EUKARYOTIC CELL, Aug. 2010, p. 1262–1271 Vol. 9, No. 8
1535-9778/10/$12.00 doi:10.1128/EC.00117-10

Uga3 and Uga35/Dal81 Transcription Factors Regulate *UGA4* Transcription in Response to γ-Aminobutyric Acid and Leucine▽

Sabrina Beatriz Cardillo, Mariana Bermúdez Moretti,* and Susana Correa García

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina

Received 14 May 2010/Accepted 14 June 2010

The *Saccharomyces cerevisiae UGA4* gene encodes a permease capable of importing γ-aminobutyric acid (GABA) and δ-aminolevulinic acid (ALA) into the cell. GABA-dependent induction of this permease requires at least two positive-acting proteins, the specific factor Uga3 and the pleiotropic factor Uga35/Dal81. *UGA4* is subjected to a very complex regulation, and its induction is affected by the presence of extracellular amino acids; this effect is mediated by the plasma membrane amino acid sensor SPS. Our results show that leucine affects *UGA4* induction and that the SPS sensor and the downstream effectors Stp1 and Stp2 participate in this regulation. Moreover, we found that the Uga3 and Uga35/Dal81 transcription factors bind to the *UGA4* promoter in a GABA-dependent manner and that this binding is impaired by the presence of leucine. We also found that the Leu3 transcription factor negatively regulates *UGA4* transcription, although this seems to be through an indirect mechanism.

The utilization of nonpreferred nitrogen sources in the absence of preferred sources requires control at the level of transcription for the synthesis of pathway-specific catabolic enzymes and permeases. This transcriptional control requires two positive signals, the first being a global signal indicating nitrogen limitation and the second being a pathway-specific signal that involves the presence of a substrate or intermediate of a metabolic pathway (35). γ-Aminobutyric acid (GABA) can be used as a nitrogen source by the unicellular budding yeast *Saccharomyces cerevisiae*, being a poor source. The *UGA4* gene encodes the GABA and δ-aminolevulinic acid (ALA) permease Uga4 in this organism. Its expression depends on nitrogen catabolite repression (NCR) and GABA induction (2, 5). Induction of this permease requires at least two positive-acting proteins, the specific Uga3 and the pleiotropic Uga35/Dal81 factors (3, 10). These factors act through a 19-bp CG-rich upstream activating sequence named UAS_{GABA}. The participation of both Uga3 and Uga35/Dal81 in *UGA4* induction was demonstrated by genetic analysis (2, 39), and the interaction of Uga3 with the UAS_{GABA} region was shown *in vitro* in terms of binding using electromobility shift assays (25). The promoter region of *UGA4* also contains four adjacent repeats of the heptanucleotide 5′-CGAT(A/T)AG-3′, which constitute a UAS_{GATA} element (12). This element, together with the GATA transcription factors, is responsible for the effect of NCR on *UGA4*.

Yeast cells assess the availability of extracellular nutrients through plasma membrane sensors. Ssy1 is a nutrient receptor that functions together with the two peripheral membrane-associated proteins Ptr3 and Ssy5 as a sensor of extracellular amino acids. Ssy1, Ptr3, and Ssy5 constitute a plasma membrane-associated complex named SPS (18). The homologous zinc finger transcription factors Stp1 and Stp2 are downstream effector components of the SPS sensor pathway. These factors are synthesized as latent cytoplasmic proteins with N-terminal regulatory domains crucial for the regulation of their activity (4, 31). In response to amino acids, Stp1 and Stp2 are activated by endoproteolytic removal of their N-terminal domains and act through specific upstream activating sequences named UAS_{aa}, present within SPS sensor-regulated promoters (14, 37). Uga35/Dal81 is required for full induction of amino acid-induced SPS sensor-dependent expression of the *AGP1*, *PTR2*, and *BAP2* genes (1, 7, 26) and increases the efficiency of Stp1 binding to the *AGP1* promoter (8).

Using whole-genome expression analysis of amino acid sensing (16, 17, 28), several groups reported that genes encoding amino acid and peptide transporters are induced by amino acids and that genes under NCR are repressed by amino acids and/or are strongly expressed in a *ssy1*Δ mutant. The *UGA4* gene could be included in both groups since it encodes a transporter and it is under the control of NCR. Previously, we demonstrated that *UGA4* induction diminished in the presence of extracellular amino acids (6).

Leu3 has been described as a regulator of five genes that belong to the branched-chain amino acid synthesis pathway (*LEU1*, *LEU2*, *LEU4*, *ILV2*, and *ILV5*), one gene (*BAP2*) which belongs to a family of permeases involved primarily with the uptake of branched-chain amino acids, and one gene (*GDH1*) mainly responsible for the assimilation of ammonia. Leu3 activity depends on the presence of α-isopropylmalate (α-IPM), an early intermediate in leucine biosynthesis (29). Leu3 acts both as a repressor and as an activator of transcription in the absence or in the presence of α-IPM, respectively. α-IPM synthesis is highly regulated, since α-IPM synthase encoded by *LEU4* is feedback inhibited by leucine and reversibly inactivated by coenzyme A (CoA) (29). On the basis of the transcriptional responses and *in vivo* binding of Leu3, Boer and

* Corresponding author. Mailing address: Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Pabellón 2, Piso 2, C1428EGA, Capital Federal, Argentina. Phone and fax: 54 11 4576 3300, ext. 483. E-mail: mariana@qb.fcen.uba.ar.

▽ Published ahead of print on 25 June 2010.

TABLE 1. Strains used in this work

Strain	Genotype	Parent	Primer	Source or reference
23344c	*mat*α *ura3*			M. Grenson
30995b	*mat***a** *ura3 ssy1*Δ::*KanMX2*			7
KW018	*mat*α *ura3 stp1*Δ			46
KW021	*mat*α *ura3 stp2*Δ			46
KW023	*mat*α *ura3 stp1*Δ *stp2*Δ			46
FA050	*mat*α *ura3 uga35*Δ::*KanMX2*			1
SBCY01	*mat*α *ura3 leu3*Δ:: *KanMX4*	23344c	F/R-leu3	This study
SBCY02	*mat*α *ura3 LEU3-3HA-kanMX6*	23344c	F/R- LEU3-Tag	This study
SBCY04	*mat*α *ura3 leu4*Δ::*loxp*	23344c	F/R-leu4	This study
SBCY05	*mat*α *ura3 leu4*Δ::*loxp leu5*Δ::*KanMX4*	SBCY04	F/R-leu5	This study
SBCY08	*mat*α *ura3 his3*::*KanMX leu4*Δ::*loxp*/*pSBC-LEU4*^{fbr}	SBCY04	Plasmid M3929	This study
SBCY10	*mat*α *ura3 6HA-UGA35*	23344c	F/R-Tag-UGA35	This study
SBCY13	*mat*α *ura3 6HA-UGA3*	23344c	F/R-Tag-UGA3	This study
SBCY17	*mat*α *ura3 uga35*Δ::*natMX4*	FA050	F/R-ME	This study
SBCY18	*mat***a** *ura3 ssy1*Δ::*natMX4*	30995b	F/R-ME	This study
SBCY20	*mat*α *ura3 uga35*Δ::*natMX4 leu3*Δ::*KanMX4*	SBCY17	F/R-leu3	This study
SBCY22	*mat*α *ura3 leu3*Δ::*KanMX4 6HA-UGA3*	SBCY13	F/R-leu3	This study
SBCY23	*mat*α *ura3 leu3*Δ::*KanMX4 6HA-UGA35*	SBCY10	F/R-leu3	This study
SBCY24	*mat***a** *ura3 ssy1*Δ::*natMX4 6HA-UGA35*	SBCY18	F/R-Tag-UGA35	This study
SBCY26	*mat***a** *ura3 ssy1*Δ::*natMX4 6HA-UGA3*	SBCY18	F/R-Tag-UGA3	This study
XK14-15D	*mat*α *LEU4*^{fbr} *his4*			G. B. Kohlhaw[a]

[a] Gently provided by Anders Brandt (Carlsberg Laboratory, Copenhagen Valby, Denmark).

collaborators identified three additional Leu3-regulated genes (*BAT1*, *GAT1* and *OAC1*). They also reported increased *UGA4* transcription, among other genes, under ammonium limitation in a *leu3*Δ mutant (9).

The transcription factors Leu3, Uga3, and Uga35/Dal81 are zinc binuclear cluster $Zn(II)_2$-Cys_6 proteins. They might interact with DNA as monomers, homodimers, or heterodimers (34). All these transcription regulators target very similar sequences; therefore, other factors are needed to ensure that each protein carries out its own specific regulatory task. The determinants of DNA binding specificity of the zinc binuclear cluster proteins are the nucleotides surrounding the CGG triplets, the orientation of these triplets, and the spacing between them (34).

It has been proposed that Leu3 and Uga3 recognize an everted CGG repeat spaced by 4 bp but that Leu3 does not recognize targets of Uga3 and vice versa, since additional specificity is provided by nucleotides located between the two CGG triplets (38). In addition to the CCG-N4-CGG motif, the nucleotides flanking this everted repeat are also essential for Uga3 *in vitro* binding and activation of transcription (25).

The target sequence of Uga35/Dal81 is controversial (34). Experiments showed that the $Zn(II)_2$ Cys_6 cluster-type DNA binding domain of Uga35/Dal81 is not required for its role in allophanate-induced transcription (10), as was described for *tamA*, an *Aspergillus nidulans* gene encoding a protein highly similar to Uga35/Dal81 (13).

The UAS_{GABA} element of the *UGA4* promoter includes 19 bp, 5′-AAAAACCGCCGGCGGCAAT-3′, with the central core of this sequence being a GC-rich region that contains a perfect 10-bp palindrome, 5′-CCGCCGGCGG-3′ (39).

This work focuses on the interplay of global and specific factors and their influence on the regulation of the catabolic pathway-specific gene *UGA4*. In order to elucidate the molecular mechanisms of the regulation by amino acids of *UGA4* transcription, we demonstrate herein, for the first time, the increased *in vivo* binding of the Uga3 and Uga35/Dal81 transcription factors to the *UGA4* promoter in response to the inducer GABA. We also find that this binding is impaired in cells preincubated with leucine prior to GABA addition in an SPS-dependent manner. Moreover, we show that *UGA4* is also strongly regulated by Leu3. Altogether, our results show the relevance of the transcription factors Uga35/Dal81, Uga3, and Leu3 as responsible for the regulation of *UGA4* by amino acids.

MATERIALS AND METHODS

Strains and media. The *Saccharomyces cerevisiae* strains used in this study, isogenic to the wild-type strain Σ1278b, are listed in Table 1. It was necessary for this work to use prototrophic strains to avoid the addition of amino acids during growth.

Cells were grown in minimal buffered (pH 6.1) medium (27), with 3% glucose as the carbon source and 10 mM proline as the nitrogen source.

Strain construction. All the strains generated in this study except for the SBCY08 strain were constructed using the PCR-based gene deletion strategy described by Wach et al. (44, 45) or modified versions of it. All the parental strains are listed in Table 1, and all primers used for PCRs are listed in Table 2.

The *leu4*Δ deletion was generated using the pUG6 plasmid (23) to amplify the *loxP-KanMX-loxP* cassette. After the strain was generated, the *KanMX* cassette was excised by recombination mediated by Cre recombinase (pSH47 plasmid).

The *leu3*Δ and *leu5*Δ strains were constructed using the pFA6a-KanMX4 plasmid as a template for PCR (45).

Strains with a C-terminal tag were generated using the pFA6a-3HA-KanMX6 plasmid (32).

Strains that express N-terminal tagged proteins under the control of its natural promoter were generated using the pOM10 plasmid as a template for PCR (20), with posterior Cre-mediated excision of the *KanMX* cassette.

The *uga35*Δ::*natMX* and *ssy1*Δ::*natMX* deletions were generated by replacing the *KanMX2* cassette of the FA050 and 30995c strains, respectively, with a *natMX* cassette. The *natMX* cassette was amplified from the pAG25 plasmid (22).

For the construction of the *leu4*Δ *his3*Δ strain, the SBCY04 strain was transformed with a BamHI-digested M3929 plasmid (43).

All yeast transformations were carried out using the lithium method (21).

TABLE 2. Primers used in this work

Primer group and name	Sequence (5′ to 3′)
Oligonucleotides for plasmid construction	
F-Del1	CGCGGAATTCGACAATTTCTTCAATCATTGAAATG
R-Del1	ACATAAAACATCTCGAAATTGGTTTTTGGCGCACGA
F-Del2	TCGTGCGCCAAAAACCAATTTCGAGATGTTTTATGT
R-Del2	CCCCAAGCTTCATACTCATTGTTAGTAATAATAAATTATAAGACCT
F-LEU4^{fbr}	CGCGGAATTCACTGCTCCTGCTTCATCG
R-LEU4^{fbr}	CGCGGAATTCCGTCACTAACCGCCAAAC
Oligonucleotides for deletion strain construction	
F-leu3	TGCAATTATGGAAGGAAGATCAGATTTTGTGGCGACTTCACACGTACGCTGCAGGTCGAC
R-leu3	GGACTTTAAACCTTGGGATTGAACGCAAATTCATTCATTAAAATCGATGAATTCGAGCTC
F-leu4	AAAGGATTCTCACACTAGAAGTTTACTGTAGACTTTTTCCCAGCTGAAGCTTCGTACGC
R-leu4	TATAGAAATAAATAGAAGCGAATAAGTCCTGAAATACAGACATAGGCCACTAGTGGATCTG
F-leu5	ACTGCTAAAATAAACACAGTTCTTAAGTATGACGCGAGATCAGCTGAAGCTTCGTACGC
R-leu5	AATTAAATGCCAAAATTCCATTTCATTCTTTCATAGACGACATAGGCCACTAGTGGATCTG
F-ME	CGTACGCTGCAGGTCGAC
R-ME	ATCGATGAATTCGAGCTC
Oligonucleotides for tagged strain construction	
F-LEU3-Tag	GTTGATATTTTAATGAATGAATTTGCGTTCAATCCCAAGGTTCGGATCCCCGGGTTAATTAA
R-LEU3-Tag	ACGTATATAGAAAATCATTTACCTCTCCTGTAGCACCGCAGTATCGATGAATTCGAGCTC
F-Tag-UGA3	CATGTATGGATGCCAAGAAAACAAAGTTTTTTAAAGTGAGGTATGTGCAGGTCGACAACCCTTAAT
R-Tag-UGA3	CATGCTTCGAATATTTCAATTTCAGCTTCTCCACGCCATAATTGCGGCCGCATAGGCCACT
F-Tag-UGA35	TGTTTAGACGAGCGGCAGAACGACAGGCAGCCATACTATCAAATGTGCAGGTCGACAACCCTTAAT
R-Tag-UGA35	CTTCGTAGGCGATGCGGCATTATCAGCTGGTGATTGGTGAGGGTCGCGGCCGCATAGGCCACT
Oligonucleotides fos qChIP[a]	
F-UGA4qPCR	AATCGCTTATCGCTTATCGTG
R-UGA4qPCR	GGAACTGATTACTGTGCCAAG
F-LEU2qPCR	TCGCCTGACGCATATACC
R-LEU2qPCR	ACGATTGCTAACCACCTATTG
F-UGA4 UCqPCR	AGTCCAATACCTCTGTCCTC
R-UGA4 UCqPCR	AGCCGCAACTTCATTCTG

[a] qChIP, quantitative ChIP.

Transformants were selected on rich medium containing 200 μg/ml G418 or 100 μg/ml nourseothricin (ClonNat; Werner BioAgents).

Plasmids. The plasmids used to analyze promoter activities were derived from the YEp357 plasmid (36). The *UGA4-lacZ* fusion gene carries the 5′ regulatory region and part of the coding region of *UGA4* (positions −583 to +15 with respect to the ATG initiation codon). Two nested 5′ deletions of *UGA4*, called $UAS_{GATA}\Delta$ (i.e., without the UAS_{GATA} sequence, positions −406 to +15) and *UAS*Δ (i.e., without the UAS_{GATA} and UAS_{GABA} sequences, positions −385 to +15), fused to the *lacZ* reporter gene were also used. The UAS_{GABA}*mut-lacZ* fusion gene contains the *UGA4* sequence, positions −583 to +15 with respect to the ATG initiation codon, with an altered UAS_{GABA} element, where the core sequence GCCGGCGGC was replaced by ATTAGTAAT (the changed positions are underlined). All these constructions were previously described by Luzzani et al. (33). The UAS_{GABA}*del-lacZ* fusion gene generated using the strategy described by Strachan and Read (38a) contains the *UGA4* sequence, positions −583 to +15 with respect to the ATG initiation codon, with the sequence GCCGGCGGC deleted from the UAS_{GABA} element. The *UGA3-lacZ* and *UGA35-lacZ* fusion genes contain the *UGA3* and *UGA35* sequences, positions −795 to +24 and −788 to +30 with respect to the ATG initiation codon, respectively. The primers used to construct UAS_{GABA}*del-lacZ* (F/R-Del1 and F/R-Del2), *UGA3-lacZ* (F/R-UGA3), and *UGA35-lacZ* (F/R-UGA35) are listed in Table 2. All constructions were verified by DNA sequence analysis.

The pSBC-*LEU4*^{fbr} plasmid was constructed cloning a fragment containing the promoter, coding region, and 3′ noncoding region (positions −996 to +2016) of the *LEU4*^{fbr} gene into the pRS413 plasmid (11). The *LEU4*^{fbr} gene was amplified from genomic DNA of the XK14-15D strain (gently provided by Anders Brandt [Carlsberg Laboratory, Copenhagen Valby, Denmark]) and was sequenced (GenBank accession no. GU598519).

β-Galactosidase assays. Cells grown on the minimal buffered medium up to an absorbance at 570 nm of 0.5 to 0.9 were harvested and transferred to fresh medium with or without 1.3 mM leucine. After a 30-min incubation at 30°C, 0.1 mM GABA was added. At the indicated time points, an aliquot (10 ml) of each culture was collected by centrifugation and resuspended in 2 ml buffer Z (Miller, 1972). β-Galactosidase activity measured according to Miller (35a) was expressed as Miller units. The results shown are the means for duplicates within a representative assay. At least duplicate assays for each of two independent transformants were performed. The deviation of these values from the mean was less than 15%.

Chromatin immunoprecipitation (ChIP) assays. Cells (a 100-ml culture) were grown to an optical density at 600 nm (OD_{600}) of 0.8 and after different treatments were fixed for 20 min at room temperature in the presence of 1% formaldehyde. Glycine was then added to give a final concentration of 125 mM and incubated for 5 min. Cells were harvested, washed with ice-cold 125 mM Tris-buffered saline (TBS)–glycine and ice-cold TBS and resuspended in 0.4 ml of FA lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (0.5 mm in diameter; Sigma) was added, and the cells were disrupted by vortexing them for 40 min at 4°C (4 × 10 min with intervals on ice). The lysates were separated from the glass beads, and the chromatin was then pelleted by centrifugation (17,000 × *g* for 30 min) and resuspended in 0.4 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3 × 10 s at 15% amplitude) and clarified by centrifugation at 17,000 × g for 30 min. Protein content was measured using the Bradford assay, and 1 mg of protein was used for each immunoprecipitation. Samples were stored at −80°C. Normal mouse IgG (Santa Cruz) or monoclonal antihemagglutinin (anti-HA) antibody (12CA5 Roche) were added to 25 μl of preblocked (1 mg/ml salmon sperm DNA and 1 mg/ml bovine serum albumin) magnetic beads coupled to protein G (Dynal). After a 5-hour incubation, beads were added to each lysate and were incubated overnight at 4°C in a rotator. Immune complexes were sequentially washed five

TABLE 3. Effect of amino acids on transcription driven by the *UGA4-lacZ* construction in wild-type and *ssy1*Δ cells[a]

Condition	Value for:	
	Wild type	*ssy1*Δ
MM	94 ± 2	38 ± 1
MM-GABA	697 ± 54	442 ± 61
MM-Leu-GABA	232 ± 8	407 ± 38
MM-Phe-GABA	329 ± 25	405 ± 47
MM-Met-GABA	396 ± 46	616 ± 68
MM-Trp-GABA	394 ± 17	512 ± 30

[a] β-Galactosidase activity was determined for wild type (23344c) and *ssy1*Δ (30995b) cells carrying the *UGA4-lacZ* fusion gene. Cells were grown in minimal medium (MM) and preincubated for 30 min, with each amino acid (1.3 mM) added before the addition of 0.1 mM GABA, or not preincubated. After 60 min, samples were taken out and β-galactosidase activity was measured. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay.

times with FA lysis buffer, four times with FA lysis buffer containing 500 mM NaCl, five times with wash buffer (10 mM Tris-Cl, pH 8, 0.25 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and two times with Tris-EDTA (TE) buffer. Bound proteins were eluted from the beads by adding 150 μl elution buffer (50 mM Tris-Cl, pH 8, 10 mM EDTA, 1% SDS) and incubating for 15 min at 65°C. Cross-linking was reversed by an overnight incubation at 65°C in the presence of proteinase K (0.25 mg/ml). DNA was purified using a QIAquick PCR purification kit (Qiagen). Real-time quantitative PCR (qPCR) was carried out with an Opticon Monitor 3 (Bio-Rad) with primers that amplified promoter regions of the *UGA4* (F/R-UGA4qPCR) and *LEU2* (F/R-LEU2qPCR) genes (Table 2). A pair of primers that amplify a region located 2.5 kb downstream of the *UGA4* promoter was used as an unbound control (F/R-UGA4 UCqPCR).

ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over an IgG control ChIP. The $\Delta\Delta C_T$ method was used to calculate the fold change of binding to the promoter of interest (30). Propagation of error was handled using standard root mean square methods.

Nucleotide sequence accession number. The sequence determined for the present study has been deposited in GenBank under accession no. GU598519.

RESULTS

Previous studies have shown that the induction of *UGA4* was inhibited by the addition of a mix of amino acids to the culture medium and that this effect was mediated by the SPS amino acid sensor system (6). To test if individual amino acids known to be SPS activators have the same negative effect on *UGA4* expression, wild-type and *ssy1*Δ cells were incubated with leucine, phenylalanine, tryptophan, or methionine or not incubated (Table 3). In a wild-type strain, GABA induction of the *UGA4-lacZ* fusion gene was significantly reduced by the addition of the amino acids tested, whereas in a *ssy1*Δ mutant, this effect was not observed, indicating that the treatment with each amino acid was sufficient to reduce *UGA4* expression and that this decrease was dependent on the activity of the SPS sensor. Similar results were obtained using *ptr3*Δ and *ssy5*Δ cells (data not shown).

To get further insights into the regulation of *UGA4* by amino acids, we tested whether the downstream effectors of the SPS signaling pathway participate in *UGA4* regulation by leucine, an amino acid commonly used as an inducer of the SPS sensor. Although in the single mutants *stp1*Δ and *stp2*Δ, GABA induction of *UGA4* diminished in the presence of extracellular leucine, this effect disappeared in the double mutant *stp1*Δ *stp2*Δ (Fig. 1), suggesting that at least one of these two factors is essential for the signaling cascade that is triggered by the extracellular amino acids and that modulates *UGA4* expression.

In order to establish the regions of the *UGA4* promoter responsible for the effect of leucine on *UGA4* transcription, we analyzed the promoter activity of different DNA fragments covering the region comprising positions −583 to +1 of this gene (Fig. 2A). The induction driven by both the complete promoter of *UGA4* and the promoter lacking the UAS_{GATA} element was repressed in the presence of leucine (Fig. 2B and D). In a *ssy1*Δ strain, the induction profiles of the *UGA4-lacZ* fusion gene were similar in the presence and absence of leucine (Fig. 2C). These results indicate that the UAS_{GATA} element is not a target sequence of the signal triggered by leucine. The construct lacking both the UAS_{GATA} and the UAS_{GABA} elements was unable to produce any significant expression (Fig. 2E). The transcription levels directed by the constructs without the central core of the UAS_{GABA} element were high and independent of GABA, but they were still sensitive to the presence of leucine (Fig. 2F and G). Altogether, these results indicate that neither the UAS_{GATA} element nor the central core of the UAS_{GABA} element participates in the regulation by leucine of the *UGA4* gene. The positive effect of altering or deleting the central core of the UAS_{GABA} element on the transcriptional activity of *UGA4* (Fig. 2, compare panel B to panels F and G) suggested the existence of a negative factor acting on this element.

In silico analysis using the databases YEASTRACT (http://www.yeastract.com) (41) and SCPD (http://rulai.cshl.edu/SCPD) revealed that there is a consensus binding site for the transcription factor Leu3 within the UAS_{GABA} region, as was already mentioned (38, 39). These findings and the whole-genome transcriptional profiles reported by Boer and collab-

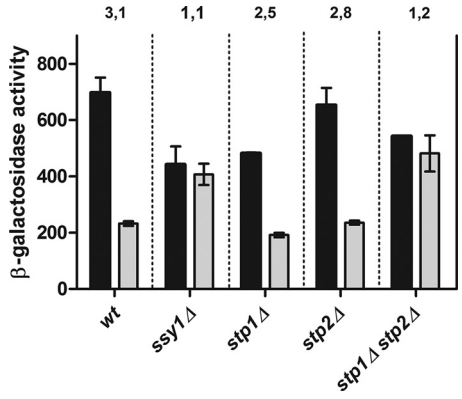

FIG. 1. Effect of leucine on the expression of the *UGA4-lacZ* fusion gene in wild-type, *ssy1*Δ, *stp1*Δ, *stp2*Δ, and *stp1*Δ *stp2*Δ cells. β-Galactosidase activity was determined for wild-type (23344c), *ssy1*Δ (30995b), *stp1*Δ (KW018), *stp2*Δ (KW021), and *stp1*Δ *stp2*Δ (KW023) cells carrying the *UGA4-lacZ* fusion gene. Cells were grown in minimal medium and preincubated with 1.3 mM leucine for 30 min (gray bars) or not preincubated (black bars). Then, cells were incubated with 0.1 mM GABA for 60 min, and samples were taken out for β-galactosidase activity measurements. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay. The numbers above the bars are the ratios between the Miller units calculated for untreated cells and the Miller units calculated for treated cells of each strain.

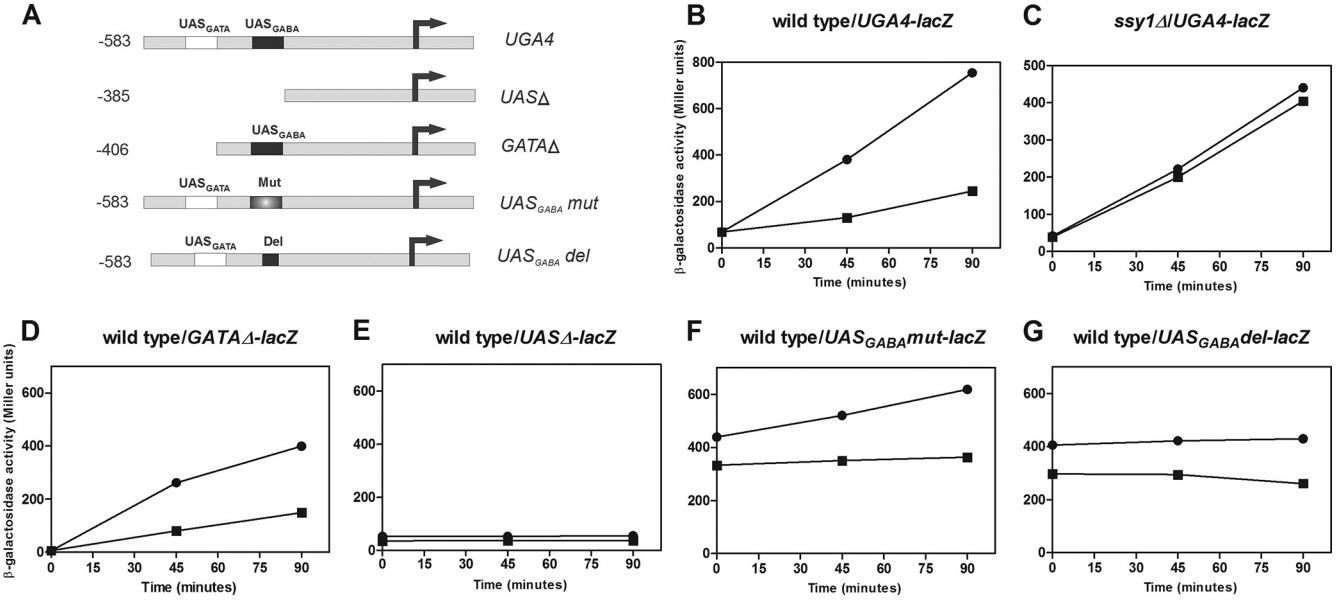

FIG. 2. Effect of leucine on transcription driven by different promoter constructions in wild-type and *ssy1*Δ cells. (A) Scheme of the fusion genes used. (B to G) β-Galactosidase activity was determined for wild-type (23344c) (B, D, E, F, and G) and *ssy1*Δ (30995b) (C) cells carrying the *UGA4-lacZ* (B and C), the UAS_{GATA}Δ-*lacZ* (D), the *UAS*Δ-*lacZ* (E), the UAS_{GABA}*mut-lacZ* (F), or the UAS_{GABA}*del-lacZ* (G) fusion gene. Cells were grown in minimal medium and preincubated with 1.3 mM leucine for 30 min before the addition of 0.1 mM GABA (squares) or not preincubated (circles). Then, samples were taken out at the indicated time points, and β-galactosidase activity was measured. The results shown, expressed in Miller Units, are the means for duplicates within a representative assay, with the deviation being less than 15%.

orators (9) prompted us to postulate that Leu3 was a putative repressor of the *UGA4* gene acting on the UAS_{GABA} region.

In order to determine whether or not Leu3 modulates *UGA4* expression, cells deficient in *LEU3* were transformed with the plasmid containing the full-length promoter region of *UGA4* fused to *lacZ*. The results depicted in Fig. 3A supported our hypothesis proposing Leu3 as a negative regulator of the *UGA4* gene since high levels of *UGA4* expression in *leu3*Δ cells were detected. The transcription of *UGA4* in the absence of Leu3 did not depend on GABA, suggesting that this factor is involved in the induction process, probably by maintaining low basal levels of *UGA4* expression. On the other hand, Leu3 seems to be participating in the regulation of *UGA4* by leucine, although it might be remarked that in cells lacking Leu3, some effect of leucine on *UGA4* expression was still detectable (Fig. 3A).

Gene regulation by Leu3 depends on the levels of α-IPM (29). To determine whether the negative effect of Leu3 on *UGA4* expression is also modulated by intracellular levels of α-IPM, we measured the expression of our *UGA4-lacZ* fusion gene in strains with different capacities for synthesizing α-IPM (Table 4). In both a wild-type strain and a mutant strain pro-

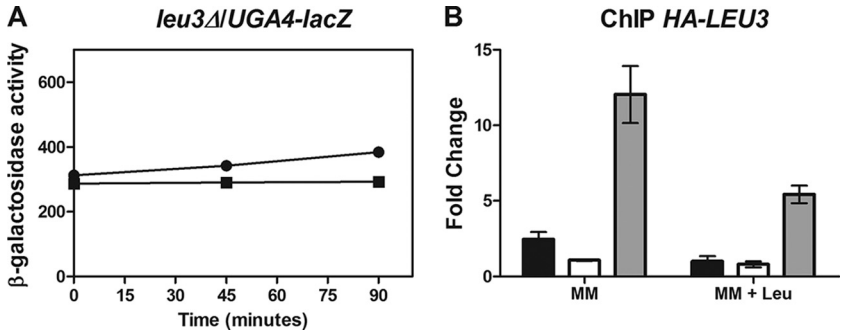

FIG. 3. Effect of Leu3 on *UGA4* regulation. (A) β-Galactosidase activity was determined for *leu3*Δ (SBCY01) cells carrying the *UGA4-lacZ* fusion gene. Cells were grown in minimal medium and preincubated with 1.3 mM leucine for 30 min before the addition of 0.1 mM GABA (squares) or not preincubated (circles). Then, samples were taken out at the indicated time points. The results shown, expressed in Miller Units, are the means for duplicates within a representative assay, with the deviation being less than 15%. (B) Wild-type cells expressing the Leu3-HA fusion protein (SBCY02) were grown in minimal medium (MM) preincubated for 30 min with 1.3 mM leucine or not preincubated. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify the *UGA4* promoter (F/R UGA4qPCR) (black bars), a region 2.5 kb downstream of the *UGA4* promoter (F/R UGA4 UCqPCR) (white bars) (used as a negative control), and the *LEU2* promoter (F/R LEU2qPCR) (gray bars) (used as a positive control). ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over an IgG control ChIP. The $\Delta\Delta C_T$ method was used to calculate the fold change of binding to the promoter of interest.

TABLE 4. Expression of the *UGA4-lacZ* fusion gene in cells with different α-IPM-synthesizing capacities[a]

Condition	Value for:			
	Wild type	*ssy1*Δ	*leu4*Δ *leu5*Δ[b]	*LEU4*^{fbr}
MM	90 ± 9	51 ± 7	39 ± 2	60 ± 5
MM-GABA	544 ± 41	454 ± 26	131 ± 2	475 ± 44
MM-Leu (2 mM)-Ile (1 mM)[c]	64 ± 1	18 ± 1	14 ± 1	76 ± 5
MM-Leu (2 mM)-Ile (1 mM)[c]-GABA	274 ± 13	547 ± 9	67 ± 5	240 ± 3

[a] β-Galactosidase activity was determined for wild-type (23344c), *ssy1*Δ (30995b), *leu4*Δ *leu5*Δ (SBCY05), and *LEU4*^{fbr} (SBCY08) cells carrying the *UGA4-lacZ* fusion gene. Cells were grown in minimal medium (MM) containing or not containing leucine and isoleucine. Each culture was divided in two, and only one half was induced with 0.1 mM GABA for 60 min. Samples were taken out, and β-galactosidase activity was measured. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay.

[b] Strain SBCY05 requires leucine (0.23 mM) for growth.

[c] Since higher concentrations of leucine alone can cause growth retardation, isoleucine was added to alleviate that effect (24).

TABLE 5. Expression of the *UGA35-lacZ* and *UGA3-lacZ* fusion genes in wild type, *leu3*Δ, and *leu4*Δ *leu5*Δ cells[a]

Gene	Value for:		
	Wild type	*leu3*Δ	*leu4*Δ *leu5*Δ
UGA35-lacZ	47.2 ± 2.8	≤10	≤10
UGA3-lacZ	128.3 ± 8.0	33.6 ± 1.6	≤10

[a] β-Galactosidase activity was determined for wild-type (23344c), *leu3*Δ (SBCY01), and *leu4*Δ *leu5*Δ (SBCY05) cells carrying the *UGA35-lacZ* and *UGA3-lacZ* fusion genes. Cells were grown in minimal medium. Samples were taken out, and β-galactosidase activity was measured. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay.

ducing feedback-resistant α-IPM synthase (*LEU4*^{fbr}), the levels of GABA-dependent expression of the *UGA4-lacZ* fusion gene were high. When these two strains were grown in the presence of leucine, a condition expected to lower the α-IPM production in the wild type but not in the *LEU4*^{fbr} strain (24), the levels of *UGA4-lacZ* induction were reduced. It is noteworthy that when a similar experiment was performed with a *ssy1*Δ strain, this reduction was not observed, indicating that such effect was caused by the signal mediated by the SPS sensor in response to leucine rather than by changes in the α-IPM levels. In a strain devoid of α-IPM synthase activity and hence α-IPM, where it is known that Leu3 acts as a strong repressor (29), the expression of *UGA4-lacZ* was 4-fold lower than in a wild-type strain, showing that the repressing activity of this factor in *leu4*Δ *leu5*Δ cells is stronger than in wild-type cells. However, the fact that the expression of the two transcription factors responsible for *UGA4* induction, Uga3 and Uga35, was almost undetectable in *leu4*Δ *leu5*Δ cells (Table 5) suggested that the regulation of *UGA4* by Leu3 might be indirect.

We confirmed that α-IPM levels were being effectively modulated in *leu4*Δ *leu5*Δ and *LEU4*^{fbr} strains by measuring the expression of *BAP2*, known to be regulated by α-IPM levels through Leu3 activity (data not shown).

UGA4 expression basal levels (i.e., before the addition of GABA) driven by the promoters lacking the central core of the UAS_{GABA} element in wild-type cells (Fig. 2F and G) and by the full-length *UGA4* promoter in *leu3*Δ cells (Fig. 3A) were very high. These data and the fact that the consensus binding site for Leu3 is within the UAS_{GABA} element pointed to Leu3 as a transcription factor negatively regulating *UGA4* transcription and probably acting through the UAS_{GABA} element. Therefore, we decided to investigate the *in vivo* binding of Leu3 to the regulatory region of *UGA4*. For this purpose, we used chromatin immunoprecipitation (ChIP) assays with cells expressing Leu3 with its C-terminal end fused to the HA epitope. We were not able to detect any significant *in vivo* binding of Leu3-HA to the *UGA4* promoter either in cells incubated in minimal medium or in cells treated with leucine (Fig. 3B). Similar results were obtained with the use of a strain that expresses an N-terminal tagged version of Leu3 (data not shown). This result supported the idea that Leu3 acts negatively on *UGA4* expression in an indirect way as mentioned above. The *LEU2* promoter, a well-known Leu3 target (29), was used as a positive control for Leu3-HA binding. The functionality of both C- and N-tagged fusion proteins was checked by measuring *UGA4* expression in these strains (data not shown).

Considering that the Uga35/Dal81 transcription factor is required for the full induction of several amino acid permeases in response to signals triggered by the SPS sensor (1, 8) and that this factor is also required for the induction of *UGA* genes by GABA (42), we decided to study the *in vivo* binding of Uga35/Dal81 to the UAS_{GABA} region of the *UGA4* promoter. For this, we performed ChIP assays using a strain expressing the HA-Uga35/Dal81 fusion protein. We found that HA-Uga35/Dal81 bound to the *UGA4* promoter in a GABA-dependent manner and that this binding was impaired by preincubation with leucine (Fig. 4A). These observations correlate with the low levels of *UGA4* induction measured in the presence of leucine (Table 3 and Fig. 2B).

In both inducible processes (transcription of genes controlled by the SPS pathway and induction by GABA of *UGA* genes), Uga35/Dal81 acts together with an inducer-specific transcription factor (1). In the case of the response to GABA of *UGA4*, this factor is Uga3. For this reason, we decided to investigate Uga3 binding to the *UGA4* promoter *in vivo* under the same conditions used to test the binding for Uga35/Dal81. Our results showed that HA-Uga3 interacted with the *UGA4* promoter similarly to the way that HA-Uga35/Dal81 did (Fig. 4B), suggesting that Uga3 bound to the *UGA4* promoter in a GABA-dependent manner and that leucine weakened this interaction.

To determine whether the effect of leucine on HA-Uga3 and HA-Uga35/Dal81 binding to the *UGA4* promoter is dependent on the SPS sensor, we performed ChIP assays using strains deficient in *SSY1* and expressing tagged versions of Uga3 or Uga35/Dal81. We detected both transcription factors bound to the *UGA4* promoter even in the presence of leucine (Fig. 4C and D), confirming that the lower binding capacity of HA-Uga3 and HA-Uga35/Dal81 in the presence of leucine observed in wild-type cells (Fig. 4A and B) was caused by the signal triggered by this amino acid through the SPS sensor system.

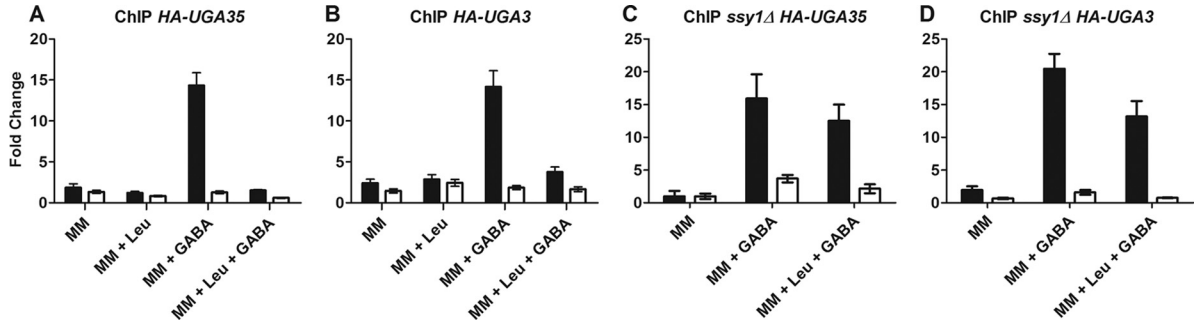

FIG. 4. Binding of HA-tagged Uga35/Dal81 and Uga3 to the *UGA4* promoter in wild-type and *ssy1*Δ cells. Wild-type (A and B) and *ssy1*Δ (C and D) cells expressing the HA-Uga35 (SBCY10 and SBCY24) (A and C) and HA-Uga3 (SBCY13 and SBCY26) (B and D) fusion proteins were grown in minimal medium (MM), preincubated for 30 min with 1.3 mM leucine or not preincubated, and then incubated with 0.1 mM GABA or not incubated. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify the *UGA4* promoter (F/R UGA4qPCR) (black bars) and a region 2.5 kb downstream of the *UGA4* promoter (F/R UGA4 UCqPCR) (white bars), used as a negative control. ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over an IgG control ChIP. The $\Delta\Delta C_T$ method was used to calculate the fold change of binding to the promoter of interest.

In our attempt to understand the events that caused the high levels of *UGA4* expression in a *LEU3*-deficient strain, we decided to study the recruitment of HA-Uga3 and HA-Uga35/Dal81 to the promoter of *UGA4* in the absence of Leu3. Although the expression level of the *UGA3-lacZ* fusion gene in a *leu3*Δ strain was significantly lower than that in a wild-type strain (Table 5), this transcription factor appeared bound to the *UGA4* promoter after the addition of GABA (Fig. 5, left panel), as was already observed in the wild-type strain (Fig. 4B). On the other hand, HA-Uga35/Dal81 binding seemed to be impaired by the *leu3* deficiency (Fig. 5, right panel). In this strain, *UGA35-lacZ* fusion gene expression was almost undetectable, explaining the low level of recruitment observed (Table 5). These results did not explain the high basal levels of *UGA4* expression observed in the absence of Leu3 (Fig. 3A); however, the lack of *UGA4* induction in this strain would be explained by the low level of availability of Uga35/Dal81. The expression levels of *UGA4-lacZ*, UAS_{GABA}*mut-lacZ* (Fig. 6), and UAS_{GABA}*del-lacZ* (data not shown) in *a leu3*Δ *uga35*Δ strain were significantly lower than those in a *leu3*Δ strain, indicating that the Uga35/Dal81 factor is in some way involved in the high levels of expression observed under both conditions (i.e., *UGA4-lacZ* expression in a *leu3*Δ strain and expression driven by the UAS_{GABA}*mut-lacZ* construction in a wild-type strain) in the absence of an inducer.

DISCUSSION

The aim of this work was to elucidate the mechanisms by which leucine regulates *UGA4* induction. Here, we demonstrate for the first time that leucine affects the GABA-mediated binding of the Uga3 and Uga35/Dal81 transcription factors to the *UGA4* promoter and that this effect depends on the SPS sensor pathway.

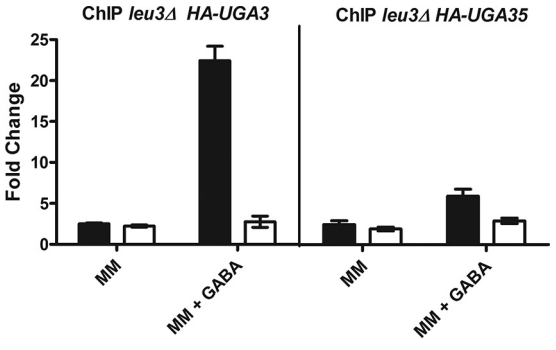

FIG. 5. Binding of HA-tagged Uga35/Dal81 and Uga3 to the *UGA4* promoter in *leu3*Δ cells. *leu3*Δ cells expressing the HA-Uga3 (SBCY22) (left panel) and HA-Uga35 (SBCY23) (right panel) fusion proteins were grown in minimal medium (MM) and then incubated with 0.1 mM GABA or not incubated. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify the *UGA4* promoter (F/R UGA4qPCR) (black bars) and a region 2.5 kb downstream of the *UGA4* promoter (F/R UGA4 UCqPCR) (white bars), used as a negative control. ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over an IgG control ChIP. The $\Delta\Delta C_T$ method was used to calculate the fold change of binding to the promoter of interest.

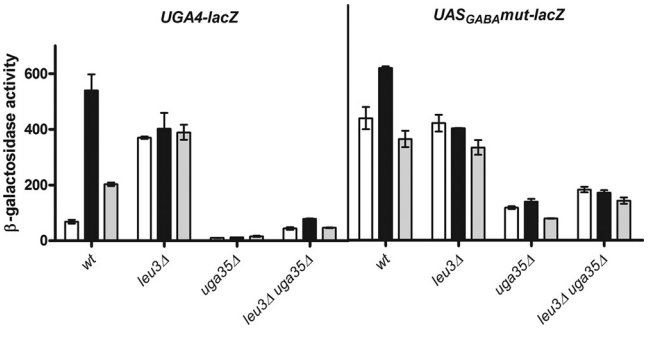

FIG. 6. Effect of leucine on the expression of the *UGA4-lacZ* and the UAS_{GABA}*mut-lacZ* fusion genes in wild-type, *leu3*Δ, *uga35*Δ and *leu3*Δ *uga35*Δ cells. β-Galactosidase activity was determined for wild-type (23344c), *leu3*Δ (SBCY01), *uga35*Δ (SBCY17), and *leu3*Δ *uga35*Δ (SBCY20) cells carrying the *UGA4-lacZ* (left panel) or the UAS_{GABA}*mut-lacZ* (right panel) fusion gene. Cells were grown in minimal medium. White bars correspond to untreated cells, black bars correspond to cells treated with 0.1 mM GABA for 60 min, and gray bars correspond to cells treated with 1.3 mM leucine for 30 min and 0.1 mM GABA for 60 min. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay.

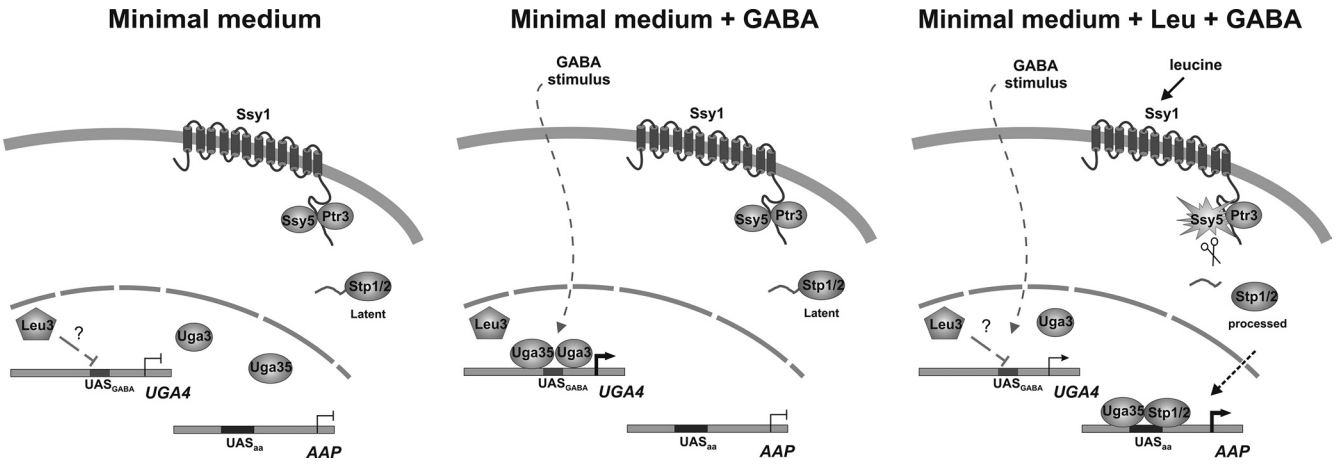

FIG. 7. Schematic representation of the molecular events triggered by leucine and GABA affecting *UGA4* gene expression. (Adapted from reference 31 with permission of the publisher.)

We previously reported that *UGA4* expression is regulated by amino acids through the plasma membrane sensor SPS (6). In this paper, we studied the effect of leucine, one of the most potent known elicitors of signaling through SPS (15, 19), on *UGA4*. The effect of leucine on *UGA4* expression was detected shortly after the addition of this amino acid, suggesting a sensor mediated response that was strictly dependent on the components of the SPS sensor.

In the *stp1*Δ and *stp2*Δ single mutants, the effect of leucine was almost indistinguishable from that observed in the wild type, while no effect of leucine was detected in the *stp1*Δ *stp2*Δ double mutant, indicating that these two factors are involved in *UGA4* regulation by leucine and confirming that they are functionally redundant (31).

On the basis of the proposal of Abdel-Sater and collaborators (1) and the findings of Boban and Ljungdahl (8), demonstrating that the binding of Stp1 to the *AGP1* promoter was dependent on the presence of Uga35/Dal81, we studied the participation of Uga35/Dal81 in *UGA4* regulation by leucine. In this work, we demonstrated that GABA induced the interaction between HA-Uga35/Dal81 and the *UGA4* promoter and that leucine impaired this interaction.

The behavior observed in the binding of HA-Uga3 to the *UGA4* promoter in response to leucine was similar to that observed for HA-Uga35/Dal81. This was an unexpected result, since Uga3 is an inducer-specific transcription factor of *UGA* genes and there were no previous reports relating Uga3 with the amino acid-responsive pathway. One possible explanation is that Uga3 would need Uga35/Dal81 to some extent to properly bind to the *UGA4* promoter. In consequence, the binding of Uga3 depending on Uga35/Dal81 would respond to leucine. *In vitro* electromobility shift assays using an *Escherichia coli*-produced version of Uga3 protein showed a Uga35/Dal81- and GABA-independent binding of Uga3 to the UAS$_{GABA}$ element (25). These results, apparently in contrast with ours, came from an *in vitro* assay, and the aim of our work was to elucidate the *in vivo* mechanism, which is probably more complex and highly regulated.

A hierarchy has been proposed for different induction processes mediated by the Uga35/Dal81 factor (1), i.e., SPS amino acid-regulated genes and GABA-induced genes. Our results showing that the decrease caused by leucine in the recruitment of HA-Uga35/Dal81 to the *UGA4* promoter depended on Ssy1 support this hypothesis. The signal triggered by the SPS sensor in response to extracellular leucine activates Stp1/Stp2, which would be recruiting Uga35/Dal81 to promote transcriptional leucine induction of other permeases and decreasing the availability of Uga35/Dal81, and consequently of Uga3, for GABA induction of the *UGA4* gene (Fig. 7).

Therefore, the element in the *UGA4* promoter involved in the response to leucine seems to be the UAS$_{GABA}$ element, since this element is in this regulatory region where both the Uga3 and the Uga35/Dal81 factors act.

Bricmont and collaborators (10) have demonstrated that the Zn(II)$_2$-Cys$_6$ cluster-type DNA domain of Uga35/Dal81 is not required for its role in allophanate-induced transcription. Our results showed that HA-Uga35/Dal81 bound to the *UGA4* promoter. Two possible explanations may account for this. The first one is that Uga35/Dal81 directly interacts with the *UGA4* promoter. The second one is that Uga35/Dal81 might be associated with the *UGA4* promoter via an interaction with another protein, such as Uga3.

The Uga35/Dal81 factor seems to be participating in two opposite processes. On the one hand, it mediates the negative effect of leucine on *UGA4* induction. On the other hand, the high basal levels of *UGA4* expression observed in *leu3*Δ cells and the high levels of expression of the *UAS$_{GABA}$mut-lacZ* fusion gene in wild-type cells seem to depend on Uga35/Dal81. This dual function of the Uga35/Dal81 transcription factor needs to be further investigated.

Furthermore, in this work, we also demonstrated that the transcription factor Leu3 negatively regulated the *UGA4* gene, irrespective of the presence or absence of α-IPM. In addition, this negative effect was stronger in cells devoid of α-IPM synthase activity, probably due to the low expression levels of Uga3 and Uga35/Dal81 factors under this condition.

The repression of *UGA4* by leucine was barely detected in cells deficient in the *LEU3* gene, suggesting that *UGA4* regulation by leucine was mediated at least in part by Leu3. However, we were not able to detect significant binding of

Leu3-HA to the *UGA4* promoter. Tang and collaborators, comparing expression and binding under both low and high levels of Leu3 activity, showed no detectable binding of Leu3 to several genes whose expression was affected by *LEU3* deletion. They proposed that this could be due to the low sensitivity of the ChIP technique or due to an indirect regulation of these genes by Leu3. Moreover, these authors were not able to detect Leu3 binding to the *UGA4* promoter even when experiments were performed under conditions of high levels of activity of Leu3 (40). It is worth remarking that the growth conditions they used are different from those used in the present report. The facts that the effect of Leu3 on *UGA4* was negative under all conditions, in contrast to what was described for the *BAP2* and *LEU2* genes (29, 37), and that we were not able to detect Leu3-HA bound to the *UGA4* promoter suggested an indirect regulation of *UGA4* by Leu3. Transcription driven by the $UAS_{GABA}mut$ and the $UAS_{GABA}del$ constructs in a *uga35*Δ/*dal81*Δ strain was still quite sensitive to leucine, and this effect was not observed when *LEU3* was also deleted. This suggests that Leu3 is further regulating *UGA4* in response to leucine besides its effect on *UGA35*/*DAL81*.

In summary, we demonstrated that the *UGA4* gene is under the regulation of the SPS signaling pathway and that its induction was inhibited by amino acids, as was the expression of the other genes under both NCR and SPS regulation (16). Leu3, Uga3, and Uga35/Dal81 play an important role in the regulation of *UGA4* transcription, and these three factors are responsible for the negative effect of leucine on GABA induction. The mechanism by which Leu3 negatively regulates *UGA4* transcription still remains unclear, although evidence presented here suggests that this effect is indirect rather than caused by the direct interaction of this factor with the *UGA4* promoter. Uga3 and Uga35/Dal81 bind to the *UGA4* promoter in a GABA-dependent manner, and this binding is impaired by extracellular leucine through the SPS sensor (Fig. 7). Some interplay between SPS signaling molecules and Leu3 has been proposed (37) since Stp1 and Leu3 are both dependent on Ssy1. However, this connection is not completely elucidated yet.

ACKNOWLEDGMENTS

This research was supported by the University of Buenos Aires (UBA) and Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina.

REFERENCES

1. **Abdel-Sater, F., I. Iraqui, A. Urrestarazu, and B. Andre.** 2004. The external amino acid signaling pathway promotes activation of Stp1 and Uga35/Dal81 transcription factors for induction of the AGP1 gene in Saccharomyces cerevisiae. Genetics **166:**1727–1739.
2. **Andre, B., C. Hein, M. Grenson, and J. C. Jauniaux.** 1993. Cloning and expression of the UGA4 gene coding for the inducible GABA-specific transport protein of Saccharomyces cerevisiae. Mol. Gen. Genet. **237:**17–25.
3. **Andre, B., D. Talibi, S. Soussi Boudekou, C. Hein, S. Vissers, and D. Coornaert.** 1995. Two mutually exclusive regulatory systems inhibit UASGATA, a cluster of 5′-GAT(A/T)A-3′ upstream from the UGA4 gene of Saccharomyces cerevisiae. Nucleic Acids Res. **23:**558–564.
4. **Andreasson, C., S. Heessen, and P. O. Ljungdahl.** 2006. Regulation of transcription factor latency by receptor-activated proteolysis. Genes Dev. **20:**1563–1568.
5. **Bermudez Moretti, M., S. Correa Garcia, E. Ramos, and A. Batlle.** 1996. delta-Aminolevulinic acid uptake is mediated by the gamma-aminobutyric acid-specific permease UGA4. Cell. Mol. Biol. (Noisy-le-grand) **42:**519–523.
6. **Bermudez Moretti, M., A. M. Perullini, A. Batlle, and S. Correa Garcia.** 2005. Expression of the UGA4 gene encoding the delta-aminolevulinic and gamma-aminobutyric acids permease in Saccharomyces cerevisiae is controlled by amino acid-sensing systems. Arch. Microbiol. **184:**137–140.
7. **Bernard, F., and B. Andre.** 2001. Ubiquitin and the SCF(Grr1) ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in Saccharomyces cerevisiae. FEBS Lett. **496:**81–85.
8. **Boban, M., and P. O. Ljungdahl.** 2007. Dal81 enhances Stp1- and Stp2-dependent transcription necessitating negative modulation by inner nuclear membrane protein Asi1 in Saccharomyces cerevisiae. Genetics **176:**2087–2097.
9. **Boer, V. M., J. M. Daran, M. J. Almering, J. H. de Winde, and J. T. Pronk.** 2005. Contribution of the Saccharomyces cerevisiae transcriptional regulator Leu3p to physiology and gene expression in nitrogen- and carbon-limited chemostat cultures. FEMS Yeast Res. **5:**885–897.
10. **Bricmont, P. A., J. R. Daugherty, and T. G. Cooper.** 1991. The DAL81 gene product is required for induced expression of two differently regulated nitrogen catabolic genes in Saccharomyces cerevisiae. Mol. Cell. Biol. **11:**1161–1166.
11. **Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:**119–122.
12. **Cunningham, T. S., R. A. Dorrington, and T. G. Cooper.** 1994. The UGA4 UASNTR site required for GLN3-dependent transcriptional activation also mediates DAL80-responsive regulation and DAL80 protein binding in Saccharomyces cerevisiae. J. Bacteriol. **176:**4718–4725.
13. **Davis, M. A., A. J. Small, S. Kourambas, and M. J. Hynes.** 1996. The tamA gene of Aspergillus nidulans contains a putative zinc cluster motif which is not required for gene function. J. Bacteriol. **178:**3406–3409.
14. **de Boer, M., P. S. Nielsen, J. P. Bebelman, H. Heerikhuizen, H. A. Andersen, and R. J. Planta.** 2000. Stp1p, Stp2p and Abf1p are involved in regulation of expression of the amino acid transporter gene BAP3 of Saccharomyces cerevisiae. Nucleic Acids Res. **28:**974–981.
15. **Didion, T., M. Grausland, C. Kielland-Brandt, and H. A. Andersen.** 1996. Amino acids induce expression of BAP2, a branched-chain amino acid permease gene in Saccharomyces cerevisiae. J. Bacteriol. **178:**2025–2029.
16. **Eckert-Boulet, N., P. S. Nielsen, C. Friis, M. M. dos Santos, J. Nielsen, M. C. Kielland-Brandt, and B. Regenberg.** 2004. Transcriptional profiling of extracellular amino acid sensing in Saccharomyces cerevisiae and the role of Stp1p and Stp2p. Yeast **21:**635–648.
17. **Forsberg, H., C. F. Gilstring, A. Zargari, P. Martinez, and P. O. Ljungdahl.** 2001. The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol. Microbiol. **42:**215–228.
18. **Forsberg, H., and P. O. Ljungdahl.** 2001. Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. Mol. Cell. Biol. **21:**814–826.
19. **Gaber, R. F., K. Ottow, H. A. Andersen, and M. C. Kielland-Brandt.** 2003. Constitutive and hyperresponsive signaling by mutant forms of Saccharomyces cerevisiae amino acid sensor Ssy1. Eukaryot. Cell **2:**922–929.
20. **Gauss, R., M. Trautwein, T. Sommer, and A. Spang.** 2005. New modules for the repeated internal and N-terminal epitope tagging of genes in Saccharomyces cerevisiae. Yeast **22:**1–12.
21. **Gietz, R. D., and R. A. Woods.** 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. **350:**87–96.
22. **Goldstein, A. L., and J. H. McCusker.** 1999. Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast **15:**1541–1553.
23. **Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann.** 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24:**2519–2524.
24. **Hu, Y., T. G. Cooper, and G. B. Kohlhaw.** 1995. The Saccharomyces cerevisiae Leu3 protein activates expression of GDH1, a key gene in nitrogen assimilation. Mol. Cell. Biol. **15:**52–57.
25. **Idicula, A. M., G. L. Blatch, T. G. Cooper, and R. A. Dorrington.** 2002. Binding and activation by the zinc cluster transcription factors of Saccharomyces cerevisiae. Redefining the UASGABA and its interaction with Uga3p. J. Biol. Chem. **277:**45977–45983.
26. **Iraqui, I., S. Vissers, F. Bernard, J. O. de Craene, E. Boles, A. Urrestarazu, and B. Andre.** 1999. Amino acid signaling in Saccharomyces cerevisiae: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol. Cell. Biol. **19:**989–1001.
27. **Jacobs, P., J. C. Jauniaux, and M. Grenson.** 1980. A cis-dominant regulatory mutation linked to the argB-argC gene cluster in Saccharomyces cerevisiae. J. Mol. Biol. **139:**691–704.
28. **Kodama, Y., F. Omura, K. Takahashi, K. Shirahige, and T. Ashikari.** 2002. Genome-wide expression analysis of genes affected by amino acid sensor Ssy1p in Saccharomyces cerevisiae. Curr. Genet. **41:**63–72.
29. **Kohlhaw, G. B.** 2003. Leucine biosynthesis in fungi: entering metabolism through the back door. Microbiol. Mol. Biol. Rev. **67:**1–15.
30. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25:**402–408.

31. **Ljungdahl, P. O.** 2009. Amino-acid-induced signalling via the SPS-sensing pathway in yeast. Biochem. Soc. Trans. **37:**242–247.
32. **Longtine, M. S., A. McKenzie III, D. J. Demarini, N. G. Shah, A. Wach, A. Brachat, P. Philippsen, and J. R. Pringle.** 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast **14:**953–961.
33. **Luzzani, C., S. B. Cardillo, M. Bermudez Moretti, and S. Correa Garcia.** 2007. New insights into the regulation of the Saccharomyces cerevisiae UGA4 gene: two parallel pathways participate in carbon-regulated transcription. Microbiology **153:**3677–3684.
34. **MacPherson, S., M. Larochelle, and B. Turcotte.** 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol. Mol. Biol. Rev. **70:**583–604.
35. **Marzluf, G. A.** 1997. Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev. **61:**17–32.

35a. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 23–56. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

36. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions. Gene **45:**299–310.
37. **Nielsen, P. S., B. van den Hazel, T. Didion, M. de Boer, M. Jorgensen, R. J. Planta, M. C. Kielland-Brandt, and H. A. Andersen.** 2001. Transcriptional regulation of the Saccharomyces cerevisiae amino acid permease gene BAP2. Mol. Gen. Genet. **264:**613–622.
38. **Noel, J., and B. Turcotte.** 1998. Zinc cluster proteins Leu3p and Uga3p recognize highly related but distinct DNA targets. J. Biol. Chem. **273:**17463–17468.

38a. **Strachan, T., and A. P. Read.** 1999. PCR, DNA sequencing and in vitro mutagenesis. *In* Human molecular genetics, 2nd ed. Garland Science, London, United Kingdom.

39. **Talibi, D., M. Grenson, and B. Andre.** 1995. Cis- and trans-acting elements determining induction of the genes of the gamma-aminobutyrate (GABA) utilization pathway in Saccharomyces cerevisiae. Nucleic Acids Res. **23:**550–557.
40. **Tang, L., X. Liu, and N. D. Clarke.** 2006. Inferring direct regulatory targets from expression and genome location analyses: a comparison of transcription factor deletion and overexpression. BMC Genomics **7:**215.
41. **Teixeira, M. C., P. Monteiro, P. Jain, S. Tenreiro, A. R. Fernandes, N. P. Mira, M. Alenquer, A. T. Freitas, A. L. Oliveira, and I. Sa-Correia.** 2006. The YEASTRACT database: a tool for the analysis of transcription regulatory associations in Saccharomyces cerevisiae. Nucleic Acids Res. **34:**D446–D451.
42. **Vissers, S., B. Andre, F. Muyldermans, and M. Grenson.** 1990. Induction of the 4-aminobutyrate and urea-catabolic pathways in Saccharomyces cerevisiae. Specific and common transcriptional regulators. Eur. J. Biochem. **187:**611–616.
43. **Voth, W. P., Y. W. Jiang, and D. J. Stillman.** 2003. New 'marker swap' plasmids for converting selectable markers on budding yeast gene disruptions and plasmids. Yeast **20:**985–993.
44. **Wach, A.** 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae. Yeast **12:**259–265.
45. **Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen.** 1994. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast **10:**1793–1808.
46. **Wielemans, K., C. Jean, S. Vissers, and B. Andre.** 2010. Amino acid signaling in yeast: post-genome duplication divergence of the Stp1 and Stp2 transcription factors. J. Biol. Chem. **285:**855–865.