Analysis of genes expressed at the infective larval stage validates utility of Litomosoides sigmodontis as a murine model for filarial vaccine development

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The rodent filarial parasite *Litomosoides sigmodontis* has recently been proposed as an important model of filariasis because it is the only filarial species in which the full developmental cycle can take place in inbred laboratory mice (1, 19). The power of murine genetics and immunology can now be brought to bear on fundamental questions in filarial biology that have previously been intractable. Perhaps the most immediate application of this model is the rigorous testing of vaccine strategies. The main objective of a filarial vaccine would be to target the incoming vector-derived larvae without inducing an immune response to later stages that might damage the host. We and others have been studying genes that are specific to the third larval (L3) stage of human filarial parasites (12, 13, 27). This work has identified several possible vaccine targets, with the abundant larval transcript 1 (*alt-1*) gene emerging as a particularly promising candidate (11).

The aim of this study was to sample genes expressed by the L3 stage of *L. sigmodontis* for comparison with gene expression in onchocerciasis and lymphatic filariasis parasites and thus to assess the utility of this rodent filaria as a model for vaccine studies. With this information, it will be possible to assess the validity of using the *Litomosoides* model to evaluate specific vaccine candidates. Further, the data will provide an important resource for comparative analysis of gene expression between species both within and outside the filariae.

For this study, we used an *L. sigmodontis* L3 cDNA library (in UniZap XR; Stratagene, La Jolla, Calif.). Randomly chosen recombinants were picked and cDNA inserts (with an average size of ~640 bp) were PCR amplified using vector primers. Two hundred thirty PCR products were prepared using shrimp alkaline phosphatase and exonuclease I (Amersham Pharmacia Biotech Ltd., Uppsala, Sweden) (6) and sequenced using the 5' vector primer SAC (GGGAACAAAAG CTGGAG) and ABI Big DYE terminators (the Perkin-Elmer Corporation, Norwalk, Conn.). Sequencing reactions were analyzed using an ABI 377 automated sequencer. There were 197 successful sequences with an average read length of 421 bp. The clones are archived and are freely available to the research community.

Sequences were edited to remove vector and poor 3' sequences and then compared to the public databases using the BLAST family of algorithms (2). Expressed sequence tags (ESTs) with no significant similarity to any protein sequences in the databases using a minimum BLASTX score of 80, with a probability of $<1 \times 10^{-9}$, were designated as novel. Sequences were clustered using AssemblyLIGN (Oxford Molecular, Oxford, United Kingdom), and where clusters contained more than one EST, an overlapping nucleotide sequence was used to generate an improved consensus sequence. Sixteen clusters with more than one EST and 136 clusters containing only one EST were defined. ESTs within a single cluster are assumed to be derived from the same gene. Each cluster is designated with an *L. sigmodontis* cluster (LSC) number. Nucleotide sequences were translated into putative peptide sequences and aligned to homologues from other species using ClustalW as implemented in MacVector.

Although small, the EST data set gave us both the information we were looking for and significant new information on the biology of this organism. None of these 152 genes have been previously identified from *L. sigmodontis*. Table 1 gives an overview of the 16 clusters containing more than one EST and their relationship to other filarial genes. A more comprehensive analysis of all the genes sequenced can be found at http://www.ed.ac.uk/~mbx/LitoWeb/LitoESTs.html.

Because extensive EST data sets are available for the human pathogens *Brugia malayi* (3) and *Onchocerca volvulus* (25), it is possible to directly compare the information from our study with gene expression data from these human pathogens. The results of EST analysis of the most abundantly expressed genes from the L3 stage of *B. malayi* and *O. volvulus* are shown in Table 2. Of the twenty genes most abundantly expressed in *B. malayi* or *O. volvulus* L3, eight were identified in the *L. sigmodontis* data set. A more extensive EST analysis will almost certainly identify more shared genes between *L. sigmodontis* and these human pathogens.

Of particular interest was the identification of a gene similar to *alt-1* of *B. malayi* as the most abundantly expressed gene in the *L. sigmodontis* dataset (EST cluster LSC00018) (Table 1).
alt-1 was originally identified as one of the most abundant spliced leader-trans-spliced mRNAs expressed by B. malayi L3 (12) and has subsequently been shown to be specific to the infective L3 stage (11). Vaccine studies with gerbils have demonstrated the highest level of protection elicited by a recombinant filarial antigen to date (11). A related gene, alt-2, has also been identified in B. malayi (12). Additional alt-like genes have been sequenced from Dipetalonema immitis (20/22 antigen) (7), Acanthocheilonema viteae (20), Brugia pahangi (GenBank accession no. AJ275489), Wuchereria bancrofti (GenBank accession no. AF084553), and O. volvulus (14). Recognition of the ALT antigen has also been associated with protective immunity in experimental models of D. immitis (7) and O. volvulus infection (14).

The EST data sets from B. malayi and O. volvulus confirm the stage specificity of expression of the alt genes identified and also reveal additional members of the alt gene family with distinct sequence and expression patterns (D. Guiliano, B. Gregory, and M. Blaxter, unpublished data). Among the L. sigmodontis ESTs in this study, only a single alt gene was identified. Comparison to published sequences shows that the L. sigmodontis ALT is most similar to that from the rodent parasite A. viteae (Fig. 1). Phylogenetic analysis shows that L. sigmodontis and A. viteae lie basal to the human infective filaria. This is consistent with analyses published previously (26) as well as our own data from small-subunit rRNA genes (not shown). The biological function of the ALT proteins is not known, but their highly regulated expression, abundance, and presence in excreted-secreted products of mammalian stage parasites have received significant attention in recent years because they may play a key role in detoxifying host oxyradicals deposited on the parasite (5, 10, 15, 17). The L. sigmodontis ESTs include both a thioredoxin peroxidase (LSC00036) and a thioredoxin (LSC00102). Proteases may mediate important processes in larval invasion of the host and establishment of the parasite, while protease inhibitors secreted by filarial L3 may play roles in the life cycle (in particular molting) (18) and in interfering with the host immune response (27). The L. sigmodontis EST data set includes clusters encoding cathepsin L-like (LSC00017 and LSC00209) and aspartyl (LSC00143) proteases and a cystatin-like protease inhibitor (LSC00180). The cystatin is most like B. malayi cystatin-2, an inhibitor synthesized throughout the B. malayi lifecycle (B. Gregory and R. Maizels, personal communication). Several clusters encode apparent structural and housekeeping genes, including those encoding nucleosome assembly protein (nap-1, LSC00014), translationally controlled tumor protein (tph-1, LSC00025), putative RNA binding protein (ral-2, LSC00108), and putative nuclear assembly protein (nap-1, LSC00014).

<table>
<thead>
<tr>
<th>Cluster identifier</th>
<th>No. of ESTs</th>
<th>Length of consensus (bp)</th>
<th>Gene name (description)</th>
<th>Species containing homologues</th>
<th>Other nematodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSC00018</td>
<td>16</td>
<td>611</td>
<td>alt-1 (abundant larval transcript)</td>
<td>B. malayi, Bp, O. volvulus, W. bancrofti, A. viteae, D. immitis</td>
<td>None</td>
</tr>
<tr>
<td>LSC00017</td>
<td>8</td>
<td>1,311</td>
<td>cpl-1 (cathepsin L-like cysteine protease)</td>
<td>B. malayi, O. volvulus, B. pahangi, D. immitis</td>
<td>None</td>
</tr>
<tr>
<td>LSC00030</td>
<td>5</td>
<td>890</td>
<td>cos-1 (mitochondrial cytochrome oxidase subunit 1)</td>
<td>B. malayi, O. volvulus</td>
<td>C. elegans, Pristionchus pacificus</td>
</tr>
<tr>
<td>LSC00036</td>
<td>3</td>
<td>588</td>
<td>tsp-1 (thioredoxin peroxidase)</td>
<td>B. malayi, O. volvulus, D. immitis</td>
<td>C. elegans</td>
</tr>
<tr>
<td>LSC00047</td>
<td>3</td>
<td>818</td>
<td>pph-1 (similar to phosphatidyethanolamine binding protein)</td>
<td>B. malayi, Onchocerca ochengi</td>
<td>C. elegans, T. canis</td>
</tr>
<tr>
<td>LSC00080</td>
<td>3</td>
<td>557</td>
<td>col (similar to cuticular collagen)</td>
<td>B. malayi, O. volvulus</td>
<td>C. elegans, Caenorhabditis briggsae, P. pacificus, N. americanus</td>
</tr>
<tr>
<td>LSC00108</td>
<td>3</td>
<td>502</td>
<td>rbp-1 (putative RNA binding protein)</td>
<td>B. malayi, O. volvulus, W. bancrofti</td>
<td>C. elegans</td>
</tr>
<tr>
<td>LSC00014</td>
<td>2</td>
<td>561</td>
<td>Novel</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>LSC00025</td>
<td>2</td>
<td>535</td>
<td>ndh-5 (mitochondrial NADH dehydrogenase subunit 5)</td>
<td>B. malayi, O. volvulus</td>
<td>C. elegans, Ascaris suum</td>
</tr>
<tr>
<td>LSC00040</td>
<td>2</td>
<td>404</td>
<td>nap-1 (nucleosome assembly protein)</td>
<td>B. malayi</td>
<td>C. elegans, P. pacificus</td>
</tr>
<tr>
<td>LSC00042</td>
<td>2</td>
<td>672</td>
<td>rat-2 (Ascaris and Onchocerca antigen homologue)</td>
<td>B. malayi, O. volvulus, W. bancrofti</td>
<td>C. elegans, P. pacificus, A. suum</td>
</tr>
<tr>
<td>LSC00055</td>
<td>2</td>
<td>689</td>
<td>tph-1 (translationally controlled tumor protein homologue)</td>
<td>B. malayi, O. volvulus</td>
<td>C. elegans</td>
</tr>
<tr>
<td>LSC00066</td>
<td>2</td>
<td>605</td>
<td>Novel (similar to C. elegans hypothetical protein)</td>
<td>B. malayi, O. volvulus</td>
<td>C. elegans</td>
</tr>
<tr>
<td>LSC00139</td>
<td>2</td>
<td>592</td>
<td>Novel (similar to C. elegans hypothetical protein)</td>
<td>B. malayi, O. volvulus</td>
<td>C. elegans, P. pacificus, T. canis</td>
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<tr>
<td>LSC00152</td>
<td>2</td>
<td>275</td>
<td>col-1 (Ancylostoma secreted protein homologue)</td>
<td>B. malayi, B. pahangi</td>
<td>C. elegans</td>
</tr>
<tr>
<td>LSC00194</td>
<td>2</td>
<td>602</td>
<td>asp-1 (Ancylostoma secreted protein homologue)</td>
<td>B. malayi, O. volvulus, A. viteae, P. pacificus</td>
<td>C. elegans, Ancylostoma caninum, N. americanus, H. contortus</td>
</tr>
</tbody>
</table>
FIG. 1. (A) Alignment of ALT homologues from filarial nematodes. The alignment was constructed using ClustalW and adjusted by eye. Residues identical in more than four of eight aligned sequences are shaded grey, while similar residues are not shaded but boxed. A five-of-eight majority rule consensus is given below the alignment. (B and C) Analysis of similarity of ALTs. Phylogenetic trees were constructed using only the conserved C-terminal portion (residues 72 to 153 of the alignment in panel A). (B) Minimum evolution, neighbor joining; (C) maximum parsimony (gaps included). Italicized numerals give bootstrap support (1,000 replicates). Trees were arbitrarily rooted by using *O. volvulus* ALT.
ing proteins and mitochondrially encoded genes (see http://www.ed.ac.uk/~mbx/LitoWeb/LitoESTs.html). For other clusters, a domain similarity can be discerned that is suggestive of function. For example, LSC00029 and LSC00181 have immune-related homologues. For instance, LSC00029 and LSC00181 have immunoglobulin-like domains, most similar to immunoglobulin domain-containing proteins from Caenorhabditis elegans. For many clusters (23 are described at http://www.ed.ac.uk/~mbx/LitoWeb/LitoESTs.html), the closest or only homologue in the public databases is a protein of unknown function predicted from the C. elegans genome project. For two such clusters, LSC00066 and LSC00139, more than one EST was sequenced, suggesting that these genes may be expressed at high levels in the vector-derived L3. Homologues of these clusters are also expressed at high levels in B. malayi. Whatever functions they perform, their abundance in the L3 data set suggests that they might be of importance to the larvae.

If L. sigmodontis is to be a model of real value in discovery and testing of vaccine candidates, it will be necessary to understand the relationship of this organism to the filarial pathogens which cause human disease. Importantly, in this study we were able to verify that, as in the human filarial nematodes, alt-1 is a highly abundant larval transcript in L. sigmodontis. However, it is important to recognize that 34% of the genes identified were unique to L. sigmodontis. This is likely to reflect the different migration patterns and host specificities of the parasites. Studies of novel genes unique to a particular parasite may further our understanding of the biology of filarial nematodes and their relationship to the mammalian host. Although it is small, the EST data set provided a substantial amount of basic information about this organism on which to build further studies and will greatly enhance our ability to use this important model system.

Nucleotide sequence accession numbers. The sequences of L. sigmodontis genes found in this study were submitted to the EST database section of GenBank (accession numbers AW152683 to AW152860 and BE140074 to BE140093).

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