Identification and sequence comparison of a cuticular collagen of *Brugia pahangi*

M. BISOFFI[1] *and* B. BETSCHART[2]*

[1] *Department of Medical Parasitology, Swiss Tropical Institute, 4051 Basel, Switzerland*
[2] *Institute of Zoology, University of Neuchâtel, 2007 Neuchâtel, Switzerland*

(*Received 25 November 1995; revised 28 February 1996; accepted 28 February 1996*)

SUMMARY

The cuticle of filarial nematodes is a specialized extracellular matrix that covers the parasite and protects it from adverse conditions of the environment. As a surface structure it is in direct contact with the host defence mechanisms and therefore plays an important role in the molecular host–parasite relationship. Using polyclonal antisera raised against the insoluble components of the cuticle of the adult filarial parasite *Brugia pahangi*, we have isolated cDNA clones encoding collagen molecules of the cuticle. The protein domain structure of cDNA clone Bpcol-1 was compared with the known structures of cuticular collagens of the nematodes *Brugia malayi*, *Caenorhabditis elegans*, *Ascaris suum* and *Haemonchus contortus*, confirming interspecies similarities. Using affinity-purified anti-Bpcol-1 antibodies we identified Bpcol-1 antigenic determinants in different nematode extracts, and determined the localization of such epitopes within the cuticle of *B. pahangi*.

Key words: *Brugia pahangi*, filarial nematode, cuticle, collagen, sequence comparison, immunolocalization.

INTRODUCTION

All nematodes so far investigated are bound by an extracellular matrix, called the cuticle (Bird & Bird, 1991). The cuticle functions as a selective barrier in nutrition uptake and metabolite secretion (Ho *et al.* 1990), plays an important role as a skeletal counteracting structure to the muscle movement (Wharton, 1986), and represents an excellent protection against unfavourable environmental conditions which, in the case of parasitic nematodes, include the various immunological responses of the host defence (Ogilvie *et al.* 1980). The successful establishment of nematode parasites within their host depends on the host–parasite interactions at their interface. Therefore, the biochemical composition of the cuticle is of great interest. Previous work has shown that the major protein components of nematode cuticles are nematode-specific collagens (Josse & Harrington, 1964). Like vertebrate collagens, they are characterized by the repeating amino acid sequence (Gly-X-Y)$_n$, where X and Y can be any amino acid, but are often proline or hydroxyproline (Cox, 1992). This motif is responsible for the triple-helical, rod-like structure of collagen molecules formed by 3 polypeptide chains. Although the cuticular collagens of parasitic nematodes are not directly exposed to the host, they are part of the nematode surface, and antibodies against cuticular collagens have been detected in patients infected with filarial parasites (Selkirk *et al.* 1989*a*). While this may be partly due to death and decay of worms within the host, it may nevertheless represent an important immunological event in the host–parasite relationship. The latter is emphasized by the fact that cuticular collagens have been discussed in the context of protective immunity in hookworm infections (Pritchard, McKean & Rogan, 1988) and infections with the sheep parasite *Haemonchus contortus* (Boisvenue *et al.* 1991).

The use of cloning techniques has led to the identification of various collagen genes coding for cuticular and non-cuticular collagens of different nematodes, including the free-living nematode *Caenorhabditis elegans* (Cox, 1992), the parasitic nematodes *Ascaris suum* (Kingston, Wainwright & Cooper, 1989) and *H. contortus* (Shamansky *et al.* 1989). Caulagi and coworkers have isolated a basement membrane collagen gene from the human filarial parasite *Brugia malayi*, which was termed BmCol1 (Caulagi, Werner & Rajan, 1991). Collagen molecules of the filarial parasites *B. malayi* and *B. pahangi* have been characterized with respect to their synthesis and immunogenicity (Selkirk *et al.* 1989*a*), and recently, the molecular cloning of a cuticular collagen of *B. malayi*, *Bmcol-2*, has been reported (Scott *et al.* 1995). In the present study we contribute to the molecular information on cuticular components of filarial parasites and report the cloning of a cDNA coding for a cuticular collagen of *B. pahangi*. The deduced protein structure of this collagen molecule is compared with other known cuticular collagens, and its precise localization within the adult parasite is presented.

* Corresponding author. University of Neuchâtel, Institute of Zoology, Rue Emile-Argand 11, 2007 Neuchâtel, Switzerland. Tel: ++41 38 23 30 45. Fax: ++41 38 23 30 01. E-mail: bruno.betschart@zool.unine.ch.

MATERIALS AND METHODS

Parasites

B. pahangi adult worms were recovered from the peritoneal cavity of jirds, following infection with 3rd-stage larvae (McCall *et al.* 1973). Adult *A. suum* worms were received from a local slaughterhouse. *C. elegans* (var. Bristol, strain N2; kindly provided by Dr H. Tobler, University of Fribourg, Switzerland) were grown and harvested according to the method described by Sulston & Brenner (1974).

Parasite protein extracts

Adult *B. malayi* and *C. elegans* were briefly washed in cold phosphate-buffered saline (PBS), homogenized and sonicated in 125 mM Tris–HCl, pH 6·8, 1 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 1% sodium dodecyl sulphate (SDS), and 5% 2-mercaptoethanol (2-ME), and stirred for 4 h at 4 °C. *A. suum* adult worms were briefly washed in PBS and subjected to multiple freezing/thawing rounds to enhance detachment of the cuticle from the body of the worms. The cuticle fragments were cut into pieces and extracted in PBS containing 5% 2-ME for 24 h at 37 °C (Fujimoto & Kanaya, 1973). All extracts were briefly centrifuged and the concentration of the supernatants determined (Bradford, 1976).

Antiserum

Cuticles from adult *B. pahangi* worms were isolated essentially as described for *C. elegans* (Cox, Kusch & Edgar, 1981). Worms were suspended in 10 mM Tris–HCl, pH 7·5, 1 mM EDTA, 1 mM PMSF, and sonicated on ice. Cuticle pieces were recovered by centrifugation, suspended in 125 mM Tris–HCl, pH 6·8, 1% SDS, and heated for 2 min at 80 °C. The suspension was turned end over end for 2 h at room temperature, the cuticles recovered by centrifugation, and extracted again as described. The remainder of the preparation was extracted further by heating at 80 °C for 2 min in 125 mM Tris–HCl, pH 6·8, 1% SDS, and 5% 2-ME, and by rotation for 2 h at room temperature. This procedure was repeated once following centrifugation. The remaining insoluble material enriched in epicuticular proteins was used to immunize rabbits according to standard protocols (Maniatis, Fritsch & Sambrook, 1982).

Immunoscreening

The production of the λgt11 cDNA library derived from mRNA of adult *B. pahangi* parasites has been described by Selkirk *et al.* (1989*b*). Immunoscreening of the library in *Escherichia coli* Y1090 was as described by Altmann, Handschin & Trachsel (1987). After induction of the *lacZ* operon, the filters were saturated with 50 mM Tris-HCl, pH 8, 150 mM sodium chloride, 0·3% Tween 20, 0·05% Triton–X100 (TBST), containing 5% dried milk powder, and incubated overnight with the polyclonal antiserum raised against epicuticular components of *B. pahangi*, at a 1/500 dilution in TBST containing 0·5% bovine serum albumin (TBST–BSA). Bound antibodies were detected with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., USA) in TBST-BSA and visualized by the substrates 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (BCIP) and *p*-nitro blue tetrazolium chloride (NBT; Kirkegaard Perry Laboratories, USA). Primary immunoreactive clones were subjected to second and third screening rounds for verification and plaque purification. Plaque purified phages were amplified in *E. coli* Y1088.

DNA preparation, blotting and hybridization

For the differential cross-hybridization experiments the inserts of the various positive clones were amplified by the polymerase chain reaction (PCR), subjected to agarose gel electrophoresis, and transferred to nylon hybridization membranes (NEN Research Products, USA). The probe used for the hybridization experiments was a 677 bp *Eco*RI fragment corresponding to bp 223–900 of Bpcol-1. The probe was labelled with ^{32}P-dATP by the random primer extension method (Feinberg & Vogelstein, 1983). Hybridization experiments were performed according to standard protocols (Maniatis *et al.* 1982) using different concentrations of standard saline citrate (SSC), 5× Denhardt's solution, and 0·5% SDS, and at various temperatures according to the different stringency conditions chosen.

DNA sequencing and sequence comparison

The cDNA inserts of the positive clones were excised from λgt11 with the restriction endonuclease *Eco*RI and subcloned into the *Eco*RI restriction site of the sequencing vector M13mp18/19 by the ligase reaction (Maniatis *et al.* 1982). The cDNA insert was sequenced by the chain termination method (Sanger, Nicklen & Coulson, 1977) using the Sequenase DNA Sequencing Kit from United States Biochemical, USA. DNA and protein sequences were analysed and compared using the GCG programs on a VAX system (Devereux, Haeberli & Smithies, 1984).

Production of Bpcol-1-β-galactosidase fusion protein

E. coli strain Y1090 was infected with the recombinant phage on plates to achieve confluent lysis and

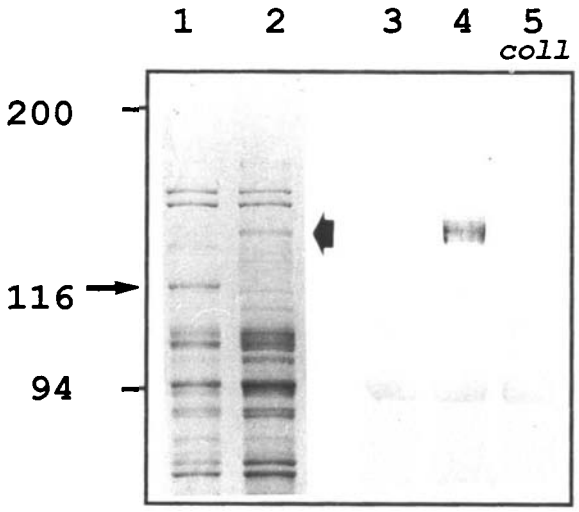

Fig. 1. Immunoblot and collagenase susceptibility of Bpcol-1. Extracts of *E. coli* infected with wild type λgt11 (lanes 1 and 3) or Bpcol-1 (lanes 2, 4, and 5) were analysed in SDS–PAGE (lanes 1 and 2) and electroblotted onto nitrocellulose (lanes 3–5). The thin arrow indicates the position of β-galactosidase at 116 kDa, the thick arrow indicates the position of Bpcol-1 fusion protein at 142 kDa. Immunoblots were performed using affinity-purified anti-Bpcol-1 antibodies; *coll* indicates collagenase treatment. Numerals on the left indicate molecular size in kDa.

the synthesis of the fusion protein was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG) as described (Young & Davis, 1983). The plates were then washed (Altmann *et al.* 1987) and proteins in the wash solution were concentrated by ammonium sulphate precipitation (50% saturation) and resuspended in either SDS-sample buffer or in PBS containing 1 mM PMSF for the collagenase digestion experiments.

Collagenase digestion

High purity collagenase from *Clostridium histolyticum* (Sigma type VII, Switzerland) was used for the digestion of fusion proteins. The enzyme was used at a concentration of 100 μg/ml and digestions performed for 30 min at 37 °C in 10 mM Tris–HCl, pH 7·5, 10 mM calcium chloride (Selkirk *et al.* 1989*a*). Reactions were terminated by the addition of protein loading buffer and boiling for SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Affinity-purification of antibodies

Bacterial lysates containing the Bpcol-1-β-galactosidase hybrid protein were size-fractionated in preparative 7·5% SDS–polyacrylamide gels and electroblotted onto nitrocellulose membranes (Towbin, Staehelin & Gordon, 1979). The size region of the nitrocellulose membranes around 140 kDa was excised and the remaining binding sites on the strips blocked with 2·5% BSA in TBST. After washing the strips in TBST they were incubated with the polyclonal antiserum raised against the insoluble components of adult *B. pahangi*, diluted 1/20 in TBST–BSA. The bound antibodies were eluted by 2-min rinses with 0·15 M glycine-HCl, pH 2·8, and quickly neutralized with 0·1 M NaOH. BSA was added to a final concentration of 0·5%.

Immunoblot analysis

Bacterial lysates, nematode extracts, and human collagens (Sigma, Switzerland) were size-fractionated electrophoretically in 7·5% or 12·5% SDS–polyacrylamide gels and transferred to nitrocellulose essentially as described by Towbin *et al.* (1979). Following electrophoretic transfer, blocking of non-specific binding was achieved by a 1 h incubation in TBST containing 5% dried milk powder. The polyclonal antiserum was used at a dilution of 1/500 in TBST–BSA. Bound antibodies were detected with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., USA) in TBST–BSA and visualized by the substrates BCIP and NBT (Kirkegaard Perry Laboratories, USA).

Immunocytochemistry

The immunocytochemical experiments were performed on ultrathin sections of female *B. pahangi* adult worms processed and embedded in Lowicryl K4M according to standard procedures (Kiefer *et al.* 1986). Immunocytochemistry was carried out using the affinity-purified anti-Bpcol-1 antibodies and normal rabbit serum as a negative control. Bound antibodies were visualized with colloidal gold-labelled protein A (Aurion, Netherlands) and analysed in a Philips 300 electronmicroscope.

RESULTS

Isolation of Bpcol-1 cDNA clone

A λgt11 cDNA library derived from mRNA of adult filarial *B. pahangi* nematodes (Selkirk *et al.* 1989*b*) was immunoscreened using a polyclonal antiserum raised in rabbits against the insoluble components of the cuticle of *B. pahangi* worms. Differential cross-hybridization experiments with the isolated clones under different stringency conditions showed that most of the clones contained cDNAs of one particular gene. The clone carrying the largest insert was further characterized by preparing β-galactosidase fusion protein in bacteria induced with IPTG. The clone was shown to produce a hybrid protein of 142 kDa, thus about 26 kDa larger than β-galactosidase (116 kDa; Fig. 1, lane 2). The poly-

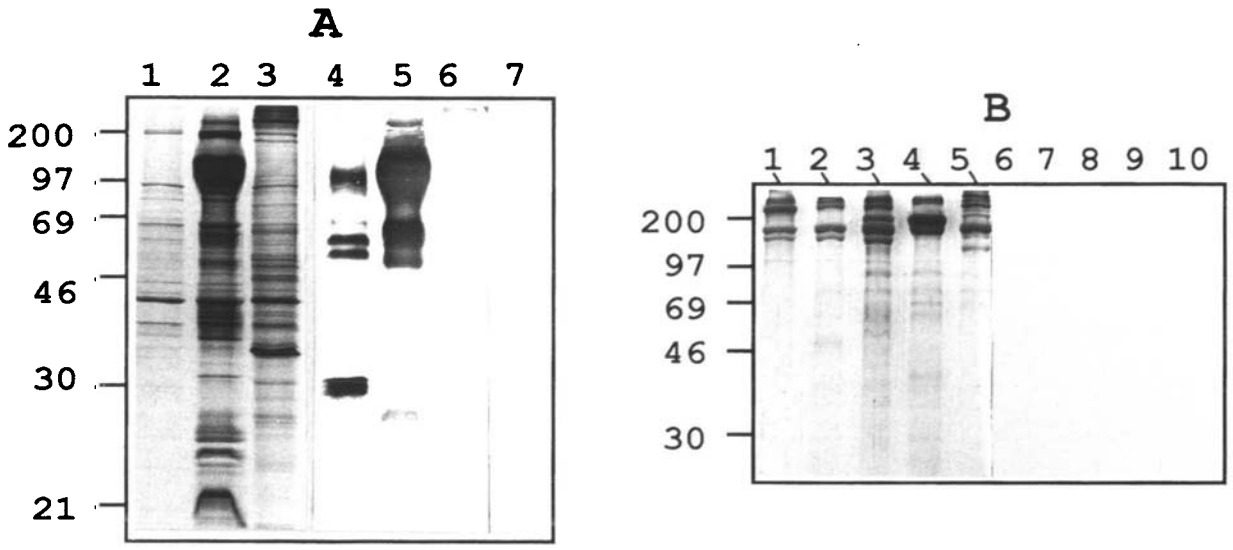

Fig. 2. Immunoblots of anti-Bpcol-1 antibodies on parasite extracts (A) and mammalian collagens (B). (A) Extracts of *Brugia malayi* (lanes 1, 4, and 7), *Ascaris suum* (lanes 2 and 5), and *Caenorhabditis elegans* (lanes 3 and 6) were analysed in SDS–PAGE (lanes 1–3) and electroblotted onto nitrocellulose (lanes 4–7). Lanes 4–6, anti-Bpcol-1 antibodies; lane 7, normal rabbit serum. (B) Mammalian collagens (lane 1, human type III; lane 2, calf skin type VI; lane 3, human type VIII; lane 4, human type IX; lane 5, human type X) were analysed in SDS–PAGE (lanes 1–5) and electroblotted onto nitrocellulose (lanes 6–10). Numerals on the left indicate molecular size in kDa.

peptide was strongly recognized by the polyclonal antiserum (Fig. 1, lane 4), whereas the reaction to the bacterial extract infected with wild type λgt11 was negative, except for the unspecific background reactivity (Fig. 1, lane 3). To test positive cDNA clones for their protein structure their expression was induced in *E. coli*, and the extracts were treated with bacterial collagenase prior to immunoblot analysis. As shown in Fig. 1, lane 5, the observed reaction with the fusion protein of the clone further analysed was completely removed by this treatment, indicating its collagenous nature. This clone was therefore named Bpcol-1, for *Brugia pahangi* collagen 1.

Identification of native parasite proteins

To identify the native proteins that share antigenic determinants with Bpcol-1, crude cuticular or total extracts were produced of different adult nematode worms and were analysed in immunoblot experiments using polyclonal antibodies affinity-purified on Bpcol-1/β-galactosidase fusion protein. Proteins at M_r 30–33 kDa, 57–61 kDa, and 100 kDa in the *B. malayi* extract were significantly stained (Fig. 2A, lane 4). These size ranges correspond well to the molecular size of *B. malayi* collagen molecules described using SDS–PAGE (Selkirk *et al.* 1989*a*). An extensive cross-reactivity of the anti-Bpcol-1 antibodies was also observed with the *A. suum* collagen groups 53–71 kDa and 90–123 kDa (Fig. 2A, lane 5) described by Betschart & Wyss (1990), while only weak reactivity was observed to large collagens (100– ⩾ 200 kDa) in *C. elegans* extracts (Fig. 2A, lane 6). Normal rabbit serum showed no reaction with the *B. malayi* protein extract (Fig. 2A, lane 7). In contrast, human collagens of different types were not recognized by the anti-Bpcol-1 antibodies (Fig. 2B, lanes 6–10), indicating the specificity of the antibodies for nematode cuticular collagens.

Sequence characterization of Bpcol-1

The sequence of the Bpcol-1 cDNA was determined after subcloning into the sequencing vector M13mp18/19 by the dedeoxy chain-termination method (Sanger *et al.* 1977). A total of 900 bp were present in the *Eco*RI restriction site. No sequence corresponding to the eukaryotic TATA promoter (Corden *et al.* 1989) or any in-frame initiator methionine were present in the 5′ part of the clone, but several termination codons and one potential eukaryotic AATAAA polyadenylation signal (Proudfoot & Brownlee, 1976) were present in the non-coding 3′ region of the clone. Sequence analysis of the 900-bp insert revealed an open reading frame encoding 218 amino acids. The deduced amino acid sequence is shown in Fig. 3. The calculated size of this polypeptide is 24 kDa, thus corresponding well to the relative mobility shift of the ß-galactosidase fusion protein observed in SDS–PAGE (Fig. 1). A total of 156 amino acids of Bpcol-1 represent $(Gly\text{-}X\text{-}Y)_n$ triple-helical motifs which are typical for collagens (shaded in Fig. 3; Miller & Gay, 1987). The proline content within the triple-helical motifs

```
  1 Thr Lys Leu  Glu Ser Ser  Arg Ser Ala  Leu Lys Lys  Glu Arg Gln  15
 16 Lys Arg Gln  Ala Val Phe  Gln Cys Cys  Ser Cys Gly  Ile         28
 29 Gly Pro Val  Gly Pro Pro  Gly Pro Pro  Gly Gln Asp  Gly Asp Asp  43
 44 Gly Arg Asp  Gly Pro Pro  Gly Lys Pro  Gly Met Pro  Gly Gln Asp  58
 59 Ala Gln Glu  Thr Gln Leu  Pro Thr Glu  Arg Asp Trp  Cys Phe Asn  73
 74 Cys Pro Ala                                                    76
 77 Gly Pro Pro  Gly Pro Arg  Gly Lys Pro  Gly Pro Lys  Gly Gln Arg  91
 92 Gly Leu Pro  Gly Asp Lys  Gly Ser Ser  Gly Gln Pro  Gly Glu Pro 106
107 Gly Pro Val  Gly Pro Gln  Gly Pro Arg  Gly Pro Asn  Gly Pro Arg 121
122 Gly Asn Pro  Gly Pro Ala  Gly Glu Pro  Gly Lys Pro  Gly Val Gln 136
137 Thr Glu Val  Pro                                              140
141 Gly Pro Pro  Gly Pro Pro  Gly Pro Pro  Gly Pro Gln  Gly Pro Pro 155
156 Gly Glu Gln  Gly Pro Ala  Gly Arg Asp  Gly Asn Pro  Gly Arg Pro 170
171 Gly Pro Arg  Gly Pro Pro  Gly Gln Asn  Gly Lys Asp  Gly Pro Pro 185
186 Gly His Asp  Gly Pro Asn  Gly Asp Gln  Gly Glu Ala  Gly Pro Asp 200
201 Gly Pro Lys  Gly Ser Cys                                       206
207 Asp His Cys  Pro Pro Pro  Arg Thr Ala  Pro Gly Tyr              218
```

Fig. 3. Predicted amino acid sequence of *Brugia pahangi* Bpcol-1 cDNA. The sequence is shown from the amino to the carboxy terminus and represents the total coding region of the clone. The standard three letter code is used. The sequence is listed such that the glycine residues within the triple-helical regions are aligned. The glycine residues are bold and the $(Gly\text{-}X\text{-}Y)_n$ regions are shaded. Cysteine residues are boxed. Numerals indicate numbers of amino acids.

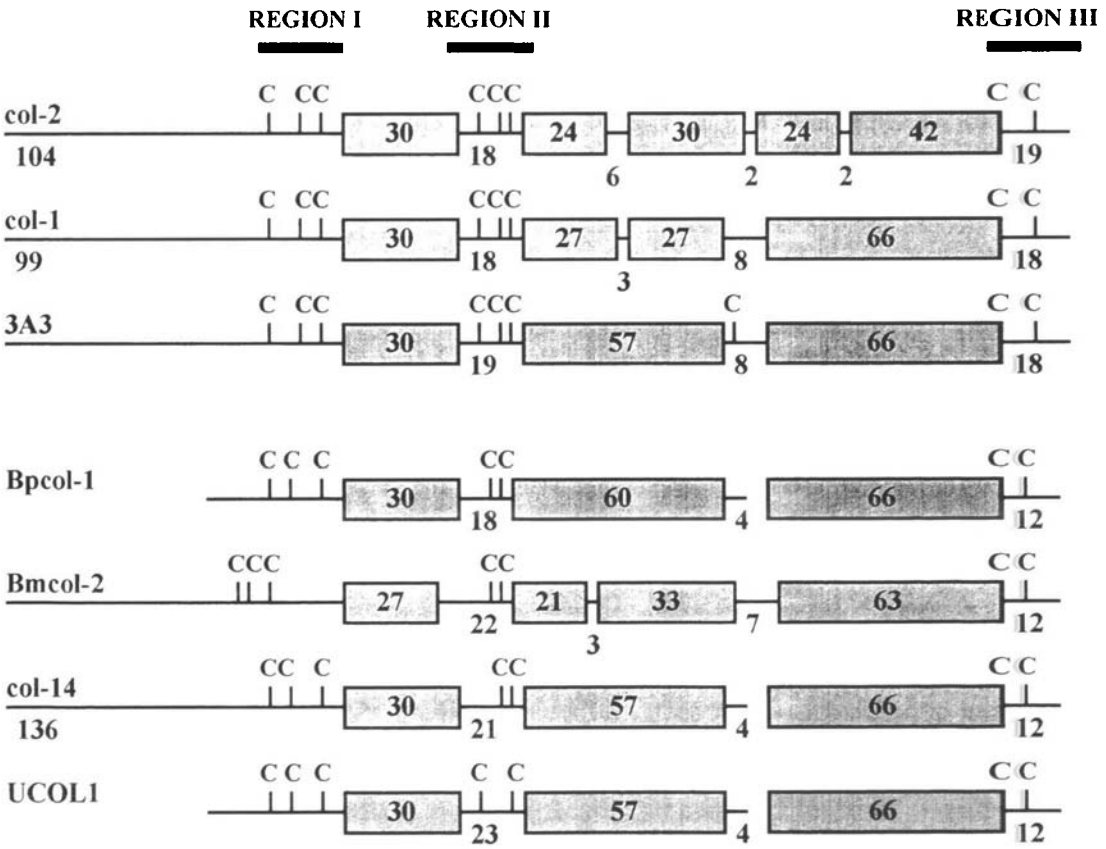

Fig. 4. Schematic representation of the protein domain organization of collagen molecules Bpcol-1 (*Brugia pahangi*), *Bmcol-2* (*B. malayi*), *col-1*, *col-2*, *col-14* (*Caenorhabditis elegans*), 3A3 (*Haemonchus contortus*), and UCOL1 (*Ascaris suum*). The proteins are grouped according to the position of cysteine residues (two major groups). The $(Gly\text{-}X\text{-}Y)_n$ domains are boxed, the non-$(Gly\text{-}X\text{-}Y)_n$ regions are represented by horizontal lines, numerals indicate number of amino acids in each region, cysteine residues are indicated by vertical lines and a C. Only the cysteine residues of region I are indicated 5′ of the first $(Gly\text{-}X\text{-}Y)_n$ region. Gaps have been introduced in Bpcol-1, *col-14*, and UCOL1 to maximize alignments of $(Gly\text{-}X\text{-}Y)_n$ domains. Regions I, II, and III, indicated by thick bars, are compared in Fig. 5.

of Bpcol-1 is 29·5 %. These motifs are interrupted by short stretches of 4–18 amino acids that depart from a repeating $(Gly\text{-}X\text{-}Y)_n$ sequence. This protein structure has been shown to be typical for collagen proteins of the cuticle of several nematode species (Cox, 1992).

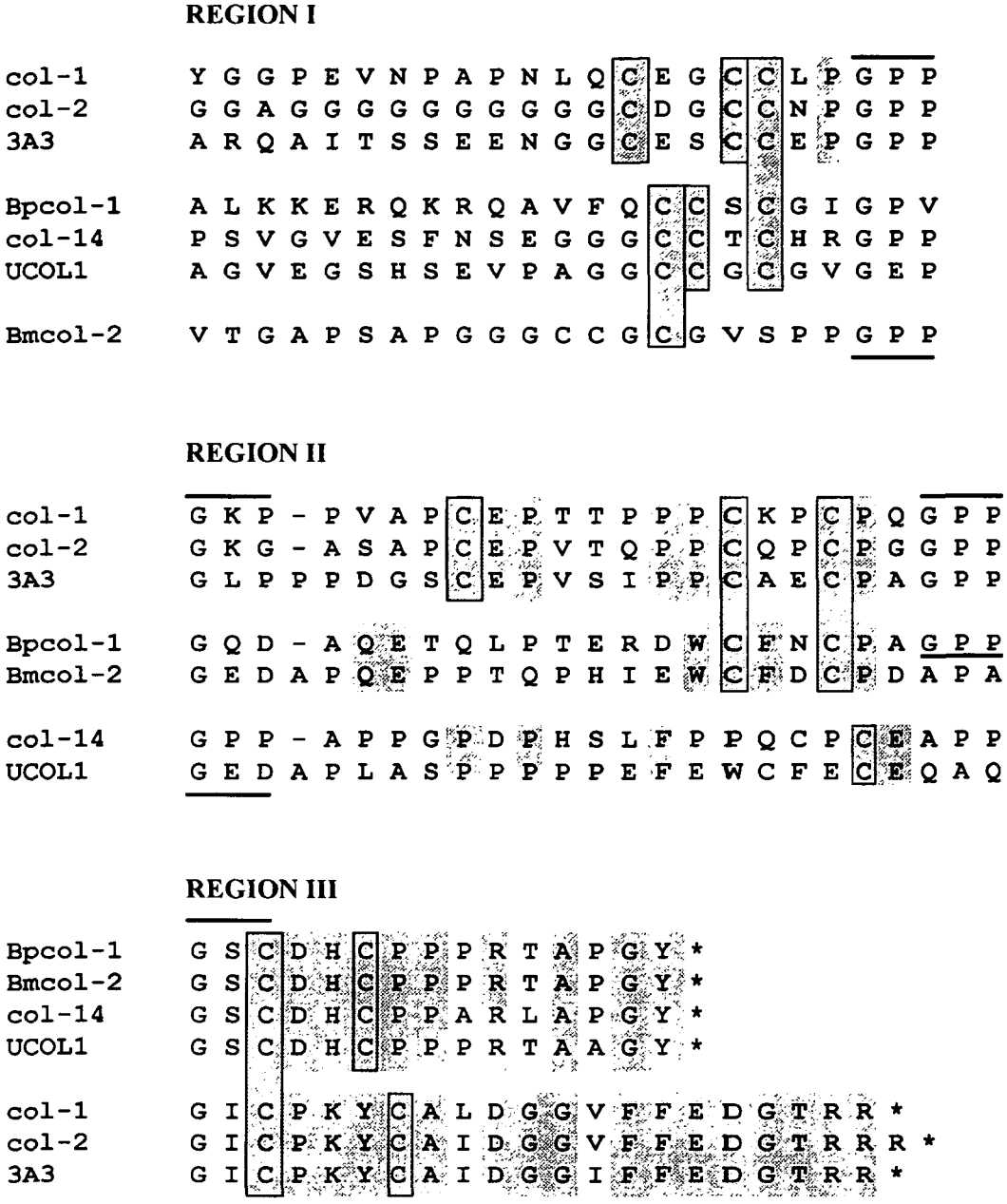

Fig. 5. Comparison of *Brugia pahangi* Bpcol-1 collagen amino acid sequences of Regions I–III (indicated in Fig. 4) with corresponding regions of *col-1*, *col-2*, and *col-14* of *Caenorhabditis elegans*, *Bmcol-2* of *B. malayi*, 3A3 of *Haemonchus contortus*, and UCOL1 of *Ascaris suum*. The standard one letter code is used. Sequences are grouped according to cysteine residues within each region. Horizontal lines denote the beginning or end of $(Gly\text{-}X\text{-}Y)_n$ domains. Conserved cysteine residues are boxed and shaded, amino acids that are identical within a group are shaded. Gaps (dashes) have been introduced in Region II to maximize homology. Asterisks indicate termination codons.

Protein domain and sequence comparison of Bpcol-1 with cuticular collagens of other nematodes

The deduced protein domain structure of Bpcol-1 collagen was compared to the structures of collagens *Bmcol-2* of *B. malayi* (Scott *et al.* 1995), UCOL1 of the intestinal parasite *A. suum* (Kingston *et al.* 1989), 3A3 of the sheep parasite *H. contortus* (Shamanski *et al.* 1989), *col-1*, *col-2*, and *col-14* of the free-living nematode *C. elegans* (Cox *et al.* 1989), as shown in Fig. 4. The proteins were aligned for maximal similarity by aligning each triple-helical region and allowing the introduction of gaps to maximize alignment. The first and last $(Gly\text{-}X\text{-}Y)_n$ regions of Bpcol-1 are identical in size to those of *col-1*, *col-14*, 3A3, and UCOL1, 30 and 66 amino acids, respectively. The organization of all domains of Bpcol-1 and *col-14* are identical, except that *col-14* has three extra amino acids between the first and the second $(Gly\text{-}X\text{-}Y)_n$ region. These three amino acids do not lack in Bpcol-1, but are part of the second $(Gly\text{-}X\text{-}Y)_n$ region, instead. UCOL1 has two additional amino acids in this domain, but is identical to Bpcol-1 and *col-14* with respect to the lengths of the other

domains. This and the position of the cysteine residues of Bpcol-1 (boxed in Fig. 3 and vertical lines in Fig. 4) allows the assignment of Bpcol-1, *col-14*, and UCOL1 to the same group (Fig. 4). In addition, the similar distribution of cysteine residues of *Bmcol-2* also allows this protein to be grouped to the same family, although the overall domain organization of *Bmcol-2* differs from that of Bpcol-1. *Col-1* and *col-2* show different organizations of the second (Gly-X-Y)$_n$ region featuring 3–6 interrupting amino acids. *col-1*, *col-2*, and 3A3 were assigned to another group, because of their obvious similarities of the domain lengths and the distribution of cysteine residues (Fig. 4).

In *C. elegans* the sequence of the nontriple-helical domains I, II, and III (bold bars in Fig. 4), and the distribution and position of cysteine residues therein are diagnostic for each of the 4 collagen gene families identified in *C. elegans* (Cox, 1990). The amino acid sequence of the corresponding regions of Bpcol-1 were further analysed in detail (Fig. 5). Bpcol-1 has 7 cysteine residues, 5 of which are at identical positions to cysteine residues of *col-14*, UCOL1, and *Bmcol-2* (boxed in Fig. 5). The amino acid sequences in regions I and II of all the listed proteins differ substantially from each other, except for the conserved cysteine residues. Region III, however, is highly conserved between certain collagen proteins, and even identical between Bpcol-1 and *Bmcol-2* (Fig. 5).

Immunolocalization of Bpcol-1

The ultrastructural localization of epitopes that share antigenic determinants with Bpcol-1 collagen was determined in immunocytochemical experiments on thin sections of female *B. pahangi* parasites tested with the affinity-purified anti-Bpcol-1 antibodies isolated from the total polyclonal antiserum raised against the insoluble material of adult *B. pahangi*. Antigenic determinants recognized by anti-Bpcol-1 antibodies were specifically stained at two different locations: in all layers of the cuticle (Fig. 6A and B), on basement membranes and secretions in the uteral lumen (Fig. 6C and D). No staining was observed with normal rabbit serum (Fig. 6E).

DISCUSSION

The cuticle of filarial nematodes is a complex, multilayered extracellular structure that features both rigid and elastic properties (Bird & Bird, 1991). Apart from its function as a selective barrier in the flow of nutrition, the most prominent aspect of the filarial cuticle is its proximity to the host defence mechanisms, and hence its role as the locus of the host–parasite interplay (Ogilvie *et al.* 1980). Insight into the molecular structure of the cuticle is therefore expected to provide a better understanding of this functional aspect.

The original aim of the present study was to identify non-collagenous structural proteins of the external layers of the epicuticle of filarial parasites. These proteins have been termed 'cuticlins' (Politz & Philipp, 1992), and are resistant to collagenase, which specifically cleaves the Gly-X bond within the (Gly-X-Y)$_n$ repetitive motif of collagens. Despite the fact that we used antisera raised against such epicuticular components to screen a cDNA library of adult *B. pahangi*, mainly collagens were identified by this approach. We hypothesize that the insoluble material used for immunization, even following exhaustive chemical and enzymatic treatment, still contained collagens masking any immunological reaction to the non-collagenous components themselves. This is consistent with earlier studies in the rodent filarial parasite *Acanthocheilonema viteae* (Betschart *et al.* 1985), and with the electron microscopical data presented by Selkirk and coworkers on 2-ME-purified cuticles of *Brugia* parasites (Selkirk *et al.* 1989*a*).

The often-mentioned interspecies conservation of the cuticle structure (Politz & Philipp, 1992) is confirmed by the present study that reports the cloning, preliminary characterization, and interspecies comparison of a collagenous molecule of the adult filarial parasite *B. pahangi*. By means of alignment searches in the GenEMBL and Swissprot databanks of the DNA and the deduced amino acid sequence of a clone isolated from the *B. pahangi* cDNA libary, we identified a collagen of the cuticle of *B. pahangi*. We termed this clone Bpcol-1 for *B. pahangi* collagen 1, in analogy with collagen genes BmCol1 (Caulagi *et al.* 1991) and *Bmcol-2* (Scott *et al.* 1992) of *B. malayi*. The highest homologies were obtained with cuticular collagens *col-14* of *C. elegans* (Cox *et al.* 1989), and *Bmcol-2* of *B.malayi* (Scott *et al.* 1995). The amino acid sequence of Bpcol-1 revealed the typical domain organization of cuticular collagens. Bpcol-1 consists of regions containing the repetitive motif (Gly-X-Y)$_n$ that is responsible for the triple-helical conformation of collagens (Miller & Gay, 1987), separated by non-(Gly-X-Y)$_n$ stretches of 4–18 amino acids. The schematic comparison of the deduced protein domain structure of Bpcol-1 with the protein structures of collagen genes *Bmcol-2* of *B. malayi* (Scott *et al.* 1995), UCOL1 of the intestinal parasite *A. suum* (Kingston *et al.* 1989), 3A3 of the sheep parasite *H. contortus* (Shamanski *et al.* 1989), *col-1*, *col-2*, and *col-14* of the free-living nematode *C. elegans* (Cox *et al.* 1989) showed a significant similarity with *col-14* and *Bmcol-2*. This structure similarity was strengthened by comparing the amino acid sequences of the nontriple-helical regions I–III, and the position of cysteine residues within these regions. Both parameters have been used to classify identified collagens of *C. elegans* into separate groups or families (Cox, 1990). Cysteine residues of nematode cuticular

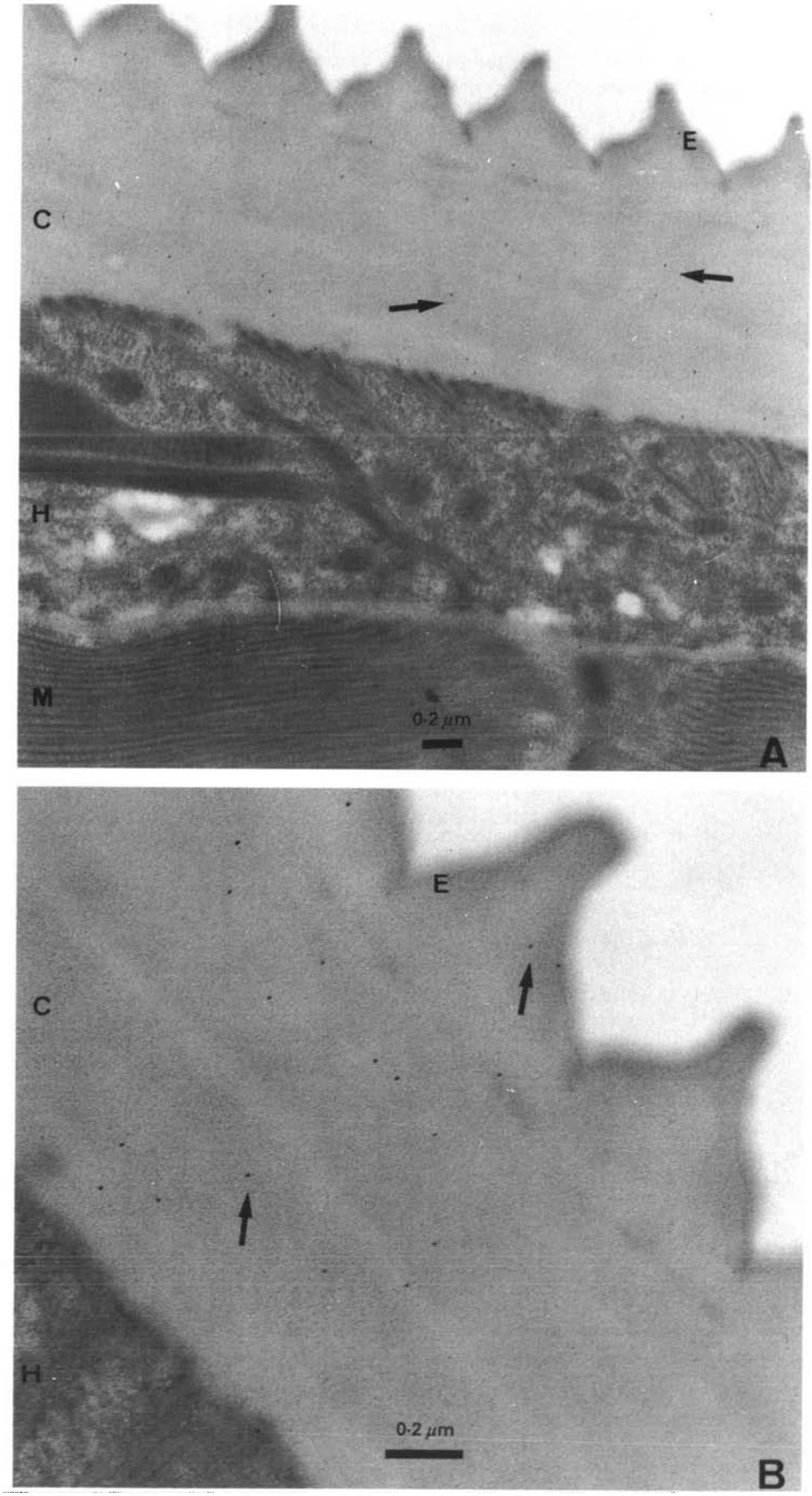

Fig. 6A, B. For legend see opposite.

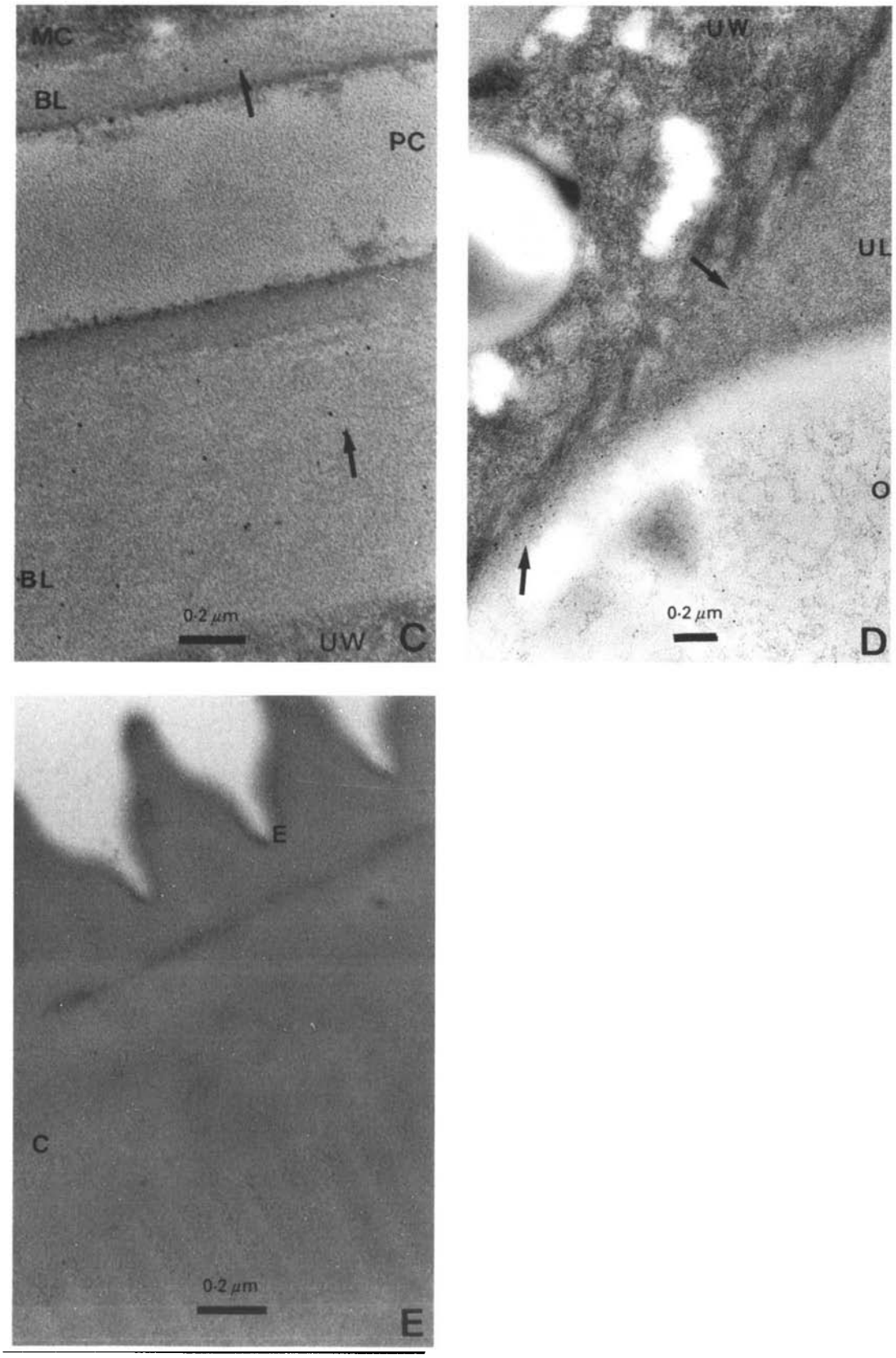

Fig. 6. Immunoelectron microscopy with the affinity-purified anti-Bpcol-1 antibodies on sections of adult *Brugia pahangi*. (A) Overview of epicuticle, cuticle, hypodermis, and muscle. (B) Labelling within the cuticle. (C) Labelling of the basal lamina of the uterus and the muscle layer. (D) Labelling on the uterus secretion. (E) Normal rabbit serum on cuticle. BL, basal lamina; E, epicuticle; C, cuticle; H, hypodermis; MC, muscle; O, ovum; PC, pseudocoel; US, uterus secretion; UW, uterus wall; arrows indicate gold particles.

collagens account for approximately 3 % of all amino acids and are involved in the formation of disulfide cross-links. These disulfides presumably form between polypeptides of a triple-helical rod (interchain cross-links), as well as between polypeptides of different collagen molecules (intrachain cross-links), and account for their insolubility in the absence of a reducing agent, such as 2-ME (Cox, 1992).

Affinity-purified anti-Bpcol-1 antibodies were used to identify corresponding antigenic determinants in nematode extracts by immunoblot analysis. In extracts of adult *B. malayi* parasites, the anti-Bpcol-1 antibodies detected proteins of molecular sizes corresponding well to collagen molecules identified not only in a previous study on collagens of *B. malayi* and *B. pahangi* (Selkirk *et al.* 1989*a*), but also of a variety of other nematodes, such as the free-living organisms *C. elegans* (Cox *et al.* 1981), *Panagrellus silusiae* (Leushner, Semple & Pasternak, 1979), and *Meloidogyne incognita* (Reddigari *et al.* 1986), the intestinal parasite *H. contortus* (Fetterer, 1989), and the filarial nematodes, *Onchocerca volvulus* and *O. gutturosa*, and *Dirofilaria immitis* (Petralanda & Piessens, 1991). In addition, we have described collagens of similar sizes in the intestinal parasite *A. suum* (Betschart *et al.* 1990). The observed cross-reactivity of anti-Bpcol-1 antibodies to collagenous molecules in extracts of *A. suum*, and in contrast, the lack of immunoreactions to mammalian collagens confirm that the antigenic structure of nematode collagens is fundamentally different from those of mammalian collagens. Nevertheless, immunological cross-reactivity between nematode-specific and mammalian collagens has been shown to occur in patients infected by filarial nematodes. Such autoreactive antibodies, however, seem to play a minor role in the context of the symptoms associated with filarial infection (Selkirk *et al.* 1989*a*).

A moderate labelling of several layers of the cuticle of adult *B. pahangi*, including the outer cortical layer, was observed in immunocytochemical experiments using affinity-purified anti-Bpcol-1 antibodies. The labelling of the basement membrane and secretions of the uterus was a rather interesting finding, which suggests the occurrence of similar epitopes in the basement membrane and the cuticle, although structural analysis of basement collagen genes of *C. elegans* (Guo & Kramer, 1989), *A. suum* (Pettitt & Kingston, 1991), and *B. malayi* (Caulagi *et al.* 1991; Caulagi & Rajan, 1995), and their encoded products, have revealed a different structure (Cox, 1992). The cross-reactivity between cuticular and basement membrane collagens observed in this study remains to be further analysed.

The authors wish to thank Dr M. E. Selkirk (Imperial College of Science, Technology and Medicine, London, UK) for providing the *B. pahangi* cDNA library and the polyclonal antiserum. We are grateful to Georg Scheidegger for technical support at the electron microscope. This work was supported by a grant from the Swiss National Research Foundation (No. 31–33961.92). Note: Nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X92099.

REFERENCES

ALTMANN, M., HANDSCHIN, C. & TRACHSEL, H. (1987). mRNA cap-binding protein: cloning of the gene encoding protein synthesis initiation factor eIF-4E from *Saccharomyces cerevisiae*. *Molecular Cell Biology* **7**, 998–1003.

BETSCHART, B. & WYSS, K. (1990). Analysis of the cuticular collagens of *Ascaris suum*. *Acta Tropica* **47**, 297–305.

BIRD, A. F. & BIRD, J. (1991). *The Structure of Nematodes*. Academic Press, San Diego.

BOISVENUE, R. J., STIFF, M. I., TONKINSON, L. V. & COX, G. N. (1991). Protective studies in sheep immunized with cuticular collagen proteins and peptides of *Haemonchus contortus*. *Parasite Immunology* **13**, 227–240.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* **72**, 248–254.

CAULAGI, V. R. & RAJAN, T. V. (1995). The structural organization of an α2 (type IV) basement membrane collagene gene from the filarial nematode *Brugia malayi*. *Molecular and Biochemical Parasitology* **70**, 227–229.

CAULAGI, V. R., WERNER, C. & RAJAN, T. V. (1991). Isolation and partial sequence of a collagen gene from the human filarial parasite *Brugia malayi*. *Molecular and Biochemical Parasitology* **45**, 57–64.

CORDEN, J., WASYLYK, B., BUCHWALDER, A., SASSONE-CORSI, P., KEDINGER, C. & CHAMBON, P. (1980). Promoter sequences of eukaryotic protein-coding genes. *Science* **209**, 1406–1414.

COX, G. N., KUSCH, M. & EDGAR, R. S. (1981). Cuticle of *Caenorhabditis elegans*: Its isolation and partial characterization. *Journal of Cell Biology* **90**, 7–17.

COX, G. N., FIELDS, C., KRAMER, J. M., ROSENZWEIG, B. & HIRSH, D. (1989). Sequence comparison of developmentally regulated collagen genes of *Caenorhabditis elegans*. *Gene* **76**, 331–344.

COX, G. N. (1990). Molecular biology of the cuticle collagen gene families of *Caenorhabditis elegans* and *Haemonchus contortus*. *Acta Tropica* **47**, 269–281.

COX, G. N. (1992). Molecular and biochemical aspects of nematode collagens. *Journal of Parasitology* **78**, 1–15.

DEVEREUX, J., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.

FEINBERG, A. P. & VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.

FETTERER, R. H. (1989). The cuticular proteins from free-living and parasitic stages of *Haemonchus contortus*-I. Isolation and partial characterization. *Comparative Biochemistry and Physiology* **94B**, 383–388.

FUJIMOTO, D. & KANAYA, S. (1973). Cuticlin: a noncollagen structural protein from *Ascaris* cuticle. *Archives of Biochemistry and Biophysics* **157**, 1–6.

GUO, X. & KRAMER, J. M. (1989). The two *Caenorhabditis elegans* basement membrane (type IV) collagen genes are located on separate chromosomes. *Journal of Biological Chemistry* **264**, 17574–17582.

HO, N. F. H., GEARY, T. G., RAUB, T. J., BARSUHN, C. L. & THOMPSON, D. P. (1990). Biophysical transport properties of the cuticle of *Ascaris suum*. *Molecular and Biochemical Parasitology* **41**, 153–166.

JOSSE, J. & HARRINGTON, W. F. (1964). Role of pyrrolidine residues in the structure and stabilization of collagen. *Journal of Molecular Biology* **9**, 269–287.

KIEFER, E., RUDIN, W., BETSCHART, B., WEISS, N. & HECKER, H. (1986). Demonstration of anti-cuticular antibodies by immunoelectron microscopy in sera of mice immunized with cuticular extracts and isolated cuticles of *Dipetalonema viteae* (Filarioidea). *Acta Tropica* **43**, 99–112.

KINGSTON, I. B., WAINWRIGHT, S. M. & COOPER, D. (1989). Comparison of collagen gene sequences in *Ascaris suum* and *Caenorhabditis elegans*. *Molecular and Biochemical Parasitology* **37**, 137–146.

LEUSHNER, J., SEMPLE, N. L. & PASTERNAK, J. (1979). Isolation and characterization of the cuticle from the free-living nematode *Panagrellus silusiae*. *Biochimica et Biophysica Acta* **580**, 166–174.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

MCCALL, J. W., MALONE, J. B., AH, H. S. & THOMPSON, P. E. (1973). Mongolian jirds (*Meriones unguiculatus*). infected with *Brugia pahangi* by the intraperitoneal route: a rich source of developing larvae, adult filariae and microfilariae. *Journal of Parasitology* **59**, 436.

MILLER, E. J. & GAY, S. (1987). The collagens: an overview and update. *Methods in Enzymology* **144**, 3–41.

OGILVIE, B. M., PHILIPP, M., JUNGERY, M., MAIZELS, R. M., WORMS, M. J. & PARKHOUSE, R. M. E. (1980). The surface of nematodes and the immune response of the host. In *The Host Invader Interplay* (ed. Van den Bossche, H.), pp. 99–104. Elsevier, Amsterdam.

PETTITT, J. & KINGSTON, I. B. (1991). The complete primary structure of a nematode $\alpha 2$ (IV) collagen and partial structural organization of its gene. *Journal of Biological Chemistry* **266**, 16149–16156.

PETRALANDA, I. & PIESSENS, W. (1991). *Onchocerca volvulus*, *O. gutturosa*, *Brugia malayi* and *Dirofilaria immitis*: A comparative study of the immunochemical properties of the cuticular proteins from filarial parasites. *Experimental Parasitology* **72**, 164–173.

POLITZ, S. M. & PHILIPP, M. (1992). *Caenorhabditis elegans* as a model for parasitic nematodes: A focus on the cuticle. *Parasitology Today* **8**, 6–12.

PRITCHARD, D. I., MCKEAN, P. G. & ROGAN, M. T. (1988). Cuticular collagens – a concealed target for immune attack in hookworms. *Parasitology Today* **4**, 239–241.

PROUDFOOT, N. J. & BROWNLEE, G. G. (1976). 3′ Non-coding region sequences in eukaryotic messenger RNA. *Nature, London* **263**, 211–214.

REDDIGARI, S. R., JANSMA, P. L., PRMACHANDRAN, D. & HUSSEY, R. H. (1986). Cuticular collagenous proteins of second-stage juveniles and adult females of *Meloidogyne incognita*: Isolation and partial characterization. *Journal of Nematology* **18**, 294–302.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463–5467.

SCOTT, A. L., YENBUTR, P., EISINGER, S. W. & RAGHAVAN, N. (1995). Molecular cloning of the cuticular collagen gene *Bmcol-2* from *Brugia malayi*. *Molecular and Biochemical Parasitology* **70**, 221–225.

SELKIRK, M. E., NIELSEN, L., KELLY, C., PARTONO, F., SAYERS, G. & MAIZELS, R. M. (1989*a*). Identification, synthesis and immunogenicity of cuticular collagens from the filarial nematodes *Brugia malayi* and *Brugia pahangi*. *Molecular and Biochemical Parasitology* **59**, 229–246.

SELKIRK, M. E., DENHAM, D. A., PARTONO, F. & MAIZELS, R. M. (1989*b*). Heat shock cognate 70 is a prominent immunogen in brugian filariasis. *Journal of Immunology* **143**, 299–308.

SHAMANSKY, L. M., PRATT, D., BOISVENUE, J. & COX, G. N. (1989). Cuticle collagen genes of *Haemonchus contortus* and *Caenorhabditis elegans* are highly conserved. *Molecular and Biochemical Parasitology* **37**, 73–86.

SULSTON, J. E. & BRENNER, S. (1974). The DNA of *Caenorhabditis elegans*. *Genetics* **77**, 95–104.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76**, 4350–4354.

WHARTON, D. A. (1986). *A Functional Biology of Nematodes*. Croom Helm, London & Sydney.

YOUNG, R. A. & DAVIS, R. W. (1983). Efficient isolation of genes using antibody probes. *Proceedings of the National Academy of Sciences, USA* **80**, 1194–1198.