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

P. J. SKUCE, D. L. REDMOND, S. LIDDELL, E. M. STEWART, G. F. J. NEWLANDS, W. D. SMITH *and* D. P. KNOX*

Moredun Research Institute, International Research Centre, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ

(*Received 27 February 1999; revised 27 April 1999; accepted 28 April 1999*)

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

* Corresponding author. Tel: +44 0131 4455111. Fax: +44 0131 4456111. E-mail: knoxd@mri.sari.ac.uk

† 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.

https://doi.org/10.1017/S0031182099004813 Published online by Cambridge University Press

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′-ACAGAATTCCAGGGICAGTGCGGITCITGGTGG-3′, 303H (3′ antisense) 5′-TTAAAGCTTCCAIGAGTTCTTIACGATCCAGTA-3′ and 509G (3′ antisense) 5′-ACAAAGCTTGTAICCICCGTTGCAICCCTC-3′, respectively], were de-

https://doi.org/10.1017/S0031182099004813 Published online by Cambridge University Press

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. 1 A), with no evidence of staining in any other tissues. The cytoplasm of the parasite's gut displayed

https://doi.org/10.1017/S0031182099004813 Published online by Cambridge University Press

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-

https://doi.org/10.1017/S0031182099004813 Published online by Cambridge University Press

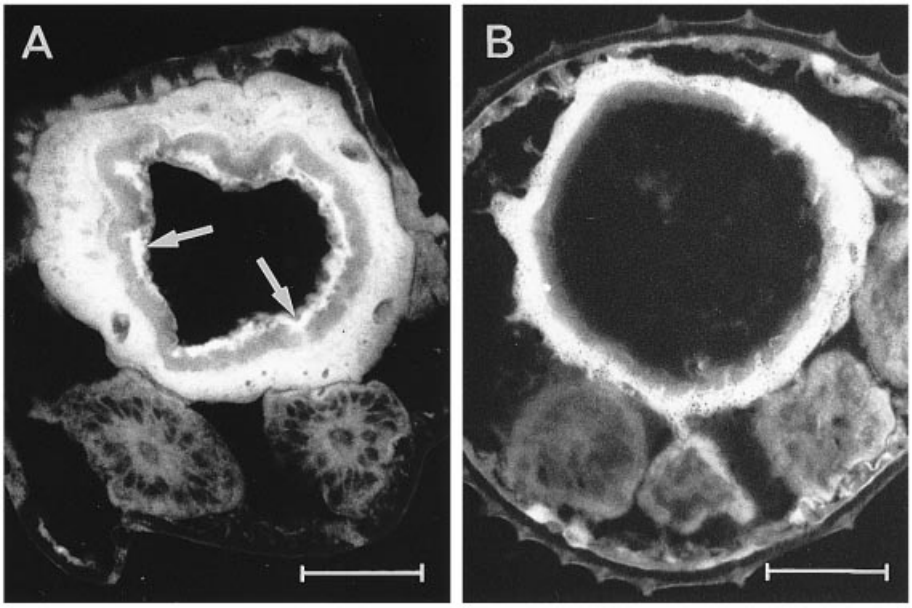

Fig. 1. (A) Cryostat section of adult *Haemonchus contortus* probed with monospecific anti-hmcp4 antibodies. Note immunofluorescence restricted to the luminal surface of the parasite's gut (arrows). (B) Control section probed with pre-immunization serum. Scale bars = 50 μm.

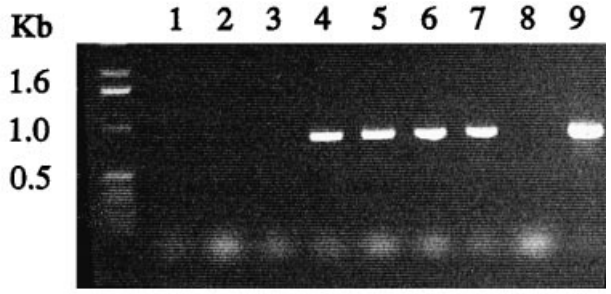

Fig. 2. Developmental expression of *Haemonchus contortus* cysteine proteinase (hmcp6). RT–PCR products obtained following amplification of sscDNA from respective life-cycle stages. PCR was carried out from eggs, L3, XL3, L4, 11, 22 and 28 day *H. contortus* and the results shown in lanes 1–7, respectively. 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.

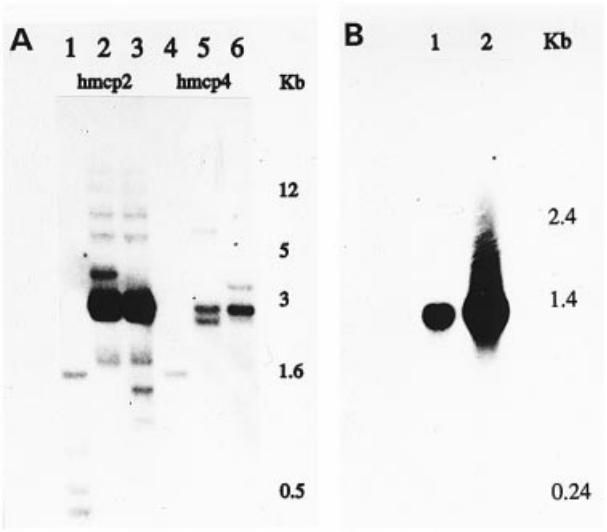

Fig. 3. (A) Representative Southern blot of *Haemonchus contortus* genomic DNA digested with *Hae*III (lanes 1 and 4), *Hin*dIII (lanes 2 and 5) and *Eco*RI (lanes 3 and 6) and probed with the respective radio-isotope labelled cysteine proteinase homologue (hmcp2 and hmcp4). Molecular weight markers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

https://doi.org/10.1017/S0031182099004813 Published online by Cambridge University Press

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.

The authors are indebted to colleagues Dr Stuart Smith and Mr Dan Pettit for the provision of S3 TSBP antisera, and to Mr Brian Easter for his photographic expertise. The financial support of the Scottish Office Agriculture, Environment and Fisheries Department (SOAEFD) and Mallinckrodt Veterinary is gratefully acknowledged.

REFERENCES

BRINKWORTH, R. I., BRINDLEY, P. J. & HARROP, S. A. (1996). Structural analysis of the catalytic site of AcCP-1, a cysteine proteinase secreted by the hookworm *Ancylostoma caninum*. *Biochimica et Biophysica Acta* **1298**, 4–8.

COLES, G. C. (1998). Drug-resistant parasites of sheep; an emerging problem in Britain? *Parasitology Today* **14**, 86–88.

COX, G. N., PRATT, D., HAGEMAN, R. & BOISVENUE, R. J. (1990). Molecular cloning and primary sequence of a cysteine protease expressed by *Haemonchus contortus* adult worms. *Molecular and Biochemical Parasitology* **41**, 25–34.

DALTON, J. P., SMITH, A. M., CLOUGH, K. A. & BRINDLEY, P. J. (1995). Digestion of haemoglobin by schistosomes: 35 years on. *Parasitology Today* **11**, 299–303.

EAKIN, A. E., BOUVIER, J., SAKANARI, J. A., CRAIK, C. S. & MCKERROW, J. H. (1990). Amplification and sequencing of genomic fragments encoding cysteine proteases from protozoan parasites. *Molecular and Biochemical Parasitology* **39**, 1–8.

HARROP, S. A., SAWANGJAROEN, N., PROCIV, P & BRINDLEY, P. J. (1995). Characterisation and localisation of cathepsin B proteinases expressed by adult *Ancylostoma caninum* hookworms. *Molecular and Biochemical Parasitology* **71**, 163–171.

ILLY, C., QURAISHI, O., WANG, J., PURISIMA, E., VERNET, T. & MORT, J. S. (1997). Role of the occluding loop in cathepsin B activity. *Journal of Biological Chemistry* **272**, 1197–1202.

KARANU, F. N., RURANGIRWA, F. R., MCGUIRE, T. C. & JASMER, D. P. (1993). Identification of proteases with diverse characteristics in adult excretory–secretory products. *Experimental Parasitology* **77**, 362–371.

KNOX, D. P., REDMOND, D. L. & JONES, D. G. (1993). Characterisation of proteinases in extracts of adult *Haemonchus contortus*, the ovine abomasal nematode. *Parasitology* **106**, 395–404.

KNOX, D. P., SMITH, S. K. & SMITH, W. D. (1999). Immunization with an affinity purified protein extract from the adult parasite protects lambs against infection with *Haemonchus contortus*. *Parasite Immunology* **21**, 201–210.

KNOX, D. P., SMITH, S. K., SMITH, W. D., REDMOND, D. L. & MURRAY, J. M. (1995). Vaccines against helminthic parasites: Thiol binding proteins. *International Patent* (#WO 95/26402).

LIDDELL, S. & KNOX, D. P. (1997). Extracellular and cytoplasmic Cu/Zn superoxide dismutases from *Haemonchus contortus*. *Parasitology* **116**, 383–394.

LONGBOTTOM, D., REDMOND, D. L., RUSSELL, M., LIDDELL, S., SMITH, W. D. & KNOX, D. P. (1997). Molecular cloning and characterisation of a putative aspartate proteinase associated with a gut membrane protein

complex from adult *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **88**, 63–72.

LOUKAS, A., SELZER, P. M. & MAIZELS, R. M. (1998). Characterisation of *Tc-cpl-1*, a cathepsin L-like cysteine protease from *Toxocara canis* infective larvae. *Molecular and Biochemical Parasitology* **92**, 275–289.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

MORT, J. S. & BUTTLE, D. J. (1997). Molecules in focus: Cathepsin B. *International Journal of Biochemical Cell Biology* **5**, 715–720.

NEWTON, S. E. & MUNN, E. A. (1999). Progress in the development of vaccines against gastrointestinal nematode parasites, particularly *Haemonchus contortus*. *International Journal for Parasitology* (in the Press).

PRATT, D., COX, G. C., MILHAUSEN, M. J. & BOISVENUE, R. J. (1990). A developmentally regulated cysteine protease gene family in *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **43**, 181–192.

REDMOND, D. L., KNOX, D. P., NEWLANDS, G. F. J. & SMITH, W. D. (1997). Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of *Haemonchus contortus*. *Molecular and Biochemical Parasitology* **85**, 77–87.

REHMAN, A. & JASMER, D. P. (1998). A tissue specific approach for analysis of membrane and secreted protein antigens from *Haemonchus contortus* gut and its application to diverse nematode species. *Molecular and Biochemical Parasitology* **97**, 55–68.

RHOADS, M. L. & FETTERER, R. H. (1995). Developmentally regulated secretion of cathepsin L-like cysteine proteases by *Haemonchus contortus*. *Journal of Parasitology* **81**, 505–512.

SAKANARI, J. A., STAUNTON, C. E., EAKIN, A. E., CRAIK, C. S. & MCKERROW J. H. (1989). Serine proteases from nematode and protozoan parasites. Isolation of sequence homologues using generic molecular probes. *Proceedings of the National Academy of Sciences, USA* **86**, 4863–4867.

SMITH, T. S., GRAHAM, M., MUNN, E. A., NEWTON, S. E., COADWELL, W. J., MCMICHAEL-PHILLIPS, D., SMITH, H., SMITH, W. D. & OLIVER, J. J. (1997). Cloning and characterisation of a microsomal aminopeptidase from the intestine of *Haemonchus contortus*. *Biochimica et Biophysica Acta* **1338**, 295–308.

SMITH, W. D. (1993). Protection in lambs immunised with *Haemonchus contortus* gut membrane proteins. *Research in Veterinary Science* **54**, 94–101.

SMITH, W. D. (1998). Prospects for vaccines against helminth parasites of grazing ruminants. *Veterinary Parasitology* **29**, 17–24.

SMITH, W. D. & SMITH, S. K. (1993). Evaluation of aspects of the protection offered to sheep immunised with a gut membrane protein of *Haemonchus contortus*. *Research in Veterinary Science* **55**, 1–9.

SMITH, W. D., SMITH, S. K. & MURRAY, J. M. (1994). Protection studies with integral membrane fractions of *Haemonchus contortus*. *Parasite Immunology* **16**, 231–241.

TORT, J., BRINDLEY, P. J., KNOX, D. P., WOLFE, K. & DALTON, J. P. (1998). Proteases and associated genes of parasitic nematodes. *Advances in Parasitology* **43**, 161–266.