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A Proline-rich Structural Protein of the Surface Sheath of Larval *Brugia* Filarial Nematode Parasites*

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Both cDNA and genomic DNA sequences have been isolated which encode a proline-rich precursor protein of the sheath from microfilariae, the first stage larvae of the filarial nematode parasites *Brugia pahangi* and *Brugia malayi*. This 22-kDa protein is soluble only under reducing conditions and is extensively cross-linked by both disulfide and nonreducible bonds. Immunogold electron microscopy shows that the protein is localized exclusively in the sheath, a vestigial remnant of the eggshell, which is retained by and encloses the mature microfilaria. Analysis by Western blotting confirms that the protein is expressed only in microfilariae and adult female worms, although transcripts are detectable only in adult females. The deduced amino acid sequence contains a short N-terminal hydrophobic putative leader sequence, a central repetitive domain that contains 14 copies of a degenerate 5-amino acid repeat with the consensus sequence Met-Pro-Pro-Gln-Gly, and a C-terminal proline-rich domain flanked by clusters of cysteine residues. These clusters can be aligned with cysteine residues implicated in cross-linking of a family of cuticular collagens originally identified in *Caenorhabditis elegans* but which extends to other nematodes.

The majority of free-living nematodes such as *Caenorhabditis elegans* and many parasitic species (e.g. *Ascaris*) give rise to progeny encased in eggshells, one of the most resistant and impermeable biological structures (Croll and Matthews, 1977). In contrast to this general plan, filarial nematode parasites are ovoviviparous, giving birth to living first stage larvae termed microfilariae. The reason for this phenomenon lies in the fact that progeny are not voided into the environment but are released into the bloodstream, where they are taken up by feeding arthropods which act as vectors for the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X58063.

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parasite. Within the filariae, a dichotomy exists in which some species (e.g. *Brugia*) release microfilariae which are enclosed by a loose baglike structure termed a sheath. Ultrastructural studies indicate that the sheath is derived from the embryonic eggshell (Rogers *et al.*, 1976; Zaman, 1987), but the reasons for the conservation of this structure in larvae of some species and not others are unclear.

Although mechanisms of immunity against nematode parasites are relatively obscure, there is a clear positive correlation of antibodies to the microfilarial sheath and clearance of larvae from the circulation (Wong and Guest, 1969; McGreevy *et al.*, 1980; Pinder *et al.*, 1988). Interest in the detailed structure of the sheath is thus not purely academic, but little is known about its biochemical composition other than that it contains protein, carbohydrate (Furman and Ash, 1983; Sayers *et al.*, 1984), sulfated proteoglycans (Simpson and Laurence, 1972), and chitin (Fuhrman and Piessens, 1985).

The general plan of a nematode eggshell is that of a multilamellate structure, which contains a central chitin-protein complex, an inner lipid layer, and outer layers derived from the vitelline membrane of the fertilized oocyte and/or secretions from uterine epithelial cells (Wharton, 1980). Given this general knowledge of the morphology of the eggshell, it is surprising that the primary structure of an eggshell protein has not been described in any nematode species, even in one so comprehensively studied as *C. elegans*. In this report, we describe a major proline-rich protein of the sheath/eggshell of *Brugia pahangi* and highlight homologies in primary sequence that point to a similar pattern of disulfide cross-linking utilized by cuticular collagens of *C. elegans*.

EXPERIMENTAL PROCEDURES

Parasites

Adult *Brugia malayi* of the zoophilic biotype (Partono and Purnomo, 1987) were recovered from the peritoneal cavity of jirds (Mongolian gerbils; *Meriones unguiculatus*) infected more than 3 months previously with 200 infective larvae from *Aedes aegypti* mosquitoes. Microfilariae were harvested from peritoneal cavities and separated from host cells by passage through Sephadex G10 (PD10) columns equilibrated with RPMI 1640 medium preheated to 37 °C (Taylor *et al.*, 1984). Infective larvae (L3) were dissected from mosquitoes and washed free of host contaminants. Adult *B. pahangi* were a kind gift from Dr. D. A. Denham (London School of Hygiene and Tropical Medicine).

Antiserum Preparation

The β -galactosidase fusion protein expressed by cDNA clone Bpa22/7 was induced and purified as previously described by electroelution from preparative SDS-polyacrylamide gels (Selkirk *et al.*, 1989a). A polyclonal antiserum was raised in rabbits by intramuscular inoculation of 200 μ g of purified protein emulsified in Freund's

complete adjuvant. Four weeks later, the rabbit was boosted with a subcutaneous injection of 50 μ g of protein emulsified in Freund's incomplete adjuvant, and the rabbit was bled 7 days later.

One- and Two-dimensional SDS-PAGE and Western Blotting

Discontinuous (7–25%) SDS-PAGE was performed according to standard protocols. Two-dimensional electrophoresis was performed according to O'Farrell *et al.* (1977) and as previously described (Selkirk *et al.*, 1989b). For one-dimensional gels, the solubility of Mf22 was examined by preparing parasite extracts via sequential homogenization in buffer a (TBS,¹ 1.5% *n*-octyl glucoside plus a mixture of protease inhibitors: 1 mM EDTA, 1 mM EGTA, 0.2 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 0.1 mM L-1-tosyl-amido-2-phenylethyl chloromethyl ketone, 1 mM *N*-ethylmaleimide, 2 mM phenylmethanesulfonyl fluoride). Following extraction on ice for 1 h, homogenates were centrifuged at 10,000 \times g for 30 min. Insoluble pellets were extracted in buffer b (the same buffer, with the addition of 5% 2-mercaptoethanol (2-ME) for 1 h at 37 °C) before centrifugation under the same conditions and recovery of the supernatant. For two-dimensional gels, parasites were homogenized in 100 mM DTT, the first dimensions run in 50 mM DTT, 6 M urea, and the second dimensions in 100 mM DTT.

Western blotting was essentially according to Towbin *et al.* (1979), with details as described previously (Selkirk *et al.*, 1989b). Specifically, following electrophoretic transfer of proteins to nitrocellulose, the blot was blocked via a 1-h incubation in 20 mM Tris-HCl, pH 8.0, 0.15 M sodium chloride (TBS) plus 5% dried milk powder. The primary antiserum (rabbit anti-Bpa 22/7 β -galactosidase fusion protein) was used at a dilution of 1/1000 in TBS, 5% milk powder, 0.1% Tween 20 overnight at 4 °C, and horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad; 172-1013) was used at 1/2000 for 2 h at room temperature in TBS alone. Binding was visualized by 4-chloro-1-naphthol (Sigma, C 8890) at a final concentration of 0.5 mg/ml.

Northern Blotting and Hybridization

RNA was isolated by sedimentation through cesium chloride following lysis of parasites in guanidinium thiocyanate (Chirgwin *et al.*, 1979), and separated on formaldehyde-based gels (15 μ g/track; Maniatis *et al.*, 1982); DNA probes were labeled by random priming (Feinberg and Vogelstein, 1983). Hybridizations were performed in 5 \times SSC at 65 °C with 10⁶ cpm/ml, and blots were washed at a final stringency of 0.1 \times SSC at 65 °C.

Immunoelectron Microscopy

Fixation and Embedding—Fixation and embedding were carried out via a modification of the method of McPhail *et al.* (1987). Adult female parasites were cut into 0.5-cm lengths and were subsequently treated in an identical manner to microfilariae. The parasites or parasite pieces were fixed in 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4 °C, followed by an overnight wash in 0.1 M phosphate buffer at the same temperature. The tissue was then dehydrated in methanol by successive incubations of 30 min in 50% methanol at 4 °C and 60 min each in 70, 90, and 100% methanol at –25 °C. The material was then infiltrated with LR Gold (London Resin Gold, London Resin Co.), first by incubation for 30 min in 1:1 methanol:LR Gold, 0.1% benzoin methyl ether (LRG/BME), then by a 1-h incubation in 3:7 methanol:LRG/BME, and finally by a 72-h incubation in LRG/BME. After embedding in gelatin capsules with fresh resin, polymerization was effected by exposure to UV light for 72 h. All infiltration and polymerization steps were carried out at –25 °C.

Immunogold Localization—Ultrathin sections (80–90 nm) of embedded worms (adult females or microfilariae) were cut on a Reichert Jung ultracut ultramicrotome and mounted on Formvar-coated nickel grids. Tissue sections were blocked for 30 min at room temperature in PBS, 1% bovine serum albumin, 0.01% Tween 20 (PBS/BT). Grids were then incubated in rabbit anti-Bpa 22/7 β -galactosidase, diluted 1/1000 in PBS/BT for 2 h at room temperature, washed three times in PBS/BT, and incubated for 30 min in goat anti-rabbit IgG-gold conjugate (Seralab, 10 nm gold particles) diluted 1/10 in the same

solution. Samples were washed once in PBS/BT, once in PBS, 0.01% Tween, and once in distilled water prior to counterstaining in 4% uranyl acetate for 10 min, followed by Reynold's lead citrate for 2 min. Control grids were treated with normal rabbit serum diluted 1/30 in PBS/BT, in place of the primary antibody. Processed samples were examined in a Jeol 100CX transmission electron microscope.

RESULTS

Mf22: Stage Specificity, Solubility, and Localization—We have recently described the physical properties, localization, and turnover of Gp29, the major surface glycoprotein of adult stage parasites of the genus *Brugia*, and raised a polyclonal antiserum to it via preparative SDS-PAGE of a 29-kDa fraction (Maizels *et al.*, 1989; Selkirk *et al.*, 1990). In using this antiserum to screen a cDNA library derived from mixed adult *B. pahangi* RNA and propagated in λ gt11, we isolated 28 clones, of which 14 represented a related family judged by cross-hybridization. The largest cDNA from this family (Bpa22/7; 649 base pairs) was lysogenized in *E. coli* Y1089 (Young and Davis, 1983), and the β -galactosidase fusion protein induced and purified by preparative SDS-PAGE as previously described for other gt11 clones (Selkirk *et al.*, 1989a). A polyclonal antiserum was raised in rabbits by immunization with the purified fusion protein in order to determine the identity of the native protein which this class of cDNAs encoded.

Surprisingly, the antiserum bound to a series of proteins expressed in first stage larvae (microfilariae) and adult female worms but not in third stage (infective) larvae or adult males (Fig. 1). Thus the cDNAs clearly did not code for Gp29, but for a protein or series of proteins which, in addition to their distinct stage specificity, were insoluble in detergent (*n*-octyl glucoside) and only soluble in reducing agents such as 2-ME or DTT. The antiserum bound to a number of proteins in microfilariae which resolved as apparent multimers of 22 kDa, and this was confirmed by Western blots of parasite extracts separated by two-dimensional gels. A series of proteins could now be visualized whose molecular mass resolved as multiples of 22 kDa up to 88 kDa, and had a number of distinct isoelectric points (Fig. 2). One obvious interpretation was that a monomeric 22-kDa protein was being cross-linked and modified to form a network of higher order products in microfilariae, and this was supported by the resolution of a major species at 22 kDa in adult females. The higher molecular mass (approximately 29 kDa) band seen in female worms in Fig. 1 was a product of incomplete reduction and was abolished

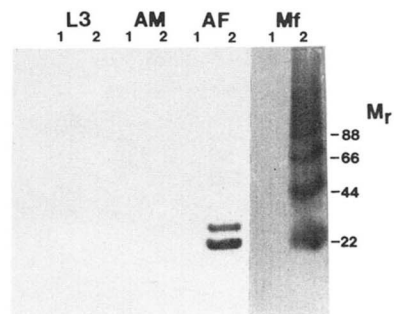


FIG. 1. Stage specificity of expression of Mf22. Extracts from third stage larvae (L3), adult males (AM), adult females (AF), and microfilariae (Mf) of *B. malayi* were run on discontinuous (7–25%) polyacrylamide gels prior to transfer to nitrocellulose and reaction with a rabbit antiserum generated to the β -galactosidase fusion protein expressed by cDNA clone Bpa22/7. Extracts were prepared by sequential solubilization of parasites in *n*-octyl glucoside (1), and 2-ME (2) (see "Experimental Procedures" for details). The position of migration of the 22-kDa monomer and polymers is indicated.

¹The abbreviations used are: TBS, Tris-buffered saline; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; PRP, proline-rich protein; EGTA, [ethylene bis(oxyethylenitrilo)]tetraacetic acid.

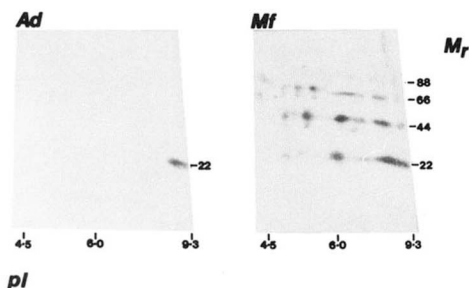


FIG. 2. **Detection of cross-linked Mf22 in microfilariae.** Extracts of adult females (*Ad*) or microfilariae (*Mf*) of *B. malayi* were separated by two-dimensional polyacrylamide gel electrophoresis, blotted, and reacted with the Bpa22/7 β -galactosidase fusion protein antiserum. Extracts were prepared by boiling in loading buffer (see "Experimental Procedures"). The migration of apparent monomers, dimers, trimers, and tetramers of Mf22 is indicated (M_r). *pI*, isoelectric point.

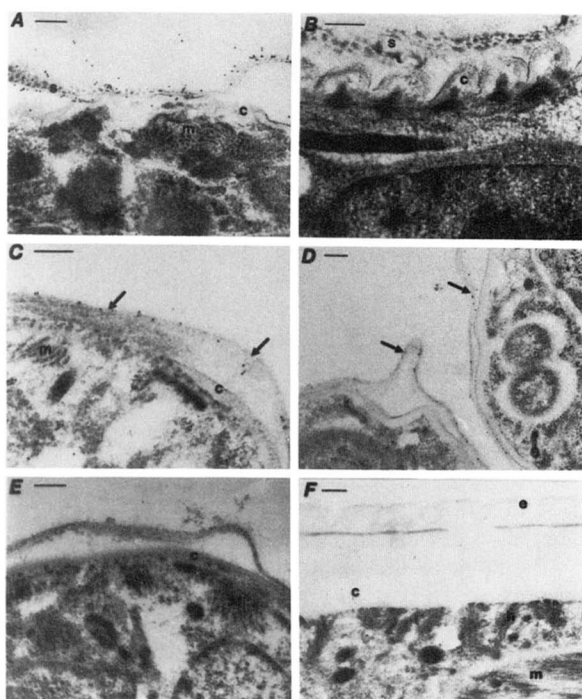


FIG. 3. **Immunogold localization of Mf22 in *B. malayi*.** Bar = 0.25 μ m. *A*, mature microfilaria stained with Bpa22/7 β -galactosidase antiserum, showing exclusive localization of the gold particles to the sheath; *B*, lack of staining of mature microfilaria with control (normal) rabbit serum; *C* and *D*, positive staining of the sheath in uterine microfilariae with the test serum; *E*, lack of staining of microfilaria *in utero* with control serum; *F*, lack of staining of an adult male worm with the test serum. Section through the body wall of the worm. Abbreviations: *s*, sheath; *c*, cuticle; *e*, epicuticle; *h*, hypodermis; *m*, muscle.

when extracts were boiled extensively (10 min) in 5% 2-ME or 100 mM DTT.

The localization of the native protein or protein complexes in microfilariae and adult worms was examined by utilizing the same antiserum generated to the Bpa22/7- β galactosidase fusion protein in immunogold electron microscopic studies. We were surprised to find that the antiserum bound solely, and at high density, to the surface sheath surrounding microfilariae, either as "mature" first stage larvae isolated from the peritoneal cavity of infected jirds, or as immature larvae developing *in utero* in sections of adult female worms (Fig. 3). Thus, Fig. 3*A* shows gold particles concentrated on the sheath in mature microfilariae, while *C* and *D* depict staining on the

same structure in developing larvae *in utero*. No staining was evident in any internal structures such as the cuticle, hypodermis, or body-wall musculature. Additionally, the antiserum did not bind to adult male worms (Fig. 3*F*), confirming the stage-specificity of expression seen in Western blots (Fig. 1). Fig. 3, *B* and *E* shows the lack of binding of control sera to microfilariae, even when used at a much higher concentration, thus confirming the specificity of the reaction seen with the test antiserum. Because of the molecular mass of the native protein and its localization to the microfilarial surface sheath, we have termed this protein Mf22 for convenience.

A Northern blot using the Bpa22/7 cDNA as a hybridization probe indicates that the Mf22 gene was not transcribed in microfilariae (Fig. 4), despite the fact that the protein was present in this stage at a high concentration on the sheath (Figs. 1–3). It thus appears that Mf22 was presynthesized in adult female worms and did not undergo significant turnover in microfilariae, consistent with a role as a structural protein. The size of the major transcript (0.9 kilobase pairs) is also consistent with a final protein product of 22 kDa, allowing for 5'- and 3'-untranslated regions.

DNA Sequence, Protein Sequence, and Structural Homologies—The cDNA clone Bpa22/7 was used as a hybridization probe in a series of restriction enzyme digests of genomic DNA from *B. malayi* and *B. pahangi*, which suggested that it was encoded by a single gene which resided on a *Hin*PI restriction fragment of approximately 1370 base pairs. Fig. 5 illustrates the sequence of the Mf22 gene from *B. pahangi*, including 5'- and 3'-flanking regions, derived from three partial cDNAs and the complete sequence of the *Hin*PI genomic fragment. The 5' end of the longest cDNA (Bpa22/7) lies at residue 23 (arrow), and this clone contains an uninterrupted reading frame through to an 18-base pair poly(A) stretch at position 749. There is no consensus polyadenylation site within Bpa22/7, but two can be located in tandem approximately 80 base pairs downstream from the 3' end (underlined), indicating that the cDNAs have been primed from the internal poly(A) tract of 20 residues seen from positions 732 to 751.

The putative initiator ATG lies 23 residues upstream from the 5' end of Bpa22/7. The sequence of this and two other shorter cDNA clones precisely matches that of the corresponding region of the genomic *Hin*PI fragment, except for

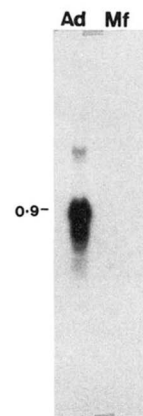


FIG. 4. **Stage specificity of transcription of Mf22.** Total RNA from adult females (*Ad*) and microfilariae (*Mf*) of *B. pahangi* was size-separated on a 1.5% agarose gel (15 μ g/track), blotted onto nitrocellulose, and hybridized with cDNA clone Bpa22/7. The post-hybridization wash was performed at 65 $^{\circ}$ C and 0.1 \times SSC. The size of the major transcript is given in kilobases. The loadings of RNA were checked by rehybridization of the same filter with a filarial heat shock protein 70 probe (Selkirk *et al.*, 1989a).

the presence of a 78-base pair intron, which is bounded by consensus splice signals that match those determined for another *Brugia* gene (Perrine *et al.*, 1988). This intron lies precisely at the 3' end of a series of 14 tandem 15-base pair repeats. The repeat is not homogeneous, and the relative identity of the most frequent variant (ATG CCA CCA-CAA GGT) falls off towards the 5' and 3' ends of the repeat block. In addition, two degenerate copies of the repeat can be discerned, in phase, upstream of a 15-base pair region with little homology to the most common repeat variant.

All of the immunoreactive cDNAs are fused with β -galactosidase in the same reading frame, which is also represented in Fig. 5. This reading frame is open for only 18 codons upstream of Bpa22/7, the longest cDNA. The first in-frame ATG (in fact a double ATG) lies 7 codons upstream of the 5' end of 22/7, and the derived sequence predicts a protein with a molecular mass of 21.5 kDa. This protein can be conceptually divided into three domains (Figs. 5 and 6). The N terminus contains a stretch of markedly hydrophobic residues, which constitute a potential signal sequence. The central repetitive domain contains 14 copies of a degenerate repeat with the consensus sequence Met-Pro-Pro-Gln-Gly, but elements of this repeat can be detected from residues 20 to 122, at precisely which point the intron is inserted. Following the intron is a stretch of 82 amino acids that is rich in proline residues (30%).

The derived protein sequence of Mf22 was used to search the NBRF/University of Wisconsin Genetics Computer Group databases using the Lipman and Pearson (1985) algorithm in the FASTA program, and considerable homology was found to a diverse array of proteins which share with Mf22 the property of having proline-rich repetitive elements. The mammalian proline-rich proteins (PRPs) constitute a set of peptides found in saliva and salivary glands. The mouse PRPs are made up of tandemly repeated 14- or 19-amino acid sequences rich in glycine, glutamine, and proline (Clements *et al.*, 1985; Ann *et al.*, 1988). The human PRPs have a more complex repeat structure, with a series of subrepeats made up predominantly of these three residues (Wong *et al.*, 1979; Wong and Bennick, 1980; Maeda *et al.*, 1985). The sequence of Mf22 compared with that of the human PRPs A and C (Wong and Bennick, 1980) showed short stretches of high homology (16/22 identical residues; 72%) ranging to longer stretches of weaker similarity (31/64 identical residues; 48%).

Examples of other proteins with similar proline-rich repetitive elements include giant octopus rhodopsin, which has a 5-amino acid N-terminal repeat (Gln-Gly-Tyr-Pro-Pro) not found in other rhodopsins (Ovchinnikov *et al.*, 1988). Bindin, a protein from the sperm of the purple sea urchin implicated in the recognition of the egg by sulfoglycan binding, has a 7-amino acid repeat (Pro-Gln-Gly-Met-Gly-Pro) at its N terminus (Gao *et al.*, 1986). It is interesting to note that all of these repeats are somewhat redundant. Thus individual members may diverge by up to 30%, but the substituted residues are often still glycine, glutamine, or proline. The same situation is observed in the repetitive elements of Mf22, and we elaborate on this in the discussion.

Repetitive sequences are common in many fiber-forming proteins such as keratin (Steinert *et al.*, 1983) and elastin (Bressan *et al.*, 1987), and the primary sequence of the repetitive region of Mf22 is most similar to that of collagens, containing glycine and proline, albeit in arrays of 5 amino acids rather than the 3 amino acids necessary for the triple-helical formation of collagen fibrils (Bornstein and Traub, 1979).

The C-terminal proline-rich domain of Mf22 is flanked by

two regions with clusters of cysteine residues (Fig. 6). This arrangement is reminiscent of the primary structure of cuticular collagens from *C. elegans* and *Haemonchus contortus* in which the proline-rich (Gly-Xaa-Yaa) repeat domains are flanked by clusters of cysteines (Kramer *et al.*, 1982; von Mende *et al.*, 1988; Shamansky *et al.*, 1989). The similarity is heightened by the fact that (a) both classes of proteins are disulfide-cross-linked and (b) disregarding residues in potential signal sequences, all of the cysteines in both molecules are clustered at these sites. A closer examination of these cysteine clusters reveals an exact alignment of the 3 residues at site 2 in the *C. elegans* collagen family, and 3 of the cysteine residues at site 2 in Mf22 (Fig. 6). In addition, several other amino acids show alignment in this region, notably 3 prolines proximal to one of the cysteine residues. The clustered cysteines of cuticular collagens are presumed to be essential for cross-linking, and LRol (left roller) phenotypes of *C. elegans* have been shown to result from mutations in *sqt-1*, a collagen gene, which result in the loss of cysteine residues. Conversely, RRol (right roller) phenotypes result from the gain of a cysteine in *sqt-1*, and collectively the data indicate that precise disulfide bond formation between collagens is essential for the correct formation of the cuticular exoskeleton (Kramer *et al.*, 1990). The observed alignment of clustered cysteine residues suggests that a similar pattern of intermolecular disulfide bridges are utilized to form a lattice of Mf22 proteins in the sheath of *Brugia*.

DISCUSSION

When released into the environment, the nematode egg represents a highly impervious self-contained unit which is resistant to desiccation, and the success of nematodes in colonizing a wide array of environmental niches must have been aided greatly by the development of the eggshell. The composition of nematode eggshells is highly variable, but a prototype structure has been outlined by Anya (1976) and Wharton (1980), which consists of an inner lipid layer, a central chitinous layer, and an outer lipoprotein or "vitelline" layer. The general impermeability of nematode eggshells is due to the inner layer, which is composed of high molecular weight glycosides termed "ascarosides" due to their isolation from *A. lumbricoides* (Tarr and Fairbairn, 1973).

Filarial nematode parasites clearly have no need for a thick impervious eggshell, and ultrastructural studies have failed to identify an inner lipid layer (McLaren, 1972; Laurence and Simpson, 1974). Tarr (1973) also found no ascarosides in eggshells of the ovoviviparous free-living nematode *Panagrellus redivivus*, and thus the environment in which the embryo develops probably determines whether this layer is formed or not. Consistent with the absence of the inner lipid layer, the permeability of filarial sheaths contrasts sharply with that of "classical" nematode eggshells, and fluorescein-tagging experiments have shown that proteins up to 45 kDa in mass can penetrate the sheath of *B. pahangi* (Devaney, 1985) and *B. malayi* (quoted in Schraermeyer *et al.*, 1987).

The central layer of nematode eggshells is frequently composed of a chitin-protein complex providing a structural backbone through the high tensile strength of chitin dispersed in the form of fibrils in a protein coat. Fibers have been described in *Trichuris suis* (Wharton, 1979) and *Porrocaecum ensicaudatum* (Wharton and Jenkins, 1978) which contain a 2.8 nm chitin microfibril core surrounded by a protein coat, an organization which had been previously observed in arthropod cuticles (Neville, 1975). The presence of chitin in the sheath of *B. malayi* has been inferred from positive staining of microfilariae with fluoresceinated lectins (*Solanum tuberosum*

origin and site of formation of Mf22 in the uterus by immunogold labeling.

The reason why some filarial species retain the sheath and others discard it is unknown. Suggestions that it may serve to protect the microfilariae against immune reactions are unconvincing in that this merely shifts the focus of an effector response onto another surface. Moreover, there is a strong positive correlation between the presence of antibodies which bind the sheath and clearance of microfilariae (Wong and Guest, 1969; McGreevy *et al.*, 1980; Pinder *et al.*, 1988). We are therefore examining the potential of Mf22 as an immunogen which would promote clearance of microfilariae and thus effectively block transmission to the mosquito vector.

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