Induction of Glyoxylate Cycle Expression in *Caenorhabditis elegans*: A Fasting Response throughout Larval Development†

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ABSTRACT: The mRNA and the bifunctional protein for the two glyoxylate cycle (GC) enzymes, isocitrate lyase and malate synthase, are expressed in a tissue- and stage-specific pattern in *Caenorhabditis elegans*. Since expression of the two enzymes for the carbon-conserving glyoxylate cycle is regulated by the availability of carbon sources in microorganisms, we have studied the bifunctional GCP gene expression under fasting conditions and in certain mutants of *C. elegans* in order to understand possible mechanisms regulating its expression during nematode development. The GCP mRNA and protein levels were elevated in early larvae which were never fed, a result consistent with previous enzyme activity measurements (Khan, F. R., & McFadden, B. A. (1982) *Exp. Parasitol. 54*, 48−54]. However, larvae of later stages also expressed higher levels of GCP mRNA and protein when they were shifted from normal to fasting growing conditions. The GCP expression appeared to be regulated primarily at the transcriptional level throughout development. Although the expression of both the GCP gene and *lin-14* peaks at about the same time during development and are induced by fasting, the regulation of the GCP gene is independent of the heterochronic *lin-14* control mechanism of postembryonic lineages, as demonstrated by the fact that there was no significant change of the GCP at both mRNA and protein levels in the heterochronic *lin-4 (lf)* and *lin-14 (gf)* mutants compared to the wild type.

The glyoxylate cycle (GC)[1] bypasses much of the tricarboxylic acid cycle (TCA cycle) to create a shunt between isocitrate and malate (Saz, 1954; Saz & Hillary, 1956; Kornberg & Krebs, 1957). The reactions catalyzed by the two glyoxylate cycle-specific enzymes, isocitrate lyase (ICL) and malate synthase (MS), are as follows:

ICL (EC 4.1.3.1)

$$\text{isocitrate} \rightleftharpoons \text{glyoxylate} + \text{succinate}$$

MS (EC 4.1.3.2)

$$\text{glyoxylate} + \text{acetyl-CoA} + H_2O \rightleftharpoons \text{malate} + \text{CoASH}$$

Nutritional regulation of the expression of ICL and MS has been studied mostly in microorganisms because of the anaplerotic function of the GC. It was found that the expression of ICL and MS is regulated by induction and repression/derepression mechanisms based on different carbon sources (Polakis & Bartley, 1965; Kornberg, 1966; Maloy & Nunn, 1982; De Lucas et al., 1994). When acetate or fatty acids are the only nutrients, the GC is induced to divert the production of acetyl-CoA to biosynthesis of carbohydrates and other macromolecules for the needs of the cell. In germinating plant seedlings, the GC participates in the conversion of stored fat to carbohydrate (Carpenter & Beevers, 1959; Canvin & Beevers, 1961; Beevers, 1969).

Nematodes have been the only animal phylum in which the presence of active GCs has been well-studied. Among nematodes, it was first established in *Ascaris lumbricoides* that fatty acid catabolism is shunted to carbohydrate biosynthesis during embryogenesis (Barrett et al., 1970). During *C. elegans* embryogenesis, the increase of the activities of both ICL and MS is found to correlate with an increasing ratio of carbohydrate to triglycerides (Khan & McFadden, 1980).

Although mechanisms underlying regulation of GC enzyme expression in *C. elegans* have not been determined as in microorganisms, previous work shows that starvation induces ICL and MS activities. We have recently cloned the cDNA from *C. elegans* which encodes ICL and MS (Liu et al., 1995). The two enzymes are encoded by a single gene and expressed as GCP, a single bifunctional polypeptide. Immunofluorescence microscopy shows that GCP first appears in intestinal and then body wall muscle cells. These cells remain the primary sites of the localization of GCP during larval development. The cloning of the *C. elegans* GCP cDNA, the use of a monoclonal antibody specific to the protein (anti-GCP mAb), and the availability of specific heterochronic mutants have allowed us to further study the response of GCP expression to fasting at both mRNA and protein levels as the first step in understanding its regulatory mechanisms during development.

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[1] Abbreviations: GC, glyoxylate cycle; GCP, glyoxylate cycle protein (bifunctional isocitrate lyase/malate synthase); mAb, monoclonal antibody; ICL, isocitrate lyase; MS, malate synthase.

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MATERIALS AND METHODS

General methods for nematode growth, immunobloting, and Northern blot hybridization have been described previously (Liu et al., 1995). For immunofluorescence microscopy, nematodes were grown on NGM plates seeded with *E. coli* strain OP50. For immunoblotting, nematodes were grown on 8P plates seeded with *E. coli* strain NA22 (Sulston & Brenner, 1974).

Immunofluorescence Microscopy of Larvae and Adults. Fresh nematodes washed free of bacteria were freeze-fractured in the same way as embryos by placing them between a 0.05% polylysine-coated glass slide and a coverslip (Liu et al., 1995; Epstein et al., 1993). Samples were then fixed in 3% freshly made paraformaldehyde for 20 min. After two washes with phosphate-buffered saline (0.29 M NaCl, 0.072 M KH_2PO_4, 0.228 M Na_2HPO_4, pH 7.2), samples were reacted with anti-GCP mAb followed by fluorescein-conjugated, affinity-purified goat anti-mouse IgG secondary antibody, and photographed as described (Liu et al., 1995).

Generation of Synchronous Embryos and Nematodes. Nematodes synchronized at specific stages were grown up from embryos which had been obtained by dissolving well-fed gravid adults in sodium hypochlorite (Epstein et al., 1993). Embryos generated this way were within a 2−3 h synchrony in developmental stages. Early embryos were those that were just released from the adults, ranging in their stages from 0 to 3 h of development. Late embryos were allowed to develop without food for 8 h on 8P plates after release from adult hermaphrodites. Later stage worms were collected after growing on 8P plates for certain periods of time. Plating of embryos released from gravid adults constituted the starting time point. The following time intervals were used for collecting worms of different stages: 0−15 h, 15−30 h, 30−39 h, 39−48 h, and 48−58 h, theoretically giving rise to L1, L2, L3, L4, and adult worms (Cassada & Russell, 1975; Byerly et al., 1976; Hirsh et al., 1979). To prepare samples for immunoblot analysis, all worms were washed 3 times in M9 buffer, dissolved in SDS buffer at 95 °C for 10−15 min, spun quickly on a microcentrifuge, frozen in liquid nitrogen, and stored at −80 °C (Liu et al., 1995). Protein concentrations were determined by the Bradford method using a kit from BioRad (Hercules, CA). Total RNA from the same samples was isolated and CsCl-gradient-purified as described (Liu et al., 1995).

Growth of Fasted Larvae. Fasted L1 larvae were obtained by plating embryos on 8P plates without bacteria and harvested after 21 h. Fasted larvae of later stages were generated by harvesting worms which had been grown for 36 h with bacteria, washed clean of bacteria, and then regrown on 8P plates without bacteria for 12 more hours. Controls were those which were regrown on 8P plates with bacteria for the same amount of time, giving populations of mixed L2−L4 larvae.

Growth of Heterochronic Mutants. Two heterochronic mutant strains, *lin-4 (e912)* II, a loss-of-function mutant, and *lin-14 (n536)* X, a gain-of-function mutant, were obtained from the *C. elegans* Genetics Center (University of Minnesota, St. Paul, MN). Worms were grown on 8P plates seeded with *E. coli* strain NA22. Embryos from gravid adults were obtained by dissolving gravid worms with sodium hypochlorite solution as described previously. Embryos were plated on 8P/NA22 plates and allowed to hatch for 32 h before harvesting. At this time point, most worms were at L2 and L3 stages. Wild-type N2 worms grown under the same conditions were obtained as controls for immunoblot and Northern blot hybridization analyses.

Quantification of Immunoblot and Northern Hybridization. The quantification method for immunoblot employed a chemiluminescence technique using a detecting system (LumiGLO Chemiluminescent Substrate Kit from Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD]. The manufacturer's procedures were followed with slight modification; 20 μg of protein in worm homogenate was separated by SDS−PAGE containing 6% polyacrylamide. The proteins were transferred to nitrocellulose filter paper, blocked, and reacted with the anti-GCP mAb as described (Liu et al., 1995). The blot membrane was then reacted with a goat anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (BioRad, Hercules, CA) at a 1:5000 dilution for 1 h at room temperature. After three washes in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5% Tween-20, the membrane was reacted with the KPL kit for 1 min at room temperature. Exposure was performed immediately after substrate reaction by using the same film for the Northern and Southern hybridization as described. Quantification was performed on an LKB UltraScan XL densitometer (Piscataway, NJ). Data in the linear range were used for quantitative analysis. The linear range was determined with a serial dilution of *C. elegans* homogenates by the same procedure. Each sample was assayed in triplicate.

GCP mRNA levels were determined by Northern blot analysis, largely following procedures described previously (Liu et al., 1995). Briefly, the RNA was run through a 1% agarose gel. Probes were prepared by hexamer-random labeling the three *Eco*RI fragments of the GCP cDNA with specific activities $>1.0 \times 10^9$ cpm/μg of DNA. Hybridization was conducted at 50 °C. Membrane washing conditions were once in 3 × SSPE [20 × SSPE: 3.0 M NaCl, 0.2 M NaH_2PO_4, and 0.025 M EDTA, pH 7.4 (Sambrook et al., 1987)]/0.1% SDS, 52 °C for 15 min, once in 1 × SSPE/0.1% SDS at 52 °C for 15 min, and finally in 1 × SSPE/0.1% SDS at 65 °C for 15 min. The radioactivity for each band was quantified using a Betascope 603 Blot Analyzer (βEtagen, Framingham, MA). Membranes were stripped of probe by two washes in 5% glycerol at 85 °C, 15 min each. Stripped membranes were reprobed with a *Pst*I restriction fragment of pCe7 plasmid corresponding to the 18S rRNA gene from *C. elegans* (Files & Hirsh, 1981). This second probe served as a control for loading error. Each sample was assayed in triplicate.

RESULTS

GCP Expression during Development. The development of *C. elegans* can be divided into embryogenesis, four larval molting, and sexually mature adult stages (Wood, 1988). In order to study possible regulatory mechanisms affecting GCP expression in *C. elegans*, we characterized the spatial and temporal pattern of this expression. *C. elegans* GCP was previously shown to be expressed in intestine and body-wall muscle cells during embryogenesis (Liu et al., 1995). In order to test whether this expression pattern continues in later stages of development, indirect immunofluorescence microscopy was performed in larval and adult worms (Figure

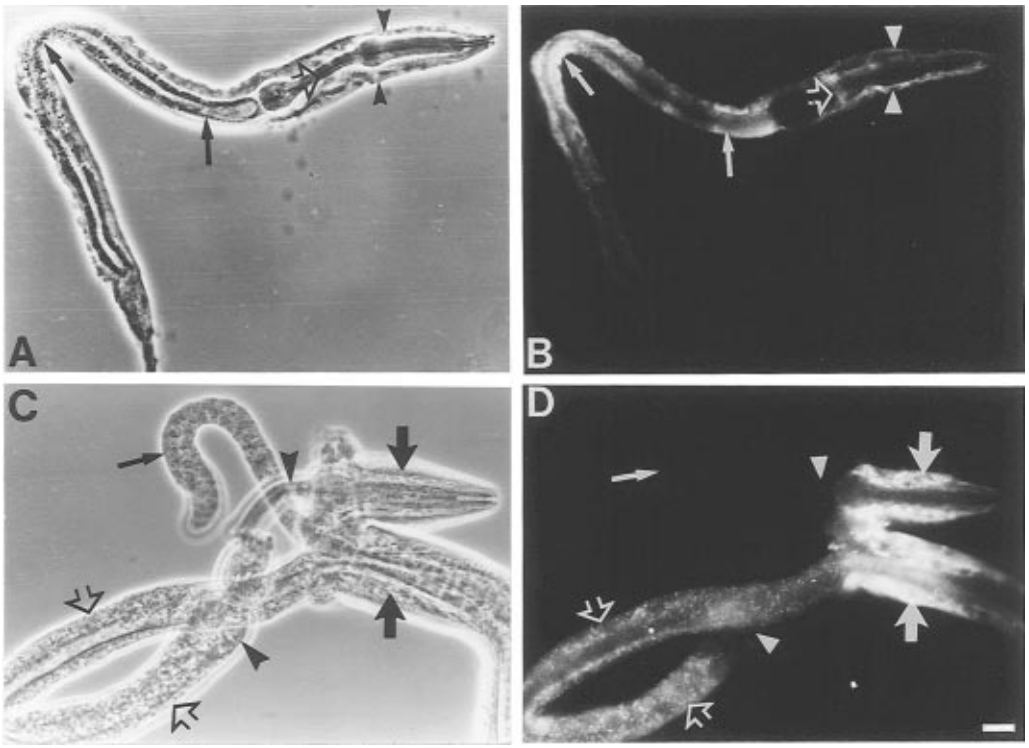

FIGURE 1: Immunofluorescence of GCP in larvae and adult. (A) Phase contrast of an early larva showing intestine (arrows), body-wall (arrowheads), and the nerve ring (open arrow). (B) Anti-GCP mAb staining showing the expression of GCP in the same animal as in (A). Note the labeling of the intestine, body-wall, and the nerve ring (or the glial-like cells). (C) Phase contrast of a young adult showing intestine (open arrows), body-wall (large arrows), gonad (small arrow), and pharynx (arrowheads). (D) Anti-GCP mAb staining showing the expression of GCP of the same animal as in (C). Note the labeling of the intestine and body-wall, but not the gonad and pharynx. Bar, 1 μm.

1). Figure 1A shows an early larva which was labeled with the specific anti-GCP mAb (Figure 1B). Prominent expression of GCP was observed in the intestine (arrows) and in body-wall muscles (arrowheads). This is very similar to the pattern of late embryos reported previously. Figure 1C shows a young adult with the intestine (open arrows), the pharynx (arrowheads), and the gonad (small arrow) squashed out of the body. In addition, the body-wall (large arrows) was also visible. Anti-GCP mAb labeled both the body-wall and the intestine but did not label pharynx or the gonad (Figure 1D).

GCP expression at mRNA and protein levels during development was also studied by Northern blotting and immunoblotting (Figure 2). Previous work demonstrates that enzymatic activities of ICL and MS increase during embryogenesis, are maximal at the early L1 larval stage, and decline as larval development progresses (Khan & McFadden, 1980, 1982; Wadsworth & Riddle, 1989). As shown by Northern hybridization, GCP mRNA expression was relatively low in early embryos. As development proceeded, GCP mRNA increased in late embryos, reached its highest level in L1 stage larvae, and then declined to a stable level in later development (Figure 2A). The expression pattern at protein level was similar to that of mRNA except for the early embryos, which contained even lower relative levels of GCP (Figure 2B), which is consistent with previous immunofluorescence observations that embryos earlier than 150 min of development did not show detectable GCP (Liu et al., 1995). Therefore, the GCP mRNA and protein expression patterns are consistent with the previous enzyme activity experiments. The relatively high level of GCP mRNA in early embryos (compared to that of protein) could be the result of either maternal or early zygotic transcription leading to a stored mRNA without translation.

GCP Expression in Fasting Larvae. In bacteria and yeast, the expression of GC enzymes ICL and MS is inducible when acetate or fatty acids are the only nutrients available. Previous work also showed that both ICL and MS activities in *C. elegans* were induced in larvae when they hatched and remained without food (Khan & McFadden, 1982). We repeated this experiment in order to establish the levels of GCP mRNA and GCP expression underlying the enzymatic activities. Both mRNA and protein levels were elevated in fasted larvae compared to well-fed ones of comparable developmental stages (Figure 3A). However, nematode development is arrested in the early L1 stage when they are hatched without food. Previous fasting experiments did not demonstrate whether nematodes of later stages could also show this fasting response. GCP expression was studied in late larvae by allowing embryos to hatch and develop past the L2 larval stage with sufficient food available. These larvae (designated as late larvae) were then transferred to 8P plates without food and grown for 12 more hours, while control nematodes continued to grow on plates with sufficient food. Both Northern hybridization and immunoblotting showed that GCP mRNA and protein levels were induced by fasting (Figure 3B). The distribution of GCP, as demonstrated by immunofluorescence, showed no obvious change in fasted larvae compared to fed ones, even though the expression levels were elevated (data not shown).

GCP Expression in Heterochronic Mutants. Figure 2 shows high GCP mRNA and protein expression in late embryos and early L1 larvae, confirming and extending previous work on enzyme activities (Khan & McFadden,

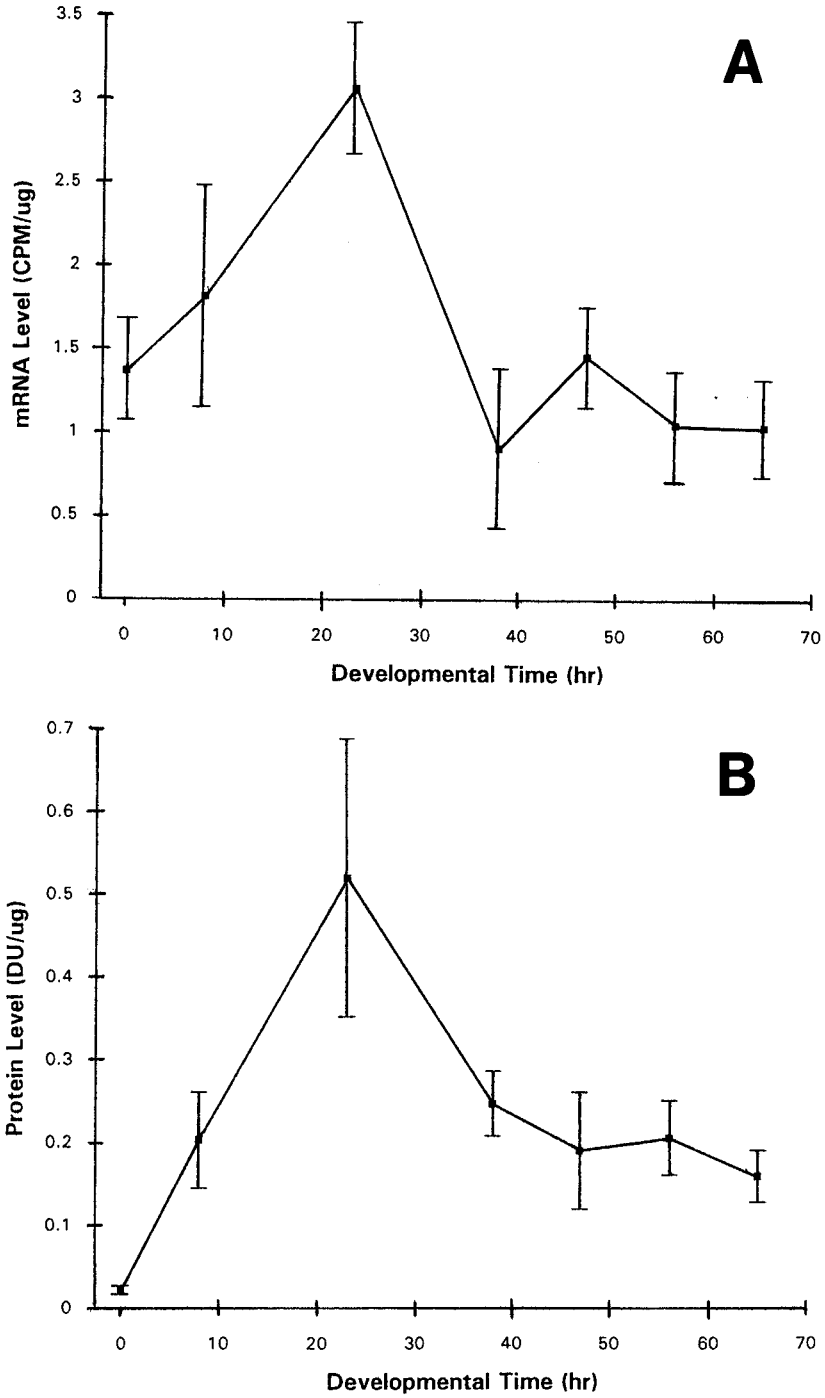

FIGURE 2: GCP expression during development. Nematodes of different developmental stages were harvested and prepared for Northern blot hybridization and immunoblotting (see Materials and Methods for details). (A) β-Counting of ^{32}P of Northern blot hybridization of total RNA isolated from three samples. (B) Densitometry data of immunoblots from the same nematode cultures used for Northern hybridization. GCP levels were quantified as per microgram of protein (DU/μg).

1982). The *lin-14*, *lin-4*, and *lin-28* genes of *C. elegans* form a regulatory hierarchy which temporally regulates cell lineages generated during postembryonic development (Ambros, 1984). *Lin-14* specifies L1 stage cell fates (Ambros & Horvitz, 1984, 1987). *Lin-4* negatively regulates the translation of *lin-14* mRNA. *Lin-14* expression has its peak level around the same period as GCP during development and is also induced by fasting (Wightman et al., 1993),

In order to examine whether GCP expression is regulated by *lin-14*, Northern hybridization and immunoblotting were performed in strains carrying a *lin-14* gain-of-function mutation and a *lin-4* loss-of-function mutation that produce heterochronic changes in postembryonically generated cell lineages. LIN-14 protein is already undetectable in N2 nematodes after the L1 stage while it is still high in both *lin-4 (lf)* and *lin-14 (gf)* mutants. In these experiments, nematodes were allowed to develop past the L1 stage. Results showed that there was no significant difference at either mRNA (Figure 4A) or protein (Figure 4B) levels among the three strains of nematodes, suggesting that regulation of GCP expression is independent of the *lin-14* translational control mechanism.

DISCUSSION

As a first step to study the developmental regulation of GCP expression in *C. elegans*, we have characterized the

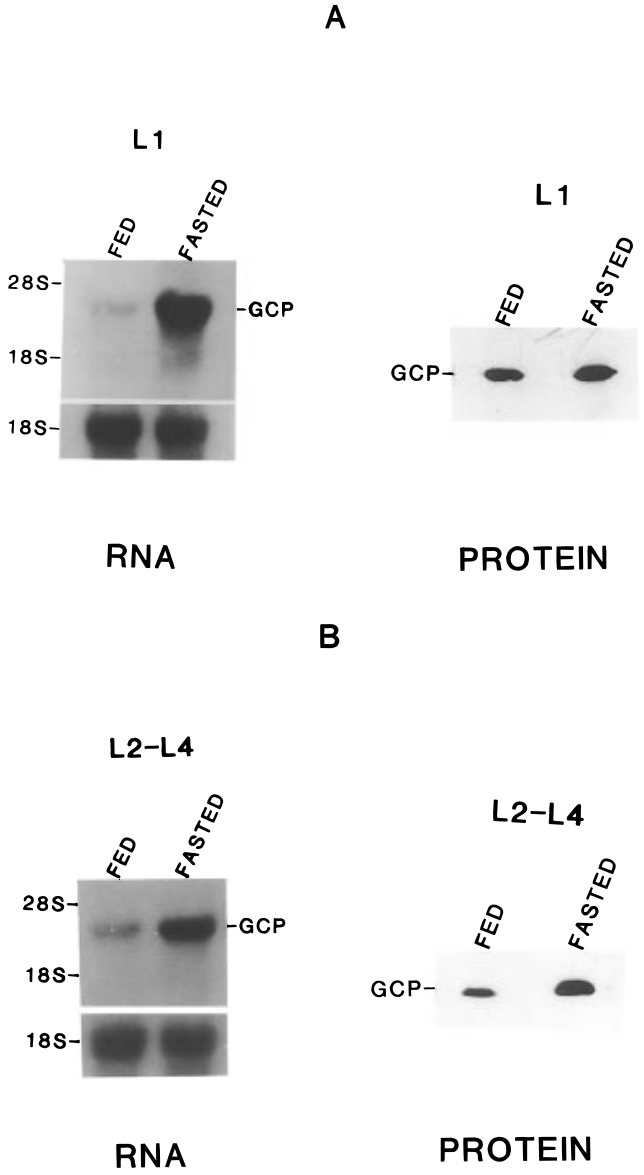

FIGURE 3: GCP expression in fasted larvae. GCP mRNA and GCP in fasted larvae were compared with fed ones of comparable stages. (A) L1 larvae showing markedly increased GCP mRNA and slightly increased GCP after fasting. (B) Larvae of mixed L2−L4 stages showing markedly increased GCP mRNA and GCP when fasted. In Northern hybridization, 10 μg of total RNA was loaded per lane. The same blots were also hybridized with a *C. elegans* 18S rRNA probe to show equal loading.

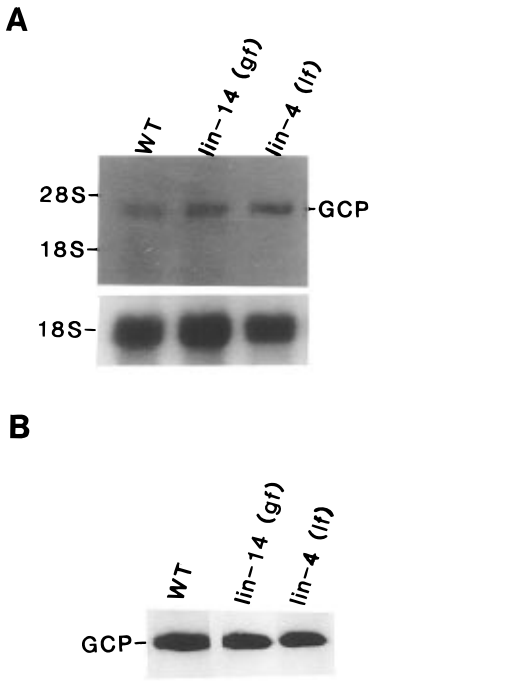

FIGURE 4: GCP expression in heterochronic mutants. Post-L1 larvae were harvested for Northern hybridization (A) and immunoblotting (B). The three strains used were the N2 wild type, the *lin-14* (*n536*) X, gain-of-function, and *lin-4* (*e912*) II, loss-of-function. Note there were no significant changes of GCP mRNA and GCP among the three strains of animals. In Northern hybridization, 10 μg of total RNA was loaded per lane. The same blots were also hybridized with a *C. elegans* 18S rRNA probe to show equal loading as in Figure 3.

developmental pattern and fasting response in the larvae of both early and late stages. The results showed that GCP expression in *C. elegans* was tissue- and stage-specific, increasing during embryogenesis, reaching maximal values in late embryos and early larvae, and then declining. Fasting experiments showed that GCP expression was induced in early fasted L1 larvae, which is consistent with a previous report (Khan & McFadden, 1982). We extended our study to examine the fasting response of GCP expression in later larvae, which also showed induction. The GCP mRNA and protein are coordinately regulated as shown by Northern and Western blot analyses of fasted larvae and nematodes of different developmental stages, suggesting that the GCP expression is mainly regulated at the transcriptional level. On the other hand, translational control may play a role during early embryonic development since only a low level of GCP is expressed in the presence of the relatively high level of GCP mRNA in early embryos. Although both the GCP gene and *lin-14* show peak expression at the same L1 stage, GCP expression is unaffected by *lin-14* and its regulatory gene *lin-4* which controls *lin-14* expression at the translational level (Wightman et al., 1993). We conclude that the mechanisms regulating GCP expression are independent of the *lin-14* mechanism.

Our early fasting results are consistent with a previous report in which ICL activities were studied in starved early larvae (Khan & McFadden, 1982). Although ICL activities were measured in that experiment for an extended period of starvation from hatching onward, the direct response of later larvae to starvation was not evaluated. Our results clearly demonstrated that GCP expression was induced by fasting even in previously well-fed larvae in stages from L2 through L4. Therefore, GCP expression appears to be induced by fasting throughout larval development. It is interesting that a previous report shows that ICL activities in predauer and dauer nematodes closely parallel those from L2 larvae, decreasing gradually throughout predauer development and into dauer stage itself (Wadsworth & Riddle, 1989). Dauer larvae represent an alternative developmental pathway to L2 larvae which can be induced by prolonged fasting. Since we have shown that GCP expression can be induced by fasting in L2 larvae, the mechanisms operating to regulate GCP expression in dauer larvae must be different from the ones during normal development. We have also showed that the regulation of GCP expression is independent of control by *lin-14*. Therefore, the fasting response is autonomous of both the *lin-14* and dauer mechanisms.

The results of these fasting experiments prompted us to consider other possible mechanisms for the regulation of

GCP expression. The carbohydrate carbon source may affect GCP expression through induction and repression/derepression mechanisms as in microorganisms (Carpenter & Beevers, 1959; Canvin & Beevers, 1961; Beevers, 1969). In mammals, carbohydrates (mainly in the form of glycogen) are consumed before fats during fasting. If the same situation holds in nematodes, fasting would then cause the decline of the carbohydrate level which, in turn, could act as a signal to derepress GCP expression. Indeed, our results showed that the expression of GCP mRNA and protein is elevated by fasting (Figure 3), consistent with the results of a previous report which showed elevated enzymatic activities of both ICL and MS (Khan & McFadden, 1982). Further testing of regulatory mechanisms is limited by the dependence of *C. elegans* as a microbiotroph upon ingestion of particles like bacteria as an optimal food source (Sulston & Hodgkin, 1988). Defined liquid media have been developed (Vanfleteren, 1980); however, the ingestion of liquid is inefficient (Sulston & Hodgkin, 1988), and a quasi-fasting state appears to be induced (F. Liu, J. D. Thatcher, and H. F. Epstein, unpublished observations).

This putative induction mechanism is also supported by the patterns of GCP expression and GC activity throughout *C. elegans* development. The existence of an active GC makes it possible for organisms to convert acetate or fat into carbohydrates because of the net production of one molecule of succinate in each round of the GC. In early nematode embryos, as in plant seedlings, there are relatively high concentrations of triglycerides and low concentrations of carbohydrates. Triglycerides contain a high density of calories and carbon skeletons relative to carbohydrates. The conversion of fat to carbohydrate correlates with increasing GC activities in both plants and nematodes. In *C. elegans*, ICL and MS activities inversely correlate with the ratio of triglyceride/carbohydrate during embryogenesis (Khan & McFadden, 1980), supporting the argument that the GC helps convert fat into carbohydrates in this species. Our present results (Figure 2) and previous experiments (Khan & McFadden, 1980, 1982; Liu et al., 1995) showed that the pattern of GCP expression is consistent with the proposed hypothesis. The initially high amounts of triglycerides and low amounts of carbohydrates in embryos (Khan & McFadden, 1980) may derepress/induce GCP expression during embryogenesis. However, the amount of the triglycerides would be gradually reduced as development proceeds, and when embryos hatch and larvae are adapted to feeding for outside nutritional sources, GCP expression would be repressed and its mRNA and protein levels decline after having reached peak expression.

A very recent report (Popov et al., 1996) shows that both isocitrate lyase and malate synthase can be detected in starved rat liver. It would be interesting to test whether the bifunctionality of the *C. elegans* GC enzymes and its transcriptional response to fasting also hold in mammalian systems.

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