The External Amino Acid Signaling Pathway Promotes Activation of Stp1 and Uga35/Dal81 Transcription Factors for Induction of the *AGP1* Gene in *Saccharomyces cerevisiae*

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Manuscript received October 30, 2003
Accepted for publication January 14, 2004

ABSTRACT

Yeast cells respond to the presence of amino acids in their environment by inducing transcription of several amino acid permease genes including *AGP1*, *BAP2*, and *BAP3*. The signaling pathway responsible for this induction involves Ssy1, a permease-like sensor of external amino acids, and culminates with proteolytic cleavage and translocation to the nucleus of the zinc-finger proteins Stp1 and Stp2, the lack of which abolishes induction of *BAP2* and *BAP3*. Here we show that Stp1—but not Stp2—plays an important role in *AGP1* induction, although significant induction of *AGP1* by amino acids persists in *stp1* and *stp1 stp2* mutants. This residual induction depends on the Uga35/Dal81 transcription factor, indicating that the external amino acid signaling pathway activates not only Stp1 and Stp2, but also another Uga35/Dal81-dependent transcriptional circuit. Analysis of the *AGP1* gene's upstream region revealed that Stp1 and Uga35/Dal81 act synergistically through a 21-bp *cis*-acting sequence similar to the UAS_{AA} element previously found in the *BAP2* and *BAP3* upstream regions. Although cells growing under poor nitrogen-supply conditions display much higher induction of *AGP1* expression than cells growing under good nitrogen-supply conditions, the UAS_{AA} itself is totally insensitive to nitrogen availability. Nitrogen-source control of *AGP1* induction is mediated by the GATA factor Gln3, likely acting through adjacent 5′-GATA-3′ sequences, to amplify the positive effect of UAS_{AA}. Our data indicate that Stp1 may act in combination with distinct sets of transcription factors, according to the gene context, to promote induction of transcription in response to external amino acids. The data also suggest that Uga35/Dal81 is yet another transcription factor under the control of the external amino acid sensing pathway. Finally, the data show that the TOR pathway mediating global nitrogen control of transcription does not interfere with the external amino acid signaling pathway.

YEAST cells possess a plasma-membrane-associated sensor for detection of a wide variety of amino acids in their external environment. This sensor comprises Ssy1p, a protein highly similar in sequence to amino acid permeases, but apparently unable to mediate amino acid uptake. Ssy1p also differs from amino acid permeases by its much larger N-terminal cytosolic tail and its relatively low expression level (Didion *et al.* 1998; Jorgensen *et al.* 1998; Iraqui *et al.* 1999; Gaber *et al.* 2003). Also essential to the response of cells to external amino acids are the Ptr3 and Ssy5 proteins (Barnes *et al.* 1998; Jorgensen *et al.* 1998; Klasson *et al.* 1999; Bernard and André 2001a) proposed to be integral parts of the sensor forming a complex with Ssy1 (Forsberg and Ljungdahl 2001a,b). The main function of this sensor complex, called SPS (Forsberg and Ljungdahl 2001b), is to respond to the presence of external amino acids by inducing expression of several genes encoding amino acid and peptide permeases. Among these are the *AGP1*, *BAP2*, and *BAP3* genes (encoding wide-range-specificity amino acid permeases) and the ditripeptide permease gene *PTR2* (Barnes *et al.* 1998; Didion *et al.* 1998; Iraqui *et al.* 1999; Regenberg *et al.* 1999). Experiments based on genome-wide microarray analyses have led to the suggestion that the Ssy1 signaling pathway influences the transcription of many other genes (Forsberg *et al.* 2001; Kodama *et al.* 2002). Another component essential for cells to respond to external amino acids is the SCF^{Grr1} ubiquitin-ligase complex (Iraqui *et al.* 1999; Bernard and André 2001b). The Ssy1 signaling pathway culminates with the proteolytic cleavage of two transcription factors, Stp1 and Stp2 (Andreasson and Ljungdahl 2002). These factors appear to be functionally redundant, contain a zinc-finger domain highly similar to that shared by the Kruppel-family transcription factors in higher eukaryotes, and play an important role in transcription of genes induced by external amino acids (Jorgensen *et al.* 1997, 1998; de Boer *et al.* 2000; Nielsen *et al.* 2001). Endoproteolytic processing of Stp1 and Stp2 is apparently required for these factors to be translocated from the cytosol into the

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nucleus (Andreasson and Ljungdahl 2002). In the *stp1 stp2* double mutant, there is no induction of the *BAP2* and *BAP3* genes by amino acids, whereas residual induction persists in both single mutants (de Boer *et al.* 2000; Nielsen *et al.* 2001). A minimal sequence (25 bp) called UAS_{AA}, found in the promoter region of the *BAP3* gene, has been shown to drive induction of reporter genes in response to external amino acids. A larger fragment (42 bp) including this sequence also displays UAS_{AA} properties, and its ability to activate reporter genes is entirely dependent on Stp1 (de Boer *et al.* 1998, 2000). A sequence of ~100 bp isolated from the *BAP2* upstream region displays similar UAS_{AA} properties (Nielsen *et al.* 2001). The UAS_{AA} elements of the *BAP2* and *BAP3* genes have in common the presence of 5′-CGGC-3′ doublets separated by three to six nucleotides. Mutagenesis experiments have confirmed that direct or inverted repeats of this tetranucleotide are crucial to UAS_{AA} function (de Boer *et al.* 1998, 2000). Furthermore, there is evidence that Stp1 and Stp2 can bind to these UAS_{AA} elements (Nielsen *et al.* 2001). In another study it was shown that yet another transcription factor, the Uga35/Dal81 protein containing a Gal4-type $Zn(II)_2$-Cys_6-cluster DNA-binding domain (Bricmont *et al.* 1991; Coornaert *et al.* 1991), is also essential for full induction of the *AGP1* gene in response to amino acids (Iraqui *et al.* 1999; Bernard and André 2001a). Whether this factor also acts through the UAS_{AA} element remains undetermined. Uga35/Dal81's role is not limited to transcription of Ssy1-regulated genes since it is also required for induction of genes involved in γ-aminobutyric acid (GABA), urea, and allantoin utilization (Turoscy and Cooper 1982; Jacobs *et al.* 1985; Vissers *et al.* 1990; Coornaert *et al.* 1991). Interestingly, experiments show that the $Zn(II)_2$-Cys_6-cluster-type DNA-binding domain of Uga35/Dal81 is not required for induction of at least some of its target genes (Bricmont *et al.* 1991). A similar situation has been described for *TamA*, an *Aspergillus nidulans* protein highly similar to Uga35/Dal81 and involved in expression of several genes in response to various nitrogenous compounds, *e.g.*, GABA (Davis *et al.* 1996).

In this study we show that induction of the *AGP1* gene by external amino acids depends mainly on the synergistic action of factors Stp1 and Uga35/Dal81, whereas Stp2 does not significantly contribute. Stp1 or Uga35/Dal81 alone can respond significantly to the Ssy1 pathway, suggesting that in addition to Stp1 and Stp2, Uga35/Dal81 is yet another transcription factor under the control of this pathway. Both Stp1 and Uga35/Dal81 act through a 21-bp sequence similar to the UAS_{AA} element. The detailed mutagenesis analysis of this element is presented. When cells grow under poor nitrogen-supply conditions, the positive effect of UAS_{AA} on *AGP1* transcription is amplified ~10-fold by yet another factor, the GATA factor Gln3. This amplification is negligible if a favored nitrogen source like ammonium is provided. We propose that Gln3 acts through neighboring 5′-GATA-3′-containing sequences, its action being conditioned by the prior action of Stp1 and Uga35/Dal81 through the adjacent UAS_{AA} element.

MATERIALS AND METHODS

Strains, growth conditions, and methods: The *Saccharomyces cerevisiae* strains used in this study are all isogenic with the wild-type Σ1278b (Béchet *et al.* 1970) except for the mutations mentioned (Table 1). Cells were grown in a minimal buffered (pH 6.1) medium with 3% glucose as the carbon source (Jacobs *et al.* 1980). To this medium, urea (5 mm), proline (5 mm), $(NH_4)_2SO_4$ (10 or 50 mm), amino acids (1–5 mm), or combinations of these compounds were added as source(s) of nitrogen. All procedures for manipulating DNA were standard ones (Ausubel *et al.* 1995; Sambrook *et al.* 1997; Susan-Resiga and Nowak 2003). The *Escherichia coli* strain used in our experiments was JM109.

Construction of *stp1*Δ, *stp2*Δ, and *uga35/dal81*Δ deletion strains: The *stp1*Δ, *stp2*Δ, and *uga35/dal81*Δ strains were constructed by the PCR-based gene-deletion method (Wach 1996). The DNA segments used to introduce these mutations were generated by using the *kanMX2* gene from plasmid pFA6a-kanMX2 (Longtine *et al.* 1998) as a template and the following PCR primers: *stp1*Δ*:kanMX2*, D5-STP1, and D3-STP1; *stp2*Δ*:kanMX2*, D5-STP2, and D3-STP2; and *uga35/dal-81*Δ*:kanMX2*, D5-UGA35, and D3-UGA35 (Table 2). The yeast strain 23344c (*ura3*) was transformed with the PCR fragment by the lithium method (Ito *et al.* 1983) as described previously (Gietz *et al.* 1992). Transformants were selected on complete medium containing 200 μg G418·ml^{-1} (Geneticin; GIBCO BRL, Gaithersburg, MD).

Formation of 5′ and internal deletions in the *AGP1* upstream region: The centromere- and *URA3*-based plasmid YCp*AGP1-lacZ* has been previously described (Iraqui *et al.* 1999). Plasmids pFA1 and pFA2 were constructed by inserting into the *Bam*HI- and *Hin*dIII-cleaved YCpAJ152 plasmid (André *et al.* 1993) DNA fragments flanked by *Hin*dIII and *Bam*H1 restriction sites and spanning the first five codons of *AGP1* preceded by 537 bp (pFA1) or 513 bp (pFA2) of upstream sequences. These DNA fragments were obtained by PCR, using plasmid p16.2 bearing the *AGP1* gene (Iraqui *et al.* 1999) as a template and the PCR primers 3-AGP1, 5-AGP1 + GA, and 5-AGP1 + GA + UASa (Table 2). Plasmid pFA3 is a derivative of YCp *AGP1-lacZ* from which the 21 bp corresponding to the UAS_{AA} element (see sequence in Table 4, line 7) have been specifically deleted. The deletion, spanning positions −516 to −538 relative to the ATG initiation codon, was introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). A similar procedure was used to delete the region spanning positions −513 to −567 (pFA4). The primers (5-Del-UASaa, 3-Del-UASaa, 5-Del-UASc, and 3-Del-UASc) used for these mutagenesis experiments are shown in Table 2. The accuracy of each mutagenized plasmid was checked by sequencing.

UAS_{AA}-driven *lacZ* reporter plasmids: The episomal pFA10 plasmid (alias YEp-*UASaa-CYC1-lacZ*) was constructed from plasmid pLG670-Z (Guarente 1983). This vector bears a *CYC1-lacZ* fusion preceded by the *CYC1* upstream region deleted of its natural UAS sequences (by excision of an internal *Xho*I-*Xho*I restriction fragment). The 21-bp sequence corresponding to UAS_{AA} (Table 4; plasmid pFA10) or the same UAS_{AA} preceded by 10 additional nucleotides (Table 4; plasmid pFA5) was inserted by site-directed mutagenesis into the reconstituted unique *Xho*I site of plasmid pLG670-Z. For

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference or source
23344c	*MAT*α *ura3*	M. Grenson
32501c	*MAT***a** *ssy1*Δ*::kanMX2 ura3*	Iraqui *et al.* (1999)
30629c	*MAT***a** *gap1*Δ*::kanMX2 ura3*	Iraqui *et al.* (1999)
30633c	*MAT***a** *gap1*Δ*::kanMX2 agp1*Δ*::kanMX2 ura3*	Iraqui *et al.* (1999)
32501d	*MAT***a** *gap1*Δ*::kanMX2 ssy1*Δ*::kanMX2 ura3*	Iraqui *et al.* (1999)
FB98	*MAT*α *ptr3*Δ*::kanMX2 ura3*	Bernard and André (2001a)
FB90	*MAT*α *ssy5*Δ*::kanMX2 ura3*	Bernard and André (2001a)
JA115	*MAT*α *grr1*Δ*::kanMX2 ura3*	Iraqui *et al.* (1999)
FA063	*MAT*α *stp1*Δ*::kanMX2 ura3*	This study
FA064	*MAT*α *stp2*Δ*::kanMX2 ura3*	This study
FA050	*MAT*α *uga35*Δ *ura3*	This study
37071a	*MAT***a** *stp1*Δ*::kanMX2 stp2*Δ*::kanMX2 ura3*	This study
37053a	*MAT*α *gap1*Δ*::kanMX2 stp1*Δ*::kanMX2 ura3*	This study
37054d	*MAT*α *gap1*Δ*::kanMX2 stp2*Δ*::kanMX2 ura3*	This study
34304b	*MAT*α *gap1*Δ*::kanMX2 uga35*Δ *ura3*	This study
37092a	*MAT***a** *gap1*Δ*::kanMX2 stp1*Δ*::kanMX2 stp2*Δ*::kanMX2 ura3*	This study
38009a	*MAT***a** *gap1*Δ *stp1*Δ *uga35*Δ *ura3*	This study
38005a	*MAT*α *gap1*Δ*::kanMX2 stp2*Δ*::kanMX2 uga35*Δ *ura3*	This study
38015c	*MAT*α *gap1*Δ *stp1*Δ *stp2*Δ*::kanMX2 uga35*Δ *ura3*	This study
30505b	*MAT*α *gln3*Δ *ura3*	André *et al.* (1995)
26854a	*MAT***a** *ure2*Δ *ura3*	Soussi Boudekou *et al.* (1997)
SBS10	*MAT***a** *gzf3*Δ*::LEU2 ura3*	Soussi Boudekou *et al.* (1997)
50036c	*MAT*α *ure2 gzf3*Δ*::LEU2 ura3*	Soussi Boudekou *et al.* (1997)

this, we used the QuikChange XL site-directed mutagenesis kit (Stratagene), oligonucleotides 5- and 3-UASaa (pFA10) or 5- and 3-UASaa2 (pFA5; Table 2), and protocols recommended by the supplier. The correct insertion of sequences into pLG670-Z was checked by sequencing. The *AGP1*-(A to S) series of plasmids (Table 7) was constructed from pFA10 by similar site-directed mutagenesis. The list of oligonucleotides used for these constructions is shown in Table 2 (5- and 3-ΔAGP1-A to -S). Each mutagenized plasmid construct was checked by sequencing.

Yeast cell extracts and immunoblotting: Crude cell extracts were prepared as previously described (Hein *et al.* 1995). For Western blot analysis, equal quantities of proteins were loaded on an 8% SDS-polyacrylamide gel in a tricine system. After transfer to a nitrocellulose membrane (Shleicher and Schüll, Keene, NH), the proteins were probed with polyclonal antibodies raised against the N-terminal tail of Agp1 (1:5000; M. El Bakkoury and B. André, unpublished data) or Pma1 (1:1000) used as a loading control. Primary antibodies were detected with horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) followed by enhanced chemiluminescence (Roche Molecular Biochemicals, Indianapolis).

β-Galactosidase assays: All β-galactosidase assays were performed on cells having reached the state of balanced growth. β-Galactosidase activities were measured as described earlier (André *et al.* 1993) and are expressed in nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Protein concentrations were measured with the Folin reagent and the standard used was bovine serum albumin.

RESULTS

Stp1 and Stp2 are not essential for *AGP1* induction: Previous studies have shown that induction by leucine of the *BAP2* and *BAP3* genes does not occur at all in a *stp1*Δ *stp2*Δ mutant, whereas residual induction subsists in *stp1*Δ and *stp2*Δ single-mutant strains (de Boer *et al.* 2000; Nielsen *et al.* 2001). We thus tested the involvement of Stp1 and Stp2 in induction by phenylalanine of *AGP1*, another amino acid permease gene under the control of the Ssy1 pathway. Phenylalanine was chosen because this amino acid is one of the most potent inducers of *AGP1*. All experiments were carried out in a *gap1*Δ mutant lacking the general amino acid permease, so that the same strains could also be used in growth tests on amino acids used as the sole nitrogen source (see below). Induction of an *AGP1-lacZ* gene carried on a low-copy plasmid was reduced by >90% in the *gap1*Δ *stp1*Δ mutant as compared to the *gap1*Δ strain, but it was essentially normal in the *gap1*Δ *stp2*Δ mutant (Table 3). In the *gap1*Δ *stp1*Δ *stp2*Δ triple mutant, induction of *AGP1* was much lower than that in the *gap1*Δ strain, but residual induction corresponding to ∼15% of the control was unexpectedly observed. Hence, the *AGP1* gene is still induced by phenylalanine, although weakly, in the total absence of Stp1 and Stp2. These results were confirmed by means of growth tests indicative of Agp1 function. When an amino acid such as phenylalanine, isoleucine, or methionine is supplied at low concentration (1 mm) as sole nitrogen source, growth of a *gap1*Δ mutant requires a functional Agp1 permease since a *gap1*Δ *agp1*Δ strain does not grow on these media (Iraqui *et al.* 1999). A similar nongrowing phenotype is displayed by the *gap1*Δ *ssy1*Δ mutant, where the *AGP1*

TABLE 2

Oligonucleotides used in this study

Reference	Sequence
D5-STP1	5′-TATGTACATAGACATATAAAGGGCATGTGGTAATTATGACGCGGCCGCCAGCTGAAGCTT-3′
D3-STP1	5′-CCAATATGATACCCTTATTTTTATCCCGTGTTATATTTAAGCGGCCGCATAGGCCACTAG-3′
D5-STP2	5′-AAGAGAGCAAAAAGTTGGGGAAGTAAGGAAATCCATTATCGCGGCCGCCAGCTGAAGCTT-3′
D3-STP2	5′-GGTTAATTCAGGTATTATTTTTATGGCGGTTTCAGGTATCGCGGCCGCATAGGCCACTAG-3′
D5-UGA35	5′-TTTAGACGAGCGGCAGAACGACAGGCAGCCATACTATCAAGCGGCCGCCAGCTGAAGCTT-3′
D3-UGA35	5′-ATTACCGTATTCCATTTTTACTTATGTGCTATTATTTATAGCGGCCGCATAGGCCACTAG-3′
5-UASaa	5′-GGAAAGCAGGAAAGGAGGGTGCCGTCTAAGCGGCACCAGATCCGCCAGGCG-3′
3-UASaa	5′-CGCCTGGCGGATCTGGTGCCGCTTAGACGGCACCCTCCTTTCCTGCTTTCC-3′
5-UASaa2	5′-GGAAAGCAGGAAAGGGTGCCGCTTGAGGGTGCCGTCTAAGCGGCACTGACAGATCCGCCAGGCG-3′
3-UASaa2	5′-CGCCTGGCGGATCTGTCAGTGCCGCTTAGACGGCACCCTCAAGCGGCACCCTTTCCTGCTTTCC-3′
5-Del-UASaa	5′-CTTCAGTGCCGCCTGATCTGCTGC-3′
3-Del-UASaa	5′-GCAGCAGATCAGGCGGCACTGAAG-3′
5-Del-UASc	5′-GACCGGCATAATAGAAGTCTGCTGCAAAAAGCTG-3′
3-Del-UASc	5′-CAGCTTTTTGCAGCAGACTTCTATTATGCCGGTC-3′
5-ΔAGP1-A	5′-GGAAAGGAGGGTACCGTCTAAGAGGCACCAGATCC-3′
3-ΔAGP1-A	5′-GGATCTGGTGCCTCTTAGACGGTACCCTCCTTTCC-3′
5-ΔAGP1-C	5′-GGAAAGGAGGGTGACGTCTAAGCAGCACCAGATCCGC-3′
3-ΔAGP1-C	5′-GCGGATCTGGTGCTGCTTAGACGTCACCCTCCTTTCC-3′
5-ΔAGP1-D	5′-GGAAAGGAGGGTGCAGTCTAAGCGACACCAGATCCGCC-3′
3-ΔAGP1-D	5′-GGCGGATCTGGTGTCGCTTAGACTGCACCCTCCTTTCC-3′
5-ΔAGP1-F	5′-GGAAAGGAGGGTGCCATCTAAGCGGAACCAGATCCGCCAGG-3′
3-ΔAGP1-F	5′-CCTGGCGGATCTGGTTCCGCTTAGATGGCACCCTCCTTTCC-3′
5-ΔAGP1-H	5′-GAGGGTGCCGACTCTAAGCGGC-3′
3-ΔAGP1-H	5′-GCCGCTTAGAGTCGGCACCCTC-3′
5-ΔAGP1-K	5′-GGAGGGTGCCGTCTCTCAGCGGCACC-3′
3-ΔAGP1-K	5′-GGTGCCGCTGAGAGACGGCACCCTCC-3′
5-ΔAGP1-L	5′-GGAGGGTGCCGAGCGGCACCAG-3′
3-ΔAGP1-L	5′-CTGGTGCCGCTCGGCACCCTCC-3′
5-ΔAGP1-M	5′-GCAGGAAAGGAGGGTACATTCTAAGCGGCACCAG-3′
3-ΔAGP1-M	5′-CTGGTGCCGCTTAGAATGTACCCTCCTTTCCTGC-3′
5-ΔAGP1-N	5′-GGGTGCCGTCTAAGACATACCAGATCCGCCAG-3′
3-ΔAGP1-N	5′-CTGGCGGATCTGGTATGTCTTAGACGGCACCC-3′
5-ΔAGP1-P	5′-GCAGGAAAGGAGACTGCCGTCTAAGCGGCTACAGATCCGCCAGG-3′
3-ΔAGP1-P	5′-CCTGGCGGATCTGTAGCCGCTTAGACGGCAGTCTCCTTTCCTGC-3′
5-ΔAGP1-Q	5′-GCAGGAAAGGAGACTGCCGTATCATCGGCTACAGATCCGCCAGG-3′
3-ΔAGP1-Q	5′-CCTGGCGGATCTGTAGCCGATGATACGGCAGTCTCCTTTCCTGC-3′
5-ΔAGP1-R	5′-GCAGGAAAGGAGGGTGCCGTATCATCGGCACCAGATCCGCCAGG-3′
3-ΔAGP1-R	5′-CCTGGCGGATCTGGTGCCGATGATACGGCACCCTCCTTTCCTGC-3′
5-ΔAGP1-S	5′-GCAGGAAAGGAGGGTGCCGTCTAAGGCCGACCAGATCCGCCAGG-3′
3-ΔAGP1-S	5′-CCTGGCGGATCTGTAGCCGCTTAGAGCCGACCCTCCTTTCCTGC-3′
3-AGP1	5′-CGCGGATCCGACTTCGACGACGACATTGT-3′
5-AGP1 + GA	5′-CCGAAGCTTTCTGCTGCAAAAAGCTGCAA-3′
5-AGP1 + GA + UASa	5′-CCGAAGCTTTGAGGGTGCCGTCTAAG-3′

gene is not induced by amino acids (Iraqui *et al.* 1999) (Figure 1). The growth test results presented in Figure 1 clearly show that the *gap1*Δ *stp1*Δ *stp2*Δ strain grows as well as the *gap1*Δ strain on all tested amino acids, indicating that the residual expression of *AGP1* recorded in this strain (Table 3) is sufficient to sustain growth under these conditions. Residual induction of *AGP1* in the *gap1*Δ *stp1*Δ *stp2*Δ strain was also confirmed in Western-blot experiments using antibodies against the Agp1 permease (Figure 2). In conclusion, Stp1 plays a major role in induction of *AGP1* by amino acids. Yet in contrast to the situation described for the *BAP2* and *BAP3* genes, Stp2 does not significantly contribute to *AGP1* expression, and significant induction of *AGP1* can still occur in the total absence of both Stp1 and Stp2. This suggests that another transcription factor besides Stp1 and Stp2 can respond to the external amino acid sensing pathway to promote some degree of transcriptional induction of the *AGP1* gene.

Stp1 acts synergistically with Uga35/Dal81 to promote *AGP1* induction: In previous articles we reported that the Uga35/Dal81 protein [containing a $Zn(II)_2$-

TABLE 3

Normal induction of *AGP1* requires Stp1 and Uga35/Dal81

Strain	*AGP1-lacZ*: β-galactosidase activity (nmol·min^{-1}·mg prot^{-1}) −Phe	+Phe
*gap1*Δ	≤10	1673
*gap1*Δ *stp1*Δ	≤10	139
*gap1*Δ *stp2*Δ	≤10	1467
*gap1*Δ *stp1*Δ *stp2*Δ	≤10	365
*gap1*Δ *uga35/dal81*Δ	≤10	174
*gap1*Δ *uga35/dal81*Δ *stp1*Δ	≤10	≤10
*gap1*Δ *uga35/dal81*Δ *stp1*Δ *stp2*Δ	≤10	≤10

Strains 30629c (*gap1*Δ *ura3*), 37053a (*gap1*Δ *stp1*Δ *ura3*), 37054d (*gap1*Δ *stp2*Δ *ura3*), 34304b (*gap1*Δ *uga35/dal81*Δ *ura3*), 37092a (*gap1*Δ *stp1*Δ *stp2*Δ *ura3*), 38009a (*gap1*Δ *stp1*Δ *uga35/dal81*Δ *ura3*), and 38015c (*gap1*Δ *stp1*Δ *stp2*Δ *uga35/dal81*Δ *ura3*) transformed with the CEN-based plasmid YCp *AGP1-lacZ* were grown on proline minimal medium with (+Phe) or without (−Phe) phenylalanine added at 5 mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were <15%.

Cys_6-cluster-type domain] is required for full induction of *AGP1* by amino acids (Iraqui *et al.* 1999; Bernard and André 2001a). We thus sought to test the relative contributions of Stp1, Stp2, and Uga35/Dal81 to *AGP1* induction. In the *gap1*Δ *uga35/dal81*Δ mutant, induction of *AGP1-lacZ* by phenylalanine was severely reduced, but weak induction persisted (Table 3). In the *gap1*Δ *uga35/dal81*Δ *stp1*Δ mutant, induction was totally abolished (Table 3). Consistently, no detectable Agp1 signal was observed on immunoblots of the *gap1*Δ *uga35/dal81*Δ *stp1*Δ strain (Figure 2). Furthermore, while the *gap1*Δ *stp1*Δ *stp2*Δ and *gap1*Δ *uga35/dal81*Δ strains grow normally on amino acids supplied as the sole nitrogen source, the triple *gap1*Δ *stp1*Δ *uga35*Δ and quadruple *gap1*Δ *stp1*Δ *stp2*Δ *uga35*Δ mutant strains do not (Figure 1). These results show that Uga35/Dal81 plays an important role in *AGP1* induction and is also responsible for residual *AGP1* expression in the *gap1*Δ *stp1*Δ *stp2*Δ mutant. It thus appears that normal *AGP1* induction requires, in addition to Stp1, activation via the Ssy1 pathway of another transcriptional activation circuit that is Uga35/Dal81 dependent. Finally, the fact that the *gap1*Δ *stp1*Δ *uga35/dal81*Δ and *gap1*Δ *stp1*Δ *stp2*Δ *uga35/dal81*Δ strains behave similarly in all experiments (Table 3; Figures 1 and 2) confirms that Stp2 does not significantly contribute to *AGP1* induction. Hence, Stp1 and Uga35/Dal81 appear as the principal transcription factors mediating induction of *AGP1* in response to external amino acids. In addition, they appear to be equally important, since induction of *AGP1* is equally impaired after deletion of the *STP1* or *UGA35/DAL81* gene (Table 1).

Identification of the UAS_{AA} element of the *AGP1* gene: We next addressed the question of whether Uga35/Dal81 and Stp1 exert their positive effects through the same or separate *cis*-acting sequences in the *AGP1* gene's upstream region. For this, we carried out experiments aimed at identifying the *cis*-acting sequences of the *AGP1* gene responsible for its induction by amino acids. Previous work has revealed in the *BAP3* gene's upstream region a minimal 25-bp sequence (called UAS_{AA}) that is sufficient to drive induction of reporter genes by multiple amino acids (de Boer *et al.* 1998, 2000). This element contains the sequence 5′-CGGCN$_6$GCCG-3′, and each 5′-CGGC-3′ quadruplet appears to be crucial to the UAS_{AA}'s induction-driving properties. Another study centered on the *BAP2* gene (Nielsen *et al.* 2001) led to the isolation of a 65-bp DNA fragment also displaying UAS_{AA} properties. This fragment contains the 5′-CGG CN$_4$CGGC-3′ sequence shown in mutagenesis experiments to determine UAS_{AA} properties. These studies indicate that direct or inverted repeats of 5′-CGGC-3′ are the core sequences of UAS_{AA} elements. Consistently, comparative analysis of the upstream sequences of several Ssy1 target genes (*AGP1*, *BAP2*, *BAP3*, *PTR2*, *TAT1*, *TAT2*, and *GNP1*) by means of the regulatory sequence analysis tools (http://rsat.ulb.ac.be/rsat/; van Helden *et al.* 1998) reveals that the most recurrent oligonucleotide within these promoter regions is the 5′-CGGC-3′ sequence. Moreover, many such tetranucleotides are separated by two to nine nucleotides. Also, in support of the view that these 5′-CGGC-3′ sequences are important for transcriptional control of these genes, most of them tend to be conserved in orthologous gene promoters of closely related yeast species (Cliften *et al.* 2003; Kellis *et al.* 2003). The upstream region of the *AGP1* gene (up to position −780 with respect to the ATG initiation codon) contains 13 copies of the 5′-CGGC-3′ sequence. Deletion of the upstream region of *AGP1* up to position −538 did not significantly reduce induction of the *AGP1-lacZ* gene by phenylalanine, but further deletion of 24 bp totally abolished this induction (Table 4, lines 1–3). This deletion eliminates the most upstream 5′-GCCG-3′ of a 5′-CGGCN$_6$GCCG-3′ sequence. We thus specifically deleted this sequence from the full-length *AGP1* upstream region. This caused strong reduction of induction, but the resulting *AGP1-lacZ* construct still responded weakly to the presence of amino acids (Table 4, lines 1 and 4). Just upstream from the deleted 5′-CGGCN$_6$GCCG-3′ is the sequence 5′-CGGCN$_9$CGGC-3′. When the latter sequence was also deleted, residual induction of the *AGP1-lacZ* was reduced another threefold (Table 4, lines 1–5). The very weak residual induction displayed by this *AGP1-lacZ* construct (~3% of the wild-type level) is likely determined by other 5′-CGGC-3′ core sequences still present in the *AGP1* gene upstream region.

A 21-bp sequence from the *AGP1* gene's upstream region (between positions −536 and −515, containing

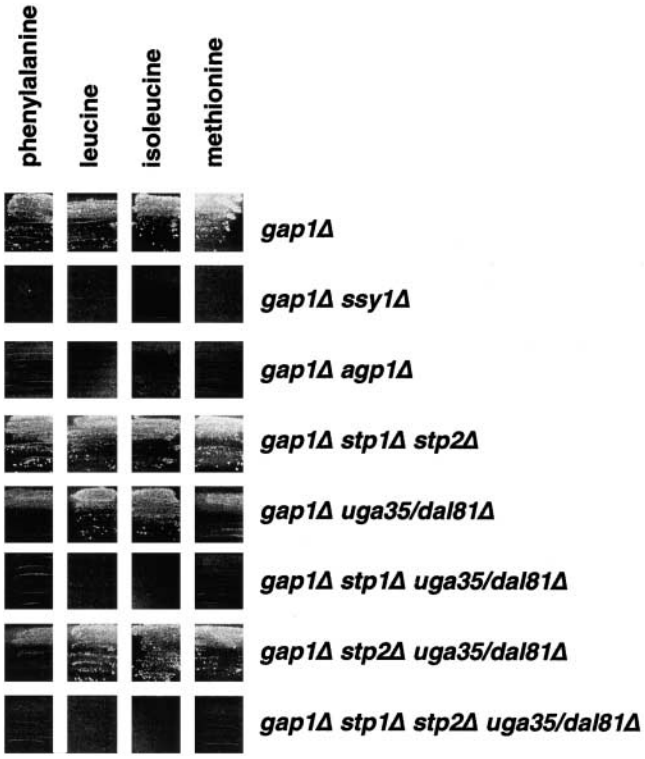


FIGURE 1.—Deletion of the *UGA35* and *STP1* genes in cells lacking the general amino acid permease (Gap1) impairs utilization of several amino acids. Cells were spread on minimal medium with the indicated amino acids as sole nitrogen sources (1 mM). The strains were 30629c (*gap1Δ ura3*), 32501d (*gap1Δ ssy1Δ ura3*), 30633c (*gap1Δ agp1Δ ura3*), 37092a (*gap1Δ stp1Δ stp2Δ ura3*), 34034b (*gap1Δ uga35/dal81Δ ura3*), 38009a (*gap1Δ stp1Δ uga35/dal81Δ ura3*), 38005a (*gap1Δ stp2Δ uga35/dal81Δ ura3*), and 38015c (*gap1Δ stp1Δ stp2Δ uga35/dal81Δ ura3*).

the 5′-CGGCN$_6$GCCG-3′ motif) was then inserted upstream from the *CYC1-lacZ* reporter gene lacking its own UAS. The resulting high-copy-number plasmid was introduced into the wild type and β-galactosidase activities were measured in cells growing either on urea or on the same medium with added phenylalanine (Table 4, lines 6–8). The results clearly show that the 21-bp sequence drives high-level transcriptional induction in response to phenylalanine (lines 6 and 7). A similar result was obtained when the 21-bp sequence was extended at its 5′ end with 10 additional nucleotides comprising a 5′-GCCG-3′ tetranucleotide (line 8). Further experiments showed that the 21-bp sequence can drive transcriptional activation in response to many other amino acids, the induction level varying markedly according to the amino acid (Table 5). This 21-bp sequence thus has the properties of a UAS$_{AA}$ element. For many amino acids like phenylalanine, threonine, leucine, or isoleucine, the responses displayed by the *lacZ* constructs under the control of the UAS$_{AA}$ alone or the entire *AGP1* upstream region (*AGP1-lacZ* gene) are similar; *i.e.*, amino acids causing strong induction of the former also cause strong induction of the latter (Table 5). However, some amino acids like valine and tryptophan are strong inducers of the *AGP1-lacZ* gene but relatively weak inducers of UAS$_{AA}$-driven transcription. In contrast, others like serine or histidine are rather good inducers of UAS$_{AA}$-dependent transcription although relatively weak inducers of *AGP1-lacZ*.

Finally, experiments showed that induction mediated by the UAS$_{AA}$ element isolated from the *AGP1* gene is entirely dependent on the Ssy1, Ptr3, and Ssy5 factors (Table 6). In previous articles, we reported that components of the SCF^{Grr1} ubiquitin-ligase complex are also required for proper induction of the *AGP1* gene (IRAQUI *et al.* 1999; BERNARD and ANDRÉ 2001b). The results of Table 6 show that the F-box protein Grr1 is also essential for UAS$_{AA}$ function. This observation is consistent with the view that SCF^{Grr1} is an integral part of the external amino acid signaling pathway.

Mutational analysis of the UAS$_{AA}$ element of the *AGP1*-gene: Site-directed mutagenesis was used to further analyze the 21-bp UAS$_{AA}$ element found upstream from the *AGP1* gene (Table 7). Single-base replacement of any of the four nucleotides within one or both of the inversely repeated 5′-GCCG-3′ quadruplets drastically reduced induction by amino acids (Table 7, lines 2–8). The same was observed when a single quadruplet was replaced with 5′-ACAT-3′ or 5′-ATGC-3′ (lines 9–11). This clearly shows that at least two copies of the 5′-GCCG-3′ sequence are needed to constitute a UAS$_{AA}$ responding strongly to amino acids. In the native UAS$_{AA}$, the quadruplets are separated by six nucleotides. When this number was reduced to two, the UAS$_{AA}$ lost its activation potency (line 12); the UAS$_{AA}$ resulting from increasing the number of separating nucleotides to eight (lines 13 and 14) was also less active. It thus seems that six nucleotides is the optimal distance between the two inversely repeated 5′-GCCG-3′ quadruplets, although derived UAS$_{AA}$ elements with greater spacing can still respond significantly to amino acids. Replacement mutations were also introduced at sites directly flanking the quadruplets, sites among the six nucleotides separating them, or both (lines 15–17). Again, both types of mutation caused a net reduction of expression, but significant induction of the *lacZ* reporter gene may subsist. When these mutations were combined in the same construct, induction was entirely lost (line 17). It thus seems that the direct environment of the repeated 5′-GCCG-3′ sequences is important for the optimal functioning of the UAS$_{AA}$. Finally, we tested whether direct instead of inverse 5′-GCCG-3′ repeats could also promote induction. Again this caused a significant reduction of expression, but significant induction was maintained (line 18). It thus seems that the orientation, spacing, and immediate context of the 5′-GCCG-3′ repeats of the UAS$_{AA}$ element of the *AGP1* gene are optimal for high-level induction in response to amino acids, but that other

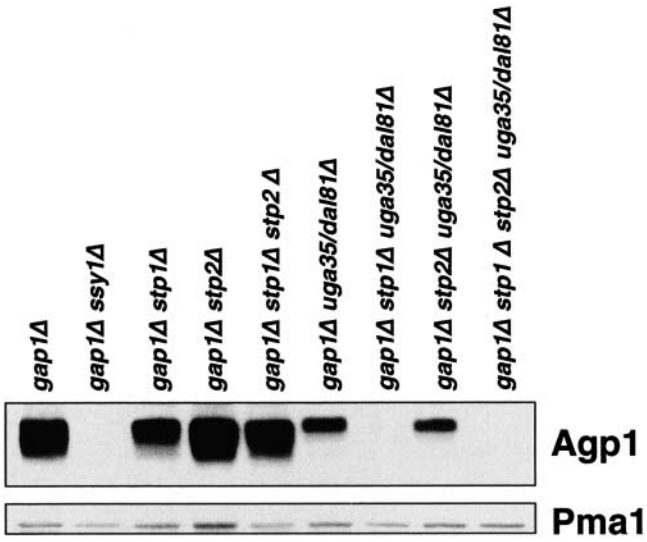


FIGURE 2.—Stp1 and Uga35/Dal81 are the main transcription factors contributing to the phenylalanine-induced synthesis of the Agp1 permease. Western blot detection of the Agp1 permease in extracts of cells grown on a minimal medium containing proline and phenylalanine (5 mM) as sole nitrogen sources is shown. Strains were: (1) 30629c (*gap1*Δ *ura3*), (2) 32501d (*gap1*Δ *ssy1*Δ *ura3*), (3) 37051a (*gap1*Δ *stp1*Δ *ura3*), (4) 37054d (*gap1*Δ *stp2*Δ *ura3*), (5) 37092a (*gap1*Δ *stp1*Δ *stp2*Δ *ura3*), (6) 34034b (*gap1*Δ *uga35/dal81*Δ *ura3*), (7) 38009a (*gap1*Δ *stp1*Δ *uga35/dal81*Δ *ura3*), (8) 38005a (*gap1*Δ *stp2*Δ *uga35/dal81*Δ *ura3*), and (9) 38015c (*gap1*Δ *stp1*Δ *stp2*Δ *uga35/dal81*Δ *ura3*). To make sure that equal amounts of proteins were loaded, Pma1 was also immunodetected.

arrangements are also possible, leading to lower but significant induction levels.

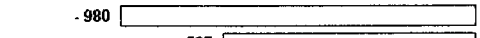
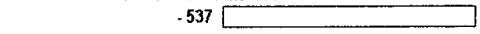
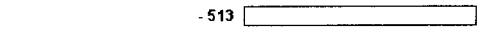
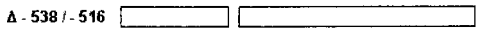
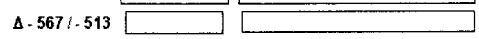
Stp1 and Uga35/Dal81 are both essential to UAS_{AA} function: We next monitored transcriptional induction driven by the native UAS_{AA} element in *gap1*Δ cells with different combinations of *STP1*, *STP2*, and *UGA35/DAL81* gene deletions (Table 8). Deletion of *STP2* did not affect induction of the reporter gene in response to phenylalanine. In contrast, deletion of *STP1* or *UGA35/DAL81* completely suppressed this induction. Hence, Stp1 and Uga35/Dal81 act through the same *cis*-acting sequence (UAS_{AA}) and both are absolutely essential to its ability to drive transcriptional induction. This situation somewhat differs from that of the *lacZ* reporter under the control of the entire *AGP1* upstream region, since in the latter case weak but significant induction persisted in cells lacking only one gene, *STP1* or *UGA35/DAL81* (Table 3). Perhaps Stp1 and Uga35/Dal81 can act alone via other 5′-CGGC-3′ sequences of lesser importance in the *AGP1* upstream region, *e.g.*, those responsible for weak residual induction of *AGP1* gene expression when the UAS_{AA} element characterized above is deleted (Table 4, lines 4 and 5).

***AGP1* induction by amino acids is under nitrogen catabolite repression:** REGENBERG *et al.* (1999) reported a two-times-higher level of *AGP1-lacZ* induction by leucine (0.23 mM) in cells growing on proline than in cells growing on ammonium as the source of nitrogen. This suggests that *AGP1* is controlled by nitrogen catabolite repression (NCR), even though the negative effect of ammonium was limited (probably because the strain used in these experiments—a derivative of S288C—is largely insensitive to this regulation (MAGASANIK and KAISER 2002). We repeated these experiments in strains derived from the Σ1278b wild type, known to respond normally to ammonium regulation. We found induction of *AGP1-lacZ* gene expression by phenylalanine to be considerably stronger in cells growing on urea (a poor nitrogen source) than in cells growing on 20 mM ammonium (a good nitrogen source; Table 9, line 1). When a fivefold higher ammonium concentration (100 mM) was used, the induction level was further halved (data not shown). Hence, induction of *AGP1* by external phenylalanine is highly sensitive to the nitrogen status of the cell. Genes subject to this global regulation (NCR) are known to contain several 5′-GATA-3′ core sequences in their upstream regions, capable of binding several transcription factors of the GATA family (COOPER 2002; MAGASANIK and KAISER 2002). The Gln3 factor is the GATA factor playing the main positive role in transcription of genes subject to NCR (STANBROUGH *et al.* 1995). On good nitrogen sources, Gln3 is sequestered in the cytoplasm, but on poor ones, it is translocated into the nucleus and activates transcription of genes via upstream 5′-GATA-3′ sequences (BECK and HALL 1999). Consistently with *AGP1* being subject to NCR, this gene's upstream region contains multiple 5′-GATA-3′ sequences. In the *gln3*Δ mutant, induction of the *AGP1-lacZ* reporter gene was severely reduced (Table 9, line 2). The Ure2 protein is required for sequestration of Gln3 in the cytoplasm under good nitrogen-supply conditions (BECK and HALL 1999). In the *ure2*Δ mutant, *AGP1-lacZ* was induced to a much higher level on ammonium (Table 9, line 3). The negatively acting GATA factor encoded by the *GZF3/DEH1/NIL2* gene is also required for normal NCR of genes (COFFMAN *et al.* 1997; ROWEN *et al.* 1997; SOUSSI BOUDEKOU *et al.* 1997). In the *gzf3*Δ mutant also, *AGP1-lacZ* was induced to a significantly higher level on ammonium, and in the *ure2*Δ *gzf3*Δ double mutant, this induction was still higher (Table 9, lines 4 and 5). Experiments were also performed with mutants lacking the two other GATA factors involved in controlling NCR-sensitive genes (*DAL80/UGA43* and *NIL1/GAT1*), but *AGP1* expression was not significantly affected in these strains (data not shown). Taken together, these data clearly show that induction of *AGP1* by external amino acids is subject to NCR and that the main *trans*-factors involved in this control are Gln3, Ure2, and, to a lesser extent, Gzf3/Deh1/Nil2.

The UAS_{AA} element is insensitive to nitrogen regulation: The same experiments were repeated with cells transformed with the *lacZ* reporter gene under the control of the UAS_{AA} element isolated from the *AGP1* gene (Table 9). The results clearly show that UAS_{AA}-driven

TABLE 4

Identification of a UAS_{AA} element from the upstream region of the *AGP1* gene

A.	Plasmid	*AGP1* upstream sequence	*AGP1-lacZ*: β-galactosidase activity (nmol·min^{-1}·mg prot^{-1}) −Phe	+Phe
1	YCpAGP1-lacZ		≤10	1510
2	pFA1		≤10	1415
3	pFA2		≤10	≤10
4	pFA3		≤10	119
5	pFA4		≤10	45

B.	Plasmid	Inserted *AGP1* upstream sequence	*CYC1-lacZ*: β-galactosidase activity (nmol·min^{-1}·mg prot^{-1}) −Phe	+Phe
6	pLG670	None	≤10	≤10
7	pFA10	5′-AGGGTGCCGTCTAAGCGGCAC-3′	≤10	513
8	pFA5	5′-CTGCCGCTTGAGGGTGCCGTCTAAGCGGCAC-3′	≤10	433

(A) Strain 23344c (*ura3*) transformed with the CEN-based plasmid YCp*AGP1-lacZ* (−980 to +5) carrying the indicated promoter deletion construct was grown on urea minimal medium with (+Phe) or without (−Phe) the inducer phenylalanine at 5 mM final concentration. (B) UAS_{AA} renders the expression of the *CYC1-lacZ* reporter gene inducible by amino acids. Strain 23344c (*ura3*) transformed with a high-copy-number plasmid carrying the *CYC1-lacZ* reporter gene without any UAS (pLG670) or under the control of UAS_{AA} elements from the *AGP1* gene (pFA10, −536 to −515; pFA5, −546 to −515) was grown on minimal urea medium with (+Phe) or without (−Phe) phenylalanine at 5 mM. The reported β-galactosidase activities are means of two to three independent experiments. Variations were <15%.

transcriptional induction is not significantly different in cells growing on urea or ammonium medium. Furthermore, a lack of Gln3, Ure2, or Gzf3 did not affect this expression. These results thus show that UAS_{AA}-driven transcription is totally insensitive to the NCR status of the cell. As target of rapamycin (TOR) kinases regulate GATA factor function in response to nutrient availability (Cooper 2002; Crespo and Hall 2002), these data also suggest that the TOR pathway does not interfere with the external amino acid signaling pathway. Induction of *AGP1-lacZ* is nevertheless much greater on urea medium than on ammonium medium, and a lack of Gln3 on urea medium leads to loss of ~85% of *AGP1* induction (Table 9, lines 1 and 2). This suggests that while Stp1 and Uga35/Dal81 play a crucial role in *AGP1* induction, Gln3 is responsible for this induction reaching much higher levels in cells grown under poor nitrogen-supply conditions.

DISCUSSION

Transcriptional induction of yeast genes in response to external amino acids requires the permease-like protein Ssy1 and the membrane-associated Ptr3 and Ssy5 factors. According to a recent model, these factors are associated in a sensor complex (SPS) able to detect external amino acids and in turn to activate by endoproteolytic processing two transcription factors, Stp1 and Stp2, which are then translocated into the nucleus to activate gene transcription (Andreasson and Ljungdahl 2002). Stp1 and Stp2 appear to be redundant since a residual transcriptional induction of the *BAP2* and *BAP3* genes in response to leucine subsists in both single *stp1*Δ and *stp2*Δ mutants whereas no induction is detected in the double *stp1*Δ *stp2*Δ strain (de Boer *et al.* 2000; Nielsen *et al.* 2001). The functional redundancy of Stp1 and Stp2 was also illustrated by the observation that the growth phenotypes of the *stp1*Δ *stp2*Δ and *ssy1*Δ mutant strains on several selective media were indiscernible, whereas the *stp1*Δ and *stp2*Δ single mutants behaved like the wild type (Andreasson and Ljungdahl 2002). In this study, we show that transcription of the *AGP1* gene encoding another amino acid permease induced by external amino acids (Iraqui *et al.* 1999) is mainly dependent on Stp1, whereas Stp2 does not significantly contribute to this expression. This means that Stp1 and Stp2 are not functionally redundant in the case of *AGP1* gene expression and this raises the question of the specific role of each Stp factor in the response of cells to external amino acids. We further show that Stp1 acts synergistically with another transcription factor, namely Uga35/Dal81: lack of Stp1 or Uga35/Dal81 dramatically reduces induction of *AGP1* by amino acids whereas lack of both transcription factors

TABLE 5

Using UAS_{AA} as a reporter to monitor the response of cells to external amino acids

No.	Nitrogen sources	β-Galactosidase activity (nmol·min^{-1}·mg prot^{-1})	
		AGP1-lacZ	*UASaa-CYC1-lacZ*
1	Urea	≤2	≤2
2	Urea + tryptophan	851	83
3	Urea + phenylalanine	838	322
4	Urea + valine	766	72
5	Urea + threonine	751	347
6	Urea + tyrosine	691	134
7	Urea + isoleucine	672	213
8	Urea + methionine	592	131
9	Urea + leucine	484	178
10	Urea + alanine	474	72
11	Urea + asparagine	116	63
12	Urea + glutamine	112	13
13	Urea + aspartate	110	17
14	Urea + glycine	110	24
15	Urea + serine	98	99
16	Urea + glutamate	81	21
17	Urea + cysteine	73	28
18	Urea + histidine	35	93
19	Urea + GABA	27	16
20	Urea + arginine	12	16
21	Urea + ornithine	5	8

Strain 23344c (*ura3*) transformed with the CEN-based plasmid YCp*AGP1-lacZ* or pFA10 plasmid was grown on urea minimal medium with or without amino acids added at a 5-mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were <15%.

results in total loss of induction. The Stp1 factor appears to be specifically involved in the transcription of genes controlled by the Ssy1 pathway. In contrast, Uga35/Dal81 is also required for induction by GABA of the *UGA* genes (utilization of GABA; Vissers *et al.* 1990; Coornaert *et al.* 1991) and for induction by allophanate of the *DUR* and *DAL* genes (degradation of urea and allantoin; Turoscy and Cooper 1982; Jacobs *et al.* 1985). In both of these induction processes, Uga35/Dal81 acts together with an inducer-specific transcription factor, namely Uga3 (GABA induction; André 1990) or Dal82/DurM (allophanate induction; Jacobs *et al.* 1985; André and Jauniaux 1990; Olive *et al.* 1991). Furthermore, each pair of transcription factors acts through regulon-specific UAS elements (Dorrington and Cooper 1993; Talibi *et al.* 1995). We here report a similar situation, *i.e.*, that Stp1 and Uga35/Dal81 mediate induction of the *AGP1* gene by acting via a UAS_{AA} element (see below). Uga35/Dal81 thus seems to act in tight conjunction with various pathway- or inducer-specific transcription factors (Stp1, Uga3, and Dal82/DurM) via specific UAS elements to promote transcriptional induction of nitrogen-utilization genes in response to various stimuli.

A significant induction of the *AGP1* gene by amino acids thus subsists in the *stp1*Δ and *stp1*Δ *stp2*Δ mutants and this induction is entirely dependent on Uga35/Dal81. Such residual induction, however, is not observed in *ssy1*Δ, *ptr3*Δ, *ssy5*Δ, or *grr1*Δ mutants. Two models may account for this observation. First, Uga35/Dal81 might act in conjunction with yet another transcription factor, which, like Stp1 and Stp2, would be

TABLE 6

The proteins of the SPS complex and the F-box protein Grr1 are essential to UAS_{AA} function

Strain	β-Galactosidase activity (nmol·min^{-1}·mg prot^{-1})			
	AGP1-lacZ		*UAS_{AA}-CYC1-lacZ*	
	−Phe	+Phe	−Phe	+Phe
Wild type	≤10	1474	≤10	505
*ssy1*Δ	≤10	≤10	≤10	≤10
*ptr3*Δ	≤10	≤10	≤10	≤10
*ssy5*Δ	≤10	≤10	≤10	≤10
*grr1*Δ	≤10	≤10	≤10	≤10

Strains 23344c (*ura3*), 30501c (*ssy1*Δ *ura3*), FB98 (*ptr3*Δ *ura3*), FB90 (*ssy5*Δ *ura3*), and JA115 (*grr1*Δ *ura3*), transformed with the CEN-based plasmid YCp*AGP1-lacZ* or the episomal pFA10 plasmid, were grown on minimal urea medium with (+Phe) or without (−Phe) phenylalanine at 5-mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were <10%.

TABLE 7

Mutagenesis of the UAS_{AA} of the *AGP1* gene

No.	Code	Sequence	β-Galactosidase activity (nmol·min^{-1}·mg prot^{-1}) −Phe	+Phe
1	*AGP1*	AGGGTGCCGTCTAAGCGGCAC	≤10	499
2	*AGP1-G*	AGGGTGCC**A**TCTAAGCGGCAC	≤10	34
3	*AGP1-E*	AGGGTGC**A**GTCTAAGCGGCAC	≤10	11
4	*AGP1-B*	AGGGT**A**CCGTCTAAGCGGCAC	≤10	13
5	*AGP1-A*	AGGGT**A**CCGTCTAAG**A**GGCAC	≤10	15
6	*AGP1-C*	AGGGTG**A**CGTCTAAGC**A**GCAC	≤10	≤10
7	*AGP1-D*	AGGGTGC**A**GTCTAAGCG**A**CAC	≤10	23
8	*AGP1-F*	AGGGTGCC**A**TCTAAGCGG**A**AC	≤10	35
9	*AGP1-M*	AGGGT**ACAT**TCTAAGCGGCAC	≤10	41
10	*AGP1-N*	AGGGTGCCGTCTAAG**ACAT**AC	≤10	≤10
11	*AGP1-O*	AGGGTGCCGTCTA**GCACAT**AC	≤10	69
12	*AGP1-L*	AGGGTGCCGAGCGGCAC	≤10	14
13	*AGP1-H*	AGGGTGCCG**AC**TCTAAGCGGCAC	≤10	61
14	*AGP1-K*	AGGGTGCCG**TC**TCTAAGCGGCAC	≤10	48
15	*AGP1-P*	AG**AC**TGCCGTCTAAGCGGC**TA**	≤10	83
16	*AGP1-R*	AGGGTGCCG**AC**C**A**TGCGGCAC	≤10	130
17	*AGP1-Q*	AG**AC**TGCCG**AC**C**A**TGCGGC**TA**	≤10	≤10
18	*AGP1-S*	AGGGTGCCGTCTAAG**GCCG**AC	≤10	107

Oligonucleotide AGP1 (line 1) corresponds to the region between −536 and −515 of the *AGP1* promoter (native 21-bp UAS_{AA} element). Derived mutant UAS_{AA} elements noted *AGP1-A, -B, -C, -D, -E, -F, -G, -M, -N, -O, -P, -Q, -R,* and *-S* are shown. In UAS_{AA}-H and UAS_{AA}-K, two nucleotides are inserted between the 5′-CGGC-*3*′ quadruplets. In UAS_{AA}-L, four nucleotides are deleted. Strain 23344c (*ura3*) was transformed with each of the plasmids YEp*AGP1*-(A → S) and grown on urea minimal medium with (+Phe) or without (−Phe) phenylalanine at 5-mM final concentration. The reported β-galactosidase activities are means of two independent experiments. Variations were <10%.

activated by external amino acids via the SPS sensor complex. The yeast proteome includes two proteins, Stp3 and Stp4, which, like Stp1 and Stp2, harbor a Kruppel-type zinc-finger domain. Yet our experiments failed to show a role of these factors in *AGP1* induction: the *stp1*Δ *stp2*Δ *stp3*Δ *stp4*Δ quadruple mutant still displays Uga35/Dal81-dependent residual induction of the *AGP1* gene in response to phenylalanine (our unpublished data). The putative factor acting with Uga35/Dal81 to induce *AGP1* transcription in the *stp1*Δ *stp2*Δ mutant would thus belong to another family of transcription factors. A second model consistent with our data is that Uga35/Dal81 itself might respond to the external amino acid signaling pathway. In other words, this factor alone might be able to activate *AGP1* transcription in response to external amino acids, its effect on gene transcription being much stronger in conjunction with Stp1. This would mean that the external amino acid signaling pathway bifurcates into two branches, one mediating activation by endoproteolytic processing of Stp1 and Stp2 and another leading to activation of Uga35/Dal81 through an unknown mechanism. This hypothesis immediately raises the question: Why would Uga35/Dal81 be involved also in GABA- and allophanate-induced transcription? Sharing of a transcription factor between different induction processes might allow the establishment of a hierarchy between them when the inducers of these pathways are simultaneously present in the medium. For instance, it is possible that upon activation by the Ssy1 pathway, Uga35/Dal81 might be recruited specifically to the promoters of genes induced by external amino acids, rather than to those responding to GABA or allophanate. In support of this view, addition of amino acids to cells growing on urea leads not only to induction of Ssy1-regulated genes but also to downregulation of the allophanate-inducible *DUR* genes involved in urea utilization (our unpublished data). Further experiments using cells growing on combinations of nitrogen compounds will be needed to test the validity of this model. Finally, three other properties of Uga35/Dal81 are worth a comment. First, whereas Stp1 is translocated into the nucleus in the presence of amino acids (Andreasson and Ljungdahl 2002), Uga35/Dal81 stays in the nucleus whether amino acids are present in the medium or not (our unpublished data). Second, experiments show 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 (Bricmont *et al.* 1991). A similar situation has been described for *TamA*, an *A. nidulans* protein highly

TABLE 8

Stp1 and Uga35/Dal81 are essential to UAS_{AA}-driven transcription

Strain	*UAS_{AA}-CYC -lacZ*: β-galactosidase activity (nmol·min^{-1}·mg prot^{-1})	
	−Phe	+Phe
*gap1*Δ	≤10	564
*gap1*Δ *stp1*Δ	≤10	≤10
*gap1*Δ *stp2*Δ	≤10	487
*gap1*Δ *stp1*Δ *stp2*Δ	≤10	≤10
*gap1*Δ *uga35/dal81*Δ	≤10	≤10
*gap1*Δ *uga35/dal81*Δ *stp1*Δ	≤10	≤10
*gap1*Δ *stp1*Δ *stp2*Δ *uga35/dal81*Δ	≤10	≤10

Strains 30629c (*gap1*Δ *ura3*), 37053a (*gap1*Δ *stp1*Δ *ura3*), 37054d (*gap1*Δ *stp2*Δ *ura3*), 34304b (*gap1*Δ *uga35/dal81*Δ *ura3*), 37092a (*gap1*Δ *stp1*Δ *stp2*Δ *ura3*), 38009a (*gap1*Δ *stp1*Δ *uga35/dal81*Δ *ura3*), and 38015c (*gap1*Δ *stp1*Δ *stp2*Δ *uga35/dal81*Δ *ura3*) transformed with the episomal plasmid pFA10 were grown on proline minimal medium with (+Phe) or without (−Phe) phenylalanine added at 5-mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were <15%.

similar to Uga35/Dal81 and involved in expression of several genes in response to various nitrogenous compounds, *e.g.*, GABA (Davis *et al.* 1996). It will thus be interesting to test the role of the $Zn(II)_2$-Cys_6 cluster domain of Uga35/Dal81 in induction of the *AGP1* gene by external amino acids. Third, among the many yeast transcription factors sharing a Gal4-type $Zn(II)_2$-Cys_6 cluster domain, Uga35/Dal81 is the factor whose cluster domain most closely resembles that of the Rgt1 transcription factor (D. Coornaert, personal communication) controlled by the external glucose signaling pathway of *S. cerevisiae* (Ozcan and Johnston 1999). In this pathway, the transporter-like glucose sensors Snf3 and Rgt2 respond to the presence of external glucose to modulate the function of Rgt1, and this signaling pathway also involves the SCF^{Grr1} ubiquitin-ligase complex (Li and Johnston 1997). Together with our data suggesting that Uga35/Dal81 could be a direct target of the Ssy1 pathway, these observations raise the possibility that the last steps of the external amino acid and glucose signaling pathways might share more structural similarities than thought thus far.

Our results show that Stp1 and Uga35/Dal81 synergistically activate *AGP1* transcription by acting through the same *cis* sequence, a UAS_{AA} element similar to those previously found upstream from the *BAP3* and *BAP2* genes (de Boer *et al.* 2000; Nielsen *et al.* 2001). Furthermore, induction by leucine of the *BAP2* gene is impaired in the *uga35/dal81* mutant (Bernard and André 2001a). It is thus likely that UAS_{AA}, Stp1 and/or Stp2, and Uga35/Dal81 are the key regulatory elements involved in transcriptional induction by external amino acids of all Ssy1-regulated genes. The UAS_{AA} of *AGP1* consists of two inverse copies of the 5′-CGGC-3′ sequence spaced by six nucleotides. Our mutagenesis experiments suggest that the situation created by the inverse orientations, spacing by six nucleotides, and flanking nucleotides of the 5′-CGGC-3′ doublet of the *AGP1* gene, is optimal for high-level induction by external amino acids. On the other hand, these experiments also showed that many mutations in this UAS_{AA} are permissible, since they strongly reduce but do not completely suppress the activating power of UAS_{AA}. Although two copies of the 5′-CGGC-3′ sequence appear crucial to UAS_{AA} function, these can be oriented as direct instead of inverse repeats and they can be spaced by more nucleotides. Furthermore, although the nucleotides between the 5′-CGGC-3′ repeats and those flanking them are also important for the function of the UAS_{AA}, their

TABLE 9

Transcription of *AGP1* is under tight nitrogen control but its UAS_{AA} is insensitive to this regulation

		β-Galactosidase activity (nmol·min^{-1}·mg prot^{-1})							
		AGP1-lacZ				*UAS_{AA}-CYC1-lacZ*			
		Urea		Ammonium		Urea		Ammonium	
	Strain	−Phe	+Phe	−Phe	+Phe	−Phe	+Phe	−Phe	+Phe
1	Wild type	≤10	1456	≤10	178	≤10	543	≤10	499
2	*gln3*Δ	≤10	265	≤10	≤10	≤10	498	≤10	567
3	*ure2*Δ	≤10	1360	≤10	764	≤10	456	≤10	554
4	*gzf3*Δ	≤10	1600	≤10	302	≤10	602	≤10	495
5	*ure2*Δ *gzf3*Δ	≤10	1885	≤10	1230	≤10	567	≤10	510

Strains 23344c (*ura3*), 30505b (*gln3*Δ *ura3*), 26854a (*ure2*Δ *ura3*), SBS10 (*gzf3*Δ *ura3*), and 50036c (*ure2*Δ *gzf3*Δ *ura3*) transformed with the CEN-based plasmid YCp*AGP1-lacZ* or YEp*UAS_{aa}-CYC1-lacZ* plasmid were grown on minimal urea or ammonium medium with (+Phe) or without (−Phe) the inducer phenylalanine at 5 mM final concentration. The reported β-galactosidase activities are means of two to three independent experiments. Variations were <15%.

replacement with other nucleotides leads to reduction rather than to complete loss of induction.

Finally, we showed that transcription dependent on UAS_{AA} alone is totally insensitive to the nitrogen status of the cell and to mutations in *GLN3, URE2*, or *GZF3/DEH1*. This suggests that the TOR signaling pathway mediating nitrogen-source control of transcription does not interfere with the external amino acid signaling pathway. Transcription driven by UAS_{AA} alone in response to amino acids (Table 5) thus likely reflects the actual external amino acid sensing capability of the cell. Nevertheless, in accord with previous conclusions (Regenberg *et al.* 1999), our results showed that *AGP1* transcription is under tight nitrogen control. This control is mediated by the GATA factor Gln3 that enhances ~10-fold the level of *AGP1* transcriptional induction when cells grow under limiting nitrogen supply conditions. Under these conditions, Gln3 is reported to be translocated into the nucleus, where it binds to 5′-GATA-3′ sequences to activate gene transcription (Beck and Hall 1999). Unlike other permease genes under Gln3 control (*GAP1*, *DAL5*, or *MEP2*), however, expression of *AGP1* absolutely requires the presence of an inducer, *i.e.*, external amino acids. Hence, Gln3 present in the nucleus is apparently unable to *trans*-activate *AGP1* expression without the prior action of Stp1 and Uga35/Dal81 through the UAS_{AA} element. These results suggest that Gln3 is somehow recruited for positive action by Stp1 and/or Uga35/Dal81. Interestingly, studies on *TamA* of *A. nidulans* show that this factor can interact with and recruit Area, a GATA-family transcription factor functionally related to Gln3 (Small *et al.* 1999). Direct interaction between Uga35/Dal81 and Gln3 might thus account for the probable recruitment of Gln3 for a positive effect on *AGP1* transcription. Such a mechanism has also been proposed to account for the much higher induction by GABA of the *UGA4* gene under nitrogen derepression than under nitrogen repression (André *et al.* 1995; Talibi *et al.* 1995). When cells are grown on a good nitrogen source, Gln3 is sequestered in the cytoplasm in a manner dependent on the Ure2 factor (Beck and Hall 1999). This unavailability of Gln3 in the nucleus is likely the main cause of the much lower *AGP1* induction in ammonium medium (Table 9), this weak induction likely reflecting the actual activating power of Stp1 and Uga35/Dal81 via the UAS_{AA} element.

In conclusion, this study shows that transcriptional induction of the *AGP1* gene in response to external amino acids involves SPS-dependent activation of two transcription factors, Stp1 and Uga35/Dal81, acting synergistically via the UAS_{AA} element. Furthermore, the amplitude of this induction is modulated according to nitrogen availability by the Gln3 factor, with poor nitrogen-supply conditions leading to much higher induction. It will be interesting to compare the physiological conditions and molecular mechanisms mediating activation of the Stp1 and Uga35/Dal81 pathways and to extend this work to other amino acid permease genes under Ssy1 control.

We gratefully acknowledge the excellent technical assistance of Catherine Jauniaux. We thank S. Vissers and D. Coornaert for a critical reading of the manuscript. We also thank members of the laboratory and also Dominique Thomas for many fruitful discussions. This work was supported by grant FRSM 3.4597.00 F for Medical Scientific Research, Belgium, and by grant 98/03-223 of the Communauté Française de Belgique, Direction de la Recherche Scientifique.

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Communicating editor: A. P. Mitchell