Identification of abundant mRNAs from the third stage larvae of the parasitic nematode, *Ostertagia ostertagi*

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Reverse transcriptase-PCR (RT-PCR) was carried out on total RNA prepared from the third-stage larvae (L3) of *Ostertagia ostertagi* in order to clone and characterize the major transcripts expressed in this larval stage, as an initial investigation of arrested larval development in the parasite. Distinct bands were visible on an agarose gel and four of these were cloned and sequenced. Three of the bands represented multiple transcripts, while the fourth band encoded the enzyme GTP cyclohydrolase I (GTP-CH), which catalyses the first and rate-limiting step in

pteridine biosynthesis. Northern blot analysis and RT-PCR demonstrated that GTP-CH is highly up-regulated in the L3 stage and undetectable in either the L2 or adult stages. Using immunogold electron microscopy, GTP-CH was shown to be predominantly localized to the cell body of the body wall muscles and the cells of the intestine in the L3.

Key words: arrested larval development, GTP cyclohydrolase.

INTRODUCTION

Many nematode parasites undergo periods of developmental arrest during their life cycles and the arrested stages are often highly significant in the epidemiology and transmission of the infection [1]. The best characterized example of an arrested stage in the phylum Nematoda is the dauer larvae of the free-living nematode, *Caenorhabditis elegans* (reviewed in [2]). Hotez et al. [3] proposed that the infective free-living third-stage larvae (L3) of some parasitic nematodes are analogous to the dauer stage of *C. elegans* in that they are both developmentally blocked and are a dispersal stage. In some parasitic nematodes, an additional period of arrest follows infection of the definitive host. This period of arrest within the host is thought to have evolved as a mechanism to ensure survival by the avoidance of adverse environmental or physiological conditions, and is often referred to as arrested larval development (ALD).

One of the best described examples of this phenomenon in parasitic nematodes is the arrested L4 of the cattle nematode, *Ostertagia ostertagi* (reviewed in [4]), the most important cause of parasitic gastritis in cattle worldwide. Under normal conditions, *O. ostertagi* has a simple direct life cycle, with cattle becoming infected by ingestion of the L3 from pasture. However, if the L3 are exposed to extremes of environmental conditions (e.g. cold in the Northern hemisphere or hot dry conditions in the Southern hemisphere), an alternative developmental pathway is followed, in which the parasite, after entry into the bovine host, arrests as an L4 [5]. This alternative development may also be induced in immune animals in response to unknown signals [6]. Although ALD in response to chilling was described many years ago, nothing is known of the molecular basis underlying this phenomenon. As part of an initial characterization of the L3 of

O. ostertagi, we have analysed abundantly expressed genes in the normal L3, itself an arrested stage, using reverse transcriptase-PCR (RT-PCR) with primers for oligo(dT) and the nematode spliced leader sequence, SL1 [7]. In the present paper, we describe the molecular characterization of abundant transcripts from the L3 of *O. ostertagi*. One of the cDNAs cloned encodes the enzyme GTP cyclohydrolase I (GTP-CH; EC 3.5.4.16), and the expression pattern and localization of this molecule in the parasite have been studied.

MATERIALS AND METHODS

RT-PCR

RNA was prepared from 10000 L3 ex-sheathed larvae using Trizol (Life Technologies) in accordance with the manufacturer's instructions, except that β -mercaptoethanol was added to a final concentration of 5%. The L3 of *O. ostertagi* are enclosed within a protective sheath, which comprises the retained cuticle of the L2. In case the sheath might impede the isolation of RNA, it was removed [8] prior to RNA extraction. Genomic DNA was removed from the RNA sample by treatment with DNaseI (Life Technologies). A 2 μ g portion of total RNA was reverse transcribed with 200 ng of oligo(dT) primer (GCCGCTCGAGT₁₇) using SuperScript II (Life Technologies) in accordance with the manufacturer's instructions. The first strand cDNA was stored in deionized water at -20°C . The majority of nematode mRNAs have a spliced leader sequence added to their 5' end post-transcriptionally; this is a 22 nt exon derived from a single gene located elsewhere in the genome. Although the percentage of mRNAs containing the SL1 sequence in *O. ostertagi* is unknown,

Abbreviations used: ALD, arrested larval development; GTP-CH, GTP cyclohydrolase I; HSP90, heat-shock protein 90; L3 etc., third-stage larva etc.; RT-PCR, reverse transcriptase-PCR; SL, spliced leader.

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it has been estimated that approx. 70–90 % of *Ascaris* mRNAs contain this sequence (reviewed in [7]). Amplification by PCR was performed on 2 µl of a 1:10 dilution of first-strand cDNA using oligo(dT) primer (see above) and the nematode SL1 primer (gccggaattcGGTTTAATTACCCAAGTTTGAG) at 95 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min, with a final extension of 72 °C for 10 min. PCR products were visualized on a 1.5 % agarose gel by staining with ethidium bromide. To isolate the distinct bands the PCR sample was separated on a 1 % NuSieve® GTG® gel and bands excised. Band fragments were melted at 65 °C and ligated into either pCRII (Invitrogen, Groningen, The Netherlands) or pTAG (R&D Systems, Oxford, U.K.) cloning vectors, and sequenced on both strands using a Licor automated sequencer. The plasmid containing GTP-CH was named pJMO10.

Reverse Northern-blot analysis

In order to ascertain the stage specificity of the various genes cloned, a reverse Northern-blot analysis was carried out. PCR was performed on each clone using vector primers M13 forward (–40) and M13 reverse, and the fragments separated on a 1.5 % agarose gel and Southern blotted using standard techniques [9]. The PCR products were then probed with cDNA derived from different life cycle stages of *O. ostertagi*. L2, L3 and adult first-strand cDNA were subjected to PCR using the SL1 primer and oligo(dT) primers as described above. The PCR products were separated on a 1 % agarose gel and purified using Spin-X columns in accordance with the manufacturer's instructions (Costar, High Wycombe, Bucks., U.K.). A 50 µg portion of each DNA sample was radiolabelled to approximately equal specific activity using the High Prime kit (Boehringer Mannheim). All subsequent pre-hybridization and hybridizations were carried out by standard procedures.

Northern-blot analysis

Northern-blot analysis was performed by separating 5 µg of total RNA from L3 and adult worms according to standard procedures [9].

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out in order to assess when the mRNA corresponding to GTP-CH was expressed. Expression levels of the mRNA were compared with that of the *O. ostertagi* actin gene (Accession number AF052043; cloned using primers based on the *C. elegans* sequence [10]). The plasmid containing the actin gene fragment from *O. ostertagi* was named pJMO1. RNA was prepared and reverse transcribed as described above with oligo(dT). The resultant first strand cDNAs were stored at –20 °C until required. Amplification by PCR was performed on 1 µl of a 1:10 dilution of first strand cDNA. The number of PCR cycles was chosen empirically in preliminary reactions with each set of primers to define conditions where reagents were not limiting. The PCR was carried out as follows: 95 °C for 5 min followed by 25 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min with a final extension of 72 °C for 10 min, using GTP-CH specific primers, GCHF (CGCggtaccGGCAAGTGAAAGCGGTTTCC) and GCHR (CGCggtaccTCACTTGTGAATGAGATTCAGGA; lower case denotes *Bam*HI site; primer positions indicated in Figure 2) and actin-specific primers Act1 (GGAGATGGTGTCACCCACACCGT) and Act2 (TCCATACCCAAGAAGGATGGAT). The PCR products were separated on a 1.2 % agarose gel and transferred

to Hybond-N (Amersham International) by standard blotting procedures [9]. Blots were then hybridized using random primed inserts derived from GTP-CH (pJMO10) and actin (pJMO1).

Expression of GTP-CH

DNA corresponding to the complete coding region of GTP-CH was amplified from pJMO10 using primers GCHF and GCHR (see above). Amplification was carried out for 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min with a final extension of 72 °C for 10 min. The DNA fragment was digested with *Bam*HI, separated on a 1 % NuSieve® GTG® gel and ligated into *Bam*HI-digested pET15b expression vector (Novagen, Madison, WI, U.S.A.) and initially propagated in NovaBlue cells. Plasmids containing insert DNA in the correct orientation were transferred to the expression host BL21(DE3). Initial small-scale expression experiments established that the recombinant protein was insoluble, thus purification was carried out according to the manufacturer's instructions in the presence of 6 M urea. In brief, inclusion bodies were solubilized in 6 M urea and the recombinant protein purified over a His-Bind column as described in the protocol, except that all buffers (binding, wash and elution) contained 6 M urea. The recombinant protein was eluted with 20 mM imidazole buffer containing urea and dialysed against PBS, but the protein was precipitated once the urea was removed. The purity of the recombinant protein was established by SDS/PAGE.

Raising of antibodies and Western-blot analysis

Approx. 100 µg of precipitated recombinant protein was emulsified in Freund's incomplete adjuvant and injected intramuscularly into two rabbits which were boosted using the same protocol 4, 8 and 12 weeks later. Blood was collected 12 days after each boost. For some of the immunolocalization experiments, the antiserum was affinity-purified as described previously [11]. In this method, the recombinant protein was separated by SDS/PAGE, blotted on to nitrocellulose paper and used to affinity-purify an IgG fraction of the antiserum. For immunoblotting, *O. ostertagi* L3 and adult soluble extracts were prepared by boiling worms in either sample cocktail buffer or sample cocktail containing 6 M urea for 5 min followed by centrifugation at 13000 g to remove insoluble material. Soluble proteins were separated on a SDS/12.5 % polyacrylamide gel, and blotted on to nitrocellulose paper using Tris/glycine/methanol buffer. GTP-CH recombinant protein, suspended in 6 M urea, was likewise run on a gel and blotted on to nitrocellulose paper. The blots were probed with a 1:100 dilution of the antiserum raised to GTP-CH recombinant protein or the pre-bleed from the same rabbit. As a positive control, parasite extracts were also probed with a 1:100 dilution of an antiserum raised to a recombinant heat-shock protein 90 (HSP90) from the filarial nematode, *Brugia pahangi*. All wash and incubation steps were carried out using standard procedures and bound antibody was visualized using an alkaline phosphatase-conjugated secondary antibody and 5-bromo-4-chloro-3-indoyl phosphate-Nitro Blue Tetrazolium as substrate.

Immunolocalization of GTP-CH

Live parasites were impact-frozen using a Leica MM80 cryofixation device, and transferred under liquid nitrogen to a Dewar-based cryosubstitution system. Specimens were then slowly dehydrated at –80 °C in acetone for 6 days prior to controlled warming (5 °C/h) to 10 °C when the cryosubstitution fluid was

replaced with fresh acetone at 20 °C. Specimens in gelatin capsules were gradually infiltrated with increasing concentrations of Unicryl acrylic resin (Unicryl, Cardiff, U.K.) in acetone at 20 °C for 3 days, rotating to provide agitation, until finally after two changes of pure resin (8 h each), they were UV (360 nm) polymerized for 2 days.

Ultrathin sections were collected on to 300 mesh nickel grids and immunostaining was performed essentially as described by Griffiths [12]. Blocking and conditioning steps using 0.2 M glycine and 1 % acetylated BSA (Aurion, Wageningen, The Netherlands) in PBS for 30 min each, were followed by incubation for 30 min in undiluted affinity-purified antibody [11]. Sections were incubated for 30 min in 1:20 goat anti-rabbit IgG/10 nm gold conjugate (Aurion) in PBS/1 % acetylated BSA. Sections were briefly counterstained in aqueous 0.5 % uranyl acetate and images examined and recorded using a Zeiss 902 Energy-Filtering Electron Microscope.

RESULTS

Identification of abundant larval transcripts

RT-PCR was carried out on RNA isolated from L3 of *O. ostertagi* as described in the Materials and methods section. After separation of the products on an agarose gel, distinct bands were visible within the size range 350 bp to approx. 1 kb (Figure 1, lane 2). Comparative RT-PCR carried out on sheathed and exsheathed larvae showed no difference in the profile of PCR products analysed on agarose gels (results not shown), suggesting that the presence of the sheath did not interfere with the extraction of RNA. Four of these bands were excised and cloned. From each ligation multiple clones were sequenced, as in most cases each band represented a composite of cDNAs (Table 1).

Within band 1, 16 separate clones were sequenced of which 12 were identical. This predominant population did not show any similarity to sequences within the databases. However, two of the four distinct cDNAs encoded ribosomal proteins. Twenty-four separate cDNAs from band 2 were sequenced; these encompassed three major populations of cDNAs containing nine, five and four members respectively. Apart from one population (of four cDNAs) encoding ribosomal protein L37a, the other two showed no similarity to sequences within the databases. There were also an additional six distinct cDNAs within band 2, all of unknown function, one of which showed similarity to a predicted protein of *C. elegans*. Within band 3, four distinct clones were sequenced which were different to one another. One represented a ribosomal protein gene while another showed a high degree of similarity to a *C. elegans* protein of unknown function.

Band 4 (pJMO10) was unlike the other bands in that it was represented by a single species of cDNA; all six cDNAs sequenced were identical, and database searches demonstrated that these encoded the enzyme GTP-CH.

A reverse Northern blot was carried out to ascertain the stage specificity of the clones isolated (Table 1). Within band 1, the predominant cDNA (pJMO5) was slightly upregulated in L2 compared with the L3 and adult stages, while the ribosomal proteins were expressed at higher levels in the larval stages compared with the adult stage. pJMO38 appeared to be an L3-specific cDNA. Of the nine separate cDNAs isolated from band 2, one was L3-specific (pJMO13) while five were elevated in the L3 (pJMO3, pJMO4, pJMO7, pJMO16 and pJMO23). pJMO21 mRNA was not detected in the L2 and the remaining two cDNAs appeared to have a constitutive pattern of expression. Of the four cDNAs isolated from band 3, three appeared to be larval-specific and the fourth was constitutively expressed. GTP-

Figure 1 Abundant SL1/oligo(dT) PCR products from the L3 stage

First-strand cDNA prepared from the L3 of *O. ostertagi* was subjected to PCR using SL1 and oligo(dT) primers, separated on a 1 % agarose gel and stained with ethidium bromide. Lane 1, molecular mass markers; lane 2, L3 cDNA; lane 3, no DNA (control). Numbers to the right of the gel denote bands excised from the gel (see Table 1 and text).

CH isolated from band 4 was expressed at high levels in the L3 stage and only barely detectable in the adult mRNA.

Sequence of *Ostertagia* GTP-CH

The cDNA encoding GTP-CH is 961 nt in length (Figure 2), the initiator methionine lying 9 nt downstream of the 3' end of the nematode transpliced SL1 sequence. An analysis of over 80 genes in *C. elegans* demonstrated a preponderance of a small number of nucleotides between these regions; approx. 63 % of genes have between 0 and 10 nt [13]. In GTP-CH all four nucleotides preceding the ATG start codon are adenines (Figure 2), which is distinct from the vertebrate consensus sequence, but similar to the consensus proposed for *C. elegans* [13] and yeast [14]. Translation of the cDNA sequence revealed an open reading frame of 642 bp (including stop codon), followed by a 3' untranslated region of 288 nt. A polyadenylation consensus sequence [15] is found between positions 925 and 930 (underlined in Figure 2), consistent with oligo(dT) priming having occurred at the polyadenylated tail of the mRNA. Sequence analysis demonstrated that there are various kinase consensus sequences in the *O. ostertagi* GTP-CH; between residues 56–60 there is a growth-associated histone H1 kinase consensus sequence, between residues 102–105 and 203–206 there is a casein kinase II consensus sequence, and between residues 139–141 there is a protein kinase C consensus sequence (all of which are underlined in Figure 2). The open reading frame encodes a polypeptide of 213 amino acids with a predicted molecular mass of 24 kDa and pI of 6.43.

A multiple alignment was carried out between the *O. ostertagi* GTP-CH protein sequence and the corresponding protein sequences from *C. elegans*, human, *Drosophila melanogaster*, *Dictyostelium discoideum*, *Saccharomyces cerevisiae* and

Table 1 Summary of sequence analysis of abundant L3 transcripts

Individual clones from four bands in Figure 1 were sequenced and compared with entries GenEMBL and SwissProt. A reverse Northern blot was carried out to ascertain the expression pattern of each gene. Briefly, PCR products prepared from L2, L3 or adult first strand cDNA using the SL1 and oligo(dT) primers were labelled by random priming and used to probe PCR fragments corresponding to each clone. Blots were exposed to autoradiography and the resulting band intensity scored as follows: + + + +, very intense; + + +, intense; + +, moderately intense; +, weak; —, no signal.

Band	Clone	Fraction of clones	Homology	Accession number	Relative expression pattern		
					L2	L3	Adult
1	pJM05	12/16	None	AF052593	+	+	+
1	pJM09	1/16	rpL38	AF052044	+	+	+
1	pJM034	1/16	rpS34	AF052590	+	+	+
1	pJM038	1/16	None	AF052591	—	+	—
1	pJM040	1/16	None	AF052592	+	—	—
2	pJM03	9/24	None	AF052738	+	+	+
2	pJM013	5/24	None	AF052045	—	+	—
2	pJM06	4/24	rpL37a	AF052737	+	+	+
2	pJM02	1/24	None	AF052739	+	+	+
2	pJM04	1/24	None	AF052049	+	+	+
2	pJM07	1/24	None	AF052735	+	+	+
2	pJM016	1/24	F48C11.3	AF052589	+	+	+
2	pJM021	1/24	None	AF052046	—	+	+
2	pJM023	1/24	None	AF052047	—	+	+
3	pJM044	1/4	None	AF052594	+	+	—
3	pJM047	1/4	rpL13a	AF052595	+	+	—
3	pJM048	1/4	K07E1.1	AF052736	+	+	—
3	pJM051	1/4	None	AF053016	+	+	+
4	pJM010	6/6	GTP-CH	AF052048	—	+	+/—

Escherichia coli (Figure 3). The amino acid sequence of *O. ostertagi* GTP-CH shows 85% identity to *C. elegans*, 73% to human, 72% to *Drosophila*, 62% to *Dictyostelium*, 69% to *S. cerevisiae* and 40% to *E. coli* sequences, demonstrating the high degree of conservation observed in this enzyme. The amino acid residues that form the GTP-binding pocket have been determined in the case of the *E. coli* GTP-CH [16]; these residues are shown by asterisks in Figure 3 and are conserved in all the species examined. Furthermore, it has been noted that a region in the rat GTP-CH shows significant similarity to the pterine ring-binding region of dihydrofolate reductases from various species [17]. The corresponding region in *O. ostertagi* GTP-CH is marked (hatched box) in Figure 3. Over this region the *O. ostertagi* GTP-CH is 35% identical to the pterine ring binding region of dihydrofolate reductase from *S. cerevisiae* [18] and 50% similar when conservative amino acids are taken into account, approximately the same level of identity as observed between rat GTP-CH and yeast dihydrofolate reductase. However, one of the two residues known to bind to the pterin ring in dihydrofolate reductase is substituted from a Trp to Asp in *O. ostertagi* GTP-CH (residue 59), although the other residue involved in binding (Phe⁶⁸) is conserved [17]. The multiple alignment reveals that the N-terminal domain is significantly different between the species examined whereas the remainder of the protein sequence is very similar between species, indicating the N-terminal domain may be involved in species-specific protein-protein interactions.

Expression pattern of GTP-CH

The expression pattern of GTP-CH was analysed by Northern blots and by RT-PCR. Northern blots of L3 and adult RNA showed that the mRNA was present in the L3 stage at relatively high abundance and was undetectable in the adult. The size of

ggtttaattaccgaagtttgaggtataaaaaATGGCAAGTGAAGCGGTTCTCTTCATCT	60
M A S E S G F L S S	10
GACAGCTCCAGCGAGGATTCGATCAGAAGATTATCGCTTTTCTAAAGACCTCAAC	120
D S S S E D C D Q K I I A F S K K T S N	30
TTGGACAAATGACGCCGCCCTATTTCATCAATATATCCCATGTTGGTGAAGACGCAAT	180
L D K M T A A Y S S I I S H V G E D V N	50
CGTCAAGGACTTCTGAAACTCCGACAGGCCGCCAAAGCAATGCTGTACTTACCAAA	240
R Q G L L K T P D E A A K A M L Y F T K	70
GGCTACGAACAACAACCTTGACGATATTTGAACGATGCCGTGTTGACGAGAATCAGAT	300
G Y E Q Q L D D I L N D A V F D E N H D	90
GAAATGGTTATCGTGAGAGATATCGAAATGTTCTCCTATGTGAACATCATCTAGTGCCA	360
E M V I V R D I E M F S L Q E H K L V P	110
TTCAATGGAAGGTTTACATTCGGTACATACCGAACAAAAAGTTCTAGGCTCTCAAAA	420
F N G K V H I G Y I P N K K V L G L S K	130
CTGGCTCGAATGTGTAGATGTTTCAGCCGACGACTTCAAGTACAAGACGACTAACAAG	480
L A R I V E M F S E E L Q V Q E R L T K	150
CAATAGCGACGCAATGGTGCAGCGGTTCAACCTGCCGGTGTGCGAGTGTCTATTGAG	540
Q I A T A M V Q A V Q P A G V A V V I E	170
GCGAGCCACATGTGTATGTTATGCTGGAGTTCAGAAAAATCAATGTACCACTCCACA	600
A S H M C M V M R G V Q K I N A T T S T	190
TCCATGATCTCGGATTTAGGGACGATCCAAAAACGAGAGAATTCCTGAATCTC	660
S C M L G V F R D D P K T E E E F L N L	210
ATTCAAGAGtgactgaagtagcagctatttaccgagtagtatttaccgacacaaatgaca	720
I H K *	213
atgtgtgatgtgtgcctatgatctgagattcacttcgctagatttttacttgatgaata	780
gggtgcattgatcaaaagctcttttctggatgtgcttatctgtatgtgctgtatt	840
gtccctaataagaagtcttcgaatgtgcttgagcagcactgcatgtcacagcttctcttg	900
aggttgtgccgttaataagaatgaaataaattgtgtggagtgaataaaaaaaaaaaaaaaaaa	961

Figure 2 Sequence of GTP-CH gene from *O. ostertagi*

Untranslated regions are in lower case and coding sequence is in upper case. The predicted protein sequence is shown below the DNA sequence and the stop codon is indicated by an asterisk. The SL1 sequence between position 1–22 is in **bold**. The position of a consensus polyadenylation sequence is underlined in the 3' untranslated region. Primers used to amplify the coding region for ligation into the expression vector pET15b, and which were also used in semi-quantitative RT-PCR, are double underlined. Individual underlined residues represent consensus sequences for growth-associated histone H1 kinase (amino acid residues 56–60), for casein kinase II (amino acid residues 102–105 and amino acid residues 203–206), and for protein kinase C (amino acid residues 139–141).

the native mRNA in the L3 sample was approx. 1 kb (Figure 4), in agreement with the size of the pJM010 cDNA (961 bp). The band was very diffuse in more than one Northern blot, indicating either that there is more than one transcript or that there is a high degree of heterogeneity in the polyadenylated tail length. RT-PCR was carried out with gene-specific primers on L2, L3 and adult first-strand cDNA in order to ascertain whether GTP-CH is larval or L3 specific. Actin-specific primers were also used on the same first-strand cDNA samples as a constitutive control. Figure 5 shows the results of one such experiment. It can be seen that there is approximately equal amounts of input mRNA in the L2, L3 and adult samples, as indicated by the actin-specific cDNA band (303 bp) in (B). However, GTP-CH is only expressed in the L3 stage, and at relatively high levels, as indicated by the 580 bp band in lane 2 of (A). The larger size band in lane 2, (A; L3 stage) does not correspond to a genomic fragment, but rather is the product of the GCHF primer (see the Materials and methods section) and residual oligo(dT) remaining from the first-strand cDNA synthesis, as deduced by sequence analysis.

Western blotting and immunolocalization

DNA encoding the complete coding region of GTP-CH was amplified from clone pJM010 by PCR and expressed in *E. coli* using the pET15b expression vector. An antiserum to the

Figure 3 Amino acid alignment of GTP-CH from various organisms

Alignment of GTP-CH from *O. ostertagi* with the homologous enzymes from *C. elegans* (Q19980), human (p30793), *D. melanogaster* (u01118), *D. discoideum* (z49706), *S. cerevisiae* (X94314) and *E. coli* (x63910). Sequences were aligned using the ClustalW program of MacVector. Identical amino acids are boxed and shown in **bold**, similar amino acids are boxed. Gaps were introduced to maximize the alignment. Numbers above the sequence include spacing. The hatched box indicates those residues predicted to bind a pteridine ring structure, while the asterisks denote those amino acids proposed to contribute to the GTP-binding pocket.

recombinant protein was then used to probe Western blots of recombinant protein, L3 and adult worm extracts. Probing recombinant protein with the GTP-CH antiserum demonstrated that it recognized the protein (26.5 kDa) used to raise it (Figure 6, lanes 1 and 2). However using the same antiserum on L3 or adult SDS-sample cocktail extracts did not reveal any bands (Figure 6, lanes 3–6). Since the recombinant protein itself required urea for solubilization, an L3 extract was prepared in sample cocktail buffer containing 6 M urea but, like the sample cocktail extract, no bands were visible on a Western blot after probing with the specific antibody (results not shown). To test the

integrity of the SDS extracts of parasite material, an antiserum raised against *B. pahangi* HSP90 was used to probe the same extracts (Figure 6, lanes 7–10). This antibody detected a specific band in the L3 extracts at approx. 90 kDa, despite the fact that it was raised to a recombinant protein from a different species of nematode.

A possible explanation for these results is that GTP-CH is not extracted from the parasites under the conditions used. For example, some proteins localizing to the cuticle of nematodes are insoluble once cross-linked into the cuticle [19]. The immune rabbit serum was used to investigate the localization of GTP-CH

Figure 4 Northern blot analysis

5 µg of RNA prepared from the L3 (lane 1) and adult (lane 2) stages were separated on 1.5% formaldehyde gel. The gel was blotted and probed with GTP-CH and subjected to autoradiography. Size markers are in nucleotides.

Figure 5 Semi-quantitative RT-PCR

RNA prepared from L2 (lane 1), L3 (lane 2) and adult (lane 3) stages was reverse transcribed with oligo(dT), and PCR was carried out with GTP-CH-specific primers, GCHF and GCHR (A) and actin-specific primers, Act1 and Act2 (B). PCR products were blotted and then hybridized using random-primed inserts derived from GTP-CH (pJMO10) and actin (pJMO1). Blots were subjected to autoradiography at -70 °C.

in the parasite by immuno-electron microscopy. Parasites were prepared for immuno-electron microscopy using a variety of methods, but due to the lack of adequate structural preservation with conventional processing protocols, it was necessary to utilize a prolonged-freeze-substitution and embedding regime, as

Figure 6 Western blot probed with antiserum against GTP-CH

An SDS/12.5% polyacrylamide gel was run with various protein fractions and probed with antiserum to GTP-CH or *B. pahangi* HSP90 or the appropriate pre-bleeds. Lanes 1 and 2, recombinant GTP-CH protein; lanes 3, 4, 7 and 8, L3 extract; lanes 5, 6, 9 and 10 adult extracts. Lanes 1, 3 and 5, probed with pre-bleed from GTP-CH immunized rabbit; lanes 2, 4 and 6 probed with antiserum raised against GTP-CH; lanes 7 and 9 probed with pre-bleed from HSP90 immunized rabbit; lanes 8 and 10 probed with antiserum raised against HSP90 from *B. pahangi*. Size markers are indicated on the right in kDa.

described in the Materials and methods section. Sections of freeze-substituted L3 were then reacted with an affinity-purified antibody (Figure 7), or with the immune serum, or a pre-bleed from the same rabbit. The upper panel of Figure 7 shows a low-power micrograph of a section through an L3 of *O. ostertagi* to demonstrate the basic morphological features of the worm. Like all nematodes, the L3 is bound by an acellular cuticle (Figure 7, labelled c) and the body, in transverse section, is organized into a series of muscle blocks (Figure 7, labelled mcb) divided by the chords, one of which is indicated in Figure 7 (labelled ch). The intestine (Figure 7, labelled int) contains many lipid droplets which presumably act as a food store in this non-feeding stage of the life cycle. The results of the experiments using the affinity-purified antibody demonstrate that GTP-CH is not a cuticular protein but is localized in a number of different sites, perhaps consistent with the multifunctional nature of this enzyme. The most intense and specific labelling was observed in the cell body of the body wall muscles of the L3 (Figure 7, lower panel), and in the cells of the intestine. When sections were reacted with the immune serum at 1:100 dilution, abundant labelling was observed in the cuticle and in the boundary between the L3 cuticle and the sheath (results not shown), with more sparse labelling in the thin layer of hypodermis overlying the muscle blocks (results not shown). However, equivalent labelling was observed with the pre-bleed, and as labelling of these areas was not observed with the undiluted affinity-purified antibody, it is likely to be non-specific.

DISCUSSION

The abundant larval transcripts cloned and characterized in this study were shown to encode a variety of genes of unknown function, four ribosomal proteins and the enzyme GTP-CH. The

Figure 7 Immunolocalization of GTP-CH in the L3 of *O. ostertagi*

L3 of *O. ostertagi* were prepared by freeze-substitution as described in the Materials and methods section. Sections of L3 were incubated with a neat-affinity-purified antibody (both panels), followed by goat anti-rabbit IgG 10 nm gold. The scale bar on the upper panel is 2.5 μ m, while that on the lower panel is 1.0 μ m. Gold particles were observed in the intestinal cells (int); the cell body of the muscle cells (mcb), but not in the chord (ch) or the cuticle (c).

observation that the majority of the cDNAs isolated have no homologies within the databases, and in particular, do not show similarity to genes from *C. elegans* (the genome sequence of which is largely complete), suggests that these clones represent parasite-specific genes. Interestingly, approx. 50% of genes identified by the *C. elegans* sequencing consortium have no similarity to other database sequences, and within the *Brugia* expressed sequence tag project approx. 55% of clones have no similarity to sequences of known function within other databases [20]. The genes isolated in the present study are likely to be specific to parasitic nematodes, and are perhaps even Trichostrongyle-specific, suggesting that they may have potential as vaccine candidates and/or drug targets. Ribosomal protein genes were highly represented within this set of clones, presumably due

to their relatively high abundance and also their small size. As part of a larger study of expressed sequence tags, Blaxter et al. [20] noted that ribosomal protein genes constituted 24% of a *B. malayi* SL1 PCR library compared with 10% in a conventionally produced library, the higher abundance in the former presumably representing preferential PCR due to the small size of their corresponding mRNAs. Even though the present study did not aim to clone L3-specific transcripts, of the 19 separate cDNAs analysed, reverse Northern-blot analysis suggests that eight are either up-regulated or exclusively expressed in the L3 stage when compared to the L2 or adult stages.

Band 4 from the initial RT-PCR reactions represented a single species of cDNA, GTP-CH. Northern blot and RT-PCR analysis revealed that the mRNA was highly abundant in the L3 and not transcribed in the L2 or the adult parasite. GTP-CH catalyses the first and rate-limiting step in the pteridine biosynthetic pathway [21]. In higher eukaryotes, mutations in the gene cause a variety of disorders [22–24]. A derivative of one of the products of the pathway, biopterin, has multiple physiological functions. It serves as a cofactor for the three aromatic amino acid hydroxylases: phenylalanine, tyrosine and tryptophan hydroxylases, that have among their functions the synthesis of melanin and neurotransmitters, such as catecholamines, serotonin and dopamine [25,26]. Besides these cofactor roles, biopterin is thought to be involved in the proliferation and growth of erythroid cells by affecting various aspects of signal transduction [27]. Pteridines are also required for the biosynthesis of riboflavin and folic acid [28]. In higher eukaryotes, the expression of GTP-CH is controlled at the level of transcription [29] and by enzyme activity levels [30]. For example, GTP-CH activity is inhibited by pterins such as tetrahydrobiopterin and dihydrofolate. The presence of a conserved pterin-binding region between amino acid residues 48–68 in *O. ostertagi* GTP-CH may indicate that it is also regulated in a similar fashion. More recently, the inhibition of GTP-CH by tetrahydrobiopterin has been shown to be mediated by a GTP-CH feedback regulatory protein [31].

In *Drosophila*, GTP-CH is the product of the *Punch* (*Pu*) locus. Mutant phenotypes of *Pu* locus are pleotropic. The pteridine pathway produces one of the two classes of pigments in the eyes of adults, where they serve a screening function [32,33]. Furthermore, along with other developmental defects, abnormalities of the cuticle have also been observed in *Drosophila* with mutations in *Pu* [34].

Attempts to identify GTP-CH in the L3 or adult stages by Western-blot analysis using an antibody raised to the recombinant GTP-CH were not successful. There are a number of possible explanations for this observation, and it may reflect a problem with sensitivity. Although the mRNA is abundant, this may not be reflected in protein abundance. Alternatively, it may reflect difficulties in solubilizing the native protein in SDS and/or urea. The recombinant protein itself was poorly soluble in SDS-sample cocktail, requiring 6 M urea for optimal solubility. These characteristics of the protein were reminiscent of the cuticlins, a family of proteins which comprise the major insoluble residue of the nematode cuticle [19]. This, together with the cuticular abnormalities observed in *Pu* mutants in *Drosophila*, and the resistance of the L3 to environmental conditions, suggested that the GTP-CH may have a cuticular localization in *O. ostertagi*. We speculated that the product of the pteridine biosynthetic pathway (tetrahydrobiopterin) may be required as a cofactor for critical enzymes involved in cuticle formation/cross-linking. The L3 of *O. ostertagi* are extremely resistant, as demonstrated by their ability to survive for prolonged periods in the environment, their resistance to chemical treatment, such as SDS (results not shown), and the problems encountered in infiltrating the larvae

for immuno-electron microscopy (this study). Their ability to withstand such environmental stresses is presumably imparted by both the cuticle and the sheath, which is the cast cuticle of the L2, and is retained by the L3. However, immunolocalization of the native protein in the L3 revealed most staining in the cytoplasm of the body wall muscle cells and the cells of the intestine. Whether its actual role/s in these locations is related to the arrested state of the L3 or reflects some hitherto unexplored aspect of the gut/muscle physiology of the worm remains largely conjectural at present. In some sections incubated with the whole serum, a sparse staining was also observed in the hypodermis (the tissue which secretes the cuticle), but the specificity of this labelling was questionable. Likewise, staining was also observed in the cuticle of the parasite, but was unlikely to be specific as, at higher concentrations, the pre-bleed also stained the same area (results not shown). Moreover, the affinity-purified antiserum did not label the cuticle. The localization of GTP-CH to a variety of sites in the L3 presumably reflects the multiplicity of possible functions of GTP-CH. As the first step in the pteridine biosynthetic pathway, GTP-CH has the potential to influence a variety of different biochemical pathways in nematodes. A similar situation exists in *Drosophila*, where the *Pu* mutant displays a complex array of phenotypes. It is not possible to isolate mutants in *Ostertagia*, but a homologous gene exists in *C. elegans* and studies on this genetically tractable organism, and particularly on the dauer stage, may help shed light on the function of GTP-CH in the parasitic worm.

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