Ferritin is a ubiquitous protein that sequesters iron and protects cells from iron toxicity. *Caenorhabditis elegans* express two ferritins, FTN-1 and FTN-2, which are transcriptionally regulated by iron. To identify the cis-acting sequences and proteins required for iron-dependent regulation of *ftn-1* and *ftn-2* expression, we generated transcriptional GFP reporters corresponding to 5’-upstream sequences of the *ftn-1* and *ftn-2* genes. We identified a conserved 63-bp sequence, the iron-dependent element (IDE), that is required for iron-dependent regulation of a *ftn-1* GFP reporter in intestine. The IDE contains two GATA-binding motifs and three octameric direct repeats. Site-directed mutagenesis of the GATA sequences, singly or in combination, reduces *ftn-1* GFP reporter expression in the intestine. In vitro DNA mobility shift assays show that the intestine-specific GATA-binding motifs and three octameric direct repeats. Site-directed mutagenesis of the GATA sequences, singly or in combination, reduces *ftn-1* GFP reporter expression in the intestine. In vitro DNA mobility shift assays show that the intestine-specific GATA-binding motifs and three octameric direct repeats. Site-directed mutagenesis of the GATA sequences, singly or in combination, reduces *ftn-1* GFP reporter expression in the intestine. Insertion of the IDE into the promoter region of a heterologous reporter activates iron-dependent transcription in intestine. These data demonstrate that the activation of *ftn-1* and *ftn-2* transcription by iron requires ELT-2 and that the IDE functions as an iron-dependent enhancer in intestine.

Iron is essential in many biological processes, including DNA synthesis, respiration, nitrogen fixation, oxygen transport, heme synthesis, and photosynthesis. At high levels, however, iron can be toxic because of its ability to react with molecular oxygen to generate free radicals that oxidize DNA and proteins and initiate lipid peroxidation, all of which can lead to cell injury and death (1). Consequently, organisms have developed mechanisms to sense, acquire and store this metal within a narrow physiologic range.

Ferritin sequesters iron in a form that is biologically available but unable to catalyze free radical formation (2, 3). Mammalian ferritin has a molecular mass of ~480,000 and consists of 24 related subunits of two types, a light subunit (L) and a heavy subunit (H). These subunits assemble to form a shell surrounding a cavity that can accommodate up to 4500 iron atoms. The H-subunits oxidize ferrous iron to ferric iron within the cavity; the L-subunits lack ferrooxidase activity and function with the H-subunits in iron nucleation. Mutations in either the ferritin H or the ferritin L gene can be deleterious. Ferritin H knockout mice die early in embryogenesis (4), while mutations in ferritin H or ferritin L genes are associated with hereditary conditions, such as hyperferritinemia cataract syndrome (5, 6), adult onset basal ganglia disease (7), and autosomal dominant iron-overload disease (8).

Ferritin induction by iron is regulated by transcriptional and post-transcriptional mechanisms that are organism-specific (9–11). In vertebrates, ferritin expression is primarily regulated at the translational level by cytosolic proteins that bind to iron-responsive elements (IREs) in the 5’- or 3’-untranslated regions of mRNAs of ferritin-H and ferritin-L mRNAs (12). When iron is scarce, these cytosolic proteins, iron-regulatory protein 1 (IRP1) and iron-regulatory protein 2 (IRP2), bind to the ferritin 5’ IREs, repressing ferritin translation and decreasing iron sequestration. When iron is abundant, IRPs lose affinity for the IREs, derepressing ferritin translation and increasing iron sequestration. IRP1 and IRP2 expression are regulated by iron. Iron causes an [4Fe-4S] cluster to assemble in IRP1, converting it from an RNA-binding protein to a cytosolic aconitase lacking RNA binding activity, whereas iron mediates the ubiquitination and proteasomal degradation of IRP2 (13, 14). In addition to iron-dependent translational regulation, iron has been shown to induce mammalian ferritin L transcription (15–19). The mechanism regulating ferritin L transcription is unclear.

In insects, ferritin is regulated by both transcriptional and post-transcriptional processes. Insects express ferritin H (HCH) and ferritin L (LCH) subunits, but only the ferritin HCH mRNA contains an IRE (10, 20). Iron is also reported to regulate ferritin LCH transcription (21) and an alternatively spliced ferritin HCH mRNA (22). Plants lack the IRE-IRP regulatory system (23), and instead utilize iron-dependent transcription as their primary mechanism for the regulation of ferritin expression (24–26). An element located in the promoters of *Zea mays* *Fer1* and *Arabidopsis thaliana* *Fer1* genes is required for transcriptional repression of ferritin during iron deficiency (26, 27).
C. elegans express two ferritins, FTN-1 and FTN-2, that are similar to mammalian ferritin H subunits, because they contain ferroxidase active-sites (28, 29). Unlike vertebrate and insect ferritins, C. elegans ftn-1 and ftn-2 mRNAs lack IRESs, and instead their transcription is regulated by iron (28, 29). A ftn-1 null strain exhibits a reduced life span when grown on excess iron, demonstrating that ftn-1 offers C. elegans protection from the toxic effects of excess iron (29).

While iron-dependent transcription has been well described in plants (24–26, 30–33) and fungi (34–37), less is known about iron-regulated transcription in animal cells. In this study, we have identified a conserved 63-bp element that activates the transcription of a ftn-1::GFP reporter in intestine. This element contains two GATA binding motifs and three direct repeats, all of which are necessary for ftn-1 reporter expression in the intestine. We found that the intestine-specific GATA protein ELT-2 binds to the GATA motifs and regulates ftn-1 GFP reporter expression. Furthermore, the 63-bp element is necessary and sufficient to activate iron-dependent transcription of a heterologous GFP reporter gene in intestine. These data indicate that the 63-bp element can function as an iron-dependent enhancer in intestine.

**EXPERIMENTAL PROCEDURES**

Worm Strains and Culture—All worm strains were cultured on nematode growth medium (NGM) agar plates seeded with Escherichia coli OP50 at 22 °C unless otherwise noted. For iron experiments, larvae were grown for 1-24 h on NGM plates supplemented with 6.6 mg/ml ferric ammonium citrate (FAC) or 0.1 mM 2,2'-dipyridyl (BP) for designated times. The pH of FAC-supplemented NGM agar was adjusted to pH 7.0. Strains used in this study are described in Table 1.

Construction of Reporter Constructs and Transgenic Lines—ftn-1::GFP-his and ftn-2::GFP-his transcriptional reporters were generated by amplification of −1.9 kb and −2.0 kb of genomic DNA (relative to their ATG start codons) from ftn-1 (C5456.14) and ftn-2 (D10370.3), respectively, using forward and reverse primers containing SalI and Nhel restriction sites. The fragments were inserted into the Sall-Nhel sites of pAP.10 (kindly provided by Susan Mango, University of Utah). pAP.10 was generated from pPD97.78 (Andrew Fire, Stanford University) by replacing GFP with GFP-histone (h2b) from pH4b52. pPD97.78 contains a minimal pes-10 promoter fused to GFP, which lacks basal transcriptional activity, but is activated by enhancers. ftn-1(Δ63)::GFP-his was generated by the deletion and the replacement of the 63-bp element in ftn-1::GFP-his with nonspecific bacterial DNA (5'-CTTAATCAAGTGAGGCACC- TATCTACGGATCTGTATTGTTGACCTGAATGAC-3'). The GATA mutants ftn-1(G1m)::GFP-his, ftn-1(G2m)::GFP-his, and ftn-1(G12m)::GFP-his were generated by mutation of GATA > CAT A in ftn-1::GFP-his using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The sequence of the 5' regulatory region of ftn-1 is shown in supplemental Fig. S1.

The pes-10(+63)::GFP-his reporter was generated by PCR-amplification of the 63-bp element in TopoZero Blunt vector (Stratagene) and inserting it into the Nhel-Sall site of pAP.10. DR1m, DR2m, DR3m, and the triple DR123m mutants were generated in pes-10(+63)::GFP-his by site-specific mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Primers used for the generation of these reporters are shown in supplemental Fig. S2. Transgenic strains were generated using a standard microinjection procedure (60). Each GFP-his construct (20 ng/μl) and a selection plasmid pBX-1 (100 ng/μl) were coinjected into pha-1 animals. Transgenic animals were obtained after growth at 20 °C. For ftn-2::GFP-his constructs, lin-15 was used as a coinjection marker. Similar GFP patterns were observed for independent strains generated for each reporter.

**Microscopy**—GFP expression in transgenic worms was visualized using either an Axioplan 2 or Axio Imager (Carl Zeiss MicroImaging, Inc, Thornwood, NY) equipped with a Chroma HQ ENDOW GFP BP filter set (excitation HQ470/40, dichroic Q495LP, emission HQ525/50) or Zeiss filter set 38HE (BP 470/40 HE, dichroic FT 495 HE, BP 525/50 HE), respectively. Images and overlays were captured using an AxioCam HRM (Carl Zeiss MicroImaging, Inc, Thornwood, NY) camera run by AxioVision software. Images were rotated, cropped, and sized using Adobe Photoshop.

**Worm Sorting**—Worms expressing ftn-1::GFP-his and ftn-2::GFP-his reporters were synchronized by treating young gravid adults with alkaline hypochlorite and allowing the eggs to hatch overnight in sterile S-basal medium (0.1 mM NaCl, 0.05 mM potassium phosphate, pH 6.0, 5 μg/ml cholesterol). Synchronized L1 larvae were harvested by centrifugation 450 g and spotted onto one 10-cm NGM plate seeded with OP50. Following 8–10 h of growth, L1 larvae were rinsed from the plate and washed by several rounds of centrifugation in M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4, 86 mM NaCl, 1 mM MgSO4). These
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L1 larvae were spotted onto control, FAC- or BP-supplemented NGM plates (10 cm) seeded with OP50 bacteria and grown for an additional 16–18 h. L2/L3 larvae were rinsed from the plates and washed with M9 supplemented with 0.1% Triton X-100 until free of bacteria and debris. Worms were analyzed using a COPAS™ (complex object parametric analysis and sorting) Biosort (Union Biometrica, Somerville, MA). Prior to data collection, a population of animals was selected (gated) based upon extinction and time of flight, sorted and verified by microscopy. The same gating parameters were used for all three experimental conditions during GFP fluorescence acquisition and subsequent analysis. Data were analyzed using FCS Express Version 3.0 Lite software (De Novo Software, Ontario, Canada).

The rescue of iron-depleted worms expressing the *ftn-1::GFP-his* construct was performed as above with the following exceptions. After 16–18 h of growth on control (untreated) or BP-supplemented NGM plates, worms were washed off the plates with M9 and the population of L2/L3 worms was divided in half. Half of each population was sorted and analyzed as described above, while the remaining populations were pelleted at 450 g and spotted onto new plates. Worms grown on BP-supplemented NGM plates were spotted onto FAC-supplemented NGM plates. Larvae were grown for an additional 24 h and then washed, sorted, and analyzed as described above.

**RNA Interference (RNAi)**—Plasmids purified from *elt-1* (W09C2.1) and *elt-2* (C33D3.1) genomic clones from the MRC RNAi feeding library (38) and *elt-2* (C33D3.1) and *elt-6* (F52C12.5) from the ORFeome-based RNAi library (39) were used as templates for PCR amplification reactions using platinum *Pfu* polymerase (Invitrogen) and T7 primers. Double-stranded RNA (dsRNA) was produced from the PCR products using the MEGAscript RNAi kit (Ambion, Inc., Austin, TX) according to the manufacturer’s protocol. dsRNA was injected at a concentration of 0.5 mg/ml into both gonads of young adult *C. elegans* hermaphrodites expressing the *ftn-1::GFP-his* reporter (control injected, *n* = 10; *elt-2* (MRC), *n* = 17; *elt-1*, *n* = 10: *elt-2* (ORF), *n* = 11; *elt-6*, *n* = 13). After recovering for 8–10-h postinjection, individual worms were picked to their own NGM plates for 12 h. F1 progeny from these 12-h broods 8–10-h postinjection, individual worms were picked to their

**Northern Blot Analysis**—Northern blot analysis was performed by fractionating total RNA (25 μg) on a 1.2% formaldehyde agarose gel followed by the transfer of the RNA to a membrane. The membrane was sequentially probed with 32P-labeled *ftn-1* and *ftn-2* probes (28). As a loading control, 28 S and 18 S ribosomal RNA were visualized using ethidium bromide.

**Electrophoretic Mobility Shift Assay (EMSA)**—Wild type and mutant IDE probes were excised from TopoZero blunt (Stratagene) with EcoRI, gel-purified and labeled with 50 μCi of 32P[α-dATP] and Klenow DNA polymerase. Recombinant ELT-2 expressed in baculovirus was a generous gift from Dr. Jim McGhee (University of Calgary) (40) and also produced in our laboratory. The EMSA reactions (20 μl) were carried out in reaction buffer (10 mM Tris-HCl, pH 7.5, 4% glycerol, 1 mM MgCl2, 1 mM dithiothreitol), 32P-labeled wild type or mutant IDE probes (0.4–1 ng), poly dI-dC (80 ng), and ELT-2 (1–2 μl). For competition experiments, unlabeled IDE DNA, or G12m DNA (10–100× molar amounts) was added to the reactions 5 min before the addition of a 32P-labeled IDE probe. The reaction was incubated at 22 °C for 25 min, and the complexes were fractionated by electrophoresis on a 5% non-denaturing polyacrylamide gel (37:5:1 cross-linking) in 0.5× TBE (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA) at room temperature. The gel was dried and exposed to a phosphorimager.

**RESULTS**

*ftn-1* and *ftn-2* mRNAs Are Transcriptionally Regulated by Iron—To determine the kinetics for *ftn-1* and *ftn-2* mRNAs response to iron and iron chelators, we grew L3 larvae on NGM plates and NGM plates supplemented with FAC or the iron chelator 2,2′-dipyridyl (BP) for 1, 5, 12, and 24 h and then analyzed *ftn-1* and *ftn-2* mRNAs by qRT-PCR and Northern blotting. Fig. 1 shows that by 5 h of FAC treatment, *ftn-1* mRNA increases 2.5-fold compared with untreated worms and remains at that level for 24 h. Iron causes a smaller but consistent elevation in *ftn-2* mRNA during the 24-h time course. In contrast, iron chelation reduces *ftn-1* and *ftn-2* mRNAs by 90 and 75%, respectively, at 5 h compared with untreated worms.

Northern blot analysis of one experiment in Fig. 1A was consistent with results obtained by qRT-PCR (Fig. 1B).

To determine how *ftn-1* and *ftn-2* mRNAs are regulated by iron, we generated transcriptional GFP reporters that contained 1.9 kb of 5′-upstream sequences of *ftn-1* and *ftn-2* fused to the initiator ATG of a nuclear-localized GFP-histone gene (Fig. 2, A and B). We chose 1.9 kb of upstream sequences because *C. elegans* genes are closely spaced, and regulatory elements are likely to be located within this region. Fig. 2 shows
that \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his} reporters are highly expressed in intestinal cells in all larval stages and in adults. Expression of \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his} in embryos is detected in late gastrulation (Fig. 2C). Weak expression of \textit{ftn-2::GFP-his} is also observed in hypodermal cells in most larval stages (data not shown).

To identify \textit{cis-} acting sequences required for iron-dependent expression of \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his}, L3 larvae were grown on NGM plates supplemented with FAC or BP for 24 h, and GFP expression was analyzed. Fig. 3 shows that expression of \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his} was reduced after BP treatment compared with untreated and FAC-treated animals. We routinely noticed a small but reproducible increase in the expression of \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his} after 24 h of FAC treatment. We therefore quantified the expression of \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his} in a large population of L4 larvae \((n = 1000)\) after FAC or BP treatment using the COPAS\textsuperscript{TM} Biosort. Fig. 3B shows that FAC significantly increases the expression of \textit{ftn-1::GFP-his} and \textit{ftn-2::GFP-his} reporters, while BP reduces GFP expression for both reporters. We conclude that the small increase in GFP expression upon addition of excess iron is due to the growth of worms on bacteria that are iron-replete. Taken together, these data demonstrate that the sequences required for iron-dependent transcriptional regulation of \textit{ftn-1} and \textit{ftn-2} genes reside within 1.9 kb of their 5'-upstream regulatory sequences.

To determine if GFP expression is reversible after BP treatment, worms expressing \textit{ftn-1::GFP-his} were grown on NGM plates or BP-supplemented NGM plates for 18 h. Half of the BP-treated worms were immediately analyzed for GFP expression using the COPAS Biosort, while the remaining BP-treated worms were spotted onto FAC-supplemented NGM plates. After 24 h, GFP expression was analyzed. Fig. 3C shows that
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FAC restored GFP expression in BP-treated worms, indicating that the reduction in GFP expression by BP is reversible.

Identification of Sequences Required for ftn-1::GFP-his Expression in Intestine—The 5′-upstream regulatory sequences of ftn-1 and ftn-2 genes have little sequence identity, but one 63-bp sequence, located at nucleotide −693 in ftn-1 and at nucleotide −1313 in ftn-2, is highly conserved (Fig. 4A). This element is composed of three direct repeats (DR1, DR2, DR3), containing the consensus sequence (CACGTA(C/G)(C/A/G)), separated by linker sequences containing a GATA consensus motif ((T/A)GATA(T/G)). GATA1 and GATA2 sequences are found in the forward and reverse orientations, respectively. The linker sequences are otherwise not highly conserved. The 63-bp element is also conserved in the 5′-upstream regulatory regions of C. briggsae ftn-1 and ftn-2 genes (Fig. 4A).

The conservation of the 63-bp element in ftn-1 and ftn-2 in C. elegans, as well as in C. briggsae, suggested that it may be important for iron regulation. To test this possibility, we deleted the 63-bp element from ftn-1::GFP-his and replaced it with nonspecific DNA (Fig. 4B). Stable transformed lines were generated (ftn-1(Δ63)::GFP-his) and assayed for GFP expression after 24 h of FAC or BP. Fig. 5A shows that deletion of the 63-bp element reduced ftn-1(Δ63)::GFP-his expression in untreated worms as well as in FAC- and BP-treated worms, confirming that the 63-bp element is required for ftn-1::GFP-his expression in the absence or presence of iron.

GATA sequences have been shown to be essential for the expression of intestinal-specific genes (40–46). GATA transcription factors bind to GATA motifs and regulate differentiation and proliferation of cells and cell fate specification (47). To determine whether GATA1 and/or GATA2 have a role in ftn-1::GFP-his expression, they were mutated to CATA either single or in combination (ftn-1(G1m)::GFP-his, ftn-1(G2m)::GFP-his or ftn-1(G12m)::GFP-his), and GFP expression was analyzed in transgenic strains. This mutation has previously been shown to reduce binding of the GATA protein ELT-2 to a GATA motif in the pho-1 gene (40). Fig. 5B shows that either single or double mutations of GATA1 and/or GATA2 sequences greatly reduced ftn-1::GFP-his expression in untreated worms. GFP expression was also reduced in worms grown on FAC- or BP-supplemented NGM plates (data not shown). These data demonstrate that both GATA1 and GATA2 sequences are required for the expression of ftn-1::GFP-his in intestine.

The 63-bp Element Directs Iron-dependent Transcription of ftn-1::GFP-his in Intestine—To determine if the 63-bp element is sufficient to activate GFP expression in intestine, it was inserted in a pes-10::GFP-his reporter upstream of a minimal promoter. This reporter contains a pes-10 promoter that has little activity on its own, but can be activated by enhancers. As expected, no GFP expression was observed in transformed worms carrying the pes-10::GFP-his reporter lacking the 63-bp element (Fig. 6A). Insertion of the 63-bp element into pes-10::GFP-his (pes-10(+63)::GFP-his) activated GFP expression in intestinal cells (Fig. 6B). Importantly, pes-10(+63)::GFP-his expression was reduced by BP. Like ftn-1::GFP-his, pes-10(+63)::GFP-his was expressed in intestine; however, expression of pes-10(+63)::GFP-his expression was weaker in the anterior intestinal cells compared with ftn-1::GFP-his (compare Fig. 3A with Fig. 6B). These data indicate that the 63-bp element can activate GFP expression of a
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ELT-2 Binds to the IDE in Vitro—

Of the 11 GATA transcription factors expressed in *C. elegans*, ELT-2, ELT-4, and ELT-7 are expressed in post-embryonic intestine (48). ELT-2 has been shown to bind to GATA motifs in the promoters of several intestinal-expressed genes (40–42). Deletion of elt-2 results in L1 larval lethality while worms carrying deletions in both elt-4 and elt-7 are wild type (46, 49), demonstrating that ELT-2 is the key regulator of intestinal gene expression. To determine whether ELT-2 binds to the GATA1 and/or the GATA2 sequences, we performed an EMSA using baculovirus-purified ELT-2 and $^{32}$P-labeled wild-type and mutant GATA IDE probes. Two ELT-2 complexes (C1 and C2) were formed with a $^{32}$P-labeled wild type IDE (Fig. 7A). We postulated that the slower mobility complex (C1) represented the occupancy of both GATA sites by ELT-2 whereas the faster mobility complex (C2) represented the binding of ELT-2 to one GATA site. To test this model, reactions were carried out in the presence of increasing ELT-2 concentrations. Fig. 7A shows that when the concentration of ELT-2 is increased, only C1 is observed, consistent with greater saturation of ELT-2-GATA binding sites.

When EMSAs were performed using $^{32}$P-labeled G1m or G2m probes and a high concentration of ELT-2, only C2 was observed, which represents the binding of ELT-2 to a single GATA site (Fig. 7B). Reduced complex formation was observed with a $^{32}$P-labeled G1m probe containing mutations in both GATA sequences (Fig. 7B). Formation of C1 and C2 was effectively competed by unlabeled IDE but not by G1m DNA (Fig. 7C). The increase in C2 at 10× molar amounts of unlabeled IDE is presumably because of the loss of ELT-2 from a single GATA site on the $^{32}$P-labeled IDE. As the concentration of unlabeled IDE is increased, C1 and C2 are both reduced. Our findings demonstrate that ELT-2 binds specifically to GATA1 and GATA2 sequences in vitro.

The reduced expression of pes-10(DR123m)::GFP-his in vivo suggested that DR123m mutations may preclude ELT-2 binding to DNA (Fig. 6F). We questioned whether ELT-2 can bind to the DR123m probe in vitro. Fig. 7C shows that when EMSAs are performed using a $^{32}$P-labeled DR123m probe and ELT-2, both C1 and C2 are formed (Fig. 7C). These data indicate that ELT-2 is capable of binding to the DR123m probe in vitro, suggesting that an additional factor(s), which binds to the DRs, is required for efficient ftn-1::GFP-his expression in vivo.
elt-2 RNAi Reduces $ftn-1::GFP$-his Expression

To determine whether ELT-2 is required for in vivo expression of $ftn-1::GFP$-his, we injected elt-2 dsRNA into transgenic $ftn-1::GFP$-his hermaphrodites and measured GFP expression in F1 larvae. For these studies, we synthesized elt-2 dsRNA using bacterial clones isolated from the MRC RNAi feeding library (38) and from the ORFeome RNAi library (39). Fig. 8A shows that the progeny of animals injected with elt-2 dsRNA are arrested at the L1 larval stage, and display intestinal defects typical of an elt-2 deletion strain (49). In worms treated with elt-2 dsRNA (ORFeome), 59% of the F1 larvae lack GFP expression while 42% express GFP. Similar results are obtained with worms injected with elt-2 dsRNA (MRC) where 64% of the F1 larvae lack GFP expression and 36% express GFP. As controls, worms were also injected with elt-6 and elt-1 dsRNAs. ELT-6 and ELT-1 are expressed in seam cells and hypodermal cells, respectively, but not in intestine (50, 51). As expected, RNAi against elt-6 and elt-1 had little effect on GFP expression (98 and 97% of worms express GFP, respectively) (Fig. 8). We conclude that ELT-2 regulates $ftn-1$ expression in vivo.

DISCUSSION

Previous studies have shown that $ftn-1$ and $ftn-2$ mRNAs are transcriptionally regulated by iron (28, 29), but the trans-acting proteins and the cis-acting sequences responsible for their regulation are unknown. Here, we identified an enhancer in the $ftn-1$ and $ftn-2$ promoters that is sufficient to activate iron-dependent transcription of a $ftn-1::GFP$-his reporter in intestine.

The IDE contains two canonical GATA motifs and three conserved direct repeats, all of which are required for $ftn-1::GFP$-his regulation. We suggest that ELT-2 functions in concert with an iron-sensitive protein partner to bind to the IDE and activate $ftn-1$ (and $ftn-2$) transcription in the presence of iron.

The regulation of $ftn-1::GFP$-his and $ftn-2::GFP$-his reporters by iron and BP recapitulates the regulation of endogenous $ftn-1$ and $ftn-2$ mRNAs. For example, BP robustly reduced $ftn-1::GFP$-his and $ftn-2::GFP$-his reporter expression and the amounts of endogenous $ftn-1$ and $ftn-2$ mRNAs. In contrast, iron caused a small but consistent increase in reporter expression and in the amounts of $ftn-1$ and $ftn-2$ mRNAs. Using the COPAS Biosort to analyze a large population of worms, we found that the increase in $ftn-1::GFP$-his and $ftn-2::GFP$-his expression by iron was highly significant. Thus, the small increase of $ftn-1$ and $ftn-2$ mRNAs and in reporter expression by iron is likely due to the iron-sufficient status of worms because they are fed bacteria that are iron-replete.

Consistent with our findings, Kim et al. (29) reported that a translational $ftn-1$ GFP reporter was iron-regulated and expressed in intestine. These same authors showed that a trans-

[FIGURE 5. GATA binding sequences are required for $ftn-1::GFP$-his expression in intestine. A, expression of a $ftn-1::GFP$-his reporter lacking the 63-bp element ($ftn-1(63)$::GFP-his) in L4 larvae after growth on NGM plates or NGM plates supplemented with FAC or BP for 18 h. B, expression of a $ftn-1::GFP$-his reporter in L4 larvae containing mutations in GATA1 (G1m), GATA2 (G2m), or GATA12 (G12m) sequences after growth on NGM plates for 18 h.]

[FIGURE 6. The 63-bp element is an iron-dependent enhancer in intestine. GFP expression of $pes-10::GFP$-his reporters lacking the 63-bp element (A) or reporters containing the 63-bp element ($pes-10(63)$::GFP-his) (B), containing single mutations (DR1m, DR2m, and DR3m) or a triple mutation (DR123m) in the direct repeats (C–F). L4 larvae are shown after growth on NGM plates or NGM plates supplemented with FAC or BP for 18 h.]
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FIGURE 7. ELT-2 binds to the GATA elements in the IDE. A, increasing amounts of baculovirus-produced ELT-2 were added to EMSA reactions with a $^{32}$P-labeled wild type IDE probe. ELT-2-DNA complexes were analyzed by non-denaturing polyacrylamide gels. Complexes are indicated as C1 and C2. B, binding of ELT-2 to a $^{32}$P-labeled wild type IDE and IDE probes containing mutations in the GATA sequences (G1m, G2m, G12m) or in the three direct repeats (DR123m). C, $^{32}$P-labeled wildtype IDE probe was incubated with increasing molar amounts of unlabeled wild type IDE or unlabeled G12m IDE DNAs.

FIGURE 8. The reduction of ELT-2 by RNAi reduces ftn-2::GFP-his expression. Double-stranded RNA for elt-2 (Orfome and MRC clones), elt-6 and elt-1 were injected into adult hermaphrodites expressing ftn-1::GFP-his. GFP expression was measured in F1 larvae. A, representative larvae are shown for RNAi against elt-2(Orf), elt-1, and elt-6. B, quantification of GFP expression in A. RNAi phenotypes are shown as the percentage of worms expressing GFP (On) or lacking GFP expression (Off) (control 1 mock injected, n = 216; elt-2(MRC), n = 291; elt-1, n = 205 and control 2 mock-injected, n = 230; elt-2 (Orf) n = 147; elt-6, n = 232). Arrows, intestinal nuclei.

In vivo and in vitro, the GATA factor Fep1 binds DNA in the presence of iron and recruits the co-repressors Tup11 and Tup12, leading to the transcriptional inactivation of iron transport genes (54). Mammalian GATA-1, a GATA transcription factor critical for erythroid differentiation, has been reported to bind iron and zinc to activate DNA-binding. In S. pombe, the GATA factor Fep1 binds DNA in the presence of iron and recruits the co-repressors Tup11 and Tup12, leading to the transcriptional inactivation of iron transport genes (54). Mammalian GATA-1, a GATA transcription factor critical for erythroid differentiation, has been reported to bind iron and zinc to activate DNA-binding in vitro (55); however, the in vivo significance of this data is unclear. In Caenorhabditis elegans, however, there is no evidence that ELT-2 is regulated by iron. Many intestinal-expressed genes are regulated by GATA sequences, including metallothionein (mtl-1, mtl-2) (41), vitellogenin (vit-2, vit-5, vit-6), an acid phosphatase (pho-1) (40), sphingosine-1-phosphate lyase (spl-1) (42), a cysteine protease (cpr-1) (43) and gut esterase (ges-1) (44), and several of these genes have been shown to be regulated by ELT-2 in vivo (40–42). Moreover, recent computational studies have found that the promoters of intestinal-specific genes are enriched for GATA sequences (45, 46). Taken together, these data have led to the proposal that most, if not all, intestine-enriched and intestine-specific genes are regulated by ELT-2 (45, 46). Because ELT-2 is considered a global regulator of intestine-specific gene expression, this suggests that ELT-2 is unlikely to be directly regulated by iron.
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A Iron sufficiency

![Diagram showing iron sufficiency with DRBP and Ftn-1]

B Iron deficiency

![Diagram showing iron deficiency with DRBP and Ftn-1]

FIGURE 9. Proposed transcriptional model for iron-dependent regulation of ftn-1 and ftn-2. In the presence of iron, a putative direct repeat-binding protein (DRBP) enhances the interaction between ELT-2 and the GATA sequences of the IDE, enabling ELT-2 to function as an activator of ftn-1 and ftn-2 transcription. As depicted in the figure, iron could directly bind DRBP to enhance its activity or iron could alter DRBP amounts or nuclear localization. During iron deficiency, DRBP activity and/or concentration are reduced leading to reduced ELT-2 transcriptional function at the ftn-1 and ftn-2 loci. Whether ELT-2 binds to the GATA sequences in vivo during iron deficiency remains to be determined.

We prefer a model whereby a protein that binds to the direct repeats (direct repeat binding protein or DRBP) interacts with ELT-2 to activate ftn-1 and ftn-2 transcription in intestine during iron sufficiency (Fig. 9). This interaction would strengthen the ability of ELT-2 to function as a transcriptional activator. This model is consistent with our data showing that the expression of ftn-1(DR123m):GFP-his reporters is reduced in vivo, whereas purified ELT-2 is capable of binding the DR123m probe in vitro. Iron could directly bind to the DRBP to affect its interaction with DNA and/or with ELT-2. Alternatively, iron could affect DRBP amounts or localization. When cells become iron-deficient, DRBP activity and/or amounts are reduced, leading to reduced ELT-2 function at the ftn-1 and ftn-2 loci. Whether ELT-2 is competent to bind to the GATA sequences in vivo during iron deficiency remains to be determined.

It is well established that GATA proteins use their zinc fingers to bind DNA as well as to mediate protein-protein interactions (56, 57). For example, the interaction of the mammalian transcription factor GATA1 with different protein partners can positively or negatively modulate its transcriptional activity (57, 58). ELT-2 contains a conserved C-terminal zinc finger that binds DNA as well as a N-terminal cysteine-rich region whose function is unknown (59). Thus, it is plausible that the zinc finger and/or cysteine-rich region in ELT-2 may mediate its interaction with a partner protein, such as the DRBP, to activate iron-dependent transcription of ftn-1 and ftn-2. The identity of the DRBP is under investigation.

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