Coordination of ges-1 Expression Between the Caenorhabditis Pharynx and Intestine

Sean D. G. Marshall and James D. McGhee
Department of Biochemistry and Molecular Biology, Genes & Development Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1

We have previously shown that the Caenorhabditis elegans gut-specific esterase gene (C.e-ges-1) has the unusual ability to be expressed in different modules of the embryonic digestive tract (anterior pharynx, posterior pharynx, and rectum) depending on sequence elements within the C.e-ges-1 promoter. In the present paper, we analyze the expression of the ges-1 homolog (C.b-ges-1) from the related nematode Caenorhabditis briggsae and show that C.b-ges-1 also has the ability to switch expression between gut and pharynx + rectum. The control of this expression switch centres on a tandem pair of WGATAR sites in the Cb-ges-1 5'-flanking region, just as it does in C.e-ges-1. We use sequence alignments and subsequent deletions to identify a region at the 3'-end of both C.e-ges-1 and C.e-ges-1 that acts as the ges-1 cryptic pharynx enhancer whose activity is revealed by removal of the 5' WGATAR sites. This region contains a conserved binding site for PHA-4 (the C. elegans ortholog of forkhead/HNF3α, βγ factors), which is expressed in all cells of the developing pharynx and a subset of cells of the developing rectum. We propose a model in which the normal expression of ges-1 is controlled by the gut-specific GATA factor ELT-2. We propose that, in the pharynx (and rectum), PHA-4 is normally bound to the ges-1 3'-enhancer sequence but that the activation function of PHA-4 is kept repressed by a (presently unknown) factor binding in the vicinity of the 5' WGATAR sites. We suggest that this control circuitry is maintained in Caenorhabditis because pharyngeal expression of ges-1 is advantageous only under certain developmental or environmental conditions.

Key Words: Caenorhabditis elegans; Caenorhabditis briggsae; ges-1; gene expression; intestine; pharynx; elt-2; pha-4.

INTRODUCTION

How is gene expression coordinated between the different components of a developing organ system? The embryonic digestive tract of the nematode Caenorhabditis elegans provides a simple experimental system in which this question can be addressed. As illustrated in Fig. 1, the four distinct modules of the C. elegans digestive tract are derived from four distinct cell lineages: anterior pharynx, posterior pharynx, and rectum are derived from the A Ba, MS, and ABp blastomeres, respectively, and the gut proper is clonally derived from the single E blastomere of the eight-cell embryo (Sulston et al., 1983). Over the past decade, a large number of detailed studies have described how these early blastomeres become distinct from each other at the level of cell fate, largely through the action of maternally provided gene products (reviewed in Schnabel and Priess, 1997; Bowerman, 1998; Rose and Kemphes, 1998). In the present paper, we wish to consider whether a later level of control also exists, after blastomere fate has been determined. In other words, can genes be controlled by regulatory networks that operate between digestive tract modules?

The C. elegans ges-1 gene encodes a nonspecific (with respect to substrate) carboxylesterase whose expression is normally restricted completely to the gut or E lineage, beginning when the gut has only four cells and continuing throughout the life of the worm (Edgar and McGhee, 1986; Kennedy et al., 1993). ges-1 shows unusual and interesting behavior that suggests that gene expression may indeed be coordinated throughout the entire digestive tract: deletion of a tandem pair of WGATAR sites (where W = A or T and R = A or G) in the 5'-flanking region abolishes ges-1 expression in the gut but simultaneously activates ges-1 expression in both anterior and posterior pharynx as well as the rectum, i.e., the three other modules of the digestive tract (Aamodt et al., 1991; Egan et al., 1995; Fukushima et al., 1998).
Our present model is that ges-1 harbors a pharynx/rectum enhancer sequence somewhere within the gene but that the activity of this enhancer is normally kept repressed by a factor that binds to, or in the vicinity, of these 5' WGATAR sites. While the ELT-2 GATA factor is currently the best (but not the only) candidate for binding the WGATAR sites and activating ges-1 expression in the gut (Hawkins and McGhee, 1995; Fukushige et al., 1998), the identity of the pharynx/rectum repressor, the pharynx/rectum enhancer, and the pharynx/rectum enhancer binding factor are presently unknown.

In the present paper, we analyze the ges-1 homolog from the related nematode Caenorhabditis briggsae. We show that the central feature of ges-1 regulation is retained in C. briggsae, namely a WGATAR site-dependent switch in expression between gut and pharynx/rectum. We use sequence comparisons and subsequent gene deletions to identify the major pharynx enhancer, which lies at the 3'-end of both C. elegans and C. briggsae ges-1 genes and whose activity is revealed by deletion of the WGATAR sequences. Within this enhancer sequence lies a conserved binding site for the PHA-4 protein. The pha-4 gene is the worm homolog of Drosophila forkhead and vertebrate HNF-3α, β, γ genes and has been shown to act as an "organ-identity-factor" during pharynx (and rectum) development (Horner et al., 1998; Kalb et al., 1998). The pharynx (+ rectum) repressor proposed normally to silence the PHA-4 transactivation function is as yet unknown.

Two of the principal factors that we propose participate in this intermodule regulatory network are shown in Fig. 1: PHA-4 in the pharynx and the rectum (red) and ELT-2 in the intestine proper (green). Indeed, we have already described an interaction between these two factors (Kalb et al., 1998) that is reminiscent of interactions between the Drosophila transcription factors serpent (which shows high sequence similarity to elt-2) and forkhead (the ortholog of pha-4) in establishing the fundamental partitions within the insect digestive tract (Reuter, 1994). Thus, elements of this intra-

FIG. 1. Cell lineage of the early blastomeres that produce the C. elegans digestive tract. All nuclei of the gut (green) are visualized by means of an elt-2::GFP transgenic reporter construct. All nuclei in the pharynx and a subset of nuclei in the rectum (red) are visualized by using an antibody against PHA-4 (Kalb et al., 1998). Arrows indicate that a blastomere produces other cells besides those of the digestive tract; only the gut or E lineage is a clone.
digestive tract network of gene control appear to have been widely conserved in evolution.

MATERIALS AND METHODS

Strains and Transformation Methods

C. elegans N2 (wild type), JM1041 (Ce-ges-1 null strain; McGhee et al., 1990), and C. briggsae AF16 (wild type) were maintained as described by Brenner (1974). Microinjections were performed essentially as described by Mello et al. (1991); unless otherwise noted, pRF4 [rol-6(su1006)] was used as a coinjection marker. Test plasmid and marker DNA were initially present at a concentration of 50 μg/ml each in 10 mM Tris, 1 mM EDTA, pH 8. For each altered construct, 2–10 stably transformed lines were produced in both C. elegans and (where possible) C. briggsae; dozens to hundreds of embryos from each transgenic line were inspected after staining for 3 min (see below). Transgenic arrays in selected strains were integrated into the genome by γ-irradiation as described in Egan et al. (1995). The C. elegans ges-1 null strain JM1041 (McGhee et al., 1990) is our transformation host of choice because ectopic C. briggsae host must be assessed above the background level of the C. elegans ges-1 gene; dozens to hundreds of embryos from each transgenic line were inspected after staining for 3 min (see below). In addition, we found (as have others; Gilleard et al., 1997) that C. briggsae could be difficult to transform and, for several constructs, transgenic strains in C. briggsae could not be produced in spite of repeated efforts.

Vectors and Constructs

All deletions of the C. briggsae ges-1 gene were made within the parent plasmid pJM102, which contains the entire Ce-ges-1 gene, the 5.9-kb insert contains 1.7 kb of 5′-flanking sequence, 2.0 kb of coding sequence (6 introns and 7 exons), and 2.2 kb of 3′-flanking sequence downstream of the poly(A) addition site. The coordinate system used in the present paper takes bp #1 as the first G residue in an upstream SalI site, such that the ATG codon of Cb-ges-1 begins at position 1697 and the poly(A) addition site is located at position 3692. Deletions within pJM102 were created by using convenient restriction sites or were produced by the PCR-based method of “splicing by overlap extension” (White, 1993). Mutated constructs, see Marshall (1998).

The vector pJM77 used to test the enhancer activity of candidate sequences for (example, multiple copies of the WGATAR sites was constructed as follows: a 446-bp Sau3A fragment from the promoter of the C. elegans heat shock gene 16–48 was isolated from plasmid pPC16.48-1 (Stringham et al., 1992) and inserted in the correct orientation into BamHI-cleaved vector pPD96.04 (kindly provided by A. Fire, Carnegie Institute of Washington, Baltimore, MD). In this construct, the heat shock elements of the 16–48 gene are intact but can be removed either by PsI digestion or by double digestion with PsI and HindIII. pJM77 contains the transcription initiation site, the 5′-UTR, the ATG codon, and the first 15 amino acids of the 16–48 heat shock protein fused to a GFP-lacZ reporter incorporating 15 synthetic introns. Sequence elements to be tested for enhancer activity are firstimerized, cloned into the EcoRV site of pBluescript and transferred as a HindIII-PsI fragment into HindIII-PsI-cleaved pJM77, thereby removing the original heat shock elements and preserving insert orientation.

Histochemistry

Histochemical staining of embryos for esterase activity was conducted essentially as described by Edgar and McGhee (1986) for the incubation times indicated in the text. Detection of β-galactosidase activity was performed as described in Fire (1992), usually for 15 h at 37°C.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift experiments were performed as described in Hawkins and McGhee (1995) and Kalb et al. (1998). For experiments with ELT-2 probe, each reaction contained 1–2 μl of either programmed or unprogrammed reticulocyte lysate (Promega TNT). Experiments with the PHA-4 protein used PHA-4C, the shortest of the three protein isoforms (Azzaria et al., 1996), produced in baculovirus and purified by affinity chromatography. Each reaction contained 50,000 cpm of end-labeled double-stranded probe, 0.1–1 μg of poly(dIdC:dIdC) as nonspecific competitor and, where appropriate, up to 100-fold molar excess of specific double-stranded competitor oligonucleotide. Reactions were incubated for 20–30 min at room temperature in a buffer containing 25 mM Hepes, pH 7.4, 50 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, 200 μg/ml BSA, 0.1% N40, and 10% glycerol. Products were separated at 4°C on a 4–5% polyacrylamide gel containing 0.5× TBE running buffer.

RNA-Mediated Interference

pha-4 RNAi was produced essentially as described by Montgomery et al. (1998). Double-stranded RNA corresponding to pha-4 cDNA isomor 1, the longest of the three transcripts (Azzaria et al., 1996), was injected at a concentration of ~500 μg/ml into gonads and/or gut/body cavity of strain JM97 (see below). Embryos were collected between 12 and 36 h after injection and stained for β-galactosidase activity.

RESULTS

Sequence Comparisons of the C. briggsae and C. elegans ges-1 Loci

Our initial description of the ges-1 genes from C. elegans and C. briggsae (Ce-ges-1 and Cb-ges-1, respectively) compared coding sequences along with limited flanking sequences (Kentner et al., 1993). We are now in a position to extend these comparisons considerably: the genomic sequence of the entire C. elegans ges-1 locus is now available (The C. elegans Sequencing Consortium, 1998) and we have extended the available sequence for the C. briggsae ges-1 gene.

A dot matrix comparison of the two ges-1 genomic regions is shown in Fig. 2. As noted previously (Kennedy et al., 1993), the coding regions are ~75% identical at the nucleotide level (83% amino acid identity) and the correspondence between exons is obvious. However, outside of the coding regions, Ce-ges-1 and Cb-ges-1 show only two regions with significant sequence conservation (labeled #1 and #2 on Fig. 2). Region #1 in the 5′-flanking region was identified previously (Kennedy et al., 1993) and contains a 17/17-bp match between the two genes. Deletion of this
sequence from the Ce-ges-1 promoter, either alone or in conjunction with the deletion of several other promoter regions, has little influence on either spatial or temporal expression of Ce-ges-1, at least in the embryo (Egan et al., 1995). As will be demonstrated below, the corresponding region from the C. briggsae gene can also be deleted without obvious influence on Cb-ges-1 embryonic expression patterns. The second region (#2), not previously identified, continues for ~300 bp downstream from the ges-1 poly(A) addition sites; the overall match in this region is ~67%. This region will be shown below to contain the cryptic pharynx enhancer.

The tandem pair of WGATAR sites controlling the Cb-ges-1 gene is indicated ~1.1 kb upstream of the Ce-ges-1 ATG codon (Egan et al., 1995). As will be described below, Cb-ges-1 is also controlled by a tandem pair of WGATAR sites, lying ~700 bp upstream of the Cb-ges-1 ATG codon. However, no significant sequence conservation between these two regions is apparent, even at higher resolution and despite wide variation of the window size and stringency (data not shown). As described in the figure legend, there is no obvious reason to expect that neighboring genes are influencing ges-1 expression patterns.

The Endogenous Cb-ges-1 Gene Shows a Low Level of Pharyngeal Activity

Figure 3A compares the esterase staining patterns observed with wild-type C. elegans (strain N2) and wild-type C. briggsae (strain AF16) at three different staining periods: 3 min (appropriate for assaying multicopy transgenes), 1 h (appropriate for detecting the single copy endogenous ges-1 gene), and 4 h (used to detect the low level of pharynx + rectum staining produced by chromosomal deletions of the endogenous Ce-ges-1 promoter; Fukushige et al., 1996). The primary conclusion is that the vast majority of staining activity at all time periods is in the intestine. However, a weak activity can be detected in the C. briggsae pharynx after 4 h staining that is not detected in C. elegans under the same condition (see also Fukushige et al., 1996). Any possible rectal staining would be obscured by the intense gut staining. Essentially all of the esterase activity in the first half of C. elegans embryogenesis derives from the Ce-ges-1 gene (MCGhee et al., 1990; Egan et al., 1995). We must assume that this also holds true for C. briggsae embryos and that the endogenous activity in the C. briggsae pharynx is indeed caused by Cb-ges-1 and not by some other esterase.

The next step is to evaluate the esterase staining activity produced by the full-length C. briggsae ges-1 transgene (construct pJM102), either when introduced into the homologous C. briggsae host (wild type for Cb-ges-1) or when introduced into the heterologous C. elegans host (strain JM1041, null for Ce-ges-1; MCGhee et al., 1990). Figure 3B shows a typical C. briggsae embryo (upper) and a C. elegans embryo (lower) transformed with pJM102 and stained for 3 min. Although the majority of transgenic esterase activity is indeed in the gut, both embryos show low but significant levels of staining in the pharynx, considerably more than is observed when the Ce-ges-1 gene is transformed back into C. elegans (where pharynx + rectum staining is low though not zero; data not shown and see also Egan et al., 1995). This strong-gut–weak-pharynx expression of the Cb-ges-1 transgene is consistent with the behavior of the endogenous Cb-ges-1 gene described above (Fig. 3A).

Deletions within the 5′-Flanking Sequences of the C. briggsae ges-1 Gene Reveal a Gut-to-Pharynx + Rectum Switch in Expression Similar to That Observed with the C. elegans ges-1 Gene

Figure 4A summarizes the results from a deletion analysis of the 5′-flanking region of Cb-ges-1. The two deletions shown above the central line produce essentially the same expression pattern as does the undeleted parent construct pJM102, i.e., intense gut staining and weak staining in the pharynx (and perhaps rectum). In contrast, for the two deletions represented below the gene line (in both C. briggsae and C. elegans hosts), expression in the gut is extinguished essentially completely (best demonstrated in the C. elegans ges-1 null mutant) but concomitantly activated in cells of the pharynx. These deletion constructs also produce staining in the area of the rectum, at least in the C. elegans ges-1 null host where it can be detected. In other words, the C. briggsae ges-1 gene, just like its Ce-ges-1 counterpart, has the cryptic ability to be expressed in other modules of the digestive tract. This unusual behavior appears to be controlled by a region 670–760 bp upstream of the Cb-ges-1 ATG.

The Gut-Activation/Pharynx + Rectum Repression of the C. briggsae ges-1 Gene Center on a Tandem Pair of WGATAR Sites

The dot-matrix comparison of Fig. 2 shows no obvious sequence conservation between the 5′ WGATAR region of Cb-ges-1 and the ~100-bp region controlling the gut-to-pharynx + rectum switch in Cb-ges-1 expression, nor could significant alignments be produced by the programs GAP or BESTFIT [Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI]. This region in the Cb-ges-1 promoter contains a total of five WGATAR sites, including one tandem pair spaced 20 bp apart. Figure 4B shows our best alignment (by eye) between this tandem pair of C. briggsae WGATAR sites and the tandem pair of WGATAR sequences, spaced 12 bp apart, that control the switch in Ce-ges-1 expression (Egan et al., 1995). Deletion of these two sites (a 35-bp deletion within the otherwise intact construct pJM102) does indeed cause the Cb-ges-1 expression pattern to switch from the gut into the pharynx (Fig. 4C). In general, rectum staining is weaker and more variable than observed with corresponding Ce-ges-1 deletions and from this point on, we focus primarily on pharynx staining.

Although the overall features of this intradigestive tract switch in expression pattern seem to be maintained be-
between Cb-ges-1 and Ce-ges-1, the genes show two clear differences. First, deletion of either the upstream or the downstream WGATAR site from Cb-ges-1 produces the complete switch in expression pattern from gut to pharynx (Fig. 4C); in contrast, deletion of either the upstream or the downstream WGATAR sites from Ce-ges-1 abolishes expression in the posterior gut, maintains expression in the anterior gut, but does not activate expression in the pharynx (Egan et al., 1995). Secondly, deletion of a 20-bp region adjoining the Ce-ges-1 WGATAR sites produces only anterior gut expression (Schroeder and McGhee, 1998); in contrast, a similar region in the C. briggsae ges-1 gene could not be found, either by sequence comparisons or by deletion (data not shown).

Properties of the Cb-ges-1 WGATAR Sites

Enhancer activity in the embryonic gut. As shown in Fig. 5A, multiple copies of either the individual or the paired Cb-ges-1 WGATAR sites, in either orientation and with either C. elegans or C. briggsae as transformation hosts, produce intense reporter gene expression specifically in the embryonic gut. In both host species, reporter expression was first observed at the 4E cell stage of development, the stage at which endogenous ges-1 expression normally can first be detected. The low-power view on the right side of Fig. 5A illustrates the uniform and reproducible expression of one particular reporter gene array, following integration into the genome.
Although these reporter constructs initiate expression at the correct stage, expression is not maintained much later than the L2 larval stage in either species (data not shown); in contrast, endogenous Ce-ges-1 expression continues to increase throughout the life of the worm (Kennedy et al., 1993). When only a single copy of either the upstream WGATAR site, the downstream WGATAR site, or the tandem pair of sites was inserted into the test vector and introduced into C. elegans, no expression in transformed embryos could be detected, either within the gut or elsewhere and even with prolonged staining for β-galactosidase activity (our most sensitive assay for expression).

**Can the WGATAR sites function as portable “gut-activators/pharynx repressors” within the context of the Cb-ges-1 gene?** To test for limits on the reversibility of the switch in ges-1 expression pattern, the tandem pair of Cb-ges-1 WGATAR sites was reinserted at either of two positions within the Cb-ges-1 construct from which the paired WGATAR sites have been deleted and the resulting constructs transformed into C. elegans. As shown in Fig. 5B, reinsertion of the paired WGATAR sites in the forward orientation at a site ~300 bp downstream from their normal location is sufficient to reconstitute normal expression patterns, i.e., strong gut, weak pharynx. However, when the
FIG. 4. (A) Deletion analysis of the 5′-flanking region of the Cb-ges-1 gene. The thick central line represents the 1.7-kb of 5′-flanking sequence present in the parent construct pJM102. Precise coordinates of the particular deletions are provided in Marshall (1998). Transformed deletion constructs depicted above the gene line produce a wild-type Cb-ges-1 staining pattern, both in a C. elegans and a C. briggsae host. Deletion constructs depicted below the gene line do not produce gut staining but rather produce staining in the pharynx and rectum. The arrow in the lower right panel indicates the low level of endogenous ges-1 staining observed in the gut of the C. briggsae host embryo after three minutes of staining. The position of the tandem pair of WGATAR sites is shown. (B) Alignment between the tandem pair of WGATAR sites from the 5′-flanking region of Cb-ges-1 (top) and Ce-ges-1 (bottom). As indicated, the Cb-ges-1 gene has an additional 8 bp inserted between the two sites. (C) Deletion of either or both of the WGATAR sites from the Cb-ges-1 5′-flanking region is sufficient to abolish Cb-ges-1 staining in the gut and activate expression in the pharynx. The sequence of the region and the precise base pairs deleted in each construct are shown at the left. The low level of endogenous Cb-ges-1 staining (3 minute incubation) in the C. briggsae host is indicated by the arrow. For constructs in which individual WGATAR sites were deleted, transformed strains could not be produced in C. briggsae (see Materials and Methods).
paired WGATAR sites were moved (in the reverse orientation) upstream ~1 kb from their normal location, wild-type Cb-ges-1 expression was only partially reconstituted, i.e., expression in the gut was weak and pharynx (+ rectum) staining persisted.

**Mutation vs. deletion of the WGATAR sites.** Mutation of all six residues in both of the Cb-ges-1 WGATAR sites or mutations in only the downstream Cb-ges-1 WGATAR site lead to the same expression pattern produced by deletion of the same sequences: gut expression is abolished and phar-
yxn (+ rectum) expression is activated (Fig. 5C). This was true with both host species. However, the upstream Cb-ges-1 WGATAR site shows a different behavior: whereas deletion of the upstream site abolishes gut expression and activates pharynx + rectum expression (Fig. 4C, above), mutation of the same site has little effect (Fig. 5C). This difference was only observed with C. elegans as a host because the corresponding C. briggsae transformed strain could not be produced.

The C. elegans ELT-2 protein binds to the C. briggsae WGATAR sites. The C. elegans gut-specific GATA-type transcription factor ELT-2 was identified based on its binding to the C. elegans ges-1 WGATAR sites (Hawkins and McGhee, 1995) and has been shown to be sufficient (but not necessary) for ges-1 activation (Fukushige et al., 1998). It seems reasonable that a C. briggsae ELT-2 homolog would also be involved in control of the Cb-ges-1 gene, and Fig. 5D shows that C. elegans ELT-2 protein does indeed bind to the C. briggsae tandem WGATAR sites. Competition with C. briggsae unlabeled double-stranded oligonucleotides indicates that the binding is "specific" but that binding to C. briggsae sites is significantly (perhaps fivefold) weaker than to the homologous C. elegans WGATAR sites.

Identification of the Pharynx Activator in the Conserved 3′ Region of the C. briggsae ges-1 Gene

The results shown in Fig. 6A identify the pharynx activator within the Cb-ges-1 gene. Deletions depicted above the line representing the Cb-ges-1 gene have no effect on the normal low level of pharynx staining observed with the intact Cb-ges-1 transgene construct, pJM102. In contrast, deletions depicted below the gene line abolish this pharynx activity (but retain gut activity). Overall, the results demonstrate that the pharynx activator must lie within several hundred base pairs downstream of the poly(A) addition site, within the conserved #2 region identified in Fig. 2.

The Conserved 3′ Region Is the Major Pharynx Activator in the ges-1 Genes

We deleted the 3′-conserved regions from Ce-ges-1 and Cb-ges-1 constructs that were also deleted for the 5′ tandem pair of WGATAR sites. Expression of these doubly deleted transgenes was highly variable and was detected in only a few cells per embryo, none of which belonged to the digestive tract (data not shown). Indeed, the expressing cells were often hypodermal, suggesting that the transgene might now be coming under the influence of the rol-6 coinjection marker (which encodes a cuticle collagen; Mello et al., 1991). We conclude that the conserved 3′-region of the ges-1 genes contains the major pharynx activator whose activity is revealed by deletion of the 5′ WGATAR sites. We show below that this sequence, from either C. elegans or C. briggsae, can drive pharynx-specific expression of a reporter gene.

A potential pharynx + rectum activator element had been tentatively identified immediately upstream of the Ce-ges-1 ATG (Aamodt et al., 1991; Egan et al., 1995). However, when two copies of this 68-bp Ce-ges-1 sequence were inserted at the 3′-end of Cb-ges-1 (within deletion pJM102.21; see Fig. 6A), pharynx expression was not restored (data not shown), suggesting that any enhancer activity that this region might possess is minor.

The ges-1 Pharynx Enhancer Sequence Interacts with PHA-4

To identify binding factors potentially responsible for the ges-1 pharynx enhancer activity, the two ~300-bp regions were aligned as shown in Fig. 6B and analyzed by the TRANSFAC program (Heinemeyer et al., 1998). Only one potential binding site appeared both significant and conserved: a strong HNF3β binding site within one of the most conserved regions. The C. elegans (and presumably C. briggsae) ortholog of HNF3β is the product of the pha-4 gene, which is expressed in all cells of the pharynx and in many cells of the rectum; moreover, pha-4 is crucial for development of the pharynx as an organ (Mango et al., 1994; Azzaria et al., 1996; Horner et al., 1998; Kalb et al., 1998). While the preferred binding site for PHA-4 has not yet been determined, a sequence has been identified within the myo-2 pharyngeal myosin promoter that binds PHA-4 in vitro and that acts as a PHA-4 target in vivo (Kubal et al., 1998); this sequence is similar to the putative site identified in the 3′ conserved region. Electrophoretic mobility shift assays (Fig. 6C) demonstrate that this conserved site from the C. elegans ges-1 3′ pharynx enhancer is indeed a binding site for pure PHA-4 protein (isoform C, produced in baculovirus); similar results were obtained by using the corresponding site from the C. briggsae gene (data not shown). In both cases, binding is "specific" in that the wild-type PHA-4 site from the myo-2 promoter competes effectively for binding, whereas an oligonucleotide containing a mutated myo-2 site, known not to bind PHA-4 in vitro (Kalb et al., 1998) and not to be effective in driving pharyngeal expression in vivo (Okkema and Fire, 1994), competes much more weakly.

Does this conserved PHA-4 binding site influence pharynx enhancer activity within the context of the ges-1 gene? Fig. 6D summarizes a more detailed deletion analysis of the 3′-region of the C. briggsae gene. As predicted, a deletion that removes the conserved PHA-4 binding site (together with neighboring conserved sequences as shown in Fig. 6B) does indeed abolish pharynx activity. However, pharynx activity is also abolished by deletions that extend from ~3791 to 3805 bp, suggesting that at least one other nearby site must also be involved; this latter region is not easily matched with a conserved sequence in the alignment shown in Fig. 6B.

Enhancer Properties of the 3′ Conserved Region

To determine whether these 3′ regions act as enhancers when taken out of the context of the ges-1 genes, single copies of either the C. briggsae or C. elegans ~300-bp
region were cloned, in either orientation, in our tester construct pJM 77 and transformed into C. elegans. In a total of 19 transformed strains (11 and 8 strains with the Ce-ges-1 and Cb-ges-1 enhancers, respectively), all but one strain expressed the GFP reporter strongly in the pharynx and only the pharynx (easily detectable, even at the level of the fluorescent dissecting microscope). One particular transformant containing the C. elegans 3' region in the forward orientation was integrated into the genome (to produce strain JM97) and examples of the staining patterns are shown in Fig. 7. Clearly, cells in both the anterior and posterior pharynx express the reporter gene. Rectal staining was never observed. Analysis of confocal sections (Fig. 7C) identify the 20–30 expressing cells as primarily muscle and

FIG. 6. (A) Deletion analysis of the 3' -flanking region of the C. briggsae ges-1 gene. The thick central line represents the Cb-ges-1 3' region in construct pJM 102, beginning immediately downstream of the poly(A) addition site. Deletion constructs depicted above the line produce the wild-type Cb-ges-1 staining pattern in both C. elegans and C. briggsae transformation hosts. Deletion constructs below the line produce a strong gut staining pattern but pharynx staining is abolished; precise coordinates of the various deletions are provided in Marshall (1998). The region suggested to contain the pharynx enhancer is indicated by the parallel black lines. The double-headed arrow labeled “B” corresponds to the sequence shown in Fig. 6B and in region #2 of Fig. 7. The sequence alignment of the proposed pharynx enhancer from the C. briggsae and C. elegans ges-1 genes (region #2 from Fig. 7) is predicted by the TRANSFAC program and is indicated by the thick lines. The dashed line represents the deletion pJM 102.25 that removes the HNF-3 site, as shown in Fig. 6D. The sequence of the oligonucleotides used for electrophoretic mobility shifts is indicated in bold. (B) Electrophoretic mobility shift assays to test binding of PHA-4C protein to the conserved oligonucleotide from the 3' -region of the C. elegans ges-1 gene. The wild-type competitor (double-stranded) oligonucleotide is from the promoter of the pharyngeal muscle-specific myo-2 gene and is designated C183 in Okkema and Fire (1994). The mutant competitor is designated C183mut2. (C) A more detailed deletion analysis demonstrating the bipartite nature of the C. briggsae 3'-pharynx enhancer. The asterisk represents the conserved PHA-4 binding site from Fig. 6B. Deletion of this site in construct pJM 102.25 abolishes the pharynx expression from Cb-ges-1. Deletion of a second region within the 3' enhancer (upstream pair of dashed lines) also abolishes pharyngeal expression.
marginal cells; staining in other classes of pharynx cells (e.g., epithelial, gland or nerve cells, as well as cells of the pharyngeal–intestinal valve) was much weaker and more variable. Interestingly, expression in muscle cells of the m6 class was much weaker than in the neighboring classes m5 and m7 (Fig. 7C); this expression pattern is reminiscent of that seen with the homeobox gene cep-22 (Okkema and Fire, 1994) and indeed, several potential CEH-22 binding sites can be identified in the enhancer sequence (P. Okkema, personal communication).

We have previously shown that the pharyngeal expression of the WGATAR-deleted Ce-ges-1 gene is abolished in pha-4 mutants (Fukushige et al., 1996). To test whether the isolated 3'-enhancer also lies (genetically) downstream of pha-4, we injected double-stranded pha-4 RNA into strain JM97 to phenocopy a pha-4 null mutation, collected the arrested (pharynx-defective) embryos, and stained for β-galactosidase activity. From 171 embryos examined, none showed reporter gene expression (data not shown). This result is consistent with PHA-4 directly acting on the ges-1 3'-enhancer. However, it must be remembered that expression of all pharyngeal genes is abolished in the absence of PHA-4 (Mango et al., 1994; Horner et al., 1998; Kalb et al., 1998).

**DISCUSSION**

In the present paper, we have analyzed the control of the gut-specific esterase gene ges-1 from the nematode *C. briggsae*. We can now compare these results to those from our previous analysis of the ges-1 homolog from *C. elegans* (Aamodt et al., 1991; Egan et al., 1995). The vast majority of endogenous ges-1 activity in both species is restricted to the intestine, beginning at approximately the 4E cell stage of development. The main features of ges-1 control also appear to be conserved between the two species: (1) expression of both genes in the intestine lineage depends on a tandem pair of WGATAR sites located in the 5'-flanking region; (2) deletion of these WGATAR sites abolishes expression in the gut lineage but concomitantly activates expression in cells of the pharynx and rectum; and (3) the cryptic pharynx enhancer, revealed when the WGATAR sites are deleted, lies in a conserved sequence at the 3'-end of ges-1 in both species and contains a conserved PHA-4 binding site.

Two differences in the behavior of the two ges-1 genes were also described: (1) the endogenous CB-ges-1 gene shows a low level of activity in the pharynx (and possibly in the rectum), whereas the Ce-ges-1 counterpart does not; this difference is carried over into transgenic nematodes, where the exogenous CB-ges-1 gene produces a significantly greater level of pharynx expression, whether the transformation host is *C. elegans* or *C. briggsae*, and (2) deletion of either one of the two C. elegans WGATAR sites or deletion of an immediately adjacent region activates Ce-ges-1 expression only in the anterior gut (Egan et al., 1995; Schröder and McGhee, 1998); in contrast, no evidence could be found for anterior gut expression produced by any of the variously modified CB-ges-1 constructs.

A schematic model for ges-1 control in the different modules of the Caenorhabditis digestive tract is shown in Fig. 8. The detailed argument why we feel justified in interpreting changes in patterns of ges-1 activity in terms of changes in ges-1 transcriptional initiation is provided in Egan et al. (1995) and Fukushige et al. (1996). In cells of the intestinal lineage, ges-1 control centers on the tandem pair of WGATAR sites in the 5'-flanking region. We propose that the gut-specific GATA factor ELT-2 binds to these paired sites and activates gut-specific ges-1 expression. The elt-2 gene was cloned on the basis of its binding to these WGATAR sites (Hawkins and McGhee, 1995). ELT-2 is completely gut-specific, is present in the gut immediately prior to ges-1 activation, and ectopic expression experiments show that ELT-2 is sufficient for ges-1 activation (Fukushige et al., 1998). However, ges-1 is still expressed in elt-2 mutants, suggesting that a second intestinal factor can compensate for the loss of ELT-2 (Fukushige et al., 1998). The obvious candidate for ges-1 activation in the absence of ELT-2 is the GATA factor END-1, which controls (possibly directly) the initial expression of elt-2 (Zhu et al., 1997, 1998). However, in experiments to be reported elsewhere (Fukushige and J. D. M.), we have found that ectopic END-1 cannot activate ectopic ges-1 expression in the background of an elt-2 mutant. Moreover, END-1 levels have declined by the 8E cell stage (Zhu et al., 1997) but ges-1 expression increases throughout the life of the worm (Kennedy et al., 1993).

In cells of the pharynx (and perhaps rectum as well), we propose that PHA-4 protein is bound to the conserved enhancer at the ges-1 3'-end. We have shown that expression of the WGATAR-deleted Ce-ges-1 transgene (Fukushige et al., 1996) as well as the activity of the Ce-ges-1 3'-enhancer (see above) lies genetically downstream of pha-4. PHA-4 is the *C. elegans* homolog of Drosophila forkhead and the vertebrate HNF-3α, β, γ transcription factors, is present in all committed pharyngeal precursor cells, and functions as a "pharynx identity factor" (Horner et al., 1998; Kalb et al., 1998). We have suggested that PHA-4 participates in all acts of transcription in the developing pharynx (Kalb et al., 1998). However, in the case of the ges-1 gene in the pharynx, activation by PHA-4 must be repressed by a factor that binds in or around the 5'-WGATAR region. Only when this WGATAR region is deleted is the full activation function of PHA-4 revealed.

Do our present results provide any insight into the nature of the pharynx repressor? The obvious candidate would be a GATA factor expressed in the other modules of the digestive tract. ELT-2 is ruled out because it is completely gut-specific (Fukushige et al., 1998). Although the *C. elegans* genomic sequence reveals 11 proteins with significant similarity to GATA factors, none appear good candidates for the ges-1 pharynx + rectum repressor. Two of these factors (MED-1 and MED-2) are expressed in the early EMS lineage (Maduro et al., 2001) but there is no evidence that...
these factors are present either in the (ABa-derived) anterior pharynx or in the (ABp-derived) rectum, nor whether they are present when the altered ges-1 genes are first expressed. The ELT-3 GATA factor (Gilleard et al., 1999; Gilleard and McGhee, 2001), whose principal locus of expression is the hypodermis, is also expressed in a few cells in the pharyngeal intestinal valve and the rectum but ELT-3 expression in these cells clearly begins later than does expression of the WGATAR deleted ges-1 genes. Of course, the pharynx repressor need not be a GATA factor but need only bind in the immediate vicinity of the WGATAR sites. Indeed, one possible explanation for the observation that mutation and deletion of the WGATAR sites produce somewhat different effects (Fig. 5C above, and Fig. 3 of Egan et al., 1995) is that the factors controlling the gut activation and the pharynx rectum repression have different and separable sequence requirements.

Several observations suggest that the ges-1 expression switch is likely to be more complex than depicted in Fig. 8. PHA-4 protein is present in all cells of the embryonic pharynx (Horner et al., 1998; Kalb et al., 1998) and yet the WGATAR-deleted ges-1 genes are only expressed in a subset of these cells (Egan et al., 1995; Fukushige et al., 1996), as are the reporter constructs driven by the ges-1 3′-enhancer (see Fig. 7C). Furthermore, expression of the 3′-enhancer driven reporter constructs initiates later than does expression of deleted ges-1 transgenes (data not shown) and expression in the rectum is never observed (Figs. 7A and 7B). Finally, our deletion analysis of the 3′-enhancer (Fig. 6D) points to a site, besides the PHA-4 binding site, that is important for pharyngeal expression.

A question that has persisted throughout our analysis of the ges-1 genes is: why should there be a cryptic switch in expression of a terminally differentiated gene? The evolutionary conservation of this behavior described in the present paper is a strong argument that the switch must provide some benefit to the worm, but what is this benefit? We discuss three possible models for maintaining this regulation throughout Caenorhabditis evolution.

(1) Our previous model (Egan et al., 1995; Fukushige et al., 1996) proposed that the ancestor of C. elegans and C. briggsae expressed ges-1 throughout the digestive tract. At some point in evolution, esterase expression in the pharynx (→ rectum) might have become detrimental to the worm and thus ges-1 repression would provide an advantage. However, if silencing of ges-1 expression in the pharynx were the only driving force for maintenance of the ges-1 expression switch, it would seem much more likely that the pharyngeal enhancer would simply be disabled by mutation, rather than by the elaboration and maintenance of a complex repression mechanism.

(2) Perhaps the selection pressure maintaining the ges-1 expression switch lies not in the pharynx but rather involves a role for pha-4 in the gut. It has been suggested that winged helix transcription factors may function by altering chromatin structure (Cirillo et al., 1998; Shim et al., 1998) and there are clear examples of HNF-3 and GATA factors cooperating to form preexpression complexes prior to turning on liver-specific genes in mouse embryos (Zaret, 1999). Thus, a second model would propose that the low and transient level of PHA-4 in the gut (Kalb et al., 1998) is actually necessary for GATA factors such as ELT-2 to

---

**FIG. 7.** The conserved 3′ region from the C. elegans ges-1 gene acts as a pharynx-specific enhancer. A single copy of the sequence from 7840 to 8160 bp of Ce-ges-1 was cloned in the forward orientation into pJM77 and one particular transforming array (using lin-15 as a transformation marker) was integrated into the genome to produce strain JM97. (A, B) A pretzel stage embryo and an L2 larva stained for β-galactosidase activity; note strong pharynx staining but absence of staining in the rectum. (C) A projection of a focal series of digitally deconvolved images taken through the pharynx of an L4 larva from strain JM97; expressing nuclei are visualized by GFP fluorescence.

**FIG. 8.** Schematic model to describe regulation of the ges-1 genes in the Caenorhabditis digestive tract. Full details are given in the text.
activate ges-1 and other gut-specific terminally differentiated genes. Two observations argue against this model. Even thoughpha-4 mutants do show a gut phenotype (Kalb et al., 1998), as far as we can detect, pha-4 expression in the wild-type gut actually begins later than does ges-1 expression. However, PHA-4 could still be involved in the maintenance of ges-1 expression in the gut. A stronger argument against this model is that transgenic ges-1 genes deleted for the 3’-enhancer nonetheless are still expressed in the gut (Fig. 6).

(3) The third model, which presently seems the most reasonable, proposes that ges-1 serves some function in the pharynx but only under particular environmental conditions or at particular developmental stages. Under normal conditions, ges-1 expression in the pharynx would be detrimental and is kept repressed, but the worm nonetheless retains the latent ability to activate ges-1 when needed. Two possible candidates for life stages when pharyngeal ges-1 might be advantageous are hatching and moulting. Lipid hydrolyzing enzymes (such as esterases) are produced by pharyngeal glands and implicated in the hatching of other nematodes (Bird, 1971, p. 296); moreover, pharyngeal gland cells are thought to be actively involved in both hatching and moulting in C. elegans (Singh, 1978). However, the pharynx enhancer is expressed weakly if at all in pharyngeal gland cells (Fig. 7C). A further possibility is that ges-1 is expressed in the pharynx of dauer larvae. However, our initial staining of cryostat sections of C. elegans dauer larvae did not reveal significant levels of ges-1 activity (data not shown). Perhaps ges-1 is expressed in the pharynx only under certain environmental conditions yet to be defined.

Our overall motivation for conducting these detailed analyses of the ges-1 promoters has been that the ges-1 gene somehow is responsive to a general high-level coordinate system that specifies anterior–posterior position throughout the Caenorhabditis digestive tract, both between the different modules of the digestive tract and within the gut lineage itself. However, the results of the present study do not support such a unified digestive-tract-wide coordinate system, at least in its simplest form. We have previously shown that anterior–posterior patterning within the intestine lineage (as revealed by the serendipitous expression patterns of particular ges-1 deletions) is regulated by elements of the Wnt/wingless signalling pathway (Schroeder and McGhee, 1998). The present study has begun to identify the molecules and the detailed regulatory circuits governing the switch in ges-1 expression between the different digestive tract modules and suggests that this pha-4-based intermodule regulatory pathway is entirely different from the Wnt pathway that patterns the E lineage.

ACKNOWLEDGMENTS

We thank Karen Lau for providing the images used in Fig. 1 and Barbara Goszczynski for help in identifying pharyngeal cells (Fig 7C). Some of the strains used in this work were supplied by the Caenorhabditis Genetics Centre, which is funded by the NIH National Centre for Research Resources. This work was funded by the Canadian Institutes of Health Research. J.D.M. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research (AHFMR).

REFERENCES


Received for publication May 8, 2001
Accepted August 22, 2001
Published online October 11, 2001