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A Hemoglobin with an Optical Function

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF138295, AF138291, AF138296, AF138297, AF138292, AF138293, AF138294, and AF140502.

[1] The abbreviations used are: Hb(s), hemoglobin(s); PCR, polymerase chain reaction; RT, reverse transcription.

Abstract

Hemoglobins are best known as oxygen transport proteins. Here we describe a hemoglobin from the parasitic nematode Mermis nigrescens (Mn-GLB-E) that has an optical, light shadowing function. The protein accumu-lates to high concentration as intracellular crystals in the ocellus of mature phototactic adult females while also being expressed at low concentration in other tissues. It differs in sequence and expression pattern from Mn-GLB-B, a second Mermis globin. It retains the struc-ture and oxygen-binding and light-absorbing properties typical of nematode hemoglobins. As such, recruitment to a shadowing role in the eye appears to have occurred by changes in expression without modification of bio-chemistry. Both globins are coded by genes interrupted by two introns at the conserved positions B12.2 and G7.0, which is in agreement with the 3exon/2intron pat-tern model of globin gene evolution.

Article

It has become evident that heme-based oxygen carriers (hemoglobins) are widespread even in the lower phyla. Hemoglobins (Hbs)[1] and Hb-like proteins have been characterized in an ever extending list of invertebrates, plants, fungi, protozoa, and bacteria. Comparisons across this protein family have revealed unexpected diversity in function and structure (1–6). Despite the great variability in primary and quaternary structure of their Hbs, the globin domains of phylogenetically widely diverged species all display the "globin fold" (5, 7). The evidence strongly supports the hypothesis that globins arose from a common ancestor long before the advent of atmospheric oxygen (1, 8).

During evolution, the globin domain evolved to assist the reversible binding to heme of small gaseous ligands (O_2, CO, H_2S, and NO) resulting in biological functions as diverse as O_2 storage, transportation, and scavenging, as well as transportation and accumulation of NO and H_2S (2, 5, 6, 9). These functions generally require large amounts of Hb. High molecular weight Hb complexes likely evolved to avoid excretion of Hb that is present in the body fluids as a solute (10). A different solution to this problem was the packaging of highly concentrated Hb in specialized cells, *e.g.* erythrocytes. Alternatively, smaller amounts of myoglobin-like proteins are present in virtually all cells, perhaps providing an efficient intracellular oxygen delivery system to the respiring mitochondria and chloroplasts (4, 11, 12).

Oxygen-binding hemoproteins may also accept electrons from suitable donors that reduce the bound dioxygen or the heme iron. These are found particularly in unicellular systems, notably bacteria in which the electron transport system and the O_2 carrying hemoproteins are not contained within separate compartments. The flavohemoglobins of *Escherichia coli, Alcaligenes euthropus*, and *Saccharomyces cerevisiae* catalyze redox reactions, with the heme playing a direct role in electron transfer much as in cytochromes (13–16). The dioxygenase activity of the *E. coli* flavohemoglobin that was recently described (17) also falls into this category. Hb-associated iron atoms or unknown proteins potentially assist electron transfer (18–21). Hemoglobins function as a terminal oxidase in *Vitreoscilla* (22) and *A. euthropus* (15) and play a central role in protection against oxidative stress in *S. cerevisiae* (23) and detoxification of NO in *E. coli* (17, 24).

Mermis nigrescens is a nematode parasite of grasshoppers and other Orthopteran insects (25, 26). From eggs ingested by the host, larvae grow to the 10-cm adult length coiled inside the body cavity. The L4-stage larvae break out of the host and burrow into the soil where the final molt takes place and the adult nematode matures. The gravid adult females emerge 1–2 years later from the soil and exhibit a positive phototaxis during the search for suitable egg-laying sites in grass (27–29). During maturation in the soil, the ocellus becomes pigmented with Hb (29–33). We here describe the structure, function and expression of a Hb used in an optical function in the ocellus of the nematode *M. nigrescens*.

MATERIALS AND METHODS

Collection and Cultivation—Egg-laying adult females were collected from vegetation in Vancouver, Canada and stored at about 8 °C in moist autoclaved soil. The desert locust host *Schistocerca gregaria* was infected by feeding a counted number of eggs, and the female fourth stage (L4) larvae were collected as they emerged 4–5 weeks later. Larvae were investigated 1–2 days after emergence. Immature adult females were investigated 2–3 months post emergence before visible amounts of eye Hb had developed. The mature adults, which had densely colored ocelli, were either field collected or cultivated for at least 10 months post emergence.

Microscopy—Anterior pieces containing the ocellus were fixed 16 h in 3% glutaraldehyde, 0.5 M phosphate buffer at pH 7.2. After washing 2 h in buffer, fixing 3 h in 2% OsO_4, and washing 2 h in buffer, the pieces were dehydrated in an ethanol series to propylene oxide and embedded

in Epon. Sections approximately 60 nm thick were mounted on Formvar-coated grids and stained for 25 min with uranyl acetate and for 6 min with lead citrate. A Phillips EM300G electron microscope was used with a eucentric tilting apparatus. Light micrographs were obtained under either bright field or laterally incident illumination.

Behavioral Experiments—Arena and test conditions were similar to those described previously (27–29). After acclimation to 21 °C and light, the motion of individual worms crawling on moistened black felt was recorded under far-red and near-infrared light (630–990 nm) with a CCD video camera (Cohu model 4915) and VCR. The test stimulus was a horizontal monochromatic (420 nm) beam at 1.33×10^{13} photons s^{-1} cm^{-2} (6.3 μW cm^{-2}), which was filtered to remove all heat radiation (27).

An active worm was placed in the arena, and the test stimulus was provided in the following sequence: 30 min at 420 nm, 5 min of darkness, 30 min at 420 nm, and 60 min of dark control period. During playback, the angular orientation of the "neck" (the 3 mm behind the 2-mm "head") was measured every 30 s during light or dark periods except for an initial 4-min recovery interval. For hypothesis testing, mean vectors of neck orientations were calculated for each worm. A mean vector is $1/n$ times the vector sum of unit vectors pointing in the sampled directions. Treating the x and y coordinates of the ends of the mean vector as samples from a bivariate normal population, Hotelling's one-sample test estimates the probability that the population of x and y points has the origin 0.0 as its center. If this null hypothesis can be rejected at $p < 0.05$, then one can conclude worm necks are significantly oriented. The mean of mean vectors indicates the average direction (34).

Protein Purification and Sequencing—Hb was isolated from 75 egg-laying females. The anterior part was cut, and the Hb was leached from 25 worms into 4 μl of distilled water (32). After SDS-polyacrylamide gel electrophoresis and transfer to a polyvinylidene difluoride membrane, a prominent band with $M_r \sim 17{,}000$ was selected for amino acid sequencing using an ABI 471-B sequencer (35).

Derivation of Degenerate Oligonucleotides—N-terminal peptide sequence was used to design a sense strand degenerate primer for PCR. MnG-d(AT) is designed to be an AT biased primer (GTWAATTTA-GATATWTTWMGIGC, a 23-mer with 64 redundancies).

Cloning of Mn-glb by RT-PCR—The first 4 mm of the *Mermis* head was dissected from 10 young adult females that had faintly visible eye spots. Two headless bodies were also kept for RNA isolation. Total RNA was made from the heads and bodies by (i) grinding the nematode sections in a sterile mortar under liquid nitrogen and (ii) extracting using the Ultraspec RNA isolation system (Biotecx) according to the manufacturer's instructions. Oligo(dT)-primed cDNA was generated using the GeneAmp kit (Perkin-Elmer). Head and body cDNAs and the degenerate primer were used in two separate reactions with an anchored oligo(dT) primer DGDT(GCGCGGATCCGCTTTTTTTTTTTTTT-TTTTT) to generate PCR fragments for cloning and sequencing. The amplified products were cloned into the pMOS t-vector (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Recombinant plasmids were sequenced using vector-derived primers and the ABI 377 sequencer. To determine the globin cDNA sequence, three separate cloned cDNAs were sequenced from the sense and antisense strands, and a consensus was generated.

Cloning of Mn-glb-e by RT-PCR—Two primers were designed based on the difference between the *Mn-glb* cDNA sequence and the amino acid sequence information obtained by Edman degradation of eye Hb. MerF1A (GTTGGCCAAATTGCCCATCAACGAGA) is a primer that matches the *Mn-glb* cDNA sequence completely. MerF1T (GTTGGC-CAAATTGCCCATCAACGAGT) is a primer that matches the "body isoform" cDNA except for the very last base, which changes the codon to one encoding Phe instead of Ile. Both primers were high pressure liquid chromatography-purified and were used together with the oligo(dT) primer in a RT-PCR with RNA from both head and body. Positive fragments were cloned and sequenced as described earlier.

The 5′ end of the *Mn-glb-e* cDNA was isolated using rapid amplification of cDNA Ends (Life Technologies, Inc.). In this procedure, first strand cDNA was synthesized using the specific primer MerR2UTR (GGAATAAGACGACGAACCATTCCC). A poly(C) tail was added to the 3′ end of the cDNA with terminal deoxytransferase. PCR was then carried out using an oligo(dG) adaptor and the specific primer MerR1INT (GGGCACCTCCTCCGGCTTGATTGC). Positive fragments were cloned and sequenced.

Sequencing the Genes Mn-glb-e and Mn-glb-b—The gene for the eye globin was amplified using the specific primers MereyeF (CAGTACT-TGTGGTTCTGGCGGT) and MerR2UTR. The gene of the body globin was amplified using the specific primers MnG-S5 (ATGGTGGTGAATT-TGGACATT) and MnG-E6 (TCACCAGCCTCCGATGTACTTC). The gene fragments were subsequently purified, cloned, and sequenced.

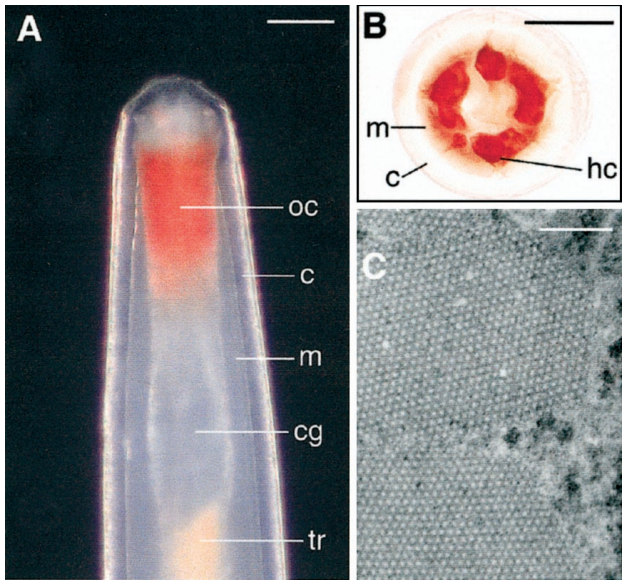

FIG. 1. **Pigmentation and crystals in the ocellus of *M. nigrescens*.** *A*, whole mount of anterior tip of *Mermis*; pigmentation is confined to the ocellus. *Bar*, 100 μm. *B*, transverse thick section; cylindrical distribution of pigment in expanded hypodermal chords. *Bar*, 100 μm. *C*, transverse section through the ocellus; crystals in cytoplasm of hypodermal chord cell. Transmission electron micrograph of section stained with uranyl acetate and lead citrate. *Bar*, 100 nm. *c*, cuticle; *cg*, cerebral ganglion; *hc*, hypodermal cord; *m*, body wall muscle; *oc*, ocellus; *tr*, trophosome (food storage body).

Analysis of Sequences—Nucleic acid sequences were analyzed using MacVector™ 6.0.1. (Oxford Molecular Ltd.) and AssemblyLIGN™ 1.0.7. (Eastman Kodak Company). Data base searches for amino acid sequence similarities were performed using the BLAST server (36) to search a collection of data bases including GenBank™ and dbEST. The *Mermis* globin sequence was aligned to other globins. The alignment was used to derive phylogenetic trees using neighbor joining as implemented in the TREECON program (37). Support for trees was assessed using the bootstrap procedure (1000 replicates).

Analysis of Transcription of Mn-glb-b and glb-e by RT-PCR—Multiplex reactions were performed in which either *Mn-glb-b* or *Mn-glb-e* was co-amplified with a fragment of the *M. nigrescens* ribosomal protein gene L6 (*Mn rpl-6*), which was sequenced by chance from the nonglobin clones isolated from the original degenerate PCR reactions. The following primers were used in the multiplex reactions: (i) for amplification of *Mn rpl-6*, MnR-1F (CTGTACTGATCGTATTAGTTG) and MnR-2R (GATCGCACCTATGATCATGCCATC); (ii) for amplification of *Mn-glb-b*, MnG-S6 (AGGGAATTCCATATGGTCGTCAATTTGGACATTA) and MnG-E7 (CGCGGATCCGCGTCACCAGCCTCCGATGTACTTC); and (iii) for amplification of *Mn-glb-e*, MereyeF and MerR2UTR. In addition to analyzing the RT-PCR results by agarose gel electrophoresis, the specificity of the PCR reactions was confirmed by digesting the PCR products with *Sau*3AI to distinguish *Mn-glb-b* products from *Mn-glb-e* products.

RESULTS AND DISCUSSION

The anterior of adult gravid females of *M. nigrescens* contains a strongly pigmented region forming a hollow cylinder (Fig. 1, *A* and *B*). The cylindrical conformation of the ocellus is due to deposition of pigment in the hypodermal cells of the region, swelling the hypodermal chords to envelop the body cavity (Fig. 1*B*). In transmission electron micrographs of thin sectioned worms, the cells are seen to be densely packed with needle-like crystals having a hexagonal symmetry in cross-section (Fig. 1*C*). In longitudinal sections, parallel stripes disappear and reappear at 60° intervals as a section is tilted. The patterns and dimensions are consistent with parallel rows of protein molecules forming hexagonal tubes with shared walls.

Microspectrophotometry through the ocellus in fresh worm heads reveals an absorption spectrum and dichroic spectrum

typical of HbO_2 and its crystals (33). Thus both the eye pigmentation and crystals are shown to consist of oxyHb. There was no detectable Hb in neighboring tissues. The heme concentration in the ~0.5 nl volume of the pigmented region is ~10 mM, nearly that of Hb in vertebrate erythrocytes (32, 33).

In other nematode ocelli, melanin plays a shadowing role. A sensory nerve ending lies anterior to a melanin pigment spot or shallow pigment cup (38–40) where it would be shadowed when the head is pointed away from a light source (41). This provides an orientation signal for negative phototaxis (40). The oxyHb in the ocellus of *Mermis* females appears to have a similar role, but its arrangement results in positive rather than negative phototaxis (27–29). We have investigated the role of Hb further, taking advantage of a period during development before the oxyHb pigmentation appears. From videotaped images recorded under infrared illumination, we measured the neck orientations of three ages of female in the presence of a 420 nm test source at 1.33×10^{13} photons s^{-1} cm^{-2} (6.3 μW cm^{-2}) (Fig. 2). Only in the pigmented, mature females was there significant phototaxis, as judged by the Hotelling's one-sample test. The nonpigmented, 2–3-month-old immature females were not significantly phototactic, nor were the nonpigmented, 1–2-day-old L4 larvae (Fig. 2). This confirms that the presence of a pigmented ocellus is necessary for positive phototaxis. We noted further that the positive phototaxis of mature adults is significant over the 350–560 nm range, which includes most of the wavelengths where the concentrated Hb in the ocellus would cast a shadow (27). Together, the spectrophotometry, morphology, and behavioral experiments strongly implicate the Hb to be involved in a shadow-casting function during positive phototaxis. Both the high absorptivity of oxyHb and the high concentration are required for the 25–35-μm-thick pigment cylinder to cast a shadow. The use of a Hb in such an optical role is, to present knowledge, unique.

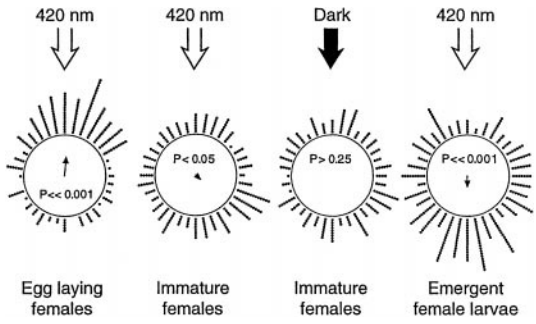

FIG. 2. **Distribution of neck orientations with respect to direction of horizontal monochromatic light.** Responses to light of different developmental stages of female *Mermis*, which contain ocellar pigmentation (mature, egg-laying adults) or lack ocellar pigmentation (immature adults and emergent L4 larvae), are compared with immature adults during dark control periods. Each *dot* indicates an orientation of the neck region 2–5 mm behind the tip (28, 29) measured at 30-s intervals. Mean vectors plotted within each circular histogram point in the mean direction, and their lengths indicate the concentration of observations in that direction. For hypothesis testing, mean vectors were calculated for independent measurement periods and the Hotelling's one-sample test was applied (see "Materials and Methods"). The sample sizes under each condition were (*left* to *right*) 11, 10, 5, and 12. The probabilities that worm necks were not oriented are <0.01 (significant orientation), >0.5, >0.5, and >0.2.

Have there been any structural modifications related to this optical function? How is this Hb related to the rest of the globin family? To answer these questions, we isolated and characterized the Hb cDNA and gDNA.

Hb was leached from cut anterior tips of nematodes, separated on SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. A prominent band, M_r ~17,000, was sequenced resulting in 33 N-terminal amino acid residues of the eye globin (*Mn*-GLB-E; Fig. 3). MnG-d(AT), a degenerate forward primer based on this sequence, and oligo(dT) were used in RT-PCR reactions using total cDNA from head as template. The amplified fragment differed by three predicted residues from the N-terminal sequence obtained from protein sequencing, suggesting the presence of a second globin isoform, which we designate *Mn*-GLB-B. Primers designed to distinguish between the different codons at residue 19 (for Phe or Ile; Fig. 3) were used in conjunction with an anchored oligo(dT) reverse primer to separately amplify and clone fragments of the *Mn*-GLB-E and *Mn*-GLB-B isoforms. Complete cDNA sequences were obtained using 5′-rapid amplification of cDNA ends. The predicted amino acid sequences of *Mn*-GLB-E and *Mn*-GLB-B are aligned with selected globins in Fig. 3.

Both cDNAs encode proteins of 146 amino acid residues with sequences typical of globins. An unambiguous alignment of the *Mermis* sequences with globins of known structure shows: (i) the correct location of Phe(CD1) and His(F8), the two absolutely conserved residues of globins, (ii) the correct alignment of Pro(C2), which determines the folding of the BC corner, and of Gly(B6) and Gly(E8), which position the cross-over of the B and E helices, (iii) congruence with the six conserved secondary structural motifs of the globin fold, and (iv) acceptable substitutions at buried and surface sites that are known to have restricted polarity and volume in vertebrate and nonvertebrate globins (7, 8, 42).

In addition, residues characteristic of nematode globins are conserved, notably Tyr(B10), and Gln(E7). These residues project into the distal heme pocket, where oxygen is bound and replace the Leu and His found in the majority of globins. They are usually associated with a high oxygen affinity because of a low rate constant for dissociation of the ligand (43–45). For *Ascaris suum* pseudocoelomic Hb it is shown that the exceptionally high oxygen affinity is due to an H bonding network that includes residues Tyr(B10), Gln(E7), and bound oxygen (46). The same residues in the *Mermis* globins may account for

FIG. 3. **Alignment of *M. nigrescens* eye globin (*Mn*-GLB-E) and body globin (*Mn*-GLB-B) with sperm whale myoglobin (*Physeter catadon*, *Pc*-GLB-M), domain 1 of *A. suum* pseudocoelomic hemoglobin (*As*-GLB-D1), and *C. elegans* hemoglobin (*Ce*-GLB-1).** Residue positions in the myoglobin fold (*Mb fold*) are identified (8, 42). The 32 buried residues are in *lowercase letters*, and the 32 surface residues in *capital letters* are *underlined*. The residues of the *Ascaris* sequence in α-helices are *underlined*. The 33 N-terminal residues identified by protein sequencing of eye globin extracts are *boxed*. *Bold letters* identify residues that differ between eye and body globin.

```
                  1   5   10   15      1   5   10   15 1   5 1       1   5 1   5   10   15   20
                  .   .    .    .      .   .    .    .  .   .       .   . .   .    .    .    .
                                                                 cCCCCCCC.
Mb fold           AAAAAAAaAAaaAAaA     BBBBBBBBbbBBbbBBCcCcCCCCdDDDDDDDDDDDDDDDEEEeEEEeEEeeEeeEEeeE

Pc-GLB-M    -VLSEGEWQLVLHVWAKVEA----DVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHGVTVLTALGAILKK
As-GLB-D1   ----NKTRELCMKSLEHAKVDTSNEARQDGIDLYKHMFENYPPLRKYFKSREEY-TAEDVQNDPFFAKQGQKILLACHVLCAT
Ce-GLB-1    SMNRQEISDLCVKSLEGRMVGTEAQNIENGNAFYRYFFTNFPPDLRVYFKGAEKY-TADDVKKSERFDKQGQRILLACHLLANV
Mn-GLB-E    ----VVNLDILRAQLAKL-P----INEFNGPKFYVHMFSSQEDWRNYFKGSEAI-KPEEVPTCPRFLRQGQRVLLSMELMIEL
Mn-GLB-B    ----VVNLDIIRAQLAKL-P----INEINGPKFYVHMFSTQPDLRNFFKGSENI-KPEEVPASARFLRQGQRLLLSMELMIEL


                        1   5   10 1   1   5   10   15          1   5   10   15   20   25
                        .   .    . FFFf.   .    .    .          .   .    .    .    .    .
Mb fold                 FFFffFFFFF GGGgGGGGgGGgGGgGGgGGG       HHHHHHHhHHhhHHhHHHHhHHHHHHHHH

Pc-GLB-M    KGH---HEAELKPLAQSHAT-KHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGYQG
As-GLB-D1   YDDRETFNAYTRELLDRHARDHVHMPPEVWTDFWKLFEEYLGKKTT-L-DEPTKQAWHEIGREFAKEINKHGR
Ce-GLB-1    YTNEEVFKGYVRETINRHRI--YKMDPALWMAFFTVFTGYLESVGC-L-NDQQKAAWMALGKEFNAESQTHLKNSNLPHV
Mn-GLB-E    ADKPQLFDAYVRDLLDKHKQ-IKGIDYDLYSAFFDVWYGYLSKIIG-M-SDKEKKEWEAFRVELFLPAVKKYIAGW
Mn-GLB-B    ADKLQLFEPYVREMLDKHKR-FKGIDYDLYNAFFDIWYGYLSKVIG-L-SDQEKKEWEAFKVELFLPAVKKYIGGW
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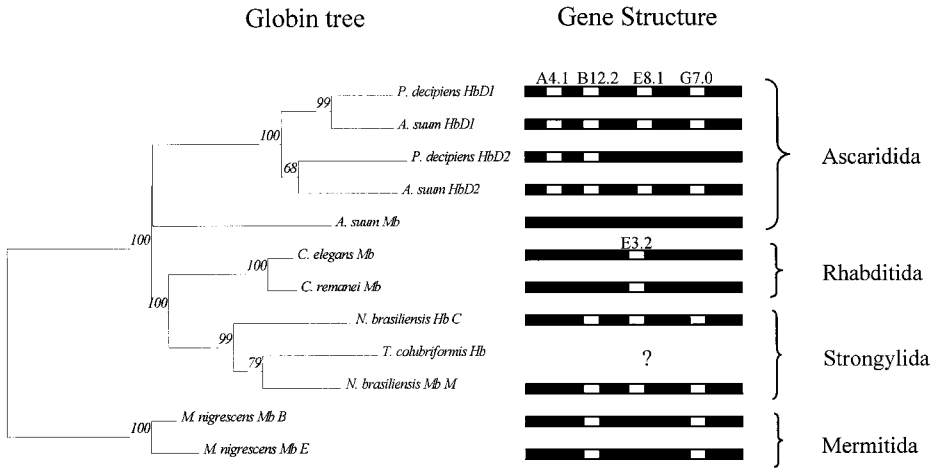

FIG. 4. **Evolutionary tree of nematode globins, with their gene structures.** The tree was inferred from distance analysis using neighbor-joining method. The *numbers* to the *right* of the nodes indicate bootstrap values. *Black boxed*, exons; *white boxes*, introns. Intron locations are labeled at the *top*. *Mb*, intracellular globin; *Hb*, pseudocoelomic isoform; *Hb C*, cuticular isoform.

the relatively high oxygen affinity observed for eye Hb (33). The presence of a ligand stabilizes Hb against oxidation of the heme and denaturation and loss of absorbance in the visible region. The high oxygen affinity of eye Hb may ensure the retention of the high absorbance necessary for its shadowing function.

Mn-GLB-E and *Mn*-GLB-B are 84% identical, indicating their origin in a relatively recent duplication event. A Cys in eye globin at the exposed position E1 could be available for an intermolecular disulphide bond. The other substitutions between the two sequences have no obvious structural or functional consequences.

Sequence comparisons suggest that the *Mermis* globins are distantly related to the other nematode globins sequenced to date (Fig. 4 and Refs. 43, 47, and 48).[2] This is consistent with a phylogeny based on small subunit ribosomal RNA sequence comparisons (50).

With exception of the human HbC variant (β6 Glu $\rightarrow$ Val), *Mermis* eye Hb is unique in forming true crystals in nature (51, 52). These are structurally different from the paracrystalline arrays of aggregated fibers formed by deoxygenated sickle cell Hb (53, 54). Our evidence indicates that the crystals consist predominantly or entirely of eye globin. Indeed, the amino acid sequence encoded by *Mn-glb-e* exactly matches the 33 N-terminal residues of the protein sequenced from eye extracts; therefore, *Mn-glb-e* is most likely the eye globin gene. *Mn-glb-b* product cannot be present in significant amounts in the eye extracts because the protein sequencing products of residues 7, 19, and 31 had no trace of the different amino acids encoded by *Mn-glb-b*. There was no evidence of residues encoded by other globin genes in the Edman degradation products of any of the residues sequenced, and no other cDNAs were isolated. Thus *Mn*-GLB-E is likely the only globin present at high concentration in the eye. Although the sequence strongly suggests that eye globin forms a globin fold, it is not possible to identify the residues that stabilize the contacts between molecules in the crystal without information from x-ray crystallography.

The genes encoding each globin were sequenced. Comparison of the genomic and cDNA sequences shows the interruption of both genes by two introns at the conserved positions B12.2 and G7.0 (Ref. 55 and Fig. 4). However, neither gene has a "central" intron, an intron found at various locations in the E helix of many nematodes, plants, and other nonvertebrates, along with introns at the invariant sites B12.2 and G7.0 (12, 47, 48, 56–62). Vertebrates and the trematode *Paramphistomum epiclitum*[3] also contain only the B12.2 and G7.0 introns. One parsimonious explanation of these observations is that the ancestral globin gene had only the two introns at B12.2 and G7.0, and the central introns at various sites arose later by insertion events (48, 61, 64),[2] which is in keeping with their being inserted at different locations (*e.g.* E8.1 and E3.2 in nematodes). In this model both B and G introns were lost from the Mb gene in an ancestor to the rhabditids (*Caenorhabditis elegans* and *C. remanei*). Intron losses likely occurred independently during the evolution of the *A. suum* Mb gene as the phylogenetically younger strongylid *(Nippostrongylus brasiliensis* Mb) retains a three-intron pattern. There is little doubt that both E and G introns were secondarily lost from the second domain of the gene encoding *Pseudoterranova decipiens* Hb (Fig. 4). It should be stressed that this two-intron hypothesis differs from an earlier proposal that a three-intron globin gene was ancestral (3, 8, 57, 65–67).

Neither globin message contains a secretory leader sequence, evidence that both expressed proteins are intracellular. This is in keeping with light and electron microscopic observations of hemoglobin color or crystals only in the cytoplasm of eye hypodermal cells (Fig. 1 and Refs. 30 and 31). Both messages are expressed in tissues outside the ocellus along the entire length of egg-laying adult females, in the ocellus-lacking L4 larvae

TABLE I
Globin expression in different life stages and body regions of female Mermis

Life stage[a]	Age[b]	Relative expression levels[c]	
		Mn-glb-b	*Mn-glb-e*
L4 larva	1	+	++
Adult molt[d]	9	+++	−
Immature adult			
	13	−	−
	23	++	−
	35	++	+++
	77	+	+++
Egg-laying adult	>365		
Anterior third		++	+++
Middle third		+	++
Posterior third		+	++

[a] Entire worms were extracted except where noted.
[b] Age is measured as the number of days post-emergence from host.
[c] The intensity of globin PCR product compared to the *Mn-rpl-6* product in the same reaction. −, No globin product when *Mn-rpl-6* product present; +, Much fainter than the *Mn-rpl-6* product; ++, As bright as the *Mn-rpl-6* product; +++, Much brighter than the *Mn-rpl-6* product. Intensities are comparable within each column but may not be strictly comparable between columns because primers and products were different. Because of the saturating kinetics characteristic of PCR reactions, +++ designations should not be interpreted as indicating equal template concentrations.
[d] Molt from L4 to adult, 4–11 days post-emergence.

[2] P. Hunt, A. H. J. Burr, and M. L. Blaxter, unpublished observations.
[3] S. Dewilde, B. Winnepenninckx, Y. Van de Peer, J. Vanfleteren, and L. Moens, unpublished observations.

(Table I) and adult males (data not shown). Several tissues run the length of the body, including muscle, hypodermis, neurons, and the trophosome (food storage body; Fig. 1*A*). Hemoglobin has been located histochemically in a section posterior to the ocellus, in a hypodermal chord cell extending between the cuticle and the trophosome where it could have an oxygen-bearing role (68).

The two *Mermis* genes are expressed at different times during development (Table I). The time of relatively high level of expression of *Mn-glb-b* correlates with the L4 to adult molt. In contrast, expression of *Mn-glb-e* at higher-levels begins 35 days post emergence, 2–3 months prior to the appearance of faint color in the eye. Visible pigment continues to accumulate gradually over at least the next 8 months to the high concentration seen in egg-laying females collected in the field. Higher levels of *Mn-glb-e* message are detected in the anterior third of maturing adult females (Table I). Both *Mermis* globins may be protected by the presence of N-terminal Val, known to be stabilizing against ubiquitin-mediated N-end rule degradation (69, 70). Also, eye globin could be protected from degradation by its crystallization.

Why was Hb recruited for a shadowing role rather than the melanin found in other nematode ocelli? Evolution often makes use of whatever is available. It is likely that a cylindrical pigmentation was needed to provide positive rather than negative phototaxis, and this could be provided by the Hb normally expressed in the cylindrically arrayed hypodermal cells. Recruitment appears to have occurred simply by changing gene regulation so that Hb accumulates in anterior hypodermal cells to amounts high enough to expand the hypodermal chords and cast a shadow.

There is growing interest in proteins that have evolved to multiple functions (71). In the multiple evolution of eyes, recruitment of proteins for their physical properties has occurred repeatedly, and the physical phenotypes have required high concentrations. Crystallins at high concentration provide the refractive index and transparency of vertebrate lenses, whereas at low concentration in other tissues their original function as stress proteins or metabolic enzymes is preserved (72, 73). Recruitment of lens crystallins has occurred by changes in gene regulation, involving Pax-6, to provide eye-specific high level expression (74, 75). In another example, reduced cytochrome *c* is found at high concentration in the inner segment of cone photoreceptors in certain fishes. It probably acts, because of its high absorbance, as a short wavelength blocking filter to modify the spectral sensitivity of the photoreceptor (49, 63). *M. nigrescens* provides us with the first example of a Hb recruited for a physical property and ability to achieve a high concentration in eye cells.

Several interesting questions remain to be answered. How is the high level expression of eye globin regulated, and is a *Mermis* homologue of Pax-6 involved? In what other tissues is it expressed and is an oxygen bearing function retained? What sequence modification has occurred to stabilize its naturally crystalline state?

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