T he \textit{clk} class of genes of the nematode \textit{Caenorhabditis elegans} affects the timing of cellular, developmental, and behavioral features of the worm (Hekimi et al. 1995; Wong et al. 1995; Lakowski and Hekimi 1996; Branicky et al. 2000). Affected features include the cell cycle, embryonic and postembryonic development, rhythmic adult behaviors, reproductive rates, and life span. The period, duration, or rate of these processes appears to be deregulated, resulting in an average slowing down (Wong et al. 1995). \textit{clk} gene mutants can be phenotypically rescued by a maternal effect, a phenomenon that has been most extensively documented for \textit{clk-1} (Wong et al. 1995). That is, homozygous mutants derived from heterozygous mothers are phenotypically wild type except for the rate of egg production. This means that most phenotypes can be observed only in the second homozygous generation. In fact, all three \textit{clk} genes were identified in a screen designed to identify viable maternal-effect mutations (Hekimi et al. 1995). However, given that adult animals are >500 times larger than the egg, it is surprising that maternal rescue is possible for physiological features of the adult. This observation, together with other data on \textit{clk-1} (Wong et al. 1995; Branicky et al. 2000), has been interpreted to suggest that the \textit{clk} genes can induce an epigenetic state during early development that is maintained throughout the life of the animal.

One aspect of the phenotype of \textit{clk} mutants that has attracted particular attention is their increased life span (Lakowski and Hekimi 1996). Both the average and maximum life spans of \textit{clk-1}, -2, and -3 mutants exceed those of the wild type. In addition, these mutations can also act synergistically, such that double mutants live substantially longer than the component single mutants (Lakowski and Hekimi 1996). Moreover, double mutants of \textit{clk-1} with \textit{daf-2}, another gene that has been implicated in life span regulation in \textit{C. elegans} (Kenyon et al. 1993), also live a very long time, from three to five times longer than the wild type (Lakowski and Hekimi 1996). We have argued that these observations, as well as other data, suggest that \textit{clk} mutants live long because they live slowly (Hekimi et al. 1998; Hekimi 2000). It should be noted, however, that the metabolic rates of \textit{clk} mutants are not reduced in any simple fashion (Braeckman et al. 1999; Felkai et al. 1999; Miyadera et al. 2001), and it remains unclear, therefore, which progeric process has been attenuated in \textit{clk} mutants.

\textit{clk-1} was cloned and found to encode a mitochondrial protein (Felkai et al. 1999) that is structurally and functionally highly conserved throughout the eukaryotes (Ewbank et al. 1997) and in those prokaryotes whose genome is most similar to that of mitochondria (Hekimi 2000). \textit{clk-1} mutants do not make ubiquinone (coen-

\section*{Regulation of Physiological Rates in \textit{Caenorhabditis elegans} by a \textit{tRNA-Modifying Enzyme in the Mitochondria}}

Jason Lemieux, Bernard Lakowski, Ashley Webb, Yan Meng, Antonio Ubach, Frédéric Bussière, Thomas Barnes and Siegfried Hekimi

\textit{Department of Biology, McGill University, Montréal, Que´ be´ c H3A 1B1, Canada}

Manuscript received January 25, 2001
Accepted for publication June 11, 2001

\textbf{ABSTRACT}

We show that the phenotype associated with \textit{gro-1(e2400)} comprises the whole suite of features that characterize the phenotype of the \textit{clk} mutants in \textit{Caenorhabditis elegans}, including deregulated developmental, behavioral, and reproductive rates, as well as increased life span and a maternal effect. We cloned \textit{gro-1} and found that it encodes a highly conserved cellular enzyme, isopentenylpyrophosphate:tRNA transferase (IPT), which modifies a subset of tRNAs. In yeast, two forms of the enzyme are produced by alternative translation initiation, one of which is mitochondrial. In the \textit{gro-1} transcript there are also two possible initiator ATGs, between which there is a sequence predicted to encode a mitochondrial localization signal. A functional GRO-1::GFP fusion protein is localized diffusely throughout the cytoplasm and nucleus. A GRO-1::GFP initiated from the first methionine is localized exclusively to the mitochondria and rescues the mutant phenotype. In contrast, a protein initiated from the second methionine is localized diffusely throughout the cell and does not rescue the mutant phenotype. As oxygen consumption and ATP concentration have been reported to be unaffected in \textit{gro-1} mutants, our observations suggest that GRO-1 acts in mitochondria and regulates global physiology by unknown mechanisms.
zyme Q), a crucial lipid cofactor in the respiratory chain (Jonassen et al. 2001; Miyadera et al. 2001), but respires by using demethoxyubiquinone instead (Miyadera et al. 2001). In an attempt to reconcile a number of disparate observations, including the mitochondrial localization of CLK-1, the maternal effect, the absence of severe metabolic phenotypes, and the general deregulation of physiological rates observed in clk mutants, we proposed a model in which clk-1 affects, directly or indirectly, the mechanisms by which the state of mitochondrial function is relayed to the nucleus to modulate gene expression (Felkai et al. 1999; Branicky et al. 2000). Such cross talk between the mitochondria and the nucleus has been observed in several systems (Biswas et al. 1999; Frolov et al. 2000; Sekito et al. 2000; Amuthan et al. 2001) and likely serves to adapt the pattern of nuclear gene expression to changes in the metabolic state of the organism.

To test our hypothesis further, we are characterizing genetically and molecularly other clk-like genes. One such gene is gro-1, which was originally isolated as a slow-growing mutant segregating from a wild-type strain (PaC1) distinct from the standard C. elegans wild-type strain N2 (Hodgkin and Doniach 1997). Partial early characterization of the gro-1 mutants also indicated that, like the clk gene mutants, they had an increased life span (Lakowski and Hekimi 1996). Furthermore, as for clk-1, the overall metabolic capacity of gro-1 mutants was found to be essentially unaffected (Braeckman et al. 1999).

Here we present further genetic characterization of gro-1, its molecular identification, subcellular pattern of expression, and the subcellular localization required for its function. We also briefly discuss how the molecular identity of GRO-1, which is the highly conserved cellular enzyme isopentenylpyrophosphate:RNA transferase, and its requirement in the mitochondria lends credence to a role for clk genes in mitochondrial/nuclear cross talk.

MATERIALS AND METHODS

Genetic mapping of gro-1: Multipoint mapping placed gro-1 on chromosome III, close to, but to the left of, dpy-17 (Hodgkin and Doniach 1997), and close to the cloned gene clk-1 (Wong et al. 1995; Ebbank et al. 1997). Although clk-1(e2519) and gro-1(e2400) map very close to each other and have very similar phenotypes, e2400 complements e2519, indicating that these mutations are in different genes (Wong et al. 1995). By picking Lon non-Dpy recombinant progeny from dpy-17 clk-1 lon-1 gro-1 animals, we had confirmed that gro-1 maps very close to clk-1 but were unable to separate clk-1 and gro-1 (Hekimi et al. 1995). We now mapped gro-1 0.03 cM to the left of clk-1. For this, the following experiments were performed: gro-1/dpy-17 unc-32 [dpy-17] A (g1-51 unc-32); dpy-17 gro-1/+ [30/200 Dpys developed quickly, p < 0.2 cm]; gro-1/lon-1/+ [2/250 Lon developed quickly, p = 0.4 cm]; dpy-17 gro-1/ma-4/unc-79 clk-1/lon-1 [unc-79 44 dpy-17 gro-1 0 0 2 ndy-14] [Sma-Non-Dpy recombinants: dpy-17 22 gro-1 13 dpy-17 27 lon-1 27 sma-4]; dpy-17 includes gro-1(e2400); sDf121 deletes gro-1(e2400).

Moving gro-1 from the PaC1 to the N2 background: To remove linked PaC1 sequences from gro-1(e2400), the gro-1(e2400) mutation was first flanked on the left and on the right by the two closest morphological markers, dpy-17(e164) and lon-1(e185), respectively. The dpy-17(e164) gro-1(e2400) lon-1(e185) triple mutant was then outcrossed three times to remove unlinked sequences. These markers were then removed in several steps. First, the closest marker, dpy-17(e164), was removed by crossing unc-79(e1030) males into the dpy-17(e164) gro-1(e2400) lon-1(e185) strain and picking Lon non-Dpy recombinants. In this way, a unc-79(e1050) gro-1(e2400) lon-1(e185) strain was generated. This strain was then outcrossed with N2 males and Unc non-Lon recombinants were picked to generate a unc-79(e1030) gro-1(e2400) strain. Finally, this unc-79(e1030) gro-1(e2400) strain was outcrossed a third time with N2 males and 340 non-Unc F2 progeny were placed individually on plates. Four of the 340 F2 broods developed slowly and segregated ¼ Uncs [putative unc-79(e1030) gro-1(e2400)/gro-1(e2400) strains]. From one of these plates non-Unc progeny were picked individually to new plates to generate a homozygous gro-1(e2400) strain, which was given the strain name MQ520. In this process, unlinked chromosomes were outcrossed nine times, presumably removing almost all unlinked PaC1 sequences.

PCR amplification: For PCR reactions using clean abundant template, a single pair of primers was used. The notation AB is used here, where A is the first primer and B the second. In cases where the template of interest is rare and nonspecific amplification is likely, nested PCR was used. The notation A/C/B is used here, where A and B are the primers in the first reaction and C and D are the primers in the second “nested” reaction. The sequences of all primers cited can be obtained from S.H. upon request.

Construction of clones for rescuing experiments: The deletion construct pMQ2 was made by deleting a 29.9 kb SpeI fragment of ZC395. The frameshift construct pMQ4 was made by cutting pMQ2 at a unique ApaI site in the second predicted exon of ZC395.7 and degrading the resulting 4 bp overhang with mung bean nuclease. pMQ5 was made by cutting pMQ2 at a unique Ndel site in the second predicted exon of ZC395.6 and filling in the resulting 2 bp overhang with the Klenow fragment of DNA polymerase. pMQ8 is a construct in which residues 27,230–27,522 of cosmid C34E10 were fused to residues 3678–3867 of cosmid ZC395. The region of C34E10 is the region immediately 5’ of the gro-1 coding sequence and encodes the entire intragenic region between gro-1 and C34E10,8, which is a transcript in the opposite direction. It must thus contain the entire promoter of the gro-1 operon. The region from ZC395 contains the entire gro-1 coding sequence as well as 23 nucleotides 5’ of the initiator ATG and 134 nucleotide 3’ of the stop codon. To construct this clone we performed recombinant PCR, using hybrid primers, that is, single primers complementary to more than one DNA fragment. The hybrid primers, which were complementary in part to the promoter sequence and in part to the gro-1 coding sequence, were SHP159 and SHP160. The flanking primers used were SHP161 and SHP162, which had the restriction site for SacI and PstI, respectively, built into their 5’ ends. Three sequential PCR reactions were performed. The first PCR reaction used the primers SHP161 and SHP160 and N2 genomic DNA as template to amplify the promoter. The second PCR reaction used the primers SHP159 and SHP162 and N2 genomic template to amplify gro-1. The third PCR reaction used the flanking primers SHP161 and SHP162 and the gel-purified products from the first and the second PCR reaction as tem-
plate to fuse the two products. The high-fidelity polymerase VENT (New England Biolabs, Beverly, MA) was used.

Construction of clones for expression experiments: The gro-1::gfp clone pMQ418 was constructed using vector pPD95.77 (a generous gift from A. Fire). The construct pMQ8 (see above) was used as template for amplification with SHP151:SHP170. SHP151 and SHP170 contain overhangs that will incorporate SpH1 and XbaI restriction sites into the PCR product, respectively, to allow for the ligation of the fragment (containing the gro-1 promoter and coding sequence) into the SpH1/XbaI sites of pPD95.77.

Clone pMQ418 was used as template for site-directed mutagenesis [Stratagene (La Jolla, CA) site-directed mutagenesis kit] to construct clones pMQ420 (using primers SHP1860: SHP1861) in which the first potential initiator ATG is changed to ATC, pMQ421 (using primers SHP1862:SHP1863) in which the second potential initiator is changed to ATC, and pMQ441 (using primers SHP1858:SHP1859) in which the A at nucleotide position 33 between the two ATGs is removed. This creates a frameshift such that the GRO-1 protein cannot be produced after initiation from the first ATG. The sequence of all clones produced by PCR was verified by sequencing.

Identifying the e2400 mutation: Genomic DNA was prepared by standard techniques and the primers SHP93:SHP92 (on the basis of the known genomic sequence) were used to amplify the gro-1 region from N2, PaC1, and gro-1(e2400) (CB4512). The PCR regimen was 94°C for 20 sec, 55°C for 1 min, and 72°C for 2 min, for 30 cycles. These sequences were performed with the following primers: SHP93, -94, -95, -96, -97, -98, -99, -100, and -92.

Establishing splicing and trans-splicing patterns: RNA was extracted by standard methods from mixed-stage worms and used to make reverse-transcribed cDNA libraries (Frohman et al. 1988). To determine which of the genes upstream of gro-1 were transcribed in the same operon, the 5′ end of each was amplified using SL1 or SL2 primers. The SL1/2 primers were used in conjunction with a gene-specific primer. A second round of PCR, using the same SL primer but a different internal primer, was then performed to obtain a very specific product. For gop-1 the internal primer pair used was SHP141/ SHP142 and the expected product size was ~570 bp. The primer pair for gop-2 was SHP143/SHP144 to produce a product of ~510 bp. The primer pair SHP145/SHP146 was used to amplify the 440-bp gop-3 5′ end. For ham-1, the primer pair SHP130/SHP119 was used in the amplification of the 465-bp 5′ end. Finally, to amplify gro-1′s 390-bp 5′ end, the primer pair SHP95/SHP99 was used.

The primer Rt, used to amplify the cDNA library, has two primer landing pads Ri and Ro built into its 5′ end. Each of the five genes of the operon was amplified from this cDNA in a number of pieces: the 5′ end, using a primer corresponding to the trans-spliced leader sequence SL2 (when the gene was trans-spliced; see below) and two internal primers, and the 3′ end using the primers Ri and Ro along with an internal primer. This allowed priming for PCR at the end of each cDNA. gop-1 was amplified in three parts: the 5′ part was amplified with the nested primers SHP190:SHP174/SHP176, the middle part with SHP172/SHP173:SHP176, and the 3′ end with SHP175:Ri/Ro. gop-2 was amplified with the primers SL2:SHP143/SHP144 and SHP180:Ri/Ro. gop-3 was amplified with the primers SL2:SHP184/SHP135 and SHP138:Ri/Ro. ham-1 was amplified with the primers SL2:SHP99/SHP100, SHP94:SHP99/SHP100, and SHP97:Ri/Ro.

In all experiments designed to establish splicing patterns, the PCR regimen used was 94°C for 20 sec, 60°C for 1 min, 72°C for 2 min, for 30 cycles for the first PCR; and 94°C for 20 sec, 60°C for 1 min, 72°C for 2 min, for 30 cycles for the second nested PCR.

hGRO-1 sequence: The human protein sequence shown in the alignment of Figure 3 was obtained by compiling information from several sources. We sequenced a publicly available clone (c-2ec05), obtained from Genome Systems, whose partial sequence (accession nos. F07677 and Z40724) encoded a protein similar to GRO-1. We then identified overlapping clones in the database and assembled a predicted mRNA sequence for a human gro-1 gene. Sequences that were used include the following accession nos.: AA332152, AA121465, AA847885, and NP_060116.

RESULTS

Genetic mapping of gro-1 and transfer into the N2 genetic background: Multipoint mapping placed gro-1 on chromosome III, close to, but to the left of, dpy-17 (Hodgkin and Doniach 1997) and close to the cloned gene clk-1 (Wong et al. 1995; Ewbank et al. 1997). Detailed mapping of the region allowed us to genetically separate gro-1 from clk-1 and map it 0.03 cM to the left of clk-1 (see MATERIALS AND METHODS).

Originally, the growth and life span of mutants carrying the gro-1(e2400) mutation were characterized in the strain CB4512 (Lakowski and Hekimi 1996; Hodgkin and Doniach 1997). To facilitate phenotypic comparisons between gro-1 and other genes that have been isolated in the N2 background, we transferred the gro-1(e2400) mutation into a background as close as possible to that of N2. To remove linked PaC1 sequences from gro-1(e2400), the mutation was first flanked on the left and on the right by the two closest morphological markers, dpy-17(e164) and lon-1(e185), respectively. These markers were then removed in several steps (see MATERIALS AND METHODS).

In this process, unlinked chromosomes were outcrossed nine times, removing almost all unlinked PaC1 sequences, while remaining linked PaC1 sequences should represent <200 kb centered around gro-1.

Phenotype of gro-1 mutants: gro-1 mutants display a general slowing down of physiological features. The affected features we scored included the duration of embryogenesis and postembryonic development, the rates of adult behaviors, and brood size (Table 1). In addition, the animals appear in general to behave sluggishly, yet they appear anatomically normal and are capable of complex behaviors such as mating for males. The most striking aspect of the phenotype, however, is the existence of a maternal effect that extends to adult phenotypes and includes development, adult behavior, and reproductive phenotypes (Table 1). In fact, homozygous gro-1 mutants derived from a heterozygous mother are essentially indistinguishable from the wild type. On the other hand, heterozygotes produced by mating of wild-type males to homozygous gro-1 mothers are mostly wild type except for the duration of embryonic development, which is clearly slower than that of wild-type or mater-
TABLE 1

The phenotype of gro-1(e2400) mutants can be rescued by a maternal effect

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Wild type (N2)</th>
<th>e2400/e2400 (MQ520)</th>
<th>e2400/+ from e2400/e2400 mothers (zygotic rescue)</th>
<th>e2400/e2400 from e2400/+ mothers (maternal rescue)</th>
<th>+/+ and +/+ e2400 from +/+ e2400 mothers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryogenesis</td>
<td>13.9 ± 1.6</td>
<td>22.5 ± 2.0</td>
<td>17.0 ± 1.43</td>
<td>13.7 ± 0.5</td>
<td>13.6 ± 0.5</td>
</tr>
<tr>
<td>(hr)</td>
<td>(n = 26)</td>
<td>(n = 115)</td>
<td>(n = 64)</td>
<td>(n = 27)</td>
<td>(n = 82)</td>
</tr>
<tr>
<td>Brood size</td>
<td>302 ± 31</td>
<td>177 ± 52</td>
<td>248 ± 75</td>
<td>291 ± 50</td>
<td>291 ± 34</td>
</tr>
<tr>
<td>(eggs)</td>
<td>(n = 20)</td>
<td>(n = 12)</td>
<td>(n = 10)</td>
<td>(n = 27)</td>
<td>(n = 56)</td>
</tr>
<tr>
<td>Postembryonic development</td>
<td>46.8 ± 1.4</td>
<td>104.9 ± 10.1</td>
<td>43.2 ± 2.4</td>
<td>47.7 ± 2.1</td>
<td>47.5 ± 2.2</td>
</tr>
<tr>
<td>(hr)</td>
<td>(n = 200)</td>
<td>(n = 70)</td>
<td>(n = 49)</td>
<td>(n = 129)</td>
<td>(n = 389)</td>
</tr>
<tr>
<td>Defecation cycle</td>
<td>54.3 ± 2.5</td>
<td>88.6 ± 7.5</td>
<td>52.1 ± 1.9</td>
<td>57.8 ± 2.8</td>
<td>54.9 ± 3.1</td>
</tr>
<tr>
<td>(sec)</td>
<td>(n = 18)</td>
<td>(n = 25)</td>
<td>(n = 10)</td>
<td>(n = 28)</td>
<td>(n = 72)</td>
</tr>
</tbody>
</table>

*All phenotypes were scored at 20° as in Wong et al. (1995).

†Progeny from heterozygous (+/e2400) hermaphrodites or putative cross-progeny were placed singly on individual plates and their phenotype was scored. Their genotype was later established from the phenotype of their clonal progeny, either slow growing (e2400/e2400) or wild-type growth rate (+/+ or +/+ e2400).
TABLE 2
The effect of gro-1(e2400) on life span in different wild-type backgrounds

<table>
<thead>
<tr>
<th>Strain</th>
<th>Life span (days) ± SEM</th>
<th>[maximum life span (days)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (wild type)</td>
<td>20.03 ± 0.3*</td>
<td>37</td>
</tr>
<tr>
<td>PaC1 (wild type)</td>
<td>23.1 ± 0.3*</td>
<td>200</td>
</tr>
<tr>
<td>MQ520 [gro-1(e2400)] in N2 background</td>
<td>22.3 ± 0.5*</td>
<td>43</td>
</tr>
<tr>
<td>CB4512 [gro-1(e2400)] in PaC1 background</td>
<td>31.3 ± 0.9*</td>
<td>56</td>
</tr>
</tbody>
</table>

*The error of the mean of two independently conducted experiments is given.

The sample size corresponds to the total number of animals examined.

To test further the significance of the difference in means of N2 and MQ520, the populations from the two independent experiments were pooled (SD, standard deviation for the pooled population), a t-test was performed, and a highly significant difference was found (P < 0.001).

Maximum life span (days).

region were used for transformation rescue. Cosmids ZC395 (that contains also clk-I) and C34E10 were capable of fully rescuing the Gro-1 phenotype, including the slow growth and behaviors. The narrow overlap between the cosmids defined a rescuing region of ~7 kb. An ~3.5-kb deletion clone (pMQ2) of ZC395 containing the left-most end of the cosmid also produced full rescue. pMQ2 contains two predicted genes, ZC395.6 and ZC395.7. To identify which corresponds to gro-1, each predicted gene was tested independently for rescuing activity by introducing frame-shift mutations in the other gene (see MATERIALS AND METHODS). The clone (pMQ4) that contains an intact ZC395.6 gene but an inactivated ZC395.7 could fully rescue the phenotype, but the clone (pMQ5) that contains an intact ZC395.7 but inactivated ZC395.6 could not.

ZC395.6 was resequenced from the mutant and by comparison to the N2 sequence the e2400 lesion was identified as a small rearrangement starting at nucleotide 2720 of ZC395 (the sequence TGCAATGTA is replaced by GC). This results in a frameshift producing a 33-amino-acid extension after the lesion in the predicted mutant protein. The lesion was not found in the gro-1(+) gene in the PaC1 background.

gro-1 lies in an operon with four other genes: Examination of genes predicted by Genefinder (http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml) in the genomic sequence upstream of gro-1 suggested that gro-1 could lie in an operon of up to five genes. For genes to be organized into such a transcriptional unit they must share the same 5’-3’ orientation and be closely positioned (Zorio et al. 1994). Approximately 70% of C. elegans genes are trans-spliced at their 5’ end to a 22-bp sequence known as spliced leader (SL1). Genes downstream in operons are unique in that they are trans-spliced to a distinct leader (SL2; Huang and Hirsh 1989). Therefore, the presence of SL2 in an mRNA can be used as a marker indicating a gene’s membership in an operon. We examined the trans-splicing of four genes upstream of gro-1, using SL1- or SL2-specific primers in conjunction with pairs of gene-specific primers to amplify the 5′ ends of these genes from a reverse transcription (RT)-PCR-generated cDNA library (see MATERIALS AND METHODS). gro-1 and the three genes upstream of it were all found to be spliced to SL2 (Figure 2). Therefore, gro-1 appears to be the fifth member of a five-gene operon. We renamed the first three genes in the operon gop-1 to -3 (for gro-1 operon). The fourth gene was named hap-1 as it is strongly homologous to the yeast gene ham1, which is involved in hydroxylaminoparaine sensitivity (Noskov et al. 1996).

We also established the splicing pattern of all five genes by sequencing their cDNAs amplified from an RT-PCR library (see MATERIALS AND METHODS). In general, the predictions made by Genefinder were correct with three exceptions. What was predicted to be the last exon of gop-3 actually splices over C34E10.9 (a small predicted gene on the complementary strand) to pick up two additional exons (Figure 2). We also found an additional intron in the predicted second exon of gro-1. Finally, the true gro-1-initiating AUG was actually 42 bp upstream of the predicted start. The sequences of all five genes are deposited in GenBank.

The molecular identity of GRO-1: The protein encoded by gro-1 is highly similar to a highly conserved cellular enzyme, isopentenylpyrophosphate: tRNA transferase (IPT; Bartz et al. 1970; Caillet and Droogmans 1988; Tolerico et al. 1999; Stanford et al. 2000). Figure 3 illustrates the sequence conservation from Escherichia coli (MiaAp) to Saccharomyces cerevisiae (Mod5p) and Homo sapiens (hGRO1). The human sequence was obtained by sequencing a publicly available clone and database searches. The residue at which the e2400 mutation truncates the protein is indicated by an asterisk in the figure. IPT catalyzes the transfer of an isopentenyl moiety to the adenosine immediately adjacent 3′ to the anticodon of tRNAs whose anticodons terminate in U. In bacteria, the gene that encodes IPT is generally called miaA and mutations in this gene have been studied extensively for their effect on growth and gene expression (Ericson and Bjork 1986; Bjork et al. 1999). In yeast, IPT is encoded by the gene mod5 (Dihanich et al. 1987), whose products are being studied mostly for
Figure 2.—gro-1 is the most downstream gene in an operon containing five genes. (A) The 5′ region of the five genes forming the putative gro-1 operon were PCR amplified from a cDNA library (see materials and methods). SL1/2 primers were used with gene-specific reverse primers. No PCR bands could be detected using a SL1 forward primer for any of the five genes. However, a specific band could be detected using a SL2 forward primer for gop-2, gop-3, hap-1, and gro-1, indicating that these genes are arranged in an operon. (B) The structure of the gro-1 operon and the intron/exon structure of all genes. The 3′ untranslated region of each gene is denoted by black boxes. The positions of gene-specific reverse primers used in A are denoted by arrows.

The mechanisms involved in their complex subcellular localization (Tolerico et al. 1999). MiaAp/Mod5p-like proteins are encoded by all sequenced bacterial and eukaryotic genomes so far but appear to be absent from archaeal genomes (Stanford et al. 2000). The prokaryotic and eukaryotic sequences are highly similar (Figure 3), and there appears to be only one gro-1-like sequence in the genome of any organism whose genome has been fully sequenced (Stanford et al. 2000). The eukaryotic sequences differ mostly by a C-terminal extension that contains a predicted zinc finger motif in the human and worm sequences (Figure 3). The sequences that allow nuclear localization in S. cerevisiae also map to this region (Tolerico et al. 1999). The other recognizable structural motif found in all IPT sequences is an ATP/GTP binding site motif (Figure 3).

Of the genes in the operon, gop-1 to -3 encode proteins that have highly conserved vertebrate homologs of unknown function. Only gop-2 encodes a protein with a recognizable motif, an ATP/GTP binding site motif of the same type as GRO-1 (p-loop). The fourth gene in the operon, hap-1, is similar to the yeast gene HAM1, mutations in which can confer resistance to mutagenesis by 6-N-hydroxylaminopurine (Noskov et al. 1996) by an unknown mechanism. However, recent work on a putative homolog in the Archaea Methanococcus jannaschii suggests that it might be an NTPase that can hydrolyze nonstandard nucleotides (Hwang et al. 1999).

Expression of GRO-1: To study the expression of gro-1 we first constructed a synthetic gene in which the gro-1 coding sequence is placed directly adjacent to, and thus under the control of, the putative promoter sequence of the operon, that is, the intergenic sequence between gop-1 and the next 5′ gene (C34E10.8; see materials and methods). This construct (pMQ8) rescues the gro-1 phenotype to wild-type (growth rate) or near wild-type (defecation cycle length) values (Tableotic and eukaryotic sequences are highly similar (Figure 3), and there appears to be only one gro-1-like sequence in the genome of any organism whose genome has been fully sequenced (Stanford et al. 2000). The eukaryotic sequences differ mostly by a C-terminal extension that contains a predicted zinc finger motif in the human and worm sequences (Figure 3). The sequences that allow nuclear localization in S. cerevisiae also map to this region (Tolerico et al. 1999). The other recognizable structural motif found in all IPT sequences is an ATP/GTP binding site motif (Figure 3).

Using pMQ8, we then constructed a gro-1:gfp reporter gene (see materials and methods). This construct (pMQ418) rescues the phenotype to the same degree as pMQ8, indicating that the fusion protein is fully active. The green fluorescent protein (GFP) expression was very mosaic but essentially every cell could be observed to express it in at least a subset of animals (data not shown). The subcellular pattern of expression was diffuse, filling the entire cell (Figure 4, A–D and F). In some cells (most frequently in neurons) the fluorescence in the nucleus was less intense than in the cytoplasm (Figure 4G). This broad subcellular localization is consistent with the findings in yeast, in which the two products of MOD5 are distributed uniformly in the nucleus, cytoplasm, and mitochondria (Gillman et al. 1991; Tolerico et al. 1999).

In yeast, the subcellular distribution of Mod5p is achieved by the production of two different proteins from the same transcript by alternative translation initiation (Gillman et al. 1991). The longer form (Mod5p-I) contains a mitochondrial targeting sequence but some of the protein remains extramitochondrial. The
shorter form (Mod5p-II) is produced by initiation from the next ATG in the open reading frame and is partitioned between the cytoplasm and the nucleolus. In Figure 3, the two first methionines of Mod5p and GRO-1 are highlighted by a double underline. To study whether the wide distribution observed with GRO-1::GFP was achieved in a way similar to that of Mod5p, we used site-directed mutagenesis to change the first or the second ATG in the coding sequence (residue 15) to ATC (isoleucine). We also introduced a frameshift between the two ATGs by deleting an A at position 33 of the nucleotide sequence, starting with the A of the first ATG. Thus, two constructs, gro-1(Met15Ile)::gfp and gro-1(DelA33)::gfp, should be able to produce only a short form of the protein starting at the second methionine, lacking the mitochondrial localization sequence. Both these constructs gave strong expression and showed a distribution that appeared identical to that of the unmodified gro-1::gfp sequence (Figure 4F). However, an absence of fluorescence in the mitochondria could probably not have been observed within a broadly fluorescent cytoplasm. Neither of the two constructs was capable of rescuing the mutant phenotype.

In contrast to the short forms, the gro-1(Met15Ile)::gfp construct appeared to be expressed exclusively in the mitochondria (Figure 4, E and I) in a pattern indistinguishable from that of other mitochondrial proteins (Figure 4H). This construct was able to rescue the mutant phenotype, including those features that were precisely quantified (Table 3). Taken together, these findings strongly suggest that GRO-1 is normally distributed throughout the cell and that two forms of the protein are produced by the use of alternative translation initiation sites. Furthermore, they indicate that it is the absence of wild-type gro-1 activity in the mitochondria that is responsible for the phenotypes we observe in the mutant.

DISCUSSION

Maternal rescue of gro-1 mutants: We show here that gro-1(e2400) mutants have a highly pleiotropic phenotype, including altered developmental, behavioral, and reproductive rates. Furthermore, gro-1(e2400) increases life span, albeit in a manner that depends in part on the genetic background. The gro-1 phenotype can be rescued by a maternal effect, so that homozygous gro-1::gfp produces a transcript that is indistinguishable from wild-type animals. There are many indications (Golovko et al. 2000; Stanford et al. 2000) that the metazoan homologs of MiaAp/Mod5p are also modifying tRNAs. tRNAs, however, are abundant molecules whose total number is likely to increase in parallel with the volume of the animal, which increases >500-fold during postembryonic development. The finding of an almost complete maternal rescue is therefore surprising. Gro-1 would have to be an ex-
extremely stable and active protein (which is not the case in bacteria; Leung et al. 1997) for the heterozygous mother’s contribution of material to the egg (mRNA or protein) to be sufficient to carry out the gene’s function adequately in the much larger adult animal. On the basis of similar observations with the clk-1 gene we suggested an alternate model: that the presence of maternally contributed GRO-1 early during embryonic development could establish an epigenetic state that can last throughout development (Wong et al. 1995; Branicky et al. 2000). Investigations of the levels of the GRO-1 protein, and of modified tRNAs, during the development of maternally rescued animals will help to distinguish between these alternatives.

Subcellular localization of GRO-1: mod5 encodes IPT in yeast (Dihanich et al. 1987), and its gene product exists in two forms: one form that is found in the mitochondria (where it modifies mitochondrial tRNAs) and in the cytoplasm and one form that is found in the cytoplasm and the nucleus (Martin and Hopfer 1982; Gillman et al. 1991; Boguta et al. 1994; Tolerico et al. 1999). These two forms differ by only a short N-terminal sequence whose presence or absence is determined by differential translation initiation at two “in frame” ATG codons (Gillman et al. 1991). The gro-1 open reading frame also contains two ATG codons at comparable positions, with the coding sequence between the two codons constituting a plausible mitochondrial sorting signal. We found that a GRO-1::GFP fusion protein is localized throughout the cell. When the first putative initiator ATG is removed by site-directed mutagenesis, GRO-1 is still abundantly expressed in the cytoplasm and nucleus, indicating that the second ATG is a good initiator codon. Furthermore, when the second initiator ATG of gro-1 is removed, GRO-1 is localized exclusively to the mitochondria. These findings strongly suggest that the cellular distribution of GRO-1 is achieved by mechanisms very similar to those observed in yeast with Mod5p.

MiaAp, the bacterial homolog of GRO-1, regulates gene expression: Mutations in miaA, the bacterial homolog of gro-1, have broadly pleiotropic consequences in various bacteria species, including effects on growth rate (Diaz et al. 1987), on the rate of spontaneous GC → TA transversions (Connolly and Winkler 1989) in E. coli, on the regulation of the biosynthesis of amino acids in Salmonella typhimurium (Ericson and Bjork 1986; Blum 1988), and on the regulation of virulence genes in Shigella flexneri (Gray et al. 1992). In Shigella, it was found that the level of the virulence-associated protein

Table 3

Transgenic expression of GRO-1, GRO-1::GFP, and GRO-1::GFP with altered distribution

<table>
<thead>
<tr>
<th>Genotypea</th>
<th>Defecation cycle length (sec)</th>
<th>Postembryonic development (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gro-1(+)</td>
<td>55.3 ± 3.6</td>
<td>60.3 ± 0.4</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td>(n = 310)</td>
</tr>
<tr>
<td>gro-1(e2400)</td>
<td>93.2 ± 9.2</td>
<td>104.9 ± 4.7</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td>(n = 126)</td>
</tr>
<tr>
<td>gro-1(+)::qmEx217[gro-1; (pMQ8)]</td>
<td>63.5 ± 6</td>
<td>64.7 ± 4.2</td>
</tr>
<tr>
<td>(n = 24)</td>
<td></td>
<td>(n = 56)</td>
</tr>
<tr>
<td>gro-1(e2400)::qmEx218[gro-1; (pMQ8)]</td>
<td>69.2 ± 7.4</td>
<td>68.1 ± 0.1</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td>(n = 53)</td>
</tr>
<tr>
<td>gro-1(+)::qmEx215[gro-1::gfp; (pMQ418)]</td>
<td>67.7 ± 8.8</td>
<td>64 ± 2.3</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td>(n = 85)</td>
</tr>
<tr>
<td>gro-1(e2400);qmEx219[gro-1::gfp; (pMQ418)]</td>
<td>64.7 ± 7.6</td>
<td>69.5 ± 6.6</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td>(n = 44)</td>
</tr>
<tr>
<td>gro-1(e2400);qmEx211[gro-1::gfp; (pMQ418)]</td>
<td>68.9 ± 6.1</td>
<td>66.3 ± 4.4</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td>(n = 81)</td>
</tr>
<tr>
<td>gro-1(+)::qmEx220[gro-1(Met1Ile)::gfp; (pMQ420)]</td>
<td>66.4 ± 7.0</td>
<td>61.4 ± 2.7</td>
</tr>
<tr>
<td>(n = 21)</td>
<td></td>
<td>(n = 87)</td>
</tr>
<tr>
<td>gro-1(e2400);qmEx214[gro-1(Met1Ile)::gfp; (pMQ420)]</td>
<td>84.7 ± 9.5</td>
<td>97.5 ± 8.9</td>
</tr>
<tr>
<td>(n = 19)</td>
<td></td>
<td>(n = 59)</td>
</tr>
<tr>
<td>gro-1(+)::qmEx221[gro-1(Met15Ile)::gfp; (pMQ421)]</td>
<td>69.9 ± 7.5</td>
<td>56.1 ± 5.9</td>
</tr>
<tr>
<td>(n = 14)</td>
<td></td>
<td>(n = 91)</td>
</tr>
<tr>
<td>gro-1(e2400);qmEx216[gro-1(Met15Ile)::gfp; (pMQ421)]</td>
<td>61.0 ± 4.9</td>
<td>63.8 ± 4.4</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td>(n = 118)</td>
</tr>
<tr>
<td>gro-1(e2400);qmEx213[gro-1(DelA33)::gfp; (pMQ419)]</td>
<td>87.2 ± 16.4</td>
<td>100.9 ± 9.9</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td>(n = 51)</td>
</tr>
</tbody>
</table>

The names of the clones microinjected to form the extrachromosomal arrays are given in parentheses. The construction of the clones is described in MATERIALS AND METHODS.
VirF was reduced 10-fold in the mutant but that the level of mRNA was unaffected. These observations together with the fact that MiaAp modifies a component of the translation machinery suggested that MiaA acts by post-transcriptional mechanisms (Bjork et al. 1999).

The function of GRO-1 in mitochondria: We found that it is the mitochondrially expressed form of GRO-1, which is important for the dramatic phenotypes we observe in the mutant. Our findings thus suggest that a broad regulatory function for IPT has been conserved from bacteria to the mitochondria of metazoans. As in most animals, the number of proteins encoded and expressed in the mitochondria of nematodes is fairly limited (Keddie et al. 1998), and all of them are elements of the respiratory chain. However, the respiration, ATP levels, and metabolic capacity of gro-1 mutants do not appear to be strongly affected (Braeckman et al. 1999). If GRO-1 exerts its effect by acting on translation only, the actual translation defect in the mutant must be relatively subtle as it does not grossly impair mitochondrial function. Thus, at present it is unclear what defect produces the major phenotypic effects we observe in the mutants.

As described in the Introduction, clk-1 is another gene that affects physiological rates and can produce a maternal effect (Wong et al. 1995). As CLK-1 is a mitochondrial protein (Felkai et al. 1999), to explain all the phenotypic features of clk-1 mutants, in particular the mito-
chondrial localization, the maternal effect, and the absence of major metabolic defects, we hypothesized that CLK-1 affects, perhaps indirectly, the cross talk between the mitochondria and the nucleus (Branicky et al. 2000). Such cross talk would be useful in allowing the pattern of nuclear gene expression to be modulated in response to the metabolic state of the cell. In this model, during early development the presence of CLK-1 or GRO-1 in the mitochondria of maternally rescued clk-1 or gro-1 mutants, respectively, would allow the presence of metabolically competent mitochondria to be signaled to the nucleus. This in turn could lead to the establishment of a stable pattern of gene expression resulting in a wild-type phenotype, that is, rapid growth, behavior, and reproduction throughout the life of the animal.

We are grateful to Alan Coulson and Andy Fire for the gift of clones and to Anne Wong for technical assistance. We are particularly grateful to Robyn Branicky for reviewing the manuscript. Some strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Center for Research Resources of the U.S. National Institutes of Health. This work was supported by a studentship from the Medical Research Council (MRC) of Canada to J.L., by a studentship from the Fonds pour la Formation de Chercheurs et l’Aide à la Recherche to B.L., and by grants from the MRC of Canada to S.H.

LITERATURE CITED


Regulation of Physiological Rates in \textit{C. elegans}


Communicating editor: P. Anderson