Functional Elements and Domains Inferred from Sequence Comparisons of a Heat Shock Gene in Two Nematodes

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Summary. Caenorhabditis elegans and Caenorhabditis briggsae are two closely related nematode species that are nearly identical morphologically. Interspecific cross-hybridizing DNA appears to be restricted primarily to coding regions. We compared portions of the hsp-3 homologs, two grp78-like genes, from C. elegans and C. briggsae and detected regions of DNA identity in the coding region, the 5' flanking DNAs, and the introns. The hsp-3 homologs share approximately 98% and 93% identity at the amino acid and nucleotide levels, respectively. Using the nucleotide substitution rate at the silent third position of the codons, we have estimated a lower limit for the date of divergence between C. elegans and C. briggsae to be approximately 23–32 million years ago. The 5' flanking DNAs and one of the introns contain elements that are highly conserved between C. elegans and C. briggsae. Some of the regions of nucleotide identity in the 5' flanking DNAs correspond to previously detected identities including viral enhancer sequences, a heat shock element, and an element present in the regulatory regions of mammalian grp78 and grp94 genes. We propose that a comparison of C. elegans and C. briggsae sequences will be useful in the detection of potential regulatory and structural elements.

Key words: Caenorhabditis elegans — Caenorhabditis briggsae — hsp70 — grp78 — Gene comparison — Evolution — Regulatory elements

Introduction

The nematode Caenorhabditis elegans is well suited to genetic and developmental studies for several reasons. A detailed genetic map has been compiled (Edgley and Riddle 1987), a complete description of the cell lineage has been achieved (Sulston and Horvitz 1977; Sulston et al. 1983), and a library of ordered cosmids clones and yeast artificial chromosomes is nearing completion (Coulson et al. 1986, 1988). With the development of a C. elegans transformation system (Fire 1986) it has become feasible to study the regulation of genes on a cellular basis as well as to identify mutations by rescue with the wild-type copy of the gene. Recently, a cross-hybridization technique has been developed to identify coding regions, aiding the alignment of a genetic map with the physical map (Snutch 1984; Prasad and Baillie 1989). Most of the cross-hybridizing DNA sequences detected between C. elegans and Caenorhabditis briggsae represent functional transcribed elements (Prasad and Baillie 1989).

To identify potential regulatory elements, it is helpful to compare species that have been separated for evolutionary periods long enough for unconstrained sequences to diverge. Such comparisons have been made using several Drosophila species, and sequences representing genes and elements important for gene regulation have been identified. For example, a comparison of the 5' flanking DNA sequences of the hsp82 genes from four different Drosophila species (Blackman and Meselson 1986) revealed that most of the conserved features detected were known to be involved in transcriptional control [for example, the TATA and heat shock element (HSE) motifs]. In the Sgs-3 genes of several Drosophila species, a number of conserved elements in...
the 5' flanking DNAs were detected (Martin et al. 1988), some of which are known to be involved in gene regulation. In addition, conserved sequences at the 5' end of the Gart-nested cuticle gene are believed to be involved in developmental expression of the cuticle gene (Henikoff and Eghtedarzadeh 1987).

Caenorhabditis elegans and C. briggsae, two closely related species, have been described as "twin species" (Nigon and Dougherty 1949). Although nearly identical morphologically, DNA aneuploidy experiments have suggested that any related sequences in C. elegans and C. briggsae must differ by at least 20% (Emmons et al. 1979) and that the two species have been evolutionarily separated for tens of millions of years. The divergence observed appears to be restricted primarily to the noncoding regions (Swintz 1984; Heine and Blumenthal 1986; Prasad and Baillie 1989). Therefore, the relatedness of and the relatively high degree of nucleotide divergence between C. elegans and C. briggsae is ideal for a comparison of homologous DNA sequences to detect potential coding regions and regulatory elements.

During analysis of the hsp70 gene family from C. elegans, we characterized a gene, hsp-3 (Heschl and Baillie 1989), that shared identity with the mammalian grp78 genes. The grp78 gene is a member of the highly conserved hsp70 multigene family (Lindquist and Craig 1988) and encodes a protein located in the endoplasmic reticulum (Lee 1987). The hsp-3 gene, encoding the hsp70C protein, is constitutively expressed, developmentally regulated, and not heat inducible (Snutch et al. 1988). We previously detected several identities to known regulatory elements including viral enhancers and an HSE (Heschl and Baillie 1989) as well as an element that shares considerable identity with one that is important for the high-level expression of the rat grp78 gene (Resendez et al. 1988; Heschl and Baillie 1989). Our present objective is to determine if a comparison of the C. elegans and C. briggsae homologs can detect, by DNA sequence conservation and divergence, potential regulatory elements. This paper characterizes part of the hsp-3 homolog from C. briggsae, including the first two-thirds of the coding region and the 5' flanking DNA, and presents a comparison of the C. briggsae hsp-3 gene with the C. elegans hsp-3 gene.

Materials and Methods

Construction of Plasmids: The isolation and characterization of the C. elegans hsp-3 gene [hsp-3(Ce)] has been described elsewhere (Snutch et al. 1988; Heschl and Baillie 1989). The phage CB10 contains the C. briggsae hsp-3 homolog [hsp-3(Cb)] (Snutch 1984). The hsp-3(Cb) coding region was delineated by hybridization of the restriction fragments back to the hsp-3(Ce) gene. CB10 was digested with HindIII and cloned into the plasmid vector Bluescript (Stratagene). The plasmid pCBS1 contains the hsp-3 coding region and 5' flanking DNAs. pCBS1 was further subcloned by a partial SauI digestion and a complete HindIII digestion to retrieve 5' flanking DNA. The 3.4-kb fragment encompassing the SauI site nearest the coding region to the HindIII 5' of the coding region was electroeluted as described in Maniatis et al. (1982) and ligated into Bluescript+ cut with SauI and HindIII to give rise to pCBS2. Overlapping plasmid deletions were made using either exonuclease III (Henikoff 1987) or restriction enzymes.

Plasmid DNA Preparation. Plasmid DNA for deletion and sequencing reactions was prepared by the mini alkalysis lysis method (Maniatis et al. 1982) except that two phenol:chloroform extractions were done, and, after the first ethanol precipitation, the air-dried pellet was resuspended in 0.25 M sodium acetate and reprecipitated with ethanol. After digestion with RNase, the plasmid DNA samples were precipitated with polyethylene glycol (Hattori and Sakaki 1986).

DNA Sequencing and Sequence Analysis. Dideoxy sequencing was performed on denatured plasmid DNA as described by Mierendorf and Pfeffer (1987). DNA sequence analysis was done using the computer program Microgenie (Beckman). Visual inspection and preparation of the DNA sequences for publication were done with the aid of the computer program ESEE (Cobat and Beckenbach 1989). Nucleotide divergence was calculated by subtracting the percent identity from 100. Correction for multiple mutational events was calculated on a Poisson distribution using the formula $-\frac{1}{e} \ln(1 - \frac{f}{100})$, where $f$ is equal to the uncorrected percent nucleotide divergence divided by 100.

Results and Discussion

Characterization of the hsp-3 Homolog from a Closely Related Species, C. briggsae

Two phage of one type containing the C. briggsae hsp-3 homolog [hsp-3(Cb)] were isolated from a partial EcoRI genomic library in Charon 4 (Snutch 1984). Only the first two-thirds of the hsp-3(Cb) homolog and 5' flanking DNA were represented in the phage isolated. These data are summarized in Fig. 1. The sequence of the hsp-3(Cb) homolog and 5' flanking DNA is in Fig. 2 and is aligned against the C. elegans hsp-3 [hsp-3(Ce)] gene (Heschl and Baillie 1989) for maximum identity.

Comparison of the hsp-3 Homologs from C. elegans and C. briggsae

The hsp-3 coding regions are highly conserved between the two species sharing 92.6% identity at the nucleotide level and 98% identity at the amino acid level (Fig. 2). A summary of the nucleotide substitutions is presented in Table 1. The C. briggsae hsp70C protein has a hydrophobic leader sequence from residue 1 to 17 similar to the C. elegans hsp70C protein (Fig. 2). Although the amino acid sequence in this region is not completely conserved, the hy-
Table 1. Summary of changes in the nucleotide sequence between the hsp-3 homologs

<table>
<thead>
<tr>
<th>DNA region</th>
<th>Nucleotide matches</th>
<th>Nucleotide mismatches</th>
<th>Total number of shared nucleotides</th>
<th>Percent identity*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 1</td>
<td>180</td>
<td>17</td>
<td>197</td>
<td>91.4</td>
</tr>
<tr>
<td>Exon 2</td>
<td>441</td>
<td>33</td>
<td>474</td>
<td>93.0</td>
</tr>
<tr>
<td>Exon 3 (partial)</td>
<td>614</td>
<td>48</td>
<td>662</td>
<td>92.7</td>
</tr>
<tr>
<td>Exon 1 + 2 + 3</td>
<td>1235</td>
<td>98</td>
<td>1333</td>
<td>92.6</td>
</tr>
<tr>
<td>Third codon position*</td>
<td>160</td>
<td>44</td>
<td>204</td>
<td>78.4</td>
</tr>
<tr>
<td><strong>Noncoding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5' Flanking</td>
<td>270</td>
<td>109</td>
<td>379</td>
<td>71.2</td>
</tr>
<tr>
<td>Intron 1*</td>
<td>18</td>
<td>14</td>
<td>32</td>
<td>56.3</td>
</tr>
<tr>
<td>Intron 2*</td>
<td>141</td>
<td>40</td>
<td>181</td>
<td>77.9</td>
</tr>
</tbody>
</table>

* Not corrected for multiple mutation events
* Includes only the codons CTN, GTN, TGN, CCN, ACN, GCN, CGN, and GGN
* Intron calculations exclude the 5' and 3' conserved splice sites; because of uncertainties in the alignments, the percent identity values shown must be regarded as approximations

Amino acid replacements appear to be randomly distributed throughout the first and second exons (5 out of 62 and 7 out of 158, respectively; Fig. 2). The partial sequence of the third exon from hsp-3(Cb) representing 221 amino acids does not show any changes in amino acid content when compared to the hsp-3(Ce) gene. However, nucleotide substitutions appear to be as prevalent in the third exon (7.3%) as in the first and second exons (8.6% and 7.0%, respectively). Functionally, this domain may be highly important and thereby less able to tolerate amino acid replacements than the domains encompassed by the first and second exons. In fact, the domain encoded by the third exon diverges quite highly from the analogous domains in other hsp70-like proteins but not the grp78-like proteins. It may be that this domain is highly specific for and unique to the grp78 subfamilies and that the analogous regions in the other hsp70 subfamilies would, by the same argument, encode domains highly specific and unique to each hsp70 subfamily.

Comparison of the hsp-3 5' Flanking DNAs

The 5' flanking DNAs of the hsp-3 homologs were aligned for maximum identity from the translation start site using Microgenie (Beckman) and visually with ESEE (Cabot and Beckenbach 1989). Both methods gave comparable alignments and results. Overall, the percent identity of the 5' untranslated region was determined to be approximately 71%. A summary of the nucleotide changes is presented in Table 1. Several conserved elements were observed and are outlined in Fig. 2. For this study, only those regions that exhibited 75% identity or more over their lengths were considered to be conserved elements. Block 5 contains an identity to the E1A enhancer and blocks 3 and 8 contain identities to the SV40 and E1A enhancers in the reverse orientation, respectively (Hearing and Shenk 1983; Weiher et al. 1983). Block 3A is a highly conserved repeat of block 3 [from –140 to –122 in hsp-3(Ce)] and also contains a copy of the SV40 enhancer in the reverse orientation. (The conservation of the viral enhancers raises a couple of intriguing possibilities. First, Caenorhabditis viruses that are related to SV40 and adenovirus may exist, or, second, the viral enhancer motifs may be functionally quite ancient and may have been recruited as enhancers for use by the mammalian viruses.) Block 5 also contains an identity to the HSE (CNNGAANNTTCNNG; Pelham...
1982, 1985), although the C. elegans hsp-3 gene is not heat inducible under the conditions tested (Snutch et al. 1988). None of these identities detected were perfect matches. An element in block 7 contains an identity detected in a comparison of the hsp-3(Ce) regulatory region with a portion of the rat grp78 regulatory region (Heschl and Baillie 1989; Resendez et al. 1988). This element, which is known to be important for the high-level expression of the rat grp78 gene, is also conserved in the human grp78 and the human and chicken grp94 genes (Chang et al. 1987; Resendez et al. 1988). Block 6 is an imperfect repeat of block 7, sharing identity with nucleotides -326 to -313 of block 7 in hsp-3(Ce).

The other blocks contain structural features including inverted repeats (block 9) and pyrimidine-rich sequences (blocks 2 and 2A). Block 1 probably represents the transcribed, untranslated region. Dot matrix analyses did not detect any identities in the reverse complement of one strand that may have been missed by our alignment analyses. On the basis of the identities to known functional elements and structural features, we believe that we have detected most, if not all, of the conserved elements at the 5' end of the hsp-3 homologs that may be involved in regulation and/or structure.

Blumenthal and Zucker-Aprison (1987) have stated that Box 1 and Box 2 elements, two heptameric sequences potentially involved in transcriptional regulation, are conserved between the C. elegans and C. briggsae vitellogenin genes. Box 2 homologies appear to be required for estrogen stimulation of Xenopus vitellogenin gene transcription in transfected human mammary carcinomas (Klein-Hitpass et al. 1986). Potential stem-forming regions at the 5' end of the vitellogenin genes are also conserved between C. elegans and C. briggsae. Even among multigene families such as the major sperm protein (MSP) genes from C. elegans, conserved putative 5' regulatory elements are observed scattered amid highly diverged sequences (Klass et al. 1988).

Comparison of Intron Sequences between C. elegans and C. briggsae

The two introns represented in the hsp-3(Cb) sequence were found at corresponding positions when compared to the first two introns of the hsp-3(Ce) sequence (Figs. 1 and 2). The second introns of hsp-3(Cb) and hsp-3(Ce) are approximately the same length (243 vs 238 nucleotides, respectively), whereas the first introns are not (74 vs 46 nucleotides, respectively). A summary of nucleotide substitutions is presented in Table 1. The percent identity of intron 2 is much higher than that of intron 1 (Table 1), suggesting that there may be conserved elements in intron 2. Therefore, we compared the intron 2 sequences further to detect any possible conserved elements (Fig. 2). Two sets of conserved elements were detected in intron 2 including the intron boundaries (5' AG/GTAAGT ... TTTT-CAG/G 3'; Blumenthal and Thomas 1988) and a noticeable region of nucleotide identity extending over most of intron 2.

In the relatively short introns of C. elegans, there are apparently few conserved elements other than the 5' and 3' splice sites. The need for specific sequences involved in splicing, such as the splice branch point, have apparently disappeared in C. elegans (Blumenthal and Thomas 1988). Therefore, it came as a surprise that a conserved element was present in the second intron of the hsp-3 homologs. If, as suggested above, conserved elements in Caenorhabditis are functionally important, it may be that this intronic element is important in the regulation of hsp-3 either as an enhancer of transcription or by affecting the splicing or stability of the hsp-3 mRNA. In fact, it has been suggested that an enhancer element in the third intron of the unc-54 gene (encoding the major myosin heavy chain) plays a major role in directing tissue-specific expression (Fire and Harrison, personal communication).

An Estimate of Divergence for C. elegans and C. briggsae

An estimate of the time of divergence between C. elegans and C. briggsae can be made by determining the nucleotide divergence of the silent third position of fourfold degenerate codons. The nucleotide divergence calculated for these sites in the hsp-3 coding region is 25.5% when corrected for multiple mutational events. Nucleotide substitution rates at the silent third codon position have been determined.
for bacteria [0.8%/million years (Myr) (Ochman and Wilson 1987)] and the nuclear genes of mammals [0.9%/Myr (Li et al. 1983a,b)], insects [1.1%/Myr (Bodmer and Ashburner 1984; Blackman and Meselson 1986)], plants [1%/Myr (Chang and Meyerowitz 1986)], and sea urchins [1.1%/Myr (Busslinger et al. 1982)]. Because all of these organisms have similar percent nucleotide substitutions/Myr, it is not unreasonable to assume that Caenorhabditis will also fall within the range of 0.8–1.1% nucleotide substitutions/Myr. Therefore, we estimate that *C. elegans* and *C. briggsae* last shared a common ancestor no less than 23–32 Myr ago.

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