoles, with the main species implicated being *T. circumcincta* although some cases of resistance in *H. contortus* have been reported, mainly in southern regions (Jackson and Coop, 2000). A recent survey showed that more than 60% of Scottish farms harbour benzimidazole resistant nematodes and that, worryingly, multiple resistance has been detected in several lowland farms (F. Jackson, personal communication). In south east England, 30% of farms have been reported to harbour nematodes resistant to both benzimidazoles and levamisole (Coles, 2002). In the UK, the prevalence of benzimidazole resistance has been shown to be higher in goats than in sheep (Jackson et al, 1992) due partly to their more rapid metabolism of anthelmintics which can lead to the equivalent of underdosing. Therefore, in areas where sheep and goats are grazed together, the potential for the more rapid evolution of anthelmintic resistance exists.

Benzimidazole resistance in helminths results from changes in the β-tubulin isotype pattern leading to the loss of high affinity receptors (Lacey and Gill, 1994). A common point mutation which results in a phenylalanine to tyrosine change at amino acid 200 in isotype 1 β-tubulin has been shown to correlate with benzimidazole resistance in *H. contortus*, *T. colubriformis* and *T. circumcincta* (Kwa et al, 1994; Elard and Humbert, 1999). Further selection results in the elimination of isotype 2 β-tubulin genes from highly resistant individuals (Condor and Campbell, 1995).

Little is known of the biochemical mechanisms of resistance to the nicotinic antagonists. However, radio-ligand binding assays have demonstrated alterations in

drug pharmokinetics at the acetylcholine receptor, with levamisole resistant *H. contortus* binding the drug less tightly at low affinity sites as compared with susceptible worms (Sangster et al, 1998).

Resistance to the macrocyclic lactones appears complex and able to manifest itself in different ways depending on the selection processes involved in its development. Molecular genetic work on the free-living nematode *Caenorhabditis elegans* has demonstrated that mutations of two members of the multigene family encoding glutamate chloride α-type subunits can result in a 7 to 10 fold increase in resistance to ivermectin, a value close to that seen for resistant *H. contortus*, although simultaneous mutations of at least 3 genes is required before high-level resistance is achieved (Dent et al, 2000). More recently, clear differences in response to ivermectin inhibition of pharyngeal pumping between ivermectin selected and susceptible *H. contortus* have been observed, indicating that resistance may be associated with changes in the pharyngeal muscle (Kotze, 1998; Sangster and Gill, 1999). P-glycoproteins, which mediate the ATP-dependent export of potentially toxic chemicals from cells (Sangster, 1994), have also been implicated in resistance to the macrocyclic lactones with resistance appearing to be determined by a number of genes that are variably expressed or overexpressed (Köhler, 2001). The fact that nematodes may have to acquire multiple mutations to develop high level resistance may explain why, so far, only isolated cases of ivermectin resistance have been reported in the field.

The fact that the gene/genes that confer resistance may already be present within the range of a nematode species has been accepted as a pre-adaptive phenomenon as has the concept that the continued use of an anthelmintic confers a survival advantage to individuals carrying the resistant gene/genes thereby increasing the selection pressure on that phenotype. Once the population is comprised of predominantly homozygous resistant worms, then little, or no reversion to the susceptible phenotype will occur (Le Jambre et al, 1981).

Management practices which can be employed to delay the development of resistance include reduction in the frequency of anthelmintic treatment, avoidance of underdosing and rotation between drug families. Development of resistance can also be effectively delayed by maintaining the number of worms in refugia, i.e that are

not exposed to anthelmintics and which therefore provide a reservoir of worms able to escape selection for resistance to produce the next generation (Martin et al, 1981). However, this would require increased investment in farm management to diagnose and treat animals only as necessary. In addition, the idea of maintaining pasture contaminated with susceptible larvae to effectively dilute out the number of resistant worms available to infect a new host is contrary to the established "clean pasture" management policies adopted by many farmers (Coles, 2002).

4. Alternative control strategies

The likelihood of a new generation of anthelmintic drugs being available on market in the near future appears remote (Geary et al, 1999) and the problems of anthelmintic resistance, combined with increasing concerns over drug residues in the food chain and the environment, mean that there is a very real need to develop alternative, sustainable strategies for the control of gastrointestinal nematode infections.

4.1 Genetic resistance

Considerable variation in resistance to parasitism exists both within and between breeds of sheep (Gray, 1991; Eady et al, 1996). Because many of these differences are heritable, breeding animals resistant to parasitic nematode infection is a possibility and long term selection experiments have established lines of sheep which are considered more resistant to challenge infection with *T. colubriformis* or *H. contortus* as compared to control animals, based on reduced faecal egg count (FEC) (Windon, 1996). FEC is the primary phenotypic trait which can reliably estimate resistance (Eady, 1995) and is the resistance marker used in the active commercial breeding programs of Australia (Nemesis, Anon, 1994) and New Zealand (WormFEC, McEwan et al, 1995). However, identification of physiological traits which will allow the prediction of resistance in non-parasitised animals has proved elusive. Genetic markers linked to parasite resistance are therefore being developed and include ovine Major Histocompatibilty Complex genes (Outteridge et al, 1986 and 1988; Schwaiger et al, 1995) and microsatellite markers (Beh and Maddox, 1996). Recently, Coltman et al (2001) reported that resistance to

T. circumcincta in a naturally parasitised population of Soay sheep was associated with an allele at a microsatellite marker locus located in an intron of the gamma interferon gene.

4.2 Host nutrition

The nutritional state of the host will affect its resilience (i.e. ability to cope with the adverse effects of parasitism) and resistance to infection (reviewed by Coop and Kyriazakis, 2001). The loss of productivity attributable to parasitic gastroenteritis is a direct result of reduction in voluntary food intake and/or reduction in the efficiency of food use, especially protein. Evidence from both experimental (van Houtert and Sykes, 1996; Abbot et al, 1988; van Houtert et al, 1995a) and natural grazing trials (van Houtert, 1995b) have shown that feeding with a dietary protein supplement can increase both host resilience and improve production responses such as weight gain, wool growth and carcass quality. Similar, but less pronounced, improvements in resilience can also be obtained by supplementation of the basal diet with urea, a source of degradable nitrogen (Knox and Steel, 1999), and this provides a cheaper alternative for use in tropical and subtropical areas where forage quality is generally poor and the cost of high-quality protein supplements prohibitive.

4.3 Tannins

Tannins are naturally occurring plant polyphenols which play a role in protecting plants from microbial and parasitic infestations and which are found in many of the plants in the diet of herbivores. Because condensed tannins are relatively stable in the digestive tract of animals, rarely with toxic effects, several plants with high tannin concentrations have been tested for their potential to control gastrointestinal nematode infections. Results have been promising with lambs naturally exposed to gastrointestinal nematodes performing better and with lower worm burdens when grazing on sulla or great trefoil compared to lambs grazing on tannin-free forage (Niezen et al, 1998). Sheep trickle infected with *T. colubriformis* larvae showed a 25-40% reduction in FEC when offered food containing condensed tannins from *Quebracho* extract for 10 weeks as compared to sheep offered a tannin-

free diet (Athanasiadou et al, 2000). However, a subsequent study showed that there was no effect on worm burden and that advantages on the performance of parasitised sheep were seen only when condensed tannins were used to supplement high-protein food (Athanasiadou et al, 2001). Because of their negative effect in inhibiting microbial and enzyme activity, thus reducing food intake and digestibility, the anthelmintic effect of condensed tannins may be best exploited by allowing sheep to feed only occassionally on tanniferous plants.

4.4 Copper oxide wire particles

Copper oxide wire particles have been developed for the treatment of copper deficiency in grazing livestock (Dewey, 1977). After ingestion, they are rapidly transferred to the rumen where the acidic environment induces the release of soluble copper which was shown by Bang et al (1990) to have anthelmintic effects, in sheep, on the abomasal parasites *H. contortus* and *T. circumcincta* reducing worm burdens by 96% and 56%, respectively. No effect was observed on the establishment of *T. colubriformis* which is parasitic in the small intestine. A further study carried out under Australian grazing conditions showed that, when used at the dose recommended for treatment of copper deficiency, copper oxide wire particles had the potential to reduce the establishment and worm fecundity of *Haemonchus* over a 10 week period, although the high efficacy achieved by Bang et al was not repeated and there was a variable response between experiments (Knox, 2002). However, copper oxide wire treatment has previously been observed to have negative effects on wool tensile strength (Masters and Mata, 1997) and the copper status of a flock, including the availability of copper accumulating plants in the forage, would need to be determined prior to use in order to avoid the risk of copper toxicity.

4.5 Nematophagous fungi

This heterogeneous group of microfungi utilise nematodes either as their main source of nutrients or supplementary to a saprophytic existence (Barron, 1977), producing nematode-trapping structures on the growing mycelium. By virtue of their unique, thick-walled resting spores (chlamydospores) produced inside the growing hyphae, *Duddingtonia flagrans* can survive passage through the gastrointestinal tract

of livestock and has been identified by workers in Denmark (Larsen et al 1991), France (Peloille, 1991) and Australia (Larsen et al, 1994) as a potential biological control agent. *D. flagrans* has been tested in full-scale field trials in Denmark against many of the important nematodes of cattle, sheep, horses and pigs where daily feeding of chlamydospores over the initial 2-3 months of the grazing season was found to prevent the build up of dangerous levels of infective larvae on pasture (reviewed by Larsen, 2000). Similar results in sheep in Australia have also been reported with a tendency for improved liveweight gain (Knox and Faedo, 2001). Although showing promise, further work to determine the impact of continuous use of nematode-trapping fungi on the environment will need to be carried out and the problem of how to deliver chlamydospores to livestock resolved.

It is unlikely that any of the control methods mentioned above will by themselves provide a realistic, alternative method of control, but rather they will form part of an integrated strategy towards the sustainable control of gastrointestinal nematode infections.

4.6 Vaccines

That host immunity can develop against nematode infections indicates that immunological-based control is a feasible method of controlling parasitic infections. Although much effort has been directed at vaccine development, the only marketed vaccine against gastrointestinal nematodes remains Dictol^{TM}, based on the attenuated, irradiated infective larvae of the cattle lungworm, *Dictyocaulus viviparus*. However, the economic and ethical issues involved with producing sufficient parasites to allow isolation of native antigens for use in vaccine preparations is a common and prohibitive factor when dealing with gastrointestinal nematodes. For this reason, attention has turned to the development of recombinant protein-based vaccines. However, the production of useful recombinant antigens is a complex process which must take into consideration several important factors. For instance, as discussed earlier, the host immune response to an invading parasite is multifaceted involving cellular, humoral and inflammatory components. Understanding the relevant host-protective responses will allow an informed decision on both the route

of immunisation and choice of adjuvant used in vaccine formulation. For example, immunisation of sheep with a purified native *H. contortus* L3 surface antigen gave significant reductions in FEC using aluminium hydroxide, which induces a predominantly Th2-type host immune response, as adjuvant whereas no reductions were observed when the same antigen was formulated in Quil A, which induces a predominantly Th1-type response (Jacobs et al, 1999). In addition, better levels of protection were obtained by rectal mucosal immunisation as compared with the intradermal route of delivery. These considerations are most pertinent to vaccination with natural antigens based on induction of effector mechanisms that are involved in the development of natural protective immunity. In addition, the possibile detrimental effects to the host of eliciting hypersensitivity-type immune reactions associated with increased levels of IgE, mast cells and eosinophils needs to be taken into account.

An alternative vaccination strategy is the use of "hidden" or covert antigens which relies on the induction of a neutralising humoral immunity against critical parasite proteins not seen by the host during the course of a normal infection. This approach has been adopted successfully in the development of a vaccine against the cattle tick, *Boophilus microplus* (Willadsen et al, 1995), and subsequently used to identify possible candidate vaccine antigens from the gut of *H. contortus* (reviewed in Munn, 1997). However, because specific host immune responses will not be boosted by subsequent natural infection, repeat vaccination may be required to maintain a protective antibody titre until natural immunity is acquired. The possibility of using recombinant cytokines to modulate the immune response (Lofthouse et al, 1996) may help to overcome this potential problem.

There are a number of systems in which recombinant proteins can be expressed. Bacteria are, in most cases, the system of choice due to ease of growth and the typically high yield of recombinant protein. However, if conformational epitopes or functional activity is deemed important for protection, then more complicated eukaryotic systems such as yeast, mammalian cell lines or insect cells may need to be considered in order to obtain pertinent post-translational modifications of recombinant antigens. These considerations will be discussed in more detail later.

However, the recent development of recombinant protein-based vaccines against the cattle tick *B. microplus* (Willadsen et al, 1995) and the sheep cestode *Taenia ovis* (Rickard et al, 1995), which elicit an effective host-protective antibody response sustainable over a significant period of time, has served to highlight the effectiveness of this approach in field conditions.

5. Parasite enzymes

Parasites contain and secrete a variety of enzymes which have evolved to accomplish some of the tasks imposed by a parasitic life style, including tissue penetration, digestion of host tissue for nutrition and evasion of the host immune response. Amongst these, proteolytic enzymes (proteinases) have been the most extensively studied. All the four major groups of proteinases, namely serine, aspartic, cysteine and metalloproteinases, have been identified in parasitic helminths, although by far the largest number reported up to now belong to the papain superfamily of cysteine proteinases (Tort et al, 1999).

5.1 Tissue penetration and niche establishment

Proteinases have long been implicated in skin penetration by the infective larvae of *Strongyloides* species (Lewert and Lee, 1954) and the rapid migration of *Strongyloides stercoralis* through the dermal extracellular matrix is mediated by a neutral metalloproteinase secreted by L3 larvae (McKerrow et al, 1990). The free-living cercariae of schistosome parasites secrete a serine proteinase from the acetabular glands which facilitates invasion of the skin and connective tissue (McKerrow et al, 1983; Newport et al, 1988). More recently, secreted cysteine proteinases of L4 and adult *H. contortus* were shown to degrade the major components of connective tissue (glycoprotein, elastin and collagen) *in vitro*, suggesting that these enzymes may be involved in breakdown of host tissue (Rhoads and Fetterer, 1995a). Two cathepsin L-like proteinases are secreted by *F. hepatica* which cleave intracellular matrix proteins such as collagen, laminin and fibronectin, indicating a possible role in the degradation of host tissue barriers (Berasain et al, 1997). Serine and metalloproteinases released by L4 and adult *T. vitrinus* can

degrade a number of macromolecules, including fibrinogen, plasminogen and fibronectin, which may indicate a role in the disruption of mucosal tissue allied to a role in parasite feeding (MacLennan et al, 1997). A cathepsin L-like cysteine proteinase present in adult *Ostertagia ostertagi* excretory/secretory (ES) extracts shows high proteolytic activity against mucin and may be important for penetrating the host and feeding in the abomasal mucus (Geldhof et al, 2000).

5.2 Evasion of the host immune system

Because of their long co-evolutionary interaction with the host immune system, long-lived helminth parasites are well adapted to immune evasion and manipulation, inducing forms of immune tolerance to permit their survival (Maizels and Lawrence, 1991).

Enzymes released by parasites can interfere with the host immune response in a number of ways, the best documented being immunoglobulin cleavage. For example, secreted cysteine proteinases from adult *H. contortus* can hydrolyse IgG within the hinge region (Rhoads and Fetterer, 1995b). Cathepsin L1 secreted by *F. hepatica* also cleaves immunoglobulin at the hinge region and can prevent the antibody-mediated attachment of eosinophils to juvenile flukes (Carmona et al, 1994; Smith et al, 1993a). The ES proteinases of the hookworm *Necator americanus* infective L3 larvae have been shown to cleave IgG , IgM and IgA, the latter being the principal immunoglobulin in the gastrointestinal tract (Kumar and Pritchard, 1992). Secreted proteinases of *Schistosoma mansoni* cleave the Fab portion of IgG after it has bound to the surface of schistosomula (Auriault et al, 1981) and an elastase-like serine proteinase of both cercarial and schistosomular extracts has been shown to cleave IgE and to render it non-functional (Pleass et al, 2000). The products of proteolytic cleavage of host immunoglobulin can have secondary effects on the host immune response. For example, peptides produced by IgG cleavage by *S. mansoni* schistosomula can modulate macrophage activity, including decreased IgE-dependent cytotoxicity against schistosomula (Aurialt et al, 1985).

A metalloproteinase present in the ES products of adult *N. americanus* degrades eotaxin, a potent eosinophil chemoattractant, and may be a strategy

employed by helminths to prevent recruitment and activation of eosinophils at the site of infection (Culley et al, 2000).

Some parasites, mainly those of the alimentary tract, although there are a few exceptions (such as the cattle lungworm *D. viviparus*), release acetylcholinesterases (AChE) into their immediate environment and this enzyme may impair a number of immune responses by hydrolysing acetylcholine which is involved in, for example, antibody dependent cytotoxicity, lysosomal enzyme secretion and neutrophil chemotaxis (reviewed by Knox, 1994). Establishment of *N. brasiliensis* in the rat jejunum results in a dramatic increase in the overall amount of AChE produced accompanied by a switch in isoform expression (Edwards et al, 1971). The amount of AChE produced appears to be regulated in part by the immune status of the host with a reduction in the level of nematode AChE occurring if parasites are transferred to an immunologically naïve host (Sanderson et al, 1972) and conversely, up-regulation of enzyme expression following passive transfer of immune serum (Jones and Ogilvie, 1972). This suggests that expression of parasite AChEs may contribute to maintenance of their position in the gastrointestinal tract. However, most of the hypotheses concerning the physiological function of parasite-derived AChE have not yet been systematically investigated.

In addition to their normal functions in detoxifying oxidants produced during aerobic metabolism, antioxidant enzymes may be required for parasites to defend themselves against reactive oxygen species (e.g. superoxide anion, hydrogen peroxide, hydoxyl radical) generated by host macrophages, neutrophils and eosinophils (Callahan et al, 1988; Brophy and Pritchard, 1992). Activated oxygen species have been shown to be toxic to various parasites *in vitro*, including *T. spiralis* (Bass and Szejda, 1979), *S. mansoni* (Mkoji et al, 1988), *Onchocerca cervicalis* (Callahan et al, 1990) and *Brugia malayi* (Ou et al, 1995). The anthelmintic agent endoperoxide may act as a free-radical generator and has been shown to kill *H. contortus* L3 *in vitro* (Bennet-Jenkins and Bryant, 1996). Increased reactive oxygen species production by peritoneal leukocytes has been correlated with the rejection of *N. brasiliensis* and *F. hepatica* (Smith and Bryant 1989a; Smith et al, 1992). Elevated levels of key antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase have been correlated with *N. brasiliensis*

persistence in rats (Smith and Bryant, 1986; Batra et al 1993). Superoxide dismutase enzyme activity has been shown to be elevated in *N. brasiliensis* capable of surviving in primed hosts as compared to worms harvested after a primary infection of naïve rats (Knox and Jones, 1992). Furthermore, *N. brasiliensis* rejection can be inhibited by the administration of anti-oxidants (Smith and Bryant, 1989b). Peroxiredoxins are an emerging family of multifunctional enzymes abundant in parasitic nematodes and which catalyse the reduction of hydrogen peroxide to water without the requirement of co-factors (Henkle-Dührsen and Kampkötter, 2001). These enzymes are present throughout the life cycle of parasitic nematodes and localisation profiles indicate that they are active in dealing with both internal and external oxidative stress. Localisation of peroxiredoxins in *Onchocerca volvulus* is predominantly in the hypodermis and cuticle, indicating a role at the host-parasite interface (Zipfel et al, 1998).

Parasites may also evade host immune responses by producing different isoforms of key enzymes as the host immune response develops. *H. contortus* retrieved from lambs immunised with parasite gut antigen extracts were shown to have modified proteinase expression, both in terms of total enzyme content and expression of different molecular forms (Chapter 2; Knox et al, 1993). Similar responses have been noted in acetylcholinesterase secreted from *N. brasiliensis* (Jones and Ogilvie, 1972) and *D. viviparus* (McKeand et al, 1994) and in superoxide dismutase expression in *N. brasiliensis* capable of surviving in primed hosts (Knox and Jones, 1992).

5.3 Nutrition

The enzymatic degradation of host tissue that occurs during parasite tissue penetration and niche establishment, discussed above, will release peptides that can be utilised as nutrients by the invading parasite.

Considerable research has focused on the role of proteinases in the maintenance of blood-feeding parasitic helminths. In schistosomes, haemoglobin digestion is attributable to cysteine proteinases, with both cathepsin L1 and cathepsin D localising to the parasite gastrodermis and being able to cleave human haemoglobin at low pH (Brady et al, 1999; Brindley et al, 2001). A role for

exopeptidases, such as dipeptidyl peptidase I (Hola-Jamriska et al, 1998) and papain-like cysteine proteinases in the terminal stages of digestion to release dipeptides or amino acids, has been suggested (Brindley et al, 1997). The cathepsin Ls of *F. hepatica* are released by the gastrodermis of the parasite and are also likely to perform a role in the digestion of ingested host proteins (Smith et al, 1993b). Hotez and Cerami (1983) described the presence of a metalloproteinase in extracts of adult *Ancylostoma caninum* hookworms. The proteinase had elastinolytic activity which inhibited fibrin clot formation, a function which may prevent the coagulation of blood during feeding (Hotez and Cerami, 1983; Hotez et al 1985). Recently, gut-derived, cathepsin D-like aspartic proteinases from *N. americanus* and *A. caninum* were shown to cleave haemoglobin in a host-specific fashion (Williamson et al, 2002). The anticoagulant activity of *H. contortus* ES is attributable to a cathepsin L-like proteinase (Rhoads and Fetterer, 1995b). In adult *H. contortus*, the majority of proteinases capable of degrading the blood proteins haemoglobin, albumin and fibrinogen were active at acidic pH and could be ascribed to the cysteine proteinase class (Chapter 2; Knox et al, 1993). In addition, a gut-expressed putative aspartyl proteinase was shown to have affinity for haemoglobin as substrate (Chapter 6; Longbottom et al, 1997) and may have a co-ordinated function with gut-expressed metalloproteinases (Chapter 5; Redmond et al, 1997) in digestion of the host blood meal.

Clearly, many of the roles suggested for parasite-derived enzymes are crucial for establishment and survival in the host and, as such, enzymes may be considered as pertinent candidate vaccine antigens.

6. Nematode vaccines – progress to 1993

In order to put the work presented in this thesis into context, the following section describes briefly the progress of vaccine antigen research up to 1993, with particular respect to gastrointestinal nematode parasites of livestock, which is summarised in Table 1.

Worm	Stage	Antigen	Protective*	Host tested	Reference
H. contortus	L3	gamma-irradiated	y	sheep	Smith and Angus, 1980
	?	tropomyosin	y	sheep	Cobon et al, 1989
	L3/L4	collagen	n	sheep	Boisvenue et al, 1991
	adult	GST	nt	-	Sharp et al, 1991
	adult	fibrinogen-degrading extract	y	sheep	Boisvenue et al, 1992
	adult	gut homogenate	y	sheep	Smith, 1993
	adult	gut homogenate	y	goats	Jasmer & McGuire,1991
	adult	contortin	y	sheep	Munn et al, 1977
	adult	H11	y	sheep	Munn et al, 1993a
T. circumcincta	L3	ES 31 kDa	y	sheep	McGillivery et al, 1992
T. colubriformis	L3	gamma-irradiated	y	sheep	Gregg et al, 1978
	L3	tropomyosin	y	guinea pig	O'Donnell et al, 1989a
	L3	ES	y	guinea pig	O'Donnell et al, 1989b
	L3	ES 94 kDa	n	guinea pig	O'Donnell et al, 1989b
	L4	homogenate	y	guinea pig	Rothwell, 1978
	L4	SDS-gel fractions	y	guinea pig	O'Donnell et al, 1985
	L4	AChE	n	guinea pig	Rothwell & Merritt, 1975
	L4/adult	ES lectin-binding (30 kDa, 37 kDa)	y	guinea pig	Savin et al, 1990
	L4/adult	ES lectin unbound	y	guinea pig	Savin et al, 1990
	L4/adult	ES 11 kDa	y	guinea pig	Dopheide et al, 1991
	L4/adult	ES 18 kDa	y	guinea pig	Frenkel et al, 1992
O. radiatum	L4	homogenate	y	calves	Keith & Bremner, 1973
	adult	homogenate	y	calves	Keith & Bremner, 1973
	adult	high Mwt filtrate	y	calves	East et al, 1989

Table 1. Gastrointestinal nematode worm antigens identified up to 1993.

* y, produced significant level of protection in vaccine trials: n, not protective: nt, not tested.

6.1 Irradiated larvae

Vaccination is considered feasible for those host-parasite interactions where acquired immunity has been shown to develop. Radiation-induced attenuation of infective L3 has been used to provide self-limiting infections which stimulate protective immunity in the host. This approach resulted in the development of an anti-nematode vaccine for the control of the lungworm *D. viviparus* in cattle, (Jarrett et al, 1958) where two doses of 10^2-10^3 gamma-irradiated L3 were shown to provide solid immunity (Poynter, 1963). A similar vaccine was developed against *D. filaria* in sheep (Sharma et al, 1981) and *A. caninum* in dogs (Miller, 1978). Large numbers ($\sim 10^4$) of gamma-irradiated L3 of *T. colubriformis* or *H. contortus* can generate protective immunity in sheep more than 6 months old, but, as in natural infection, immature animals which are most at risk from nematode infection are not protected (Gregg et al, 1978; Smith and Angus, 1980).

Subsequent work focused on the fractionation of larval and worm extracts to specifically identify parasite antigens which stimulate protective immunity.

6.2 Somatic antigens

Whole homogenates of both L4 and adult *Oesophagostomum radiatum*, a pathogenic parasite of cattle which inhabits the large intestine, induced protective immunity in calves (Keith and Bremner, 1973). Further fractionation of adult homogenates by gel filtration yielded a high molecular weight fraction which caused significant reductions in both worm burden and FEC of 57% and 55%, respectively (East et al, 1989). Similarly, whole L4 homogenates of *T. colubriformis*, and SDS-gel isolated subfractions of these homogenates, caused accelerated expulsion of worms from outbred guinea pigs, used as a laboratory model for sheep (Rothwell 1978; O'Donnell et al, 1985). Sodium deoxycholate extracts of *T. colubriformis* L3 were shown to contain a much simpler set of antigens with only 4 major protein bands (O'Donnell et al, 1989a). Of these proteins, only the 41 kDa band proved protective, resulting in a 45-51% reduction in worms when used to vaccinate guinea pigs. Partial amino acid sequencing identified this 41 kDa protein as tropomyosin (O'Donnell et al 1989a) and allowed the design of an oligomer to facilitate the isolation of full-length cDNA by library screening (Frenkel et al, 1989). A 27 kDa

subunit, expressed in bacteria as a β-galactosidase (β-gal) fusion protein, elicited accelerated worm expulsion in guinea pigs following challenge infection (Cobon et al, 1989). DNA hybridisation isolated a *H. contortus* tropomyosin homologue. A significant level of protection was achieved in sheep by vaccinating with recombinant *H. contortus* tropomyosin and was comparable to the protection achieved with native protein (Cobon et al, 1989).

Native collagen and an 18 aa cuticle collagen peptide isolated from L3 and L4 *H. contortus* were tested in sheep, but neither induced reproducible levels of protection, possibly because antibody or host immune cells were not able to reach the collagen epitopes in the nematode cuticle without prior removal of the surface coat (Boisvenue et al, 1991).

6.3 Soluble antigens

By culturing infective *T. circumcincta* L3 *in vitro* in medium supplemented with ^{35}S-methionine, a 31 kDa glycoprotein was shown to be one of the major components of the ES complex (McGillivery et al, 1990). Gel purified, stage-specific 31 kDa antigen significantly reduced FEC in immunised sheep (McGillivery et al, 1992), demonstrating that this protein conferred partial protection against L3 *T. circumcincta* larvae.

Antisera raised against glutathione S-transferase (GST) purified from adult *H. contortus,* although found to inhibit enzyme activity, had no effect on parasite survival *in vitro*, even though a specific GST inhibitor reduced parasite survival in a parallel experiment (Sharp et al, 1991). The efficacy of the purified *H. contortus* GST protein was not tested directly in a protection trial. Significant protection against *H. contortus* in sheep has been obtained using a soluble, fibrinogen-degrading extract from the intestinal tract of adult parasites (Boisvenue et al, 1992). Chromatographic fractionation of this extract identified a predominant 35 kDa cysteine proteinase, as determined by active site labelling (Cox at al, 1990). This protein was shown to be a member of a developmentally regulated cysteine proteinase gene family of which cDNAs encoding five members have been isolated and their nucleotide sequences determined (Pratt et al, 1990; 1992).

Guinea pigs vaccinated with a soluble, fractionated extract from *T. colubriformis* L4 enriched for AChE were protected against homologous challenge (Rothwell and Merritt, 1975). However, this fraction was contaminated with an undefined low molecular weight allergen. Purified *T. colubriformis* AChE, free from this allergen, did not stimulate protective immunity (Rothwell and Merritt, 1975).

Several antigens purified from ES of larval and adult *T. colubriformis* have shown considerable potential in vaccination trials. The ES products of exsheathed L3 conferred some protection (46% in terms of worm counts) to guinea pigs against homologous challenge (O'Donnell et al, 1989b). The dominant component of the L3 ES was a strongly antigenic 94 kDa glycoprotein. Sephacryl column fractions enriched in the 94 kDa antigen were not correspondingly enriched in their protective capacity. Similarly, preparations in which the 94 kDa antigen had been removed by immunoaffinity absorption retained their capacity to induce host-protection, indicating the presence of more than one host protective antigen in *T. colubriformis* L3 ES (O'Donnell et al, 1989b). ES material from L4 and adult *T. colubriformis* has been fractionated by passage through a lentil lectin column and, although results of protection trials are unpublished, Savin et al (1990) amongst others, have reported that both the lectin-bound and unbound fractions display host-protective activity. Complimentary DNA corresponding to two of the lectin-binding proteins has been cloned and characterised. The major secreted glycoprotein in *T. colubriformis* L4 and adult ES is a 30 kDa protein which shares limited sequence similarity with porcine valosin, suggesting an influence on host gut physiology (Savin et al, 1990). A second 37 kDa protein is the major ES product recognised by lymph from naturally infected sheep, although nucleotide sequence database searches failed to find a match which could point to a functional role for this molecule (Verkuylen et al, 1993). Two components of the non-glycosylated, lectin unbound fraction of L4 and adult *T. colubriformis* ES have also been isolated. The major component of this fraction is an 11 kDa protein found to be highly protective (50-100%) in guinea pigs (Dopheide et al, 1991). An oligomer based on partial amino acid sequence analysis of this 11 kDa protein was used to isolated a full-length encoding cDNA, which showed homology with a human IFNγ-induced protein. This cDNA was expressed as a recombinant protein in *Escherichia coli* (Dopheide et al, 1991). An 18 kDa protein

was also isolated and induced 60-84% protection of guinea pigs from challenge infection following a single intraperitoneal injection (Frenkel et al, 1992). Although nucleotide sequence analysis of isolated cDNA encoding the 18 kDa protein showed only low overall identity with human and insect globin, conservation between invariant globin residues suggests that the parasite component may encode a globin and that it may play a role in oxygen transport.

6.4 Hidden antigens

The concept of using hidden, or covert antigens to stimulate a protective humoral immune response was successfully demonstrated against the cattle tick *B. microplus* (Rand et al, 1989) and this approach was employed by Munn et al (1987) to isolate an integral gut membrane fraction from adult *H. contortus* enriched for contortin, a helical, polymeric protein located between the microvilli of the parasite gut (Munn, 1977). Vaccination with a contortin-rich fraction of adult *H. contortus* yielded a mean reduction in worm burdens of 78% (Munn et al, 1987). Subsequently, a second integral gut membrane extract enriched for a 110 kDa protein, termed H11, was obtained from adult *H. contortus* and shown to reduce worm burdens by up to 89% and FEC by up to 92% in sheep vaccination trials, protection correlating with host serum antibody titre (Munn et al, 1993a & 1993b). This represents the best level of protection achieved with a native nematode antigen. At the same time, the ability of gut antigens to induce a protective immune response against blood-feeding nematodes in goats (Jasmer and McGuire, 1991) and sheep (Smith, 1993), was determined by vaccinating with a detergent extract of dissected-out guts of *H. contortus*. Serum from sheep hyperimmune to *H. contortus* did not react with the gut membrane extract (Smith, 1993), suggesting that these antigens are normally hidden from the host during the course of a natural infection.

Clearly, up to 1993, considerable progress had been made in the fractionation of parasite extracts and identification of the protective antigen(s) within them. However, only limited progress had been made in the molecular cloning and characterisation of the genes encoding these proteins and in the expression of

recombinant parasitic antigens, with a view to evaluating their efficacy in host protection trials.

7. Aims of project

The aims of the work presented in this thesis are:

1. to characterise the proteinases present in extracts of adult *H. contortus* in terms of their substrate specificity, inhibitor sensitivity and molecular size with a view to identifying potential vaccine antigen candidates;
2. to clone and characterise full-length cDNAs encoding these proteinases and to express them in appropriate bacterial vectors to allow evaluation of recombinant proteins in sheep vaccination trials and;
3. to evaluate the potential of the free-living nematode *C. elegans* as a vector for expression of recombinant nematode antigens.

8. Overview

The work presented in this thesis forms a coherent body of work which contributes significantly toward the overall aim of developing a recombinant vaccine against *H. contortus* through the identification of potential vaccine candidate antigens, their molecular cloning, characterisation and expression in recombinant form for evaluation in vaccine trials. In addition, the work described with the free-living nematode *C. elegans*, highlights its potential both as a vector for expression of candidate vaccine antigens from parasitic nematodes and as a model system for studying parasite gene expression and control.

As discussed previously (5.1-5.3), parasite enzymes have evolved to accomplish some of the tasks imposed by a parasitic life style, many of which are considered crucial for establishment and survival in the host. If accessible to the host immune system, then parasite enzymes are potential candidate vaccine antigens. Chapter 2 describes the initial characterisation of the proteinases present in extracts of adult *H. contortus* in terms of their substrate and inhibitor sensitivities, pH optima and molecular size. Serum inhibition studies were used to define parasite enzymes of potential importance at the parasite/host interface.

Subsequently, an enzymatically active integral gut membrane glycoprotein complex, namely thiol sepharose-binding proteins (TSBP), was isolated from adult *H. contortus* and shown to confer significant levels of protection against homologous challenge in sheep. The TSBP fraction was greatly enriched for cysteine proteinases. Following fractionation of TSBP by anion-exchange chromatography, only subfractions containing cysteine proteinase activity afforded protection in a subsequent sheep vaccination trial. *H. contortus* cysteine proteinases were shown to be encoded by a multigene family and Chapter 3 describes the isolation and molecular characterisation of cDNAs encoding several members of this gene family using a combination of cDNA library immunoscreening and the polymerase chain reaction (PCR).

A second host-protective protein complex isolated from adult *H. contortus*, *Haemonchus* galactose-containing glycoprotein (H-gal-GP), also shows marked proteinase activity. Inhibitor sensitivity studies showed that aspartyl-, metallo- and

cysteine proteinase activity could be ascribed to this complex, Chapter 4. Chapter 4 also describes analysis of the polypeptide composition of H-gal-GP using a combination of polyacrylamide gel electrophoresis (PAGE) and N-terminal amino acid sequencing. As with TSBP, protection tends to correlate with proteinase-containing fractions of H-gal-GP. Full-length cDNAs encoding both the metallo- and aspartyl-proteinase (pepsinogen) components of H-gal-GP were isolated using a combination of cDNA library immunoscreening and PCR, described in Chapters 5 and 6, respectively. Both cDNAs were cloned into appropriate bacterial vectors to allow expression of recombinant proteins.

Expression of cDNAs encoding the enzyme components of TSBP and H-gal-GP in bacterial systems resulted, typically, in good yields of recombinant proteins. However, they were expressed as insoluble, enzymatically inactive inclusion bodies. Sequential denaturation and reduction of H-gal-GP resulted in loss of its protective capacity, indicating that conformational epitopes and/or enzyme activity may be important for protection. Therefore, in order to obtain appropriate protein folding and post-translational modification of recombinant proteins, expression in a eukaryotic system is probably required. The type of N-glycans present in *H. contortus* is similar to that seen in other invertebrates, and occurs also in the free-living nematode *C. elegans*. Together with the fact that *C. elegans* is closely related to *H. contortus* in evolutionary terms, this suggests that *C. elegans* would be an appropriate system for expression of functionally active *H. contortus* candidate vaccine antigens. Chapter 7 describes the isolation and structure of the gene encoding the pepsinogen component of H-gal-GP and its expression in *C. elegans* under the control of a *C. elegans* promoter.

The potential of using *C. elegans* as a heterologous transformation system to examine activity and specificity of parasitic nematode gene promoters is addressed in Chapter 8. The *H. contortus* pepsinogen promoter was isolated using a two-step PCR technique which was developed to allow amplification of unknown DNA sequences. The pepsinogen promoter, together with two other parasite promoters, was tested for the ability to drive correct spatial and temporal expression of a reporter gene in *C. elegans*. This transformation system was also used to analyse deletion constructs of the *H. contortus* aspartyl proteinase promoter with the aim of identifying

regulatory motifs which direct protein expression to the parasite gut. Such motifs, if present, could be used in the construction of gut-specific cDNA libraries, or as the basis for gene-specific PCR. As part of the detailed analysis of the *H. contortus* pepsinogen promoter, primer-extension analysis of mRNA was carried out and showed low-level splicing to a 22 nucleotide SL2-like sequence, the first observed case of SL2 *trans*-splicing in *H. contortus*. The PCR isolation of the *H. contortus* SL2 gene and its nucleotide sequence analysis are described in Chapter 9.

Unresolved questions arising from this body of work are discussed in Chapter 10.

Chapter 2: Characterization of proteinases in extracts of adult *Haemonchus contortus*, the ovine abomasal nematode.

Knox DP, Redmond DL and Jones DG. 1993. *Parasitology*, **106**: 395-404.

A previous study examined proteinase activities in gastrointestinal nematodes of ruminants and demonstrated the release of elastolytic and chymotryptic activities from L3 and adult *H. contortus* (Knox and Jones, 1990). However, non-specific protein substrates were used and no conclusions could be drawn regarding the number and specificity of these proteinases, nor was evidence of their antigenicity sought. Fibrinogen-degrading activity in a PBS/glycerol soluble extract from adult *H. contortus* was associated with a predominant 35 kDa cysteine proteinase (Cox et al, 1990), strong evidence for a potential role of proteinases in the blood feeding habit of adult parasites. Furthermore, significant protection against *H. contortus* in sheep was achieved by immunising with this fibrinogen-degrading extract (Boisvenue et al, 1992), highlighting the potential of parasite-derived enzymes as candidate vaccine antigens.

The aim of the work presented in this paper was to characterise the proteinases in extracts of adult *H. contortus* in terms of their substrate specificities (particularly in relation to feeding and digestion of the host blood meal), pH optima, inhibitor sensitivities, molecular size and immunogenic potential.

Triton X-100 extracts of adult *H. contortus*, comprising the water soluble, membrane-associated and membrane-bound proteins, were evaluated for enzyme activity over a broad pH range against the general protein substrate azocasein and the blood proteins fibrinogen, albumin and haemoglobin. Peak enzyme activities were detected at pH 5 (vs albumin, fibrinogen and haemoglobin), pH 7 (vs albumin) and pH 9 (vs fibrinogen, haemoglobin and azocasein). These enzyme activities were further characterised using low molecular weight substrates in analyses which demonstrated tryptic and aminopeptidase activities at pH 5 and elastase activity at pH 9. At pH 7, activity could not be clearly ascribed to any particular proteinase type.

Incubation of *H. contortus* Triton X-100 extracts with specific proteinase inhibitors prior to azocasein degradation analysis showed that proteolysis at pH 5 was almost completely due to cysteine proteinase activity and at pH 9 to serine and, to a lesser extent, metalloproteinase activity.

Gelatin-substrate gels were used to determine the molecular size of the proteinase activities at the 3 different pH optima described above. Zones of substrate proteolysis are visualised as clear bands against a blue background following Coomassie Blue staining. At pH 5, activity was evident at 55 kDa and >200 kDa while at pH 9, a smear of activity >100 kDa was observed. Again, proteolysis at pH 5 was almost completely abolished by incubation of the gel with the cysteine protease inhibitor, L-trans-epoxysuccinyl-L-leucylamide-(4-guanidino)-butane (E64), prior to staining. Proteolysis at pH 9 was highly sensitive to the serine proteinase inhibitor phenyl-methylsulphonylfluoride (PmsF), and to the metalloproteinase inhibitor ethylenediaminotetra-acetic acid (EDTA), with slight sensitivity to cysteine and aspartyl proteinase inhibitors indicating the presence of multiple enzyme classes.

Proteinases eluted from polyacrylamide gel slices following fractionation on the basis of molecular size were also analysed for their ability to hydrolyse blood proteins. This analysis was conducted in an attempt to identify proteinases involved in blood meal digestion. Haemoglobin degradation occurred almost exclusively at acidic pH and was sensitive to the cysteine proteinase inhibitor E64. A dominant peak of fibrinogen degradation at pH 5 associated with cysteine proteinase activity was noted in the molecular size range 48.5-58.5 kDa. Further analysis of breakdown products by polyacrylamide gel fractionation showed that degradation was restricted to the fibrinogen α-chain peptide. This activity is in contrast to the dominant *H. contortus* 35 kDa fibrinogen-degrading cysteine proteinase previously described by Cox et al (1990) which is active at neutral pH. This anomaly may reflect strain divergence and is an important point to consider as it may have implications for the production of a globally effective *H. contortus* vaccine.

In an attempt to define parasite enzymes of potential importance at the parasite/host interface, parasite extracts or gel-eluted protein fractions were incubated with serum harvested from lambs demonstrably refractory to *H. contortus* challenge following either hyperimmunisation by repeat infection, or by

immunisation with a *H. contortus* gut antigen extract, prior to determination of the degree of inhibition of proteolysis. Sera from lambs immunised with gut-antigen extracts were shown to inhibit the predominant 55 kDa cysteine proteinase activity at pH 5 in gelatin-substrate gels, whereas serum from hyperimmune lambs had no effect in this system. Fibrinogen degrading proteinase activity was also inhibited by both types of sera, in particular low molecular weight enzyme activity (<58.5 kDa) at acidic pH. Enzyme activity between 58.5 and 84 kDa was inhibited only by hyperimmune serum and, contrastingly, activity <26.5 kDa only by antisera against gut-antigen extracts. These observed inhibition profiles may reflect the different proteinases accessible to host immune attack during the course of a natural infection as compared to those contained in a gut antigen extract. The total proteinase activity in Triton X-100 extracts of worms recovered from *H. contortus* gut-antigen immunised lambs was found to be 2-3 fold higher than that of worms recovered from control animals, with a marked increase in expression of proteinases >60 kDa, as determined by azocasein degradation and gelatin-substrate gel analysis. This observation may indicate that parasites are able to modify their proteinase expression in response to host immune attack.

In conclusion, the work presented here expands the information available on the proteolytic enzymes expressed by adult *H. contortus*, demonstrating the expression of multiple enzymes with differing substrate specificities and pH optima, some of which are likely to play a role in the degradation of the host blood meal. Cysteine proteinases were found to be dominant at acidic pH and to catalyse the degradation of the blood proteins haemoglobin and fibrinogen. Evidence of immune inhibition provides preliminary information on those enzymes which may be important at the parasite/host interface and may, therefore, be appropriate candidate vaccine antigens.

Contribution to work

Having just joined the research group, my input was in a technical capacity, contributing to enzyme assays and gelatin-substrate gel analysis of protein extracts.

Characterization of proteinases in extracts of adult *Haemonchus contortus*, the ovine abomasal nematode

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SUMMARY

The degradation of several protein substrates, including the blood proteins haemoglobin, albumin and fibrinogen, by proteinases present in extracts of adult *Haemonchus contortus* was examined over a broad pH range. These proteinases were further characterized on the basis of substrate specificity, inhibitor sensitivity and molecular size by spectrophotometric and substrate gel analysis. The majority of the proteinases capable of degrading the blood proteins tested were active at acidic pH and could be ascribed to the cysteine proteinase class. In addition, evidence is presented that these proteinases are differentially recognized and inhibited by immune sera and that parasites capable of withstanding protective host immune responses exhibit modified expression of proteinases.

Key words: *Haemonchus contortus*, proteinases, biochemical characterization.

INTRODUCTION

Haemonchus contortus is a highly pathogenic nematode parasite infecting the abomasal glands primarily of sheep and other small ruminants, although it also affects cattle. The blood-feeding habits of the adult parasite can induce severe anaemia, weight loss and, occasionally, death. Following the description of a secreted proteolytic anticoagulant with fibrinogenolytic properties by *Ancylostoma* hookworms (Hotez & Cerami, 1983) there has been considerable interest in the role of proteinases in the maintenance of blood-feeding parasitic helminths. 'Haemoglobinase' activity has been demonstrated in the hookworms *Necator americanus* and *Ancylostoma* spp. (Pritchard *et al.* 1992) and in the trematode *Schistosoma mansoni* (Chappell & Dresden, 1986). Digestive proteases have been characterized extensively in schistosomes (reviewed by McKerrow & Doenhoff, 1988), in particular those proteases involved in haemoglobin digestion by the adult parasite. The major proteinase responsible for haemoglobin digestion has been classified as a cysteine proteinase (Chappell & Dresden, 1986) although several other peptidases have been identified in the schistosome gut using histochemical assays (e.g. Bogitsch & Dresden, 1983). After initial degradation by the haemoglobinase further processing is assumed to be carried out by other peptidases in a way similar to that seen in vertebrates (Bogitsch & Kirschner, 1986).

Until recently, evidence for the involvement of proteinases in the blood feeding of adult *H. contortus* has been limited. Gross histological evidence suggests that bleeding continues from damaged mucosal capillaries for extended periods of time following detachment of the adult parasite, possibly indicative of anticoagulant release by the parasite. Recently, we provided evidence that products of adult *H. contortus* released *in vitro* contained elastinolytic activity and we postulated that the activity could act as an anticoagulant to facilitate blood feeding (Knox & Jones, 1990). A recent series of reports has described the molecular cloning and sequencing of a 35 kDa cysteine proteinase with putative fibrinogen degrading properties from adult *H. contortus* (Cox, Milhausen & Hageman, 1990*a*, *b*) as well as the structure and organization of the coding gene (Pratt *et al.* 1990). Further experiments indicated that adult *H. contortus* express mRNAs for several distinct cysteine proteinases (Pratt *et al.* 1992) although there is, as yet, no information on the substrate specificity or physiological roles of these enzymes. Moreover, it was claimed that lambs could be protected against challenge infection with *H. contortus* by immunization with extracts of the adult parasite enriched on the basis of fibrinogen-degrading activity (Cox *et al.* 1990*a*).

In the present study the degradation of several protein substrates by extracts of the adult parasite has been examined over a broad pH range. Proteinases detected have been further characterized on the basis of substrate specificity and inhibitor sensitivity and their molecular size determined. In addition, the effects of serum, harvested from lambs demonstrably immune to challenge *H. contortus* infection, on proteinases in extracts of the adult parasite have been evaluated as an indication of their immunogenic potential.

Table 1. Hydrolysis of a variety of peptide substrates by proteinases in Triton X-100 extracts of adult *Haemonchus contortus*

(Each figure represents the mean of at least 3 separate determinations. The maximum coefficient of variation observed was 5·2%. All readings were performed at 405 nm except for elastin–orcein, the insoluble elastase substrate, which was read at 520 nm. CBZ, carbobenzoxy; Bz, benzoyl; NPE, nitrophenol ester; NA, nitroanilide; Arg, arginine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Succ, succinyl; Try, tryptophan; Tyr, tyrosine.)

		Change in absorbance/h	
Specificity	Substrate	pH 5	pH 9
Trypsin	CBZ-L-Lys-4NPE	0·43	0·02
	CBZ-L-Arg-4NPE	0·22	0·03
	Bz-DL-Arg-4NA	0·13	0·01
Chymotrypsin	CBZ-L-Tyr-4NPE	—	0·14
	CBZ-L-Try-4NPE	—	0·65
	CBZ-L-Phe-4NPE	0·13	0·11
	Bz-Tyr-4NA	0·18	0·12
	Succ-Phe-4NA	0·14	0·04
Elastase	CBZ-L-Ala-4NPE	0·21	1·28
	Elastin–orcein	0·05	0·16
Aminopeptidase	Leu-4NA	1·16	0·34

MATERIALS AND METHODS

Preparation of parasite extracts

Adult *H. contortus* were harvested from worm-free lambs which had been infected individually with 10000–30000 infective L3 as described previously (Knox & Jones, 1990). For routine enzyme analyses, adult parasites (500 mg wet weight) were homogenized in 2 ml of 1% aqueous Triton X-100 in water at 4 °C using a 1 ml glass/glass homogenizer (Jencon's Scientific, UK). Homogenates were briefly centrifuged at 3000 ***g*** to pellet particulate material and supernatant fractions either used immediately for proteinase determinations or stored at −70 °C prior to analysis.

Enzyme activity

The protein substrates azocasein, bovine fibrinogen and bovine serum albumin, as well as the peptide substrates and inhibitors used in this study, were purchased from the Sigma Chemical Company, Poole, Dorset, UK. Ovine haemoglobin was prepared from sheep red blood cells which had been washed 3 times in 10 volumes of ice-cold phosphate-buffered saline before lysis with an equal volume of distilled water. The haemoglobin content of the lysate was adjusted to approximately 5 mg/ml on the basis of an estimated whole blood haemoglobin concentration of 10 mg/ml. All buffers used contained penicillin (500 U/ml) and streptomycin (5 mg/ml). Proteinase activity with azocasein as substrate was determined as previously described (Knox & Jones, 1990). Proteolysis of fibrinogen, albumin and haemoglobin was monitored by measuring the release of α-amino nitrogen using the ninhydrin reaction essentially as described by Matthews (1977). Usually, 10 μl of homogenate (or H_2O for blanks) was incubated with 100 μl of substrate (1 mg/ml in buffer) for 16 h, after which undigested protein was precipitated by addition of an equal volume (110 μl) of 5% trichloroacetic acid and the supernatant fraction harvested by centrifugation for ninhydrin analysis. Low molecular weight substrate solutions (1 mg/ml) were prepared in dimethyl sulphoxide (Me_2SO). Substrates used and the proteinase specificities indicated are listed in Table 1. Reaction mixtures routinely comprised 10 μl of homogenate (or H_2O for blanks), 150 μl of buffer, 25 μl Me_2SO and 5 μl of substrate solution. Initial (0 min) and final (60 min) absorbance readings were determined using an I. L. Multistat III microcentrifugal analyser (Instrumentation Laboratory, Warrington, Cheshire, UK) as described previously (Knox & Kennedy, 1988). All activities were corrected for non-enzymic hydrolysis by subtraction of the appropriate reagent blanks.

pH optima of proteinase activities

The effect of pH on the hydrolysis of fibrinogen, haemoglobin and albumin was monitored over the range 3·0 to 11·0 using the following buffers, all at 0·1 M: pH 3·0–5·5, acetate; pH 5·5–7·5, phosphate; pH 7·5–9·0, Tris and pH 9·0–11·0, carbonate–bicarbonate. All activities were corrected for non-enzymic hydrolysis by subtraction of the appropriate buffer blank. In addition, the effect of pH on

hydrolysis of the general protein substrate, azocasein, was determined for use in routine analyses.

Inhibitor sensitivity of proteinase activity

Proteinase inhibitors, purchased from Sigma, were tested for their effects on the hydrolysis of azocasein at pH 5, 7 and 9. Routinely, 10 μl of homogenate (or H_2O for blanks) were pre-incubated with 150 μl of inhibitor/buffer solution, at the final concentrations listed in Table 2, for 15 min before determining the remaining enzyme activity by addition of 20 μl of substrate solution (5 mg/ml). Following incubation of the reaction mixture for 16 h at 37 °C, undigested protein was precipitated by addition of 180 μl of 1·0 M perchloric acid and the supernatant fraction harvested following centrifugation at 13000 *g* for 5 min using a microfuge. The absorbance (405 nm) of the supernatant fraction was determined and corrected for non-enzymic hydrolysis by subtraction of the appropriate inhibitor blank. Inhibition was expressed as the percentage activity remaining compared to a control incubated in the absence of the inhibitor. Proteinase activity was not significantly inhibited by the solvent/buffer proportions used in the final reaction mixes. In addition, the proteolytically active fractions prepared by gel elution, described below, were tested for their sensitivity to phenylmethylsulphonylfluoride, (PmsF), 4-hydroxymercuribenzoate (4HMB) L-trans-epoxysuccinyl-leucylamido-(4-guanidino)butane (E64), ethylenediaminotetra-acetic acid (EDTA) and pepstatin.

Gelatin–substrate gel analyses

Extracted *H. contortus* proteins (usually 20 μg/track) were fractionated in discontinuous sodium dodecyl sulphate–polyacrylamide gel slabs (SDS–PAGE) under non-reducing conditions. Gelatin (0·1 % w/v) was incorporated into the 7·5 % separating gel and electrophoresis performed using a BioRad Mini-Protean system (BioRad Ltd, UK) with buffer formulations as recommended by the manufacturers. Samples (20 μl) were mixed with 20 μl of SDS–PAGE sample buffer (0·05 M Tris, pH 7·5 containing 5 % SDS, 20 % glycerol and 0·01 % bromophenol blue) and applied to the gel without boiling. Following electrophoresis at 200 V, constant voltage, for 45 min at 4 °C gels were washed in 3 changes of 2·5 % Triton X–100 over a 30 min period to remove SDS before being sliced and the gel slices incubated in buffer alone or in the presence of inhibitor for 24 h at 37 °C. Zones of proteolysis were visualized as clear bands against a blue background by Coomassie Blue staining.

Serum inhibition of H. contortus *extract proteinases*

Parasite extracts (20 μg protein in 20 μl of Triton X–100) or gel eluted proteins (see below) were pre-incubated with 2 μl of various sera for 1 h at 37 °C before determination of proteolysis, either spectrophotometrically with azocasein as substrate or by gelatin–substrate analysis. Sera used were obtained either from worm-free lambs, lambs which had been immunized with an adult *H. contortus* gut antigen preparation and were highly refractory to challenge infection with the homologous parasite (kindly provided by Dr W. D. Smith) or from animals demonstrably immune to challenge infection following repeated infection with the parasite. Spectrophotometric determinations of serum inhibition were calculated after subtraction of the appropriate blank containing serum, substrate and buffer alone. For substrate gel analyses, parasite extracts were pre-incubated with serum as described above and then mixed with an equal volume of sample buffer prior to electrophoresis and visualization of enzyme activity.

Gel elution of proteinases

Triton X–100 extracts of adult *H. contortus* (500 μg protein) were fractionated by 7·5 % non-reducing SDS–PAGE using the Mini-Protean gel system and a preparative gel comb with a capacity for 500 μl of sample and flanked with separate sample wells for molecular weight markers. Following electrophoresis for 45 min at 200 V at 4 °C, the marker tracks were excised and briefly stained using 0·5 % Coomassie Blue in 10 % methanol to visualize the molecular weight markers. SDS was eluted from the remainder of the gel by washing in 3 changes of 2·5 % Triton X–100 for a total of 30 min and the gel was sliced following alignment with the marker tracks. Protein was eluted by homogenization of gel slices in ice-cold 1 % Triton X–100 using a glass/glass homogenizer. Gel material was pelleted by brief centrifugation in a microfuge and the supernatant fraction (usually 500 μl) was retained for determination of proteolytic activity using the blood proteins fibrinogen, haemoglobin or albumin as substrates.

Aliquots (usually 10–50 μl) of the gel-eluted proteins were mixed with 1 % solutions of fibrinogen, haemoglobin or albumin (100 μl) prepared in buffers over the pH range 3·0 to 10·0 as described above. Following overnight incubation at 37 °C protein degradation was determined using the ninhydrin assay. Inhibition of proteolysis by PmsF, E64, EDTA and pepstatin was subsequently evaluated at the pH optima determined in these experiments. Further aliquots (50 μl) of these gel eluates were mixed with 50 μl of 1 % bovine fibrinogen solution in buffers at pH 5 or 7, formulated as described above. Following incubation for 2 h at 37 °C, samples were mixed with an equal volume of SDS sample buffer, boiled for 5 min, and 30 μl aliquots fractionated on 7·5 % SDS–PAGE slabs

Table 2. The effect of various inhibitors on the hydrolysis of azocasein by proteinases in Triton X-100 extracts of adult *Haemonchus contortus*

(The degree of inhibition is expressed as the percentage activity remaining in comparison to a control in the absence of inhibitor. The maximum coefficient of variation observed was 8·7%. PmsF, phenylmethylsulphonylfluoride; Tos-Phe, tosyl-phenylalanine halomethylketone; Tos-Lys, tosyl-lysine halomethylketone. NEM, *N*-ethylmaleimide; 4-HMB, 4-hydroxymercuribenzoate; E64, L-trans-epoxysuccinyl-L-leucylamide-(4-guanidino)-butane; DTT, dithiothreitol; EDTA, ethylenediaminotetra-acetic acid; 1,10 Phe, 1,10 phenanthroline.)

Inhibitor	Final reaction concentration (mM)	Proteinase class indicated	Activity remaining (%) pH 5	pH 7	pH 9
PmsF	1·0	Serine	99	80	12
Tos-Phe	1·0	Serine*	89	ND	31
Tos-lys	1·0	Serine/* cysteine	45	ND	78
NEM	1·0	Cysteine*	31	0	63
4HMB	0·2	Cysteine*	19	68	86
E64	0·2	Cysteine	17	57	92
DTT	2·0	Cysteine/* metallo-	157	100	88
EDTA	2·0	Metallo-	106	64	42
1,10 Phe	1·0	Metallo-	83	72	51
Pepstatin	0·2	Aspartate	62	78	86
			(n = 5	3	4)

* Inhibition was not class-specific.

under reducing conditions. Degradation products were visualized by Coomassie Blue staining.

RESULTS

pH optima

Proteolysis of the general protein substrate, azocasein (data not shown) and the blood proteins fibrinogen, haemoglobin and albumin by Triton X–100 extracted proteinases of adult *H. contortus* over a broad pH range is shown in Fig. 1. The pH profiles were determined on 4 occasions from freshly prepared parasite extracts and the same peaks of activity were detected on each occasion. At acidic pH conditions albumin and fibrinogen both showed relatively sharp activity peaks at pH 5 in comparison to haemoglobin which showed a slightly lower, more diffuse profile. All three blood proteins and azocasein exhibited biphasic pH responses but, in contrast to the others, albumin had a second strong peak of activity at pH 7. Fibrinogen, haemoglobin and azocasein had much weaker biphasic responses with alkaline pH optima of 8–9.

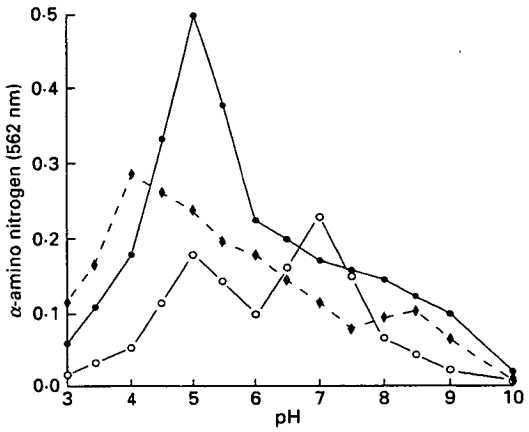

Fig. 1. The effect of pH on the degradation of the blood protein substrates haemoglobin (◆), albumin (○) and fibrinogen (●) by proteinases in Triton X–100 extracts of adult *Haemonchus contortus*.

Substrate specificity

To obtain further information on the variety of proteinases present in Triton X–100 extracts of the adult parasite, hydrolysis of a variety of low molecular weight peptide substrates was examined at pH 5, 7 and 9. While tryptic and aminopeptidase activities were dominant at pH 5 there was a clear shift in substrate specificity at pH 9 (Table 1). At that pH elastase activity, indicated by a combination of degradation of insoluble elastin-orcein and cleavage of the low molecular weight peptide substrate CBZ-L-Ala-4NPE, and chymotryptic activity predominated. Activity at pH 7 (not shown) could not be clearly ascribed to a particular proteinase type.

Inhibitor sensitivity

The sensitivity of azocasein degradation to a variety of proteinase inhibitors was examined at pH 5, 7 or

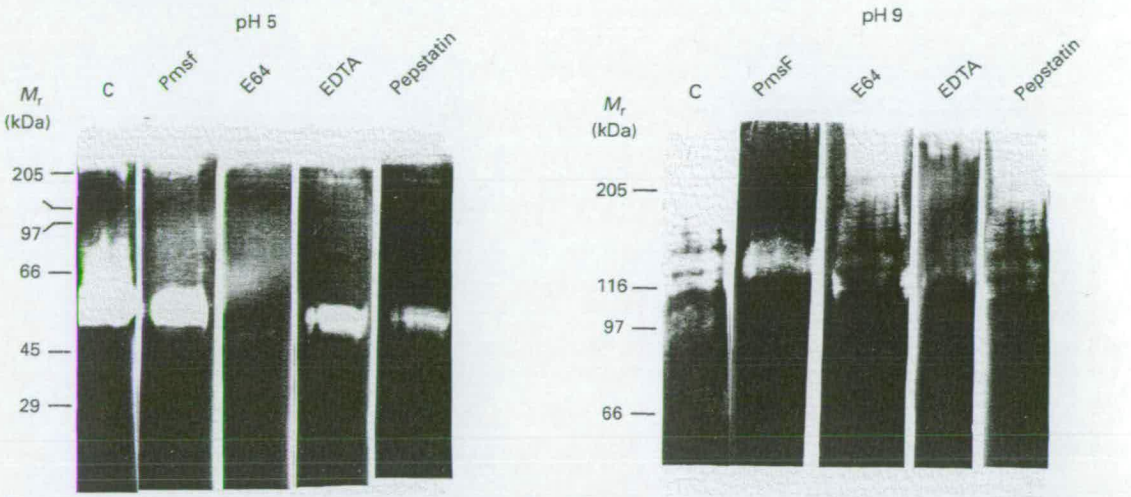

Fig. 2. Gelatin–substrate gels of proteinases in Triton X–100 extracts of adult *Haemonchus contortus* and the inhibition of proteolysis by various proteinase inhibitors. Following electrophoresis gels were sliced and incubated in either buffer alone (C), buffer containing PmsF (1·0 mM), E64 (200 μM), EDTA (2·0 mM) or pepstatin (200 μM) at either pH 5 or 9 before visualizing zones of proteolysis by Coomassie staining. Molecular weight markers are marked in kDa.

9 (Table 2). At pH 5 proteolysis was almost completely eliminated in the presence of the class-specific cysteine proteinase inhibitor, E64, as well as the less specific 4 HMB, and markedly reduced by the alkylating agent, NEM. In addition, proteolysis at this pH was markedly enhanced by the thiol-protecting agent, DTT. Activity was diminished in the presence of the halomethylketone, Tos-Lys, but unaffected by the serine proteinase inhibitor, PmsF, and the metallo-proteinase inhibitors, EDTA and 1,10 phenanthroline, although significantly reduced in the presence of the carboxyl proteinase inhibitor, pepstatin. In contrast, proteolysis at pH 9 was relatively unaffected by cysteine proteinase inhibitors but was notably reduced by PmsF and, to a lesser extent, by Tos-Phe, EDTA and 1,10 phenanthroline. The inhibition profile obtained at pH 7 was not conclusive although enzyme activity was reduced in the presence of the EDTA and 1,10 phenanthroline and NEM to a greater extent than was evident at either pH 5 or pH 9.

Gelatin–substrate gel analysis

The molecular size of proteinases active in extracts of adult *H. contortus* was estimated by gelatin–substrate gel analysis (Fig. 2). At pH 5, a sharply defined zone of proteolysis was evident at 55 kDa and a smear of activity was observed above 200 kDa when gel slices were incubated in buffer alone (Fig. 2, Track C, pH 5). In the presence of the cysteine proteinase inhibitor, E64 (0·1 mM), activity at 55 kDa was completely abolished (Fig. 2, Track E64, pH 5) and reduced in the presence of EDTA (Track EDTA, pH 5, Fig. 2) and pepstatin (Track pepstatin, Fig. 2). The high molecular weight activity was diminished in the presence of E64, EDTA and pepstatin indicating the presence of more than one proteinase activity in this region. By contrast, at pH 9, a dominant smear of proteolysis was evident above 100 kDa with individual banding faintly discernible (Fig. 2, Track C, pH 9). This activity was highly sensitive to the serine proteinase inhibitor, PmsF (1·0 mM, Fig. 2, Track PmsF) but only slightly reduced by E64 and pepstatin. In addition, activity at this pH was markedly reduced in the presence of EDTA (Track EDTA, pH 9, Fig. 2). Activity at pH 7 could not be consistently demonstrated in this gel system.

pH optima and inhibitor sensitivity of gel-eluted proteinases

The sensitivity of proteolysis of fibrinogen, albumin and haemoglobin by gel-eluted proteins (subsequently referred to as gel-eluted proteinases) to pH change and to class-differentiating proteinase inhibitors was evaluated (Fig. 3).

Extracts of the adult parasite contained a variety of proteinases with differing specificity for the natural potential protein substrates tested and these enzymes had differing pH optima (Fig. 3). Haemoglobin degradation was generally observed at more acidic pH than that determined for either fibrinogen or albumin. High molecular weight (> 84 kDa) proteolysis of fibrinogen and albumin was detected at alkaline pH. A dominant peak of fibrinogenolysis was noted in the molecular size range 48·5 to 58·5 kDa. Albumin degradation was detected only in reaction mixtures containing proteinases with mol-

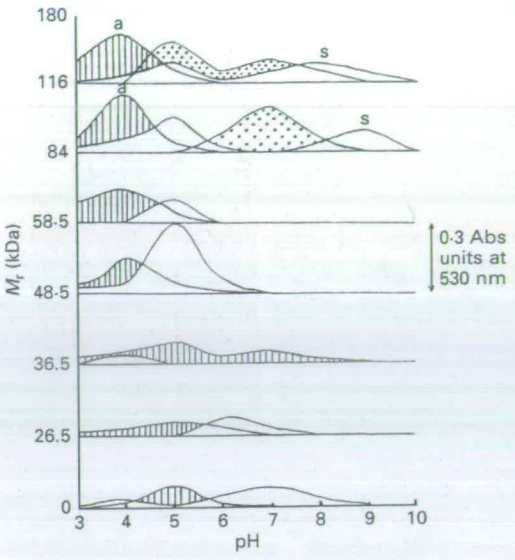

Fig. 3. pH optima and inhibitor sensitivity of gel-eluted proteinases. The ability of proteinases, eluted from polyacrylamide gels following fractionation on the basis of molecular size, to hydrolyse the protein substrates fibrinogen (□), albumin (▨) and haemoglobin (▥) was assessed over a broad pH range. All activities were sensitive to the cysteine proteinase inhibitor, E64, except for those indicated. a, aspartate; s, serine. The Y-axis shows the size range of the eluted proteins in kDa.

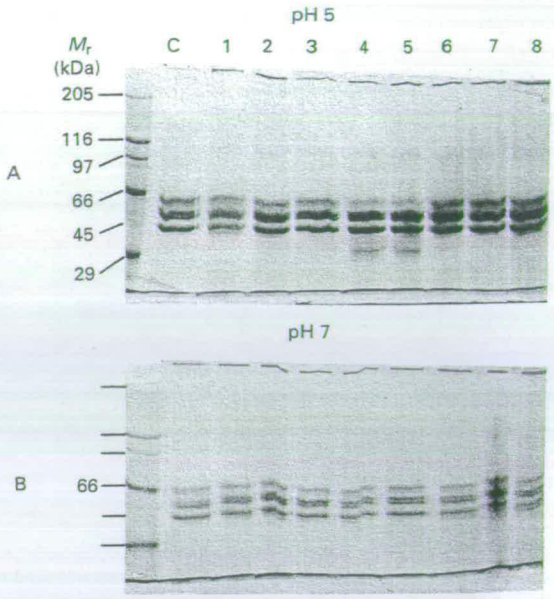

Fig. 4. Degradation of bovine fibrinogen by gel-eluted proteinases. Degradation products were fractionated using SDS–PAGE and were most evident when fibrinogen was incubated with eluted proteinases in the size range 48·5 to 84 kDa. Only the upper α-chain appeared to be degraded (compare to control C).

ecular weights between 84 and 180 kDa. Material above 205 kDa was not evaluated in this assay system because of the poor gel resolution of proteins in this region.

Most of the eluted proteinase activities were sensitive to the cysteine proteinase inhibitor, E64, although serine and aspartate proteinases were also evident in the high molecular weight components with specificity for both fibrinogen and haemoglobin. Albumin degradation was partially inhibited by EDTA.

Fibrinogen degradation studies

Fibrinogenolysis by proteins present in adult *H. contortus*, which had been fractionated by SDS–PAGE and subsequently eluted from the gel on the basis of molecular size, was evaluated by further gel electrophoresis.

Following fractionation of reaction mixtures by SDS–PAGE under reducing conditions degradation products were visualized by Coomassie Blue staining (Fig. 4). Under these conditions undegraded fibrinogen (Fig. 4A, Track C) is resolved into three peptides which, in descending order of size, represent the α, β and γ peptide chains. Fibrinogenolysis was observed by eluted proteins in the range 36·5 to 84 kDa (Fig. 4A, Tracks 3–5) at pH 5 with maximal activity between 58 and 84 kDa (Fig. 4A, Track 5). Degradation appeared to be restricted to the α-chain peptide. No degradation was detected at pH 7 using these methods (Fig. 4B).

Serum inhibition experiments

In the first experiment gel-eluted proteinases were pre-incubated with sera from lambs of defined immune status (see Materials and Methods section). Inhibition (Table 3) was calculated as the percentage enzyme activity remaining in comparison to the pre-immunization lamb sera, these lambs having been maintained worm-free before use. Assays were performed at the optimum pH previously estimated for gel-eluted proteinases (Fig. 3) with fibrinogen as substrate. Apparently preferential inhibition of *H. contortus* proteinases was evident with both types of immune sera tested. Enzyme activity in the size ranges 26·5 to 36·5 kDa and 48·5 to 58·5 kDa was particularly susceptible to inhibition by both immune sera. Of interest was the marked inhibition of activity between 58·5 and 84 kDa by the hyper-immune serum but not by the gut antigen antiserum. In contrast, activity in the range 0 to 26·5 kDa was inhibited only by the gut antigen antiserum. Activity above 116 kDa was not significantly inhibited by either immune serum type.

Sera from lambs immunized with a gut antigen preparation from adult *H. contortus* inhibited pro-

Table 3. Inhibition of *Haemonchus contortus* proteinases by sera from lambs immune to challenge infection with the homologous parasite

(Proteinases in extracts of *H. contortus* were eluted from polyacrylamide gels on the basis of molecular size. Eluted proteinases were incubated with sera from lambs which had been repeatedly infected with the parasite (hyper-immune) or had been immunized with a gut antigen extract (Gut Ag). Following this incubation proteolytic activity remaining was monitored using fibrinogen as substrate. All data were corrected using pre-immunization serum from the Gut Ag lamb as blank. Data presented are from 2 separate experiments.)

Proteinase size range (kDa) and assay (pH)	Activity remaining (%)	
	Gut Ag	Hyperimmune
0–26·5 (5)	29, 35	62, 76
26·5–36·5 (6)	0, 11	35, 53
36·5–48·5 (5)	82, 75	31, 47
48·5–58·5 (5)	26, 19	0, 9
58·5–84·0 (5)	100, 96	30, 28
84·0–116 (5)	77, 82	35, 54
84·0–116 (9)	65, 88	100, 101
116–180 (5)	72, 76	110, 93
116–180 (9)	85, 74	69, 71

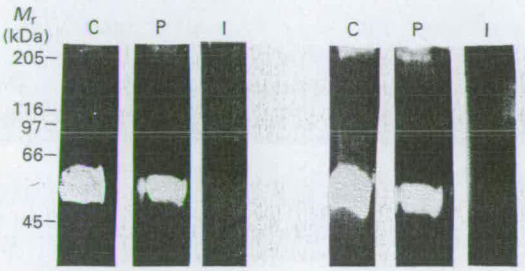

Fig. 5. Inhibition of proteinases in Triton X–100 extracts of adult *Haemonchus contortus* by immune lamb sera. Following incubation of extract proteins with either pre-(P) or post-(I) immunization sera from two separate lambs, proteolysis was only inhibited by sera harvested from immune lambs.

teolysis in gelatin-substrate gels (Fig. 5). Sera from two lambs were tested and both post-immunization sera abolished activity in the 55 kDa region while pre-immunization sera from the same lambs had no effect. The hyperimmune serum described above had no discernible effect in this assay system.

Total proteinase activity, assessed by azocasein degradation and expressed as a function of total protein (O.D. 405/mg) in extracts of adult *H. contortus*, was 2–3 times higher (21·9–37·3 O.D. 405/mg) in parasites harvested from lambs which had been immunized with gut antigen extracts and rendered refractory to challenge infection compared with activity in worms from control lambs (9·5–14·2 O.D. 405/mg). This trend was confirmed by

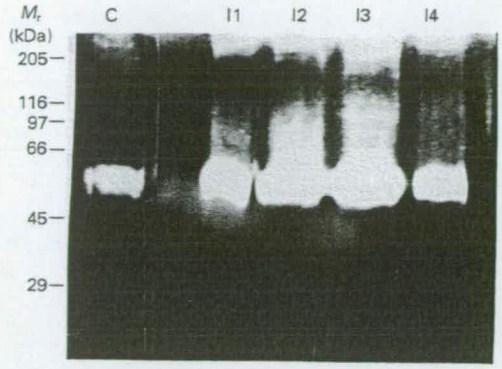

Fig. 6. Adaptive proteinase expression by adult *Haemonchus contortus* harvested from immune lambs. The figure shows the proteinase activity present in *H. contortus* harvested from control (C) lambs compared with those from immune (I1, I2, I3, I4) lambs following gelatin–substrate gel analysis.

gelatin–substrate gel analysis (Fig. 6). It was notable that expression of higher molecular weight (> 60 kDa) proteinases was more marked in parasites harvested from immune lambs than was observed in controls (compare in particular lanes I2 and I3 with lane C, Fig. 6).

DISCUSSION

The present study expands the information available on proteolytic enzymes (proteinases) present in somatic extracts of the adult *H. contortus* and evidence is provided that these enzymes were differentially recognized by sera from lambs immune to *H. contortus* infection either following repeated natural infection or by immunization with parasite extracts.

The differing pH optima observed for proteolysis of fibrinogen (pH 5) and haemoglobin (pH 4) as well as the biphasic profile observed with albumin as substrate (optima pH 5 and 7), when these substrates were degraded by Triton X–100 extracts of the adult parasite, suggested the presence of multiple proteinases, perhaps with selective specificity for the potential native substrates tested here. This impression was confirmed by gelatin–substrate gel analysis and the characterization of gel-eluted proteinases. Both analyses indicated the presence of a number of proteinases of differing molecular size, inhibitor sensitivity and substrate preference in extracts of the adult parasite and, in agreement with the general pH profiles, the majority of enzymes were active at acidic pH.

Acidic haemoglobinase activity is in accord with the cysteinyl haemoglobinase described in *Schistosoma mansoni* (Chappell & Dresden, 1986) and acidic haemoglobinases in the hookworms *Necator americanus* and *Ancylostoma caninum*

(Pritchard *et al.* 1991). It was notable that the optimal pH of fibrinogenolysis (pH 5) observed here contrasts with the pH (7) used in defining the putative 35 kDa fibrinogen-degrading proteinase previously described in extracts of adult *H. contortus* (Cox *et al.* 1990*b*). In addition, when fibrinogenolysis by gel-eluted proteinases was assessed at pH 5 or 7 using SDS–PAGE degradation products were evident only at pH 5 and were associated with proteinases eluted in the size range 48·5 to 84 kDa. These discrepancies could be due to methodological differences or, alternatively, the difference could be due to strain divergence as evidenced by the inability to amplify any of the gene sequences encoding cysteine proteinases from adult *H. contortus* described by Pratt *et al.* (1992) from the strain of the parasite used at Moredun (D. L. Redmond and D. P. Knox, manuscript in preparation). In particular, attempts to isolate the published AC1 sequence (Cox *et al.* 1990*a*) by polymerase chain reaction amplification (PCR) using specific primers based on that sequence have been unsuccessful. Thus, although a PCR amplification product of the correct predicted size was obtained its sequence showed only 70% homology with AC1. A third possibility for differences in expressed enzymes might be parasite adaptive responses to the host environment as discussed below.

Because the pH of the ingested host blood would, presumably, be essentially neutral it appears, at first sight, anomalous that the dominant proteinases present in adult *Haemonchus*, with the capability to degrade host blood proteins, should have acidic pH optima. However, between blood meals the adult parasite moves freely on the abomasal mucosal surface and, therefore, is in a highly acidic environment. Because of this the gut lumen of the parasite might also be acidic and, hence, the proteinases involved in digesting the blood meal would be required to be active at acidic pH. Moreover, the gut luminal pH of the blood-feeding trematode *Schistosoma mansoni* is reportedly acidic (Senft, 1976).

The present results indicate that proteolysis at acidic pH values was primarily attributable to the action of cysteine proteinases, proteolysis at this pH being almost totally inhibited by the class-specific cysteine proteinase inhibitor, E64. With the limited panel of synthetic peptide substrates used it is not possible to define, precisely, the type of cysteine proteinase(s) responsible for this activity although a clear preference for peptides featuring basic amino acids was indicated. This substrate preference would be in accord with cathepsin B-type cysteine proteinase action (Barrett, 1977) and is in agreement with recent DNA sequence analyses which showed that adult *H. contortus* express a number of cysteine proteinases with homology to cathepsin B (Cox *et al.* 1990*a*; Pratt *et al.* 1990, 1992). Elastinolytic activity, demonstrated by the hydrolysis of insoluble elastin–orcein and the low molecular weight peptide substrate CBZ-L-Ala-4NPE, observed at alkaline pH, is in agreement with the pH optima reported for purified elastases (e.g. Barrett, 1977; Hotez *et al.* 1985). Also, proteolysis at this pH was sensitive to PmsF, an inhibitor of serine proteinases. In addition the anticoagulant properties of excretory/secretory proteins from *Ancylostoma caninum* were related to elastolytic activity capable of degrading plasminogen (Hotez & Cerami, 1983; Hotez *et al.* 1985). Previous studies provided evidence for the release of elastase activity by adult *H. contortus* during *in vitro* culture (Knox & Jones, 1990) and it is possible that the elastase activity detected in somatic extracts of the adult parasite, in part, reflects a secretory type anticoagulant.

The proteinases present in Triton X–100 extracts of the adult parasite were further defined by gelatin–substrate gel analysis. Parasites harvested from lambs, which were maintained worm-free prior to infection, exhibited a relatively simple profile in this gel system. At pH 5, proteolysis observed at 50 kDa and above 180 kDa was almost completely inhibited by E64, confirming the predominance of cysteinyl proteinases at this pH which had been indicated by the inhibitor data described above and Table 2. Moreover, proteinases active at pH 9 were resolved into a relatively high molecular weight (> 150 kDa) zone of proteolysis which was, as expected from the data in Table 2, markedly inhibited by PmsF and EDTA indicating the action of both serine and metallo-proteinases.

The characterization of parasite proteinases by a procedure involving gel fractionation, elution and the subsequent detection of proteolysis using the ninhydrin technique, although more tedious than substrate-gel analysis, was very sensitive and enabled the rapid definition of individual proteinases within a complex mixture. Here this approach was used to examine the specificity of *Haemonchus* proteinases for potential natural blood protein substrates over a broad pH range. This analysis revealed a greater variety of proteinases active at acidic pH than had been indicated by substrate-gel analyses. Haemoglobin degradation occurred, almost exclusively, at acidic pH and was primarily catalysed by cysteine proteinases, observations which are consistent with current hypotheses concerning haemoglobin digestion in schistosomes (McKerrow & Doenhoff, 1988). Moreover, the sensitivity of this technique provided more information on parasite proteinases targeted by the host immune response than was attainable using substrate gel analysis.

Recent reports have shown that the gut of blood-feeding parasites can be a source of potential protective antigens (Smith & Munn, 1990; Jasmer & McGuire, 1991). Lambs immunized with gut antigen extracts, which included a proteinase component

(D. P. Knox, unpublished observations), of adult *H. contortus* were demonstrably refractory (90%) to challenge infection with the homologous parasite (Smith, 1992). In view of the protective response described in lambs immunized with anticoagulant extracts prepared from adult *H. contortus* (Cox *et al.* 1990*b*) and the previous observations that *Nippostrongylus brasiliensis* harvested from primed rats show modified acetylcholinesterase (Jones & Ogilvie, 1972) and superoxide dismutase (Knox & Jones, 1992) expression in response to host immune attack, it was pertinent to compare the proteinase content of the parasites harvested from gut antigen-immunized lambs with that of parasites harvested from naive lambs. The data indicated that the parasites retrieved from immunized lambs had modified proteinase expression, both in terms of total enzyme content and expression of differing molecular forms. These observations may reflect heterogeneity in the normally expressed proteinases within the parasite population. Alternatively, they might indicate that the parasite is able to modify proteinase gene expression in response to alterations in the local gut environment generated by the host immune response or to specific, possibly antibody-mediated, immune inhibition of normally expressed proteinases. In a recent experiment (Suttle *et al.* 1992) dietary molybdenum supplementation enhanced rejection of *H. contortus* from lambs. Proteinase activity, as judged by azocasein degradation, in and secreted by adult parasites retrieved from molybdenum-supplemented lambs was increased in comparison to the controls. The mechanism(s) underlying these changes were unclear but they, and the observations described above, indicate that modifications in proteinase expression may have a central role to play in parasite survival of host immune attack. Pratt *et al.* (1992) suggested that mRNA transcripts of some members of the cysteine proteinase gene family encoding fibrinogenase activity might be present in the adult parasite but not translated. The trigger for translation may be provided by elements of the host immune response.

The variable proteinase profiles observed in adult *Haemonchus* might, in part, be a survival response by the parasite to host immune, possibly antibody-mediated, inhibition of normally expressed activities. Sera from lambs which had been immunized with gut-antigen extracts completely inhibited the prominent 55 kDa enzyme active at pH 5 as judged by gelatin–substrate gel analysis, while the effect was less marked with sera from hyperimmune lambs (not shown). Using the more sensitive gel elution method there was apparently selective inhibition of extracted proteinases by both types of sera tested. However, there were differences in inhibition profiles obtained and these could reflect the differing proteinases accessible to host immune attack during the course of natural infection compared to those contained in the gut antigen preparation. For example, activity in the size range 58·5 to 84 kDa was markedly inhibited by hyperimmune sera yet relatively unaffected by sera from lambs immunized with gut antigen extracts. It is possible that this activity is secreted by the parasite, or in some way exposed on the parasite surface, during the course of a natural infection but is not present in the parasite gut.

The observed differential inhibition of *Haemonchus* proteinases may have been mediated by antibody but the presence of proteinase inhibitors in the sera used cannot be discounted. However, fibrinogen-degrading proteinases in the size range (up to 116 kDa) susceptible to inhibition were all apparently members of the cysteine proteinase class. If the inhibition observed was due to non-specific circulatory proteinase inhibitors such as alpha-2-macroglobulin then a more general non-specific inhibition of parasite proteinases would have been anticipated. Antibody-mediated inhibition of parasite proteinases has previously been described in the rabbit-*Ascaris suum* model (Knox & Kennedy, 1988) and, of more relevance to natural infections, calves immune to challenge infection with the lung worm, *Dictyocaulus viviparus*, either from natural infection or following vaccination with the commercial vaccine, Dictol (Britton *et al.* 1992).

In conclusion, the present report expands the information available to date on the proteinases expressed by the blood-feeding nematode, *Haemonchus contortus*. Using a combination of techniques for characterizing these enzymes it has been possible to provide some insight into the variety of these expressed proteinases and to present evidence for their immune inhibition. This type of approach enables the rapid definition of parasite enzymes of potential importance at the host/parasite interface, information which can enable the design of specific purification techniques and the direct isolation of the genes encoding these enzymes, for example by PCR amplification (Sakanari *et al.* 1989). Finally, the suggestion that parasitic nematodes might modify proteinase expression in response to host immune attack has considerable potential for the development of helminth control strategies based on immunologically mediated inhibition of parasite enzyme systems.

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Chapter 3: Molecular cloning and characterization of gut-derived cysteine proteinases associated with a host protective extract from *Haemonchus contortus*.

Skuce PJ, Redmond DL, Liddell S, Stewart EM, Newlands GFJ, Smith WD and Knox DP. 1999. *Parasitology*, **119**: 405-412.

This paper won the 1998/1999 Urquhart prize, awarded by the British Association of Veterinary Parasitology, (see Appendix 1).

A vaccine against the haematophagous cattle tick *B. microplus* has been developed successfully using an antigen which is not normally seen by the host during the course of a natural infection and which is therefore termed a "hidden" or covert antigen (Willadsen et al, 1995). The success of this approach is reliant on:

1. the generation of a specific, systemic host antibody response and
2. exposure of the parasite target antigen to host antibody ingested with the blood meal.

Although they may be exposed to host blood or lymph, hidden antigens are only accessible to natural host immune responses if the worm becomes disrupted (Munn, 1997). Therefore, the value of hidden antigens in terms of vaccine development, is that there is no selection pressure for their evolution to facilitate parasite evasion of the host immune response.

Detergent extracts of dissected-out guts of adult *H. contortus* were capable of inducing a host protective response against homologous challenge in both goats (Jasmer and McGuire, 1991) and sheep (Smith, 1993), demonstrating the applicability of the hidden antigen approach to the development of a *H. contortus* vaccine. Furthermore, lectin and affinity chromatography were used to purify a 110 kDa integral membrane protein, termed H11, from gut extracts of adult *H. contortus* (Munn and Smith, 1990; Smith et al 1993c), subsequently identified as a microsomal aminopeptidase (Smith et al, 1997). This hidden antigen afforded high levels of protection (~92% reduction in FEC and up to 89% reduction in worm burdens) against homologous challenge in sheep vaccination trials, protection correlating both with host serum antibody titre (Munn et al 1993a & 1993b) and the degree of

inhibition of H11 aminopeptidase activity by immunoglobulin from vaccinated lambs (Munn et al, 1997).

The work presented in Chapter 2 showed that cysteine proteinases predominate in somatic extracts of adult *H. contortus* and that these enzymes could degrade fibrinogen and haemoglobin, indicating a possible role in digestion of the host blood meal. Sera from lambs immunised with a gut membrane antigen extract from adult *H. contortus* (Smith, 1993), and protected against a single homologous challenge infection, were shown to inhibit cysteine proteinase activity as determined by gelatin-substrate gel analysis. This suggests that antibody-mediated inhibition of cysteine proteinases expressed in the parasite gut may contribute to the protection stimulated by vaccination with *H. contortus* gut membrane extracts.

Passage of *H. contortus* membrane-bound protein extracts over a thiol sepharose affinity chromatography column resulted in a 24 fold enrichment of cysteine proteinase activity, this fraction being termed TSBP. TSBP provided significant protection (70% reduction in FEC, 50% reduction in worm burden) against *H. contortus* challenge infection in sheep vaccination trials (Knox et al, 1995 & 1999).

The aim of this study was to clone and characterise the cysteine proteinases associated with the host-protective TSBP extract of adult *H. contortus*.

Immunoscreening of a cDNA library with pooled sera from sheep immunised with TSBP and which were refractory to homologous challenge, identified several immunopositive clones. PCR amplification of these clones, nucleotide sequence analysis and GenBank database searching revealed that three clones represented full-length cathepsin B-like homologues, designated hmcp1, 4 and 6. Further analysis of the gene sequences showed that they would be expected to encode soluble enzymes, with no evidence of a membrane anchor or regions of significant hydrophobicity.

Antibodies eluted from lawns of plaque purified clones of hmcp1, 4 and 6 were used to probe cryostat sections of adult *H. contortus* and, in all cases, showed intense immunolocalisation to the microvillar surface of the parasite intestine, demonstrating their accessibility to antibody ingested with the host blood meal. In

addition, the intestines of worms harvested from TSBP-vaccinated animals, but not controls, were coated in sheep immunoglobulin.

The developmental expression of the *H. contortus* cysteine proteinase mRNA was investigated by Reverse Transcriptase-PCR (RT-PCR) and provided further evidence for their potential role in parasite feeding with all three enzymes being expressed only in the blood feeding L4 and adult stages.

In order to identify the predominant cysteine proteinases expressed in the Moredun strain of *H. contortus*, a generic PCR approach was adopted using primers directed to the consensus sequences flanking the active site cysteine (aa 29), histidine (aa 199) and asparagine (aa 219) residues. This resulted in the reamplification of hmcp1 and 4 sequences, but also amplification of three novel, partial cDNA sequences designated hmcp2, 3 and 5. High stringency PCR, using a freeze-thaw lysate of the cDNA library as template and primers specific to the vector arms, was employed to extend these fragments to full-length sequences. Amino acid similarities between the six hmcp cathepsin B-like proteinases ranged from ~44%–71% identity. Although the predicted amino acid sequences of the cysteine proteinases isolated from the Moredun strain could be readily aligned with members of the AC gene family identified in the USDA BPL1 strain of *H. contortus* (Pratt et al, 1990), no members or homologues of the AC gene family of cysteine proteinases were specifically identified. Furthermore, a specific attempt to PCR-amplify the AC-1 gene proved unsuccessful. These results are in keeping with the cysteine proteinase profile of extracts of the Moredun strain of adult *H. contortus* described in Chapter 2, which showed a different pH optimum and molecular size to that previously described for the USDA BPL1 strain of *H. contortus*.

Southern blots of *H. contortus* genomic DNA using hmcp-encoding DNA fragments as probes were indicative of multi-copy genes with the exception of hmcp4 which produced a simple banding profile more consistent with that expected for a single copy gene. Northern blot analysis showed that, in all cases, the cysteine proteinase genes were encoded by a 1.3 kb transcript.

Three developmentally regulated cysteine proteinases, associated with a host-protective *H. contortus* integral gut-membrane fraction, TSBP, and postulated to play a role in degradation of the host blood meal, have been identified and shown to

localise specifically to the parasite gut. These proteinases represent potential candidate vaccine antigens. Three further homologues were isolated using a generic PCR approach. However, all six Moredun *H. contortus* cysteine proteinase homologues were distinct from those previously reported in the USDA BPL1 strain of the parasite highlighting stain differences and potential implications for the development of a generic *H. contortus* vaccine.

Contribution to work

Experimental design. All steps involved in the generic PCR amplification of *H. contortus* cysteine proteinases, their cloning, sequence analysis and identification through homology database searching. Northern and Southern blot analysis and preparation of cDNA extracts used in analysis of developmental expression. Analysis and interpretation of results and their communication at relevant scientific meetings. I prepared this work for publication in 1992, but the manuscript was not submitted due its commercial sensitivity. Subsequent cDNA library immunoscreening and immunolocalisation was carried out by P Skuce, with whom I am equal co-author of this manuscript.

In addition to the data presented here, two recent sheep vaccination trials have provided further evidence that the cysteine proteinase activity is the protective component of adult *H. contortus* TSBP. In the first trial, TSBP was fractionated by anion-exchange chromatography on MonoQ. Only subfractions containing cysteine proteinase activity, as determined by gelatin-substrate gel analysis, afforded protection against homologous challenge infection (unpublished results). In a second trial, the TSBP cysteine proteinases were further purified by affinity chromatography using purified recombinant *H. contortus* cystatin (Newlands et al, 2001), a potent inhibitor of cysteine proteinases, coupled to cyanogen bromide-activated sepharose. Although representing only 1-1.5% of total TSBP proteins, the cystatin-binding fraction contained 100% of the cysteine proteinase activity. When used in a sheep vaccination trial, immunisation with <3μg of this cystatin-binding fraction conferred a degree of protection against challenge infection similar to that attained with unfractionated TSBP (unpublished results).

Molecular cloning and characterization of gut-derived cysteine proteinases associated with a host protective extract from *Haemonchus contortus*

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SUMMARY

Cysteine proteinases have been implicated in the protection conferred by vaccination with detergent-soluble extracts of *Haemonchus contortus*. In the present study, antisera from sheep refractory to *Haemonchus* challenge following vaccination with a 'proteinase-enriched' *Haemonchus* gut membrane extract, were employed to screen a cDNA expression library of the adult parasite. This resulted in the isolation of 3 cDNAs (designated hmcp1, 4 and 6) encoding cathepsin B-like cysteine proteinases. Immunocytochemical studies specifically localized the products of these genes to the microvillar surface of the parasite's gut and RT–PCR experiments revealed that these were developmentally regulated, being expressed exclusively during the blood-feeding parasitic stages. In addition, a generic PCR approach was adopted in order to identify the predominant cysteine proteinases in a UK strain of *Haemonchus*. A panel of 5 cDNAs, including hmcp1 and 4, was amplified in this way. Genomic Southern blot analysis indicated that some of these enzymes were encoded by single-copy genes, whereas others were encoded by multi-copy genes. Subsequent sequence analysis revealed that the proteases identified in this study were distinct from those previously reported in USA strains of the parasite.

Key words: *Haemonchus contortus*, cysteine proteinase, cathepsin B, cDNA cloning, polymerase chain reaction.

INTRODUCTION

Haemonchus contortus is an economically important and highly pathogenic nematode parasite of small ruminants, especially sheep.† The symptoms of ovine haemonchosis are a direct result of the blood-feeding habit of the adult parasite. At present, *Haemonchus* infections are controlled by the strategic use of anthelmintic drugs, however, the emergence of resistant strains of the parasite and increasing concern about drug residues in the food chain and the environment have highlighted the need for alternative control strategies (Coles, 1998; Newton & Munn, 1999). Much effort has been directed recently towards the development of a vaccine against *Haemonchus* and a number of promising candidate vaccine antigens have been identified (Smith, 1998). Significant amongst these are the so-called 'hidden' antigens, such as H11 (Smith *et al.* 1997) and H-gal-GP (Smith, Smith & Murray, 1994). These are typically located on the surface of the parasite's gut and are hidden from the immune system of the host during the course of a natural infection. However, they are accessible to host antibodies ingested by the parasite. Thus, gut antigen-based vaccination takes advantage of the fact that *Haemonchus* is an obligate blood feeder.

Classically, hidden antigens have been purified from detergent-soluble integral membrane protein extracts of adult worms or dissected *Haemonchus* guts (Smith, 1993). Such extracts possess marked proteinase activities, for example, H11 is an amino-peptidase (Smith *et al.* 1997) and H-gal-GP exhibits both aspartyl- and metalloproteinase activities (Longbottom *et al.* 1997; Redmond *et al.* 1997). Possibly the most intense proteinase activity associated with crude *Haemonchus* gut membrane extracts is attributable to cysteine proteinases (Knox, Redmond & Jones, 1993). In a previous study in our laboratory, a membrane-bound thiol-binding protein fraction of *H. contortus*, enriched for cysteine proteinase activity (designated S3 TSBP), proved to be highly host protective, capable of reducing faecal egg output by up to 95% and adult worm burdens by up to 50% in a number of vaccination trials in sheep (Knox *et al.* 1995; Knox, Smith & Smith, 1999). As such, the cysteine proteinases associated with this membrane-bound fraction of *H. contortus* represent candidate vaccine antigens.

In the present study, we have employed antisera from lambs protected against *Haemonchus* challenge following vaccination with S3 TSBP material to

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† Nucleotide sequence data reported in this paper are available in the GenBank data base under the accession numbers Z69342, Z69343, Z69344, Z69345, Z69346 and Z81327.

screen a cDNA expression library of the adult parasite. The cysteine proteinases identified in this way were subsequently characterized in terms of their localization within the parasite, the temporal pattern of their expression and their possible role in protection against *Haemonchus*. A number of cysteine proteinase genes have previously been isolated from USA strains of *H. contortus* using a combination of library screening and PCR. These comprise the AC-family [AC1-5] (Cox *et al.* 1990; Pratt *et al.* 1990) and the recently discovered gcp7 (Rehman & Jasmer, 1998). We have, therefore, also employed a generic PCR approach to amplify the predominant cysteine proteinases in UK *Haemonchus* (Moredun strain) to aid comparison with those previously identified in a USA strain of the parasite.

MATERIALS AND METHODS

cDNA library screening

The construction of an adult (28 day) *H. contortus* cDNA library in λgt11 has been described elsewhere (Longbottom *et al.* 1997; Redmond *et al.* 1997). In addition, an 11 day *Haemonchus* cDNA library was constructed in UniZAP-XR according to the manufacturer's instructions (Stratagene). The primary titre of the 11 day library was *ca* 1×10^6 plaque-forming units (pfu)/ml with $> 98\%$ recombinants. An aliquot (*ca* 50 000 pfu) of the unamplified λgt11 library in the first instance was screened with pooled sera from lambs which had been protected from *Haemonchus* challenge following immunization with S3 TSBP material. Immunopositive clones were re-screened to plaque purity. Insert DNA was PCR amplified using primers specific for the vector arms and cloned into the plasmid pCRII (TA Cloning System, Invitrogen) for sequencing. Plasmid DNA was isolated by alkaline lysis as outlined by Maniatis, Fritsch & Sambrook (1982), and sequenced using the Pharmacia T7 Sequenase sequencing kit. Nucleotide and deduced amino acid sequences were analysed and compared to existing sequences in the GenBank and SwissProt databases using the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package version 8.0-UNIX, 1994.

Immunolocalization studies

Cryostat sections of adult *H. contortus* were prepared as previously described (Smith & Smith, 1993) and probed with (undiluted) monospecific antibody fractions affinity purified on lawns of plaque-pure cysteine proteinase-encoding immunopositive clones, essentially as described by Cox *et al.* (1990). Antibodies were eluted from sera taken from lambs that had been vaccinated with S3 TSBP and that were demonstrably protected against *Haemonchus* challenge. Antibody fractions eluted from the same source sera on non-recombinants were used as controls, as were pre-immunization sera. Fluorescein-conjugated anti-sheep/goat IgG (Scottish Antibody Production Unit, SAPU) was used as secondary antibody.

Developmental expression of H. contortus *cysteine proteinases*

The developmental expression of hmcp1, 4 and 6 mRNA was evaluated by reverse transcriptase (RT)–PCR. Single stranded cDNA (sscDNA) was prepared from representative life-cycle stages using Superscript II reverse transcriptase (Gibco–BRL) and the oligo(dT) primer supplied. Gene-specific primers were designed on the basis of a GCG PILEUP alignment of the full-length sequences. Fifty ng sscDNA from the respective life-cycle stages was used as template in PCR under the following conditions. Initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55–60 °C for 2 min and 72 °C for 3 min, followed by a final extension at 72 °C for 7 min. A reaction containing 100 ng *H. contortus* genomic DNA as template was included as a negative control to verify that PCR products were not the result of contamination of sscDNA preparations with genomic DNA. Plasmid DNA, prepared from the respective cysteine proteinase-encoding clones, was used as a positive control. The integrity of the respective transcripts was evaluated by PCR amplification of the constitutively expressed *H. contortus* extracellular superoxide dismutase (SODe) gene (Liddell & Knox, 1997). In order to confirm identity, amplified PCR products were separated on 0·8% (w/v) agarose gels, alkali-blotted onto Hybond N^+ nylon membrane (Amersham) and probed with the relevant cysteine proteinase, which had been DIG-labelled by random priming using the Boehringer–Mannheim DIG Labelling Kit, under conditions of high stringency (overnight at 42 °C in DIG Easy Hyb buffer and washed to 0·1 × SSC/0·1% SDS) [1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate, pH 7·0].

Generic PCR amplification of H. contortus *cysteine proteinases*

In order to identify the predominant cysteine proteinases in a UK strain of *Haemonchus*, a panel of degenerate PCR primers [508G (5′ sense) 5′-ACAGAATTCCAGGGICAGTGCGGITCITG GTGG-3′, 303H (3′ antisense) 5′-TTAAAGCTT CCAIGAGTTCTTIACGATCCAGTA-3′ and 509G (3′ antisense) 5′-ACAAAGCTTGTAICCIC CGTTGCAICCCTC-3′, respectively], were de-

signed to target the consensus sequences flanking the active site residues, namely Cys-29, His-199 and Asn-219, on the basis of previously published sequences (Cox *et al.* 1990; Eakin *et al.* 1990). An additional pair of primers [550J (3′ antisense) 5′-TGTTCCACGGCATTCCCCGTA-3′ and 699N (5′sense) 5′-ATGAAATACTTGGTICTIGCICT ITGC-3′] were designed to target AC-1, part of the protective 'fibrinogenase' complex identified in a USA strain of *H. contortus* (Cox *et al.* 1990). Finally, a poly(T) primer (5′-GAGTCGAGTCGACATC GATTTTTTTTTTTTTTTTTT-3′) was designed to exploit the polyadenylation of eukaryotic mRNA and allow amplification of the extreme 3′ end of gene sequences. In order to reduce degeneracy, inosine was incorporated at positions where any of the 4 bases could encode a given amino acid, and restriction sites were introduced at the 5′ end of primers (except 550J) to facilitate subsequent cloning of PCR products.

Single-stranded cDNA (sscDNA) was prepared as template from adult *H. contortus* using the Amersham cDNA synthesis Plus kit with an oligo(dT) primer according to the manufacturer's instructions. Fifty ng sscDNA was used as template in each reaction under the following conditions. Initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 25 °C for 2 min and extension at 72 °C for 3 min with a final extension at 72 °C for 7 min. Products of the predicted size were excised from 1 % agarose gels and purified using a GeneClean kit as instructed by the manufacturer (Bio 101, Inc.). Following digestion with *Eco*RI and *Hin*dIII/*Xho*I, fragments were cloned into the pBluescript vector (Stratagene Ltd) and sequenced as described previously. High stringency PCR was employed in order to extend the resultant fragments to full-length sequences. Briefly, an aliquot (100 μl at *ca* $4{\cdot}5 \times 10^9$ pfu/ml) of the 1 × amplified 11 day λZAP library was diluted 1:1 with distilled H_2O and subjected to a series of freeze–thaw cycles to lyse the phage particles. The lysate was then used as template in PCR using primers specific for the vector arms (T3, T7, m-13 Forward and m-13 Reverse) in combination with gene-specific primers based on the sequences obtained above. After further sequence analysis, full-length coding sequences were isolated using gene-specific 5′ and 3′ primer combinations under conditions of high stringency (primer annealing at 55–60 °C). Identity was confirmed by subsequent amplification and sequence analysis of full-length cDNAs.

Southern and Northern blot analysis

Nucleic acids were extracted from adult *H. contortus* exactly as described previously (Longbottom *et al.* 1997; Redmond *et al.* 1997). For Southern blotting, genomic DNA (2 μg) was digested with either *Eco*RI, *Hin*dIII or *Hae*III for 5 h at 37 °C and the digestion products separated on a 0·8 % agarose gel. DNA was blotted onto Hybond N nylon membrane (Amersham) using standard conditions (Maniatis *et al.* 1982). Hybridizations were performed at 42 °C in 2 × SSC, 0·5 % (w/v) SDS, 5 × Denhardt's solution [5 ×: 1 % (w/v) Ficoll, 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) BSA, 0·1 mg/ml salmon sperm DNA, 50 % (v/v) formamide] using the above-mentioned PCR products, which had been labelled with [α-^{32}P]dATP by random priming, as probes. Membranes were washed in 1 × SSC/0·1 % (w/v)SDS for 10 min at room temperature with 1 change of buffer, followed by 2 × 15 min washes in 0·1 × SSC/0·1 % (w/v) SDS at 68 °C. Membranes were autoradiographed for 48–72 h at −70 °C. For Northern blotting, adult worm total RNA (4 μg) or mRNA (2 μg) were fractionated on a 1 % (w/v) denaturing formaldehyde gel as described by Maniatis *et al.* (1982), and blotted onto Hybond N nylon membrane (Amersham) according to the manufacturer's recommendations. Hybridizations were carried out as described above.

RESULTS

cDNA library screening and characterization of clones

Three weakly immunopositive clones were isolated by screening the λgt11 library with sera from lambs protected following vaccination with S3 TSBP material. Nucleotide sequencing, combined with a GCG FastA search of the GenBank database, revealed that all 3 clones (designated hmcp1, 4 and 6) represented full-length cathepsin B-like cysteine proteinase homologues. All possessed potential stop codons and complete 3′ ends including putative polyadenylation signals (AATAAA) and *ca* 18–20 bp of the poly(A) tail. Table 1 displays the homologies at the amino acid level for the full-length versions of hmcpl, 4 and 6 identified by immunoscreening, together with hmcp2, 3 and 5 subsequently identified by generic PCR, AC1-5 and gcp7 from a USA strain of *Haemonchus*, and human cathepsin B.

Immunolocalization

Cysteine proteinase-specific antibody fractions were affinity purified from sera taken from S3 TSBP vaccinates on lawns of λ clones (hmcp 1, 4 and 6) and used to probe cryostat sections of adult *H. contortus*. Representative sections are shown in Fig. 1. In all cases, intense immunofluorescence was only evident along the microvillar surface of the parasite's gut (Fig. 1A), with no evidence of staining in any other tissues. The cytoplasm of the parasite's gut displayed

Table 1. Amino acid homologies between *Haemonchus contortus* cysteine proteinases (hmcp1–6, ac1–5, gcp7) and human cathepsin B

	Protein comparison (% identity/ % similarity)											
	hmcp1	hmcp2	hmcp3	hmcp4	hmcp5	hmcp6	AC-1	AC-2	AC-3	AC-4	AC-5	gcp7	Humcp
hmcp1	—	70·8/82·1	47·5/63·3	48·8/63·3	47·2/60·1	46·7/60·1	47·7/68·7	47·4/62·3	47·0/60·6	48·6/63·2	44·7/58·2	53·7/68·8	41·3/60·8
hmcp2		—	46·7/62·3	47·2/63·4	45·8/59·4	44·3/59·8	44·7/59·5	45·2/59·7	45·8/63·0	47·1/64·7	45·6/59·9	51·6/67·8	43·4/59·8
hmcp3			—	69·1/80·4	64·2/76·8	62·3/77·2	55·8/70·1	55·8/70·7	60·0/74·3	53·3/70·3	55·9/71·4	46·7/61·2	41·1/60·9
hmcp4				—	67·9/80·9	61·6/74·6	58·4/71·5	58·5/71·5	60·6/75·2	60·0/74·0	58·3/71·3	45·7/60·5	49·5/65·1
hmcp5					—	56·7/73·6	59·2/70·8	58·9/70·2	58·2/71·9	56·3/69·7	55·7/71·2	44·1/59·4	43·4/62·6
hmcp6						—	53·6/67·5	53·6/67·6	54·6/66·6	50·6/65·0	53·6/68·5	39·8/53·7	41·0/60·5

an element of autofluorescence. No immunostaining was evident in sections probed with control sera (Fig. 1B).

Developmental expression of H. contortus *cysteine proteinases*

The RT–PCR profiles observed for hmcp1, 4 and 6 were identical and representative results are shown in Fig. 2. Specific PCR products, as judged by Southern blotting, were evident exclusively in L4, 11 day, 22 day and 28 day worms. PCR product intensities appeared to be relatively constant throughout. The integrity of the respective sscDNA pools was demonstrated by the successful amplification of the *H. contortus* SODe transcript in all life-cycle stages, essentially as described by Liddell & Knox (1997).

Generic PCR amplification of H. contortus *cysteine proteinases*

A series of 5 PCR products (designated hmcp1–5), ranging in size from 114 bp to 742 bp, were generated from adult *H. contortus* sscDNA using different combinations of 5′ and 3′ consensus cysteine proteinase primers and low stringency primer annealing (25 °C). Two of these cysteine proteinases, namely hmcp1 and 4, had previously been identified by immunoscreening. PCR performed at high stringency was successful in amplifying full-length versions of each cDNA. By analysis of their open reading frames, the predicted amino acid sequences of the *H. contortus* cysteine proteinase-encoding cDNA fragments could be readily aligned with each other, with members of the AC-family of cysteine proteinases and gcp7 from a USA strain of *Haemonchus* (Cox *et al.* 1990; Pratt *et al.* 1990; Rehman & Jasmer, 1998) and with cathepsin B homologues from a range of other species. No members of the AC-family or gcp7 were specifically identified in this experiment.

Southern and Northern blot analysis

The cysteine proteinase-encoding cDNA fragments (hmcp1–5) described above, were used as probes on Southern blots of adult *H. contortus* genomic DNA. All produced distinct hybridization profiles, a representative example of which is shown in Fig. 3A. While hmcp2 hybridized strongly at *ca* 3Kb in *Eco*RI and *Hin*dIII digests, other bands were evident in a ladder pattern, indicative of a multi-copy gene. In contrast, the banding observed with hmcp4 was relatively simple and more consistent with that expected for a single copy gene. The size of the transcripts encoding the respective cysteine pro-

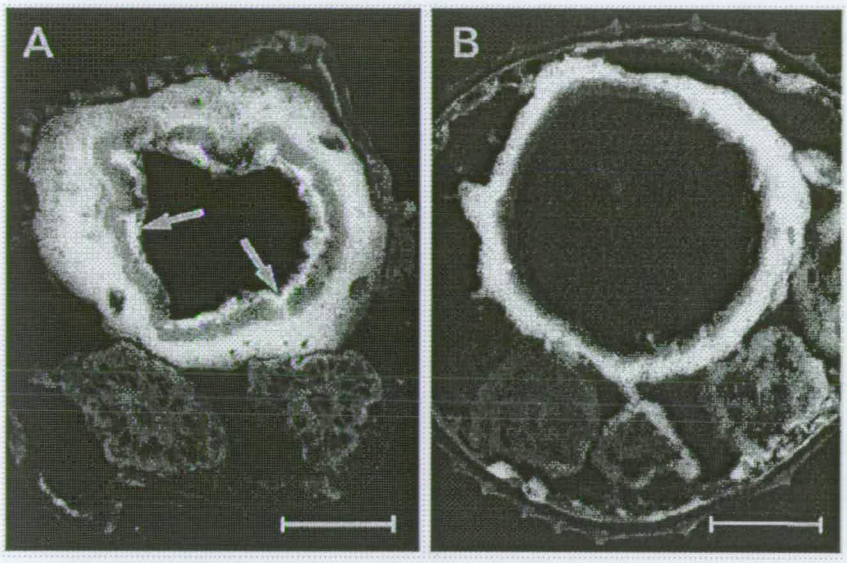

Fig. 1. (A) Cryostat section of adult *Haemonchus contortus* probed with monospecific anti-hmcp4 antibodies. Note immunofluorescence restricted to the luminal surface of the parasite's gut (arrows). (B) Control section probed with pre-immunization serum. Scale bars = 50 μm.

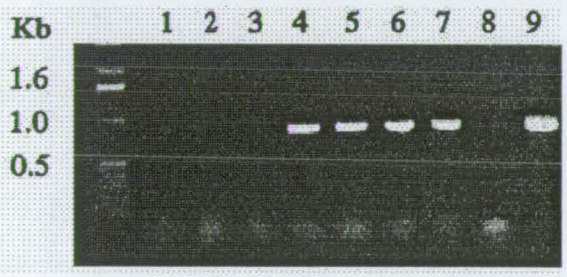

Fig. 2. Developmental expression of *Haemonchus contortus* cysteine proteinase (hmcp6). RT–PCR products obtained following amplification of sscDNA from respective life-cycle stages. PCR was carried out from eggs, L3, XL3, L4, 11, 22 and 28 day *H. contortus* and the results shown in lanes 1–7, respectively. Amplifications from genomic DNA (lane 8) and hmcp6 plasmid DNA (lane 9) are included as negative and positive controls, respectively. Molecular weight markers migrated as indicated.

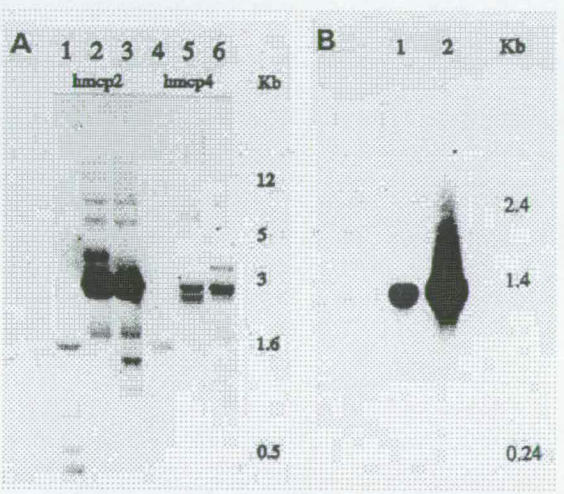

Fig. 3. (A) Representative Southern blot of *Haemonchus contortus* genomic DNA digested with *Hae*III (lanes 1 and 4), *Hin*dIII (lanes 2 and 5) and *Eco*RI (lanes 3 and 6) and probed with the respective radio-isotope labelled cysteine proteinase homologue (hmcp2 and hmcp4). Molecular weight markers migrated as indicated. (B) Representative Northern blot of total RNA (lane 1) and messenger RNA (lane 2) probed with radio-isotope labelled cysteine proteinase homologue hmcp4. Molecular weight markers migrated as indicated.

teinase homologues was assessed by Northern blot analysis. All fragments produced a hybridization signal at *ca* 1·3 kb as illustrated in Fig. 3B.

DISCUSSION

The long-term aim of our current research programme is the development of a subunit vaccine against the blood-feeding nematode, *H. contortus*. Previous research has identified a fraction (S3 TSBP), derived from a parasite gut membrane extract and enriched for cysteine proteinase activity, that is highly host protective and represents a strong candidate vaccine antigen (Knox *et al.* 1995, 1999). In the present study, we have identified 3 cysteine proteinases (hmcp1, 4 and 6) that are specifically localized to the gut of the parasite and are associated with this protective fraction having been identified through immunoscreening with antisera from S3 TSBP vaccinates.

Analysis of nucleotide and deduced amino acid sequences for hmcp1, 4 and 6, the gut-associated cysteine proteinases identified by immunoscreening,

clearly shows that these are not simply UK homologues of the AC- and gcp7 genes found in USA strains of the parasite, sharing at best *ca* 60% identity at the amino acid level. It is possible that AC- and gcp-homologues do exist in UK strains and have simply not been detected by our immunoscreen. Therefore, a generic PCR approach (Sakanari *et al.* 1989) was adopted in an effort to clone the predominant cysteine proteinases in a UK strain of *Haemonchus* and aid comparison with those described from USA strains. This approach failed to amplify any of the AC- or gcp-genes from a UK strain of *Haemonchus*. Instead, a panel of 4 cysteine proteinase homologues (designated hmcp1–4), including hmcp1 and 4 previously identified by immunoscreening, was amplified. Indeed, a concerted attempt to clone the putative UK homologue of AC-1, associated with the protective 'fibrinogenase' complex described in USA strains of *Haemonchus* (Cox *et al.* 1990), only succeeded in the amplification of a novel cathepsin B homologue, designated hmcp5. This molecule shared most homology with hmcp3 and 4 (*ca* 68%), yet was more similar to AC1-5 (*ca* 58%) than to hmcp1 or 2. In fact, hmcp1 and 2 shared, on average, only *ca* 45% identity with the rest of the hmcp series and were more similar at the protein level to gcp7 (*ca* 54%). It is unlikely that the cysteine proteinase genes from the respective UK and USA strains have diverged to such an extent that their protein products occupy distinct families. Rather, they represent distinct, yet related groups of cysteine proteinases. Indeed, phylogenetic analyses indicate that the AC- and hmcp-proteases are sufficiently related to occupy a single clade (Tort *et al.* 1998). Diversity among *Haemonchus* cysteine proteinases has been observed between USA and Kenyan isolates of the parasite and is even reflected in differences in the apparent molecular mass of the predominant enzyme activity (Karanu *et al.* 1993).

All the cysteine proteinase-encoding cDNAs isolated from *H. contortus*, both in this and previous studies (Cox *et al.* 1990; Pratt *et al.* 1990; Rehman & Jasmer, 1998), encoded homologues of the vertebrate lysosomal cysteine proteinase, cathepsin B. Residues Cys-29, His-199 and Asn-219 (mammalian numbering) form the catalytic triad and are completely conserved in hmcp1–6. They typically possess an insertion in their primary sequence that encodes a structural element known as the occluding loop, which is peculiar to cathepsin B-like cysteine proteinases (Illy *et al.* 1997). The mature forms of vertebrate cathepsin Bs possess 14 cysteine residues, which form at least 6 disulphide bridges and contribute to the 3-dimensional structure of the enzyme. All 14 cysteines are completely conserved throughout the sequences of hmcp1–6. In addition, a significant number of the proline residues in the vertebrate homologues are completely conserved in the *Haemonchus* sequences, suggesting that these molecules share a similar tertiary structure despite sharing only *ca* 40% homology at the amino acid level. This is borne out by theoretical 3-dimensional modelling of the respective homologues based on known mammalian cathepsin B crystal structures (data not shown). All 6 homologues possess at least 2 potential *N*-linked glycosylation sites (designated *N*-X-S/T), which are often in register with those of their vertebrate counterparts. The fact that the S3 TSBP proteins are capable of binding a variety of lectins, particularly concanavalin A (Knox *et al.* 1995, 1999), is evidence that these proteins may be glycosylated in their native form.

Despite the fact that all the *Haemonchus* cysteine proteinase sequences identified to date are cathepsin B-like, excretory–secretory (E–S) and gut-derived proteinases of adult *Haemonchus* have the potential to cleave synthetic cathepsin L-specific substrates (Rhoads & Fetterer, 1995; Torte *et al.* 1998). The same is true for the S3 TSBP cysteine proteinases (D. P. Knox, unpublished observations). Similarly, the cathepsin B-like proteinases secreted by the dog hookworm, *A. caninum*, cleave the cathepsin L-specific substrate, Z-Phe-Arg-AMC, in preference to the cathepsin B-specific substrate, Z-Arg-Arg-AMC (Harrop *et al.* 1995). This observation has been explained by closer examination of the residues around the active site of one such homologue (AcCP-1) (Brinkworth, Brindley & Harrop, 1996). Key residues involved in determining substrate specificity have been replaced, thereby promoting a more cathepsin L-like substrate specificity. A similar analysis of the active sites of the *Haemonchus* homologues (hmcp1–6) revealed that the same key residues were retained in some, yet replaced in others. Therefore, hmcp1–6 probably represent a panel of essentially cathepsin B-like cysteine proteinases with broad substrate specificities that cover the spectrum from cathepsin B to cathepsin L. Conversely, a cysteine proteinase isolated recently from *Toxocara canis* shows cathepsin B-like residues in the active site cleft despite being essentially cathepsin L-like in sequence (Loukas, Selzer & Maizels, 1998). This paradoxical substrate specificity may actually be a feature of nematode cysteine proteinases.

Structural analysis of the cDNAs for the gut-expressed cysteine proteinase homologues (hmcp1, 4 and 6) reveals that they would be expected to encode essentially soluble enzymes, yet strong detergent is required to solubilize the cysteine proteinases associated with S3 TSBP material (Knox *et al.* 1999). There is no evidence of a membrane anchor or any significant regions of hydrophobicity which could account for their retention in the detergent-soluble phase. It is interesting to note that the predicted molecular masses of the mature enzyme forms of the respective cysteine proteinase homologues are in the order of 28 kDa, whereas the

predominant cysteine proteinase activity demonstrable in gelatin substrate gels of *Haemonchus* membranes or S3 TSBP material is in the order of 38–52 kDa (Knox *et al.* 1993, 1995, 1999). It is possible that these molecules are complexed to a genuine integral membrane protein(s) and are thereby retained in the membrane fraction.

Immunolocalization studies have revealed that the protein products of the hmcp1, 4 and 6 genes (at least) are expressed on the luminal surface of the parasite's gut. Only 1 of the cysteine proteinases isolated thus far from a USA strain of *Haemonchus*, namely gcp7, has been definitively associated with the gut, although its precise localization has not been determined (Rehman & Jasmer, 1998). The expression of the hmcp1, 4 and 6 genes, as revealed by RT–PCR, coincides with the onset of blood-feeding during the parasitic phase of the life-cycle, that is, exclusively from 4th-stage larvae (L4) onwards. Cysteine proteinases have been implicated in the feeding of a number of parasitic species, most notably the haemoglobinase activity of the human blood fluke, *S. mansoni* (Dalton *et al.* 1995).Taken together, these observations would suggest a similar role for the cathepsin B homologues on the gut surface of *Haemonchus*, in digestion of the bloodmeal. The fact that *Haemonchus* possesses a battery of gut-derived cysteine proteinases may reflect subtle differences in their respective pH optima and/or substrate specificities. It is perhaps noteworthy that, in contrast to the membrane-bound thiol Sepharose-binding proteins of *H. contortus* (S3 TSBP), the soluble thiol Sepharose-binding proteins (designated S1/S2 TSBP) proved not to be protective in vaccination trials in sheep (Knox *et al.* 1995, 1999). This observation could imply that the cysteine proteinases associated with the parasite gut membranes and retained in the detergent-soluble phase are different from those released by the parasite.

Therefore, in conclusion, we have identified and characterized a number of developmentally regulated, gut-derived *H. contortus* cysteine proteinase homologues that have potential as candidate vaccine antigens. Moreover, using a generic PCR approach, we have identified a further 3 homologues in a UK strain of *Haemonchus*. This brings the total number of individual cysteine proteinase homologues described in *Haemonchus* thus far to 12. All 6 UK homologues were distinct from those previously reported in USA strains of the parasite. Indeed, the strain differences highlighted in the present study may have implications for the development of a generic vaccine against *Haemonchus*. However, much work still remains in order to completely characterize the protective fractions from which these cysteine proteinase homologues were isolated and to optimize expression and routes of administration if any of these molecules is to find utility as a subunit vaccine against this important blood-feeding parasite.

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Chapter 4: Further immunization and biochemical studies with a protective antigen complex from the microvillar membrane of the intestine of *Haemonchus contortus*.

Smith SK, Pettit D, Newlands GFJ, Redmond DL, Skuce PJ, Knox DP and Smith WD. 1999. *Parasite Immunology*, **21**: 187-199.

In addition to the TSBP fraction, a second host-protective, hidden integral membrane protein antigen complex, H-gal-GP, was isolated from adult parasites (Smith et al, 1994a). Following differential extraction of whole worm adult *H. contortus* homogenates to remove both water soluble and membrane associated proteins, H-gal-GP was subsequently purified from an integral membrane Triton X-100 extract by peanut lectin affinity chromatography which shows specificity for intestinal brush border glycoproteins as determined by lectin probing of *H. contortus* cryostat sections (Smith et al, 1994a). Over the course of several sheep vaccination trials, H-gal-GP proved to give solid protection against homologous challenge, reducing FEC by up to 93% and worm burdens by 72%. Passive transfer of immune serum to infected lambs resulted in a decline in FEC in recipients, indicating that immunoglobulin was the effector mechanism (Smith, 1993). Furthermore, immunostaining of periodate-treated cryostat sections of surviving worms showed the luminal surface of the parasite gut was coated in sheep immunoglobulin. It was suggested that sheep antibody may act by either neutralising functional proteins such as enzymes, or as a physical barrier to nutrient absorption (Smith, 1993). The survival of some worms may indicate that a critical amount of worm gut antibody coating is necessary to be effective.

A wide variety of techniques has been employed in attempts to further fractionate the host protective component(s) of the H-gal-GP complex (Smith et al, 1993d). However, to date, these techniques have proved unsuccessful. Taken together with the fact that individual protein components of H-gal-GP are present in consistent, reproducible proportions in different antigen preparations (unpublished results), this indicates that H-gal-GP does exists as a complex in adult worms rather than being an artefact of the purification methodology.

The aim of this paper was threefold:

1. to investigate the relationship between antibody titre and degree of protection in sheep immunised with H-gal-GP
2. to identify the constituent H-gal-GP polypeptides
3. to describe the biochemical properties of the H-gal-GP complex

Immunisation of sheep with H-gal-GP was shown not only to reduce the overall FEC and worm burden, but to be significantly more effective against female rather than male worms, as determined by the sex ratio from vaccinated compared to adjuvant control groups. The application of an enzyme-linked immunosorbent assay demonstrated a significant, positive correlation between total IgG titre and the level of protection in vaccinated animals, again suggesting that antibody is a main effector mechanism in protection with regards to immunisation with H-gal-GP.

The polypeptide composition of H-gal-GP was analysed under different electrophoretic conditions. Using native PAGE, the mass of H-gal-GP was estimated to be in the region of 1000 kDa. With non-reducing SDS-PAGE, H-gal-GP could be readily dissociated, indicating that the complex is held together electrostatically, and resolved as four main protein zones A-D (A ~230 kDa, B ~170 kDa, C ~40 kDa, D ~31 kDa). Following electroelution from gel slices, each of these protein zones resolved further into between 2 and 5 disulphide linked components under reducing SDS-PAGE conditions. N-terminal amino acid sequence data was obtained for all but 2 of these protein subunits. Results of sequence database homology searches identified several metallopeptidases (MEP) associated with zones A (MEP1, 2 and 4) and B (MEP 3), an aspartyl proteinase associated with zone C and a prominent cysteine proteinase component in zone D, which was similar to the *H. contortus* cysteine proteinase gene family described previously in Chapter 3. Western blots of H-gal-GP were probed with affinity purified antibodies raised to bacterially expressed *H. contortus* recombinant MEP1 (see Chapter 5) and pepsin (see Chapter 6). MEP1 antibodies recognised both zones A and B while pepsin antibodies recognised only zone C, confirming the N-terminal amino acid sequence results, above.

Western blots of H-gal-GP separated under reducing SDS-PAGE conditions and probed with biotinylated lectins showed all but one subunit to be glycosylated.

Evidence for N-linked glycosylation was obtained from the shift in electrophoretic mobility of some of the protein subunits associated with zones B and C following incubation with N-glycosidase F prior to electrophoresis.

Sequential extraction of water soluble (PBS), membrane associated (Tween 20) and integral membrane proteins (Triton X-100) from intestines of adult *H. contortus* showed the four protein zones A-D to be concentrated in the Triton X-100 extract, confirming that all components of the H-gal-GP complex are present in the parasite gut. Phase-separation experiments with Triton X-114 separated H-gal-GP into the aqueous phase, which would not be expected of integral membrane proteins with a hydrophobic transmembrane region. Incubation of *H. contortus* membranes for up to 24 hours in phosphatidylinositol-specific phospholipase C or D did not release any components of H-gal-GP into the supernatant, suggesting that the complex is not connected to the membrane by a phosphatidylinositol phospholipase anchor.

The pH optima of the proteinase activity of H-gal-GP were determined against the general substrate azocasein and the blood protein haemoglobin using methods described previously, (see Chapter 2). Proteolysis of azocasein was maximal at pH 6 and was identified as a neutral endopeptidase through its marked inhibition by chelating agents and phosphoramidon. Haemoglobin degradation peaked at pH 4 and was almost completely inhibited by incubation with the aspartyl proteinase inhibitor pepstatin. In addition, hydrolysis of a specific aspartyl proteinase peptide substrate was measured spectrophotometrically showing a pH optimum of 5. That hydrolysis of haemoglobin at pH 4 was approximately seven-fold more efficient than hydrolysis of azocasein, or the blood proteins fibrinogen and albumin, demonstrates a specificity of the H-gal-GP aspartyl proteinase for haemoglobin as substrate. Gelatin-substrate gel analysis of H-gal-GP identified cysteine proteinase proteolysis associated with zone D. These biochemical characteristics of H-gal-GP further confirm the N-terminal amino acid sequencing results, above.

This work has shown that the protection afforded by immunisation of sheep with the *H. contortus* antigen H-gal-GP correlates with the production of a systemic IgG antibody response. The H-gal-GP complex is located in the parasite gut and consists of a number of disulphide and electrostatically linked subunits. How the

complex attaches to the parasite membrane is still unknown because it lacks an anchoring transmembrane hydrophobic region or a phosphatidylinositol phospholipase anchor. N-terminal amino acid sequence analysis of the protein components, together with specific antibody probing and biochemical analysis of the proteinase activity of H-gal-GP have shown that the complex comprises principally metallopeptidase, aspartyl and cysteine proteinases. The aspartyl proteinase shows affinity for haemoglobin as substrate at acidic pH and may, therefore, play a role in degradation of the host blood meal.

Contribution to work

The identification and isolation of full-length cDNAs encoding components of the H-gal-GP complex through cDNA library immunoscreening, sequence homology database searching and matching to N-terminal sequence data obtained from individual protein components of the complex. Active participation in group discussions regarding results and future direction of work.

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Further immunization and biochemical studies with a protective antigen complex from the microvillar membrane of the intestine of *Haemonchus contortus*

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SUMMARY

Immunization of sheep with a protective antigen complex from the intestinal cells of Haemonchus contortus *in Freund's adjuvant stimulated individually variable antibody responses but still conferred significant protection against parasite infection. Correlation between antibody concentration and degree of protection was suggestive of antibody being the effector mechanism. The antigen is known as* Haemonchus *galactose-containing glycoprotein complex (H-gal-GP) because it binds to lectins with a specificity for N-acetyl-galactosamine. Polypeptide composition analysis by polyacrylamide gel electrophoresis indicated an apparent molecular weight of about 1000 kDa and SDS gels revealed four major polypeptides, containing between 2 and 5 disulphide linked subunits, nearly all being glycosylated. N-terminal amino acid sequence was obtained from 12 subunits, ten showing homologies with cDNAs from* Haemonchus *encoding either pepsin, metalloprotease or cysteine protease-like enzymes. pH optima, inhibitor and various substrate studies confirmed that the native complex possessed proteolytic activities in agreement with the sequence data. Although the cDNAs predicted water soluble enzymes, little of the complex was solubilized from worm membranes without the use of a detergent, such as Triton X-100. It is hypothesized that H-gal-GP is a gut membrane associated multiprotease complex which is involved in the digestion of the blood meal and which can be neutralized by specific antibodies with drastic consequences for the parasite.*

Keywords Haemonchus contortus, *protection, antibodies, aspartyl protease, metallopeptidases, cysteine proteases*

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INTRODUCTION

Substantial protection against the economically important nematode *Haemonchus contortus* has been achieved by immunising sheep with a glycoprotein fraction isolated from the intestinal membranes of this parasite (Smith *et al.* 1994). This fraction has been termed *Haemonchus* galactose-containing glycoprotein (H-gal-GP) complex since it binds selectively to lectins with a specificity for N-acetyl-galactosamine. Lambs immunized with H-gal-GP complex and challenged with Haemonchus were protected relative to controls by up to 93% as measured by faecal egg counts and up to 72% in terms of worm numbers (Smith *et al.* 1994).

H-gal-GP complex can be extracted from membrane preparations of whole Haemonchus or isolated worm intestines by nonionic detergents such as Triton X-100. However, although H-gal-GP can readily be separated from other components of such extracts under native conditions, including for example the aminopeptidase complex known as H11 (Smith *et al.* 1994), attempts to fractionate it further in its native state have not been successful. Techniques tried have included affinity chromatography with a wide variety of lectins, gel filtration, ion-exchange, hydrophobic interaction and hydroxylapatite chromatography as well as preparatory free-flow isoelectrophoresis (Smith *et al.* 1993). Similarly, extraction of *Haemonchus* membranes with different nonionic detergents (e.g. Triton X-100, octylglucoside, CHAPS) or under variable ionic strength or pH did not lead to differential solubilization of components (unpublished data). For this reason H-gal-GP is thought to exist as a complex. Encouragingly, however, the complex did retain its protective capacity when dissociated with SDS, especially under nonreducing conditions, although the results of an experiment designed to fractionate the protective component by SDS-PAGE were not clear cut (Smith & Smith 1996).

The aim of the present paper was to investigate the relationship between antibody titre and degree of protection in sheep immunized with H-gal-GP and to describe some of the biochemical properties of this protective antigen complex and its constituent polypeptides in more detail.

MATERIALS AND METHODS

Sheep

These were Suffolk-Greyface crosses reared and housed indoors in conditions designed to exclude accidental infection with nematode parasites.

Parasites

Infective larvae were from a strain of *H. contortus* which has been maintained at Moredun for several years.

Parasitological techniques

The methods for faecal egg counting, enumeration of worm burdens and for obtaining clean adult parasites for fractionation have been described before (Smith & Smith 1993).

Preparation of H-gal-GP

H-gal-GP complex was isolated from Triton X-100 extracts of adult (21 day) *H. contortus* membranes by affinity chromatography with peanut lectin as detailed elsewhere (Smith *et al.* 1994). The complex was buffer exchanged to 10 mM Tris, 0·1% CHAPS, pH 7·4, by passage through a Sephadex G-25 column and then concentrated by filtration (Centriprep 10 – Amicon).

ELISA for antibodies to H-gal-GP

Microtitre plate wells were filled with 50 μl H-gal-GP (1 μg/ml in 50 mM bicarbonate buffer, pH 9·6) and incubated overnight at 4°C. The plates were emptied, given a standard wash (filled and emptied four times with the wash buffer used for immunoblotting) and incubated with 10% w/v skimmed milk powder overnight at 4°C. After re-washing, the wells were filled (50 μl) with serum from the immunized sheep (diluted 1/4000 in wash buffer) and incubated for two h at room temperature. After another wash, 50 μl of peroxidase conjugated antisheep IgG (1/500 in wash buffer/10% milk powder) was added for one h at room temperature. The plates were washed again, 50 μl OPD substrate was added to each well and after 30 min in the dark, the colour reaction was stopped by the addition of 50 μl sulphuric acid. The plates were read at 492 nm.

Each test sample was assayed in triplicate. Duplicate serial dilutions of a standard serum from a sheep protected by immunization with H-gal-GP were included on each plate and the test samples were expressed as a percentage of this.

Design of protection experiments

Two immunization-challenge trials were conducted, each consisting of a group of 14 sheep immunized with H-gal-GP and a group of seven controls which were injected with adjuvant alone. The groups were balanced for sex and weight. At the time of first immunization the sheep were aged five and six months in expts 1 and 2, respectively.

The dose of H-gal-GP antigen was 100 μg protein per immunization. Primary injections were given in Complete Freund's Adjuvant at four subcutaneous sites per sheep each of 0·5 ml. Two booster immunizations were made subsequently three and six weeks later. These injections were with Incomplete Freund's and were administered intramuscularly as two 1 ml doses into each hind leg.

All sheep were bled at weekly intervals and challenged with 5000 *Haemonchus* larvae two weeks after the third injection. Faecal egg counts were monitored three times a week from day 16 after challenge and the animals were killed on day 34 or 35 and the worms counted.

Preparation of extracts of Haemonchus intestines

Intestines were dissected from 35-day-old worms, homogenized in phosphate buffered saline, pH 7·4 (PBS) containing 1 mM EDTA and 1 mM PMSF as protease inhibitors and the membrane pellet was sequentially extracted with PBS containing Tween 20 and Triton X-100 as described before (Smith 1993).

Phase separation of Triton X-114 extracts of Haemonchus membranes

One gram of adult (21 day) *H. contortus* was homogenized in ice-cold PBS with protease inhibitors (Smith *et al.* 1994). The homogenate was centrifuged and the resulting pellet washed three more times in homogenising buffer by centrifugation. The pellet was then mixed with an excess of 2% Triton X-114 (Pierce) in homogenising buffer for one h and then centrifuged (100 000 g for one h), all at 4°C. The supernate was layered over 1 ml of 0·25 M sucrose in a conical bottomed centrifuge tube and the detergent and aqueous phases separated by warming to 23°C for 10 min followed by centrifugation at 2000 g for five min (Pryde 1986). The process was repeated twice more by adding further cold Triton X-114 and PBS to the aqueous and

detergent phases, respectively, then mixing, cooling and re-warming.

Phosphotidylinositol specific-phospholipase (PIPL C) digestion of *Haemonchus* whole worm membranes.

Suspensions of washed *Haemonchus* membranes were divided in to equal aliquots. Different aliquots were incubated at 37°C for two, four, six or 24 h with 120 mU PIPLC (Boehringer) in 70 mM triethanolamine, 5 mM EDTA, pH 7·5. Controls were incubated in buffer alone. The samples were then spun for five min in a microfuge and analysed by SDS-PAGE and/or immunoblotting. As a positive control, the same batch of enzyme was used to cleave acetylcholinesterase from bovine red cell membranes as described by Taguchi & Ikezawa (1981).

Polyacrylamide gel electrophoresis (PAGE). Proteins were separated on continuous or gradient (5–15%) minigels (Biorad) for analytical purposes or on gradient preparative gels (1·5 mm × 14 cm × 12 cm).

Blue Native PAGE with Serva Blue G was conducted according to the method of Von Jagow & Schägger (1994) using horse spleen ferritin, urease and bovine serum albumin (Sigma Chemical Co.) or bovine heart mitochondria membrane proteins (gift from Dr David Apps of Edinburgh University) as molecular weight markers.

For SDS-PAGE (Laemmli 1970) samples were heated at 100°C for five min in an equal volume of sample buffer (63 mM Tris-HCl pH 6·8 containing 5% SDS and bromophenol blue) and 50 mM dithiothreitol (DTT) was included if reduction was desired. Molecular weight markers (Sigma Chemical Co.) were run on each gel and the relative molecular weights and molar ratios of the unknown polypeptides were estimated on dried, Coomassie Blue-stained gels with an imaging densitometer equipped with appropriate software (BioRad model GS 670).

Gelatin-substrate gel analysis was conducted under non-reducing conditions as described previously (Knox *et al.* 1993). After electrophoresis, the gels were washed extensively in several changes of 2·5% Triton X-100 over a one-h period and then incubated overnight at 37°C in 0·1 M acetate buffer, pH 5 or 50 mM Tris-HCl pH 7·5, with or without the addition of 5 mM DTT. Zones of proteolysis were visualized by Coomassie blue counterstaining.

Electroelution

For recovery of individual polypeptides from acrylamide gels, nonreduced H-gal-GP was fractionated by SDS-PAGE with Coomassie blue in the cathode buffer, then gel slices containing each band were excised and electroeluted according to Von Jagow & Schägger (1994).

N-terminal amino acid sequencing

Proteins were electrophoresed from gels to ProBlot membranes (Applied Biosystems) and stained with Coomassie blue. The bands were excised from the membrane and their N-terminal amino acid sequence was determined on an Applied Biosystems Model 477 A Peptide sequencer. The sequences were compared with existing sequences in the GenBank and EMBL databases.

Antibodies and antisera

The preparation of biotinylated, affinity purified sheep antibodies specific for either recombinant *Haemonchus* metallopeptidase 1 fused to glutathione-S-transferase (GST) from *Schistosoma japonicum* (anti-MEP1) or for recombinant *Haemonchus* aspartyl protease (anti-Pepsin)

Table 1 Protective effects stimulated against a *Haemonchus* challenge by vaccination with H-gal-GP in expts 1 and 2. Group mean (± standard error) parasitology and protection data

Experiment	Antigen	*n*	Eggs		Worms		
			Epg*	Protection (%)	Total	%male	Protection (%)
1	H-gal-GP	14	1384 (367)	69·7	1523 (230)	68 (2·3)	40·0
	control	7	4215 (384)		2359 (177)	54 (2)	
2	H-gal-GP	14	1367 (321)	56·5	1195 (207)	72 (3·9)	53·5
	control	7	3030 (404)		2255 (140)	53 (2·2)	

* Group mean of egg counts averaged for each sheep over days 18–34 after challenge. Within each experiment all parameters were significantly different ($P < 0·01$) between vaccinated and control sheep.

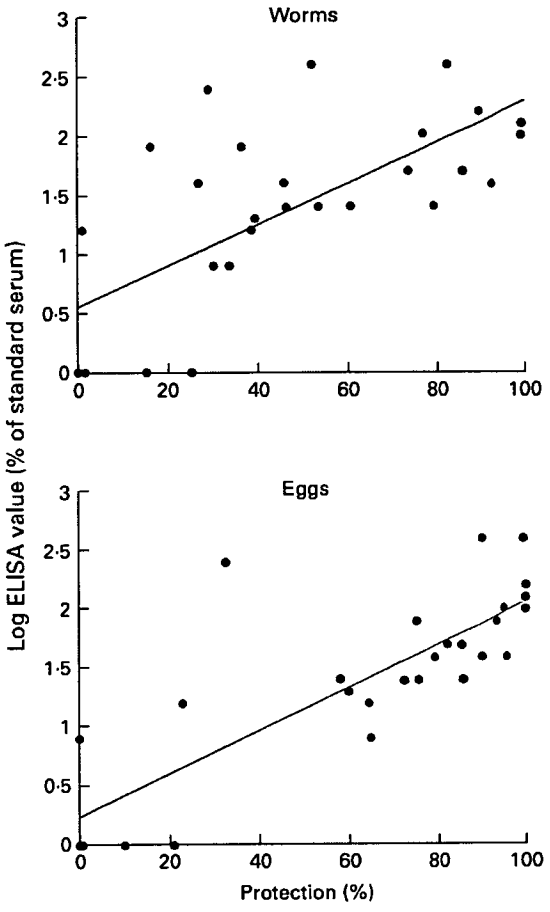

Figure 1 Correlation between anti-H-gal-GP antibody titre and degree of protection, measured against final worm count (upper panel) or egg output averaged over day 18–34 after challenge.

has already been described (Longbottom et al. 1997; Redmond et al. 1997).

Antiserum raised in sheep to *E. coli*-expressed recombinant H11, an intestinal microvillar aminopeptidase from *Haemonchus* (Smith *et al.* 1997), was gifted by Dr E. Munn of the Babraham Institute. Antibodies isolated from this serum by affinity chromatography using a native H11-sepharose column were biotinylated (Goding 1983).

Whole sheep antiserum was used to probe blots for the presence of either recombinant GST alone, native H-gal-GP, or Zone D, one of the components of H-gal-GP (Table 1). The latter was excised following SDS-PAGE of H-gal-GP under nonreducing conditions. Sheep were injected subcutaneously with 50–100 μg of protein three times at three week intervals. The first inoculation was an emulsion with Freund's complete adjuvant but subsequent injections were in incomplete Freund's. Serum separated from blood collected two weeks after the final injection was used to probe blots.

Blotting with antisera and lectins

Proteins were electrophoresed from gels onto Immobilon P membranes (Millipore).

Membranes to be probed with antisera were optionally treated with 50 mM $NaIO_4$ to disrupt carbohydrate epitopes (Woodward *et al.* 1985) and blocked for one h with 10% skimmed milk powder in wash buffer (10 mM Tris, 0·5 M Na Cl, 0·05% Tween 20, 0·01% thiomersal). Membranes to be reacted with biotinylated lectin (Vector Laboratories) were blocked with 2% globin-free bovine serum albumin (Sigma Chemical Co.) in wash buffer.

The membranes were then cut into strips and incubated individually with an excess of sheep antiserum (1/200), biotinylated antibody (1–2 μg/ml) or biotinylated lectin (10 μg/ml) for two h. The strips were re-washed and incubated with an excess of peroxidase-conjugated horse anti-sheep immunoglobulin or streptavidin-peroxidase (Pierce) for those first treated with antiserum or biotinylated ligand, respectively. After further washing all blots were developed with 1 mM 3,3 diaminobenzidene.

Protein concentrations. These were estimated by the bicinchoninic protein assay reagent according to the manufacturer's (Pierce) instructions.

Proteolytic activity of H-gal-GP

This was assessed, initially, with azocasein or haemoglobin as substrates, over a broad pH range using previously described methods (Knox *et al.* 1993), haemoglobin being prepared directly from sheep red blood cell lysates. The protein substrate preference of H-gal-GP was assessed using additional blood proteins fibrinogen and albumin and buffer at pH 5·0. Reaction mixtures comprised H-gal-GP (2·5 μg in 25 μl elution buffer), substrate (25 μg in 25 μl water), 25 μl buffer and 10 μl penicillin/streptomycin. Buffers used, all 0·1 M, were acetate pH 3·5–5·0 and phosphate pH 5·0–7·0. After overnight incubation at 37°C, undigested protein was precipitated by the addition of 85 μl of ice-cold trichloroacetic acid (5%w/v), incubation on ice for 30 min and subsequent centrifugation at 10 000 g for ten min using a microfuge. The supernatants were retained for further analysis. Proteolysis was monitored indirectly using the ninhydrin protocol (Knox *et al.* 1993) with the final absorbance (562 nm) being determined using a Beckman DU650 spectrophotometer. Blanks, containing water in place of sample were included for each observation.

Inhibitor sensitivity of H-gal-GP proteolytic activity

The sensitivity of H-gal-GP proteolysis to a panel of class-differentiating proteinase inhibitors was tested at pH 4·0 using haemoglobin as substrate and at pH 6·0 using azocasein as substrate according to the protocol outlined above. The reaction was stopped by acid precipitation, the supernatant retained for ninhydrin development and the OD_{562} determined.

Hydrolysis of a peptide substrate with specificity for aspartyl proteinases

The ability of H-gal-GP to hydrolyse H-Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu-OH, a synthetic peptide substrate with specificity for pepsin and other aspartyl proteinases (Dunn *et al.* 1984), was assessed over the pH range 2·5–6·0 using 0·1 M acetate buffer and pH 6·0–7·0 using 0·1 M phosphate buffer. H-gal-GP (10 μg) was mixed with 100 μl of buffer and the reaction started by addition of substrate (1 mg/ml in 1·0% acetic acid) and the change in absorbance, 310 nm, monitored over a five-min period at room temperature using a Beckman DU650 spectrophotometer.

Statistical methods

Arithmetic group means are shown with standard errors. Individual percentage protection (% P) values were calculated as percentage $P = 1 - (\text{individual vaccinate count}/\text{mean control count}) \times 100$, where count was either the total number of worms recovered *post mortem* or the egg count averaged over the sampling period. Negative protection values were assigned the value 0. Data from both trials were pooled and a regression analysis was conducted between the antibody titre of vaccinated sheep measured at the time of challenge and percentage P, for both eggs and worms, using Minitab, a statistics program.

RESULTS

Immunization experiments

In each experiment immunization with H-gal-GP significantly reduced the number of eggs and worms recovered from the vaccinated sheep compared to the controls (Table 1). Immunization was also significantly more effective against female than male worms as shown by the abnormal sex ratio of the populations recovered from each vaccinated group (Table 1). Examination of the individual data showed that there was considerable individual variation in the degree of protection conferred on the vaccinates (Figure 1).

When the degree of protection, measured either by egg counts or worm numbers, was plotted against prechallenge

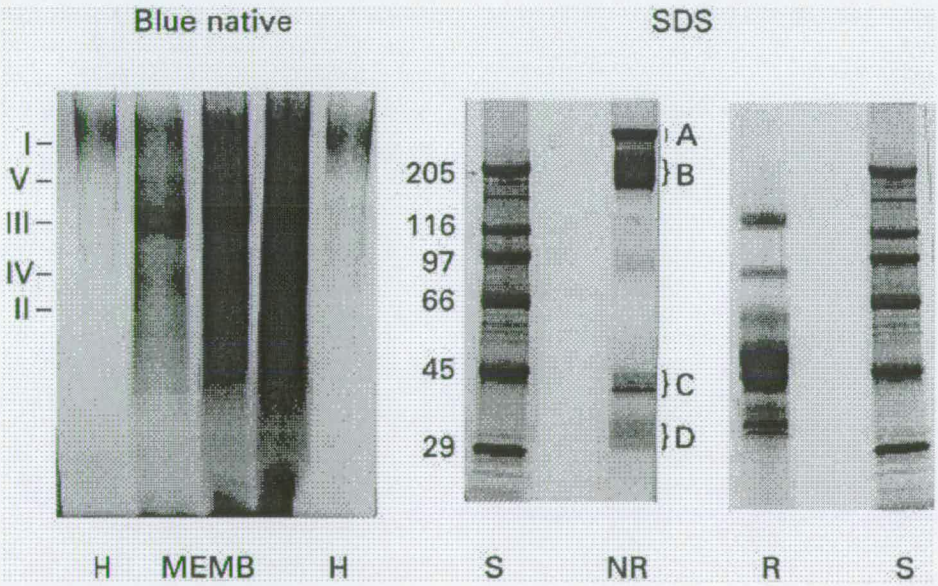

Figure 2 Blue Native and SDS PAGE analysis of H-gal-GP. Blue Native gel: H = H-gal-GP; MEMB = three tracks loaded from left to right with increasing amounts of a lauryl maltoside extract of *Haemonchus* membranes. Roman numerals show the relative mobility of bovine mitochondrial complex components which were used as molecular weight markers. I = 880 kDa; V = 600 kDa; III = 485 kDa; IV = 200 kDa; II = 30 kDa. SDS gel: S = molecular weight standards; NR = H-gal-GP under non reducing conditions, the main protein zones are labelled A to D; R = H-gal-GP under reducing conditions.

Table 2. Estimated molecular weights (kDa) and relative quantities of the major proteins of dissociated, nonreduced H-gal-GP

Protein zone	A	B	C	D
Mean mol wt (SD) of main band in zone	233 (23·9)	172 (12·9)	40 (2·7)	31 (2·8)
Mean OD of zone as % of total OD (SD)	16·2 (0·94)	38·2 (2·51)	14·0 (1·14)	14·1 (0·93)

serum antibody concentrations significant correlations were observed (Figure 1). Regression analysis yielded the following relationships: Log elisa $= 0{\cdot}287 + 0{\cdot}18\%P$ for eggs ($R^2 = 65{\cdot}5\%$, $P < 0{\cdot}001$) and Log elisa $= 0{\cdot}629 + 0{\cdot}017\%P$ for worms ($R^2 = 43{\cdot}9\%$, $P < 0{\cdot}001$).

Biochemical analysis of H-gal-GP

A. Polypeptide analysis

1. Native PAGE.

Under Blue Native PAGE purified H-gal-GP was visualized as a single diffusely staining band (H, Figure 2) with a relative mobility somewhat slower than bovine mitochondial Complex 1, which has an estimated molecular mass of 880 kDa (Von Jagow & Schägger 1994). A band of identical relative mass and appearance was observed in the polypeptide profile of Haemonchus membranes extracted with lauryl maltoside as recommended by Von Jagow & Schägger (1994) (MEMB, Figure 2).

2. Non reducing SDS-PAGE.

SDS-PAGE under non-reducing conditions resolved H-gal-GP into four main protein zones (Figure 2, lane NR) which were termed Zones A to D. Zone A appeared as a single band. Zone B was a diffusely staining region with a prominent band apparent at its anodic margin. Zone C contained two, sometimes three, relatively sharp bands, the smallest of which was the most abundant. Zone D stained rather diffusely, although two bands could be detected, the smaller of which was the more prominent. Five different preparations of H-gal-GP were electrophoresed and scanned, each revealing five peaks which corresponded to the three prominent bands within Zones A, B and D plus the two bands within Zone C. The respective molecular masses and percentage of total OD of each peak were calculated (Table 2). The coefficient of variation for these parameters was less than 10%, indicating that the composition of H-gal-GP complex was consistent between preparations.

3. Reducing SDS-PAGE and identification of bands by sequence homology.

When subjected to SDS-PAGE under reducing conditions most of the H-gal-GP proteins migrated as a zone of unresolved bands between 40 and 55 kDa, although distinct polypeptides with calculated molecular masses of 130, 90, 60, 33, 31 and 29 kDa could also be seen (Figure 1, lane R and Figure 3). To further analyse the composition of the complex, protein Zones A to D were individually electro-eluted from nonreducing Coomassie-blue SDS-PAGE gels, then re-subjected to SDS-PAGE under reducing conditions (Figure 3). The profiles of two preparations of H-gal-GP treated in this way were scanned and the mean molecular masses of the bands detected in Zones A to D were calculated. For convenience these bands are subsequently referred to by the letter of their zone of origin and their estimated relative kDa (e.g. A.130). N-terminal amino acid sequences were determined for all bands except B.62 and C.52.

Zone A separated into five main bands designated A.130, A.91, A.45, A.41 and A.29 according to their calculated molecular weights (Figure 3, lane A reduced). Sixteen of the 20 predominant amino acids in the N-terminal data obtained for band A.130 matched those predicted from a cDNA recently isolated from a *Haemonchus* expression library

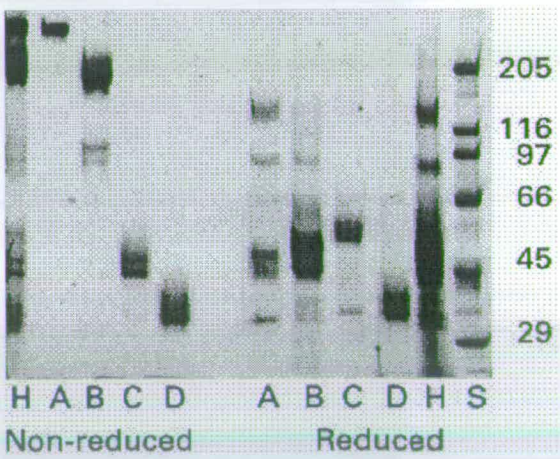

Figure 3 SDS PAGE analysis of H-gal-GP and its major polypeptide components under non reducing and reducing conditions. H = H-gal-GP. Tracks A to D contain protein zones A to D, the major components of the complex, excised and electroeluted from nonreduced gels. S = molecular weight standards.

Table 3 N-terminal amino acid sequences of Zone A bands which showed homology with Haemonchus metalloprotease-like cDNAs

Band	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	cDNA	Genbank accession number (or ref.)
A.130	M	D	D	I	R	S	K	H	E	S	P	P	P	V	G	P	I	P	P	K		
			S	P	N	L			P	I	H	L	W			A		L		V		
				H	P																	
	M	D	D	I	R	S	K	H	E	S	H	T	T	V	M	P	I	P	P	K	MEP-4	Skuce *et al.* (unpublished)
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**		
A.91	A	S	I	H	A	S	S	S	K	Y	D	P	S?	N	A	E	A	L	G	F		
			P		S	D				P				E				T				
A.41	A	S	I	H	S	S	S	Y	L	P	S	P	Q	Y	A	L	N	L	P	D		
		H	A	E	A	E	F		A	Y	N	Y	A	E	I	A	T	V	Q	T		
				P	D	L	P															
	46	*47*	*48*	*49*	*50*	*51*	*52*	*53*	*54*	*55*	*56*	*57*	*58*	*59*	*60*	*61*	*62*	*63*	*64*	*65*		
	A*	S	I	H	V	N	S	S	K	Y	C	P	S	Y	G	E	A	L	F	T	MEP-1	Z75054 or AF047416
	106	*107*	*108*	*109*	*110*	*111*	*112*	*113*	*114*	*115*	*116*	*117*	*118*	*119*	*120*	*121*	*122*	*123*	*124*	*125*		
	A*	S	I	H	A	N	S	S	K	Y	C	P	S	Y	G	E	A	L	N	T	MEP-2	AF080117
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**		
A.29	D	X	S	A	P	T	I	L	S	I	L	A	Y	G	D	E	N	R	E	D		
														G		E					MEP-4	Skuce *et al.* (unpublished)
	G	G	S	F	P	T	I	L	S	I	L											

Zones of identity between derived and predicted amino acid sequences are highlighted in grey. Derived N-terminal amino acid sequences are numbered in bold. Predicted amino acid sequences are numbered in italics from the start of the open reading frame of the cDNA.
* Predicted pre/proenzyme cleavage site.

by screening with antisera to H-gal-GP (Table 3). This cDNA, termed MEP-4, showed 59% identity at the DNA level with a previously described putative metalloprotease cDNA from *Haemonchus* (MEP-1, Redmond *et al.* 1997). Although sequencing of MEP-4 has not yet been completed, significant homology was also detected between the N-minus of band A.29 and that deduced from MEP-4 able 3) in a region of the cDNA calculated to be the uivalent of 651 amino acids C terminal from the start of 130.

N-terminal amino acid sequence analysis also showed nificant homology between A.91 and A.41 (Table 3), en though the data from A.41 indicated the presence of re than one component. These sequences also partially responded to those predicted from two other closely ated (more than 70% identical) putative metallopepti-es termed MEP-1 and MEP-2. The N-terminus data from 45 indicated the presence of more than one component, so far a corresponding cDNA has not been isolated.

The size of the MEP-1 and MEP-2 cDNAs suggested t A.91 was the full length product of either or both l that A.41 was a fragment of A.91. Similar reasoning gested that A.29 represented a fragment of A.130 oded by MEP-4.

Reduction resolved Zone B into four major bands, B.90, B.62, B.47 and B.41, although B.47 and B.41 were much more abundant than the others (Figure 3, lane B reduced). Sequence analysis revealed that the N-terminus for B.41 matched almost exactly that deduced from a *Haemonchus* cDNA clone encoding a further metalloprotease homologue designated MEP-3 (Table 4). The data from band B.47 suggested the presence of more than one component, but, by selecting the appropriate alternative and inserting a gap of 2 after number 15, it was possible to match 17 out of the 20 amino acids predicted from a section of the MEP-3 cDNA starting 386 amino acids C-terminal from the B.41 N terminus (Table 4). So far a cDNA with sequence corresponding to B.90 has not been isolated.

When the major band in Zone B was excised, electro-eluted and electrophoresed nonreduced, a minor proportion of it ran as an additional pair of bands of about 100 and 91 kDa (Figure 3 lane B non reduced). These bands increased in intensity if the protein was run partially reduced by incubation with reducing buffer at 25°C for 30 min (not shown). Moreover, if these 100 and 91 kDa bands were excised and electroeluted then analysed fully reduced, components identical in size to B.47 and B.41 were revealed (not shown), suggesting that the 100 and 91 kDa bands were intermediate forms.

Zone C was usually observed as a pair of bands of 42 and

Table 4 N-terminal amino acid sequences of Zone B bands which showed homology with *Haemonchus* metalloprotease-like cDNAs

Band	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	cDNA	Genbank accession no.
B41	V	D	N	V	F	C?	P	N	V	G	N	A	C?	R	S	X	E	W	K	D				
	71	*72*	*73*	*74*	*75*	*76*	*77*	*78*	*79*	*80*	*81*	*82*	*83*	*84*	*85*	*86*	*87*	*88*	*89*	*90*			MEP-3	AF080172
	V	D	N	V	F	C	P	N	V	G	N	A	N	R	S	K	E	W	K	N				
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**			**16**	**17**	**18**	**19**	**20**		
B.47	D	N	R	T	D	A	P	M	K	E	V	I	M	K	H			M	P	Y	G	P		
			D	R		I	R		L		L	D	T	I	T				Y					
	457	*458*	*459*	*460*	*461*	*462*	*463*	*464*	*465*	*466*	*467*	*468*	*469*	*470*	*471*	*472*	*473*	*474*	*475*	*476*	*477*	*478*	MEP-3	AF080172
	D	E	R	T	D	A	R	M	K	C	V	D	T	I	T	T	Y	M	P	Y	G	T		

Zones of identity between derived and predicted amino acid sequences are highlighted in grey. Derived N-terminal amino acid sequences are numbered in bold. Predicted amino acid sequences are numbered in italics from the start of the open reading from of the cDNA.

40 kDa when analysed without reduction (Figure 3, lane C nonreduced) although sometimes an intermediate band was also detected (Figure 2, lane NR). Sequence analysis revealed that the N-termini of the 42 and 40 kDa bands were identical except that C.42 had an extra four amino acids at the N-terminus as well as an additional tyrosine and aspartic acid (Table 5). These sequences showed very close homology to that predicted for the product of an aspartyl protease gene recently isolated from a *Haemonchus* cDNA expression library by means of antiserum specific for H-gal-GP (Longbottom et al. 1997). Reduction increased the apparent molecular weight of most of Zone C doublet from 40/42 to 52 kDa, although about half of it resolved at 31 kDa (Figure 3, compare lanes C, reduced and nonreduced). This increase in apparent molecular weight following reduction may have been caused by the disruption of internal disulphide bonds leading to expanded polypeptides with reduced mobility through the gel matrix. A database search did not reveal any significant homology with the N-terminal data derived for C.31.

The prominent band in Zone D had an apparent molecular weight of 31 kDa but resolved into a 33 and 31 kDa doublet after reduction (Figure 3, compare lanes D reduced and nonreduced). All 3 bands had the same N-terminal amino acid sequence which showed some homology with the predicted protein sequences of a family of *Haemonchus* cysteine proteinase genes (Table 5).

Table 5 N-terminal amino acid sequences of Zone C and D bands which showed homology with *Haemonchus* pepsin or cysteine protease-like cDNAs

Band	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	cDNA	Genbank accession no.
C.42	A	E	P	T	V	F	P	H	P	I	Y	D	Y	D	Y	Q	D	T	E	Y	L	A					
C.40					V	F	P	H	P	I	Y	D	Y			Q	D	T	E	Y	L	A	K	I	T		
	55	*56*	*57*	*58*	*59*	*60*	*61*	*62*	*63*	*64*	*65*	*66*	*67*			*68*	*69*	*70*	*71*	*72*	*73*	*74*	*75*	*76*	*77*		
	A	N	Q	T*	V	F	P	H	P	I	Y	D	Y			Q	D	T	E	Y	L	A	K	I	T	Pep1	Z72490
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**												
D.31 or	D	P	D	I	P	E	N	Y	D	P	R	L	I	W	P												
D.33	*93*	*94*	*95*	*96*	*97*	*98*	*99*	*100*	*101*	*102*	*103*	*104*	*105*	*106*	*107*												
	N	E	D*	I	P	E	S	F	D	S	R	E	V	W	K											HMCP1	Z69342
	N	E	D*	I	P	E	S	F	D	S	R	I	V	W	K											HMCP2	Z69343
	G	D	D*	I	P	E	S	F	D	A	R	T	H	W	P											HMCP3	Z69344
	G	E	D*	I	P	E	S	F	D	A	R	T	K	W	P											HMCP4	Z69345
	G	D	D*	I	P	E	S	F	D	A	R	T	H	W	P											HMCP5	Z69346
	D	D	D*	I	P	E	S	F	D	A	R	T	H	W	A											HMCP6	Z81327

Zones of identity between derived and predicted amino acid sequences are highlighted in grey. Derived N-terminal amino acid sequences are numbered in italics from the start of the open reading frame of the cDNA.

* Predicted pre/proenzyme cleavage site.

The data for band C has already been published by Longbottom *et al.* 1997. It is shown here for completeness.

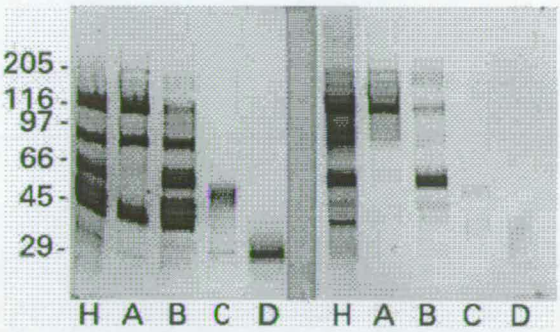

Figure 4 Reaction of H-gal-GP and protein zones A to D separated by SDS-PAGE under reducing conditions with Con-A (left panel) and wheatgerm (right panel) lectins.H = H-gal-GP. Tracks A to D contain protein zones A to D, the major components of the complex, excised and electroeluted from non-reduced gels.

4. Glycosylation of H-gal-GP polypeptides.

(i) Lectin binding. Blots of H-gal-GP and protein Zones A to D separated by reducing SDS-PAGE were reacted with a panel of biotinylated lectins. Some, like Con-A, bound almost all the bands, albeit at varying levels of intensity, whereas the reaction of others was restricted to relatively few components (Figure 4). For example the reaction of wheatgerm was largely confined to bands in Zones A and B (Figure 4).

(ii) Treatment with de-glycosylating enzymes. The appearance and relative mobility on SDS-PAGE of Zones A, B and C following incubation with either N-glycosidase F, O-glycosidase or neuraminidase was compared with control samples incubated under the same conditions without enzyme. Treatment with O-glycosidase had no effect. However, incubation with N-glycosidase F increased the electrophoretic mobility of B.62, B47, B.41, C.52 and C.31 by about 2 kDa each, although those from band A were unaffected (not shown). Sub-components B.47 and B.41 appeared to be the only ones affected by neuraminidase treatment which reduced their intensity and gave rise to additional bands of about 32 and 24 kDa.

5. Antigenic relationship between H-gal-GP polypeptides.

When H-gal-GP was probed with anti recombinant *Haemonchus* pepsin antibody, only Zone C bands were detected (Figure 5). Similarly, when the complex was incubated with anti Zone D serum, it was clear that the reaction was mainly directed at that zone, even though faint cross reactions could also be detected (Figure 5). In contrast, anti recombinant *Haemonchus* metallopeptidase1-GST did not discriminate between Zone A and B proteins (Figure 5). This reaction was specific because Zones C and D were not stained and because control anti-GST serum did not react with any component of H-gal-GP (Figure 5).

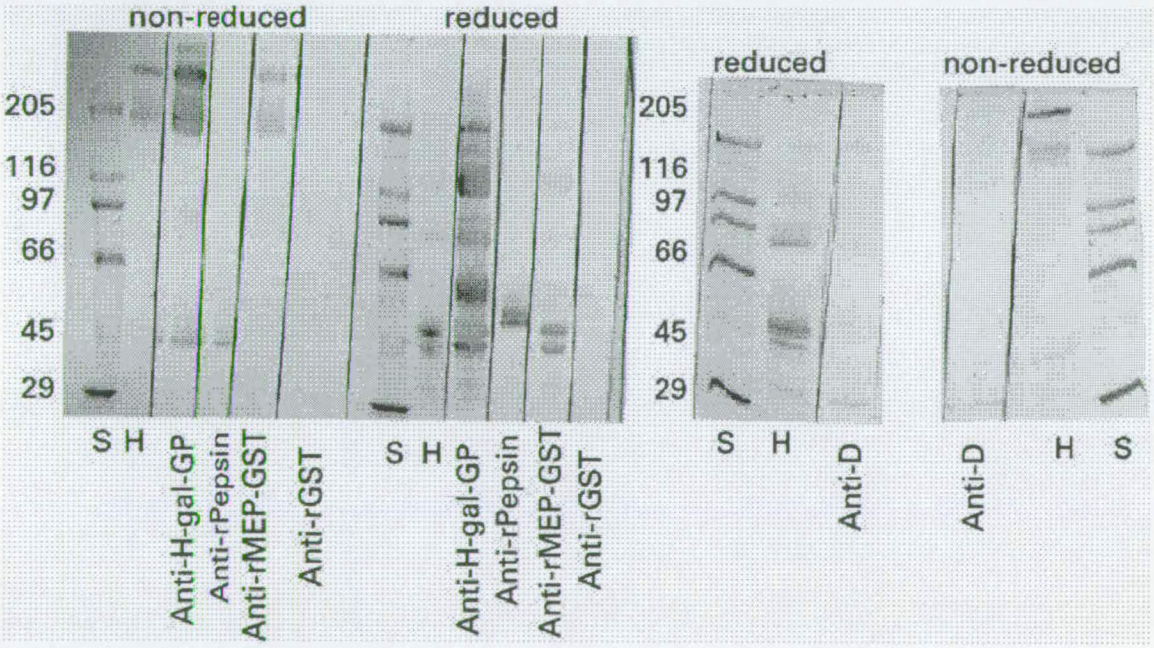

Figure 5 Blots of H-gal-GP following SDS PAGE under reducing or non-reducing conditions probed with antiserum to native H-gal-GP or Zone D proteins or with affinity purified antibodies specific for recombinant *Haemonchus* pepsin, recombinant *Haemonchus* metallopeptidase-GST fusion protein or GST alone. S and H = Coomassie stained molecular weight standard markers and H-gal-GP, respectively.

B. Association of H-gal-GP with intestinal cell membranes

1. Experiments with dissected out *Haemonchus* intestines.
SDS-PAGE analysis of the PBS soluble fraction of homogenized *Haemonchus* intestines and subsequent, consecutive Tween and Triton X-100 extractions of the membrane pellet showed that protein Zones A to D were all present and most concentrated in the Triton extract (not shown). High speed centrifugation (100 000 g for one h) deposited most, though not all, of the H-gal-GP from the PBS and Tween extracts, but not from the Triton one.

2. Phase separation experiments with Triton X-114.
H-gal-GP partitioned mainly into the aqueous fraction when *Haemonchus* membranes were extracted and subsequently phase-separated with Triton X-114 (Figure 6). In contrast, H11, the intestinal microvillar aminopeptidase with a known hydrophobic transmembrane region (Smith *et al.* 1997), showed relatively more affinity for the detergent fraction. However, prolonged dialysis of the aqueous phase against Tris buffer resulted in precipitation of most of the H-gal-GP (not shown).

Probing with anti-rPepsin confirmed that the aspartyl protease was more concentrated in the aqueous than the detergent phase (Figure 6). Interestingly, anti-rMEP1 showed that whereas Zone A proteins were almost entirely confined to the aqueous phase, a proportion of Zone B separated with the detergent. An unknown avidin peroxidase-reacting band of about 60 kDa was also detected in the aqueous fraction of the membrane extract (Figure 6).

3. Comparison and relative yields of H-gal-GP recovered from PBS or Triton extracts of *Haemonchus*.

The PBS extract of a 20-g batch of Haemonchus was spun at 100 000 g, the supernate was passed through a peanut lectin column and the bound fraction was analysed by SDS-PAGE. H-gal-GP protein Zones A to D were all identified, together with some additional unknown components. Attempts to separate protein Zones A to D of PBS soluble H-gal-GP either by Blue Native PAGE or by gel filtration chromatography were unsuccessful.

H-gal-GP was also prepared following Triton X-100 extraction of the PBS insoluble fraction of the same batch of worms by the standard method. It was estimated that the yield of PBS soluble H-gal-GP was less than 5% of that from the Triton extract.

4. Experiments with PIPL anchor cleaving enzymes.
Incubation of *Haemonchus* membranes with PIPL C or D for between two and 24 h did not release any components of H-gal-GP into the supernate (not shown).

C. Proteolytic activity of H-gal-GP

The objective was to determine whether native H-gal-GP possessed proteolytic properties and if so to characterize it by measuring the pH optimum, the sensitivity to a panel of inhibitors, the activity on gelatin substrate gels and the effect on selected substrates.

1. pH optima.
The results are shown in Figure 7 where each data point

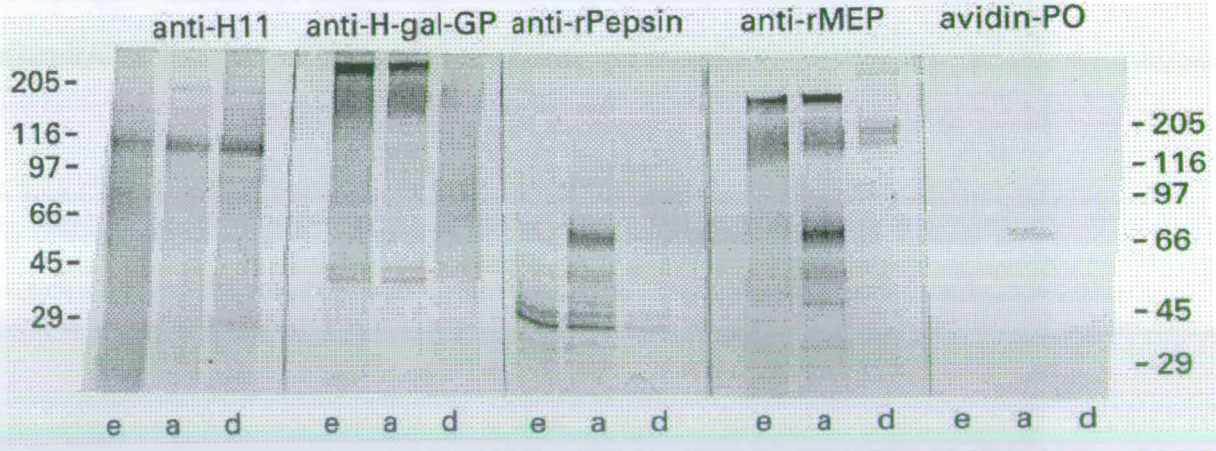

Figure 6 Triton X-114 extracts of adult *Haemonchus* membranes were prepared and separated into aqueous and detergent phases. Following SDS PAGE under non-reducing conditions, samples were blotted and probed with peroxidase conjugated anti-H11 or anti-H-gal-GP sera, with biotinylated antibodies specific for recombinant pepsin or metallopeptidase or with avidin peroxidase alone. The six most left hand tracks were derived from the same gel which had electrophoresed less distance than the remaining nine tracks which were run together on a different gel. e = extract of whole worm membranes; a = aqueous phase fraction; d = detergent phase fraction. Note the presence of an avidin peroxidase binding protein in the aqueous phase.

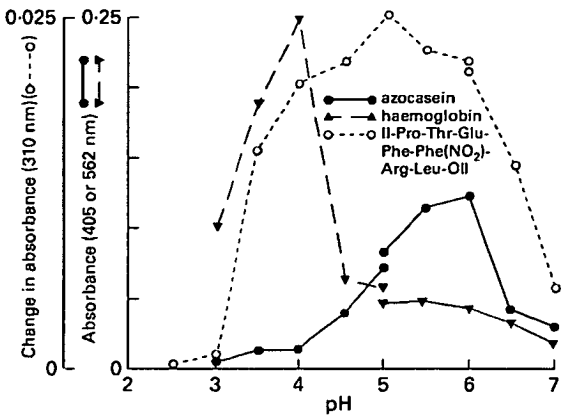

Figure 7 The pH optima of the proteinase activity of H-gal-GP with three substrates.

represents the mean of three separate observations on the same batch of H-gal-GP and the maximum coefficient of variation observed was 7·3%. Similar profiles were observed when different batches of H-gal-GP were tested.

Proteolysis of azocasein and haemoglobin was maximal at pH 6·0 and 4·0, respectively. In comparison hydrolysis of the specific aspartyl proteinase peptide substrate H-Pro-Thr-Glu-Phe-Phe(NO2)-Arg-Leu-OH occurred between pH 3·5–6·5 with a peak at 5·0 as previously described (Longbottom *et al.* 1997).

2. Inhibitor sensitivity.

The sensitivity of H-gal-GP proteinase activity to a panel of proteinase inhibitors is shown in Table 6.

Table 6 Mean (±SD) protease activity remaining after treating replicates of H-gal-GP with various inhibitors

	% activity remaining			
	pH 4.0		pH 6.0	
Inhibitor	Mean	(SD)	Mean	(SD)
Control	100		100	
PmsF	100	1·91	101	3·50
E64	88	1·26	99	3·59
Dithiothreitol	88	5·38	47	2·38
Cysteine	94	2·16	2	2·36
Glutathione	100	2·65	86	1·50
4-hydroxymercuribenzoate	95	2·22	90	1·41
N-ethylmaleimide	105	3·30	77	2·08
EDTA	94	4·57	13	8·22
1,10 phenanthroline	100	1·29	23	1·71
Pepstatin	12	3·50	85	7·14
Phosphoramidon	100	2·06	10	2·65

Proteolysis of haemoglobin at pH 4·0 was almost completely inhibited by pepstatin and relatively unaffected by the other inhibitors tested. By contrast, proteolysis of azocasein at pH 6·0 was markedly inhibited by the sulphydryl reagents DTT and cysteine, by the chelating agents EDTA and 1,10 phenanthroline and by phosphoramidon, but was relatively unaffected by pepstatin.

3. Gelatin substrate gel analysis.

Proteolysis was consistently associated with Zone D. This effect was more pronouced at pH 7·5 than 5 and was enhanced by the presence of DTT (not shown).

4. Protein substrate specificity.

The affinity of H-gal-GP for the natural blood proteins haemoglobin, fibrinogen and albumin, as well as the general protein substrate azocasein, was evaluated at pH 4·0 and 7·0 and the results are shown in Table 7.

At pH 4·0, haemoglobin was hydrolysed at least sevenfold more efficiently than the other substrates tested, but at pH 7·0 azocasein hydrolysis predominated.

These findings suggested that native H-gal-GP possessed proteolytic activity consistent with the presence of more than one type of enzyme. The activity with the pH optimum of 4–5, which hydrolysed the substrate H-Pro-Thr-Glu-Phe-Phe(NO2)-Arg-Leu-OH and which was inhibited by pepstatin, clearly indicated the presence of an aspartyl protease. The finding that Zone C proteins showed sequence homology with mammalian pepsins endorsed these results. Similarly, that part of the activity with a pH optimum of 6·0 which was sensitive to EDTA and phosphoramidon was consistent with metal dependent neutral endopeptidases and was confirmed by the sequence homologies detected between Zone A and B proteins and mammalian metallopeptidases. Equally, the remaining part of this activity was inhibited by cysteine and dithiothreitol indicative of cysteine proteases also subsequently identified by substrate gels and sequence homology to be present in Zone D.

Table 7 Relative proteolytic activity of H-gal-GP with different substrates

Substrate	pH 4·0	pH 7·0
Fibrinogen	0*	0·64
Haemoglobin	5·12	0·27
Albumin	0·77	1·32
Azocasein	0·55	5·63

* Absorbance at 562 nm.

DISCUSSION

Mean levels of protection observed in each of the present sheep experiments were lower than those from two previous trials with native H-gal-GP, where values of greater than 90% for eggs were recorded (Smith *et al.* 1994). The most obvious difference between the two sets of experiments was in the adjuvant regime. Freund's Complete Adjuvant was used for all three immunizations in the earlier trials but, in order to comply with changes in British Home Office animal licence regulations, this powerful but toxic adjuvant was used only for the first injection in the present experiments and in smaller, more widely dispersed amounts. Although the resulting immunization regime stimulated antibody concentrations which varied considerably between individual sheep, the titres correlated with the degree of protection observed against challenge, a result which suggested that antibody was the protective mechanism. This notion is supported by previous vaccine studies with different gut membrane antigens of *Haemonchus* which have shown that protection correlates with antibody titre (Munn *et al.* 1997) and can be passively transferred between sheep by immune serum (Smith 1993) or colostrum (Andrews *et al.* 1995). Subsequent immunization trials with H-gal-GP in our laboratory, using QuilA as adjuvant have resulted in consistent, high concentrations of antibody and have restored the levels of protection to those originally obtained with Complete Freunds (Smith *et al.* submitted for publication).

Native PAGE showed that H-gal-GP complex was large with an apparent relative molecular weight of about 1000 kDa. The finding that a component of similar size and appearance was present in extracts of worm membranes suggested that the size of the complex was not an artefact of the purification procedure. The observation that H-gal-GP migrated as a single band on native gels was consistent with its failure to separate by a variety of chromatography techniques under native conditions. The complex readily dissociated in the presence of SDS either by PAGE or by gel filtration chromatography (unpublished observations) into four main protein zones, indicating that its major polypeptide components were held together electrostatically. The finding that the ratio of these components was consistent between preparations argues for a genuine complex rather than for a random mixture of molecules, which would have been expected to yield a more variable profile. Three of the four protein zones resolved by nonreducing SDS-PAGE contained between two to five disulphide linked subunits each. With one exception, all of these subunits were glycosylated as detected by lectin binding, the glycan being sensitive to PNGase F in 5 cases, and therefore N-linked. There was no evidence for any O-linked carbohydrate. A much more detailed analysis of the glycan architecture of *Haemonchus* integral membrane proteins (Haslam *et al.* 1997) showed that not all the N-linked oligosaccharides were actually susceptible to PNGase F and revealed the presence of several novel core structures.

Initial experiments showed that H-gal-GP possessed proteolytic properties. Inhibitor and pH optima studies indicated the presence of aspartyl and neutral endopeptidase-like activities and substrate gels suggested the presence of cysteine proteases. These observations were confirmed when N-terminal amino acid sequence data from subunits of all the 4 major protein zones showed clear homology with the deduced products of pepsin, metallopeptidase or cysteine protease-like cDNAs recently isolated from *Haemonchus* (Tables 3, 4 and 5, Longbottom *et al.* 1997; Redmond *et al.* 1997).

As expected, antibodies specific for the recombinant product of the pepsin-like gene reacted only with both Zone C bands, the N-termini of which had the corresponding sequence. Similarily, a polyclonal antiserum raised against polypeptides in Zone D was by and large specific for that zone. Conversely, antibodies raised against a recombinant metallopeptidase (MEP1) which had sequence that matched two bands from Zone A recognized most of the bands in Zones A and B. Since these antibodies were raised against bacterially expressed recombinant proteins, the cross-reaction could not have been caused by common carbohydrate epitopes. This was explained when it was found that 4 of the 5 subcomponents of Zone A and the two most abundant subcomponents of Zone B could be identified by their N-terminal sequences as being members of a closely related family of four *Haemonchus* metallopeptidases which show at least 60% similarity to each other. A more detailed description of the molecular architecture of this gene family is being prepared.

Since all the components of H-gal-GP were found in extracts of dissected out *Haemonchus* intestines, it was clear that the complex was present in the gut. This finding endorsed previous histological studies where N-acetyl-galactosamine specific lectins or antibodies specific for recombinant MEPs or pepsin specifically illuminated the worm intestinal membrane or its lumen. (Smith *et al.* 1994; Longbottom *et al.* 1997; Redmond *et al.* 1997). The mechanism of the association of H-gal-GP with the intestinal membrane is not clear, however. On the one hand the complex behaved like an integral membrane protein in that it was much more readily extracted by nonionic detergents than by saline; on the other, H-gal-GP separated into the aqueous phase with Triton X-114 and the pepsin, metallopeptidase and cysteine protease gene sequences all predict water soluble molecules. The possibility that the complex was connected to the membrane by a phosphotidylinositol phospholipase anchor (Jasmer *et al.* 1996)

seemed to be have been ruled out, although it is just possible that the anchor cleaving enzyme might have been inhibited by some unknown substance in the parasite membrane preparation. Further analysis of the composition of the individual components should provide additional insight on the matter, but our working hypothesis is that H-gal-GP is a gut membrane associated multiprotease complex which is involved in the digestion of the blood meal and which can be neutralized by specific antibodies with drastic consequences for the parasite.

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Chapter 5: Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*.

Redmond DL, Knox DP, Newlands G and Smith WD. 1997.
Molecular and Biochemical Parasitology, **85**: 77-87.

As already discussed in Chapter 4, the integral gut membrane glycoprotein complex H-gal-GP, isolated from adult *H. contortus*, affords a high degree of protection against homologous challenge when used to immunise sheep. Under non-reducing SDS-PAGE conditions, the H-gal-GP complex can be dissociated into major protein zones. In an attempt to identify the protective component(s) of the H-gal-GP complex, gel slices representing these zones were used in a sheep vaccination trial (Smith and Smith, 1996). Although protection was variable, the 190-200 kDa protein zone protected four of six sheep, and the dyefront proteins (<55 kDa) three of six sheep. From the detailed H-gal-GP protein analysis described in Chapter 4, the higher molecular weight zone would be expected to comprise metallopeptidases and the dyefront zone, the aspartyl and cysteine proteinase components of H-gal-GP. That neither of these individual zones gave a level of protection equivalent to unfractionated H-gal-GP may indicate that more than one sub-component is necessary to obtain a consistent effect, or that protective epitopes were damaged by gel fractionation and elution. It has been shown that the protective efficacy of H-gal-GP diminished with dissociation and reduction (Smith and Smith, 1996).

The aim of the work presented in this chapter and in Chapter 6 was to clone and characterise the potentially protective metallopeptidase and aspartyl proteinase components of H-gal-GP.

An adult *H. contortus* cDNA library was immunoscreened with pooled sera from lambs which had been immunised with H-gal-GP, and which were demonstrably refractory to subsequent challenge with *H. contortus*. Six immunoreactive clones were identified. Insert cDNAs were amplified by PCR using oligonucleotide primers directed to the vector arms, subcloned into a plasmid vector,

and their nucleotide sequences determined. Two clones proved to be identical and were termed metallopeptidase1 (MEP1) after database searching with their nucleotide and deduced amino acid sequence showed them to share similarity with mammalian neutral endopeptidases. Full-length MEP1 sequence was obtained by extending the 5' and 3' sequences by PCR using generic spliced leader (SL1) and oligo(dT) primers, respectively, in combination with MEP1 gene-specific primers. Alignment of the deduced full-length MEP1 amino acid sequence with rabbit neutral endopeptidase showed conservation of critical amino acids and the active site region, even though the overall sequence similarity was very low showing only 25% identity. Six potential N-linked glycosylation sites were identified, at least one of which is utilised as the MEP components of H-gal-GP have been shown previously to bind lectins (see Chapter 4).

As predicted from the nucleotide sequence, Northern blot analysis showed MEP1 to be encoded by a 2.6 kb transcript. The complex profile of hybridising bands seen by Southern blot analysis is indicative of a multi-copy gene family and is consistent with the identification of 4 different MEP-encoding N-terminal amino acid sequences in H-gal-GP described in Chapter 4, and with the identification of a second MEP-encoding clone isolated from the cDNA library immunoscreen described above.

The full-length MEP1 coding sequence was subcloned into the pGEX-2T bacterial expression vector to form an in-frame fusion with GST and to allow inducible expression of recombinant protein. Enzymatic lysis of induced bacterial cultures and SDS-PAGE analysis of soluble and insoluble fractions showed good levels of expression of recombinant GST-MEP1 fusion protein, although it proved to be highly insoluble. Following separation of insoluble bacterial extracts by SDS-PAGE, recombinant GST-MEP1 was purified from excised gel slices by electroelution and was injected into a single sheep to raise an antiserum. Specific sheep GST-MEP1 antibody was purified by affinity-chromatography. Paraffin wax sections of adult *H. contortus* probed with biotinylated MEP1 affinity-purified antibodies showed strong staining of the microvillar surface of the gut, demonstrating the probable accessibility *in vivo* of the *H. contortus*

metallopeptidases to antibody ingested with the host blood meal, and was consistent with the localisation of the H-gal-GP complex (see Chapter 4).

Developmental expression of *H. contortus* MEP1 steady state mRNA levels was investigated by RT-PCR, expression being detected only in the blood-feeding L4 and adult stages. The pattern of MEP1 protein expression was determined by probing Western blots of worm membrane extracts with affinity-purified GST-MEP1 antibodies. The temporal pattern of MEP1 protein expression was also found to be restricted to the L4 and adult stages of *H. contortus*.

The MEP1 sequence identified and cloned here represents one member of a family of metallopeptidases identified as being part of the host protective H-gal-GP antigen complex. Localisation of MEP1 to the parasite gut and its expression only in the blood-feeding and adult stages may be indicative of a critical role in parasite feeding and/or survival. *H. contortus* MEPs may function in a co-ordinated role with other parasite gut enzymes in the digestion of the host blood meal. Conversely, and by analogy to mammalian neutral endopeptidase function (Menozzi et al, 1991), the *H. contortus* MEPs may act to maintain parasite gut homeostasis through the active degradation of host-derived neuropeptides which may otherwise activate parasite opioid receptors leading to a reduction in parasite gut motility.

Contribution to work

I carried out all the work presented in this paper with the exception of raising antibody to recombinant protein in sheep (WD Smith) and subsequent affinity-purification and biotin-labelling of specific antibody (GFJ Newlands). I was responsible for experimental design, interpretation of results and their communication at relevant scientific meetings, and preparation of the manuscript for publication.

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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*[1]

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Abstract

Antisera from lambs immunised with the *Haemonchus contortus* integral membrane protein complex, *Haemonchus* galactose-containing glycoprotein (H-gal-GP), the lambs being refractory to subsequent challenge, were used to identify several clones from an adult *H. contortus* λgt11 cDNA library. Using gene-specific oligonucleotide primers in conjunction with primers directed to a conserved nematode Spliced Leader (SL) sequence and to the polyA$^+$ tail of mRNA, the remaining 5′ and 3′ sequences of one of these clones, metallopeptidase-1 (MEP1), were amplified. The 2.4 kb full-length coding sequence was subsequently amplified in a single reaction. Sequence analysis identified MEP1 as encoding a putative zinc metallopeptidase, which shared limited homology with the mammalian type II integral membrane protein neutral endopeptidase (NEP). Southern blotting indicated that MEP1 belonged to a multigene family. MEP1 was expressed in bacteria as a glutathione-*S*-transferase (GST) fusion protein, and a specific antiserum raised in sheep. This antiserum recognised several polypeptide components of H-gal-GP. Immunolocalisation studies showed that MEP1 encoded a protein located on the luminal surface of the nematode gut. Both MEP1 mRNA and protein are developmentally regulated with expression being limited to the blood-feeding stages of *H. contortus*.

Keywords: *Haemonchus contortus*; Metallopeptidase; Neutral endopeptidase; Polymerase chain reaction

Abbreviations: GST, glutathione-*S*-transferase; H-gal-GP, *Haemonchus* galactose-containing glycoprotein; NEP, neutral endopeptidase; RT-PCR, reverse transcribed polymerase chain reaction; SL, spliced leader.

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[1] *Note:* Nucleotide sequence data reported in this paper is available in the GenBank data base under the accession number Z75054.

1. Introduction

Haemonchus contortus is a highly pathogenic blood-feeding nematode which resides in the abomasum of sheep, goats and other small ruminants. In the ovine host, infection can induce anaemia,

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weight loss and, in severe cases, death [1] and is, consequently, of considerable worldwide economic importance.

Current control of haemonchosis is dependent on the use of anthelmintic drugs. However, the rapid and widespread emergence of anthelmintic resistant strains of *H. contortus* [2] has resulted in increased pressure to develop alternative means of control.

Substantial protection against *H. contortus* infection in both sheep and goats has been achieved by immunisation with either crude extracts of intestines dissected from the adult parasites [3,4] or by using individual antigens or antigen complexes purified from such extracts [5,6]. As these proteins are not recognised serologically by sheep which have acquired immunity following natural infection [4], they are often termed 'hidden' antigens.

Recently, Smith et al. [7] described the isolation of a galactose-containing glycoprotein complex (H-gal-GP), from integral membrane protein extracts of adult *H. contortus*. The complex was shown to be highly protective against homologous challenge in sheep protection trials, reducing mean challenge worm burdens by up to 72% and mean faecal egg counts by up to 93% [7]. The glycosylated H-gal-GP complex is located on the luminal surface of the parasite's intestinal cells and can be fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) into four or eight major bands under non-reducing and reducing conditions, respectively [7]. In addition, enzyme analysis of H-gal-GP showed it to contain both aspartyl proteinase and neutral endopeptidase (NEP) activity [8].

Here we report the isolation, characterisation and expression of a full-length cDNA encoding a protein which exhibits limited homology with mammalian NEP and which is a component of the protective H-gal-GP protein complex.

2. Materials and methods

2.1. Parasites

Clean *H. contortus* were harvested from the abomasa of donor lambs 7 to 28 days post-infection as described previously [9]. Parasites were stored in liquid nitrogen until required.

2.2. Isolation of nucleic acids

RNA was isolated from adult *H. contortus* using guanidinium thiocyanate and phenol-chloroform extraction [10]. Polyadenylated mRNA was isolated as described by Sambrook et al. [11] except that SDS was omitted from the buffers. Genomic DNA for Southern blot analysis was extracted from adult parasites according to standard protocols [11].

2.3. Construction and screening of cDNA library

Single (ss cDNA) or double stranded cDNA (ds cDNA) was synthesised according to the manufacturer's instructions using the Amersham 'cDNA synthesis plus' kit and an oligo dT primer. An adult *H. contortus* λgt11 library was constructed as per manufacturer's instructions (Stratagene) and subsequently screened using pooled sera from lambs which had been immunised with H-gal-GP and which were demonstrably protected against challenge infection with *H. contortus*.

2.4. DNA sequencing

Insert DNA from λgt11 immunopositive clones was amplified from plaque pure DNA stocks using the polymerase chain reaction (PCR) essentially as described in [12], digested with *Eco*RI and ligated into the Bluescript SK$^+$ (Stratagene) plasmid vector. Plasmid DNA was isolated using the alkaline lysis method [11] and sequencing performed using the Pharmacia T7 sequenase sequencing kit. Nucleotide and deduced amino acid sequences were compared to existing sequences in the GenBank and SwissProt databases respectively using the University of Wisconsin Genetics Computer, Sequence Analysis Software Package Version 8.0-UNIX, 1994.

2.5. PCR amplification and cloning of full-length cDNA

The PCR strategy and primer sequences are summarised in Fig. 1. Fifty ng adult *H. contortus*

ss cDNA were used as target under the following reaction conditions: after initial denaturation at 94°C for 1 min, DNA was amplified in 30 cycles of 94°C, 1 min; 45°C, 1 min; 72°C, 3 min, with a final 8 min extension at 72°C, with the exception of reaction P2 to dT where annealing was performed at 25°C. The Spliced Leader-1 (SL1) primer [13] and gene-specific P1 primer were used to obtain the 5′ end of cDNA corresponding to clone MEP1. The gene-specific primer P2 and an primer directed to the PolyA$^+$ tail were used to obtain the 3′ end of the transcript. Sequence analysis allowed the translational start and stop signals to be determined. Oligonucleotide primers P3 and P4 were designed to these regions and *Bam*HI and *Sma*I restriction enzyme recognition sites added to the 5′ ends of the primers respectively to facilitate in-frame cloning of the full-length P3/P4 amplified product. Following digestion with *Bam*HI and *Sma*I, the PCR product was ligated into *Bam*HI/*Sma*I digested pGEX-2T (Pharmacia) bacterial expression vector and the construct transformed into competent JM109 *E. coli* cells using standard methods.

2.6. Southern and Northern blotting

For Southern blot analysis *Eco*RI, *Hin*dIII or *Bam*HI digested genomic DNA was fractionated on a 0.8% (w/v) agarose gel and transferred onto a Hybond-N nylon membrane (Amersham) under standard conditions [14]. For Northern blot analysis, adult *H. contortus* mRNA was fractionated on a 1.3% denaturing formaldehyde gel as described [15] and blotted as above. P3/P4 PCR amplification product was used as probe and was labelled according to the manufacturer's instructions using the 'DIG-labelling' kit (Boehringer Mannheim). Hybridisations were performed under conditions of high stringency (at 42°C overnight in DIG Easy Hyb buffer [Boehringer Mannheim]). The membranes were washed in 1 × SSC (150 mM NaCl, 15 mM sodium citrate, pH7)/0.1% (w/v) SDS for 30 min at room temperature with one change of buffer followed by 2 × 15 min washes in 0.2 × SSC/0.1% (w/v) SDS at 65°C and developed according to the manufacturer's instructions using the Boehringer Mannheim 'DIG Nucleic Acid Detection' kit.

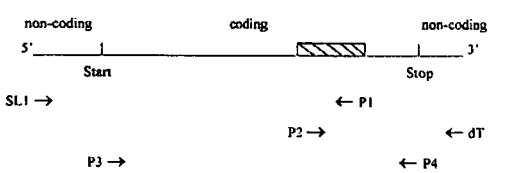

Fig. 1. Location of PCR primers used in amplification of the 5′, 3′ and full-length coding region of the *H. contortus* gene MEP1. Arrows indicate the direction in which DNA amplification was initiated from each primer. The boxed region corresponds to the relative position of the insert cDNA from immunopositive clone MEP1. Primer sequences (5′–3′) were as follows: P1, GTCCTCCTATGTCAGCAACG; P2, GCTCGCCTGTGGTCCTGATA; P3, GTCGGATCCATGCGTTACCTGGATGAC; P4, GTCCCGGGCTAGTTGTCGACCCAGACC; dT, ACAGAATTC T_{15}; SL1, GGTTTAATTACCCAAGTTTGAG.

2.7. Expression and Purification of Recombinant Protein

Expression of the recombinant protein was induced with IPTG (1 mM final concentration) for 3 h at 37°C. Cells were pelleted by centrifugation at 1200 × *g* for 10 min, the supernatant removed and the cells lysed by resuspending in 1/10 vol. 10 mM Tris–HCl pH 7.4, 1 mM EDTA pH 8.0 (T.E.) containing 100 μg ml^{-1} lysozyme and 0.1% (v/v) Triton X-100. After incubation at 30°C for 15 min, samples were held on ice for 30 min and nucleic acids degraded by the addition of DNase and RNase A to a final concentration of 10 μg ml^{-1} in the presence of 8 mM $MgCl_2$. Soluble and insoluble fractions were separated by recentrifugation and analysed in 7.5% discontinuous SDS-PAGE gels [16] under reducing conditions. Proteins were visualised by coomassie blue staining.

2.8. Antibody production and purification

Using the method of Shagger and von Jagow [17], insoluble fractions containing the recombinant GST-MEP1 fusion protein were run on 6–12% preparative gradient gels (BioRad Protean II

vertical slab gel apparatus), a gel slice containing the recombinant protein excised and the protein electro-eluted (Atto AE3590). A single sheep was given an initial injection of 10 μg of purified recombinant protein followed by two injections of 30 μg in QuilA (Superfos) intramuscularly at three weekly intervals. Serum was collected one week after the final injection. Electro-eluted recombinant *H. contortus* GST-MEP1 fusion protein (1 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer's instructions. Sheep antiserum (1 ml) was applied to the column and bound immunoglobulin eluted with 6 M urea in 0.1 M citrate buffer containing 0.5 M NaCl, pH 2.2. Eluted material was dialysed overnight against phosphate buffer saline (PBS) containing 0.01% (w/v) NaN_3. Affinity-purified sheep antibody was then labelled with biotin using the method of Goding [18] and dialysed overnight against PBS containing 0.01% (w/v) NaN_3.

2.9. Immunolocalisation

Twenty one day old *H. contortus* were chopped into small segments on ice cold PBS, embedded in 3% (w/v) agar, fixed overnight in Modified Bouins fluid (4.9% (v/v) 40% formaldehyde, 92.7% (v/v) saturated aqueous picric acid, 2.4% (v/v) glacial acetic acid) overnight and subsequently processed and embedded in paraffin wax. Sections 5 μm thick were probed with biotinylated antibody for 1 h at room temperature followed by optimally diluted streptavidin-horse radish peroxidase (HRPO [Sigma]). Immunoreactivity was revealed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide as substrate.

2.10. Western blotting

Integral membrane proteins were prepared from 0.25–0.5 g (wet weight) adult *H. contortus* by successive extractions with PBS, Tween 20 and TritonX-100 [7], the supernatant from the latter being used for Western blots. *H. contortus* membrane protein extracts were separated on a 10% discontinuous SDS-PAGE gel under reducing conditions and electrophoretically transferred onto Immobilon P membrane (Millipore). After periodate treatment to block carbohydrate epitopes [19], blots were probed with affinity-purified GST-MEP1 antibody (1 μg ml^{-1}) overnight at room temperature. Following extensive washing in 25 mM Tris, 137 mM NaCl, 2.7 mM KCl pH 7.4, 0.05% (v/v) Tween 20 (TBST), the membrane was incubated in HRPO-conjugated donkey anti-sheep IgG (Scottish Antibody Production Unit) at a 1/500 dilution in TBST for 1 h, re-washed and developed with DAB and hydrogen peroxide.

2.11. Developmental PCRs

H. contortus genomic DNA or ss cDNA was amplified in a PCR reaction using the MEP1 sequence specific primers P1 and P5 (5'-GAATGCTCAAACGATTTGCC-3') and reaction conditions as described above. Amplification from the pGEX-2T GST-MEP1 construct was included as a positive control. Amplified products were separated on a 0.8% (w/v) agarose gel and blotted onto Hybond-N^+ nylon membrane (Amersham) in 0.4 M sodium hydroxide according to the manufacturer's recommendations. The membrane was probed with DIG-labelled full-length MEP1 under high stringency conditions as described above.

3. Results

3.1. cDNA library screening and analysis of immunopositive clones

Screening with anti-H-gal-GP serum and subsequent antibody elution experiments identified six clones encoding components of H-gal-GP. Subsequent sequence analysis showed that two of these clones were identical and that their deduced amino acid sequence shared 42% identity with human, rabbit and rat NEP. One of these clones, MEP1, was used in the subsequent PCR cloning strategy.

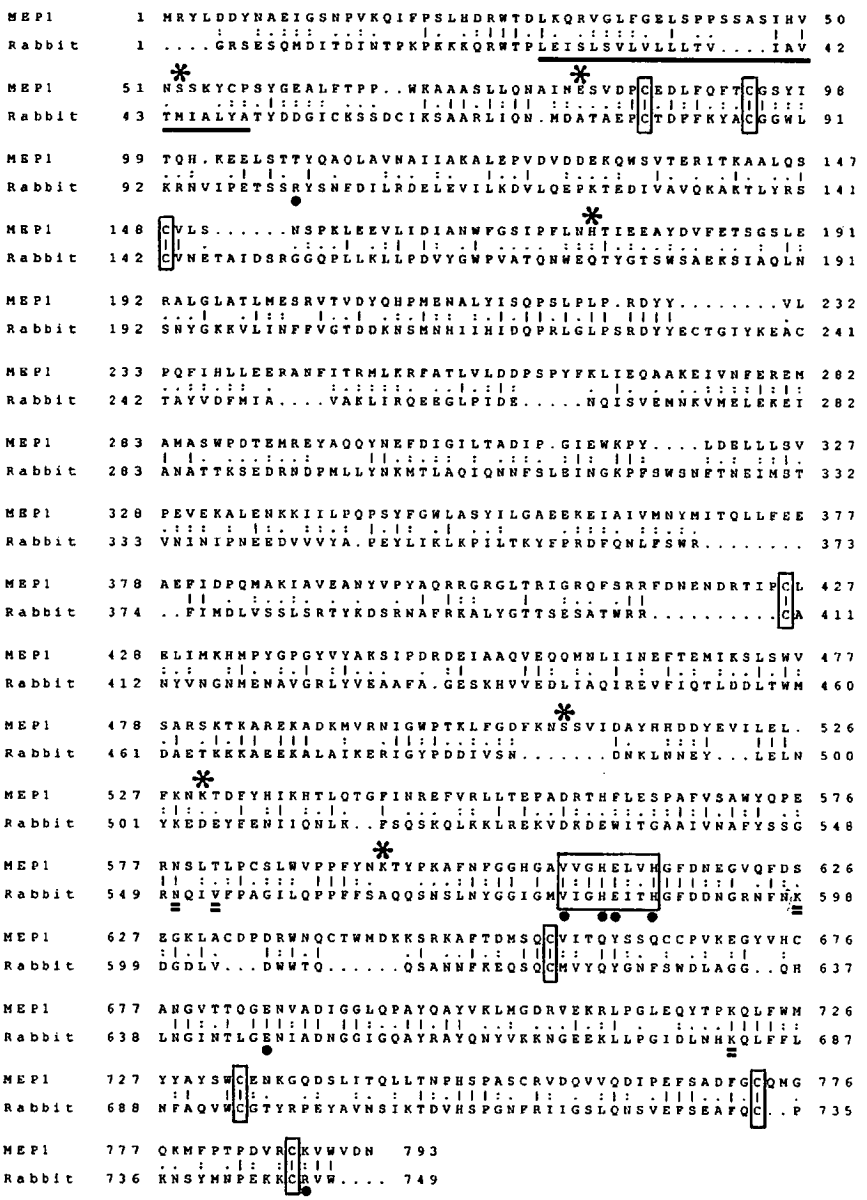

Fig. 2. Alignment of the predicted amino acid sequence of the full-length coding region of gene MEP1 with rabbit NEP (Genebank accession No. P08049). The six potential N-linked glycosylation sites (N-X-T/S) present in MEP1 are highlighted with asterisks. Eight cysteine residues conserved between the two sequences are boxed as is the putative neutral zinc metallopeptidase active site consensus sequence (VxxHExxH). The 24-aa transmembrane region of rabbit NEP is underlined. Conserved aa identified as critical for mammalian NEP-substrate binding and /or catalytic activity (Val 580, His 583, 587, Glu 584, 646, Arg 102, 747) are noted (•). The four amino acid residues thought to act as hydrogen bond donors during NEP-substrate catalysis are double underlined.

3.2. Amplification and cloning of full-length cDNA encoding MEP1

The relative positions of the PCR primers used to amplify the 5′ and 3′ sequences of the selected recombinant and the subsequent amplification of the full-length coding region are shown in Fig. 1. The products of SL1 to P1 (2.17 Kb) and P2 to dT (0.68 Kb) were sequenced and a single base pair change noted in the 191 bp region where these sequences overlapped. To ensure that the products from the separate 5′ and 3′ PCR reactions were encoded by the same cDNA, primers P3 and P4 were designed to the consensus coding sequences at the predicted translational start and stop codons, respectively, to allow the full-length coding region to be amplified in a single reaction. As predicted from the sequences already obtained, a specific product of 2.4 Kb was amplified.

3.3. DNA sequence analysis

The deduced amino acid sequence of the 2.4 Kb PCR amplification product (designated MEP1) and its alignment with rabbit NEP is shown in Fig. 2. Amino acids 624–728 were found to be identical to the cDNA insert of the original selected immunopositive clone. The sequence encoded an open reading frame of 793 amino acids and contained six potential N-linked glycosylation sites. A database search showed that the predicted amino acid sequence of MEP1 shared 50% similarity and 25% identity with human, rabbit and rat NEP sequences. Amino acid differences were distributed along the gene length although several residues required for enzyme activity (marked in Fig. 2) were conserved between MEP1 and the mammalian NEP sequences including a putative neutral zinc metallopeptidase active site consensus sequence (VxxHExxH) encoded by amino acids 608–615. In addition, the relative positions of eight cysteine residues was common to both the MEP1 and mammalian NEP sequences.

3.4. Northern and Southern blot analysis

A random primed probe corresponding to the full-length coding region of MEP1 hybridised to a single mRNA transcript of approximately 2.6 Kb under high stringency conditions (data not shown). Southern blotting revealed a complex profile of hybridising bands with each restriction enzyme (Fig. 3).

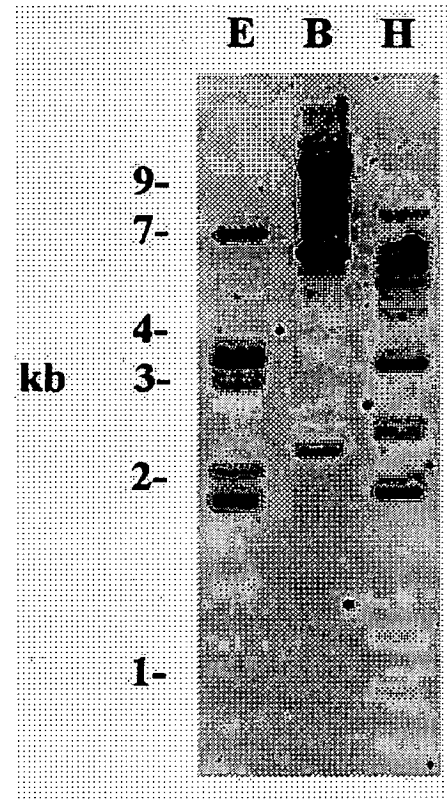

Fig. 3. Autoradiograph of Southern blot in which adult *H. contortus* genomic DNA digested with *Eco*RI (E), *Bam*HI (B) and *Hin*dIII (H) was probed with DIG-labelled PCR product corresponding to the full-length coding region of MEP1. Migration of 1 kb DNA molecular standards (Gibco BRL) was as indicated.

3.5. Expression of recombinant MEP1

Following induction of the recombinant GST-MEP1 fusion protein, soluble and insoluble fractions were analysed by SDS-PAGE. A prominent band of approximately 105 kDa was evident in the insoluble preparation (data not shown).

3.6. Immunolocalisation

The recombinant GST-MEP1 protein was purified, specific antiserum raised in sheep and affinity purified as described in Section 2. The affinity-purified antibodies reacted strongly with four polypeptides of reduced H-gal-GP by Western blotting (Fig. 4) and, on *Haemonchus* sections stained the microvillar surface of the gut strongly, with fainter staining being observed in the gut lumen (Fig. 5).

3.7. Developmental expression of MEP1

The developmental pattern of MEP1 expression was determined using gene-specific internal oligonucleotide primers and RT-PCR. The results are shown in Fig. 6A. A specific 1.3 Kb product was obtained from L4, 11 day, 22 day and 28 day *H. contortus* only. The pattern of protein expression within these stages was determined by probing Western blotting extracts of worm membranes with affinity-purified sheep anti-recombinant GST-MEP1 (Fig. 6B). The temporal pattern of protein expression was identical to mRNA expression in that reactivity was observed in the L4, 11, 22 and 28 day worms only. This reactivity was similar to that observed with H-gal-GP with bands at approximately 130, 91, 47 and 41 kDa, except that an additional component of 115 kDa was also recognised.

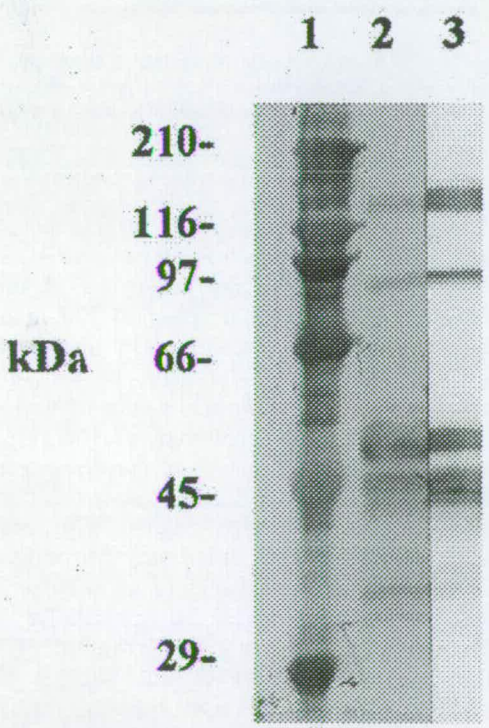

Fig. 4. Western blot of reduced H-gal-GP with the coomassie blue-stained profile of the protein complex shown in lane 2. A Western blot strip probed with affinity purified anti-recombinant GST-MEP1 is shown in lane 3. Molecular weight markers are shown in lane 1.

4. Discussion

We report here the isolation of a cDNA encoding a putative metallopeptidase (MEP1) expressed by *H. contortus* which is a component of the protective gut glycoprotein complex H-gal-GP.

The 2.4 kb coding region of MEP1 predicted a 793 amino acid protein with a polypeptide core of approximately 80 kDa. MEP1 showed maximal homology (50% similarity, 25% identity) to rabbit (Fig. 2), human and rat NEP. The metallopeptidase NEP is a type II integral membrane protein which exhibits a diverse tissue distribution and which is present in microvillar structures such as the brush border of kidney [20] and intestine [21]. NEP is responsible for the degradation of a variety of pharmacologically active peptides both in nervous and peripheral tissues, with enzyme activity being zinc-dependent. The zinc metallopeptidase family of proteinases also includes carboxypeptidase, angiotensin converting enzyme, collagenases and bacterial thermolysin. Although individual sequences are comparatively divergent, they all show conservation in the region which is involved in the binding of zinc and in catalysis [22]. This consensus active site sequence VxxHExxH is encoded by amino acids 608–615 in MEP1 as VVGHELVH. By analogy to bacterial thermolysin [23], the two histidine residues are zinc-coordinating ligands while the glutamine plays a role in catalysis through polarising a water molecule.

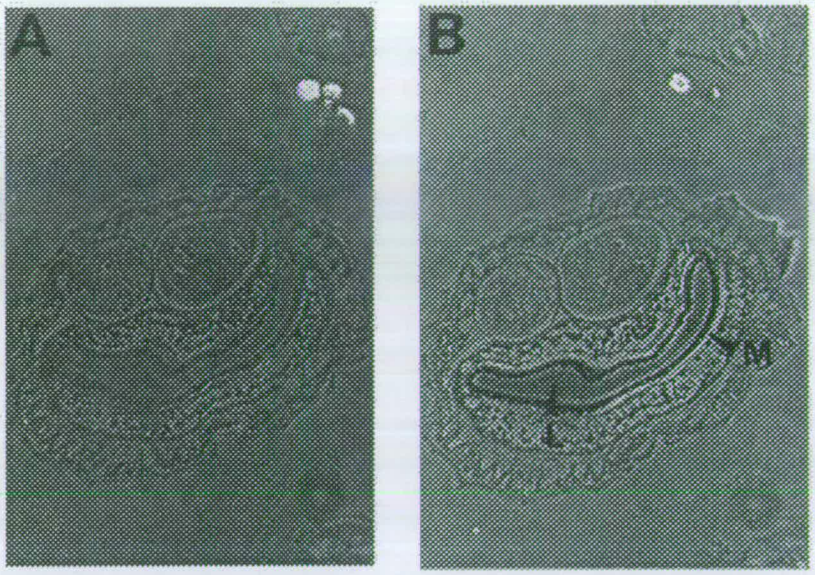

Fig. 5. Immunolocalisation of the gene product of the *H. contortus* gene MEP1. Micrographs (phase contrast microscopy, × 200 magnification) of transverse sections through adult *H. contortus* probed with either pre-immune control serum (A), or with affinity-purified anti-recombinant GST-MEP1 serum (B). Immunostaining is evident on the microvillar surface of the gut (M) and in the gut lumen (L). Diameter of sections is approximately 300 μm.

Although showing only weak homology with other zinc metallopeptidases, the mammalian NEP sequences share greater than 90% primary amino acid sequence identity and 100% conservation of critical amino acids and functional motifs [24]. A best-fit alignment with rabbit NEP (Fig. 2) indicates that, in addition to the conserved active site consensus sequence, some of these residues are present in MEP1. For example, of four NEP residues thought to act as hydrogen bond donors stabilising the intermediate enzyme-substrate complex [24], two, Asn 550 and Lys 683, aligned with Asn 578 and Lys 721 in the MEP1 sequence. Glu 646, which functions as a zinc coordinating residue in mammalian NEP [24], aligned with Glu 685 in MEP1. The active site Arg 747 in mammalian NEP, which interacts with the carbonyl amide group of the hydrophobic residue of the substrate where cleavage occurs [25], aligned with a lysine residue (789) in MEP1, although it is interesting to note that there is an Arg at position 786 in MEP1 which may be functional in the active site. A second active site Arg residue at position 102 in mammalian NEP was replaced by Thr in the MEP1 sequence. This Arg is thought to function in substrate binding although its contribution is very dependent on the nature of the substrate and has been shown not to be essential in mammalian NEP [26]. MEP1 contains sixteen cysteine residues, eight of which aligned with cysteine residues in NEP and which may form the four disulphide bridges necessary to maintain enzyme structure and activity [27]. Six potential N-linked glycosylation sites were identified in MEP1, consistent with the mature enzyme being a glycoprotein. MEP1 does not contain the short NH_2-terminal cytoplasmic and 23 amino acid hydrophobic transmembrane domains found in mammalian NEPs [24]. No regions of particular hydrophobicity were identified in MEP1, suggesting that the protein is not anchored into the

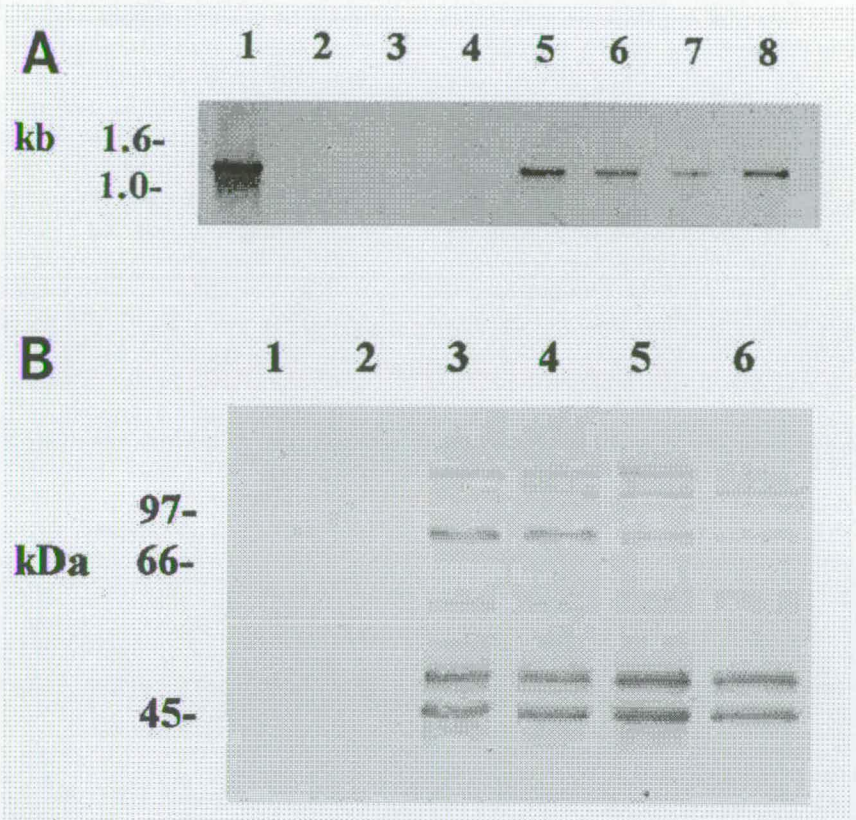

Fig. 6. Developmental expression of the *H. contortus* MEP1 gene. (A) cDNA from different developmental stages was used as template in a PCR amplification using MEP1 gene-specific primers to determine the pattern of mRNA expression. PCR products were blotted and probed with DIG-labelled probe corresponding to the full length coding region of MEP1. A PCR reaction using *H. contortus* genomic DNA as template (lane 2) was included as a control to confirm that the PCR products obtained from the developmental cDNA preparations were not derived from contamination with genomic DNA. Amplification from vector containing MEP1 was included as a positive control (lane 1). PCR was carried out from eggs, L3, L4, 11, 22 and 28 day *H. contortus* and the results shown in lanes 3–8, respectively. Migration of 1 kb DNA markers was as indicated. (B) The pattern of protein expression was determined by Western blotting. Membrane extracts of eggs, L3, L4, 11, 22, and 28 day *H. contortus* were probed using affinity-purified anti-recombinant GST-MEP1 serum and the results shown in lanes 1–6, respectively. Molecular weight markers migrated as indicated.

membrane itself but may form a complex with another membrane-anchored protein, as has been reported for proteins of the general secretory pathway in *E. coli* [28]. Immunolocalisation studies using affinity-purified antibody to the GST-MEP1 fusion protein showed the native protein to be located principally on the surface of the gut with specific staining of the microvilli, although some fainter staining was also observed in the gut lumen (Fig. 5). The latter might indicate a soluble form of the enzyme such as has been detected in human physiological fluids including plasma, cerebral spinal fluid and seminal plasma [22].

Southern blot analysis of *Haemonchus* genomic DNA probed with DIG-labelled MEP1 (Fig. 3) showed multiple hybridising bands, indicating that the peptide is probably encoded by a member of a multi-gene family. Using the same initial immunoscreening procedure, we have recently isolated a second cDNA fragment (MEP2) which encodes an open reading frame of 218 amino acids and which shares 71% homology with

MEP1 and 25% identity with mammalian NEPs. This cDNA hybridised to a mRNA transcript of 3.0 kb in size under Northern blot analysis (data not shown). When Western blot strips of H-gal-GP, fractionated under reducing conditions, were probed with an affinity purified antibody to the recombinant GST-MEP1 construct, several bands were recognised including bands of 91 kDa and 130 kDa (Fig. 4). These proteins may correspond to the glycosylated forms of the proteins encoded by the 2.6 and 3.0 kb mRNA transcripts described above. In addition, two bands at 41 and 47 kDa were strongly recognised. Presumably these were degradation products as antiserum raised to GST alone did not recognise any peptide components of H-gal-GP (data not shown), demonstrating that the reactivity was specific. However, the posibility that they are themselves distinct NEP-like sequences cannot be excluded. Shorter NEP mRNA transcripts, probably derived by alternative splicing, have been detected in brain tissue [29], human lung [30] and in B- and T-cells [31]. Their location, whether or not these proteins are expressed and if they are enzymatically active is, however, unclear.

MEP1 expression was shown to be developmentally regulated, mRNA transcript production and translation being restricted to the L4 and adult stages of the parasite (Fig. 6A,B). The appearance of a faintly reacting band of 115 kDa, which is not seen in H-gal-GP, may indicate expression of other MEP gene products in the parasite. This pattern of expression may indicate that the encoded protein is necessary for blood-feeding, the enzyme possibly being involved in proteolytic digestion of the blood meal.

However, in the mammalian gastro-intestinal tract, neuropeptide activation of opioid receptors can lead to a reduction in gut motility, especially through changes in ion and fluid transport across the mucosal cell membrane [32]. Opioid receptors are located in close proximity to NEP and aminopeptidases in the mammalian gut suggesting that all three may be required for neuropeptide degradation [33]. It is worthy of note that another highly protective gut membrane protein isolated from *Haemonchus* is an aminopeptidase (Munn et al., 1994, ICOPA VIII abstract S26.3 (1404), pp. 98). The enzyme encoded by MEP1, its potential homologues, as well as the H11 aminopeptidase, may have a co-ordinated function to digest the blood meal and/or to degrade ingested biologically active host-derived peptides in order to maintain gut homeostasis in the parasite.

The similarities noted to mammalian NEP, as well as the fact that the H-gal-GP protein complex has been shown to contain NEP-like enzyme activity as judged by the inhibition of azocasein hydrolysis at neutral pH by phosphoramidon, chelating agents and sulphydryl reagents [7], suggests that the sequence described here encodes an NEP homologue expressed on the surface of the nematode gut. However, there are sufficient fundamental differences between MEP1 and the mammalian equivalents for the enzyme to merit detailed analysis to assess its function in the gut and to determine if gut localisation is unique to *Haemonchus* and to other blood-feeding helminths. The results presented in this paper clearly demonstrate that MEP1 has potential as a sub-unit vaccine component or as a novel target for chemotherapeutic inhibition.

Acknowledgements

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Chapter 6: Molecular cloning and characterisation of a putative aspartate proteinase associated with a gut membrane protein complex from adult *Haemonchus contortus*.

Longbottom D, Redmond DL, Russell M, Liddell S, Smith WD and Knox DP. 1997. *Molecular and Biochemical Parasitology*, **88**: 63-72.

As discussed previously (see Chapter 4; Smith et al, 1999), H-gal-GP possesses marked proteinase activity, exhibiting metallo-, aspartyl and a sometimes detectable cysteine proteinase activity. A sheep vaccination trial using gel-eluted subfractions of H-gal-GP, separated on the basis of molecular size, showed that a degree of protection could be achieved by immunising with either a high molecular weight fraction (190-200 kDa) containing metallopeptidases, or a dyefront fraction (<55 kDa) expected to contain the aspartyl proteinase component of H-gal-GP (Smith and Smith, 1996). The H-gal-GP aspartyl proteinase has been identified as having a molecular size of ~40-42 kDa (under non-reducing conditions), as determined by N-terminal amino acid sequence analysis of the H-gal-GP subunit components (see Chapter 4; Smith et al, 1999). The molecular cloning and characterisation of an immunogenic metallopeptidase (MEP1) component of H-gal-GP was described in Chapter 5.

The aim of this paper was to clone and characterise the potentially protective aspartyl proteinase component associated with the dyefront fraction of H-gal-GP.

Biochemical analysis of H-gal-GP, as reported in Chapter 4, identified optimal aspartyl proteinase-associated haemoglobinase activity at pH 4. Here, that >90% inhibition of H-gal-GP haemoglobin hydrolysis was obtained by pre-incubation with the specific inhibitor pepstatin, demonstrated that the aspartyl proteinase component of H-gal-GP was primarily responsible for this activity. H-gal-GP was also shown to hydrolyse a specific aspartyl proteinase peptide substrate in a dose-dependent manner.

Immunoscreening of an adult *H. contortus* cDNA library with pooled sera from lambs immunised with H-gal-GP, and which were demonstrably protected from

challenge infection, identified six immunopositive clones. Nucleotide sequence analysis of insert cDNA identified two clones encoding MEP1 (see Chapter 5) and a third encoding a related MEP sequence. Nucleotide sequence analysis of a further clone showed it to contain a full-length sequence with homology to the mammalian aspartyl proteinase precursor, pepsinogen. Alignment of the deduced amino acid sequence of the *H. contortus* pepsinogen (PEP1) with mammalian sequences revealed conservation of critical elements, including the two active site regions. *H. contortus* PEP1 contains three potential N-linked glycosylation sites. The aspartyl proteinase components of H-gal-GP bind both wheatgerm and lentil lectin (Smith et al, 1994b) indicating that at least one of these sites is utilised. N-terminal amino acid sequence data obtained from the 40 kDa component of H-gal-GP (see Chapter 4) corresponded to the PEP1 sequence commencing at the predicted signal cleavage site, providing confirmation that PEP1 encodes the aspartyl proteinase component of H-gal-GP.

PEP1 hybridised to a transcript of 1.5 kb on Northern blots and gave a simple banding profile, consistent with a single copy gene, by Southern blot analysis.

Oligonucleotide primers were designed to PCR amplify cDNA encoding both full-length pepsinogen precursor and the predicted mature enzyme, and to facilitate in-frame cloning into the pET22b bacterial expression vector. Both recombinant proteins were expressed as insoluble inclusion bodies, as determined by SDS-PAGE analysis of bacterial fractions. When used individually in a sheep protection trial, neither recombinant protein afforded any protection against subsequent *H. contortus* challenge (unpublished results). Recombinant mature *H. contortus* pepsin was solubilised in urea and used to raise antibodies in both rabbits and sheep. Specific antibodies were purified from sheep serum by affinity chromatography and used to probe paraffin wax sections of adult *H. contortus*. Immunostaining was localised to the surface of the adult gut but was also evident in the gut lumen, suggesting liberation of a soluble form of the enzyme which is not complexed to H-gal-GP.

When used to probe a Western blot profile of H-gal-GP after non-reducing SDS-PAGE, antibodies to recombinant PEP1 recognised two bands of 40 and 42 kDa. As mentioned above, N-terminal amino acid sequence analysis of the 40 kDa band corresponded to the PEP1 amino acid sequence commencing at the predicted

signal peptide cleavage site. Sequence data for the 42 kDa band was very similar, but possessed an additional 4 amino acids at the N-terminus, and an additional internal DY, perhaps indicating alternative processing or the presence of minor variants within the worm population.

The temporal pattern of *H. contortus* pepsinogen steady-state mRNA levels and protein expression was analysed by RT-PCR, and by probing Western blots of membrane-bound extracts of the different developmental stages of *H. contortus* with rabbit anti-PEP1 serum. In both cases, expression was restricted to the blood-feeding L4 and adult stages.

In conclusion, a full-length cDNA sequence (PEP1) encoding a *H. contortus* protein with homology to the mammalian aspartyl proteinase precursor, pepsinogen, has been isolated. That the PEP1 sequence contains N-terminal amino acid sequence obtained from a peptide subunit of H-gal-GP and that antiserum raised to bacterially expressed recombinant PEP1 binds strongly to two bands of ~40 kDa on Western blots of H-gal-GP, previously identified as the aspartyl proteinase component of this complex by virtue of their binding to pepstatin-agarose, is evidence that PEP1 encodes the potentially protective aspartyl proteinase component of the H-gal-GP complex. The *H. contortus* pepsinogen shows high affinity for haemoglobin as substrate, localises to the parasite gut and is expressed only in the blood-feeding L4 and adult stages, strongly suggesting that this enzyme plays a critical role in digestion of the host blood meal and is, therefore, a pertinent candidate vaccine antigen.

Contribution to work

Library construction, immunoscreening and preliminary sequencing of the pepsinogen clone were carried out by D Longbottom, M Russell and S Liddell. All other work presented in this paper was initiated and carried out by myself. Communication of results at scientific meetings. Preparation of "Materials and Methods" and "Figures" for publication.

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Molecular cloning and characterisation of a putative aspartate proteinase associated with a gut membrane protein complex from adult *Haemonchus contortus*[1]

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Abstract

A cDNA was isolated from an adult *Haemonchus contortus* cDNA expression library the deduced amino acid sequence of which showed significant homology to mammalian pepsinogen sequences. The library was screened with antisera raised against *Haemonchus* galactose-containing glycoprotein complex, a gut membrane protein complex with aspartyl proteinase activity which has shown considerable potential as a protective antigen. The amino acid sequence obtained corresponded very closely in part to the N-terminal amino acid sequences of two polypetides within the complex. The enzyme was shown to be almost exclusively expressed by the blood-feeding parasite stages. The cDNA was expressed in *E. coli*, and antibody produced to the recombinant protein bound to the luminal surface of the gut in the adult parasite. The proteinase may play a central role in digesting the blood meal and is considered a potential sub-unit vaccine candidate. © 1997 Elsevier Science B.V.

Keywords: *Haemonchus contortus*; Aspartyl proteinase; Pepsinogen; H-gal-GP; Gut expression

1. Introduction

A recent report [1] described the isolation of a galactose-containing glycoprotein complex, termed H-gal-GP, from integral membrane protein extracts of the adult *Haemonchus contortus*. Sheep immunisation trials showed that the complex was highly protective against homologous challenge, reducing mean worm bur-

Abbreviations: H-gal-GP, *Haemonchus* galactose-containing glycoprotein; Pep, pepsinogen; RT-PCR, reverse transcribed polymerase chain reaction; SL, spliced leader; IPTG, isopropyl-thio-β-D-galactoside.

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[1] *Note*: Nucleotide sequence data reported in this paper are available in the EMBL and GenBank™ databases under the accession number Z72490.

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dens by up to 72% and mean faecal egg counts by up to 93% [1]. This complex is glycosylated, selectively binding to lectins with specificity for *N*-acetylgalactosamine, and is located on the luminal surface of the intestinal cells of the parasite. It can be fractionated into four or eight major bands using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing or reducing conditions respectively [1]. In addition, H-gal-GP was shown to contain weak proteinase activity towards the general protein substrates azocasein and gelatin and strong activity towards haemoglobin [2]. Furthermore, it was found that two components of H-gal-GP, each with an apparent molecular mass under non-reducing conditions of approximately 40 kDa, bound very avidly to the pepstatin-agarose. The present report describes the isolation of a cDNA from *Haemonchus* which encodes these components of H-gal-GP and the deduced protein sequence shows homology to mammalian pepsinogens.

2. Materials and methods

2.1. Purification of Haemonchus galactose-containing glycoprotein and proteinase activity determinations

H-gal-GP was purified from integral membrane protein extracts of adult *H. contortus* using lectin-affinity chromatography [1]. Haemoglobin degradation was monitored from pH 3.5–7.0 [3] and aspartyl proteinase activity sought using the peptide substrate, H-Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu-OH (Novabiochem; [4]).

2.2. Isolation of nucleic acids

RNA was extracted from 11-day old *H. contortus* using guanidinium isothiocyanate and phenol-chloroform extraction [5]. Polyadenylated mRNA was isolated by chromatography on oligo(dT)-cellulose [6] except that SDS was omitted from all the buffers. Genomic DNA for Southern blot analysis was extracted from 28 day parasites according to standard protocols [6].

2.3. cDNA synthesis and λgt11 library screening

cDNA synthesis and λgt11 library construction were performed as described previously [7]. A portion (50 000 plaque forming units) of the resultant library was immuno-screened using pooled sera from lambs which had been immunised with H-gal-GP [1] and which were demonstrably protected against challenge infection with *H. contortus*.

The H-gal-GP peptide component encoded by selected recombinant phage was identified using affinity purified antibody selected on a lawn of plaque-pure recombinant phage [8]. The antibody solutions were used to probe Western blot strips of H-gal-GP, fractionated using reducing SDS-PAGE.

2.4. DNA sequencing

Insert DNA from a selected recombinant, designated Pep1, was amplified by polymerase chain reaction (PCR) [9] and sub-cloned into the plasmid vector pCRII (TA cloning system, Invitrogen). The DNA sequence was determined using the M13 forward and reverse primers and specific primers directed to internal sequences of the insert using the Pharmacia T7 sequenase sequencing kit. The nucleotide and deduced amino acid sequences were compared to existing sequences in the GenBank and EMBL databases using the University of Wisconsin Genetics Computer Group, Sequence Analysis Software Package Version 8.0-UNIX, 1994.

2.5. Northern and Southern blotting

Nucleic acids were prepared by standard methods and transferred to a Hybond-N nylon membranes under standard conditions (Amersham, UK; [6]) and the blots hybridised with denatured Pep1 labelled using the T7 QuickPrime kit (27-9252-01, Pharmacia, UK) with [^{32}P]dCTP (Amersham, UK). Hybridisation was carried out overnight at 65°C [6] with two high stringency washes in 0.1 × SSC/0.1% SDS at 65°C, each for 15 min. Blots were analysed by autoradiography at −70°C using a Cronex lightening plus intensifying screen.

2.6. N-terminal amino acid sequencing

H-gal-GP (5 μg) was fractionated under non-reducing conditions by SDS-PAGE and transferred to ProBlot membranes (Applied Biosystems). After Coomassie blue staining, two distinct bands of about 40 and 42 kDa were excised from the membrane and the N-terminal amino-acid composition of each was determined on an Applied Biosystems Model 477A peptide sequencer.

2.7. Sub-cloning into pET plasmid expression vector

cDNAs encoding both the full-length pepsinogen precursor and the predicted mature enzyme were amplified using the primer pairings P1/P3 and P2/P3 respectively with primer annealing at 45°C. The primer positions are indicated in Fig. 2 and their sequences given in the figure legend. The sense primers, P1 and P2, contained *Pst*1 and *Nde*1 restriction enzyme recognition sites while the antisense primer, P3, included a *Bam*H1 site to facilitate in-frame sub-cloning into the vector.

The amplification products were purified from a 0.8% agarose gel and, following digestion with *Bam*H1 and *Nde*1, the 1.1 kb (mature enzyme) and 1.3 kb (proenzyme) fragments were ligated into the plasmid pET 22b (+) and used to transform competent BL21 *E. coli* (Novagen).

2.8. Induction of recombinant protein expression in pET

Liquid bacterial cultures (50 ml in L-broth/ampicillin; $OD_{600} = 0.5$) were induced by the addition of IPTG to 1 mM final concentration and incubated for a further 3 h. Recombinant protein expression was confirmed by SDS-PAGE analysis as described previously [7]. Bacterial cell pellets were suspended in 3 M deionised urea to solubilise contaminating bacterial proteins and, after centrifugation at 11 000 × *g*, the recombinant protein pellet was solubilised in 5 M urea/50 mM β-mercaptoethanol, denatured and rapidly diluted in 50 mM Tris pH 8.5 [10]. The recombinant protein was concentrated using Centricon-10 devices (Amicon) to give final pooled protein concentrations of about 840 μg ml^{-1}. Aliquots of this solution were diluted 1–4 with 50 mM Tris, pH 8.

2.9. Anti-pET 1.1 pep sera and affinity purification of antibodies

Recombinant pET 1.1 pep (50–100 μg) in Freund's complete adjuvant was administered subcutaneously to two rabbits and six sheep in 0.25 ml doses at two sites. The animals were boosted with antigen in Freund's incomplete adjuvant 3 and 6 (sheep) or 4 and 7 weeks after primary immunisation and blood samples were removed 14 days after each boost.

Insoluble fractions containing recombinant pET 1.1 pep protein were solubilised in SDS-PAGE sample buffer and subjected to blue SDS-PAGE [11]. Recombinant protein was harvested by electroelution and then coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Sheep antiserum (1 ml) was applied to the column and bound antibody was eluted with 6 M urea in 0.1 M citrate buffer, pH 2.2, containing 0.5 M NaCl. After dialysis against PBS, the antibody was biotinylated [12].

2.10. Immunohistology

Adult *H. contortus* sections were prepared as described previously [7] and probed with biotinylated antibody for 1 h at room temperature followed by optimally diluted streptavidin-HRPO (Sigma). Immunoreactivity was revealed using 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide as substrate. The sections were then counterstained with haematoxylin.

2.11. Developmental reverse transcribed polymerase chain reactions and Western blots

Single-stranded cDNA (ssDNA, 50 ng) was prepared from each parasite stage and used as template for a PCR reaction with Pep1 sequence-specific primers P2 and P3 and the reaction conditions described above. Amplified products were

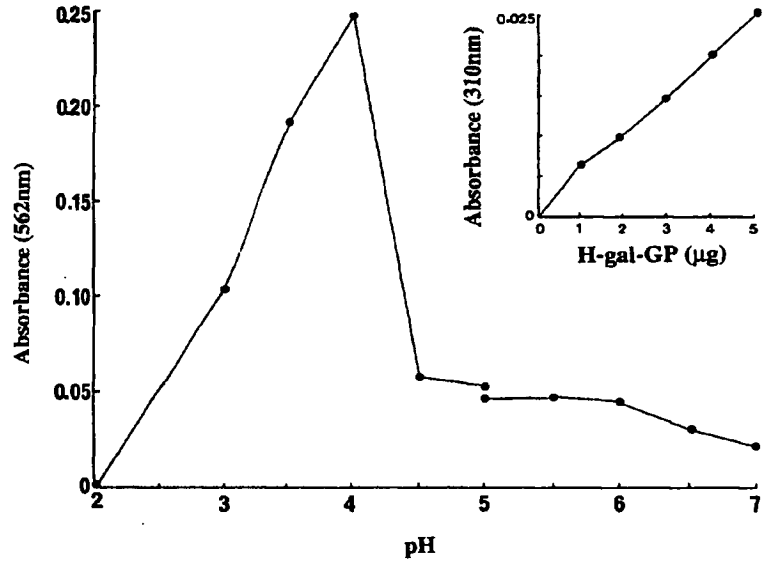

Fig. 1. Aspartyl proteinase activity of H-gal-GP. Haemoglobin degradation by H-gal-GP was monitored using 0.1 M acetate buffer (pH 2–5) and 0.1 M phosphate buffer (pH 5–7). The effect of adding increasing amounts of H-gal-GP on the rate (change in absorbance /min.) of hydrolysis of the pepsin substrate H-Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu-OH at pH 4 is shown in the inset panel.

separated on a 0.8% agarose gel (w/v), blotted onto Hybond N^+ nylon membrane (Amersham) and the blot probed under high stringency conditions with DIG-labelled Pep1 probe prepared from the portion encoding the putative mature enzyme.

Membrane-bound extracts of the different developmental stages of *H. contortus* were prepared and electrophoretically transferred to Immobilon-P membrane (Millipore) as described previously [7]. The blot was probed with rabbit anti-pET1 recombinant protein.

3. Results

3.1. *Haemonchus galactose-containing glycoprotein proteinase activity*

H-gal-GP hydrolysed haemoglobin over a relatively narrow acidic pH range with optimal activity being evident at pH 4 (Fig. 1). This activity was reduced by 91% (range 88–95%, $n = 5$) by the specific aspartyl proteinase inhibitor, pepstatin (1 μM). H-gal-GP hydrolysed a peptide substrate with specificity for aspartyl proteinases in a dose dependent manner (Fig. 1, inset).

3.2. *Sequence analysis*

A number of the selected recombinants contained the same sized insert (1500 bp), and antibody eluted from all these recombinants recognised a 40–42 kDa doublet in H-gal-GP fractionated under non-reducing conditions (not shown).

One of the selected recombinants, Pep1, contained a 1287 base pair open reading frame encoding a predicted 428 amino acid peptide. The deduced amino acid sequence showed highly significant homology to human (57.7% similarity, 33.5% identity) and porcine (56.6% similarity, 33.8% identity) pepsinogen A precursors. The alignment of the Pep1 deduced amino acid se-

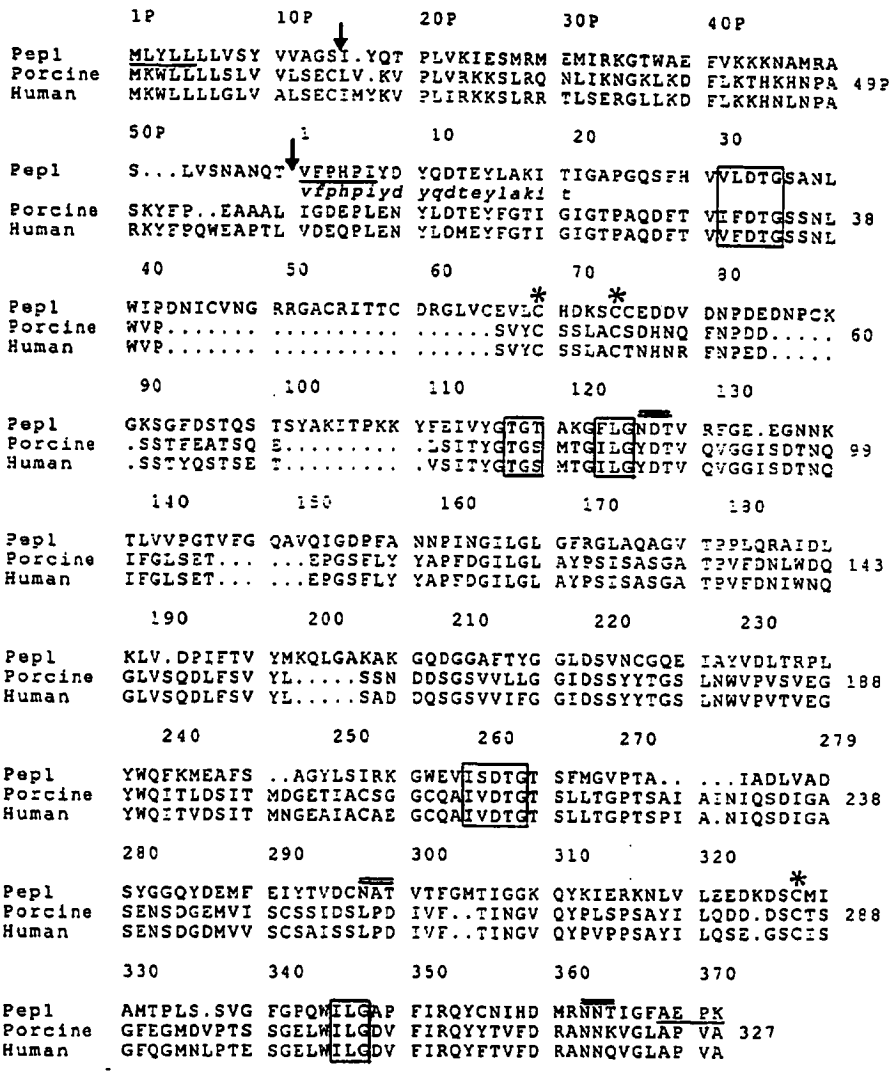

Fig. 2. Amino acid sequence alignment of Pepl with human and porcine pepsinogen. Amino acid numbering above the presented sequences are according to Pepl with the putative prepro segment of the sequence being followed by the suffix P. Numbering to the right of the porcine sequence is the numbering conventionally used for this sequence. The two active site X-X-D-T-G domains as well as other residues which may be involved in catalysis are boxed. Conserved cysteines (*) and potential N-linked glycosylation sites (=) are indicated. The N-terminal amino acid sequence of the 40 kDa component of H-gal-GP is shown in italics below amino acids 1–19. The positions of the primers used to amplify products corresponding to the preproenzyme (P1–P3) and the mature enzyme (P2–P3) are underlined. The full primer sequences including the restriction enzyme sites to facilitate subcloning were P1-*gtcctgcagcatatgctatatttattgctc*, P2-*gtcctgcagcatatggttttttcctcatccaatc* and P3-*gtcggatccatgttatttcggctccgc*

quence with human and porcine pepsinogen A precursor sequences is shown in Fig. 2. The amino acid residues comprising the predicted pre-proenzyme are numbered 1P, 2P etc. and the numbering for the putative mature enzyme commences from Val1. The Pep1 sequence contained several features consistent with a pepsinogen precursor including two putative active site regions (VLDTG, aa 30–34; ISDTG, aa 259–263), two potential peptidase cleavage sites (indicated by arrows in Fig. 2) and two cysteine residues (aa 68–73) involved in conformational disulphide bonding. It also contained 3 potential N-linked glycosylation sites.

Further confirmation that Pep1 encoded a pepsinogen-like component of H-gal-GP was obtained from the N-terminal amino acid sequence of the two major peptide components of the complex which migrate at 40 and 42 kDa using non-reducing SDS-PAGE (Fig. 3, lane 3) and were strongly recognised by affinity purified antibody

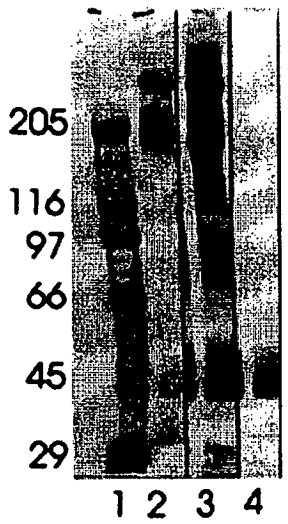

Fig. 3. Western blot identification of the peptide components of H-gal-GP. The H-gal-GP peptide profile obtained after non reducing SDS-PAGE and visualised by Coomassie staining is shown in lane 2. Lane 3 was probed with a sheep antiserum raised to H-gal-GP while Lane 4 was probed with affinity purified sheep antibody to the Pep1 recombinant protein. Molecular mass markers are shown in lane 1.

to recombinant Pep1 (Fig. 3, lane 4). Analysis of the 40 kDa band gave a sequence which commenced at the predicted signal peptide cleavage site and totally corresponded to the amino acid sequence predicted for Pep1 (Fig. 2). The sequence data for the 42 kDa band was very similar, but possessed an additional 4 amino acids (ANQT) at the N terminus and an additional DY after position 8 (Fig. 2).

3.3. Northern and Southern blot analysis

Radiolabelled probe DNA prepared from Pep1 hybridised to mRNA at approximately 1.5 kb on Northern blots under high stringency conditions (not shown).

Using *H. contortus* genomic DNA which had been digested with *Hin*dIII, *Bam*H1 or *Eco*R1 as target DNA, the Pep1 probe gave a simple profile of no more than two bands with only a single 2.1 kb signal in *Eco*R1 digests (not shown).

3.4. Expression of recombinant pepsinogen

Abundant peptides at approximately 43 and 45 kDa, strongly recognised by sheep anti-H-gal-GP serum, were evident in induced insoluble preparations from pET 22(b) + carrying the 1.1 and 1.3 kb cDNA inserts respectively but were not observed in either the soluble extracts or the uninduced preparations from either recombinant, or in any of the vector control preparations (not shown).

3.5. Immunohistochemical localisation

Affinity-purified antibody to pET 1.1 pep recombinant protein recognised, evident as brown staining, components on the microvillar surface and in the lumen of the gut in transverse sections of the adult parasite (Fig. 4, panel A). No reactivity was observed in other tissues such as the reproductive tract, body wall musculature and the cuticle (Fig. 4, panel B). No specific staining was evident in parasite sections probed with a pre-immunisation serum (Fig. 4, panel C).

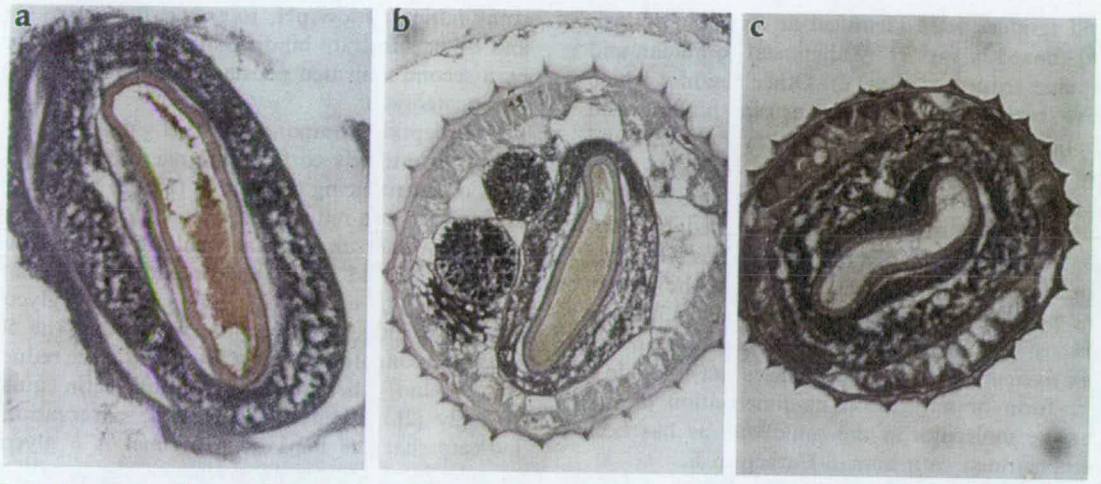

Fig. 4. Localisation of the peptide encoded by pET1 in adult *H. contortus*. A transverse section of the gut of adult *H. contortus* (panel A) and of the whole worm (panels B and C) were probed with either affinity purified biotinylated sheep antiserum to recombinant Pep1 (panels A and B) or with pre-immunisation serum from the same sheep (panel C) and then counterstained with haematoxylin. Strong brown staining was evident in the gut lumen and in the gut microvillar surface (panel A). Other worm structures were not immunostained (panel B). Each section is approximately 300 μm in diameter.

3.6. Developmental regulation of expression

Production of the mRNA transcript for Pep1 and the protein encoded was restricted largely to the late larval and adult stages (Fig. 5B), the quantitative production of both being relatively even from L4 through to 28 day adult parasites. The mRNA transcript was detectable faintly in eggs (Fig. 5B) and a very faint protein band of the correct size was also detected on a Western blot (Fig. 5A). By contrast, the transcript was not detected in L3 although a number of protein bands of the incorrect size were detected in L3 after Western blotting.

4. Discussion

A full-length cDNA (Pep1) is described encoding a pepsinogen-like aspartyl proteinase expressed almost exclusively by the blood-feeding stages of *H. contortus*. N-terminal peptide sequence analysis as well as Western blotting showed that the pepsinogen was a precursor of two polypeptide components of H-gal-GP, the gut antigen complex previously identified on the luminal surface of the adult parasite gut [1]. Biochemical analyses of H-gal-GP indicated that it contained a haemoglobinase with aspartyl proteinase properties (Fig. 1).

Pep1 encodes a pre-proenzyme with, by analogy to porcine pepsinogen processing [13], cleavage of the first 15 amino acids to give the proenzyme followed by cleavage between Thr 58P and Val1 to yield the mature enzyme (See Fig. 2). That the latter cleavage can occur during enzyme processing is indicated by the identity observed between the N-terminal peptide sequence obtained for the 40 kDa (shown in Fig. 2) antibody-positive component of H-gal-GP (Fig. 3, lane 4) and the predicted start point of the mature protein described above. The N-terminal sequence for the 42 kDa peptide contained four additional residues at the N-terminus which may indicate alternative processing. The internal DY addition is more difficult to explain but may be due to the presence of minor variants within the worm population or be an artifact of the N-terminal sequence analysis.

Two putative active site domains comprising hydrophobic-hydrophobic-Asp-Thr-Gly amino

acid residues were identified at Asp 32 and Asp 261 (boxed in Fig. 2) by alignment to human and porcine sequences (Fig. 2). Other residues which have been implicated in substrate binding and peptide folding [13–15] are conserved in Pepl and are indicated in Fig. 2.

In general terms, the predicted amino acid sequence of the mature enzyme encoded by Pepl contains a number of cysteine residues which do not align with equivalent residues in the human and porcine sequences. It was proposed [13] that a SH group contributed by Cys 210 in a *Plasmodium falciparum* aspartyl proteinase may exist in a free form or may result in dimerisation of two enzyme molecules in the same way as has been demonstrated with human Cathepsin E.

The digestive proteinases identified to date in *H. contortus* have mainly acidic pH optima [3] and are similar to those reported for schistosomes in which the gut luminal pH is reportedly acidic [16]. By analogy with pepsinogen [15], the proenzyme encoded by Pepl may be activated by a combination of low pH, to expose the active site and other substrate binding sites, and proteolysis by a second activated pepsinogen or, possibly, by autoproteolysis.

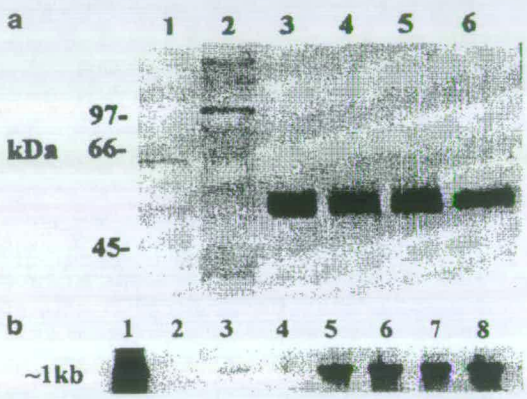

Fig. 5. Developmental regulation of expression of the Pepl encoded protein. In panel A, extracts from eggs, L3, L4, immature (11 days) and mature (22 and 28 days) *H. contortus* (lanes 1–6 respectively) were fractionated using reducing 10% SDS-PAGE and Western blots then probed with antiserum to recombinant Pepl. In panel B, Pepl specific RT-PCR products from each parasite stage were probed with DIG-labelled Pepl. Amplification from vector containing Pepl was included as a positive control (lane 1) and from *H. contortus* genomic DNA (lane 2) to confirm the specificity of the amplification products. Amplification products obtained from eggs, L3, L4, 11d, 22 and 28 day worms are shown in lanes 3–8 respectively.

The peptide components of H-gal-GP recognised by antibody to Pepl migrated at about 50 kDa under reducing conditions (Fig. 5) and at 45 kDa under non-reducing conditions (Fig. 3). Reduction will break internal disulphide bonds and this may 'unfold' the peptide retarding migration in the gel. The native Pepl gene product is glycosylated as judged by the observation that the 50 kDa components of H-gal-GP evident after reduction bound wheatgerm and lentil lectin quite strongly [2]. Taken together, these observations indicate that the Pepl gene product is a glycoprotein expressed on the surface of the intestinal cells as demonstrated by immunolocalisation (Fig. 4).

Southern blot analysis indicated that Pepl was encoded by a single copy gene and the gene is expressed, almost exclusively, by the fourth larval and adult stages of the parasite as judged by probing Western blots of parasite extracts from a variety of time points in the life-cycle (Fig. 5A) and RT-PCR from the same parasite stages (Fig. 5B). This pattern of expression could indicate that the Pepl gene is required for blood digestion, a suggestion supported by the affinity for haemoglobin as substrate of H-gal-GP proteinase activity [2] and the observation that the Pepl gene product was localised exclusively to the surface of the gut in the adult parasite (Fig. 4). Immunostaining was evident in the gut lumen also indicating that the enzyme was released into the lumen and it was weakly evident in the in vitro excretions/secretions (ES) as judged by Western blotting (not shown). Aspartyl proteinase activity has been detected in adult *Haemonchus* ES [17] and in extracts of the adult parasite [3] and it is possible that this activity is due to the Pepl gene product.

As well as the pepsinogen-like aspartyl proteinase described here, adult *H. contortus* released cathepsin L-like cysteine proteases during in vitro maintenance [18] which degraded haemoglobin amongst other protein substrates tested. *Haemonchus* contains a multigene family encoding cathepsin B-like cysteine proteases [19],

one member of which (AC1, [20]) is possibly functional in fibrinogen degradation.. Moreover, cysteine proteinases in somatic extracts of the adult parasite degraded blood proteins and these proteinases differed from AC1 as judged by size differences [3]. It is clear that, like schistosomes, digestion of the blood meal is likely to be a complex process involving a number of different steps catalysed by different proteases [21]. Recently, an aspartic proteinase was cloned from *Schistosoma japonicum* and this enzyme was also encoded by a single copy gene. The authors suggested that it may play a pivotal role in haemoglobin digestion in this parasite [21]. The proteinase showed closest identity with mammalian cathepsin Ds and less identity to renins, pepsinogens and cathepsin E [22]. There is some debate concerning the proteases involved and sequence of events which lead to the digestion of the blood meal in schistosomes [21,23] but it is becoming clear that *H. contortus* contains an equivalent array of potential digestive proteases.

Acknowledgements

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Chapter 7: Expression of *Haemonchus contortus* pepsinogen in *Caenorhabditis elegans.*

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The isolation of full-length cDNAs encoding the potentially protective enzyme components of the adult *H. contortus* integral gut membrane complexes, TSBP and H-gal-GP, has been described previously (see Chapters 3, 5 and 6). Expression of these cDNAs in bacterial systems resulted, typically, in good yields of recombinant proteins expressed as insoluble, enzymatically inactive inclusion bodies. When assessed individually in sheep vaccination trials, these recombinant proteins afforded no protection against *H. contortus* challenge infection (unpublished results).

Increasing denaturation of the *H. contortus* hidden antigen H11, led to a progressive loss of its protective capacity (Munn et al, 1997). Similarly, sequential dissociation and reduction of the H-gal-GP complex resulted in less consistent levels of protection (Smith and Smith, 1996), suggesting that conformational epitopes and/or enzymatic activity are important for protection. For this reason, it is considered that expression of potentially protective components of H-gal-GP in a eukaryotic system will be necessary in order to obtain appropriate protein folding and post-translational modifications.

Several eukaryotic expression systems are available commercially, such as yeast (*Saccharomyces, Pichia)*, mammalian cell lines and insect cells. However, because of its short life cycle, ease of growth in the laboratory, well defined genetic manipulation techniques, and close evolutionary relationship to *H. contortus* - the Strongylida and Rhabditida comprising the same clade in nematode classification (Blaxter et al, 2000) - *C. elegans* is a tractable option for potential exploitation as a vector for the expression of candidate *H. contortus* vaccine antigens. *C. elegans* has already been used successfully as a model system for the elucidation of anthelmintic drug resistance in veterinary parasites (Driscoll et al, 1989; Dent et al, 2000), and as a host for the analysis of parasite gene function through the microinjection and

expression of *H. contortus* β-tubulin alleles in the study of benzimidazole resistance (Kwa et al, 1995).

A detailed study of adult *H. contortus* glycosylation (Haslam et al, 1996) revealed high mannose content and complex N-glycans, both of which have been shown to occur in *C. elegans* (Cipollo et al, 2002). In addition, in *H. contortus*, major N-linked glycans with up to three fucose residues attached to the chitobiose core were detected. Difucosylation of the 3 and 6 positions of the distal reducing N-acetylglucosamine (GlcNAc) can occur in invertebrates and was shown also to be present in *H. contortus*. Fucosylation at the 3 position of the distal GlcNAc has been shown to be a highly antigenic epitope in both insect (Prenner et al, 1992) and plant glycoproteins (Wilson et al, 1998). However, unique stage-specific fucosylation of the proximal GlcNAc was detected in adult *H. contortus* (Haslam et al, 1996 and 1998). Whether or not this high degree of N-glycan fucosylation occurs in H-gal-GP proteins, or in *C. elegans*, remains to be determined. However, the demonstration of conservation of some types of N-glycans between the two nematodes, together with their close evolutionary relationship, would suggest that more relevant glycosylation of recombinant *H. contortus* proteins is likely to occur in *C. elegans* as opposed to other eukaryotic expression systems.

The aim of the work presented here was to evaluate the potential of the free-living nematode *C. elegans* as a vector for expression of *H. contortus* candidate vaccine antigens.

Its potential critical role in blood feeding identifies the H-gal-GP aspartyl proteinase, pepsinogen, as a prime candidate vaccine antigen. A specific, high affinity antibody to recombinant *H. contortus* PEP1 exists (see Chapter 6), which can be used to analyse its expression in transgenic *C. elegans*. That there is no close homologue of PEP1 in *C. elegans* circumvents the need to produce either a knockout line of *C. elegans*, or to evaluate recombinant protein expression against an endogenous background level. These facts make *H. contortus* PEP1 an ideal gene for evaluation of expression in *C. elegans*.

The presence of intronic sequences has been shown to enhance the expression of some heterologous genes in *C. elegans* (Fire et al, 1990). Therefore, a genomic

copy of *H. contortus* pepsinogen was isolated by PCR using primers targeting the coding sequences surrounding the translational start and stop codons based on the previously determined cDNA sequence (see Chapter 6). Nucleotide sequence analysis of genomic pepsinogen and alignment with its corresponding PEP1 cDNA sequence allowed identification of intron/exon slice donor and acceptor sites. Eight introns, 54 to 1475 bp in size, were identified, the consensus splice donor and acceptor sequences being similar to those of eukaryotes in general, with the exception of intron 7, which started with the rare GC nucleotide pairing. Conservation of G at position 5 in all introns, considered a stringent requirement for correct splicing (Esumi et al, 1983), was observed.

Expression of a gene in *C. elegans* is achieved through microinjection of DNA containing all the elements necessary for its own expression, i.e. a functional promoter, sequences necessary for mRNA formation and a polyadenylation signal sequence. Because little is known regarding the mechanisms regulating gene expression in parasitic nematodes, it was decided to express the *H. contortus* pepsinogen under the control of a *C. elegans* cysteine proteinase promoter, *cpr-5*, shown previously to direct expression to the gut. Separate PCR reactions were used to amplify the *C. elegans cpr-5* cysteine proteinase promoter and a genomic copy of the *H. contortus* pepsinogen gene using oligonucleotide primers containing restriction enzyme recognition sites to facilitate their sequential, directional cloning. The 3' non-coding region of the pepsinogen gene was also PCR amplified with the introduction of a consensus polyadenylation signal sequence achieved through the use of a specially designed antisense primer. These three amplification products were cloned in order (promoter, gene, 3' untranslated sequence) into the pBluescript plasmid. This construct was microinjected into an *unc-76* mutant strain of *C. elegans* together with a rescue plasmid, and transgenic worms identified through rescue back to the wild type phenotype. The *unc-76* mutation affects the locomotory nervous system resulting in a slow growing mutant strain. Phenotypic rescue back to the wild type phenotype means that rapid accumulation of transgenic worm material can be achieved. Two transgenic lines of *C. elegans*, which demonstrated semi-stable transmission rates of ~60%, were established.

PCR analysis of single transgenic worms using pepsinogen-specific primers verified the presence of the injected construct. RT-PCR using mRNA extracted from cultures of transgenic *C. elegans* as template, and again using *H. contortus* pepsinogen-specific primers, was used to demonstrate transcription of the *H. contortus* gene. A specific product of the predicted size, as determined from cDNA nucleotide sequence data, was amplified. Sequence analysis of this amplified product verified correct splicing of intronic sequences.

Antibodies to bacterially expressed recombinant PEP1 (see Chapter 6) were used to probe freeze-fractured whole worm preparations. Strong, specific immunostaining was evident in the gut of transgenic worms only. Reactivity was evident in the larval and adult stages, correlating with the temporal pattern of expression of the *C. elegans cpr-5* cysteine proteinase gene, whose promoter was used here to drive expression of the *H. contortus* pepsinogen protein.

Although only low level expression of the *H. contortus* pepsinogen gene was detected in transgenic lines of *C. elegans*, this work demonstrates the potential of *C. elegans* as a vector for the expression of candidate vaccine antigens from *H. contortus* and other related parasitic nematodes. In addition, it serves to indicate a degree of conservation regarding the mechanisms regulating gene expression between *C. elegans* and *H. contortus*, further emphasising the potential role of *C. elegans* as a model system for parasitic nematodes.

Contribution to work

With the exception of transformation of *C. elegans* by microinjection and the initial establishment of transgenic lines (C Clucas), all work detailed in this paper, its design and interpretation, was carried out by myself. Communication of results at scientific meetings. Preparation of the manuscript for publication.

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Expression of *Haemonchus contortus* pepsinogen in *Caenorhabditis elegans*☆

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Abstract

A genomic copy of a gut-expressed *Haemonchus contortus* candidate vaccine antigen, pepsinogen, was isolated using the polymerase chain reaction (PCR). The isolated sequence was 4 kb in length and contained eight introns ranging in size from 54 to 1475 base pairs. This sequence, together with its 3′ non-coding DNA region containing a polyadenylation signal sequence, was cloned into the Bluescript SK$^+$ vector immediately downstream of the *Caenorhabditis elegans cpr-5* gene promoter. This promoter has been shown previously to direct protein expression to the gut of *C. elegans*. The construct was micro-injected into DR96 *unc-76(e911)* mutant *C. elegans* together with a rescue plasmid and transgenic worms identified by reversion back to wild-type phenotype. Two transgenic lines of *C. elegans* were established. The presence of the injected construct and of the *Haemonchus* pepsinogen transcript in transgenic worms was confirmed by PCR analysis. Correct splicing of intronic sequences was observed. Immunohistochemistry showed expression of the *Haemonchus* pepsinogen protein in the gut of transgenic *C. elegans*, with reactivity evident in the larval and adult stages. Expression of the *Haemonchus* pepsinogen in *C. elegans* affirms the role of *C. elegans* as a model for parasitic nematodes and demonstrates its potential as a vector for expression of candidate vaccine antigens from parasitic nematodes.

Keywords: *Haemonchus contortus*; Vaccine antigens; *Caenorhabditis elegans*; Expression; Pepsinogen

1. Introduction

The rapid and widespread emergence of anthelmintic resistant strains of the highly pathogenic blood-feeding ovine parasitic nematode *Haemonchus contortus* and other economically important parasites [1] has highlighted the need to develop alternative control strategies, such as vaccination. Recombinant protein-based vaccines have recently been developed against the cattle tick *Boophilus microplus* [2] and the sheep cestode *Taenia ovis* [3], highlighting the effectiveness of this approach in field conditions.

In the case of *H. contortus*, attention has focused on the fractionation of protein extracts in an attempt to identify antigens that induce protective immunity. This has resulted in the identification of a number of promising candidates [4,5] including the so-called 'hidden' antigens expressed on the microvillar surface of the gut in the blood-feeding fourth larval stage and adult worms, not normally seen by the host immune system during the course of a natural infection. These include H11 [6], *Haemonchus* galactose-containing glycoprotein (H-gal-GP) [7] and a thiol-Sepharose binding protein fraction [8]. These antigens are purified from detergent-soluble membrane protein extracts of adult worms, are typically glycosylated and show marked proteinase activity, H11 being an aminopeptidase [6], H-gal-GP ex-

Abbreviations: PCR, polymerase chain reaction; gDNA, genomic DNA; wt, wild type; H-gal-GP, *Haemonchus* galactose-containing glycoprotein; RT-PCR, reverse transcriptase polymerase chain reaction; PBST, phosphate-buffered saline containing Tween-20.

☆ *Note:* Nucleotide sequence data reported in this paper have been submitted to the GenBank™, EMBL and DDJB databases with the accession number AF076608.

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hibiting both aspartyl- and metalloproteinase activities [9,10], and the thiol-Sepharose binding proteins being enriched for cysteine proteinases [8].

Increasing denaturation of H11 led to a progressive loss of its protective capacity [11] while dissociation and reduction of H-gal-GP resulted in less consistent levels of protection [12], results that suggest that conformational epitopes, glycosylation and/or enzyme activity may be involved in protection. For this reason, expression of these potential vaccine antigens in a eukaryotic system will probably be required in order to obtain appropriate post-translational modifications of recombinant proteins.

For several years, the free-living nematode *Caenorhabditis elegans* has been advocated as a model for parasitic nematodes [13] and has been used to study the biology of the cuticle and surface antigens of parasitic nematodes [14,15]. More recently, *C. elegans* has been used as an expression host to study the mechanism of benzimidazole resistance in *H. contortus* [16]. The fact that injected *H. contortus* genes were expressed in a biologically active form demonstrates the potential use of *C. elegans* as an expression host.

The potential advantages of using *C. elegans* as a vector for expression of *Haemonchus* proteins as compared with other eukaryotic expression systems, such as yeast, baculovirus or mammalian cell lines, are numerous: it is a higher eukaryote closely related in evolutionary terms to *H. contortus* [13]; *trans*-splicing is performed using the same spliced leader (SL1) as *H. contortus* [17,18]; gene expression and function can be studied through transformation by micro-injection [19,20]; it has a rapid life-cycle; it can be easily maintained *in vitro* on agar plates or in liquid culture, an alternative that allows for the rapid accumulation of large quantities of worm material.

In the present study, we describe the structure of the *Haemonchus* pepsinogen gene, the aspartyl proteinase component of the host protective H-gal-GP complex [9], and its expression in *C. elegans* in an attempt to evaluate its potential as a vector for expression of *Haemonchus* candidate vaccine antigens.

2. Materials and methods

2.1. Isolation of genomic DNA

Clean *H. contortus* was harvested from the abomasa of experimentally infected donor lambs 28 days post-infection as described previously [21] and stored in liquid nitrogen. Approximately 0.5 g (wet weight) worms were ground in a pestle and mortar, and genomic DNA (gDNA) prepared using the method described by Herrmann and Frischauf [22].

2.2. Isolation of the H. contortus pepsinogen gene

The sequence of a full-length pepsinogen cDNA has been previously determined [9]. Primers P3 and P4 (Fig. 1), corresponding to the coding sequences surrounding the translational start and stop signals, and containing *Eag*I and *Eco*RV restriction enzyme recognition sites, respectively, were used in a polymerase chain reaction (PCR) with 1 μg adult *H. contortus* gDNA as target and the following reaction conditions. After initial denaturation at 94°C for 5 min, DNA was amplified in 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 3 min, with a final 7 min extension at 72°C. The amplified product was purified from a 0.8% (w/v) agarose gel using a Geneclean kit according to the manufacturer's instructions (Bio 101, Inc.), cloned into the pCRII vector (TA Cloning System; Invitrogen) and sequenced (Advanced Biotechnology Center, Imperial College, London).

2.3. C. elegans strains and media

Wild-type (wt) *C. elegans* strain N2 and the mutant strain DR96 *unc76(e911)* were obtained from the *Caenorhabditis* Genetics Centre. Nematodes were maintained and harvested according to standard methods [23].

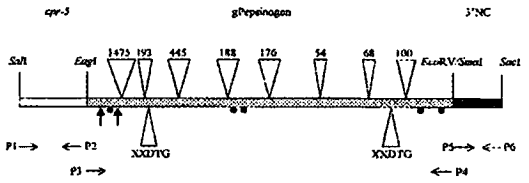

Fig. 1. Cloning strategy and location of PCR primers used in construction of the *Haemonchus* pepsinogen construct for micro-injection into *C. elegans* and organisation of the *H. contortus* pepsinogen gene. White shading, the *C. elegans cpr-5* promoter; dotted shading, the 1284 bp coding region of the *H. contortus* pepsinogen gene, with the relative size and position of intronic sequences shown above. The position of a putative signal peptide and precursor cleavage site is arrowed. Five potential N-linked glycosylation sites (●) and the two active site domains (XXDTG) are also shown. Black shading, the non-coding 3′ flanking region of the *Haemonchus* pepsinogen gene. Arrows indicate the direction in which DNA amplification was initiated from each primer. Primer sequences (5′–3′) were as follows: P1, TTAAGTCGACGTGGAATTGACATGCACTCC; P2, GTCCGGCCGTATGAGAGAAGTGTCTGCGAAG; P3, GTCCGGCCGATGCTATATTTATTGCTCTTG; P4, GTCGATATCTTATTTCGGCTCCGCAAAG; P5, GTCCCGGGCATTTTGAATGCTCTGATTAGAA; P6, GTCGAGCTCTTTATTCAGGGCTCTTGTACTC. Compatible restriction enzyme recognition sites were included in the primer sequences to facilitate directional, sequential cloning of amplification products.

2.4. Haemonchus pepsinogen construct

The general cloning strategy and location of PCR primers are summarised in Fig. 1. The 1 kb *C. elegans* cysteine protease promoter, *cpr-5*, was amplified from an existing clone [24] using primers P1 and P2, which contained *Sal*I and *Eag*I restriction enzyme recognition sites, respectively. Fifty nanograms of plasmid DNA was used as template under the following PCR conditions: initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension of 7 min at 72°C. A genomic copy of the *H. contortus* pepsinogen gene was amplified as already described (Section 2.2). In order to ensure correct processing of the mRNA, a polyadenylation signal sequence was incorporated by PCR amplification of the 3′ non-coding region of the *Haemonchus* pepsinogen from the previously isolated full-length cDNA. DNA was amplified using primers P5 and P6, which contained *Sma*I and *Sac*I restriction enzyme recognition sites, respectively. Primer P6 was directed to the PolyA$^+$ tail and was modified to contain the consensus AATAAA polyadenylation signal sequence. Fifty nanograms of target plasmid DNA was amplified essentially as already described except that annealing was carried out at 60°C. Amplification products were purified from agarose gels using a Geneclean kit (Bio 101, Inc.). Following digestion of each of the purified products with the appropriate restriction enzymes, they were ligated to each other and into *Sal*I/*Sac*I digested pBluescript SK$^+$ (Stratagene) in a single ligation reaction.

2.5. DNA transformation of C. elegans

Transformation of *C. elegans* was performed by microinjection of plasmid DNA into the distal arm of the DR96 *unc76(e911)* hermaphrodite gonad as described previously [20]. All plasmid DNA for microinjection was prepared using the Quiagen method. The *Haemonchus* pepsinogen construct at a final concentration of 50 μg ml^{-1} was co-injected with the repair plasmid p76-16B at a final concentration of 100 μg ml^{-1}. This plasmid contains a 10.7 kb *Xba*I genomic fragment that rescues the *unc76* phenotype [25], and that therefore allows transformants to be identified through reversion back to the wt phenotype. Lines in which the F2 and subsequent generations showed the wt phenotype were maintained for further analysis.

2.6. PCR analysis of transgenic C. elegans

The presence of the injected construct in transgenic worms was verified by PCR analysis of single worms as described by Kwa *et al.* [16] using the *Haemonchus* pepsinogen-specific internal primer pairing 5′-GAATGGTCGAAGAGGGGCTTGCAG-3′ (sense) and 5′-CTGCACTGCCTGACCAAATACC-3′ (antisense), complementary to nucleotides 1980–2003 and 2907–2928, respectively, under the following reaction conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 68°C for 30 s, and 72°C for 1 min, with a final 7 min extension at 72°C. PCR analysis of wt *C. elegans* was included as a control. Amplification products were separated on a 0.8% (w/v) agarose gel and visualised by ethidium bromide staining.

2.7. Analysis of expression of injected DNA

Evidence of transcription of the injected construct was sought by reverse transcriptase PCR (RT-PCR) using mRNA prepared from approximately 200 μg wet weight of worms and a commercial kit (Stratagene). Approximately 300 ng mRNA was reverse transcribed with Superscript™ II reverse transcriptase (Gibco BRL) using an oligo(dT) primer and reaction conditions as outlined in the Smart™ RACE cDNA Amplification kit (Clontech). The resultant cDNA was used as the target in a PCR reaction using the *Haemonchus* pepsinogen-specific primer pairing P3 and P4, described in Section 2.2, and the following reaction conditions. Initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min, with a final extension at 72°C for 7 min. Amplifications from the micro-injected construct and from a clone of the full-length *Haemonchus* pepsinogen cDNA were included as controls.

Integrity of the cDNA preparations was established by amplification of a *C. elegans* cathepsin L protease (gene T03E6.7) fragment using the internal primer pairing 5′-GTCTCCGTGCTCTGGGTCGGTTCCGTATC-3′ (sense) and 5′-CCATGGTGTCGACACCGAGGAGTCATAC-3′ (antisense), and the PCR conditions of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, and 72°C for 1 min, with a final 7 min extension at 72°C. Amplification products were separated on agarose gels and visualised by staining with ethidium bromide.

2.8. Immunohistochemistry

Worms were washed from agar plates with water and concentrated in the bottom of a Falcon tube by placing on ice for ~5–10 min. Worms were then transferred onto microscope slides coated with Poly-$_L$-lysine according to the manufacturer's instructions (Sigma), covered with a coverslip and excess liquid removed. Slides were immediately placed on a metal block standing in dry ice for a few minutes until frozen. Worms were fractured by simply flicking off the coverslip and fixed by immediate immersion in methanol at −20°C

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The general cloning strategy and location of PCR primers are summarised in Fig. 1. The 1 kb *C. elegans* cysteine protease promoter, *cpr-5*, was amplified from an existing clone [24] using primers P1 and P2, which contained *Sal*I and *Eag*I restriction enzyme recognition sites, respectively. Fifty nanograms of plasmid DNA was used as template under the following PCR conditions: initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension of 7 min at 72°C. A genomic copy of the *H. contortus* pepsinogen gene was amplified as already described (Section 2.2). In order to ensure correct processing of the mRNA, a polyadenylation signal sequence was incorporated by PCR amplification of the 3′ non-coding region of the *Haemonchus* pepsinogen from the previously isolated full-length cDNA. DNA was amplified using primers P5 and P6, which contained *Sma*I and *Sac*I restriction enzyme recognition sites, respectively. Primer P6 was directed to the PolyA$^+$ tail and was modified to contain the consensus AATAAA polyadenylation signal sequence. Fifty nanograms of target plasmid DNA was amplified essentially as already described except that annealing was carried out at 60°C. Amplification products were purified from agarose gels using a Geneclean kit (Bio 101, Inc.). Following digestion of each of the purified products with the appropriate restriction enzymes, they were ligated to each other and into *Sal*I/*Sac*I digested pBluescript SK$^+$ (Stratagene) in a single ligation reaction.

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for 10 min followed by 10 min in acetone at −20°C. Slides were blocked in phosphate-buffered saline (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.9% (w/v) NaCl; pH 7.4) + 0.2% (v/v) Tween-20 (PBST) + 1% (w/v) dry milk at room temperature for 20 min before washing for several minutes in PBST to remove excess milk. Slides were incubated with affinity-purified antibodies to *Haemonchus* pepsinogen [9], diluted to a final concentration of 0.5 μg ml^{-1} in PBST, overnight at +4°C in a humid chamber. After 2 × 15 min washes in PBST, slides were incubated in Fluorescein-conjugated anti-rabbit IgG (Sigma) for 1 h at room temperature, washed as already described and mounted in a 50% (v/v) glycerol solution in phosphate-buffered saline containing 2.5% (w/v) 1,4-diazabicycol[2.2.2]octane (DABCO; Sigma).

3. Results

3.1. Isolation and analysis of the H. contortus pepsinogen gene

A 3986 base pair (bp) band was amplified from *Haemonchus* gDNA using primers to the coding sequences surrounding the translational start and stop codons of the pepsinogen cDNA sequence reported previously [9]. The pepsinogen gene contained eight introns ranging in size from 54 to 1475 bp, the relative positions of which are shown in Fig. 1. The splice donor sequences of all introns have GT as the first two nucleotides, with the exception of intron 7, which has GC. All of the introns terminate with an AG nucleotide pair.

3.2. Transformation of C. elegans with a Haemonchus pepsinogen construct

In *H. contortus*, the pepsinogen protein is expressed as part of the integral gut-membrane glycoprotein complex H-gal-GP. Because no information was available regarding the *Haemonchus* pepsinogen promoter sequence itself, the PCR-amplified genomic copy of the *Haemonchus* pepsinogen gene was fused immediately downstream of a *C. elegans* cysteine protease promoter, *cpr-5*, which has been shown previously to direct expression to the gut of *C. elegans* [24]. A polyadenylation signal sequence was added by ligation of a PCR generated copy of the 3′ non-coding region of the *Haemonchus* pepsinogen cDNA downstream of the genomic copy of the gene. Sequence analysis of this 3′ non-coding region showed it to contain the variant AATGAA polyadenylation signal sequence. This sequence is rare and has been associated with defective 3′ processing in other eukaryotes [26]. For this reason, a consensus polyadenylation signal sequence, AATAAA, was also included through design of the antisense primer, P6.

Fig. 2. PCR analysis of wt *C. elegans* (lane 3) and transgenic lines AI286 (lane 4) and AI288 (lane 5) for presence of the micro-injected construct using a *Haemonchus* pepsinogen-specific internal primer pairing. A negative control reaction with no template DNA was also included (lane 2). Migration of molecular weight markers is shown in lane 1.

The slow growing DR96 *unc76(e911)* mutant strain of *C. elegans* was used for microinjection experiments. Co-injection of the rescue plasmid p76-16B resulted in phenotypic rescue of transgenic worms back to the wt phenotype that grow more quickly than the DR96 *unc76(e911)* worms, therefore allowing rapid accumulation of transgenic worm material. Two transgenic lines, AI286 and AI288, were established, both of which demonstrated semi-stable transmission rates of 50–60%.

3.3. PCR analysis of transgenic lines

Single worms were picked and analysed for the presence of the injected construct by PCR under stringent conditions and using internal primers specific to the *H. contortus* pepsinogen gene. Representative PCR reactions are shown in Fig. 2. As predicted from nucleotide sequence analysis of the *H. contortus* pepsinogen gene, a product of 0.9 kb was amplified from all of the transgenic *C. elegans* analysed, whereas no product was amplified from wt control animals.

3.4. RT-PCR analysis of expression of the injected construct

Evidence of transcription of the *H. contortus* pepsinogen gene was sought in an RT-PCR reaction as described in Section 2.7. Control amplifications using either a cloned copy of the *Haemonchus* pepsinogen gene or cDNA as target gave products of 4 and 1.3 kb, respectively (Fig. 3A), consistent with the predicted size as determined from nucleotide sequence data. No amplification product was obtained from wt worms, whereas a band of 1.3 kb was amplified from both transgenic lines of *C. elegans*. The integrity of the

cDNA preparations was checked by amplification of an internal fragment of a *C. elegans* cathepsin L protease (Fig. 3B). A band of the predicted size, 280 bp, was amplified from all three cDNA preparations.

3.5. Immunohistochemistry

The affinity purified antibodies reacted strongly with the gut of transgenic *C. elegans* (Fig. 4), with reactivity being evident in all the larval and adult stages. No staining was evident in wt worms (data not shown).

4. Discussion

In this paper, we report the isolation and molecular organisation of a genomic copy of the *H. contortus* aspartyl proteinase gene, pepsinogen, and its expression in the free-living nematode *C. elegans*, so demonstrating the potential of *C. elegans* as a vector for the expression of parasitic nematode vaccine antigens. The gene was expressed under the control of a *C. elegans* gut-expressed cysteine protease gene promoter that directed expression to the gut of transgenic worms. This expression did not induce any apparent adverse phenotypic effects affecting worm survival.

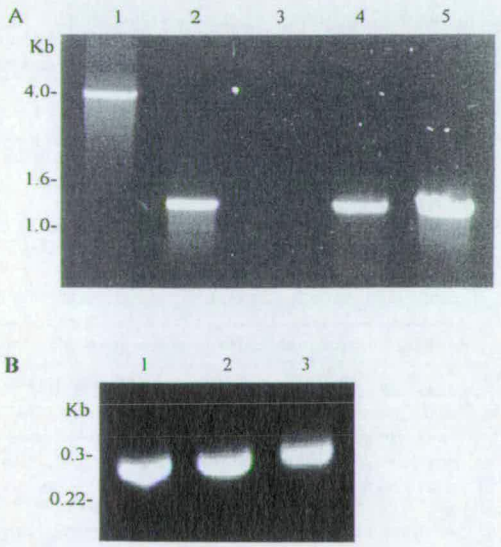

Fig. 3. (A) RT-PCR to detect transcripts of the micro-injected *H. contortus* pepsinogen gene using primers directed to the translational start and stop codons. Positive control amplification reactions using the injected construct and a clone of the full-length *Haemonchus* pepsinogen cDNA as target are shown in lanes 1 and 2, respectively. Amplification of cDNA derived from wt *C. elegans* and the transgenic lines AI286 and AI288 is shown in lanes 3–5. Migration of molecular weight markers was as indicated. (B) RT-PCR amplification of a *C. elegans* cathepsin L protease fragment using wt (lane 1) and transgenic lines AI286 (lane 2) and AI288 (lane 3) cDNA as target. Migration of molecular weight markers is indicated.

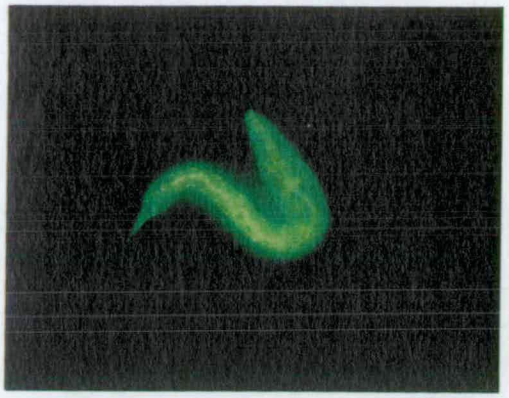

Fig. 4. Analysis of transgenic lines of *C. elegans* for expression of the *H. contortus* pepsinogen protein. The wt controls (not shown) or transgenic lines were probed with affinity-purified anti-*Haemonchus* recombinant pepsinogen serum. Immunofluorescence was observed only in the gut of transgenic worms.

H. contortus pepsinogen/pepsin is a component of the highly host-protective integral membrane protein complex H-gal-GP, isolated from the guts of adult worms [7]. The enzyme shows a high affinity for haemoglobin as a substrate and is expressed almost exclusively in the parasitic, blood-feeding stages of *H. contortus*, suggesting that it may function in digestion of the host bloodmeal and may be essential for parasite survival [9]. *Haemonchus* pepsinogen is, therefore, a potential vaccine antigen candidate. Pepsinogen is encoded by a single copy gene in *Haemonchus* [9] and has no close homologue in *C. elegans* as determined by nucleotide sequence database searching (data not shown), making it an ideal target for evaluating the potential of *C. elegans* to express candidate vaccine antigens of parasitic nematodes.

Expression of a gene in *C. elegans* is achieved through micro-injection of DNA containing all the sequences necessary for its own expression [27], i.e. a functional promoter, sequences necessary for mRNA formation and a polyadenylation signal sequence. The presence of intronic sequences has been shown to greatly enhance the expression of some heterologous genes in *C. elegans* [28].

The *Haemonchus* pepsinogen gene was isolated by PCR. It contains eight introns (Fig. 1), which is higher than average when compared with *C. elegans* [29] but not unprecedented in *H. contortus*, the cysteine proteinase AC-2 gene having previously been found to contain 11 introns [30]. The consensus splice donor and acceptor sequences were found to be similar to those of *C. elegans* [31] and of other eukaryotes in general [32]. The splice donor sequence of the introns start with the nucleotides GT, with the exception of intron 7, which starts with GC. Point mutation analysis of a β-globin

gene has shown that the GC pairing does not significantly impair splicing [33] and, although rare, a cytosine at the second position does occur naturally in eukaryotic introns [30,34,35]. It was previously suggested that there may be a stringent requirement for G at position 5 [36], and there is conservation of this nucleotide in all introns of the *Haemonchus* pepsinogen gene. All of the introns terminate with the strictly conserved AG sequence. Three-quarters of the *Haemonchus* introns are less than 200 bp in length, which is considerably smaller than introns of higher eukaryotes. Conversely, intron 1 is unusually long for a nematode intron [31], although large introns have been previously reported in *Haemonchus* cysteine proteinase genes [30,37].

In the two transformed lines of *C. elegans*, the injected construct was not integrated into the genome but maintained as extrachromosomal arrays. Both *H. contortus* β-tubulin genes [16] and a *Trichostrongylus colubriformus* collagen gene [27] have been expressed successfully in *C. elegans* in this manner. RT-PCR analysis using *Haemonchus* pepsinogen-specific primers confirmed that the injected gene was being transcribed (Fig. 3A). A product of 1.3 kb was amplified from transgenic worm populations, which corresponds in size with the cDNA control, suggesting that correct splicing of intronic sequences is occurring. This was confirmed by sequence analysis of the amplified products (data not shown).

Expression of the *Haemonchus* pepsinogen protein was detected in the gut of transgenic worms (Fig. 4). With the exception of eggs, expression was observed in all stages of the nematode. This correlates with the temporal expression pattern of the *C. elegans* cysteine protease *cpr-5*, which shows an increase in abundance post-hatch and remains high throughout the larval stages with a slight decline in adults [38]. The *cpr-5* promoter was used here to drive expression of the *Haemonchus* pepsinogen. The *Haemonchus* protein was occasionally detected by Western blotting as a weak, additional band in extracts of some, but not all, batches of transgenic worms tested (results not shown), indicating that expression levels were low. This result may be attributable to a 'dilution' of the *Haemonchus* protein on preparation of worm protein extracts, although it is also possible that expression of the *Haemonchus* protein has a deleterious effect on transgenic worms.

The results presented in this paper demonstrate the potential of *C. elegans* as a vector for expression of candidate vaccine antigens from parasitic nematodes further emphasising the degree of conservation between it and *H. contortus*, and affirming the role of *C. elegans* as a model for parasitic nematodes. Although only low levels of expression of the *Haemonchus* pepsinogen were achieved, future work will focus on attempting to optimise expression levels by integration of injected DNA into the *C. elegans* genome or by micro-injection of varying concentrations of construct. Higher levels of expression may be also be attained through using different promoter sequences and promoter manipulation. Good levels of protein expression and nematode-like post-translational processing allied to the capability of growing *C. elegans* in large-scale liquid cultures emphasises the potential of this approach for the expression and analysis of parasitic nematode genes.

Acknowledgements

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Chapter 8: Identification of promoter elements of parasite nematode genes in transgenic *Caenorhabditis elegans*.

Britton C, Redmond DL, Knox DP, McKerrow JH and Barry D. 1999.
Molecular and Biochemical Parasitology, 103: 171-181.

C. elegans has become established as a model system for the study of developmental and functional biology of metazoans. Significant advantages are its simplicity, both in anatomy and genomic organisation, rapid life cycle and ease of growth and maintenance (Wood, 1988). In addition, genetic manipulation can be performed readily in *C. elegans* with techniques having been developed to allow the creation and rescue of mutant phenotypes, genetic crossing and transformation. *C. elegans* can be transformed easily by microinjection of DNA into the hermaphrodite gonad to produce stable lines carrying the exogenous DNA as extrachromosomal arrays (Mello et al, 1991). This transformation technique has been used to study the mechanism of *H. contortus* benzimidazole resistance through expression of β-tubulin alleles in *C. elegans* (Kwa et al, 1995) and to evaluate *C. elegans* as a potential vector for expression of parasite candidate vaccine antigens through expression of *H. contortus* pepsinogen (see Chapter 7; Redmond et al, 2001).

Although little is known regarding transcription factors and the mechanisms regulating gene expression in parasitic nematodes, that functional expression of *H. contortus* genes can be achieved in *C. elegans* (Kwa et al, 1995) suggests a degree of conservation between these two nematodes. It also serves to highlight the possibility of using *C. elegans* as a heterologous transformation system to examine the activity and specificity of parasitic nematode gene promoters. Conventional transformation systems allow only quantitative analysis of promoter activity. In addition to identifying potential upstream regulatory motifs from nematode parasite genes, the use of *C. elegans* as a transformation system would allow the temporal and spatial expression of a reporter gene to be determined and would thus provide a powerful system for studying helminth parasite gene expression.

The aim of the work reported in this paper was to evaluate *C. elegans* as a heterologous transformation system for the identification and study of parasitic nematode promoter activities.

Three parasite promoters were examined, namely the gut expressed *H. contortus* cysteine proteinase AC-2, a member of the cysteine proteinase gene family identified in the USDA BPL1 strain of *H. contortus* (Pratt et al, 1990), and pepsinogen *pep-1* gene promoters (*pep-1* is used to denote the gene encoding the PEP1 cDNA described in chapters 6 and 7) and the cuticular *Ostertagia circumcincta* (also known as *Teladorsagia circumcincta*) *colost-1* collagen promoter. The *H. contortus* AC-2 upstream sequence was isolated from a genomic library by high stringency hybridisation to an AC-2 specific probe based on previously published sequence, and by subsequent PCR amplification. A novel, two-step PCR technique, outlined in Figure 2, was developed to isolate the upstream sequence of the *H. contortus* pepsinogen gene, *pep-1*. The upstream region of the *O. circumcincta colost-1* collagen gene was generated by PCR using primers based on previously published sequence and using an *O. circumcincta* genomic library as template.

All three putative promoter sequences, comprising sequence immediately upstream of the ATG start codon, were cloned into a *lac Z* vector to generate *lac Z* transcriptional fusions, verified by DNA sequence analysis of constructs. The inclusion of a nuclear localisation signal in the vector leads to accumulation of the β-gal reporter protein in the nuclei of producing cells, thus facilitating their identification in complex tissues. Constructs were microinjected into *C. elegans* together with a plasmid containing the *rol-6* gene and transformants identified by their roller right phenotype. Whole worm mounts of stable transformed lines were stained for β-gal expression.

Transformation with constructs containing the promoters for the *H. contortus* gut expressed AC-2 and *pep-1* genes resulted in expression of the β-gal reporter gene exclusively in the gut cells of transformed *C. elegans*, with strong staining observed predominantly in the anterior and posterior regions of the gut with the AC-2 promoter. As predicted for a cuticular collagen gene, specific expression of the β-gal reporter gene in hypodermal cells of *C. elegans* was observed following transformation with the *O. circumcincta colost-1* promoter. This strict tissue-specific expression demonstrates conservation of the regulatory mechanisms controlling spatial expression of these genes in these related nematode species.

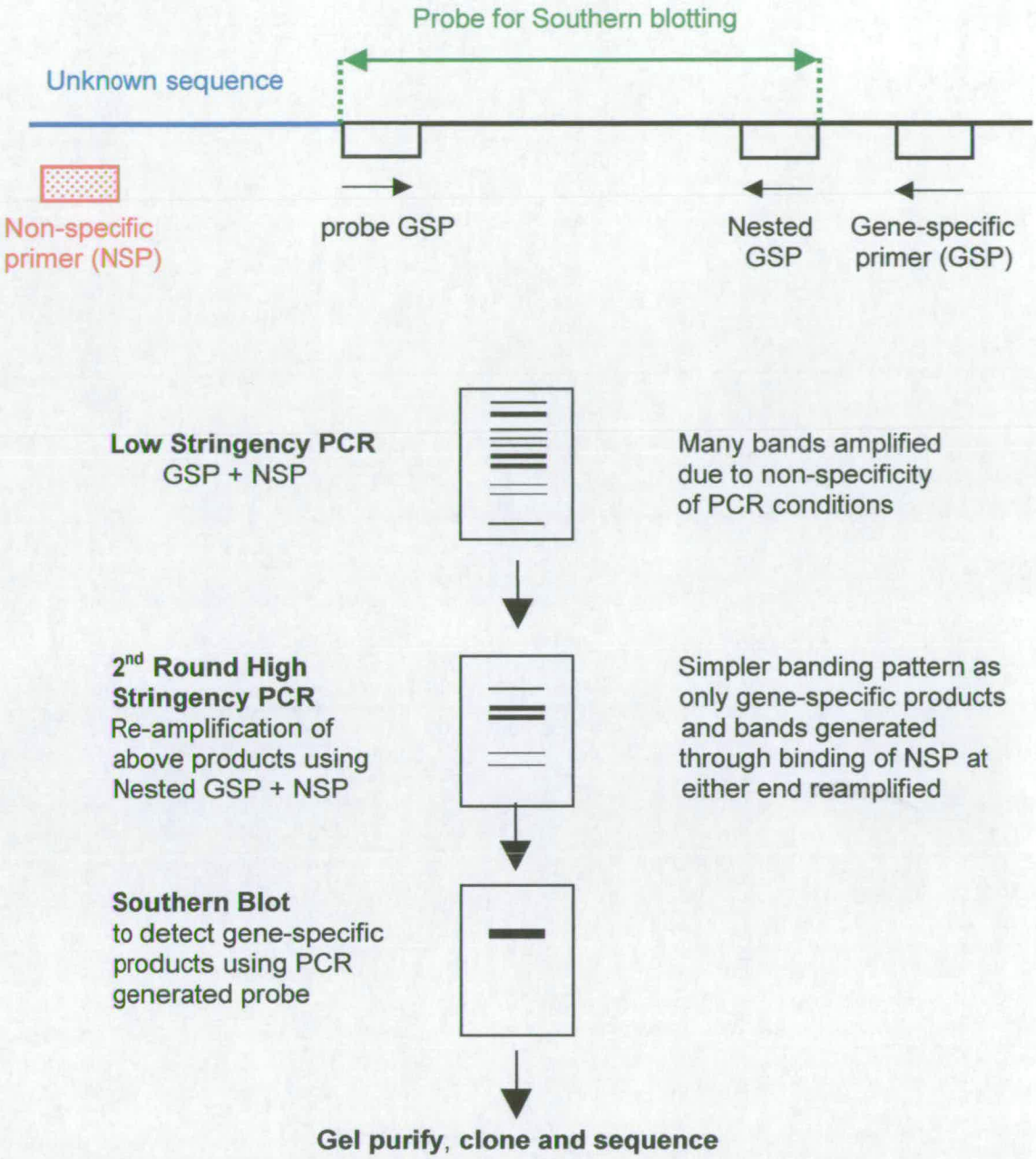

Figure 2. Schematic diagram of the gene walking technique showing the relative positions and orientations of gene-specific primers for amplification of upstream flanking sequences. Gene-specific primers are designed ~200 bp from the end of known sequence to allow amplification of a significantly large region of flanking sequence whilst still maintaining sufficient sequence overlap to confirm specificity of the amplified product. A panel of non-specific primers, each used in a separate PCR reaction, is used to increase the chances of amplifying a large region of unknown flanking sequence. The technique can also be used to obtain downstream sequence information by simply reversing primer order and orientation and can be applied to both genomic and cDNA templates.

In *H. contortus*, the temporal expression of cysteine proteinase and pepsinogen genes is restricted to the blood-feeding L4 and adult stages. However, in transformed *C. elegans*, expression of the reporter gene under the control of these *H. contortus* promoters was observed in all stages except embryos, although the strongest and most frequent expression occurred in L4s and adults. Although expression of the *colost-1* collagen gene is detected in the early larval (L1-L3) stages of *O. circumcincta*, expression of the *colost-1/lac Z* construct in *C. elegans* was restricted to the L4 stage. These observations suggest that species-specific differences occur in the fine tuning of regulatory mechanisms.

Deletion constructs of the *H. contortus* AC-2 promoter were generated in an attempt to identify potential regulatory elements. Specific restriction endonuclease digestion of the AC-2 promoter generated a 5' deletion construct leaving 697 bp sequence upstream of the ATG start codon. No significant effect on frequency or tissue-specificity of the β-gal reporter gene was observed, indicating that sequence further upstream is not essential for gene expression. Similarly, a PCR-generated deletion of the first 46 bp at the 3' end of the AC-2 promoter had no effect on expression. A second PCR-generated 3' deletion removing the first 86 bp resulted in loss of all *lac Z* reporter activity, indicating that the region between -48 and -86 bp is essential for expression. This region contains two GATA-like motifs, transcriptional elements shown previously to be necessary for correct gut expression of several *C. elegans* genes. PCR-induced mutation of the AC-2 GATA element closest to the start site resulted in loss of reporter gene expression in transformed *C. elegans*, indicating that this site is essential for *H. contortus* AC-2 gene expression.

In conclusion, this work demonstrates the potential of *C. elegans* as a surrogate transformation system to identify promoter activities and determine tissue specificity of expression of nematode parasite genes. In addition, this transformation system can be used to identify parasite promoter elements and transcription factors involved in the regulation of gene expression. Interfering with expression of critical parasite genes has potential as a novel mechanism of parasite control. Further studies may help to identify parasite homologues of essential *C. elegans* transcription factors that may be targeted with consequential deleterious effects on parasite survival.

Contribution to work

Establishment of a collaboration with C Britton. The development of a novel two-step PCR method for amplification of unknown flanking DNA sequences and its application to amplify the promoter region of the *H. contortus* pepsinogen gene. Detailed sequence analysis of the pepsinogen promoter and cloning into a *lac Z* reported plasmid for microinjection into *C. elegans*. Active discussion and analysis of results with C Britton. Initiation and execution of further collaborative work to analyse the pepsinogen promoter regulatory motifs. Preparation of relevant "Materials and Methods" section of manuscript.

Further to the results presented here, deletion constructs of the *H. contortus* gut expressed pepsinogen, *pep-1*, promoter were analysed using the heterologous *C. elegans* transformation system in an attempt to identify its minimal promoter region and potential regulatory motifs.

Sequential deletion of 5' sequence to leave 800 bp of sequence upstream of the ATG translational start signal had no effect on β-gal expression, with strong staining in the gut cells of transformed animals being observed, (Figure 3A). However, further 5' sequence deletions to leave 600 bp and 400 bp of upstream sequence resulted in a progressive decrease in the level of β-gal expression (not shown). This suggests that expression levels may be influenced by a number of activators bound to the promoter sequence. A construct containing 200 bp of upstream promoter sequence showed only low level staining in the posterior gut cells of transformed *C. elegans*, (Figure 3B). Motifs located within the first 200 bp of the *H. contortus* promoter are, therefore, sufficient to direct gut expression, indicating that tissue-specific factors bind within this region. That no staining of anterior gut cells was observed suggest that additional motifs in the –400 to –200 region may be required to direct protein expression in these cells. Ten GATA-like motifs were identified within the first 200 bp of the *H. contortus* pepsinogen promoter which may, by analogy to their function in *C. elegans*, be responsible for directing protein expression to the parasite gut. However, mutational analysis will be necessary to further identify critical motifs within the *H. contortus* pepsinogen promoter.

A

B

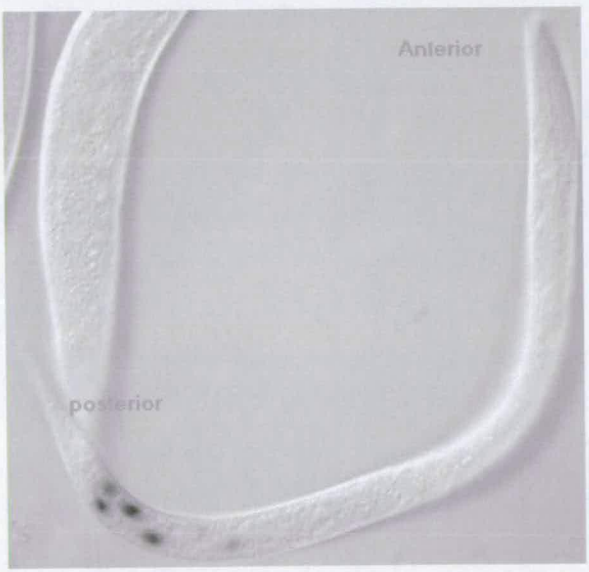

Figure 3. Expression of *H. contortus* pepsinogen promoter/*lacZ* reporter deletion constructs in transgenic *C. elegans*.
A. construct containing 800 bp of sequence upstream of the ATG translational start sequence showing strong staining in gut cells.
B. construct containing 200 bp of upstream sequence showing only low level expression in the posterior gut cells.

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Identification of promoter elements of parasite nematode genes in transgenic *Caenorhabditis elegans*☆

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Abstract

Transformation of the free-living nematode *Caenorhabditis elegans* with promoter/reporter gene constructs is a very powerful technique to examine and dissect gene regulatory mechanisms. No such transformation system is available for parasitic nematode species. We have exploited *C. elegans* as a heterologous transformation system to examine activity and specificity of parasitic nematode gene promoters. Using three different parasite promoter/*lac Z* reporter constructs strict tissue-specific expression is observed. Upstream sequences of the *Haemonchus contortus* gut pepsinogen gene *pep-1* and cysteine protease gene AC-2 direct expression exclusively in gut cells, while promoter sequence of the *Ostertagia circumcincta* cuticular collagen gene *colost-1* directs hypodermal-specific expression. Mutation analysis indicates that AC-2 promoter function is dependent on a GATA-like motif close to the translation start site, similar to our findings with the *C. elegans cpr-1* cysteine protease gene. While the spatial expression of these parasite promoters in *C. elegans* correlates with their expression in the parasite, the exact timing of expression does not. This suggests that regulatory mechanisms influencing the timing of expression may have evolved more rapidly than those controlling spatial expression of structural genes.

Keywords: Nematode; Parasite; Promoter; *Caenorhabditis elegans*; Transformation

Abbreviations: RT-PCR, Reverse transcriptase polymerase chain reaction.

☆ *Note*: Nucleotide sequence data for the *Haemonchus contortus* pepsinogen gene promoter and *H. contortus* AC-2 gene promoter are available in the GenBank database under accession numbers AF076608 and AF116182. Sequence data for the *Ostertagia circumcincta colost-1* upstream region has been added as an update to the sequence in EMBL accession number X96731.

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1. Introduction

Multicellular organisms express many genes in specific tissues at specific stages of development. Interfering with the control of such expression is likely to have deleterious effects on the organism and, for parasitic species, has potential as a novel mechanism of parasite control. At present, there is very little known of gene regulatory mechanisms and transcription factors in parasitic helminths, with studies being limited by the complex parasite life cycles and absence of a suitable transformation system. However, several recent studies have made use of mammalian cell transformation systems to identify potential regulatory regions in genes of the trematode parasite *Schistosoma mansoni* [1–4]. The expression obtained with these parasite promoters in mammalian cell systems implies that there is sufficient conservation in regulatory DNA motifs and associated transacting factors between these diverse phyla to allow examination of at least basal transcription mechanisms. It seems highly probable, therefore, that a transformation system using a more closely related species would provide a powerful, new system for studying helminth parasite gene expression. Moreover, the use of such a system might help identify not only important basal regulatory regions, but also regions involved in spatial and developmental gene expression in these multicellular organisms.

As the basic structural organisation and development are very similar between different nematode species, it is pertinent to make use of the wealth of information on the development, genetics, anatomy and gene organisation and expression of the free-living soil nematode *Caenorhabditis elegans* to study these aspects of parasitic nematode biology. In addition, *C. elegans* is easy to grow and maintain and, importantly, can be transformed easily by microinjection of DNA into the hermaphrodite gonad to produce stable lines carrying exogenous DNA in extrachromosomal arrays [5]. This transformation technique was used by Kwa et al. [6] to demonstrate functional activity of a β-tubulin gene from the sheep parasitic nematode *Haemonchus contortus* in *C. elegans*. While clearly showing expression of a parasite gene in *C. elegans*, their study did not examine the temporal and spatial expression of the parasite gene. Here we extend this approach using a *lac Z* reporter vector to identify promoter activities, determine tissue specificity of expression and identify potential upstream regulatory motifs from nematode parasite genes. The results demonstrate tissue-specific promoter activity for three genes from two parasite species.

2. Materials and methods

2.1. Construction of lac Z reporter constructs

All parasite promoters examined in this study were cloned as transcriptional fusions in *lac Z* reporter vector pPD95.03 (kindly supplied by Andy Fire; A. Fire, S. Xu, J. Ahnn, G. Seydoux, personal communication). This vector encodes a nuclear localisation signal, targeting β-galactosidase to the nucleus, thus aiding in the identification of expressing cell types. In addition, this vector contains an upstream decoy sequence to minimise readthrough transcription and additional introns within the *E. coli lac Z* sequence to increase *lac Z* expression level (Fire, unpublished). For *C. elegans* transformation, pPD95.03 was used in preference to pPD90.28, which was used for the initial cloning step and is a first generation *lac Z* reporter vector without the decoy sequence and additional introns [7].

A clone containing upstream sequence of the *H. contortus* cysteine protease gene AC-2 was isolated from an EMBL3 *H. contortus* genomic library (kindly provided by Tim Geary, Pharmacia & Upjohn) following high stringency hybridisation with a ^{32}P-labelled probe generated by PCR using primers to the 5′ and 3′ ends of the previously published AC-2 256 bp upstream sequence [8] (AC-2 sense primer 1: 5′CACAGCATGCACATATTTGGTTTAAAG3′, positions −244 to −228 relative to the ATG translational start codon; underline indicates *Sph* I site introduced for cloning; AC-2 antisense primer 1: 5′TATTGGATCCGATTTTTGAGATGTCG3′, comple-

mentary to positions −7 to −25; underline indicates *Bam*H I site introduced for cloning). A 2.55 kb *Xba* I fragment from a hybridising clone, containing 2318 bp of upstream sequence as well as 229 bp of sequence downstream of the ATG start codon (indicated in Fig. 1), was subcloned into *lac Z* vector pPD90.28 (kindly supplied by A. Fire). This generated plasmid pAC1, which contains the AC-2 *Xba* I fragment in reverse orientation (Fig. 1). Like the *C. elegans* family of cysteine proteases (CPR's) [9,10], the AC-2 protease contains an N-terminal hydrophobic region [8] which may act as a signal peptide and could potentially interfere with nuclear localisation of *lac Z* expression from translational reporter constructs. To prevent this being a potential problem, a transcriptional fusion construct, pAC2, was generated for use in transformation. Primer extension analysis has previously indicated that, like the *C. elegans* cysteine protease (*cpr*) genes [9,10], the AC-2 gene is not transpliced to SL1 nor SL2, and that transcription starts approximately 10 bp upstream of the ATG start codon [8]. As the transcription start site has not been mapped exactly, we included nucleotides just prior to the ATG translational start codon. This was achieved by PCR using Vent Polymerase (New England Biolabs) on pAC1 template DNA using AC-2 gene specific antisense primer 2 (5'TACCATCTAGACTCCGATTTTTGAGATG3', complementary to positions −6 to −22; *Xba* I site introduced for cloning is underlined) and a *lac Z* vector primer, 1912 (5'TCCAAGGGTCCTCCTGAAAATG3'). The resulting 2.3 kb upstream fragment was digested with *Xba* I and cloned into *lac Z* vector pPD95.03 to generate construct pAC2, containing the AC-2 promoter in forward orientation (Fig. 1).

A 5' end deletion construct of the AC-2 promoter region containing 697 bp of immediate upstream sequence was generated by digestion of pAC2 with *Xba* I and *Sph* I (see Fig. 1) and cloning of the proximal 697 bp *Sph* I-*Xba* I promoter fragment directly into pPD95.03. 3' end deletion constructs 1 and 2 were generated by PCR on pAC1 with antisense primers DelR1 (5'TTGGTCTAGACCACCTGTCAAAG3', *Xba* I restriction site underlined), which is complementary to positions −48 to −61, and DelR2 (5'GATATCTAGAAAGCGAGAGGAATC3', *Xba* I restriction site underlined), complementary to positions −86 to −102, in conjunction with *lac Z* vector primer 1912. Positions of primers DelR1 and DelR2 are indicated in Fig. 3(A). Mutagenesis of the GATA motif closest to the ATG start codon of the AC-2 gene was achieved

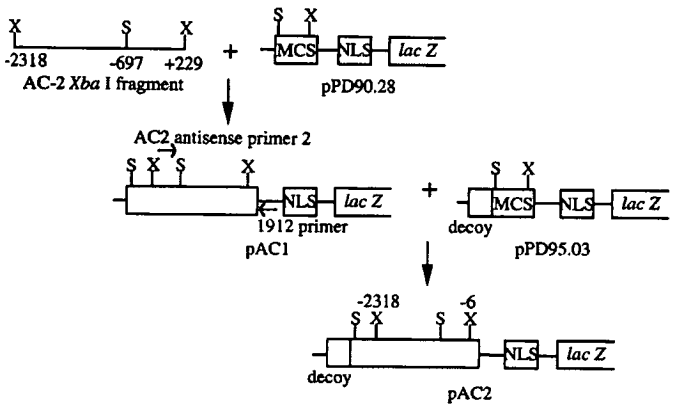

Fig. 1. Cloning strategy for AC-2/*lac Z* reporter construct. Cloning procedure is described in Section 2. X and S indicate *Xba* I and *Sph* I restriction sites in the AC-2 gene fragment and *lac Z* cloning vectors, pPD90.28 and pPD95.03. Numbers indicate positions of restriction sites in AC-2 relative to the ATG start codon. NLS indicates nuclear localisation signal and MCS indicates multiple cloning site in the vectors.

by PCR on pAC1 using antisense primer Mut1 (5'TTGGTCTAGACCACCTGTCAAATCGCCCTACTTTGTC3'; *Xba* I restriction site underlined; bases altered to induce mutagenesis of the −61 GATA motif are shown in bold type) in conjunction with *lac Z* vector primer 1912.

Upstream sequence of the *H. contortus* gut-expressed pepsinogen gene *pep-1* [11] was isolated by a gene-walking technique involving two rounds of PCR. The initial PCR was carried out at low stringency using a random primer (5'AATGTTATGTTGGCATTTG3') in conjunction with a *pep-1* specific antisense primer (5'AGAGACGCACGCTAGCATTC3', complementary to bases +132 to +152 relative to *pep-1* ATG translational start codon) with 500 ng *H. contortus* adult genomic DNA as target and the following PCR conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 30°C for 1 min, 72°C for 3 min, final extension at 72°C for 7 min. Of this reaction 1 μl was then used as template in a second round, high stringency PCR using the same random primer as above and a nested *pep-1* specific antisense primer (5'CTTTACGAATTCGGCCCATGTT3', complementary to bases +105 to +126 relative to the translational start codon). PCR conditions were as described above with the exception that annealing was carried out at 50°C. Second round products were separated on a 0.8% (w/v) agarose gel, Southern blotted and probed at high stringency using a DIG-labelled (Boehringer Mannheim) *pep-1* gene fragment corresponding to bases +1 to +152. Hybridising bands were eluted from an identical gel, cloned into the PCRII vector (Invitrogen) and sequenced (Advanced Biotechnology Center). A 2 kb fragment corresponding to the region of *pep-1* immediately upstream of the ATG translational start codon was cloned into *lac Z* vector pPD95.03 for injection into *C. elegans*.

Upstream region of the *colost-1* collagen gene was generated by PCR on an *O. circumcincta* genomic library (kindly provided by Iain Johnstone, University of Glasgow) using primers designed to the previously published sequence of this gene [12]: *colost-1* sense primer (5'CGGGGATCCGAGAGCCAGGGCAGTGTAG3') and *colost-1* antisense primer (5'GCCGTCGACGCTTGATCTGCTTGTGGACAC3'; underline indicates *Sal* I site introduced for cloning). This generated a 3 kb product, a *Sal* I-*Hin*d III 1.3 kb fragment of which was subcloned into *lac Z* vector pPD95.03 to generate a *lac Z /colost-1* transcriptional fusion extending at its 3' end to just prior to the *colost-1* ATG start codon. All constructs described here were verified by DNA sequencing.

2.2. DNA transformation of C. elegans

Transformation of *C. elegans* was performed by microinjection of plasmid DNA into the distal arm of the N2 hermaphrodite gonad as described previously [5]. All plasmid DNA for microinjection was prepared using the Qiagen method. *lac Z* reporter constructs at a final concentration of 50 μg ml^{-1} were co-injected with plasmid pRF4 [5,13] at a final concentration of 100 μg ml^{-1}. Plasmid pRF4 contains a dominant mutant allele of the *rol-6* gene and allows transformants to be identified by their right roller phenotype. Lines in which F2 and subsequent generations showed the roller phenotype were stained for β-galactosidase expression.

2.3. Fixation and staining for β-galactosidase activity

The staining procedure was carried out essentially as described by Fire et al. [7]. Nematodes were fixed in 1.25% (v/v) glutaraldehyde, washed and dried onto glass slides. They were then fixed for four minutes in acetone at −20°C before incubating in stain solution containing 0.01% (w/v) X-gal. Staining was carried out overnight at room temperature in a humid chamber. Co-staining with DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 0.1% (w/v) aided in the identification of stained nuclei.

3. Results

3.1. Expression of H. contortus AC-2 promoter/lac Z construct in C. elegans

To test whether heterologous promoters could function in *C. elegans*, transformation of the free-living nematode was carried out with a construct containing upstream sequence of a cysteine protease gene, AC-2, from *H. contortus*. AC-2 is a member of a family of Cathepsin B-like protease genes [8,14,15] that show strong sequence identity with a family of cysteine protease (*cpr*) genes from *C. elegans* [9,10]. The *H. contortus* protease genes are expressed at high levels only in the blood-feeding L4 and adult stages [8] and some of the encoded enzymes have been immunolocalised to the gut (David P. Knox, unpublished), suggesting that they may be involved in blood feeding. The *C. elegans cpr* protease genes are also expressed exclusively in the gut, suggesting a possible role in digestion. These genes are expressed in all developmental stages except the embryo [9,10].

To examine expression from the AC-2 promoter, transgenic *C. elegans* lines were generated carrying 2.3 kb of AC-2 immediate upstream sequence linked to a *lac Z* reporter gene. Because of the way in which extrachromosomal arrays are transmitted in *C. elegans*, with between 10 and 90% of progeny inheriting the array [5], as well as random mitotic loss of arrays, not all worms in a stable line show expression. Because of this random loss and the inherent variability between independent transformed lines, it is difficult to quantitate the frequency of *lac Z* expression in an accurate manner. Expression is, therefore, referred to as being present (+) or absent (−), as indicated in Fig. 3(B).

In lines transformed with the AC-2/*lac Z* construct (pAC2), strong *lac Z* expression was observed in a high number of worms, exclusively in nuclei of gut cells. Staining was observed predominantly in the anterior and posterior regions of the gut. Expression occurred in all stages except the embryo, with strongest and most frequent expression in L4 and adult worms (Fig. 2). Control worms, transformed with the pPD95.03 *lac Z* vector with no AC-2 insert, showed no *lac Z* expression, indicating that this gut-specific expression is being driven by the *H. contortus* AC-2 promoter.

3.2. Effect on expression of 5′ and 3′ deletions of the AC-2 promoter

To identify potential regulatory elements within the AC-2 promoter involved in spatial and temporal expression, deletion and mutation constructs were generated (Fig. 3(A–B)). Deletion of the 5′ region, leaving 697 bp of sequence upstream of the ATG start codon, had no significant effect on the frequency or tissue-specificity of expression: *lac Z* staining was still observed exclusively in gut cells, indicating that further upstream sequence is neither essential for expression nor is it likely to contain repressor binding sites preventing expression in other tissues. Strong expression was still observed in the posterior gut region in all stages except the embryo. There was, however, a noticeable decrease in the number of transformants showing expression in the most anterior gut cells. This could be a quantitative effect, with insufficient activation occurring to detect expression in these cells, or a qualitative effect resulting from deletion of a promoter activator element(s) necessary for expression in these cells.

Deletion of the first 47 bp at the 3′ end of the AC-2 flanking region (3′ end deletion 1, Fig. 3(B)), removing the putative TATA box, had no effect on expression. This indicates that the TATA box is not essential for expression and transcription may depend on another activator motif, such as a GATA element or other initiator sequence. The sequence TCACTCT starting at position −129 of the AC-2 gene (numbering relative to the ATG start codon; see Fig. 3(A)) fits the initiator consensus sequence PyPyANT/APyPy and may substitute for the TATA box, as has been shown for other TATA and non-TATA containing promoters [16–18]. A second deletion was made to the AC-2 promoter 3′ end, to position −86 (3′ end deletion 2, Fig. 3(B)). This resulted in loss of all *lac Z* reporter gene activity, indicating that the region between −48 and −86 is essential for expression.

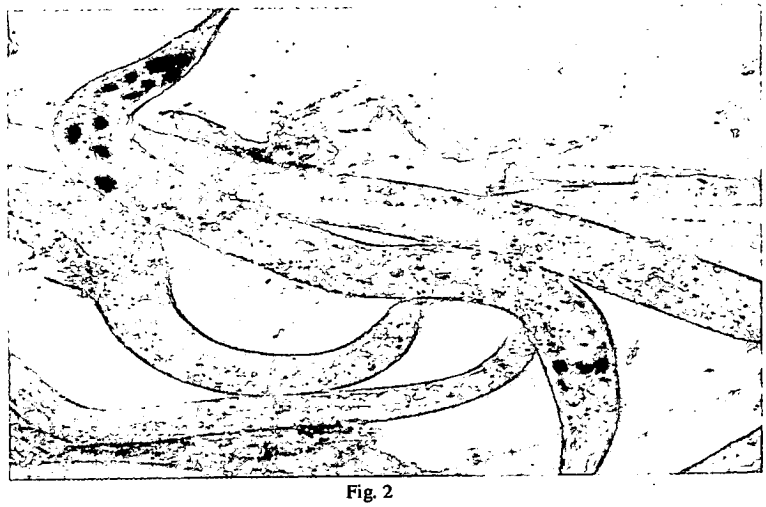

Fig. 2

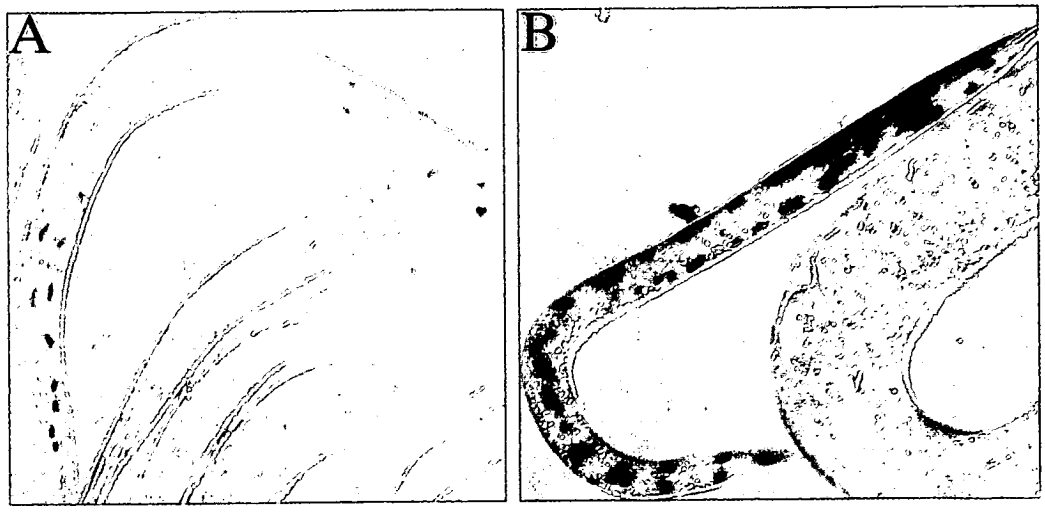

Fig. 4

Fig. 2. Expression of *H. contortus* AC-2/*lac Z* reporter construct in gut cells of transgenic *C. elegans*. This shows expression in adult stage *C. elegans*, with strong expression in anterior and posterior gut cells and weaker expression in mid gut cells. No expression is seen in any other cell type.

Fig. 4. Cell specific *lac Z* expression following transformation of *C. elegans* with other parasite promoter constructs. Panel A shows staining in anterior and posterior gut cells of adult *C. elegans* following transformation with a *lac Z* reporter vector containing 2 kb of upstream sequence of *H. contortus* pepsinogen gene *pep-1*. In Panel B, expression is observed in hypodermal cells of L4 stage *C. elegans* following transformation with an *O. circumcincta* cuticular collagen gene *colost-1* promoter/*lac Z* construct.

3.3. Mutation of AC-2 promoter GATA element

Proximal end deletion of the AC-2 promoter indicated that an essential element was present between positions −48 and −86. Examination of this region revealed that it contains an E-box motif (CANNTG) and two GATA-like elements in inverse orientation (Fig. 3(A)). Such elements

can operate in either orientation [19,20]. A GATA motif is present in a similar position in the *C. elegans cpr-1* protease gene promoter and we have shown recently that it is important for *cpr-1* expression [21]. GATA elements are also present in the immediate upstream region of the other *C. elegans cpr* genes [10] and have been shown to be necessary for correct gut expression of *C. elegans* genes *ges-1*, a gut esterase gene [22], and *vit-2*, a vitellogenin gene [23]. To determine whether this motif is involved in AC-2 promoter function, the GATA element at position −61 (numbering relative to the AC-2 ATG start codon; see Fig. 3(A)) was mutated by PCR and the resulting mutated construct injected into *C. elegans*. Worms transformed with the mutated promoter construct (GATA mut 1, Fig. 3(B)) showed no reporter gene expression, indicating that this GATA element close to the start site is essential for AC-2 promoter function. The results of the AC-2 deletion/mutation studies are summarised in Fig. 3(B).

3.4. Expression pattern of *H. contortus pep-1/lac Z construct in C. elegans*

The gut-specific expression obtained with the AC-2 promoter region was as expected for this protease gene. To further demonstrate that the spatial pattern of expression observed in *C. elegans* is consistent with expression in the parasite, the promoter region of another parasite gene, the *H. contortus* pepsinogen gene *pep-1* [11], was tested in a similar manner. The *pep-1* gene is expressed in the blood feeding L4 and adult stages of the parasite. PEP-1 protein has been immunolocalised exclusively to the gut surface, suggesting that, like AC-2, it may be involved in blood digestion. Several aspartyl proteases with sequence similarity to PEP-1 have been identified by the *C. elegans* genome project, but their expression patterns have yet to be characterised.

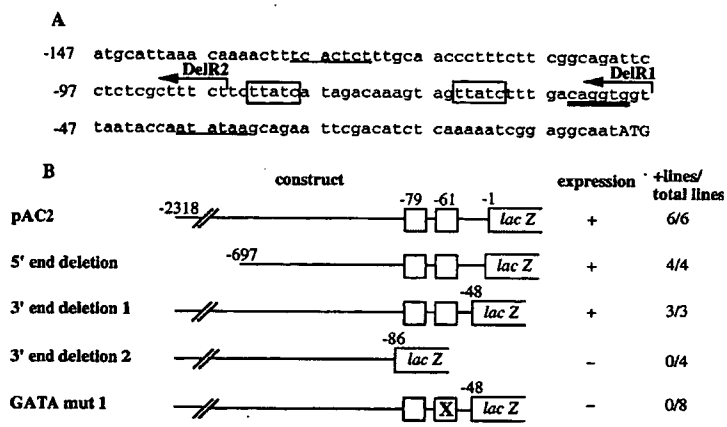

Fig. 3. Promoter analysis of *H. contortus* AC-2 gene. (A) Immediate upstream sequence of the AC-2 gene indicating putative regulatory motifs. The putative TATA box at position −39 and initiator element at position −129 are indicated (both underlined), as are the inverse GATA motifs at positions −61 and −79 (boxed) and E-box (CANNTG) motif (double underlined). Numbering is relative to the ATG start codon (shown in upper case). Also shown are the positions of proximal end deletion antisense primers DelR1 and DelR2 used in PCR to generate the corresponding 3′ end deletion constructs 1 and 2. (B) Presence (+) or absence (−) of gut cell staining following transformation with AC-2 deletion and mutation constructs. GATA motifs are indicated by boxes in all constructs and mutation of the GATA motif at position −61 in construct GATA mut 1 is shown by X. The number of transformed *C. elegans* lines showing expression from the total number of stable lines generated with each construct is shown on the right hand column.

To examine *pep-1* promoter activity, transformation of *C. elegans* was carried out with a *lac Z* reporter construct containing 2 kb of upstream sequence of the *pep-1* gene. Transformants showed *lac Z* expression exclusively in gut cells, consistent with expression in the parasite. Strong and frequent expression was observed in L4 and adult worms (Fig. 4(A)) and in the early larval stages (L1, L2 and L3). This latter expression is not detected in the parasite by RT–PCR analysis [11]. No expression was observed in embryos.

3.5. Expression of a parasite cuticular collagen gene promoter in C. elegans

To confirm that the tissue specificity of expression was dependent on the parasite gene promoter and not on the particular reporter vector used, we examined the expression pattern of a construct containing 5′ flanking sequence of a parasite gene expected to be expressed in non-gut cells. Upstream region of the cuticular collagen gene *colost-1* [12] from *Ostertagia circumcincta*, another parasitic strongyle nematode of sheep, was examined in *C. elegans*. *colost-1* is closely linked to an almost identical gene, *colost-2*, and these genes show strong sequence similarity to cuticular collagen genes *col-12* and *col-13* from *C. elegans*, which are also almost identical to one another and arranged in tandem [24]. These strong similarities in sequence and gene organisation suggest that *colost-1* and *colost-2* are orthologs of *col-12* and *col-13*. They are, therefore, predicted to encode collagens present in the cuticular layer [12], which is synthesised by the underlying hypodermis. Transformation of *C. elegans* with a *lac Z* reporter construct containing 1.3 kb of *colost-1* upstream sequence resulted in expression specifically in hypodermal cells (Fig. 4(B)), as predicted for a cuticular collagen gene. While the *C. elegans* putative orthologs *col-12* and *col-13* are expressed in all larval stages and at an increased level in adult worms, as detected by semi-quantitative RT–PCR [25], expression of the *O. circumcincta colost-1/lac Z* construct in *C. elegans* was observed only in the L4 stage.

4. Discussion

This study has demonstrated, using three different parasite gene promoters, that *C. elegans* can be used as a surrogate transformation system to examine nematode parasite gene regulation. The conservation of regulatory elements and transcription factors across widely divergent groups [26] suggests that, for many genes, there is likely to be sufficient conservation of regulatory mechanisms between *C. elegans* and parasitic nematodes to detect promoter function using this heterologous system. We have demonstrated promoter function from two animal parasitic nematode species and it has recently been reported that a putative gene promoter from the potato cyst nematode *Globodera rostochiensis* can function in *C. elegans* [27].

The advantage of this approach over other tested transformation systems is that it also provides information of the spatial and temporal expression pattern. This is true for the genes examined here and, most probably, pertinent to other genes conserved to some degree between parasites and *C. elegans*. Strict tissue-specific expression was observed for the parasite gene promoters examined. Promoters of the *H. contortus* gut-enzyme encoding genes AC-2 and *pep-1* were expressed exclusively in gut cells of *C. elegans*, while the *colost-1* cuticular collagen gene promoter directed expression exclusively in hypodermal cells. This conservation of spatial expression is probably to be expected considering the anatomical conservation between these closely related nematode species. In these nematodes, the gut is where digestion and absorption of nutrients occur and is, therefore, the tissue in which enzymes involved in these processes are expected to be expressed, no matter how diverse they may be in substrate specificity. Similarly, as the nematode cuticle is conserved structurally and functionally, cuticular collagen gene expression would be expected to occur in the hypodermal tissue in different species. Our findings confirm the conserved function of these tissues in the different species examined and indicate evolutionary constraint in the regulatory mechanisms controlling spatial expression of these structural genes. It will be inter-

esting to examine the spatial expression pattern of genes encoding similar digestive enzymes of other nematode species such as the filarids, in which a significant part of their nutrition is achieved transcuticularly [28,29]. Are such homologous enzymes expressed in the hypodermal and cuticular regions?

While spatial expression of the parasite promoter/*lac Z* constructs in *C. elegans* correlated with their expression in the parasites, the exact timing of expression differed. In *C. elegans*, the *H. contortus* AC-2 and *pep-1* promoters were active in all larval stages and in adult worms. In the parasite, mRNA and protein from these genes are detected only in the L4 and adult stages [8,11]. One possible explanation for this difference is that these genes are expressed in the early larval stages of the parasite, but at levels too low to be detected in previous studies. Alternatively, the exact mechanisms regulating temporal expression of these genes may not be conserved between these nematode species. The expression pattern observed in *C. elegans* may reflect the expression pattern of homologous *C. elegans* genes and the activity of the transacting factors involved in their regulation: AC-2 may be regulated by factors involved in *C. elegans* cysteine protease (*cpr*) gene expression and thus be activated in all stages except the embryo. The differences in temporal expression observed here could result from a greater level of activator in *C. elegans* driving expression over a threshold not reached in the parasite, or may result from earlier expression of a homologous factor or expression of a different factor(s) directing larval expression in *C. elegans*. Another possibility is that repressor factors may be present in the parasite to prevent expression in the early larval stages. Band shift studies should identify qualitative or quantitative differences in the regulatory factors involved.

Expression from the *O. circumcincta colost-1* promoter showed a temporally restricted pattern in *C. elegans*, being observed only in the L4 stage. In *O. circumcincta* this gene is detected in the early larval (L1–L3) stages by RT–PCR [12]. It is not known if the level of expression increases in the parasite L4 stage, which could explain the present finding. However, this expression pattern differs from that of the proposed *C. elegans* orthologs, *col-12* and *col-13*, which are expressed in all larval stages and at an increased level in the adult worm [25]. This again suggests differences in the temporal control of the parasite and *C. elegans* genes. Our findings indicate species-specific differences in the fine tuning of regulatory mechanisms and suggest that, while constraint is placed on spatial expression of terminal genes, regulators or regulators of regulators involved in exact timing of expression have undergone more rapid evolutionary change. It has been argued that alteration to gene regulation is the major mechanism of evolution [30,31]. We require a greater knowledge of parasite transcriptional regulators, how they are developmentally controlled and interact with other factors to influence the timing of developmental events. Comparison of these factors to their *C. elegans* homologs will be important to our understanding of nematode parasite evolution and adaptation.

The transformation system used here can identify parasite promoter elements involved in spatial and temporal gene expression. In the present study, mutation analysis of the *H. contortus* AC-2 gene promoter identified a GATA element close to the start site which is essential for promoter activity. This is very similar to our previous findings for the *C. elegans* cysteine protease gene *cpr-1* [21] and indicates that both genes are regulated by similar mechanisms, possibly involving a gut-expressed GATA-like transcription factor. In the present study we observed strong, tissue-specific expression from transcriptional fusion constructs, indicating that any motifs present downstream of the translational start site are not essential, at least for the expression observed here. We have previously found that a Box 1 type motif present in the first intron of the *C. elegans cpr-1* protease gene [9] is not essential for reporter gene expression, and that upstream sequence directs the same expression pattern as that observed in in situ hybridisation and Northern blot analysis of this gene [21]. However, we cannot rule out the possibility that, although not essential, downstream motifs may play some role in regulation of the parasite genes examined here.

As well as dissecting the elements involved in gene expression, we can make use of information

from the *C. elegans* genome project to begin to identify parasite homologs of *C. elegans* transcription factors. In *C. elegans* the gut-specific GATA factor ELT-2 is speculated to act as a key regulator of a number of genes encoding structural proteins, such as the gut esterase *ges-1*, the vitellogenins and the *cpr* cysteine protease genes [32]. Loss of ELT-2 function is lethal, with L1 larvae dying with gut malformation and degeneration. Other GATA-like transcription factors have also been shown to play essential roles in *C. elegans* tissue development and maintenance [33,34]. Interfering with specific interaction of homologous parasite factors with their DNA binding site could be an important, novel method of parasite control, not only inhibiting structural gene expression but also affecting parasite development.

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Chapter 9: *Haemonchus contortus* SL2 trans-spliced RNA leader sequence.

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In bacterial genomes, functionally related genes are often clustered together in operon structures and are co-transcribed as polycistronic pre-mRNAs from a single upstream promoter (Lewin, 1990). *C. elegans* and its relatives are unique among animals in having operons (Speith et al, 1993). At least 1,000 operons exist in *C. elegans*, each 2-8 genes long, constituting ~15% of all *C. elegans* genes, and with related genes occurring together at a frequency greater than would be expected by chance (Blumenthal et al, 2002). More than 80% of *C. elegans* mRNAs are *trans*-spliced to the common spliced leader 1 (SL1) sequence (Nilsen, 1995), which has been shown to be required for *C. elegans* survival (Ferguson et al, 1996). Cell-free extracts from synchronously developing embryos of *Ascaris lumbricoides* have been used to demonstrate a role for *trans*-splicing in nematode gene expression, with *trans*-splicing of the SL sequence and its associated hypermethylated cap structure functionally collaborating to enhance the translational efficiency of a specific mRNA (Maroney et al, 1995).

Polycistronic pre-mRNAs are processed into monocistronic units through 3' end formation and *trans*-splicing of the specialised spliced leader 2 (SL2) small ribonuclear particle to downstream mRNAs (Huang and Hirsh, 1989). In essence, *trans*-splicing to an SL2 sequence is considered as evidence of an operon structure (Blumenthal, 1995).

Through the characterisation of the *H. contortus* pepsinogen gene promoter in an attempt to identify its minimal promoter region (see Chapter 8), primer extension analysis identified a proportion of clones extended to –14 bp in relation to the ATG start codon. Of the ten extended sequences, two were *trans*-spliced to a 22 nucleotide SL2-like sequence which differed from the *C. elegans* SL2 sequence by only a 2 bp inversion.

The aim of this work was to clone and characterise the *H. contortus* SL2 gene sequence.

The *H. contortus* SL2 gene was isolated by PCR using a primer based on the *H. contortus* 22 bp SL2 sequence in conjunction with an antisense primer directed to a conserved region of *C. elegans* SL2 RNA sequence and using *H. contortus* genomic DNA as template under high stringency conditions. The 3' end of the *H. contortus* SL2 gene was subsequently obtained by application of the two-step PCR technique developed for the amplification of unknown flanking regions described in Chapter 8. Nucleotide sequence analysis of the amplified *H. contortus* SL2 gene showed it to be 113 bp in length and to share 66% identity with the *C. elegans* SL2 gene and even greater identity, 74%, with *Dolichorhabditis* SL2α, β and γ gene sequences. Computer modelling of the *H. contortus* SL2 gene sequence showed the characteristic, three stem-loop structure of nematode SL2 RNAs, including an Sm-binding consensus sequence located between the second and third stem-loops, and conservation of the top of the third SL2 stem-loop, shown to be necessary for polycistronic pre-mRNA processing.

The results presented here represent the first observed case of SL2 *trans*-splicing in *H. contortus*. However, the 2 kb of sequence immediately upstream of the pepsinogen gene has been shown to have a true promoter function (Britton et al, 1999; Chapter 8) suggesting that the *H. contortus* pepsinogen gene does not represent a downstream gene in an operon structure. Therefore, it is likely that the observed pattern of *trans*-splicing to *H. contortus* pepsinogen is analogous to the low-level *trans*-splicing of SL2 to the first gene of an operon reported to occur in both *Dolichorhabditis* and *C. elegans*.

Contribution to work

The inception, execution and analysis of this work and preparation of the manuscript for publication were carried out solely by myself. Communication of results at scientific meetings.

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Short communication

Haemonchus contortus SL2 trans-spliced RNA leader sequence☆

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In bacteria, genes whose products perform related functions are often clustered together in operon structures [1] and are co-transcribed from a single upstream promoter as polycistronic mRNAs. In contrast, genes of higher eukaryotes are generally transcribed as monocistronic mRNAs, each gene being individually regulated under the control of its own promoter and translational initiation site.

However, the free-living nematode *Caenorhabditis elegans* and, more recently, the rhabditid nematode *Dolichorhabditis* have been shown to use both the prokaryotic and eukaryotic patterns of gene organisation and transcription [2,3]. Approximately 25% of *C. elegans* genes are arranged in operons [4]. The resulting transcribed polycistronic, multigene pre-mRNAs are processed into monocistronic units as they are synthesised by cleavage and polyadenylation and by trans-splicing [2]. Trans-splicing was first described in trypanosomatid protozoans [5,6] and later shown to occur also in *Euglena* [7], flatworms [8] and nematodes [9]. In nematodes, trans-splicing involves the addition of a 22 nucleotide spliced leader (SL), including a trimethylguanosine cap, from a small nuclear RNA to the 5′ ends of pre-mRNAs [10–12].

Two SL sequences are found in *C. elegans*. The first sequence, SL1, is trans-spliced onto more than 80% of *C. elegans* mRNAs [13]. The second SL sequence, SL2, is reserved for splicing to downstream genes in polycistronic pre-mRNAs derived from operons [2], and its presence on the 5′ end of a mRNA is, therefore, considered as evidence of an operon [14].

Trans-splicing to SL1 has been shown to occur in the ovine parasitic nematode *Haemonchus contortus* [15] although, to date, trans-splicing to SL2 and the presence of operons have not been observed. Here, we report low-level splicing of an SL2-like sequence to *Haemonchus* aspartyl proteinase (pepsinogen) [16] mRNA, isolation of the corresponding SL2 gene and its characterisation.

The 5′ ends of the *H. contortus* pepsinogen mRNAs were determined by primer-extension analysis. This analysis showed that 10 of the 14 clones sequenced were extended to −14 bp in relation to the ATG translational start codon. Of these 10 clones, one was not trans-spliced, seven were trans-spliced to SL1, and two were trans-spliced to a 22 nucleotide SL2-like sequence. With the exception of a 2 bp inversion, the 22 nucleotide *Haemonchus* SL2 sequence is identical to *C. elegans* SL2 (Fig. 1A). Typically, the first gene in an operon is either trans-spliced to SL1 or not trans-spliced at all, whereas the downstream genes are trans-spliced exclusively to SL2 or to a mixture of SL2 and SL1 [2,4]. That the *Haemonchus* pepsinogen mRNAs were predominantly trans-spliced to SL1 with low levels of trans-splicing to SL2 being observed may indicate that the *Haemonchus* pepsinogen represents a downstream gene in an operon. However, analysis of 2 kb of sequence directly upstream of the *Haemonchus* pepsinogen gene has failed to identify the presence of an upstream gene (data not shown). It has also been shown previously that this 2 kb upstream sequence,

Abbreviations: PCR, polymerase chain reaction; SL, spliced leader.

☆ *Note:* Nucleotide sequence data reported in this paper have been submitted to the GenBank™, EMBL, and DDJB data base with the accession number AF213679.

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cloned in the correct orientation as would be expected to drive transcription of the pepsinogen open reading frame, can drive correct spatial expression of the β-galactosidase reporter gene in transgenic *C. elegans* [17], thus demonstrating a true promoter function. Low-level splicing of SL2 to the first gene of an operon has been reported recently in both *Dolichorhabditis* [3] and *C. elegans* [18]. The results reported here indicate an analogous situation in *H. contortus*, although analysis of genomic sequence downstream of the *Haemonchus* pepsinogen gene will be necessary to determine whether or not a second gene is present.

The *Haemonchus* SL2 gene was isolated by PCR. It is 113 bp in length and shows 66% sequence homology when aligned with *C. elegans* SL2α and SL2β genes and 74% homology with *Dolichorhabditis* SL2α, SL2β and SL2γ genes (Fig. 1A). The secondary structure of the *Haemonchus* SL2 gene as determined by computer-generated RNA folding (Fig. 1B) shows the characteristic, conserved structure of nematode SL RNAs. This structure consists of three stem-loops with the SL base paired in the most 5′ stem-loop and the GG splice donor site positioned adjacent to the turn of this loop [11]. An Sm-binding consensus sequence [21] is found between the second and third stem-loops. The importance of the Sm-binding site has been demonstrated in *Ascaris lumbricoides* where mutations within this region that prevent binding of SL ribonucleoprotein-specific

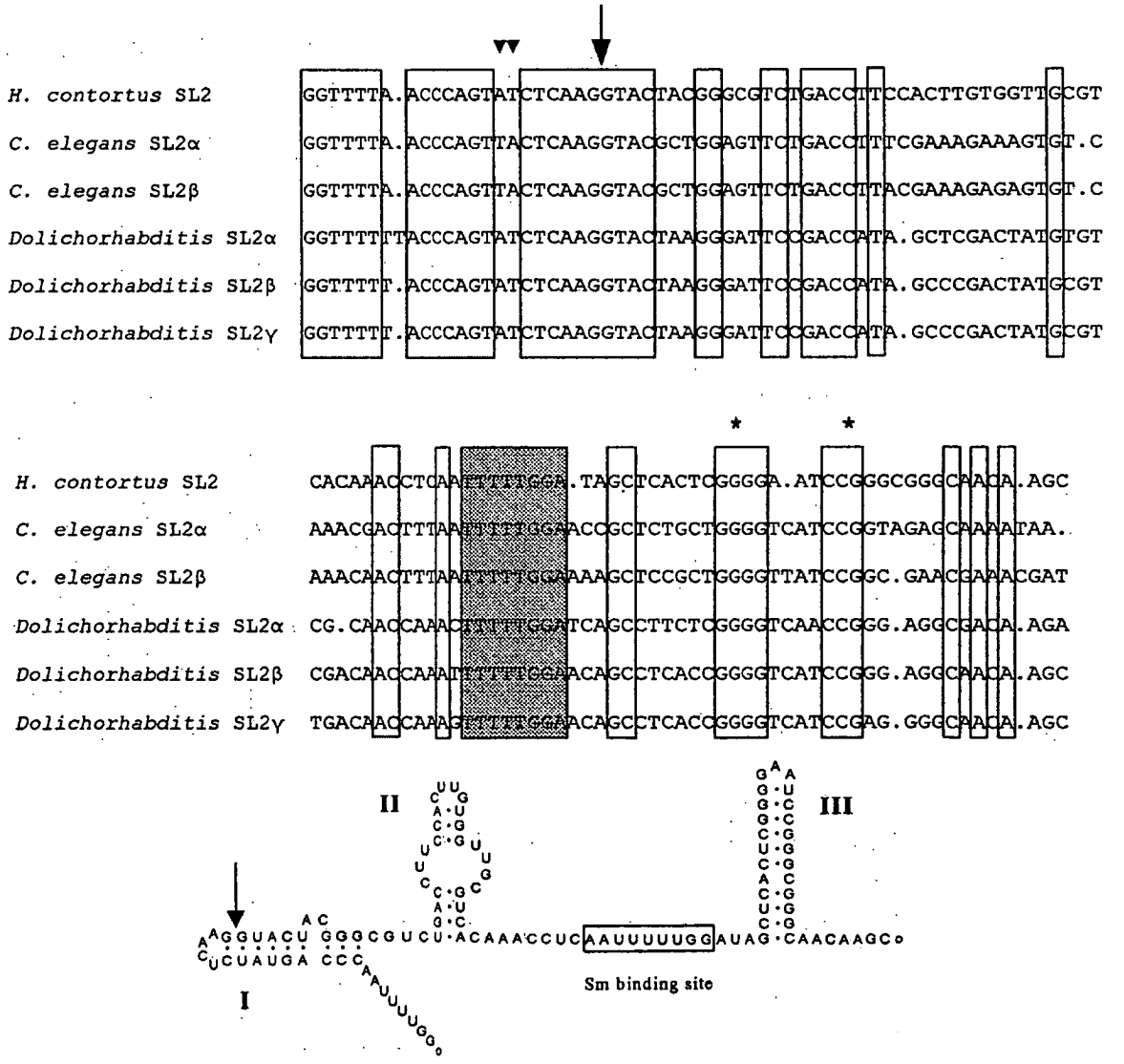

Fig. 1.

proteins result in failure to trans-splice in vitro [22]. Finally, there is conservation in the top of the SL2 third stem-loop, which is not seen in SL1 RNA. It has been suggested that this region of SL2 RNA may contribute to the determination of SL2 specificity, perhaps by binding an SL2 RNA-specific polypeptide [3].

In conclusion, we report here the discovery of SL2 trans-splicing in *H. contortus*. The *Haemonchus* SL2 gene shows homology with both *C. elegans* and *Dolichorhabditis* SL2 gene sequences with the predicted secondary structure showing the characteristic, conserved features of nematode SL RNAs. The fact that the sequence immediately upstream of the *Haemonchus* pepsinogen gene shows a true promoter function suggests that it is not a downstream gene in an operon structure. Analysis of genomic sequence downstream of the *Haemonchus* pepsinogen gene will be necessary, therefore, to determine whether or not a second gene is present and to determine the presence of an operon structure in *H. contortus*.

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Fig. 1. (A) Multiple sequence alignment of SL2 gene sequences from *H. contortus*, *C. elegans* and *Dolichorhabditis*. Primer extension analysis to determine the 5′ ends of the *H. contortus* pepsinogen mRNAs was performed on messenger RNA extracted from adult worms using a commercial kit (Stratagene). Approximately 750 ng of mRNA was reverse-transcribed with Superscript™II reverse transcriptase (Gibco BRL) using an oligo(dT) primer and reaction conditions as outlined in the Smart™RACE cDNA Amplification kit (Clontech). The resultant cDNA was used as target in a polymerase chain reaction (PCR) using the UMP primer provided in the Smart™RACE cDNA Amplification kit together with the *Haemonchus* pepsinogen-specific primer 5′-CTGCACTGCCTGACCAAATACC-3′ and the following reaction conditions. Initial denaturation at 94 °C for 30 s followed by 25 cycles of 94 °C, 30 s; 68 °C, 30 s; 72 °C, 3 min, with a final extension at 72 °C for 2 min, in a reaction volume of 50 μl. One microlitre of this reaction was then re-amplified using the UMP primer together with the nested pepsinogen-specific primer 5′-CACTGCCAGTATCTAGAAC-3′ and the PCR conditions: 94 °C, 5 min followed by 30 cycles of 94 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min, with a final extension at 72 °C for 7 min. Amplification products were purified using a Geneclean kit (Bio 101, Inc.), cloned directly into the pCRII vector (TA Cloning System, Invitrogen) and sequenced using Applied Biosystems Big Dye technology. The *Haemonchus* SL2 gene sequence was obtained by PCR using 500 ng of genomic DNA prepared by the method of Herrmann and Frischauf [19] as target. Initial amplification was done using a primer based on the *Haemonchus* SL2 sequence (5′-GGTTTTAACCCAGTATCTCAAG-3′) in conjunction with an antisense primer complementary to nucleotides 90–110 of *C. elegans* SL2 RNA (5′-TTTGCTCTACCGGATGACCCC-3′). PCR conditions were as follows: initial denaturation at 94 °C, 5 min, followed by 30 cycles of 94 °C, 30 s; 45 °C, 30 s; 72 °C, 1 min, with a final 7 min extension at 72 °C. PCR products were purified, cloned and sequenced as described above. The 3′ end of the *Haemonchus* SL2 gene was obtained using a targetted gene-walking technique (Redmond et al., submitted for publication). The *Haemonchus* SL2 primer, above, was used together with the nested gene-specific primer 5′-ACTCAAGGTACTACGGGC-3′ and the non-specific primer 5′- AGAGACGCACGCATAGCATTC-3′. PCR conditions were as described with the second-round, high-stringency amplification being carried out at 56 °C. Nucleotides that are conserved in all of the sequences are boxed. The 2 bp inversion between the *H. contorus* and *C. elegans* trans-spliced SL2 sequences is denoted by ▼▼. The trans-splice site is indicated by an arrow and the Sm-binding site highlighted by the shaded box. An asterisk denotes the conserved sequence at the top of the third stem-loop. (B) Proposed secondary structure of the *H. contortus* SL2 gene generated using the RnaViz 1.0 computer program [20] under the constraint that the Sm-binding region remains single-stranded. The three stem-loops are numbered. The trans-splice site is arrowed and the Sm-binding site boxed.

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Chapter 10: Unresolved Questions

Although the work presented in chapters 2-9 has significantly advanced progress towards the production of a *H. contortus* vaccine, several questions remain unresolved. The application of new technology, such as functional genomics, expressed sequence tag (EST) analysis and RNA interference (RNAi), may help to address some of these outstanding issues.

For example, preliminary characterisation of the enzymes present in extracts of adult *H. contortus* identified cysteine, serine, metallo- and aspartyl proteinase activities with different molecular size, substrate specificity and pH optima (see Chapter 2). However, how many proteinases contribute to the observed enzyme activities was not established. This question could be addressed by active site labelling experiments. Alternatively, identification of specific proteins in worm extracts can be achieved through a combination of 2D gel electophoresis, mass spectrometric analysis of individual protein spots to obtain amino acid sequence data, and protein/nucleotide database searching. This approach would also allow more precise definition of enzyme activity. For example, EST analysis of adult *H. contortus* has identified the presence of a distinct pepsinogen-like sequence and a cathepsin D, both of which may contribute to the observed aspartyl proteinase activity. In addition, the relative abundance of different proteins can also be obtained from EST database analysis.

Although polyacrylamide gel electophoresis and N-terminal amino acid sequence analysis has identified the major components of the integral membrane protein H-gal-GP complex, further analysis will be required to fully characterise all its protein components. 2D gel electrophoresis combined with more sensitive silver staining would help to identify the relatively less abundant protein components of the complex. This approach may also help to identify a component(s) of H-gal-GP which could function to hold the complex together, or to anchor it to the parasite gut membrane, a question currently unresolved since the major components identified thus far contain neither an anchoring transmembrane hydrophobic region or a phosphatidylinositol phospholipase anchor. This approach would also be useful in defining the protein components of TSBP. Three cysteine proteinases, namely

hmcp1, 4 and 6, have been associated with TSBP through immunoscreening of a cDNA library (see Chapter 3), although identification of the specific protein bands encoding each protein remains undefined. Again, this could be resolved using proteomic approaches and which may also identify new, immunogenic, low abundance proteins.

Partial, variable protection has been achieved in sheep immunised with either of two different molecular weight gel slices of H-gal-GP. A vaccine trial using a combination of these two fractions would determine whether or not they can act synergistically to produce levels of protection comparable to that achieved with whole H-gal-GP. Combination trials using recombinant proteins should also be evaluated.

The presence of multigene families of both cysteine and metallo-proteinases (see Chapters 3 and 5) has been confirmed by EST analysis. Importantly, the lack of homologous cysteine proteinases reported in the USDA BPL1 and Moredun strains of *H. contortus* (see Chapters 2 and 3) is borne out by EST database analysis and highlights potential implications for the development of a generic *H. contortus* vaccine.

The question of *in vivo* function of the enzyme components of both H-gal-GP and TSBP remains unresolved. That they function in digestion of the host blood meal is based on their localisation to the surface of the parasite gut and restriction of their temporal expression to the blood-feeding L4 and adult parasitic stages. An alternative role for *H. contortus* MEP in maintenance of parasite gut homeostasis has been postulated (see Chapter 5). This hypothesis could be tested by analysing the ability of MEP-enriched parasite fractions to hydrolyse pharmacological concentrations of host enkephalin *in vitro*. Expression of enzymatically active recombinant proteins would also allow analysis of substrate specificity, e.g. blood proteins, both individually and in combination *in vitro*. Where gene homologues exist in *C. elegans*, functionality and their potential importance for parasite survival and development could be studied using RNAi technology. Moreover, the possible application of RNAi to *H. contortus* is an interesting concept. During the course of hybridisation studies, Le Jambre (1979) showed that small numbers of adult *H. contortus* recovered from the abomasum of donor sheep can establish infection

when introduced directly into the abomasum of a worm-free recipient sheep. Recently, ballistic DNA transfer has been used to transform several different parasite species (Jackstadt et al, 1999). That RNA can be fixed to gold particles means that ballistic transfer offers a potential, although costly, method of gene knockout to study the gross effects of loss of function on the establishment and maintenance of infection of adult *H. contortus* in the natural host.

Bacterially expressed recombinant *H. contortus* pepsinogen and MEP1 have both proved to be enzymatically inactive which suggests that expression in a eukaryotic system may be required to obtain appropriate glycosylation and protein folding. Low level expression of *H. contortus* pepsinogen has been achieved in *C. elegans* (see Chapter 7). The interesting possibility of optimising expression levels through use of a strong promoter is still to be investigated. Similarly, it remains to be established whether or not the recombinant *H. contortus* protein is expressed in an enzymatically active form in transgenic *C. elegans*. This question could be addressed through purification of expressed recombinant *H. contortus* pepsinogen by affinity chromatography using antibodies raised to bacterially expressed recombinant PEP1 protein and testing its ability to degrade a specific low molecular weight substrate. Additionally, it may then be possible to compare the pattern of glycosylation between the native and *C. elegans*-expressed pepsinogen using mass spectrometry.

The observed *trans*-splicing to SL2 (see Chapter 9) is indicative of the presence of operon structures in *H. contortus*, although their presence has not been conclusively demonstrated. This could be investigated by analysing downstream sequence from the *H. contortus* pepsinogen gene by either gene walking or hybridisation screening of a genomic DNA library to determine whether or not a second gene is present.

Chapter 11: General Discussion

The work presented here has helped to identify potentially protective enzyme components of two host protective protein complexes, namely TSBP and H-gal-GP, isolated from the intestines of adult *H. contortus*. Together with their molecular cloning and characterisation, this is a significant advancement towards the ultimate aim of producing a recombinant vaccine against *H. contortus* infection. The successful expression of *H. contortus* pepsinogen in the free-living nematode *C. elegans*, demonstrates its potential as a novel eukaryotic system for expression of candidate parasite vaccine antigens in which glycosylation and/or conformational epitopes are considered important for protection. In addition, the potential of *C. elegans* as a heterologous transformation system for the identification of parasite promoter elements and transcription factors has also been demonstrated. The low-level *trans*-splicing of an SL2 sequence to pepsinogen mRNA represents the first observed case of SL2 *trans*-splicing in *H. contortus*, although evidence of an operon structure was not determined.

Further advances in these areas of work are discussed below.

11.1 TSBP

Immunoscreening of an adult *H. contortus* cDNA library with pooled sera from sheep vaccinated with TSBP and which were demonstrably protected against subsequent homologous challenge, identified the 60 kDa major component of TSBP (Figure 4) as glutamate dehydrogenase (Skuce et al, 1999). That glutamate dehydrogenase is localised to the cytoplasm of parasite intestinal cells suggests that it is unlikely to be accessible to host antibody and, therefore, despite being the most prominent and antigenic protein in the fraction, is unlikely to be the protective component of *H. contortus* TSBP. This was confirmed in a sheep vaccination trial where immunisation with a MonoQ fraction of *H. contortus* TSBP, enriched for glutamate dehydrogenase, failed to afford any degree of protection (unpublished results).

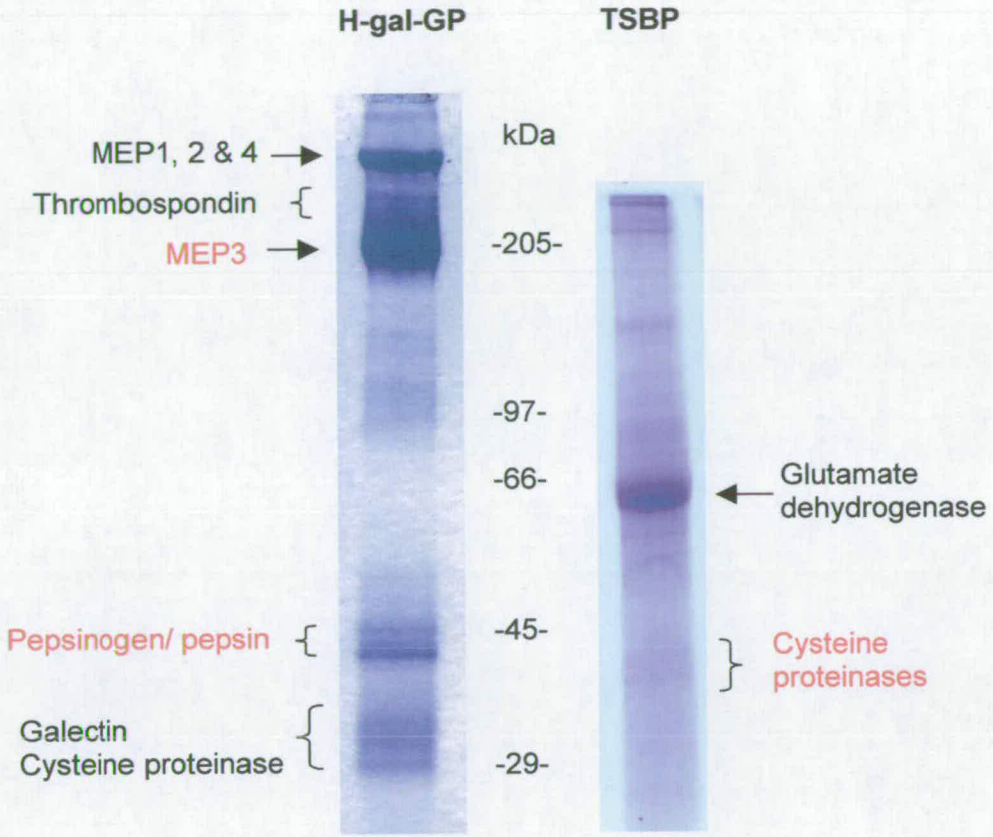

Figure 4. Summary of identified protein components of the host-protective H-gal-GP and TSBP antigens isolated from adult *H. contortus* and separated under non-reducing SDS-PAGE conditions. Proteins associated with protection are shown in red.

H-gal-GP: all identified proteins components are glycosylated as determined by lectin-binding and nucleotide sequence analysis.

TSBP: of the three identified cysteine proteinase components of TSBP, hmcp4 is not glycosylated. Amino acid sequence analysis will be necessary to specifically identify the protein bands corresponding to hmcp1, 4 and 6.

As mentioned previously (see Chapter 3), further to the molecular isolation and characterisation of the cysteine proteinases associated with adult *H. contortus* TSBP, namely hmcp1, 4 and 6, evidence that the cysteine proteinase activity is the protective component of TSBP has been provided from further vaccination trials. TSBP was fractionated by either anion-exchange chromatography on MonoQ or by affinity chromatography using recombinant *H. contortus* cystatin, a potent and specific cysteine proteinase inhibitor (Newlands et al, 2001), bound to sepharose. In both cases, only subfractions containing cysteine proteinase activity afforded any degree of protection against homologous challenge when used to immunise sheep (unpublished results). Importantly, the degree of protection conferred with the cystatin-binding fraction of TSBP, even though it represented only 1-1.5% of total TSBP proteins, was repeatable and was similar to that attained with whole TSBP.

The cDNAs corresponding to the predicted mature forms of hmcp1, 4 and 6 have been cloned into the pGEX-6P bacterial expression vector to form GST fusions. In all three cases, although high levels of bacterially-expressed recombinant GST-fusion proteins were obtained, they proved to be insoluble. Following the methodology of Rothel et al (1997), and with respect to preparation of the *T. ovis* 45W vaccine antigen, recombinant fusion proteins were solubilised in urea and dithiothreitol, and a cocktail of recombinant GST-hmcp1, 4 and 6 in equal proportions was used to immunise sheep. Sheep were immunised three times at three-weekly intervals with 100μg protein per injection using QuilA as adjuvant, and challenged with 5,000 infective *H. contortus* L3 seven days after the final immunisation. Preliminary results have proved highly encouraging. Although no significant reduction in cumulative FEC (34 days post challenge) was observed compared to adjuvant control animals, and the male to female ratio of surviving worms at necropsy was unaffected, a highly significant 38% reduction in worm burdens ($P< 0.01$) was observed (unpublished results) which was comparable to the 43% reduction in worm burdens obtained with whole TSBP in this experiment. This data implies that conformational epitopes are not a prerequisite for protection induced by TSBP. This contention could be addressed directly by determining the efficacy of denatured TSBP in a sheep vaccination trial. A reduction in worm burdens with no significant reduction in FEC, or male to female worm ratios,

suggests an overall increase in egg production by surviving female worms. The viability of these eggs was not determined but would be an important parameter to measure in repeat trials.

With respect to other *H. contortus* "hidden" antigens, H11 and H-gal-GP, protection has been found to correlate with host serum antibody titre (Munn et al, 1993a and 1993b; Smith et al, 1999, Chapter 4). However, enzyme-linked immunosorbent assay analysis of sera from the cysteine proteinase fractionation and recombinant vaccine trials has, so far, failed to identify a similar trend, although the number of animals analysed is still relatively small. Nevertheless, host serum IgG may not be the only effector mechanism involved in immunity with respect to the hidden antigen vaccination approach. For example, Karanu et al (1997) demonstrated that $CD4^+$ T lymphocytes contribute to immunity induced by *H. contortus* gut antigens in sheep and goats possibly working synergistically with antibody. $CD4^+$ T lymphocyte-derived Type 2 cytokines can promote tissue eosinophilia (IL-5), gut permeability and mucosal mastocytosis (IL-4 and IL-13), functions associated with immunity to gastrointestinal nematodes. Eosinophil-mediated killing of *H. contortus* L3 has been shown to occur *in vitro* (Rainbird et al, 1998). IL-4 and/or IL-13 enhance gut epithelial fluid secretion (Shea-Donohue et al, 2001; McDermott et al, 2003), mucus secretion and smooth muscle contraction (Zhao et al, 2003) which may function to dislodge worms from the mucosal surface of the gut. Recently, mucosal mast cells have been shown to directly enhance intestinal epithelium permeability and to mediate parasite expulsion in *T. spiralis* infected mice (McDermott et al, 2003).

11.2 H-gal-GP

The continued progress on H-gal-GP is attributable to the work of colleagues at Moredun. Immunoscreening of an adult *H. contortus* cDNA library has identified a further two components of H-gal-GP, galectin and thrombospondin (Figure 4), which had not been identified previously from the N-terminal amino acid sequence analysis of the poypeptide components of this complex (Smith et al, 1999; Chapter 4). Native galectin could be competitively extracted from H-gal-GP using lactose-agarose affinity chromatography without dissociation of the complex (Newlands et

al, 1999). When used in a sheep vaccination trial, purified native *H. contortus* galectin afforded no protection against subsequent homologous challenge, despite the induction of a high, specific antibody response to the native protein. This demonstrated that the protection conferred by vaccination with H-gal-GP was not due to its galectin moiety (Newlands et al, 1999). These results contrast with work elsewhere on *T. circumcincta*, in which extracts enriched for galectins were shown to be protective (Meeusen et al, 1995).

The thrombospondin component of H-gal-GP is associated with a diffuse zone of ~190-210 kDa under non-reducing SDS-PAGE conditions (Skuce et al, 2001). This unusual *H. contortus* glycoprotein contains six thrombospondin glycoconjugate-binding domains, together with a predominance of serine/glycine residues, suggesting that it is adhesive by nature. In addition, *H. contortus* thrombospondin contains six Kunitz-type serine proteinase inhibitor domains which share homology with anticoagulant peptides and suggest a potential role in blood-feeding through inhibition of blood coagulation. However, that native thrombospondin is not processed into its respective domains in *H. contortus* (Skuce et al, 2001) suggests that these serine proteinase inhibitor domains are more likely to function as structural spacers. Together with the fact that a close homologue exists in the free-living nematode *C. elegans* and that expression of thrombospondin appears to be down-regulated in the blood-feeding L4 and adult stages of *H. contortus*, a role in blood-feeding seems unlikely. The relatively low abundance of *H. contortus* thrombospondin, and its apparently weak immunogenicity, argue against it being a protective component of H-gal-GP (Skuce et al, 2001).

Recently, jacalin lectin was used to purify a complex from a detergent extract of adult *H. contortus* membranes (Smith et al, 2000a). SDS-PAGE and Western blot analysis showed that this complex (H-sialgal-GP) appeared to be similar to H-gal-GP, although substantially depleted in the 230 kDa band. Two vaccination trials with H-sialgal-GP showed it to be equally efficacious as H-gal-GP (Smith et al, 2000a), suggesting that the components of the 230 kDa band, defined previously as the metallopeptidases MEP1, 2 and 4 (Smith et al, 1999; Chapter 4) are not essential for protection. A previous gel cut-out vaccination trial demonstrated that partial protection against *H. contortus* challenge infection in sheep could be imparted using

fractions containing metallopeptidases and pepsinogen (Smith and Smith, 1996). That significant protection can be attained by immunisation with H-sialgal-GP, has, therefore, focused attention on MEP3 as the likely protective metallopeptidase polypeptide component of H-gal-GP.

Subsequently, work has concentrated on expression of recombinant *H. contortus* pepsinogen and MEP3 for evaluation in sheep vaccine trials. Sequential reduction and denaturation of H-gal-GP results in loss of its protective capacity (Smith and Smith, 1996), suggesting that expression of these proteins in a eukaryotic system is required to obtain appropriate protein folding and post-translational modifications. The *H. contortus* MEP3, but not pepsinogen, has been successfully expressed in the *Pichia* yeast system and lectin-purified recombinant MEP3 is currently being evaluated in a sheep protection trial. Expression of pepsinogen in baculovirus is also being investigated.

11.3 *C. elegans*

The potential of *C. elegans* as a host for expression of candidate parasite antigens has been endorsed recently through the expression of an *O. volvulus* extracellular GST (Krause et al, 2001). Post-translational modifications of the *O. volvulus* GST, including cleavage of the signal peptide and N-glycosylation, were performed successfully. The structure of the N-glycans was examined using endoglycosidase H and α-mannosidase cleavage, and the outcome was in agreement with data for native *O. volvulus* GST. Moreover, *H. contortus* cathepsin L has recently been shown to be expressed as a functionally active proteinase in *C. elegans* (Britton and Murray, 2002). The loss of *C. elegans* cathepsin L was compensated for through transgenic expression of the homologous *H. contortus* cathepsin L gene. These observations (Krause et al, 2001; Britton and Murray, 2002), together with the successful expression of pepsinogen described here (Redmond et al, 2001; Chapter 7), suggest that *C. elegans* is an appropriate vehicle for the expression of correctly processed nematode proteins.

The expression of the *O. volvulus* GST was achieved without any observed effect of survival or growth of transgenic *C. elegans* (Krause et al, 2001), suggesting that it may be possible to markedly increase recombinant protein expression levels

through the use of a strong promoter. Should the recombinant protein prove toxic, expression under the control of an inducible promoter, such as *C. elegans* heat shock protein promoters, may overcome this. Similarly, it would be interesting to establish whether or not essentially gut expressed parasite antigens could be excreted/secreted in correctly processed forms into the supernatant of transgenic *C. elegans* cultures through use of appropriate *C. elegans* promoters, although, as yet, no information is available regarding the nature of *C. elegans* ES proteins (C. Britton, personal communication). This would be advantageous in simplifying the purification of potential parasite vaccine antigens from bulk cultures, an important consideration in the production of any commercially viable vaccine.

Studies on the use of *C. elegans* as a heterologous transformation system to identify potential parasite regulatory regions have been extended through the preliminary analysis of both the *O. volvulus* GST promoter region (Krause et al, 2001) and the glyceraldehyde-3-phosphate-dehydrogenase putative promoter region from the potato cyst nematode *Globodera rostochiensis* (Qin et al, 1998). In both cases, correct spatial expression of the green fluorescent protein reporter gene was observed, indicating that certain regulatory elements in these nematode promoter regions are recognised by *trans*-acting factors from *C. elegans* and affirming the role of *C. elegans* as a model for parasitic nematodes.

Parasitic nematodes are highly complex organisms with well developed defence mechanisms which have evolved to facilitate evasion of the host immune system. For this reason, it is likely that development of a multi-component recombinant vaccine will be required. For example, although vaccination with a single recombinant antigen, Bm86, against the cattle tick *B. microplus*, has proven to evoke a protective host immune response, this response is less effective than that mounted to a partially purified Bm86 preparation (Willadsen et al, 1996). Inclusion of a second recombinant antigen, Bm91, as an adjunct to Bm86 resulted in greater vaccine efficacy, reducing tick fecundity and corresponding reproductive capacity (Willadsen et al, 1996). Similarly, in cattle, vaccination trials conducted with purified, native *F. hepatica* antigens, a combination of cathepsin L1 and haemoglobin gave significantly higher levels of protection as compared to animals

immunised with either antigen alone (Dalton et al, 1996). Therefore, although recombinant *H. contortus* candidate vaccine antigens administered individually may not achieve the protection levels necessary for an effective *H. contortus* field vaccine, a cocktail of recombinant antigens may work in a synergistic manner to provide a viable and efficient vaccine. Currently, only a limited set of defined, protective *H. contortus* antigens are being expressed for evaluation in recombinant form.

In addition to the antigens described here, the three known isoforms of the *H. contortus* hidden antigen H11 (Smith et al 1997), identified as an aminopeptidase, have been expressed as enzymatically active recombinant proteins in the insect baculovirus system. The outcome of protection trials with these antigens are awaited. Importantly, with a view to the production of a cross-protective vaccine, homologues of H11 have been identified in *T. circumcincta* and *O. ostertagia* (McMichael-Philips et al, 1995; Smith et al, 2000b). In addition, sheep vaccinated with a leucine aminopeptidase purified from adult *F. hepatica* have also been shown to be highly protected against metacercarial challenge (Piacenza et al, 1999). The cDNAs encoding the host-protective 15 kDa and 24 kDa antigens purified from adult *H. contortus* ES (Schallig and van Leeuwen, 1997), have both been expressed in bacteria (Vervelde et al, 2002). In an initial sheep trial, vaccination with a combination of the two bacterially expressed proteins gave 49% and 55% reductions in FEC and worm burdens, respectively. Unfortunately, however, protection could not be reproduced in a second trial (Vervelde et al, 2002).

Other host protective *H. contortus* candidate vaccine antigens have been defined, including contortin (Munn, 1977), Hc40 (Sharp et al, 1992), the GA1 polyprotein (Jasmer et al, 1993) and a stage-specific L3 surface antigen (Bowles et al, 1995). However, work on these antigens has either not yet progressed to the stage of their expression in recombinant forms, or remains unpublished.

The practicalities of antigen delivery should also be considered with respect to the production of a recombinant vaccine. This will necessitate an understanding of the relevant host-protective immune responses to allow an informed decision on both the route of immunisation and choice of adjuvant used. For example, protection to the *H. contortus* hidden antigens H11 and H-gal-GP correlates with host serum

antibody titres (Munn et al, 1993a and 1993b; Smith et al, 1999, Chapter 4). Therefore, formulation in QuilA, which will induce a high antibody titre, is demonstrably appropriate for these antigens. However, $CD4^+$ T lymphocytes have been shown to contribute to the immunity induced by *H. contortus* gut antigens in sheep and goats (Karanu et al, 1997). If the cytokine profile of these $CD4^+$ T lymphocytes in protected animals can be determined (i.e. Th 1 or Th 2), then it may be possible to modulate the host immune response appropriately, perhaps through the use of recombinant cytokines (Lofthouse et al, 1996), pertussis toxin, which can induce strong IgE and IgG1 antibody isotypes in mice (Sekiya, 1983), or cholera toxin, which is a potent mucosal adjuvant in mice and humans (Holmgren et al, 1993). When used as a fusion partner for the *S. haemotobium* GST antigen in a recombinant *Salmonella* vaccine, an atoxic fragment of tetanus toxin was found to modulate the immune response in immunised mice (Lee et al, 2000). A marked enhancement of serum anti-GST IgA and GST-neutralising antibodies, both of which are important correlates of protection in schistosomiasis, was induced as compared to control animals which received GST alone.

A characteristic Th2-dominated host immune response is associated with nematode infections. Therefore, adjuvants which stimulate this type of response may be more appropriate when immunising with natural parasite antigens. Indeed, significant protection with the *H. contortus* stage-specific L3 surface antigen, was achieved only with aluminium hydroxide, which induces a predominantly Th2-type host immune response (Jacobs et al, 1999). The route of immunisation was also found to be important with better levels of protection obtained by rectal mucosal immunisation as compared with intradermal delivery (Jacobs et al, 1999).

The *C. elegans* genome sequencing project and EST analyses, have produced a wealth of readily available information (The *C. elegans* sequencing consortium, 1998) which can be used to identify homologous genes in parasitic nematodes and to identify new potential vaccine antigens. For example, >40% of *B. malayi* and *O. volvulus* genes show significant matches to predicted *C. elegans* genes (The filarial genome project,. 1999; Lizotte-Waneiwski et al, 2000) and in a limited *H. contortus* EST dataset, ~70% of ESTs showed homology with *C. elegans* genes (Hoekstra et al, 2000). The development of RNAi in *C. elegans* (Fire et al, 1998)

means that, where a homologue of a parasite gene is shown to exist in *C. elegans*, its potential as a candidate vaccine antigen can be readily assessed without the need for conventional gene mutants.

In a recent study, Britton and Murray (2002) were able to demonstrate the ability of a *H. contortus* cathepsin L gene to functionally rescue the early embryonic arrested RNAi phenotype associated with its *C. elegans* homologue. This result suggested that the *H. contortus* cathepsin L gene was a functional homologue of the *C. elegans* gene. Homologous cathepsin L genes were also identified in *D. viviparus*, *T. circumcincta* and *Ascaris suum* and, as such, this enzyme may be a potentially important target for the control of several parasitic nematode species. In general, the functional importance of a gene is assessed by its ability to induce a pronounced phenotypic change or lethal RNAi phenotype. As such, RNAi knockouts which produce more subtle effects will not be detected. Therefore, when assessing potential parasite candidate vaccine antigen gene homologues by RNAi in *C. elegans*, measurement of parameters such as growth and fecundity should also be considered.

EST projects have been recently undertaken for *H. contortus* and *T. circumcincta*. Currently, >18,000 *H. contortus* sequences and >4,300 *T. circumcincta* sequences encompassing different life cycle stages have been deposited in the Nembase (www.nembase.org) and dbEST (www.ncbi.nlm.nih.gov/dbEST) databases. Importantly, EST analysis has shown that a proportion of parasite genes have no homologues in the current GeneBank databases and, therefore, may have roles which are specific to the parasitic life cycle (Lizotte-Waneiwski et al, 2000). A limited EST study of *H. contortus* (Hoekstra et al, 2000) demonstrated that the proportion of cDNAs with matches to genes of other species decreased significantly with the successive life stages of the parasite, with ~50% of adult ESTs showing no significant database matches. Comparison of ESTs from different parasite stages can be used to define gene sequences which may be associated with specific events in the life-cycle, including those genes which are switched on or upregulated in the parasitic stages, and offers a more comprehensive alternative to subtractive cDNA library analysis. That a high degree of differential regulation of gene expression exists has been demonstrated by RNA

arbitrarily-primed PCR analysis of L3 and adult *H. contortus*, with ~30% of products being unique to either stage (Hartman et al, 2001).

The relatively new development of Serial Analysis of Gene Expression (SAGE) allows the quantitative and simultaneous analysis of a large number of transcripts and may be considered an alternative to EST analysis (Velculescu et al, 1995). SAGE is based on the fact that a 10 bp tag sequence contains sufficient information to uniquely identify a transcript. Short diagnostic tags are generated, concatenated, cloned and sequenced. For parasitic nematodes where complete genome sequences are not available, tag assignment may not be possible. In a preliminary SAGE analysis of adult *H. contortus* recently undertaken at Moredun, >1,000 tags representing ~700 different sequences have been analysed to date, of which ~50% generate no hit when used to search available *H. contortus* EST databases (unpublished results). However, upregulated or differentially expressed tags of interest can be used as primers to PCR amplify both 3' and 5' specific gene sequence for further analysis. Microarray technology, where immobilised cDNAs can be screened with RNA or cDNA pools representing different life-cycle stages or different parasites, can also be used for the evaluation of gene expression, although there is little immediate prospect of the development of a parasitic nematode array.

The lack of parasite transformation systems, due principally to the fact that none can complete a parasitic life-cycle outwith the host and that few, once removed from the host, can be maintained *in vitro*, means that functionality still cannot be assessed for those genes which have no homologue in *C. elegans*. Encouragingly, RNAi has been recently extended to the study of secreted AChEs in adult *N. brasiliensis* (Hussein et al, 2002), and was pivotal in demonstrating the applicability of this technique to the study of parasitic nematodes. A procedure for RNAi in the plant parasitic nematodes *Globodera pallida* and *Heterodera glycines* has also now been established (Urwin et al, 2002). More recently, RNAi knockdown of target genes has been achieved in the filarial nematode, *B. malayi*, and may be a useful technique in the identification and assessment of novel drug target genes (Aboobaker and Blaxter, 2003).

The cloning and analysis of potential candidate vaccine antigens has demonstrated the presence of multigene families in *H. contortus*. Moreover, EST

analysis would suggest that allelic polymorphisms also occur. That such heterogeneity exists within a parasite population, together with the potential for the parasite to modify its expressed protein profile, may have potential implications for the development of a generic *H. contortus* vaccine.

The concept of DNA vaccination is quite new and would apparently offer the development of multivalent vaccines which can effectively activate both humoral and cell-mediated immunity, although they are generally less immunogenic (reviewed in Kofta and Wedrychowicz, 2001). Recently, intramuscular injection of cDNA encoding a cysteine proteinase from adult *F. hepatica* has been shown to protect rats from experimental challenge (Kofta et al, 2000). With respect to nematode infections, cDNA vaccination has been investigated for *O. volvulus* and *T. ovis*. Mice were immunised with DNA plasmids expressing either *O. volvulus* tropomyosin or the nematode-specific OvB20 antigen, and both the intramuscular (Th1 dominant response) and epidermal (GeneGun, Th2 dominant response) routes of DNA immunisation investigated (Harrison and Bianco, 2000). Despite the induction of good humoral responses, neither DNA construct promoted statistically significant levels of protection against challenge infection. DNA vaccines encoding three *T. ovis* antigens (45W, 18k and 16k) proved immunogenic in mice, generating significant titres of antigen-specific antibody (Drew et al, 2000). However, in outbred sheep their efficacy was poor, seroconversion occurring in only two out of five sheep immunised with the 45W construct and no antigen-specific antibody responses detectable in sheep immunised with either the 18k or 16k DNA constructs. The problems associated with inconsistency of results between different vaccine formulations at different doses and in different animals (Kofta and Wedrychowicz, 2001), together with potential consumer concerns regarding the safety of DNA vaccines, may prove inhibitory to the advancement of this approach with respect to the immunisation of food animals.

However, it should be remembered that the production of any successful vaccine will not be based solely on the most effective way of inducing immunity. The technical feasibility of antigen production, its formulation in an acceptable adjuvant, ease and frequency of delivery and stability are all factors that will need to

be considered in order to produce what must ultimately constitute a commercially viable, cost-effective product.

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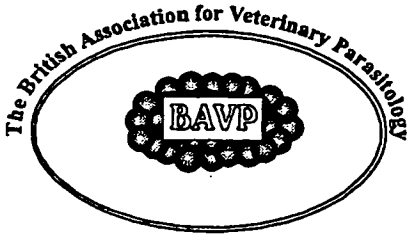
Appendix 1 – letter of award of 1998/1999 Urquhart prize



Affiliated to WAAVP

Dr P J Skuce
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3 March, 2000

Dear Philip,

BRITISH ASSOCIATION OF VETERINARY PARASITOLOGY
URQUHART PRIZE

The Urquhart Prize for Veterinary Parasitology is a recently established prize awarded by the BAVP to encourage younger people active in our discipline. It is intended to reward the best scientific publication in any aspect of Veterinary Parasitology during the previous year by a younger colleague working in a British university or other institution. The prize is named in honour of the late Professor George Urquhart, the first honorary member of the BAVP, who inspired and encouraged so many young people during his life-time.

The Executive Committee of BAVP has considered nominations and I am pleased to be able to inform you that you have been selected to receive the 1998/1999 award for your publication in Parasitology entitled 'Molecular cloning and characterization of gut-derived cysteine proteinases associated with a host protective extract from *Haemonchus contortus*'.

Please accept the congratulations of the BAVP membership.

Yours sincerely,

D E Jacobs
BAVP President