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Multiple *cis* elements and GATA factors regulate a cuticle collagen gene in *C. elegans*

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Abstract

The cuticle of the nematode *Caenorhabditis elegans* is a specialized extracellular matrix whose major component is collagen. Cuticle collagens are encoded by a large multi-gene family consisting of more than 150 members. Cuticle collagen genes are expressed in epidermis (hypodermis) and may be stage-specific or cyclically expressed. We identified cuticle collagen genes as transcriptional targets of the DBL-1 TGF-β-related signaling pathway. These studies prompted us to investigate the *cis*-regulatory sequences required for transcription of one of the target genes, *col-41*. We generated reporter constructs that reproduce stage- and tissue-specific expression of fluorescent markers. We identify four conserved sequence elements that are required for transcription of reporters. Finally, we provide evidence that *col-41* expression is controlled by a sequence element containing two GATA sites and by the epidermal GATA transcription factors ELT-1 and ELT-3.

Keywords

TGFβ; GATA; hypodermis; epidermis

Introduction

In the nematode *C. elegans*, the cuticle serves as an exoskeleton required for proper growth and defense of the animal (Page and Johnstone, 2007). Collagen is the major component of the cuticle and is encoded by a large multi-gene family. Cuticle collagen genes are expressed in the epidermis (hypodermis), a large multinucleate tissue that surrounds the nematode body. Collagen is secreted from the apical surface of the epidermal cells to the exterior of the body. Cuticle collagen genes show highly specific temporal expression patterns that may be stage-specific or cycle in phase with the cuticle molts (Jackson et al., 2014; Johnstone and Barry, 1996; Park and Kramer, 1994). Additionally, levels and timing of cuticle collagen gene expression respond to cell signaling pathways, such as the TGFβ and Wnt pathways (Jackson et al., 2014; Liang et al., 2007; Roberts et al., 2010).

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In our studies of the transcriptional response to the TGFβ family member DBL-1, we identified *col-41* and other cuticle collagen genes as transcriptional targets whose expression levels depend on the activity of the signaling pathway (Liang et al., 2007). This finding prompted us to investigate the *cis*-regulatory requirements for accurate transcription of the *col-41* gene. Although expression studies of cuticle collagen genes have been previously performed, little is known about the sequence elements that contribute to collagen gene regulation. In this study, we use transcriptional reporters to characterize *col-41* regulatory sequences. We identify a 0.5kb promoter region that directs stage- and tissue-specific regulation of a fluorescent marker and additional upstream sequences that contribute to robust, reproducible expression. Within these regions, we identify four conserved sequence motifs that are required for optimal activation of the reporter, two of which are essential for any detectable expression. One of the essential elements contains two GATA motifs. We provide evidence that *col-41* may be regulated by the epidermal-specific GATA factors ELT-1 and ELT-3.

Results and Discussion

Cuticle collagen genes were identified via microarray analysis as transcriptional targets of the DBL-1 signaling pathway (Liang et al., 2007). DBL-1 regulates multiple developmental and homeostatic functions in *C. elegans*, one of which is body size (Savage-Dunn et al., 2003; Wang et al., 2002). Since cuticle collagen genes are known to regulate body size and morphology, we were interested in these target genes as potential mediators of DBL-1 regulation of body size. Furthermore, recent evidence suggests that defects in the cuticle underlie multiple abnormalities seen in DBL-1 pathway mutants (Schultz et al., 2014). To investigate collagen gene expression *in vivo*, we generated transcriptional GFP reporters using upstream genomic sequences for the target genes *col-37*, *col-41* and *col-141*. GFP expression was undetectable for *col-37* and *col-141* (data not shown), suggesting either that our constructs were missing critical regulatory sequences, or that expression levels were below our level of detection.

In contrast, transcriptional fusions for *col-41* containing 1.6kb of upstream sequences fused to GFP or to nuclear localized 2xNLS-mCherry showed detectable expression. We chose 1.6kb since this was the distance to the nearest upstream transcript in the genome annotation at the time. (Current annotations show the presence of three small exons in this region from a gene transcribed in the opposite orientation). Reporter constructs were microinjected into the *C. elegans* gonad to create transgenic lines followed by transgene integration using γ-irradiation. Integrated *col-41p::2xNLS::mCherry* transgenes are expressed in a tissue-specific and stage-specific manner. mCherry expression is observed in epidermal nuclei in L3 and early L4 larval stage animals (Fig. 1a). mCherry is also expressed in the vulval precursor cells, a ventral epidermal lineage, during the L3 stage (Fig. 1b). Expression is absent from the lateral epidermal seam cells; this absence is more apparent in the GFP reporter, which is not nuclear localized and therefore shows diffuse fluorescence throughout the cytoplasm (Fig. 1c). The spatial expression pattern is very similar to that reported for the cuticle collagen gene *dpy-7* (Gilleard et al., 1997). Temporally, however, COL-41 appears to be a larval-specific cuticle collagen, with expression absent from embryos and adults. This result is consistent with RNAseq analyses that demonstrate a peak of *col-41* expression in

L2 (Gerstein et al., 2010; Jackson et al., 2014). Expression of the fluorescent marker may be visible later than the peak in mRNA expression due to the time lag for protein synthesis and maturation, and/or perdurance of the mCherry protein. The observed expression timing is consistent with *col-41* being identified as a target of the DBL-1 pathway at the L2 stage (Liang et al., 2007) but not at the L4 stage (Roberts et al., 2010).

Next, we analyzed the putative regulatory sequences of *col-41* in more detail. We aligned *col-41* upstream sequences from three closely related nematodes, *C. briggsae*, *C. remanei* and *C. elegans*. Sequence conservation was most prominent in the proximal 0.5kb (Fig. 2). We therefore created separate mCherry reporter constructs containing the proximal 0.5kb and distal 1.1kb *col-41* promoter fragments to generate transgenic animals as described above. *In vivo*, the reporter construct with the distal 1.1kb promoter fragment is not sufficient for expression (data not shown). In contrast, mCherry expression driven by the proximal 0.5kb promoter fragment results in tissue-specific (epidermal) and stage-specific (early larval) expression (Fig. 1d). However, expression appeared to be less robust from the 0.5kb construct, since in multiple integrated lines only ~60% of transgenic animals showed expression (four integrated lines were analyzed). We conclude that sequences in the proximal 1.1kb region contribute to reproducible expression of *col-41*. There is precedent for such mechanisms for producing robust and reproducible expression via redundant *cis*-acting elements and “shadow” enhancers (Lagha et al., 2012).

From the sequence alignment, we identified 6 conserved sequence elements (Fig. 2). We deleted the three proximal elements in the context of the 0.5kb promoter fragment and the three distal elements in the context of the 1.6kb promoter (Fig. 3). Deletion constructs were microinjected into nematodes, transgenes were integrated, and expression was analyzed by fluorescence microscopy. Images in Fig. 4 were taken on the same day with the same exposure times. Deletions 1 and 2 show no detectable mCherry expression (data not shown). Deletions 3 and 6 have reduced levels of mCherry expression (Fig. 4b,e). Deletions 4 and 5 show levels of expression similar to the wild-type 1.6kb construct (Fig. 4a,c,d). None of these deletions produced any ectopic expression, either temporally or spatially. Taken together, we conclude that elements 1, 2, 3, and 6, but not elements 4 and 5, are required for *col-41* expression. Additionally, since deletion 6 in the context of the full 1.6kb promoter has a lower level of expression than the 0.5kb promoter (that lacks element 6), we conclude that the 1.1kb region also contains an undefined repressor element.

We examined sequence elements 1, 2, 3, and 6 for potential transcription factor binding sites (Fig. 2). Since we identified *col-41* as a transcriptional target of DBL-1, a TGFβ-related ligand (Suzuki et al., 1999), we first searched for sequences related to TGFβ signaling. TGFβ responses are mediated by Smad transcription factors, which bind two types of sequences: GTCT Smad Binding Elements (SBEs) or GRCGNC GC-rich sequences (Gao et al., 2005; Shi et al., 1998; Zawel et al., 1998). Element 6 contains one potential GTCT Smad Binding Element (SBE). Most Smad target genes, however, contain more than one SBE, and the GTCT upstream of *col-41* is not conserved in *C. remanei* (Fig. 2). Thus, at this time, we do not have evidence to distinguish between *col-41* as a direct or indirect target of Smads.

Element 2 contains two potential GATA sites. The GATA motif is a common binding site for transcription factors, including GATA factors. In *C. elegans*, specific GATA factor genes act as master regulators of intestinal and epidermal fates: *elt-2* and *elt-7* in the intestine (Sommermann et al., 2010) and *elt-1* and *elt-3* in the epidermis (Gilleard and McGhee, 2001). We therefore tested whether GATA factors play a role in the transcriptional regulation of *col-41*. We used RNAi by feeding to inactivate the GATA factor genes *elt-1, elt-2,* and *elt-3* in animals carrying the 1.6k *col-41* reporter construct. We found that inhibition of the intestinal GATA factor gene *elt-2* had no effect on expression, as expected (Fig. 5b). On the other hand, inhibition of the epidermal GATA factor gene *elt-1* caused a reduction in mCherry expression (Fig. 5a). Somewhat surprisingly, inhibition of *elt-3*, another epidermal GATA factor gene, resulted in increased mCherry expression (Fig. 5c). We therefore conclude that ELT-1 plays a role in transcriptional activation of *col-41* in the epidermis. ELT-3, on the other hand, may be a transcriptional repressor of *col-41* or have a more complex regulatory interaction with *col-41*. Interestingly, the GATA factor GATA-4 has been identified as a transcriptional repressor for human *COL1A2* (Antoniv et al., 2005), which could indicate a conserved regulatory mechanism for invertebrate and vertebrate collagens.

To clarify these regulatory interactions, we used qRT-PCR to determine transcript levels of the endogenous *col-41* gene upon RNAi of *elt-1* and *elt-3. elt-1* is required in embryos to specify epidermal fates and is a transcriptional activator of *elt-3* (Gilleard et al., 1999; Page et al., 1997*).* Null mutations in *elt-1* are lethal, but partial loss of function by RNAi is viable. As expected, *elt-1(RNAi)* caused reduced transcript levels for both *elt-1* and *elt-3* (Fig. 5d). Additionally, consistent with the transgenic reporter results, we found that *col-41* transcript levels are reduced in *elt-1(RNAi)* animals (Fig. 5d). *elt-3* is required for maintenance of epidermal fates and null alleles are viable (Gilleard et al., 1999). Due to the lack of lethality, *elt-3(RNAi)* efficiently reduced *elt-3* transcript levels (Fig. 5d). It also reduced *elt-1* transcript levels, suggesting that ELT-3 is required for maintenance of *elt-1* expression, which has not previously been reported. Unexpectedly, we saw a slight reduction in *col-41* transcript levels in *elt-3(RNAi)* animals (Fig. 5d), as opposed to the increase seen in the transgenic reporter. This result suggests that our transgenic reporter may be missing sequences required for the correct direction of the regulatory interaction. Consistent with the hypothesis of direct regulation of *col-41* by ELT-3 is the identification of ELT-3 binding upstream of *col-41* by ChIP-seq analysis (Niu et al., 2011).

We were interested to determine whether regulation of cuticle collagen genes by GATA factors is a general phenomenon. Expression analyses of *dpy-7* collagen have identified a GATA sequence in the minimal promoter region (Gilleard et al., 1997). We therefore determined *dpy-7* transcript levels in *elt-1* and *elt-3* knockdowns. We find that ELT-3, but not ELT-1, is required for normal expression of *dpy-7* (Fig. 5d). Furthermore, like *col-41*, the *dpy-7* locus is associated with direct binding of ELT-3 by ChIP-seq (Niu et al., 2011).

In conclusion, we have generated transcriptional reporters for *col-41* that can be used to mark larval epidermal and vulval precursor nuclei in *C. elegans*. We have identified four conserved elements that are required for expression of *col-41*. One of these elements contains two GATA sites. In vertebrates, GATA factors play critical roles in specification of

hematopoietic and endoderm lineages (Burch, 2005; Wolff and Humeniuk, 2013). Similarly, in *C. elegans* GATA factors can specify intestinal and epidermal fates (Gilleard and McGhee, 2001; Sommermann et al., 2010). In our experiments, depletion of the epidermal-specific GATA factor ELT-1 reduced expression of *col-41*, suggesting that the cuticle collagen gene is a direct target of a tissue-specific master regulator. Furthermore, expression of the cuticle collagen gene *dpy-7* is dependent on the GATA factor ELT-3, suggesting that this may be a widespread mechanism for the tissue-specific regulation of this multi-gene family.

Methods

Molecular cloning and sequencing

The *col-41* transcriptional GFP reporter was generated by fusion PCR (Hobert, 2002) using 1.6kb upstream promoter region and GFP coding region from pPD117.01 as the template. The *col-41* 2xNLS::mCherry transcriptional reporter was generated by PCR cloning of *col-41* upstream sequences into 2xNLS-mCherry (optimized) vector (converted from pPD95.75). The *col-41* deletion series constructs were generated by using Phusion Site-Directed Mutagenesis Kit (NEB) and confirmed by sequencing.

Transgenic animals

Transgenic nematodes were generated by microinjection of plasmid DNA (10 ng/μl) with *myo-2::GFP* (5 ng/μl) as a marker (Mello et al., 1991). Arrays were integrated into chromosomes using γ-irradiation. 2–3 independent lines were generated for each construct, and all showed comparable results.

qRT–PCR

Total RNA was isolated by Trizol (GibcoBRL) and using the Qiagen RNeasy Mini kit from N2 (wild-type) worms treated with L4440 (empty vector control), *elt-1(RNAi)* and *elt-3(RNAi)*. Reverse transcription was done using enzyme from SUPERSCRIPT™ III Two-Step qRT–PCR (Invitrogen). Real-time PCR was performed using PWR SYBR Green PCR Master Mix on Applied Biosystem StepOne 48 well machine (Lifetechnologies). Data Analysis software used was StepOne Software version2.2.2. Gene *act-1* was used as endogenous control. The intron primer of *act-1* gene was used to test for the presence of genomic DNA. N2 animals were synchronized to late L2 stage with a 4 hour synchronization. Three biologically independent samples were collected. Each sample was analyzed in two technical replicates. A total of 4 genes (*col-41*, *dpy-7, elt-1*, and *elt-3*) was examined in L4440, *elt-1*, and *elt-3* RNAi backgrounds. Results are shown as relative quantitation compared with L4440 control.

RNAi

RNAi feeding was performed as described in (Kamath et al., 2003; Liang et al., 2013). For transgene reporter experiments, 12 L4 animals were transferred to feeding plates, incubated overnight, transferred to fresh RNAi feeding plates to lay eggs for 4 hours and the stage-synchronized progeny were scored. For qRT-PCR, 50 L4 animals were transferred to RNAi plates and allowed to lay eggs overnight. Adults were washed off the plates, leaving

unhatched embryos. After 4 hours, newly hatched L1 animals were transferred to new RNAi plates and allowed to develop until late L2 stage.

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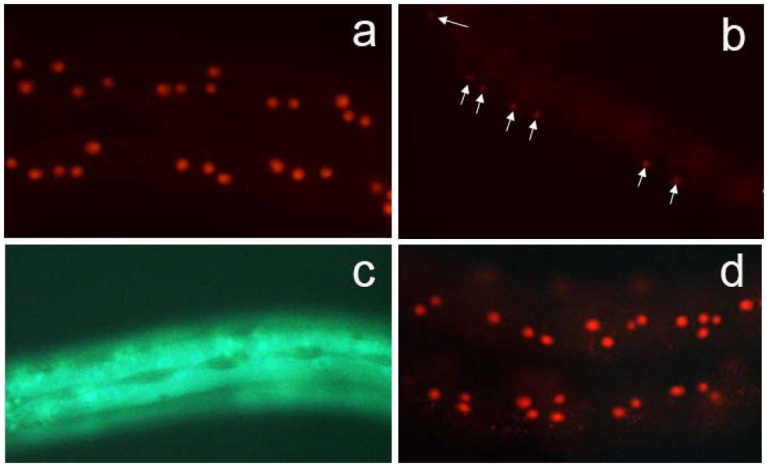

Fig. 1.

col-41 transcriptional reporters direct expression in the epidermis. **(a)** Expression of 1.6kb *col-41p::2xNLS::mCherry* reporter in epidermal nuclei during L3 stage. **(b)** Expression of 1.6kb *col-41p::2xNLS::mCherry* reporter in vulval precursor cells (marked by arrows) during L3 stage. **(c)** Expression of 1.6kb *col-41p::GFP* reporter in the epidermis is excluded from the lateral epidermal seam cells. **(d)** The 0.5kb *col-41p::2xNLS::mCherry* reporter directs expression in a similar stage- and tissue-specific manner.

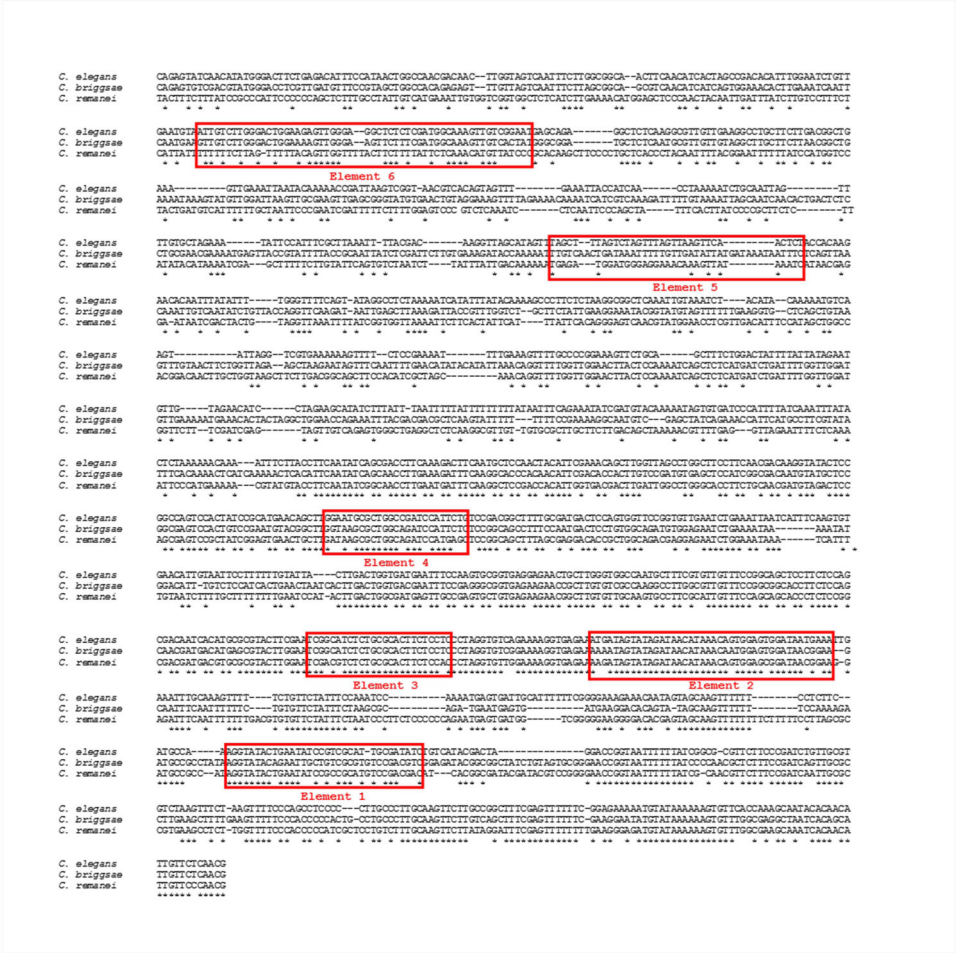

Fig. 2.

Alignment of upstream sequences of *col-41* orthologs from *C. elegans*, *C. briggsae*, and *C. remanei*. Alignment was performed using Clustalw. * indicates identical nucleotides. Red boxes indicate conserved regions that were deleted in reporter constructs.

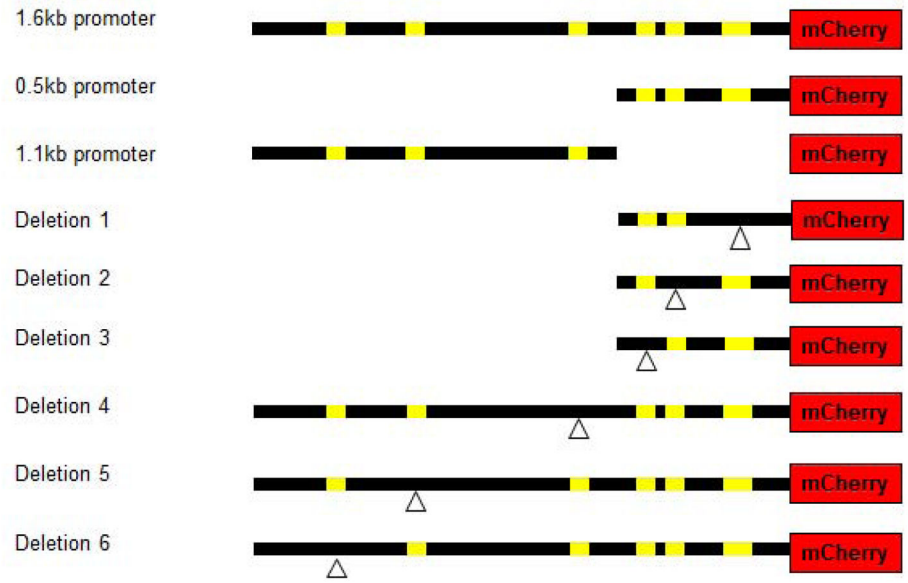

Fig. 3.

col-41 promoter constructs. Yellow bars indicate conserved regions. Deletions are indicated by the delta (inverted triangle) symbol.

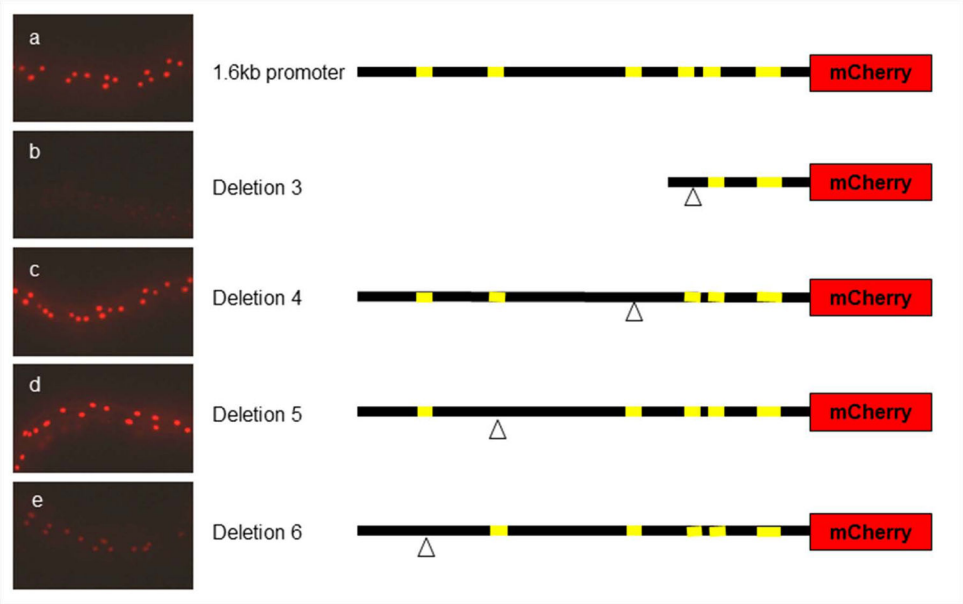

Fig. 4.

Expression of *col-41* deletion series reporters. **(a)** 1.6kb promoter control; **(b)** Deletion 3 has no detectable expression; **(c)** Deletion 4 has normal levels of expression; **(d)** Deletion 5 has normal levels of expression; **(e)** Deletion 6 has reduced levels of expression.

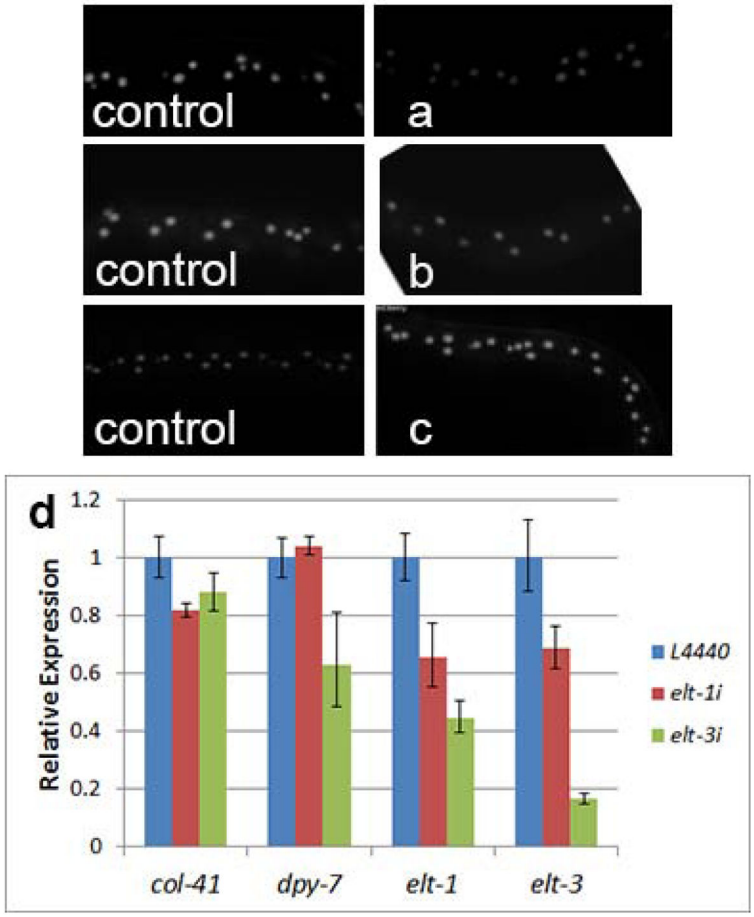

Fig. 5.

Depletion of epidermal, but not intestinal, GATA factors results in altered expression of cuticle collagen genes. **(a–c)** Expression of *col-41* transcriptional reporter. The respective empty vector control for each experiment is shown to the left. **(a)** *elt-1* RNAi (epidermal); **(b)** *elt-2* RNAi (intestinal); **(c)** *elt-3* RNAi (epidermal). **(d)** Quantitative RT-PCR analysis of *col-41*, *dpy-7*, *elt-1* and *elt-3* upon *elt-1* and *elt-3* RNAi. *col-41* expression is dependent on ELT-1 and, to a lesser extent, ELT-3. *dpy-7* expression is dependent on ELT-3. Data are averages of trials from three biologically independent replicates; error bars show standard deviation. Transcript levels are determined in L2 stage; note that *elt-1* transcript levels and functional activity are higher during embryogenesis.