THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 18, Issue of May 6, pp. 18403–18410, 2005
Printed in U.S.A.

Regulation of Sphingosine-1-phosphate Lyase Gene Expression by Members of the GATA Family of Transcription Factors*

Received for publication, September 22, 2004, and in revised form, February 9, 2005
Published, JBC Papers in Press, February 25, 2005, DOI 10.1074/jbc.M410928200

Babak Oskouian‡, Jane Mendel§, Ellyn Shocron‡, Michael A. Lee, Jr.‡, Henrik Fyrst‡, and Julie D. Saba‡¶

From the ‡Children's Hospital Oakland Research Institute, Oakland, California 94609-1673 and §California Institute of Technology, Division of Biology, Pasadena, California 91125

Sphingosine-1-phosphate is a bioactive sphingolipid that regulates proliferation, differentiation, migration, and apoptosis. Sphingosine-1-phosphate is irreversibly degraded by the highly conserved enzyme sphingosine-1-phosphate lyase. Recent studies have suggested that sphingosine-1-phosphate lyase expression affects animal development and cell fate decisions. Despite its crucial role, mechanisms affecting expression of sphingosine-1-phosphate lyase remain poorly understood. In this study, regulation of sphingosine-1-phosphate lyase gene expression was investigated in *Caenorhabditis elegans*, where lyase expression is spatially restricted to cells of the developing and adult gut and is essential for normal development. Deletion analysis and generation of transgenic worms combined with fluorescence microscopy identified a 350-nucleotide sequence upstream of the ATG start site necessary for maximal lyase expression in adult worms. Site-specific mutagenesis of a GATA transcription factor-binding motif in the promoter led to loss of reporter expression. Knockdown of the gut-specific GATA transcription factor ELT-2 by RNA interference similarly led to loss of reporter expression. ELT-2 interacted with the GATA factor-binding motif *in vitro* and was also capable of driving expression of a *Caenorhabditis elegans* lyase promoter-β-galactosidase reporter in a heterologous yeast system. These studies demonstrate that ELT-2 regulates sphingosine-1-phosphate lyase expression *in vivo*. Additionally, we demonstrate that the human sphingosine-1-phosphate lyase gene is regulated by a GATA transcription factor. Overexpression of GATA-4 led to both an increase in activity of a reporter gene as well as an increase in endogenous sphingosine-1-phosphate lyase protein.

Sphingosine 1-phosphate (S1P)[1] is a sphingolipid signaling molecule that regulates cell proliferation, migration, and apoptosis and has been shown to play a role in mediating complex physiological processes, including vascular maturation, immune cell responses, and lymphocyte trafficking (1–7). S1P mediates many of its effects through activation of a subgroup of the Edg family of G protein-coupled receptors (8). Other effects, especially those related to cell survival, appear to be mediated through both receptor-dependent and receptor-independent mechanisms (9). Intracellular S1P levels are determined by the balance between its synthesis by phosphorylation of sphingosine, catalyzed by sphingosine kinase, and its recycling back to sphingosine catalyzed primarily by S1P phosphatase or its irreversible degradation to ethanolamine phosphate and hexadecenal, catalyzed by sphingosine-1-phosphate lyase (SPL) (10). Altered expression of each of the three major enzymes controlling S1P metabolism has been shown to affect mammalian cell fate decisions and survival (11–16). Understanding the physiological mechanisms by which the expression and activity of these enzymes are regulated may be useful in further elucidating the role of S1P in physiology and disease and in providing novel approaches to manipulate S1P signaling for therapeutic purposes (17, 18).

SPL is a pyridoxal 5′-phosphate-dependent enzyme that localizes to the endoplasmic reticulum (11, 19). Until recently, SPL was presumed to be constitutively active and to serve a housekeeping function in the cell. However, it has become clear that SPL expression is influenced by a variety of factors. For example, although SPL expression has been found in most mammalian tissues with the exception of blood platelets, the degree of expression demonstrated in mouse and human tissues is quite variable, with the highest levels in liver, kidney, and intestine and lesser amounts in muscle and brain (20, 21). SPL expression in mouse embryonal carcinoma cells is upregulated during differentiation (22). In addition, SPL expression in the ecdysozoan protostomes *Drosophila melanogaster* and *Caenorhabditis elegans* is developmentally regulated, restricted to the gut, and required for normal development (23, 24). Despite this evidence of regulated SPL gene expression, no mechanism for transcriptional regulation has yet been defined nor have specific transcription factors involved in SPL expression been identified.

GATA transcription factors are a family of structurally related DNA-binding proteins first identified as key regulators of mammalian erythropoiesis (25). Members of this family have been shown to regulate gene expression in a wide variety of tissues, and functional homologs have been identified in fungi, *Drosophila,* and *C. elegans*. Vertebrate GATA transcription factors have been subdivided into two functional groups, GATA-1, -2, and -3, which are expressed in hematopoietic stem cells and neurons and regulate thymocyte, erythroid, megakaryocyte, and neuronal differentiation, and GATA-4, -5, and -6, which are expressed in tissues of mesodermal and endodermal origin and are involved in lung, liver, cardiac, intestinal, and gonadal development and

* This work was supported by National Institutes of Health Grant 1R01CA77528 (to J. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 510-450-7690; Fax: 510-450-7910; E-mail: jsaba@chori.org.

[1] The abbreviations used are: S1P, sphingosine 1-phosphate; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GST, glutathione *S*-transferase; MT, metallothionein; PBS, phosphate-buffered saline; SPL, sphingosine-1-phosphate lyase; RNAi, RNA interference; dsRNA, double-stranded RNA.

TABLE I
Primers used in these experiments

Name	Sequence
5′-120Y66	5′-TTTCTGCAGACTTTGTTTTTGAAGGGTAT-3′
5′-200Y66	5′-CGACTGCAGTCAATTGAAAAGGAGTG-3′
5′-250Y66	5′-TTTCTGCAGTGAAGTTGGTTTCATTTCC-3′
5′-275Y66	5′-CCGCTGCAGTGTTTCTGAAAAAAGTT-3′
5′-300Y66	5′-CGTCTGCAGTTCTTATCAGTATACTT-3′
5′-330Y66	5′-GTTCTGCAGCGATTTTTGTGATAATG-3′
5′-350Y66	5′-ATTCTGCAGATCCATGATTTTTCGTTTCT-3′
3′-SmaIY66H1B.4	5′-CGAATCCCGGGTAACCTGAAAA-3′
5′-Y66GATAMut	5′-TCGATTTTTGCGATCCTGACCACGTATCATATTC-3′
3′-Y66GATAMut	5′-GAATATGATACGTGGTCAGGATCGCAAAAATCGA-3′
5′-ELT2-Start	5′-TCTAGAACTAGTCCCGGGAGATCTATGGATAATAACTACAATGATAATG-3′
5′-Met-WT	5′-GATCTGTCAATAACTGATAAAATCAGAAA-3′
3′-Met-WT	5′-GATCTTTCTGATTTTATCAGTTATTGACA-3′
5′-Met-Mut	5′-GATCTGTCAATAACGGATCCAATCAGAAA-3′
3′-Met-Mut	5′-GATCTTTCTGATTGGATCCGTTATTGACA-3′
5′-T7-ELT2	5′-TAATACGACTCACTATAGGAATGGATAATAACTACAATG-3′
3′-T7-ELT2	5′-TAATACGACTCACTATAGGAGAATCTCCGTCGACCGCTTC-3′
5′-TripleGATA	5′-TCGAGTTTGTGATAATGACCATTTGTGATAATGACCATTTGTGATAATGACCAG-3′
3′-TripleGATA-Xho	5′-TCGACTGGTCATTATCACAAATGGTCATTATCACAAATGGTCATTATCACAAAC-3′
5′-SPL-WT	5′-GATCCGTTTCTTTTCGATTTTTGTGATAATGACCACGTATCATATTC-3′
3′-SPL-WT	5′-GATCGAATATGATACGTGGTCATTATCACAAAAATCGAAAAGAAACG-3′
5′-SPL-Mut	5′-GATCCGTTTCTTTTCGATTTTTGCGATCCTGACCACGTATCATATTC-3′
3′-SPL-Mut	5′-GATCGAATATGATACGTGGTCAGGATCGCAAAAATCGAAAAGAAACG-3′
5′-ELT-7	5′-GATATCCTCGAGGAATTCATGCTCCCTGAAACTACTACTCTTCAACCA-3′
3′-ELT7	5′-GATATCGAATTCCTCGAGCTATTCTTTTTTTTGCTTGACGCCGCTTTCG-3′

function (26). GATA transcription factors contain either one or two structural domains with the zinc finger motif $CXNCX_{17}CNXC$, which confers the ability to bind specifically to the consensus site (A/T)GATA(A/G). GATA transcription factors appear capable of serving both repressor and enhancer functions and have been shown to repress, activate, and/or spatially restrict the expression of various genes. Multiple GATA transcription factors have been identified in *C. elegans*, where they affect genes involved in cell migration, fusion, and cell fate specification in gut, hypodermis, vulva, and nervous tissue development (27–32).

Here we have explored mechanisms of SPL gene regulation in *C. elegans*, where a relatively small intergenic DNA sequence appears sufficient to promote maximal expression of a GFP reporter construct in intestinal cells of the developing and adult gut. A combination of deletion analysis and site-directed mutagenesis defines a 350-bp regulatory region containing a GATA transcription factor-binding site that is required for normal SPL expression. We show that the *C. elegans* GATA factor ELT-2 binds specifically to this site *in vitro*, is required for expression of SPL in the gut, and is capable of enhancing expression of the SPL promoter::β-galactosidase fusion construct in a heterologous yeast system. These results provide novel insights into the cellular mechanisms that control S1P metabolism and coordinate it with the complex developmental cascades organizing embryogenesis. We also show that GATA factors regulate SPL expression in human cells, indicating that this and potentially other mechanism(s) governing the expression of genes of sphingolipid metabolism may be conserved throughout evolution.

EXPERIMENTAL PROCEDURES

Generation and Expression of Reporter Gene Constructs for SPL Deletional Analysis—An intergenic region of 512 nucleotides of upstream untranslated sequences corresponding to a position −3 to −515 relative to the SPL ATG start codon was utilized as the full-length promoter region, as described previously (24). Deletional analysis of this promoter was performed by generating constructs containing 350, 330, 300, 275, 250, 200, and 120 nucleotides upstream of the ATG start codon. These were generated by PCR from the full-length promoter template DNA using Vent DNA polymerase (New England Biolabs, Beverly, MA), the upstream primers 5-120Y66, 5-200Y66, 5-250Y66, 5-275Y66, 5-300Y66, 5-330Y66, and 5-350Y66 and the downstream primer 3′-SmaIY66H1B (Table I). Following restriction enzyme digestion, these PCR products were cloned into PstI and SmaI sites of the GFP reporter plasmid pPD95.75 (gift of Andrew Fire). The resulting constructs were subjected to DNA sequence analysis and used to generate transgenic *C. elegans* lines as described below.

Creation of Transgenic Animals—Worms were grown under standard conditions in nematode growth medium (33). Generation of *dpy-20*(*e1362*) worms containing transgenic arrays was performed by microinjection of reporter construct DNA into the distal arm of the hermaphrodite gonad, as described previously (34, 35). Reporter construct DNA was co-injected with plasmid pMH86, allowing the recognition of transformants by rescue of the Dpy phenotype (36). Expression of the reporter was examined in both the rescued *dpy-20* and *rol-6*(*su1006*) backgrounds and found to be similar in both pattern and intensity. In the case of the 350-nucleotide reporter, reporter DNA was co-injected with the plasmid pRF4, which contains *rol6*(*su1006*), and transformants were identified by the dominant roller phenotype (37). Two independently derived stable lines for each construct were analyzed. GFP was visualized by mounting live transgenic nematodes on 5% agarose, 0.2% tricaine, 0.02% tetramisole in M9 and viewed by fluorescence microscopy (Zeiss Axioskop) using a Chroma High Q GFP long pass filter set (450 nm excitation and 505 nm emission).

Fluorescence Intensity Measurements and Imaging—Fluorescence and Nomarski images were captured using a CCD digital camera (Hamamatsu ORCA-ER) on a Zeiss Axioskop compound microscope. Mean fluorescence intensity of GFP was determined for a 200 × 250 pixel box surrounding either the int1DL/VL or int1DR/VR cell pair in the anterior gut and a 200 × 200 pixel box surrounding the int9L/R cell pair in the posterior gut using the Openlab software package (version 3.1.5, Improvision). Autofluorescence was determined using wild type (N2) hermaphrodites lacking transgene expression and subtracted from measurements obtained from transgenic hermaphrodites. All worms measured were in the mid-L4 stage of development to control for size of the expressing cells. More than 20 animals having GFP expression in both members of measured cell pair were examined for each transgene. Statistical analysis was performed using InStat software (version 3.0b, GraphPad Software Inc.). Two-tailed p values were calculated using a Mann-Whitney test.

RNA Interference by Injection—A DNA fragment containing the full-length *elt-2* open reading frame and T7 promoter sequences at 5′ and 3′ ends was generated by PCR from the pJM68 plasmid template (a full-length *elt-2* clone in pBluescriptSK(+), gift of James McGhee) using Vent DNA polymerase and the primer pair 5′-T7-ELT2 and 3′-T7-ELT2 (Table I). Double-stranded RNA (dsRNA) was synthesized using the MEGAscript RNAi *in vitro* transcription kit (Ambion, Inc., Austin, TX). The dsRNA was injected as described (35) into the distal gonads of adult *C. elegans* hermaphrodites of stable lines that express the 350-nucleotide SPL reporter construct. Eight hours after injection, each hermaph-

rodite was transferred to a fresh culture plate, and 12-h cohorts of F1 progeny were established. Broods were assessed for L1 arrest and the "gut-obstructed" phenotype as an indicator of effective knockdown of ELT-2 expression. Broods with greater than 90% arrest in L1 were examined daily with a dissecting microscope and by Nomarski microscopy (Zeiss Axioscop) (38). Control RNAi experiments were carried out simultaneously employing dsRNA corresponding to the *acn-1* (C42D8.5) gene generated as described (39) from bacterial clones derived from a *C. elegans* RNAi feeding library (40, 41). Broods were assessed for L1 arrest and evaluated for SPL reporter expression as described above.

Mutational Analysis of the SPL TGATAA Element—Site-directed mutagenesis of the TGATAA motif located at −320 to −315 in the SPL promoter was performed using the Stratagene Quikchange kit (La Jolla, CA) and the primer pair 5′-Y66GATAMut and 3′-Y66GATAMut (Table I), according to the manufacturer's instructions. The resulting PCR fragment containing the sequence change from TGATAA to CGATCC was cloned into the PstI and SmaI sites of pPD95.75, and the mutation was confirmed by nucleotide sequencing.

Electrophoretic Mobility Shift Assay (EMSA)—An ELT-2-GST fusion protein was constructed by digesting pJM68 (28) with SmaI and XhoI, isolating the ELT-2 fragment and ligating it into the SmaI/XhoI-digested vector pGEX-4T, which places ELT-2 in-frame with GST. This construct was expressed in *Escherichia coli*, and cells were harvested after induction with isopropyl 1-thio-β-D-galactopyranoside, washed with PBS, and sonicated three times with 20-s intervals. Triton X-100 was added to 1% final concentration, gently mixed in with the cells for 30 min, at which time 0.5 ml of glutathione-agarose (Sigma) was added and incubated for another 30 min. After washing with PBS containing 1% Triton X-100, bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris, pH 8. The eluted proteins were used for EMSA with a 47-nucleotide DNA probe containing the wild type TGATAA motif. The probe was labeled with polynucleotide kinase and [γ-^{32}P]ATP, followed by purification on a G-50 Sepharose column. DNA/protein binding was performed using ELT-2-GST or purified ELT-2 protein (gift of James McGhee) in 25 mM HEPES, 50 mM KCl, 1 mM dithiothreitol, 10 μM zinc sulfate, 1 μg of poly(dI/dC), 10% glycerol, followed by electrophoresis on a 5% nondenaturing acrylamide gel. To determine the specificity of the interaction between ELT-2 and the SPL promoter, competition assays were performed using unlabeled oligonucleotides corresponding to DNA elements of *C. elegans* SPL and metallothionein (MT) promoters (42). These oligonucleotides contained either a wild type or mutated GATA-binding site that ELT-2 was previously shown to bind, in the case of the MT probe (Table I). Similarly, EMSA was performed employing an ELT-7-GST fusion construct (ELT-7 cDNA was kindly provided by Joel Rothman).

Transcriptional Activation of Reporters Using a Heterologous Yeast System—SPL reporter plasmids were constructed by inserting double-stranded oligonucleotides (complementary oligomers 5′-tripleGATA and 3′-tripleGATA-Xho; Table I) containing three tandem repeats of the conserved motif TGATAA and surrounding sequences from the *C. elegans* SPL promoter into the XhoI site of plasmid pLGΔ178 (gift of Kyle Cunningham) (43, 44). Clones containing inserts in forward and reverse orientations were identified by PCR. In these constructs, *lacZ* expression is under the control of a minimal *CYC1* promoter, and the SPL promoter elements serve as upstream activating sequences. A construct containing the *elt-2* cDNA sequence in the YCpGAL vector allows ELT-2 expression after galactose induction in yeast. A similar construct containing the *elt-2* cDNA sequence in antisense orientation serves as a negative control. Co-transfection of *elt-2* and SPL reporter constructs was performed in yeast strain RE1006 (*MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52*) using the lithium acetate method (45). Double transformants were selected on synthetic complete medium containing glucose and lacking leucine and uracil (Leu$^-$ Ura$^-$). Cells containing *elt-2* and SPL reporter constructs were grown to log phase at 30 °C in Leu$^-$ Ura$^-$ medium containing 2% raffinose as carbon source. To induce transcription factor expression, galactose was added to a final concentration of 2%, and cultures were incubated for an additional 6–8 h. Quantitative assays of β-galactosidase activity were performed as described previously (46). Similar studies were performed using a construct driving expression of ELT-7.

Human SPL Luciferase Reporter Construction—A human BAC clone containing the entire human SPL gene (RPCI11 432J9) was obtained from CHORI BACPAC resources (bacpac.chori.org). A 13-kb fragment from this BAC containing the SPL promoter region was cloned in plasmid pBlueScript SKII(+), and two different size fragments were generated by restriction digestion with BamHI and KpnI, which were then cloned into the plasmid pGL3-Basic (Promega). The resulting constructs, pGL3-BamHI and pGL3-KpnI, harbor 7708 and 1825 bps, respectively, of sequences upstream of the ATG start codon of the SPL gene driving the expression of a firefly luciferase reporter. SPL gene expression was examined by co-transfection of reporter constructs along with a plasmid expressing human GATA-4. A control plasmid, pRL-CMV (Promega), which provides constitutive expression of the sea pansy luciferase, was utilized for normalizing the differences in transfection efficiency. Human GATA-4 plasmid was a gift of Xiang-Xi Xu, Fox Chase Cancer Center, Philadelphia (47). Transfections were performed using Lipofectamine 2000 (Invitrogen, Inc.) or FuGENE 6 (Roche Applied Sciences). Luciferase assays were performed on a Turner Designs Luminometer, model TD-20/20 using Dual Luciferase Reporter Assay System (Promega).

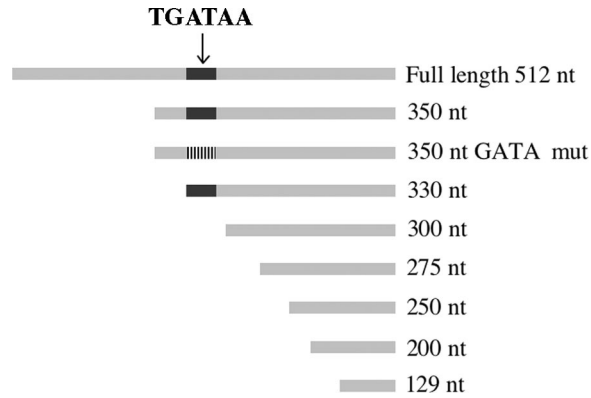

FIG. 1. **Deletion analysis of *C. elegans* SPL promoter.** Promoter fragments containing variable lengths of sequence upstream of the ATG start site as designated above were generated from the full-length 512-nucleotide promoter by PCR and cloned into the pPD95.75 GFP reporter plasmid. Site-directed mutagenesis of the conserved TGATAA to CGATCC was performed as described under "Experimental Procedures." *nt,* nucleotides.

Human Cell Line Propagation and Endogenous Human SPL Expression—The HEK293 embryonic kidney cell line (ATCC, Manassas, VA) was propagated in Dulbecco's modified Eagle's medium H-21 plus 10% fetal bovine serum under standard conditions. The MDA-MB-435 breast cancer cell line (gift of Janet Price, MD Anderson Cancer Center, Houston, TX) was propagated in RPMI plus 10% serum. For detection of endogenous human SPL by immunoblotting, a peptide matching the C terminus of the human SPL was synthesized and used to immunize a rabbit for the production of polyclonal antibodies, as described previously (11). The antisera were pooled, and an equal volume of saturated ammonium sulfate was added slowly while stirring on ice. The pH was adjusted to 7.0 with 0.5 N HCl, and the solution was left with gentle stirring overnight at 4 °C. After centrifugation at 3000 × *g* for 30 min, the pellet was dissolved in PBS, pH 7.0, and dialyzed against PBS, pH 7.0, overnight at 4 °C. After centrifugation at 10000 × *g* for 10 min, the supernatant was applied to an affinity column of 10 mg of peptide antigen coupled to 1 g of CNBr-activated Sepharose 4B. Coupling was carried out as recommended by the manufacturer (Sigma). Antibodies were applied to this column by cycling overnight with a flow of 6 ml/h at 4 °C and eluted from the column with 100 mM glycine, pH 2.0. The pH of the eluted antibody solution was immediately neutralized by adding 1 volume of 1 M Tris, pH 8.0, and dialyzed overnight against PBS, pH 7.0, containing 0.02% NaN_3.

RESULTS

Deletion Analysis of the SPL Promoter—In previous studies, we showed that a 512-nucleotide intergenic region between the *C. elegans* SPL open reading frame and the upstream gene T07A9.1 is sufficient to drive gut-restricted and developmentally regulated expression of an SPL reporter (24). The reporter expression pattern emulates the timing and spatial distribution of endogenous SPL expression, as determined by *in situ* hybridization (48). To identify specific regions of this promoter sequence implicated in the regulation of SPL gene expression, deletion analysis was performed. Promoter fragments containing variable lengths of sequences upstream of the ATG start site were generated from the full-length promoter by PCR amplification and cloned into the pPD95.75 GFP reporter plasmid (Fig. 1). These reporter constructs were then used to generate transgenic animals by simultaneous microinjection of

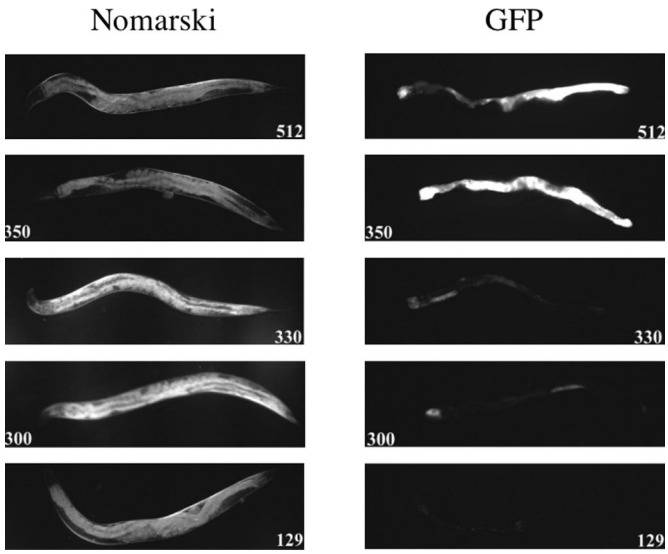

FIG. 2. **Expression pattern of SPL GFP reporters.** Transformation of worms was performed by microinjection with plasmid DNA as described under "Experimental Procedures." Several stable lines were generated for each construct and visualized by mounting live transgenic nematodes on 5% agarose and viewing by Nomarski optics and by fluorescence microscopy with a Chroma High Q GFP long pass filter. Transgenic worms containing constructs of 200, 250, and 275 nucleotides upstream of the start codon demonstrated background fluorescence similar to the 129-nucleotide construct and are not shown. In each case >20 worms were analyzed.

reporters and *dpy-20+* plasmid pMH86 into *dpy-20*(*e1362*) worms and subsequent identification of transformants by rescue of the Dpy phenotype, as described previously (24, 36). As shown in Fig. 2, stable lines in which transcription of the GFP reporter was under the control of a fragment containing 350 nucleotides of the sequences upstream of the ATG start site demonstrated a pattern of expression indistinguishable from that observed in transgenics expressing the full-length reporter with regard to timing, tissue distribution, and intensity. Maximal expression occurred at larval stage L1 and later and was maintained through adulthood. Expression was present throughout the gut, with highest intensity notable in regions just posterior to the pharynx (first intestinal ring) and just anterior to the anus (ninth intestinal ring). In contrast, worms containing transgenes harboring fragments with 275 or fewer nucleotides upstream of the ATG start site did not yield detectable expression (Fig. 2). Constructs containing 300 nucleotides upstream of the start site drove expression at ~15% of normal levels, thus defining a minimal promoter sequence. Promoters containing 330 nucleotides upstream of the start site exhibited an intermediate level of expression. These findings, which are represented quantitatively in Table II, demonstrate that *C. elegans* SPL transcription is controlled by a basal promoter contained within the 300-nucleotide sequence upstream of the ATG start site, with increasing intensity and distribution of expression throughout the gut provided by sequences between 300 and 350 nucleotides from the ATG start site.

A GATA Element Is Required for Normal SPL Expression—Inspection of the full-length 512-nucleotide intergenic sequence between SPL and T07A9.1 revealed a GATA factor-binding motif at −315 to −320 with respect to the start codon of the lyase gene. Furthermore, the gut-restricted expression pattern, developmental timing, and adult persistence of *C. elegans* SPL expression were similar to that reported for the GATA transcription factor ELT-2 (49). These observations suggested that SPL gene expression might be regulated by a GATA transcription factor. To determine whether the TGATAA motif located at −315 to −320 is involved in regulating SPL gene expression *in vivo*, site-directed mutagenesis was utilized to convert the motif to CGATCC in the 350-nucleotide construct. Transgenic worms expressing this construct were then generated as described under "Experimental Procedures" and examined for GFP expression. As shown in Fig. 3, mutation of the TGATAA motif led to a significant reduction in expression. The intensity of the GFP signal in the 350-nucleotide mutant promoter construct was reduced by 86% in the anterior gut and 25% in the posterior (Table II). These results suggest that the GATA factor-binding motif is required for maximal gut-specific expression and that mutation, deletion, or loss of sequences immediately adjacent to this motif lead to reduced GATA factor binding and expression.

Binding of ELT-2 to SPL GATA Elements—To establish the role of ELT-2 in SPL gene expression, we next examined whether the ELT-2 protein is capable of binding to the SPL promoter GATA element. Toward that end, an ELT-2-GST fusion protein was constructed, expressed in *E. coli,* and semi-purified by glutathione affinity chromatography. ELT-2-GST was shown to bind specifically to a 47-nucleotide DNA probe containing the TGATAA motif in an electrophoretic mobility shift assay, whereas GST alone was unable to bind the probe (Fig. 4*A*). To determine the specificity of ELT-2 binding, competition for binding was performed using an unlabeled oligomer corresponding to sequences within the promoter of the *C. elegans* MT gene. *C. elegans* MT transcription is known to be regulated by ELT-2 via a GATA motif present in the MT oligomer (42). As demonstrated in Fig. 4*A*, both 10- and 100-fold excess of an oligomer representing the wild type MT promoter competes successfully with the SPL promoter probe for ELT-2 binding. However, mutation of the GATA-binding motif prevents competition for ELT-2 binding even at 100-fold excess. Similar experiments performed with purified ELT-2 protein and competition using unlabeled oligomers corresponding to sequences within the promoter of the SPL gene confirm that successful competition for binding requires the presence of a wild type GATA-binding motif (Fig. 4*B*). These findings demonstrate that ELT-2 binds specifically to the GATA-binding motif of the SPL promoter *in vitro*. In contrast to our results with ELT-2, EMSA performed using ELT-7, a second putative GATA factor expressed in the nematode gut,[2] resulted in no detectable binding to the SPL promoter (data not shown). These findings demonstrate that the interaction between ELT-2 and the SPL promoter is specific and that at least one other gut-specific nematode GATA factor is unable to substitute for ELT-2 in this interaction.

ELT-2 Is Required for SPL-driven Reporter Expression—We sought to determine whether interactions between the ELT-2 transcription factor and GATA elements in the SPL promoter are required for normal SPL expression in *C. elegans*. Toward that end, RNAi was used to knock down expression of ELT-2 in worms expressing the 350-nucleotide SPL-driven reporter. Inhibition of ELT-2 expression produces a distinctive gut-obstructed phenotype, characterized by lack of patency of the gut lumen (49). In the absence of ELT-2 expression, animals arrest at the L1 larval stage and die within several days due to starvation. In order to rule out the possibility that inhibition of SPL reporter expression observed in the ELT-2 RNAi-treated worms might result from a general effect of L1 larval arrest, control experiments were simultaneously performed in SPL reporter worms using RNAi against *acn-1*, which results in L1 arrest due to a hypodermal defect (50). As shown in Fig. 5, L1 arrested progeny of *acn-1* RNAi-treated worms demonstrated strong reporter expression throughout the gut, similar to the

[2] J. Rothman and K. Strohmaier, personal communication.

TABLE II
Quantitative analysis of reporter fluorescence intensity

Reporter	Fluorescence	No. pixels	No. Fluorescence/pixel	% 350	*vs.* 350	*vs.* GATA	*vs.* N2
			Anterior gut (int1DL/VL or DR/VR)				
350	2515.35	50,000	0.0503	100.00			
350 GATA	348.68	50,000	0.0070	13.86	$p < 0.0001$[a]		
330	1436.87	50,000	0.0287	57.12	$p < 0.0001$[a]	$p < 0.0001$[a]	
300	409.25	50,000	0.0082	16.27	$p < 0.0001$[a]		
275	48.05	50,000	0.0010	1.91	$p < 0.0001$[a]		$p > 0.05$ NS[b]
N2	0	50,000	0.0000	0.00			
			Posterior gut (int9L/R)				
350	1507.17	40,000	0.0377	100.00			
350 GATA	1125.59	40,000	0.0281	74.68	$p > 0.05$ NS[b]		
330	486	40,000	0.0122	32.25	$p < 0.0001$[a]	$p < 0.005$[c]	
300	185.36	40,000	0.0046	12.30	$p < 0.0001$[a]		
275	9.15	40,000	0.0002	0.61	$p < 0.0001$[a]		$p < 0.05$[c]
N2	0	40,000	0.0000	0.00			

[a] Values indicate extremely significant.
[b] NS indicates not significant.
[c] Values indicate highly significant.
[d] Values indicate significant.

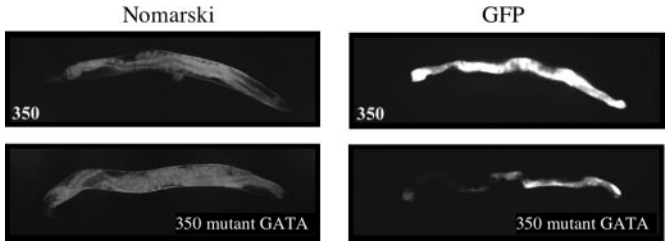

FIG. 3. **Mutation of the GATA factor-binding site leads to diminished SPL expression.** Transgenic worms containing a GFP reporter under regulation of a 350-nucleotide SPL promoter in which the GATA factor-binding motif TGATAA has been changed to CGATCC were generated and visualized by Nomarski optics and fluorescence microscopy. Worms in which this GATA motif has been mutated demonstrate a marked reduction in reporter expression compared with those containing the wild type sequence.

pattern observed in the 350-nucleotide reporter worms shown in Fig. 2. In marked contrast, worms treated with *elt-2* RNAi demonstrated little or no SPL reporter expression. These findings suggest that ELT-2 is necessary for SPL expression in the developing intestine.

In Vivo Activation of SPL Transcription by ELT-2 Expressed in Yeast—To assess the ability of ELT-2 to drive SPL expression *in vivo*, we took advantage of the fact that ELT-2, when expressed in a heterologous yeast system, is still capable of activating transcription of its target reporter genes (28, 44). Yeast cells were co-transfected with a construct containing *elt-2* under regulation of a galactose-inducible promoter along with *lacZ* reporters under the control of the SPL GATA elements (Fig. 6*A*). As shown in Fig. 6*B*, GATA motifs from the SPL promoter in either forward or reverse orientations promoted reporter expression *in vivo* in response to the ELT-2 presence, as demonstrated by significant β-galactosidase activity upon ELT-2 induction. In contrast, a construct containing ELT-2 in the antisense orientation did not promote reporter expression regardless of the orientation of GATA motifs. In addition, ELT-7 did not lead to activation of SPL in this system (Fig. 6*C*).

GATA Factors Induce Expression of Human SPL in Vivo—The ELT-2 protein sequence is homologous to GATA-4/5/6 transcription factors, which are important regulators of mammalian development and gene expression in heart, liver, gonad, gut epithelium, and lung. Analysis of the human SPL promoter using the MATInspector software (www.genomatix.de) revealed several GATA sites at −2550, −2014, −1760 and −170 nucleotides with respect to the ATG start codon. To address whether human SPL gene expression is regulated by GATA factors, the human SPL promoter was cloned; luciferase re-

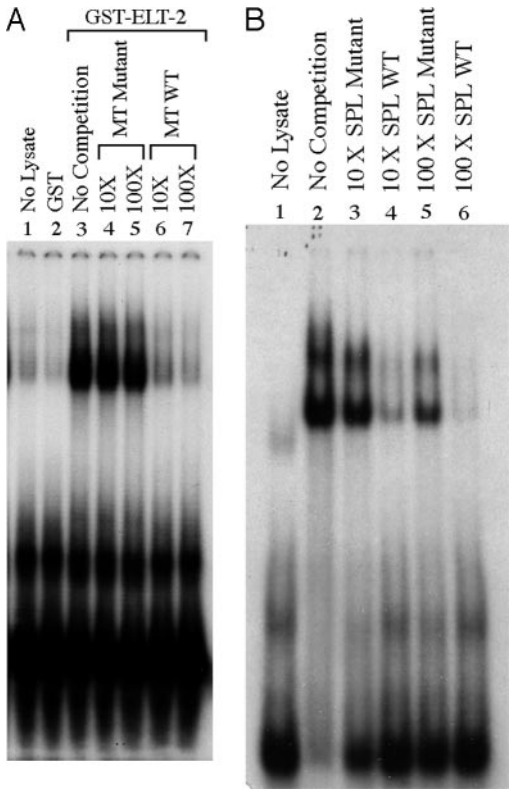

FIG. 4. **ELT-2 interacts with the SPL promoter *in vitro*.** A radiolabeled probe corresponding to a DNA sequence in the SPL promoter containing the conserved GATA factor-binding motif (Table I) was incubated with semi-purified ELT-2-GST (*A*), as described under "Experimental Procedures." Competition for binding was performed using 10- or 100-fold concentration of oligonucleotides corresponding to a sequence of the wild type *C. elegans* MT promoter (*MT WT*) shown previously to bind ELT-2 or a mutant MT promoter sequence lacking the TGATAA motif (*MT Mutant*). purified ELT-2 protein was used, rather than the ELT-2-GST fusion, and competition for binding was performed using 10- or 100-fold concentration of oligonucleotides corresponding to a sequence of the wild type SPL promoter (*SPL WT*) or a mutant SPL promoter sequence lacking the TGATAA motif (*SPL Mutant*) (*B*).

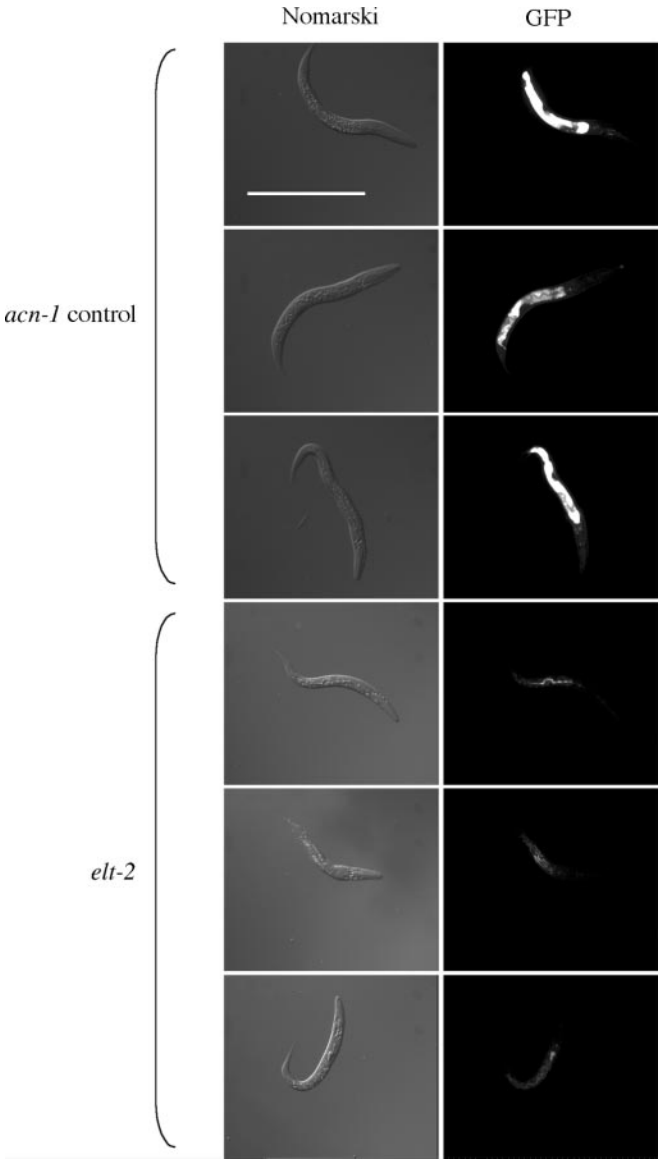

FIG. 5. **ELT-2 is required for SPL expression *in vivo*.** Adult hermaphrodites expressing transgenic arrays of a 350-nucleotide SPL promoter-driven reporter were injected with dsRNA corresponding to *elt-2* (*lower panel*) or *acn-1* (*upper panel*). The F1 progeny of worms injected with *elt-2* RNAi were evaluated for L1 arrest and "gut-arrested" phenotype, indicating loss of ELT-2 function. Worms demonstrating a phenotype consistent with ELT-2 loss of function exhibit minimal fluorescent SPL reporter expression, as shown in *lower panel*. In contrast, the L1-arrested F1 progeny of worms injected with *acn-1* RNAi strongly express the fluorescent SPL reporter in a pattern similar to the untreated reporter worms shown in Figs. 2 and 3. *Scale bar* represents 100 μm.

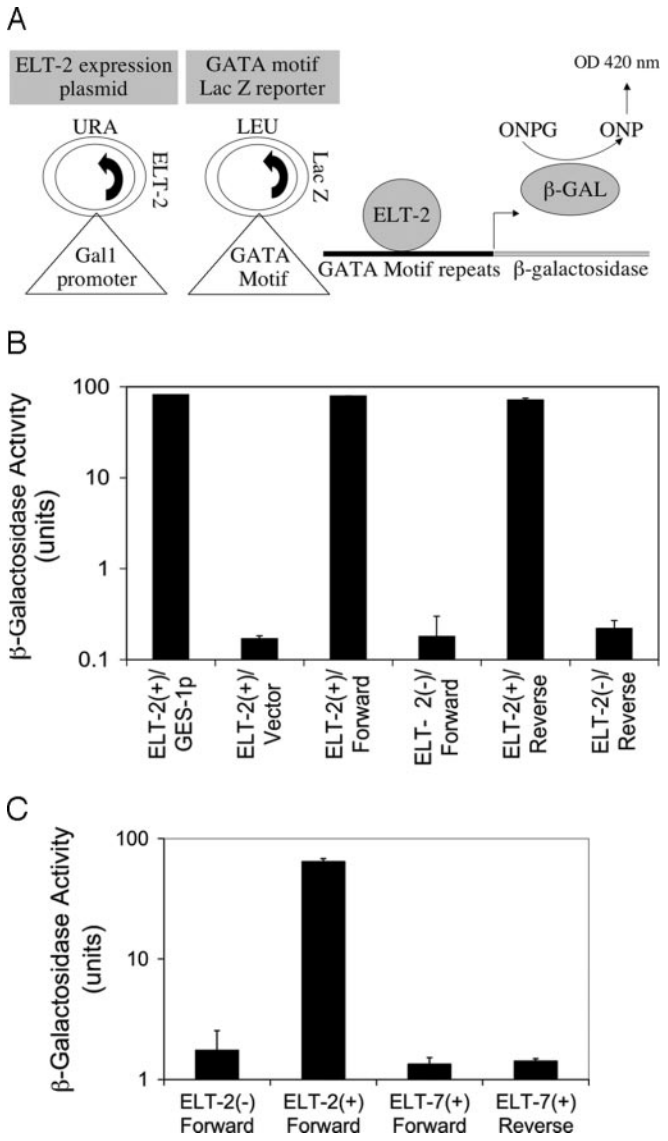

FIG. 6. **ELT-2 induces expression of SPL *in vivo*.** In the heterologous yeast expression system, *C. elegans* GATA transcription factors are expressed under regulation of a galactose-inducible promoter and evaluated for their ability to activate expression of a β-galactosidase reporter driven by a *C. elegans* target gene promoter (*A*). *ONP*, *o*-nitrophenyl; *ONPG*, *o*-nitrophenyl β-D-galactopyranoside. Expression of ELT-2 from a construct in which it is in the sense orientation (+) activates an SPL β-galactosidase (*β-GAL*) reporter with a GATA motif in either forward or reverse orientations in yeast (*B*). In contrast, expression of ELT-7 does not activate an SPL reporter (*C*). The promoter of the *ges-1* gene contains GATA elements known to be responsive to ELT-2 and was used as a positive control for reporter expression.

porter constructs were generated, and the effect of GATA factor expression on reporter induction was evaluated. As shown in Fig. 7, *A* and *B*, SPL reporter constructs were strongly induced by GATA-4 expression in HEK293 cells and in MDA-MB-435 breast cancer cells. GATA-4 transfection also led to increased expression of endogenous SPL, as determined by immunoblotting with a polyclonal antibody specific for mammalian SPL (Fig. 8) and an increase in SPL enzymatic activity (data not shown).

DISCUSSION

S1P has emerged as an important signaling molecule that regulates mammalian cell fate decisions, motility, differentiation, and angiogenesis (51). Although S1P induces signals through both extracellular and intracellular mechanisms, the putative role of S1P as a second messenger remains controversial, mainly due to the lack of identified downstream intracellular targets. The genomes of *Saccharomyces cerevisiae*, *D. melanogaster,* and *C. elegans* contain genes involved in S1P and long chain base metabolism (52). However, extracellular S1P receptors of the Edg family have not been found in simple metazoans and do not appear to have evolved prior to chordates. Thus, these non-mammalian models may present ideal systems in which to elucidate receptor-independent S1P functions, mechanisms of action, and metabolism.

Toward that end, we have explored the role of the S1P-catabolizing enzyme SPL in the developmental programs of two genetically tractable metazoan models. We found that the SPL genes of both *C. elegans* and *Drosophila* are developmentally

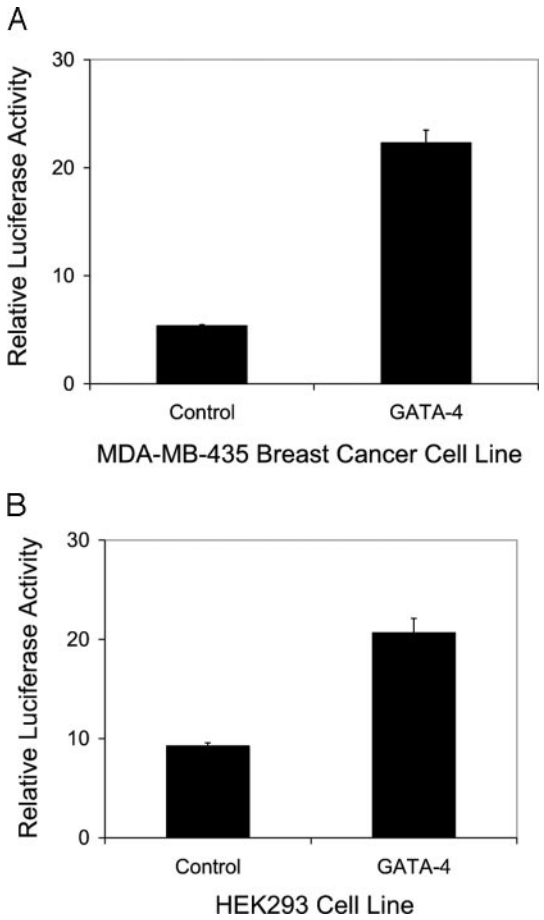

FIG. 7. **Human SPL gene expression is induced by GATA factors.** Cells were co-transfected with human SPL reporters and constructs driving constitutive expression of GATA-4. Luciferase activity was measured as described under "Experimental Procedures." Results with pLG3-KpnI constructs are shown. Similar results were obtained with pLG3-BamHI constructs. *A,* MDA-MB-435 breast cancer cells; *B,* HEK293 cells.

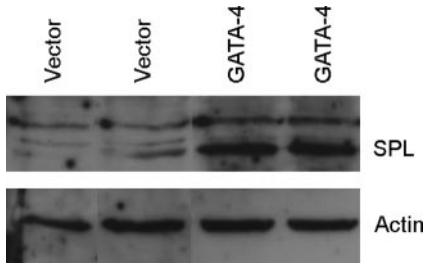

FIG. 8. **Endogenous expression of human SPL is induced by GATA factors.** HEK293 cells were transiently transfected with either pcDNA3.0 vector control or GATA-4 cDNA as described under "Experimental Procedures." Whole cell extracts were obtained at 48 h after transfection, and immunoblotting was performed with polyclonal antibody specific for mammalian SPL. Immunoblotting with an actin-specific antibody was performed as loading control. The results from two separate transfection experiments are depicted in this figure.

regulated, demonstrate gut-restricted expression, and are essential for normal development, viability, and reproduction (23, 24). In *C. elegans*, a relatively small DNA sequence upstream of the SPL open reading frame appears sufficient to drive expression of a reporter gene in a temporally and spatially regulated manner that is consistent with the expression pattern of the endogenous gene. Therefore, we chose this system to explore mechanisms of SPL gene regulation. The similarity between the expression patterns of *Drosophila* and *C. elegans* SPL genes suggests that mechanisms of gene regulation identified in *C. elegans* might be relevant to the regulation of SPL genes of other species especially, but not necessarily, limited to the ecdysozoa (animals with a cuticle that is molted) (53). Furthermore, our results as well as recent work from the laboratory of Ruth Lehmann indicate that the temporal and tissue-specific expression patterns of additional *Drosophila* genes affecting long chain base metabolism, including sphingosine kinase, ceramidase, and ceramide kinase, are similar to that of SPL (54, 55). These observations suggest the potential for coordinated regulation of these genes in metazoans.

In the current study, we investigated the mechanisms of SPL gene regulation in *C. elegans*. Deletion analysis of the *C. elegans* SPL promoter defined a 350-nucleotide sequence sufficient to drive maximal and temporally and spatially regulated expression of a GFP reporter. Analysis of additional deletion constructs identified different regions within this sequence necessary for both basal and enhanced expression, suggesting that multiple DNA cis-elements and their cognate binding factors orchestrate SPL expression within the context of the developing nematode gut. Abolition of the GATA factor binding site at −315 to −320 in this promoter led to markedly diminished expression, while the developmental timing of expression was maintained. These findings suggest that a GATA transcription factor regulates the level of SPL expression through interactions with the DNA at this site, and that other regulatory elements may contribute to the pattern and timing of expression. Although reporter expression was largely ablated in the anterior gut cells of the GATA mutated reporter, some expression remained in the posterior gut cells. This may represent a nonspecific signal, since this anatomical region has been reported to be a frequent site of nonspecific expression, possibly due to weak promoter and/or enhancer signals contained within the vectors (56). Alternatively, other cis-elements within the SPL promoter may contribute to expression in the posterior gut.

DNA binding assays along with corresponding competition assays using wild type and mutant ELT-2-binding sequences from both the SPL and the MT promoter (a known target of ELT-2) demonstrate direct and specific binding of ELT-2 to sequences in the SPL promoter *in vitro*. Inhibition of ELT-2 expression using RNAi led to loss of GFP expression in transgenic reporter worms, demonstrating that SPL expression is regulated by ELT-2 in the developing gut. In addition to demonstrating that ELT-2 is required for *C. elegans* SPL expression, the heterologous yeast expression system established that ELT-2 is capable of inducing SPL expression *in vivo*.

Most interestingly, a second GATA factor-binding motif exists in reverse orientation in the SPL promoter at −291 to −296, in close proximity to the motif we have analyzed. This motif contains the sequence ACTGATAA, which is found in several *C. elegans* gut-specific genes including the acid phosphatase *pho-1*, recently identified as a target of ELT-2 transcriptional regulation (39). This 8-nucleotide motif may be important for coordinated regulation of gene expression during intestinal development in nematodes. Furthermore, SPL cDNAs containing both SL1 and SL2 leader sequences have been identified in the *C. elegans* genomic data base Wormbase WS130 (www.wormbase.org), indicating that SPL, in addition to being driven by its own proximal promoter, may be a member of an operon. Future studies should clarify whether these factors play a significant role in the regulation of SPL expression.

Formation of the digestive system during *C. elegans* embryogenesis is regulated by a developmental program involving a cascading network of endoderm-specific transcription factors required for gut formation and the maintenance of gut integrity (27). Among these, a series of GATA transcription factors is

sequentially expressed within the developing endoderm beginning with the END-1 protein (29, 57) (expressed in E founder cells that give rise to all endodermal tissue) and including the GATA transcription factor, ELT-2, identified through its ability to bind two canonical GATA sites that regulate expression of the gut-specific esterase (*ges-1*) gene (30). ELT-2 expression is restricted to intestinal cells in embryonic and post-embryonic developmental stages, in a pattern similar to that observed for *C. elegans* SPL. END-1, END-3, ELT-2, and ELT-4 are GATA transcription factors expressed in the developing gut. Whereas END-1 and END-3 are expressed early and are involved in initiating the entire program of endodermal development, ELT-2 expression occurs later and is required for intestinal differentiation but not endodermal specification. The DNA binding domain of ELT-2 is homologous to the corresponding domains of gut-associated GATA transcription factors including vertebrate GATA-4, -5, and -6 and *Drosophila serpent* (58). *C. elegans* GATA-4 is a very small protein of 74 residues that may play a minor role in gut development (28).

We showed recently that overexpression of SPL in human cells potentiates stress-induced apoptosis through its influence on the intracellular levels of S1P and ceramide, two biochemically related bioactive sphingolipid metabolites with generally opposing effects on cell fate and survival (11). The ability of SPL to lower S1P levels and simultaneously elevate ceramide levels suggests that this enzyme may provide a novel target for diseases such as cancer in which these biomodulators are dysregulated and cell fate decisions are pathologically affected. By elucidating the mechanisms that regulate expression of SPL and other genes of S1P metabolism, it may become feasible to manipulate expression of these genes, their protein products, and subsequently intracellular S1P and ceramide levels in order to influence cell fate *in vivo* for therapeutic purposes. It is interesting to note that GATA-4, -5, and -6 have been implicated in the regulation of apoptosis and may contribute to tumorigenesis (47, 59–62). Thus, our findings may provide insight regarding the potential role of SPL in the regulation of cell fate, stress responses, and carcinogenesis.

In summary, the current study demonstrates that GATA transcription factors regulate SPL expression in nematodes and humans. Our findings thus appear to represent a conserved regulatory mechanism operational in the control of SPL expression. Further study of SPL gene regulation in *C. elegans* and other organisms is warranted and may potentially provide insight into how S1P metabolism adjusts and responds to changing cellular conditions and is coordinated with the complex signaling programs involved in animal development.

Acknowledgments—We thank James D. McGhee for generosity with reagents, critical reading of the manuscript, and for many helpful discussions; Joel Rothman and Keith Strohmaier for the use of ELT-7 constructs; Paul Sternberg for helpful discussions, and Betsy Lathrop for expert administrative assistance.

REFERENCES

1. Spiegel, S., and Milstien, S. (2003) *Nat. Rev. Mol. Cell. Biol.* **4,** 397–407
2. Xie, J. H., Nomura, N., Koprak, S. L., Quackenbush, E. J., Forrest, M. J., and Rosen, H. (2003) *J. Immunol.* **170,** 3662–3670
3. Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., and Rosen, H. (2002) *Science* **296,** 346–349
4. Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C. A., Zollinger, M., and Lynch, K. R. (2002) *J. Biol. Chem.* **277,** 21453–21457
5. Karliner, J. S. (2002) *Biochim. Biophys. Acta* **1582,** 216–221
6. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S. S., Lee, M. J., Liu, C. H., Hla, T., Spiegel, S., and Proia, R. L. (2000) *J. Clin. Investig.* **106,** 951–961
7. Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. (1999) *Cell* **99,** 301–312
8. Hla, T. (2001) *Prostaglandins Other Lipid Mediat.* **64,** 135–142
9. Spiegel, S., and Milstien, S. (2000) *FEBS Lett.* **476,** 55–57
10. Pyne, S., and Pyne, N. J. (2000) *Biochem. J.* **349,** 385–402
11. Reiss, U., Oskouian, B., Zhou, J., Gupta, V., Sooriyakumaran, P., Kelly, S., Wang, E., Merrill, A. H., Jr., and Saba, J. D. (2004) *J. Biol. Chem.* **279,** 1281–1290
12. Olivera, A., Kohama, T., Edsall, L., Nava, V., Cuvillier, O., Poulton, S., and Spiegel, S. (1999) *J. Cell Biol.* **147,** 545–558
13. Edsall, L. C., Cuvillier, O., Twitty, S., Spiegel, S., and Milstien, S. (2001) *J. Neurochem.* **76,** 1573–1584
14. Mandala, S. M. (2001) *Prostaglandins Other Lipid Mediat.* **64,** 143–156
15. Le Stunff, H., Galve-Roperh, I., Peterson, C., Milstien, S., and Spiegel, S. (2002) *J. Cell Biol.* **158,** 1039–1049
16. Johnson, K. R., Johnson, K. Y., Becker, K. P., Bielawski, J., Mao, C., and Obeid, L. M. (2003) *J. Biol. Chem.* **278,** 34541–34547
17. Spiegel, S., and Kolesnick, R. (2002) *Leukemia* **16,** 1596–1602
18. Radin, N. S. (2003) *Biochem. J.* **371,** 243–256
19. Van Veldhoven, P. P. (2000) in *Sphingolipid Metabolism and Cell Signaling* (Merrill, A. H., Jr., and Hannun, Y. A., eds) Vol. 311, pp. 244–254, Academic Press, New York
20. Zhou, J., and Saba, J. (1998) *Biochem. Biophys. Res. Commun.* **242,** 502–507
21. Van Veldhoven, P. P., Gijsbers, S., Mannaerts, G. P., Vermeesch, J. R., and Brys, V. (2000) *Biochim. Biophys. Acta* **1487,** 128–134
22. Kihara, A., Ikeda, M., Kariya, Y., Lee, E., Lee, Y., and Igarashi, Y. (2003) *J. Biol. Chem.* **278,** 14578–14585
23. Herr, D. R., Fyrst, H., Phan, V., Heinecke, K., Georges, R., Harris, G. L., and Saba, J. D. (2003) *Development* (*Camb.*) **130,** 2443–2453
24. Mendel, J., Heinecke, K., Fyrst, H., and Saba, J. D. (2003) *J. Biol. Chem.* **278,** 22341–22349
25. Orkin, S. (1992) *Blood* **80,** 575–581
26. Molkentin, J. (2000) *J. Biol. Chem.* **275,** 38949–38952
27. Maduro, M. F., and Rothman, J. H. (2002) *Dev. Biol.* **246,** 68–85
28. Fukushige, T., Goszczynski, B., Tian, H., and Mcghee, J. D. (2003) *Genetics* **165,** 575–588
29. Zhu, J., Hill, R., Heid, P., Fukuyama, M., Sugimoto, A., Preiss, J., and Rothman, J. (1997) *Genes Dev.* **11,** 2883–2896
30. Hawkins, M., and McGhee, J. (1995) *J. Biol. Chem.* **270,** 14666–14671
31. Koh, K., Peyrot, S., Wood, C., Wagmaister, J., Maduro, M., Eisenmann, D., and Rothman, J. (2002) *Development* (*Camb.*) **129,** 5171–5180
32. Koh, K., and Rothman, J. (2001) *Development* (*Camb.*) **128,** 2867–2880
33. Epstein, H., and Shakes, D. (1995) *Methods Cell Biol.* **48,** 3–29
34. Montgomery, M., Xu, S., and Fire, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15502–15507
35. Fire, A., Xu, S., Montgomery, M., Kostas, S., Driver, S., and Mello, C. (1998) *Nature* **391,** 806–811
36. Han, M., and Sternberg, P. (1991) *Genes Dev.* **5,** 2188–2198
37. Kramer, J., French, R., Park, E., and Johnson, J. (1990) *Mol. Cell. Biol.* **10,** 2081–2089
38. Guo, S., and Kemphues, K. (1995) *Cell* **81,** 611–620
39. Fukushige, T., Goszczynski, B., Yan, J., and McGhee, J. (2005) *Dev. Biol.* **279,** 446–461
40. Kamath, R., and Ahringer, J. (2003) *Methods* **30,** 313–321
41. Ashrafi, K., Chang, F., Watts, J., Fraser, A., Kamath, R., Ahringer, J., and Ruvkun, G. (2003) *Nature* **421,** 268–272
42. Moilanen, L., Fukushige, T., and Freedman, J. (1999) *J. Biol. Chem.* **274,** 29655–29665
43. Guarente, L., and Mason, T. (1983) *Cell* **32,** 1279–1286
44. Shim, Y. H., Bonner, J. J., and Blumenthal, T. (1995) *J. Mol. Biol.* **253,** 665–676
45. Ito, H., Iukada, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
46. Guarente, L. (1983) *Methods Enzymol.* **101,** 181–191
47. Capo-chichi, C. D., Roland, I. H., Vanderveer, L., Bao, R., Yamagata, T., Hirai, H., Cohen, C., Hamilton, T. C., Godwin, A. K., and Xu, X. X. (2003) *Cancer Res.* **63,** 4967–4977
48. Kohara, Y. (2001) *Tanpakushitsu Kakusan Koso* **46,** 2425–2431
49. Fukushige, T., Hawkins, M., and McGhee, J. (1998) *Dev. Biol.* **198,** 286–302
50. Brooks, D., Appleford, P., Murray, L., and Isaac, R. (2003) *J. Biol. Chem.* **278,** 52340–52346
51. Ohanian, J., and Ohanian, V. (2001) *Cell. Mol. Life Sci.* **58,** 2053–2068
52. Oskouian, B., and Saba, J. (2004) *Cell. Dev. Biol.* **15,** 529–540
53. Shoichet, S., Malik, T., Rothman, J., and Shivdasani, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4076–4081
54. Herr, D. R., Fyrst, H., Creason, M. B., Phan, V. H., Saba, J. D., and Harris, G. L. (2004) *J. Biol. Chem.* **279,** 12685–12694
55. Renault, A. D., Starz-Gaiano, M., and Lehmann, R. (2002) *Gene Expression Patterns* **2,** 337–345
56. Krause, M., White Harrison, S., Xu, S., Chen, L., and Fire, A. (1994) *Dev. Biol.* **166,** 133–148
57. Zhu, J., Fukushige, T., McGhee, J., and Rothman, J. (1998) *Genes Dev.* **12,** 3809–3814
58. Reuter, R. (1994) *Development* (*Camb.*) **120,** 1123–1135
59. Aries, A., Paradis, P., Lefebvre, C., Schwartz, R., and Nemer, M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6975–6980
60. Akiyama, Y., Watkins, N., Suzuki, H., Jair, K. W., van Engeland, M., Esteller, M., Sakai, H., Ren, C. Y., Yuasa, Y., Herman, J. G., and Baylin, S. B. (2003) *Mol. Cell. Biol.* **23,** 8429–8439
61. Suzuki, Y., and Evans, T. (2004) *Life Sci.* **74,** 1829–1838
62. Shureiqi, I., Jiang, W., Fischer, S., Xu, X., Chen, D., Lee, J., Lotan, R., and Lippman, S. (2002) *Cancer Res.* **62,** 1178–1183