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Notch-GATA synergy promotes endoderm-specific expression of *ref-1* in *C. elegans*

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The Notch signaling pathway is involved in a wide variety of cell-fate decisions during development. The diverse behavior of Notch-activated cells is thought to depend on tissue- or cell-type-specific transcription factors, yet the identities of such factors and the mechanism of cooperation with the Notch pathway are largely unknown. We identify here an enhancer in the promoter of *ref-1*, a *C. elegans* Notch target, which promotes Notch-dependent expression in mesodermal and endodermal cells. The enhancer contains predicted binding sites for the Notch transcriptional effector LAG-1/CSL that are essential for expression, a non-CSL site required for mesodermal expression, and four predicted binding sites for GATA transcription factors that are required for endodermal expression. We show that endodermal expression involves the GATA transcription factor ELT-2, and that ELT-2 can bind LAG-1/CSL in vitro. In many types of Notch-activated embryonic cells, ectopic ELT-2 is sufficient to drive expression of reporters containing the enhancer.

KEY WORDS: Notch, GATA, *ref-1*

INTRODUCTION

Cell interactions during animal development are mediated by relatively few signaling pathways, one of the most important being the Notch pathway. The basic framework of Notch signal transduction is well conserved, and has been elucidated from genetic and molecular studies in several systems (for reviews, see Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000; Greenwald, 2005; Bray, 2006). In the absence of Notch signaling, target genes can be repressed by sequence-specific DNA binding proteins in the CSL family [for vertebrate CBF1, *Drosophila* Su(H) and *Caenorhabditis elegans* LAG-1] in combination with corepressors. Contact with ligand activates the transmembrane receptor Notch, initiating processing events that eventually release the Notch intracellular domain (NICD). NICD then translocates to the nucleus where it binds CSL, displacing the corepressor complex and forming a ternary complex with an essential coactivator in the mastermind-like (MAML) protein family. The NICD-CSL-MAML ternary complex is thought to recruit chromatin-modifying enzymes that promote target gene expression.

Notch-regulated cell fate decisions have been described in ectoderm, mesoderm, endoderm and germline tissues. Examples include germ cell mitosis and vulval development in *C. elegans*, specification of the ectoderm/endoderm boundary in sea urchins, and binary cell fate decisions in neural, vascular and lymphoid precursors in vertebrates (Austin and Kimble, 1987; Robey, 1997; Sherwood and McClay, 2001; Iso et al., 2003; Greenwald, 2005). Individual Notch targets typically are expressed in one or a few interactions, but are not expressed in all interactions. For example, the *Drosophila cut* and *sim* genes are Notch targets in the wing and the ventrolateral ectoderm, respectively (Morel and Schweisguth, 2000; Guss et al., 2001). Similarly, the *C. elegans ref-1* gene is a direct target of multiple Notch interactions during embryogenesis, but does not appear to be expressed in several postembryonic Notch interactions (Neves and Priess, 2005). This specificity suggests that CSL proteins must cooperate directly or indirectly with tissue-restricted factors to control target gene expression. Multimerized binding sites for CSL proteins are sufficient to promote in vitro transcription using purified NICD, CSL and MAML proteins, and multimerized CSL binding sites can promote Notch-dependent reporter expression in cultured cells (Furukawa et al., 1995; Wallberg et al., 2002). However, multimerized CSL sites are insufficient to drive reporter expression in Notch-activated cells in vivo (Guss et al., 2001), and some endogenous Notch targets contain only one or two CSL-binding sites (Yoo and Greenwald, 2005) (our unpublished results). Furthermore, fusing CSL proteins to a strong activation domain like VP16 or a constitutively active form of Notch (NICD) does not, in general, suffice for target gene expression (Cooper et al., 2000; Morel and Schweisguth, 2000). These results together suggest that the regulation of endogenous Notch targets involves additional combinatorial factors, and transcriptional codes that are more complex than simply the presence of CSL-binding sites.

The promoters of some Notch-regulated genes contain conserved, paired CSL-binding sites that are oriented head to head and spaced 15-22 bp apart (Bailey and Posakony, 1995; Nellesen et al., 1999; Nam et al., 2007). Inverting the orientation of either, or both, of the CSL paired sites significantly impairs the expression of reporter genes (Cave et al., 2005). Recent structural and biochemical studies have shown that a CSL-paired site is able to promote cooperative interactions between ternary complexes of mammalian CSL, MAML and the NICD ankyrin domain (Nam et al., 2007). A *Drosophila* CSL-paired site, called SPS [Su(H) paired site], functions in conjunction with a binding site for Daughterless (Da), a bHLH transcription factor that can bind directly to Su(H) (Kramatschek and Campos-Ortega, 1994; Cooper et al., 2000; Cave et al., 2005). Thus, SPS + A, where A is the Da-binding site, appears to represent a transcriptional code for Notch-bHLH synergy. Other Notch transcriptional codes must exist, as some Notch-regulated genes with CSL-paired sites lack predicted bHLH

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binding sites, and many Notch targets do not contain CSL-paired sites (Cave et al., 2005). For example, recent studies have provided evidence that the *C. elegans egl-43* gene is regulated by Notch signaling and the bHLH protein HLH-2, but elements required for expression do not contain obvious CSL-paired sites (Hwang et al., 2007). Previous studies have shown that expression of the *Drosophila* Notch targets *cut* and *sim* requires the transcription factors Scalloped/TEF-1 and Dorsal/NF-κB, respectively, but how these factors couple with Notch signaling is not known (Kasai et al., 1998; Guss et al., 2001).

In the present study, we used the *ref-1* gene to study tissue-specific, Notch-dependent gene expression. The *ref-1* gene is a direct target of many, though not all, Notch-mediated cell interactions in embryogenesis, and in addition is expressed in several cells independently of Notch signaling (Neves and Priess, 2005; Ross et al., 2005; Lanjuin et al., 2006). Expression of *ref-1* in some cells involves DNA sequences located over 8 kb upstream of the start codon (Ross et al., 2005), in contrast to most *C. elegans* genes that have very small promoter regions (Okkema and Krause, 2005). These and other results suggest that *ref-1* has a complex promoter, with separable, dispersed elements controlling expression in different cell types (Neves and Priess, 2005; Ross et al., 2005; Lanjuin et al., 2006). Here, we present the first characterization of a Notch-dependent enhancer element from the *ref-1* promoter. The enhancer is highly conserved in other *Caenorhabditis* species, and confers Notch-specific expression in a cell interaction in the mesoderm, and in two interactions in the endoderm. Notch-dependent expression in the endoderm, but not mesoderm, requires predicted binding sites for GATA transcription factors. We show that ELT-2/GATA is necessary for one of the two endodermal interactions, and is sufficient for ectopic, Notch-dependent expression in multiple cell types. Endoderm expression does not require CSL-paired sites, but instead involves CSL sites closely flanked by predicted GATA-binding sites. We demonstrate that LAG-1/CSL can interact directly with ELT-2/GATA in vitro, providing a possible basis for Notch-GATA synergy.

MATERIALS AND METHODS

Nematodes

Standard techniques were used to maintain and manipulate nematodes (Brenner, 1974). The following extrachromosomal arrays were created for this study. *zuEx132*, [*ref-1*^{600bp}*::gfp(pKG55)*]; *zuEx133*, [*ref-1*$^{600bp(WCATAR)}$*::gfp(pKG58)*]; *zuEx141*, [*ref-1*$^{600bp(WCATAR;1-GATA)}$*::gfp*]; *zuEx150*, [*ref-1*$^{600bp(WCATAR;2-GATA)}$*::gfp*]; *zuEx139*, [*ref-1*$^{600bp(WCATAR;3-GATA)}$*::gfp*]; *zuEx144*, [*ref-1*$^{600bp(WCATAR;4-GATA)}$*::gfp*]; *zuEx151*, [*ref-1*$^{600bp(RAGGCAA;1-CSL)}$*::gfp*]; *zuEx148*, [*ref-1*$^{600bp(RAGGCAA;2-CSL)}$*::gfp*]; *zuEx154*, [*ref-1*$^{600bp(RAGGCAA;3-CSL)}$*::gfp*]; *zuEx149*, [*ref-1*$^{600bp(RAGGCAA;4-CSL)}$*::gfp*]; *zuEx142*, [*enh*^{A}*::gfp(pAN20)*]; *zuEx162*, [C.b. *enh*^{A}*::gfp*]; *zuEx178*, [C.r. *enh*^{A}*::gfp*]; *zuEx191*, [*enh*$^{A(RAGGCAA)}$*::gfp(pAN29)*]; *zuEx172*, [*enh*$^{A(1-CSLinverted)}$*::gfp*]; *zuEx177*, [*enh*$^{A(2-CSLinverted)}$*::gfp*]; *zuEx171*, [*enh*$^{A(3-CSLinverted)}$*::gfp*]; *zuEx179*, [*enh*$^{A(3-CSL and 3-GATA interchanged)}$*::gfp*]; *zuIs173 (pKG82)*. *wIs88 (hs-med-1)* was provided by Morris Maduro (Maduro et al., 2001) and *caIs7 (hs-elt-2)* was provided by Jim McGhee (Fukushige et al., 1998).

Transgenics

Standard techniques were used to manipulate DNA. All enhancer elements were fused to a *pes-10* minimal promoter to drive expression of a GFP::HIS2B fusion protein (*pAP10*) (Gaudet and Mango, 2002). Constructs were injected at 40 ng/μl with 100 ng/μl *rol-6* DNA. At least two independent lines were analyzed for each transgene, and at least 20 embryos were examined per line. Promoter/enhancer mutagenesis and inversion of binding sites was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Predicted CSL sites were inverted by reversing individual RTGGGAA or TTCCCAY sequences. The inverted *1-CSL* and *2-CSL* sequences would conform with the YRTGRGAA consensus CSL-binding site described previously (Yoo et al., 2004).

RNAi

RNAi against embryonically expressed genes were typically performed by injection. Briefly, young hermaphrodites were injected with dsRNA produced by in vitro transcription (0.5-2 μg/μl) and the progeny analyzed after 24 hours. For *elt-2* RNAi, injected animals were allowed to develop for 24 hours at 22°C, then an additional 12 hours at 15°C before scoring progeny. RNAi targeting maternally expressed genes was performed by feeding (Timmons and Fire, 1998).

Ectopic expression of ELT-2/GATA

For ectopic expression during AB interactions, two- to eight-cell embryos were heat-shocked at 33°C for 30 minutes then allowed to recover at 20°C for 1-1.5 hours. For ectopic expression during the pm8 interaction, E16 stage embryos were heat-shocked for 30 minutes at 33°C, allowed to recover for 2-3 hours, and then analyzed.

GST pulldowns

GST pulldowns were performed essentially as described (Poortinga et al., 1998). Briefly, full-length ELT-2 was expressed from the T7 promoter in the pCITE vector using the Promega TnT in vitro expression kit (Promega, Madison, WI) with 0.01 mM $ZnCl_2$ and labeled with ^{35}S. In vitro translated proteins were first pre-cleared by incubation with GST alone on glutathione-Sepharose beads, then incubated with GST, GST-LAG-1 (Kovall and Hendrickson, 2004) or GST-Cad ICD proteins. Beads were washed four times with PBS + 0.5% Nonidet P40 and potential protein-protein complexes were resolved by SDS-PAGE and visualized by autoradiography. GST-Cad ICD is a GST fusion to the C-terminus (200 aa) of the intracellular domain of *Drosophila* DE-cadherin, and was kindly provided by Susan Parkhurst (Fred Hutchinson Cancer Research Center, Seattle, WA).

DNA binding

Electrophoretic mobility shift assays (EMSA) were performed essentially as described (Stroeher et al., 1994). The ^{32}P-labeled probe used for ELT-2 EMSA was from the *pho-1* gene: catcgagtagccaACTGATAAagacat-tactacaa. ELT-2 was synthesized in vitro from the T7 promoter in the pCITE 4a vector (pAN47) using the Promega TnT in vitro expression kit (Promega, Madison, WI, USA) and labeled with ^{35}S. The ^{32}P-labeled probe used for GST-LAG EMSA was: gaattctcgcgactCGTGGGAAaatgggcggaagggcac-CGTGGGAAaatagttccaggaattc. This sequence has been used previously for EMSA with LAG-1 (Hwang et al., 2007), and the underlined region has been used for EMSA with CBF1/CSL (Zimber-Strobl et al., 1994). GST-LAG-1(192-663) has been described previously (Kovall and Hendrickson, 2004). EMSA reactions used 25 ng GST-LAG-1 or 3 μl ELT-2 TnT lysate and 0.1 μg of poly(dI-dC) per reaction. Probes were at 0.02 μM [LAG-1 EMSA] and 0.1 μM [ELT-2 EMSA]. Binding reactions were incubated for 30 minutes at 22°C.

Genomic sequence analysis

DNA searches were performed using Genome enhancer (www.genomeenhancer.org). SPS searches used a 36 bp window to find both RTGGGAA and TTCCCAY sequences located within 3 kb of predicted genes. A 50 bp window was used to search for clusters of two TTCCAY and TGATAR sequences.

RESULTS

A conserved *ref-1* enhancer drives Notch-specific endoderm expression

The Notch target *ref-1* is the only member of the *ref-1* gene family expressed in two Notch interactions that occur during the embryonic development of the endoderm (Neves and Priess, 2005; Hermann et al., 2000). Briefly, the entire endoderm in *C. elegans* is derived from a single embryonic blastomere called E; successive stages of the endodermal primordium are called E2, E4, etc., corresponding to the number of E descendants (Fig. 1A). All E4 cells express the receptor

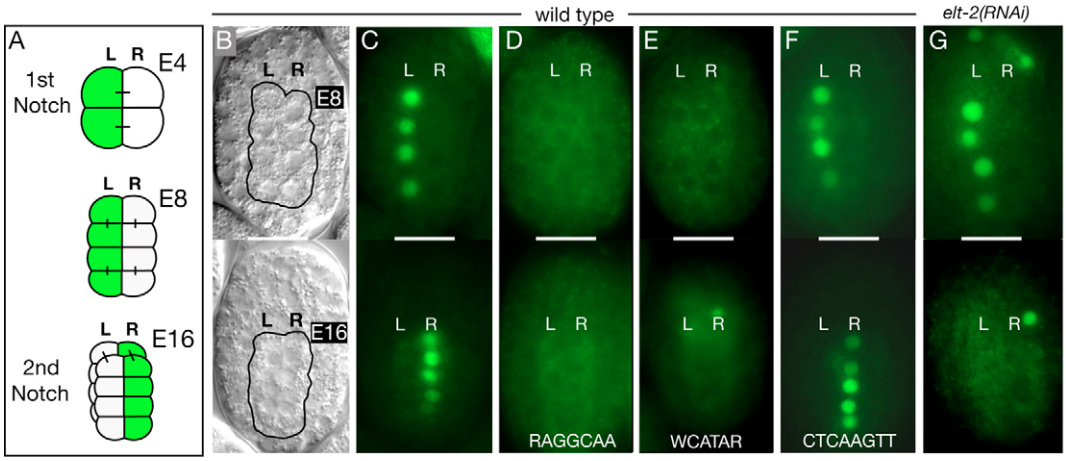

Fig. 1. Control of Notch-dependent endoderm expression. (**A**) Diagram of the two Notch interactions in the endoderm. Cells in the endoderm primordium that are activated by Notch signaling are shown in green. (**B-G**) The top and bottom panels show embryos at the E8 and E16 stages, respectively, with anterior at top. Images were taken through the plane of the intestinal primordium (outlined in black); the E16 primordium appears smaller than the E8 because the posterior end curves downward, out of the focal plane. (B) Differential interference contrast (DIC) micrographs. (C-F) Fluorescence micrographs of embryos with wild-type or mutant *enh^{A}*::*GFP* transgenes; mutations were introduced into either pAN20 or pKG55 (Fig. 2A). (C) Wild-type *enh^{A}*::*GFP* (pAN20); the onset and pattern of GFP expression in the endoderm is identical to *ref-1::REF-1::GFP* expression described previously (Neves and Priess, 2005). (D) CSL sites mutated to RAGGCAA (pAN20). (E) WGATAR sequences mutated to WCATAR (pKG55). (F) Candidate NK site mutated to CTCAAGTT (pAN20). (G) Wild-type embryos depleted for ELT-2/GATA by RNAi (GFP reporter is ANPCR in Fig. 2A).

LIN-12/Notch, but only the left two E4 cells contact ligand-expressing cells outside the primordium. The E4-Notch interaction induces *ref-1* expression in the left side of the E4 and E8 primodium, where REF-1 functions to downregulate LIN-12/Notch expression. Because LIN-12/Notch persists only in the right primordial cells, a subsequent interaction at the E16 stage induces *ref-1* expression on the right side of the primordium (Neves and Priess, 2005; Hermann et al., 2000) (Fig. 1A). Thus, *ref-1* expression switches from the left E4 and E8 cells to the right E16 cells (Fig. 1A). The E4 and E16 interactions cause a handed rotation in the positions of anterior endodermal cells, resulting in an asymmetrical twist in the developing intestine (Hermann et al., 2000).

We showed previously that a 1.8 kb region 5′ of the *ref-1* initiator ATG was sufficient for Notch-dependent *ref-1* expression in both the first and second endodermal interactions (Neves and Priess, 2005). In the present study, DNA fragments from this region were fused to a GFP reporter containing a heterologous minimal promoter, and transgenes were injected into wild-type worms to test for endoderm expression (Fig. 2A). These experiments identified a 153 bp element (called *enh^{A}*; Fig. 2B) that was sufficient to recapitulate the normal pattern of *ref-1* expression for both Notch interactions in the endoderm (Fig. 1B,C and Fig. 2A; Table 1); smaller fragments showed no expression (Fig. 2A).

Table 1. ELT-2 is required for *enh^{A}::GFP* expression in E16 endodermal cells

	enh^{A}::GFP expression			
Embryo type	AB	E8	E16	pm8
Wild type	0%(67)	42%(45)	49%(127)	38%(98)
elt-2(RNAi)	0%(54)	48%(42)	3%(106)	42%(96)

Expression of the *enh^{A}::GFP* transgene was scored in AB descendants at the 24-cell to 28-cell stage, in the left set of E8 cells, in the right set of E16 cells and in the mesodermal cell pm8. The transgene is not integrated and has a germline transmission frequency of 28% (*n*=339).

In the course of this study, we discovered that *ref-1* was expressed in a few cells in the pharyngeal primordium of the embryo; one of these pharyngeal cells, called pm8 (pharyngeal muscle group 8), is specified by Notch signaling (our unpublished results). Both the *ref-1$^{(1.8kb)}$::GFP* and *enh^{A}::GFP* transgenes showed Notch-dependent expression in the mesodermal cell pm8 in addition to Notch-dependent expression in the endoderm (Fig. 3A,B,G,H; Table 1; data not shown). Expression in pm8 occurs at about 325 minutes after the two-cell stage of embryogenesis, whereas expression in the second endodermal Notch interaction begins at about 250 minutes.

Part of the *enh^{A}* element overlaps a sequence we noted previously as being highly conserved in promoters of *ref-1* orthologues in *Caenorhabditis briggsae* and *Caenorhabditis remanei* – strains that are thought to have diverged from *C. elegans* by about 100 million years (Fig. 2C) (Neves and Priess, 2005). Recent genomic sequence from a fourth nematode species, *Caenorhabditis brenneri* (Washington University Genome Sequencing Center) includes a predicted orthologue of *ref-1* with a similar highly conserved upstream sequence (Fig. 2C). The conserved region occurs at varying distances from the predicted initiator ATG (from about –300 bp in *C. elegans* to –1400 bp in *C. briggsae*) and in both orientations with respect to coding regions (data not shown). We constructed GFP transgenes using the conserved regions from *C. briggsae* or *C. remanei*, and found that each transgene was expressed in *C. elegans* in the Notch-activated E8, E16 and pm8 cells (Fig. 3C,D; data not shown). Each of the conserved sequences contains multiple RTGGGAA sequences that are predicted LAG-1/CSL-binding sites (labeled *1-CSL* to *4-CSL* in Fig. 2B,C) (Christensen et al., 1996). A region from the *C. elegans enh^{A}* sequence that includes *2-CSL* and *3-CSL* (bold line in Fig. 2B) was tested for in vitro binding of LAG-1/CSL (Fig. 4A). In these experiments, wild-type or mutated *enh^{A}* DNA was used to compete binding between LAG-1 and a labeled probe containing two CSL sites that has been described previously and which binds LAG-1 in vitro (Fig. 4A) (Hwang et al., 2007). The wild-type sequence from *enh^{A}* competed effectively for LAG-1

binding; competition was not observed when both *2-CSL* and *3-CSL* were mutated from RTGGGAA to RAGGCAA, and LAG-1 appeared to bind both sites comparably (Fig. 4A). A GFP transgene was constructed where all four of the predicted CSL-binding sites in enh^A were mutated from RTGGGAA to RAGGCAA. This transgene was not expressed in the Notch-activated E4, E8, E16 or pm8 cells (Fig. 1D; Table 2), but was expressed in several Notch-independent cells (data not shown). For these several reasons we conclude that enh^A is a Notch-regulated enhancer for endodermal and mesodermal gene expression.

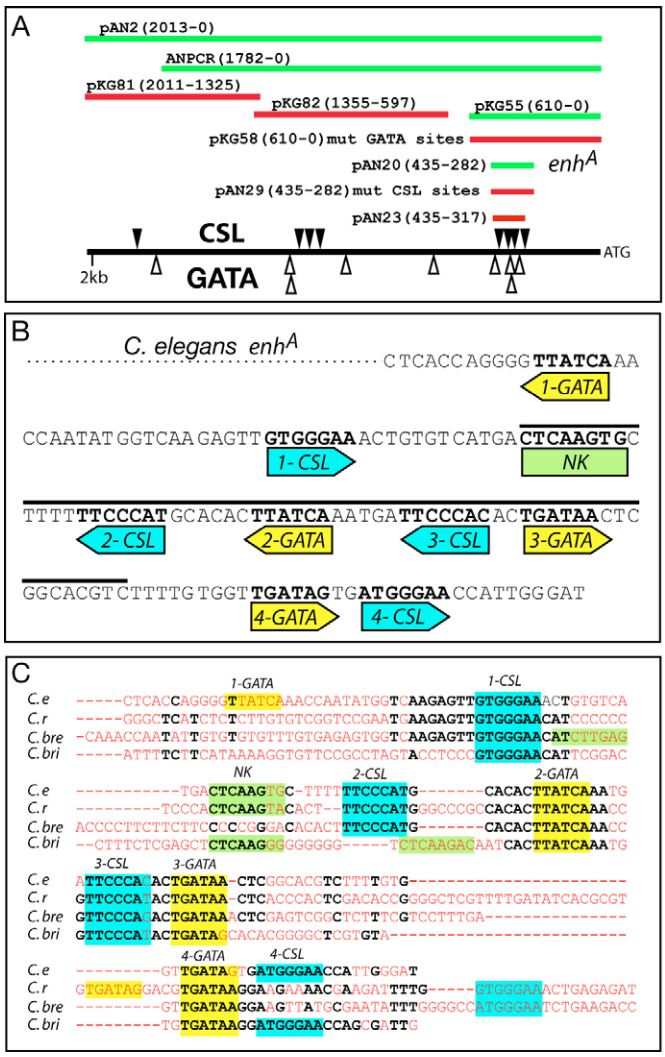

Fig. 2. Identification of a Notch-regulated endoderm/mesoderm enhancer. (**A**) The *ref-1* promoter regions shown were fused to GFP and assayed for expression in Notch-activated endodermal cells (green, expressed; red, not expressed). Transgenes were generated as plasmids (e.g. pAN20) or PCR products (e.g. ANPCR); pAN2 and ANPCR have been described previously (Neves and Priess, 2005). Numbers indicate distance from the initiator ATG (position 0). Inverted black and white triangles represent CSL- and GATA-binding sites, respectively. (**B**) Diagram of *C. elegans* enh^A. Cyan and yellow arrows indicate orientation of CSL and GATA sites, respectively. The green box represents a possible NK-binding site. Bold line indicates region used for EMSA experiments (see Fig. 4). (**C**) Sequence alignment of the enh^A element from *C. elegans* (C.e), *C. remanei* (C.r) *C. briggsae* (C.bri) and *C. brenneri* (C.bre). Black lettering indicates that the nucleotide is conserved in at least three species. Note that the *3-CSL* sequence in *C. brenneri* (TCTGGGAA) differs from a consensus CSL binding sites (YRTGRGAA) (Yoo et al., 2004); this sequence was confirmed by PCR amplification and analysis of genomic DNA from *C. brenneri*.

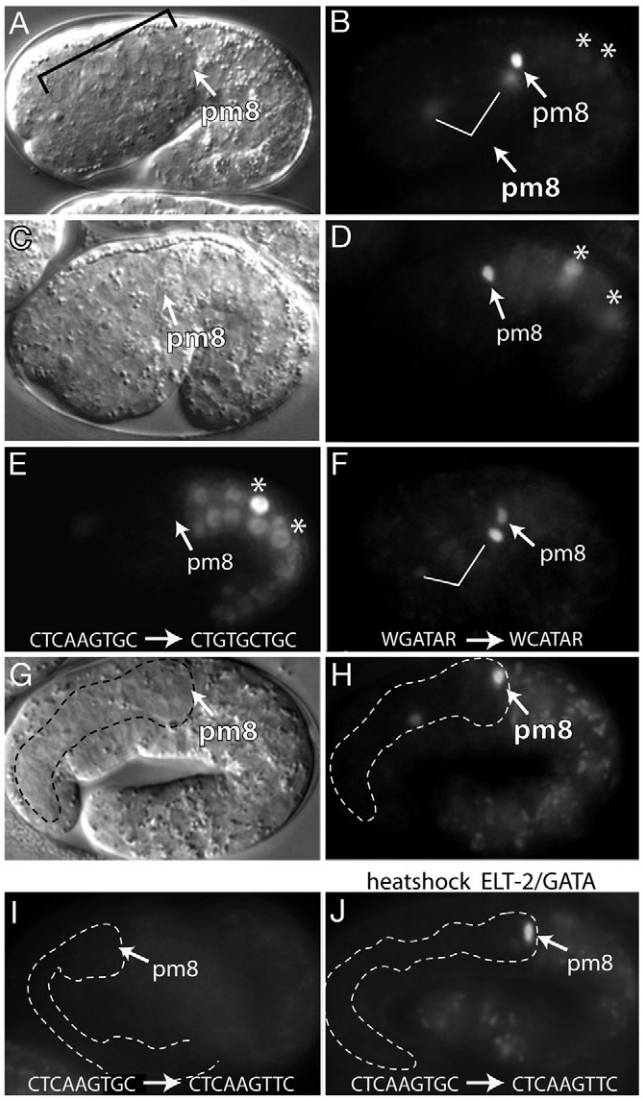

Fig. 3. Notch-regulated gene expression in pm8. (**A**) DIC image of wild-type embryo at the 1.5-fold stage focused at the plane of the pharyngeal primordium (bracket); anterior is left. (**B**) Same embryo as in A, showing GFP expression from enh^A *::GFP* (pKG55). The bent line indicates a pair of sister cells in the pharynx that express *ref-1* independent of Notch signaling; these cells are below the focal plane shown, and appear faint. Asterisks here and in panels D and E indicate intestinal cells with faint GFP expression persisting from the second endodermal Notch interaction. (**C**,**D**) DIC and fluorescence image of 1.5-fold stage embryo showing *C. briggsae* enh^A *::GFP* expression. (**E**) GFP expression after mutating the potential NK site in enh^A to CTGTGCTGC. (**F**) GFP expression after mutating all WGATAR sequences in enh^A to WCATAR. (**G**,**H**) DIC and fluorescence images of a 3-fold stage embryo showing enh^A *::GFP* expression in pm8. Small spots appearing posterior to the pharynx in H and below in J are autofluorescent granules in the intestine not related to transgene expression. (**I**) Lack of GFP expression in pm8 after mutating the NK site to CTCAAGTTC. (**J**) Expression of the same transgene used for I after heat-shock expression of ELT-2/GATA.

enh^{A} does not contain either of two sequence motifs noted previously in the promoters of some Notch-regulated genes expressed in postembryonic development (Yoo et al., 2004). However, the *enh*^{A}-related sequences from all four *Caenorhabditis* species share two motifs in addition to CSL sites (Fig. 2C). The first, CTCAAG, is a core binding motif for some NK-type homeodomain transcription factors such as mammalian TTF1 (thyroid transcription factor) and *Drosophila* Tinman (Guazzi et al., 1990; Kelly et al., 1996; Gajewski et al., 1997; Li et al., 1998). The extended CTCAAGTGC sequence in *enh*^{A} is nearly identical to a repeated CTCAAGTGG binding site for Tinman in the *D-mef2* gene (Gajewski et al., 1997). We found that mutating the CTCAAGTGC sequence to CTGTGCTGC or CTCAAGTTC abolished or markedly reduced expression in pm8 (Fig. 3E,I; Table 2). The *C. elegans* genome encodes four NK-type homeobox transcription factors: CEH-22, CEH-24, CEH-27 and CEH-28. At least one of these factors (CEH-24) is expressed in pm8, but does not appear essential for at least some aspects of pm8 differentiation (Harfe and Fire, 1998). *ceh-24* is a direct target of Notch signaling in pm8, but we do not yet know whether CEH-24 has a redundant role in regulating *ref-1* expression in pm8 (our unpublished results).

The *enh*^{A} element also contains multiple, conserved WGATAR sequences that are predicted binding sites for GATA transcription factors (for a review, see Patient and McGhee, 2002). Mutating all of the WGATAR sequences in *enh*^{A} to WCATAR did not prevent expression in pm8 (Fig. 3F, and Table 2), but abolished expression in both sets of Notch-regulated endodermal cells (Fig. 1E and Table 2). Mutating only the fourth site (*4-GATA*) or the non-conserved first site (*1-GATA*) had little if any effect on expression levels in the endodermal cells (Table 2). However, mutating either *2-GATA* or *3-GATA* markedly reduced endoderm expression (Table 2 and data not shown). These results together suggest that *enh*^{A} contains distinct sequences that collaborate with CSL-binding sites to drive Notch-dependent expression in either mesodermal or endodermal cells.

Table 2. Contribution of different CSL and GATA sites for Notch-regulated expression of *enh*^{A}*::GFP*

Mutations in *enh*^{A}	*enh*^{A}*::GFP* expression	
	Endoderm (E4,E8,E16)	Mesoderm (pm8)
None	++++	++++
RTGGGAA to RAGGCAA*		
1,2,3,4-CSL	–	–
1-CSL	+++	+
2-CSL	+	+
3-CSL	+/–	++++
4-CSL	+++	++++
1-CSL inverted*	++++	++++
2-CSL inverted*	+/–	++++
3-CSL inverted*	+/–	++++
4-CSL inverted*	++++	++++
3-CSL interchange *3-GATA*	+/–	++++
WGATAR to WCATAR		
1,2,3,4-GATA	–	++++
1-GATA	+++	++++
2-GATA	+	++++
3-GATA	+/–	++++
4-GATA	+++	++++
CTCAAGTGC to CTGTGCTGC*	++++	–
CTCAAGTGC to CTCAAGTTC*	++++	+/–

Expression of *enh*^{A}::GFP in Notch-activated endodermal cells (middle column) or the pm8 mesodermal cell (right column). Mutations were made in individual sites as listed (left column). Mutations indicated by asterisks were made in the parental plasmid pAN20, all others were made in pKG55 (Fig. 2A). Expression was scored as follows: ++++, indistinguishable from wild-type; +++, less than wild-type expression in many embryos; +, low levels of expression; +/–, very low expression; –, no apparent expression. Embryos from multiple lines containing the wild-type transgenes consistently showed high levels of expression (++++, *n*>30). Mutated transgenes showed more variable expression. For example, mutating *2-GATA* to WCATAR resulted in 12/24 embryos with no detectable expression, 9/24 with faint expression, and 3/24 embryos with approximately wild-type expression.

ELT-2/GATA regulates Notch-dependent expression in the endoderm

The finding that WGATAR sequences in *enh*^{A} are crucial for endoderm expression suggests that GATA transcription factors cooperate with Notch signaling in the endoderm. Genetic and molecular studies have shown that the specification of the E blastomere and the differentiation of intestinal cells are controlled by a regulatory cascade of multiple GATA transcription factors (for reviews, see Maduro and Rothman, 2002; McGhee, 2007). The GATA factors have overlapping patterns of expression, beginning in the parent of the E blastomere (MED-1, MED-2), in the E blastomere (END-1, END-3), in the daughters of the E blastomere (ELT-2, ELT-7) or in their descendants (ELT-4). These GATA factors are expressed in all endodermal cells, in contrast to the left-right asymmetric, Notch-dependent expression of *ref-1*. We were unable to assign a role to any of the GATA factors in the first endodermal Notch interaction using RNAi or mutant analysis (data not shown); this analysis was complicated by the requirement for END-1 and END-3 in endoderm specification, such that many affected embryos lacked endoderm (Maduro et al., 2005). However, we found that ELT-2/GATA was involved in the second (E16) endodermal interaction: most embryos lacked E16 expression of the integrated *ref-1*$^{(1.8kb)}$*::GFP* transgene (24/32 embryos) and of the non-integrated *enh*^{A}*::GFP* transgene (Table 1 and Fig. 1G, bottom panel) after depletion of ELT-2 by RNAi. Moreover, many of the *elt-2(RNAi)* embryos showed intestinal morphogenesis defects similar to those described previously in *ref-1* or *lin-12/Notch* mutants [34% of embryos (*n*=82) lacked intestinal twist] (see also Hermann et al., 2000; Neves and Priess 2005).

Previous studies have analyzed the regulation of the *pho-1* gene by ELT-2/GATA (Hawkins and McGhee, 1995). All endodermal cells express *pho-1*, and expression is not known to involve Notch signaling. Endodermal expression requires a *pho-1* promoter element that contains three WGATAR sequences, and which lacks predicted CSL-binding sites (Fukushige et al., 2003). The GATA site most critical for *pho-1* expression in the endoderm has the extended sequence ACTGATAA, and DNA from the *pho-1* promoter containing this sequence binds ELT-2 in vitro (Fig. 4B) (Hawkins and McGhee, 1995). The *3-GATA* site in *enh*^{A} has the identical sequence – ACTGATAA – and our above results showed that *3-GATA* and *2-GATA* are critical for endodermal expression of *enh*^{A}*::GFP*. To test whether ELT-2 could bind *2-GATA* and/or *3-GATA*, we used *enh*^{A} DNA (bold line in Fig. 2B) as competitor for labeled *pho-1* DNA (Fig. 4B). These results demonstrate that ELT-2 can bind both *2-GATA* and *3-GATA* in vitro, and that binding to *3-GATA* is reproducibly stronger than to *2-GATA* (Fig. 4B). These in vitro and in vivo experiments suggest that ELT-2/GATA cooperates with Notch signaling to regulate *ref-1* expression in the E16 endodermal cells.

ELT-2/GATA and *enh*^{A} are sufficient to drive Notch-regulated expression in non-endodermal cells

enh^{A} promotes Notch-dependent transgene expression in endodermal (E8, E16) and mesodermal (pm8) cells, yet *enh*^{A}*::GFP* is not expressed in numerous Notch interactions that occur either earlier or later in embryogenesis, or occur in ectodermal cells [see

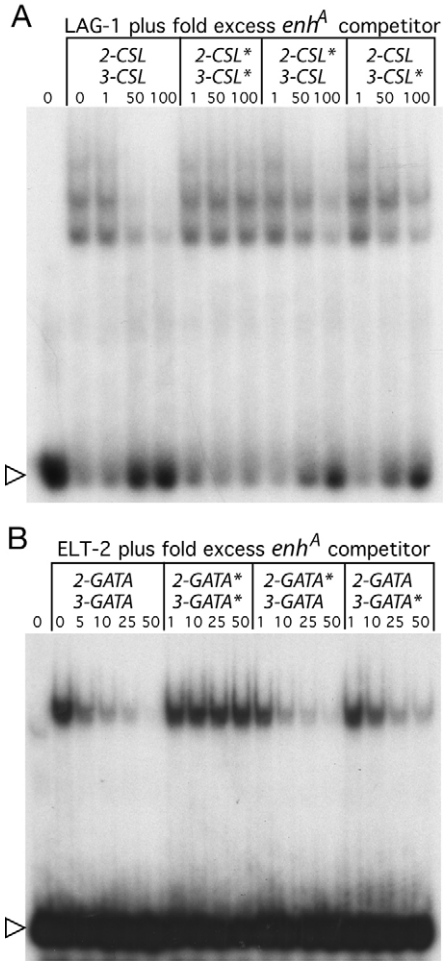

Fig. 4. In vitro binding of LAG-1/CSL and ELT-2/GATA to *enh^A* sequences. (**A**) Electrophoretic mobility shift assay (EMSA) using GST::LAG-1 and a previously described probe that binds LAG-1 through two CSL-binding sites (Hwang et al., 2007); arrowhead indicates free probe. Competitor DNA is from *enh^A* (see Fig. 2B). *2-CSL* and *3-CSL* indicate wild-type sites; *2-CSL** and *3-CSL** are ATGGGAA to AAGGCAA and GTGGGAA to GAGGCAA mutations, respectively. (**B**) EMSA using in vitro translated ELT-2 and a labeled probe from the *pho-1* endodermal enhancer (Fukushige et al., 2003). Competitor DNA is the same as in A. *2-GATA* and *3-GATA* are the wild-type sites; *2-GATA** and *3-GATA** are mutations from GATA to CATA. Note that competition with the [*2-GATA 3-GATA**] probe is approximately fivefold less than with the [*2-GATA* 3-GATA*] probe.

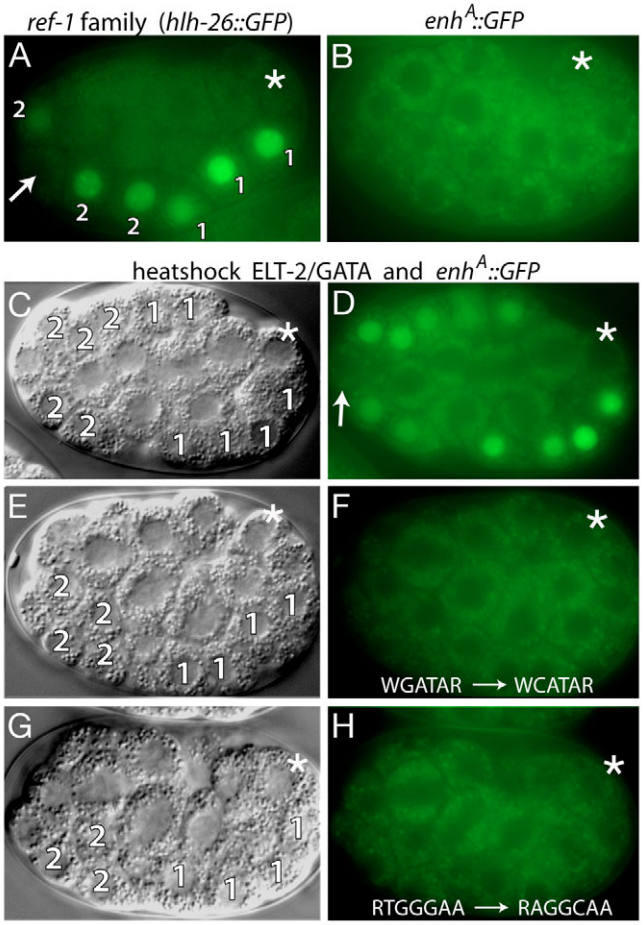

Fig. 5. ELT-2/GATA is sufficient for *enh^A::GFP* expression in non-endodermal cells activated by Notch. (**A**) Expression of a *ref-1* family member, *hlh-26*, following Notch interactions in AB descendants. The embryo is at the 26-cell stage, anterior to left. Cells labeled 1 are AB descendants activated by Notch signaling beginning at the four-cell stage of embryogenesis; cells labeled 2 are AB descendants activated by Notch signaling beginning at the 12-cell stage of embryogenesis. The arrow indicates an AB descendant that does not contact any ligand-expressing cells, and so is not activated by either the first or second interaction. The asterisk here and below indicates the P4 blastomere; this blastomere is transcriptionally quiescent, and thus serves as a negative control for expression levels. (**B**) Lack of *enh^A::GFP* expression in AB descendants in wild-type embryos. Here and below, the presence of the transgene was confirmed by either Notch-dependent or Notch-independent expression at later embryonic stages. (**C-H**) DIC and fluorescent images of similarly oriented embryos at the same developmental stages following heat shock of ELT-2/GATA. Because cell division patterns are invariant in early *C. elegans* embryos, the Notch-activated AB descendants (sets 1 and 2) can be identified solely by position. (E,F) Lack of GFP expression after mutating all WGATAR sequences to WCATAR. (G,H) Lack of GFP expression after mutating all CSL sites to RAGGCAA.

Priess (Priess, 2005) for a review of Notch interactions in the embryo]. At the 4-cell stage, for example, a daughter of the AB blastomere that is an ectodermal precursor undergoes a Notch interaction, and at the 12-cell stage a different set of AB descendants undergo a distinct Notch interaction. Notch targets in the *ref-1* family, such as *hlh-26*, are expressed in both sets of Notch-activated AB descendants (numbered 1 and 2 in Fig. 5A), but not in other AB descendants (arrow in Fig. 5A); the *enh^A::GFP* transgene is not expressed in any of these AB descendants (Table 1). To test the hypothesis that *enh^A* plus ELT-2/GATA is sufficient for Notch-specific expression, we used heat shock to misexpress ELT-2 during early embryogenesis. The *enh^A* transgene was expressed strongly in all of the AB descendants that are activated by Notch signaling at the 4-cell and 12-cell stages, but was not expressed in any of the other AB descendants, or in any other cells in the embryo (Fig. 5C,D). To confirm that the predicted GATA-binding sites in *enh^A* were important for ectopic Notch-specific expression, the heat shock experiment was repeated using a transgene with all four WGATAR sequences mutated to WCATAR. This modified transgene was not expressed in any of the early embryonic cells following heat-shock expression of ELT-2/GATA (Fig. 5E,F).

As a second test of the sufficiency of *enh*^{A} and ELT-2/GATA, we asked whether ELT-2 could cooperate with Notch signaling in the pm8 interaction; this interaction occurs in mesodermal tissue at approximately the 500-cell stage of embryogenesis, in contrast to the second endodermal interaction at approximately the 360-cell stage (our unpublished results). For this experiment, we used *enh*^{A} with the CTCAAGTTC mutation, which eliminates or markedly reduces pm8 expression (Fig. 3I). We found that heat-shock expression of ELT-2 during the period of the pm8 interaction resulted in robust transgene expression in pm8, but only occasional and weak expression in a few other embryonic cells (Fig. 3J). We conclude that *enh*^{A} plus ELT-2/GATA are sufficient to drive Notch-activated expression in multiple tissues and at multiple stages of embryogenesis.

Synergy between Notch signaling and ELT-2/GATA

Heat shock can drive expression of transgenic ELT-2/GATA in all the early somatic cells of the embryo (Fukushige et al., 1998), yet we observed *enh*^{A}::GFP expression only in the Notch-activated AB descendants following heat shock. The canonical model for Notch signaling provides a possible explanation for the restricted expression of *enh*^{A}::GFP; CSL proteins are complexed with co-repressors on Notch target genes, preventing expression in the absence of Notch signaling (see Introduction). Consistent with this view, LAG-1/CSL is present in all of the early embryonic nuclei (Christensen et al., 1996; Hermann et al., 2000). If LAG-1/CSL-mediated repression prevents expression of *enh*^{A}*::*GFP in cells that do not receive Notch signals, depleting LAG-1/CSL should allow expression in all cells with heat-shock ELT-2/GATA. Instead, we found that no cells expressed *enh*^{A}*::GFP* in *lag-1(RNAi)* embryos following heat shock (0/54 embryos scored between the 28- and 44-cell stages). To address the possibility that depleting LAG-1/CSL by RNAi indirectly prevented gene expression, we performed a complementary experiment by mutating all of the predicted LAG-1/CSL binding sites in the *enh*^{A}::GFP transgene and analyzing expression in an otherwise wild-type background. Heat-shock ELT-2/GATA failed to induce expression of the mutated transgene, despite the fact that the transgene retained multiple WGATAR sequences, and the transgene was present as a multi-copy array (Fig. 5G,H). Together, these results suggest that Notch signaling in AB descendants does not simply alleviate repressive functions of LAG-1/CSL, thereby allowing ELT-2/GATA to independently activate gene expression. Instead, these results argue that Notch signaling and ELT-2/GATA must synergize to promote gene expression; neither is sufficient in the absence of the other.

Notch-GATA synergy does not require a CSL-paired site

The first and second predicted LAG-1/CSL binding sites (*1-CSL* and *2-CSL*) in *enh*^{A} are conserved with the same inverted orientation and approximate spacing in three of the *Caenorhabditis* species (Fig. 2C). The orientation and spacing are similar to CSL-paired sites that are found in some Notch-regulated genes in both *Drosophila* and vertebrates (see Introduction). We therefore asked whether *1-CSL* or *2-CSL* were essential for Notch-regulated expression. Mutating either sequence from RTGGGAA to RAGGCAA markedly reduced pm8 expression (Table 2). However, inverting *1-CSL* or *2-CSL* did not noticeably affect expression in pm8. Thus, *1-CSL* and *2-CSL* are important for pm8 expression in combination with a possible NK site, but do not appear to function as a CSL-paired site. Mutating or inverting *1-CSL* did not noticeably affect the level of endodermal expression. By contrast, mutating either *2-CSL* or *3-CSL* markedly decreased endodermal expression (Table 2). Combined with our results on mutating individual GATA sites (above), these results suggest that sites *2-CSL*, *2-GATA*, *3-CSL* and *3-GATA* provide the major input to Notch-regulated endodermal expression (Fig. 2B).

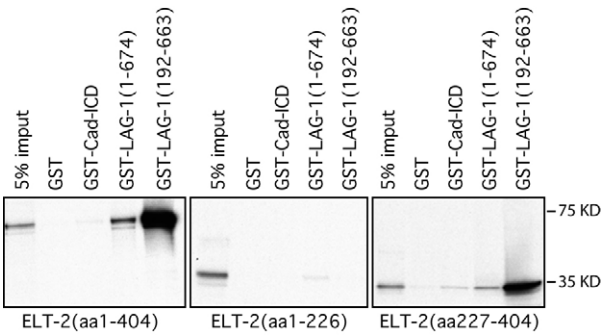

Fig. 6. LAG-1/CSL and ELT-2/GATA bind directly in vitro. Full-length or truncated ^{35}S-labeled ELT-2/GATA was tested for binding GST-LAG-1(1-674) and GST-LAG-1(192-663). Negative controls are GST alone and a GST-fusion to a cadherin domain (see Materials and methods).

Although in this study we have not examined in detail the possible spacing or orientation requirements for the sites, we found that inverting *2-CSL* or *3-CSL* markedly reduced endoderm expression, as did interchanging the positions of *3-CSL* and *3-GATA* (Table 2). A potential function of oriented CSL- and GATA-binding sites would be to facilitate physical interactions between LAG-1/CSL and ELT-2, or proteins associated with these transcription factors. To test whether LAG-1 could bind ELT-2 in vitro, GST fusion proteins were generated with full-length LAG-1 (residues 1-674) or with the LAG-1 core (amino acids 192-663); the core is conserved among CSL orthologues and has been used for crystal studies of LAG-1-DNA complexes (Kovall and Hendrickson, 2004; Wilson and Kovall, 2006). We found that both LAG-1 fusion proteins bound full-length ELT-2 (aa 1-434), with much stronger binding by the LAG-1 core (Fig. 6). In similar experiments, neither full-length nor core LAG-1 showed significant binding to the N-terminal half of ELT-2 (aa 1-226), but both reproducibly bound a C-terminal region (aa 227-404) that contains the ELT-2 DNA-binding domain (Fig. 6).

DISCUSSION

In this report, we identified a highly conserved element, *enh*^{A}, from the *ref-1* promoter that can drive Notch-dependent expression in both endodermal and mesodermal tissues. The enhancer contains four predicted binding sites for CSL proteins that are essential, but not sufficient, for expression in Notch-activated cells. This result contrasts with cell culture systems where CSL sites can be sufficient for expression, but is consistent with some in vivo studies in *Drosophila* (Furukawa et al., 1995; Guss et al., 2001). In addition to CSL sites, *enh*^{A} contains highly conserved WGATAR and CTCAAG sequences. WGATAR is a predicted binding site for GATA transcription factors, and CTCAAG sequences can function as bindings sites for members of the NK family of homeodomain proteins (Gajewski et al., 1997). The fact that these sites have remained in close proximity in all four *Caenorhabditis* species, despite considerable variation in the position and orientation of the enhancer, suggests that they have a functional relationship. Vertebrate GATA-4 and Nkx2-5 function synergistically in activating expression of cardiac genes such as atrial natriuretic factor (ANF); the ANF promoter has conserved binding sites for both factors, and Nkx2-5 and GATA-4 proteins interact in vitro and

in vivo (Durocher et al., 1997). In *C. elegans*, the mesodermal cell pm8 expresses both the NK transcription factor CEH-24 (Harfe and Fire, 1998), and the GATA factors ELT-5 and ELT-6 (our unpublished results) (Koh and Rothman, 2001). However, our studies suggest that the CTCAAG and WGATAR sequences in *enh^A* have at least partially separate roles with respect to Notch interactions in the mesoderm and endoderm, respectively. For example, mutations in all of the WGATAR sequences abolish Notch-dependent expression in the endoderm, but have no apparent effect on Notch-dependent expression in the mesoderm. It remains possible that GATA and NK proteins function together in Notch independent events in embryonic or postembryonic development, or in physiological contexts not addressed in our study.

GATA factors and Notch interactions in the endoderm

The predicted GATA-binding sites in *enh^A* are essential for two Notch interactions that occur in the endoderm, and we present evidence that ELT-2/GATA acts in concert with Notch signaling in the second interaction. The finding that heat shock ELT-2/GATA is sufficient to activate reporter expression in diverse Notch interactions argues that there are no additional endoderm-specific factors that are essential for Notch-regulated expression. Indeed, the combination of inducible ELT-2/GATA with the *enh^A*::GFP reporter might be a useful tool to identify new Notch interactions in *C. elegans* or in other animals. There are six GATA factors, including ELT-2, that are expressed before or during the first endodermal Notch interaction (Maduro and Rothman, 2002). We consider the atypical GATA factors MED-1 and MED-2 to be unlikely candidates for combinatorial factors in the first interaction, because their predicted binding sites are not present in *enh^A*, and heat shock MED-1 is unable to drive ectopic *enh^A* transgene expression in any of several Notch-activated cells (our unpublished results). Thus, ELT-2 and/or the remaining GATA factors are candidates for functionally redundant combinatorial factors in the first endodermal Notch interaction.

Notch interactions in the endoderm are required to generate a morphological twist in the intestine, but Notch mutants have an otherwise well-differentiated intestine (Hermann et al., 2000). By contrast, ELT-2/GATA appears to regulate the expression of numerous intestine-specific genes in both embryonic and postembryonic development (Pauli et al., 2006; McGhee et al., 2007). Analysis of the ELT-2 target *pho-1* has identified a 79 bp element that is important for endoderm expression; this element contains three WGATAR sequences, but no predicted CSL-binding sites (Fukushige et al., 2005) (our unpublished observations). Multi-copy arrays of transgenes with the *pho-1* element can drive endoderm-specific expression, but only if the element is multimerized in each transgene to contain between 12 and 24 WGATAR sequences. In contrast, *enh^A* transgenes with CSL-binding sites showed strong reporter expression with only three (*C. briggsae*) or four (*C. elegans*) WGATAR sequences. Heat-shock-induced expression of ELT-2/GATA was capable of driving *enh^A*::GFP expression either before or after ELT-2-dependent expression of *ref-1* normally occurs in the endoderm. However, in all cases, expression was restricted to Notch-activated cells. Similarly, heat shock ELT-2/GATA was unable to drive transgene expression when the CSL-binding sites were mutated in *enh^A*, or when LAG-1/CSL activity was depleted by RNAi. From these results we conclude that ELT-2/GATA is insufficient to drive expression *enh^A*::GFP in the absence of Notch signaling, and that this insufficiency is not caused by CSL-mediated repression.

The most critical WGATAR sequence in the promoter of the ELT-2 target *pho-1* has the extended sequence ACTGATAA; this same sequence is critical for expression of the ELT-2 target *ges-1*, and ELT-2 binds this sequence in vitro (Egan et al., 1995; Hawkins and McGhee, 1995; Fukushige et al., 2005). We showed here that an ACTGATAA sequence (*3-GATA*) is one of the two most important WGATAR sequences required for endodermal expression of *enh^A::GFP*, and that ELT-2 binds this sequence in vitro. However, the second critical GATA site in *enh^A* is TTTGATAA (*2-GATA*), and is completely conserved in each of the four *Caenorhabditis* species. This sequence differs from 19 experimentally confirmed WGATAR sequences important for ELT-2-dependent intestine-specific expression in *C. elegans*, and is very rare among potential GATA-binding sites found in a survey of over 70 intestine-specific or -enriched genes that are likely to be regulated by ELT-2 (McGhee et al., 2007). Moreover, our in vitro competition experiments suggest that ELT-2 has a lower affinity for the *2-GATA* site than for the *3-GATA* site. A possible explanation for the conservation of *2-GATA* is that it is a preferred binding site for GATA factors other than ELT-2 that presumably regulate expression in the first endodermal Notch interaction. If so, transgenes containing only *2-GATA* might be expected to show higher levels of expression after the first endodermal interaction than transgenes with only *3-GATA*. We did not observe this difference in expression; however, both transgenes were assayed as multi-copy arrays that may have masked such differences. An alternative possibility for the conservation of *2-GATA* is that presence of the NICD-CSL-MAML ternary complex facilitates binding of ELT-2 to the site, thus allowing gene expression to be dependent on Notch signaling. We have shown that the C-terminal half of ELT-2, that contains the DNA-binding domain, can bind LAG-1/CSL in vitro. Interestingly, core LAG-1 shows much stronger binding to ELT-2 in vitro than does full-length LAG-1, raising the possibility that in vivo binding to ELT-2 might be modulated by protein interactions that alter the conformation of LAG-1. In future experiments, it will be important to determine whether CSL sites or components of the ternary complex impact the recruitment of ELT-2/GATA to *2-GATA* or *3-GATA* in vivo.

Studies in several systems have shown that GATA factors often act in combination with other types of transcription factors. For example, vertebrate GATA3 recruits Smad3 to regulate target gene expression during T-cell development, and GATA4 recruits STAT proteins in the developing heart (Blokzijl et al., 2002; Wang et al., 2005). *Drosophila* studies have shown synergy between Rel/NF-κB type transcription factors and the GATA factor Serpent for immunity gene expression (Senger et al., 2004). The structures of the N- and C-terminal domains of LAG-1/CSL are remarkably similar to the N- and C-terminal Rel-homology regions of Rel/NF-κB proteins (Kovall and Hendrickson, 2004). It will be of interest to determine whether Serpent/GATA binds Rel/NF-κB proteins directly, and whether these interactions have structural parallels with ELT-2/GATA binding to LAG-1/CSL.

Towards a Notch-GATA transcriptional code

RTGGGAA and RTGAGAA sequences that are potential binding sites for CSL proteins occur frequently in the *C. elegans* genome (>80,000 copies), and are often found near genes that are unlikely to be Notch targets, such as genes encoding metabolic enzymes (Genome Enhancer, http://genomeenhancer.org/; our unpublished observations). What sequence motifs determine whether a gene will respond to Notch signaling? CSL-paired sites provide a paradigm for a Notch transcriptional code by facilitating cooperative binding of CSL ternary complexes (Nam et al., 2007) (see Introduction).

Cooperative binding involves residues in the Notch ANK domain that are conserved in vertebrates and *Drosophila*, but that are not present in the ANK domains of the *C. elegans* Notch proteins GLP-1 or LIN-12 (Nam et al., 2007). In computer searches, we identified nine *C. elegans* genes that contained a potential CSL paired site within 3 kb of the predicted initiator ATG (see Materials and methods). However, none of the promoters of the orthologous *C. briggsae* genes appeared to contain potential CSL paired sites, with the exception of the enh^A element from *ref-1*. Because our results show that the 'paired' orientation of CSL sites in enh^A is not essential for Notch-dependent expression, it is unlikely that CSL-paired sites represent an important transcriptional code for Notch regulation in *C. elegans*.

enh^A appears to promote Notch-specific expression in the endoderm primarily through two GATA sites (*2-GATA* and *3-GATA*) and two CSL sites (*2-CSL* and *3-CSL*); point mutations in any one of these sites, or changing the orientation of either CSL site, markedly reduces or eliminates enh^A::*GFP* expression. The spacing and orientations of the *2-GATA*, *3-CSL* and *3-GATA* sites have been completely conserved in all four *Caenorhabditis* species. In contrast, sequences outside the enhancer are highly divergent, and the position and orientation of the enhancer varies considerably with respect to the initiator ATG of the various *ref-1* orthologues. These observations argue against the possibility that the multiple sites have independent and equivalent functions, and simply contribute quantitatively to increase gene expression. Because ELT-2/GATA and LAG-1/CSL can interact in vitro, it is possible that some of the oriented sites in enh^A facilitate a similar interaction between these proteins in vivo. It is also possible that the sites orient CSL and GATA proteins to create unique binding surfaces for coactivators, analogous to the recruitment of the coactivator CBP by multiple proteins that bind the mammalian IFNβ enhancer (Merika et al., 1998).

GATA factors appear to have conserved roles in endoderm development in many animals, and Notch signaling is involved in the development of several endoderm-derived tissues (Contakos et al., 2005; Fre et al., 2005; Matsuda et al., 2005; Morrisey et al., 1998; Reiter et al., 1999; Reuter, 1994; van Es et al., 2005; Zhu et al., 1997). Thus, we consider it likely that examples of Notch-GATA synergy will occur in other animals. Our computer searches of genomic DNA from the four sequenced *Caenorhabditis* species do not reveal genes other than *ref-1* that have orthologues with the identical number, spacing, and orientation, of the CSL and GATA sites found in enh^A (J. Rasmussen, A.N. and J.R.P., unpublished results). It is possible that all of these sequence features are crucial for Notch-GATA synergy, but that some of the orthologous genes are not regulated by Notch signaling. Alternatively, a subset of sites in enh^A might promote a core, conserved interaction between CSL and GATA factors, with additional sites optimizing the transcriptional response. Within about 25 minutes after first contacting a signaling cell, a Notch-expressing embryonic cell in *C. elegans* can process the Notch receptor, activate transcription of a *ref-1::GFP* reporter, translate the mRNA, then produce and fold GFP into a visibly fluorescent protein (Neves and Priess, 2005). This is a remarkably short interval, and allows Notch-mediated cell fate decision to occur within a typical embryonic cell cycle lasting 30 minutes or less. Postembryonic cell cycles can extend to several hours in *C. elegans* and other animals, and thus might allow enhancers regulated by Notch and GATA more time to assemble transcriptionally-competent protein complexes.

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