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# Transcriptional and Posttranslational Regulation of the General Amino Acid Permease of *Saccharomyces cerevisiae*

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**The cellular level and activity of the general amino acid permease, the product of the *GAP1* gene of *Saccharomyces cerevisiae*, are regulated at the level of transcription by two systems, the products of *URE2*/*GLN3* and *NIL1* in response to the nitrogen sources of the growth medium and inactivation in response to the presence of glutamine or glutamate. Active permease is phosphorylated. The addition of glutamine causes rapid dephosphorylation and inactivation of the permease with the same kinetics, which is followed by slower disappearance of the protein. These results suggest that inactivation of the permease results from its dephosphorylation.**

The minimal medium which supports the most rapid growth of the yeast *Saccharomyces cerevisiae* is one in which glutamine, asparagine, or ammonia serves as the sole source of nitrogen. The presence of one of these compounds results in the repression of enzymes and permeases required for the utilization of other nitrogen sources that support growth at slower rates. An important element in this nitrogen regulation is control of the synthesis of the general amino acid permease, the product of the *GAP1* gene (21), which results in high levels of Gap1p activity during the initial stages of nitrogen starvation and during growth on a poor source of nitrogen and low levels of Gap1p activity during growth on glutamine or glutamate (8). Control of Gap1p activity appears to be very complex, involving multiple transcriptional and posttranscriptional systems which act in response to the nitrogen source. One reason that Gap1p activity is so tightly regulated is that the lack of Gap1p activity caused by growth on good nitrogen sources in turn results in secondary regulatory effects due to inducer exclusion, thus preventing induction of the catabolic enzymes needed to utilize poor nitrogen sources (26).

The general amino acid permease can transport all of the naturally occurring L-amino acids found in proteins and related compounds, such as ornithine and citrulline, and several D-amino acids and toxic amino acid analogs (44). Grenson and colleagues first observed Gap1p activity in yeast strains that lacked certain specific high-affinity amino acid permeases, such as specific arginine, lysine, or methionine permeases, and were resistant to the toxic effects of corresponding amino acid analogs when the nitrogen source was ammonia (16). These strains regained sensitivity to analogs through 10- to 100-fold increases in the rates of uptake of corresponding amino acids when the poor nitrogen source proline was used in place of ammonia, a condition which was exploited to select the first *gap1* mutants. *GAP1* has previously been cloned and sequenced; the deduced peptide sequence is homologous to that of other fungal amino acid permeases (21).

Gap1p permease is inactivated by the addition of a rich nitrogen source such as glutamine, glutamate, or ammonia to cells growing on a poor nitrogen source. A *per1* strain which lacked the ammonia inactivation response also had 60-fold-higher levels of Gap1p activity during growth on ammonia than those of the wild-type strain (8), suggesting that posttranscriptional controls can, in effect, negate transcriptional control.

Grenson and Hou have described several mutations, none of which appears to be *per1*, that affect ammonia-mediated permease inactivation (13–15). Their results suggested that the product of *NPR1* was required for Gap1p activity, even in the absence of ammonia, unless the ammonia inactivation system was defective, as in a *npi1* or *npi2* mutant. *NPR1* has previously been cloned, and its deduced amino acid sequence was shown to be homologous to those of protein kinases (42). It is not known whether the product of *NPR1* produces its effect directly by interacting with Gap1p or indirectly by counteracting the function of Npi1p and Npi2p.

There appears to be coordinate expression of a number of nitrogen-regulated genes which requires the product of *GLN3*, a transcriptional activator which contains a zinc finger that is homologous to those in GATA factors (33, 41) and to the fungal nitrogen regulators encoded by *areA* (23) and *nit-2* (11, 12). Normal nitrogen regulation requires the product of *URE2* to prevent transcriptional activation by Gln3p during growth on a repressing nitrogen source like glutamine. Both *GLN1* and *GDH2* are transcriptionally activated by the Ure2p/Gln3p system so that their expression is lowest during growth on glutamine and highest during growth on glutamate (6, 30, 31). Gap1p activity in glutamate-grown cells has been shown to be higher than that in glutamine-grown cells; this difference in activity did not exist in *gln3* cells, although the highest Gap1p activities occurred during growth on poor nitrogen sources such as proline in both *GLN3* and *gln3* strains (7).

Here we present evidence that Gln3p activates the transcription of *GAP1* during growth on glutamate, that Ure2p prevents the activation of *GAP1* transcription by Gln3p during growth on glutamine, and that an activator other than Gln3p is responsible for the activation of *GAP1* transcription during growth on ammonia or urea and in the absence of any source of nitrogen. We used epitope-tagged Gap1p to show that active Gap1p is phosphorylated and that its inactivation by triggered glutamine coincides with its dephosphorylation.

## MATERIALS AND METHODS

**Strains and media.** The *S. cerevisiae* strains used in this work are listed in Table 1. Minimal yeast medium was composed of Difco yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories, Detroit, Mich.)

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TABLE 1. Yeast strains and plasmids used in this work

| Strain or plasmid | Genotype | Source |
|---|---|---|
| Strains | | |
| JT113 | *MAT*α *ura3-52* | Thomas |
| BC14 | *MAT***a** *gap1 ade1* | Courchesne (7) |
| MS3 | *MAT*α *ura3-52 can1 gap1* | This study |
| MS138 | *MAT***a** *ura3-52 leu2-3,112 GAP1* | This study |
| MS143 | *MAT***a** *ura3-52 leu2-3,112 gap1::LEU2* | This study |
| P40-2c | *MAT***a** *ura3-52 leu2-3,112 ade2-102 gln3::LEU2* | Coschigano (6) |
| P40-3c | *mat*α *ade2-102 ura3-52 leu2-3,112 ure2Δ11::LEU2* | Coschigano (6) |
| P40-2a | *mat*α *his4-619 leu2-3,112 ure2Δ11::LEU2* | Coschigano (6) |
| Plasmids | | |
| Bluescript KS+ | | Stratagene |
| pMS1 | *GAP1* clone | This study |
| pMS16 | *Sal*I-*Bam*HI fragment from pMS1 ligated into *Sal*I-*Bam*HI-digested Bluescript KS+ | This study |
| pMS20 | *gap1::LEU2* disruption, *Bgl*II digest liberates a fragment from the vector for integration | This study |
| pMS29 | *GAP1-lacZ* translational fusion at amino acid 53 in vector pBL101 (*ARS CEN URA3*) | This study |
| pPL247 | *Sal*I-*Spe*I fragment of *GAP1* in pRS316 | Ljungdahl (25) |
| pPL257 | *GAP1::FLU1* in pRS316 | Ljungdahl (25) |

with 2% glucose as the carbon source and with the nitrogen source added to 0.1% (amino acids) or 0.2% (ammonium sulfate). Nutritional supplements were added at the concentrations specified by Sherman et al. (37). Yeast genetic techniques were performed according to the methods of Sherman et al. (37), and DNA transformation of *S. cerevisiae* was accomplished by the lithium acetate protocol (20).

**DNA manipulations and preparations.** DNA manipulations were performed as described by Maniatis et al. (27), and enzymes were used according to the recommendations of the supplier (New England Biolabs, Beverly, Mass.). Bacterial plasmids were prepared by the alkaline lysis method for minipreparations and large-scale preparations (1). CsCl ethidium bromide centrifugation of large-scale preparations was performed as described by Maniatis et al. (27). Plasmids were recovered from *S. cerevisiae* strains by a teeny prep method (39).

**Selections for resistance to canavanine.** Selection for *can1* mutants was performed by growing a yeast extract-peptone-dextrose culture of strain JT113 to saturation, washing it twice with sterile water, and plating ca. $10^7$ cells per minimal glucose-ammonia-uracil–50 μM L-canavanine plate. Selection for *gap1* mutants was performed with strain MS1 by following the protocol described above and plating on minimal glucose-proline-uracil–50 μM L-canavanine plates.

**Permease assays.** Permease assays were performed as described by Courchesne and Magasanik (8). Twenty-five-milliliter cultures were grown to 80 to 100 Klett units (green filter). Cells from a 5-ml aliquot were harvested by filtration through a 0.8-μm-pore-size, mixed cellulose ester filter (Millipore, Bedford, Mass.) and washed with distilled water, and cells were resuspended in 5 ml of prewarmed (30°C), aerated minimal medium with 2% glucose and no nitrogen source or other supplements. A 1-ml sample was filtered through a cellulose filter, washed with distilled water, and resuspended in 1 ml of water for a determination of protein content by the Lowry assay (9). Forty microliters of 100× $^{14}$C-amino acid was added; the culture was vortexed; and five samples (0.5 ml each) were taken at 20-s intervals, filtered through no. 30 glass fiber filters (Schleicher and Schuell, Keene, N.H.) in a filter manifold, and immediately rinsed with 0.1% unlabelled amino acid. Filters were dried under a heat lamp and counted in 5 ml of Scintiverse (Fisher Scientific, Fair Lawn, N.J.). Units of specific activity were calculated as nanomoles of amino acid accumulated per minute per milligram of protein. The specific activity of each amino acid used in uptake assays was 0.5 mCi/mmol, and the final concentrations employed in assays were 0.2 mM for $^{14}$C-proline (Put4p assays) and 0.1 mM for $^{14}$C-citrulline (Gap1p assays).

**Lowry protein assays.** Fifty-microliter samples of yeast cells were prepared for assays by adding 50 μl of water and 100 μl of 1 M NaOH and heating to 95°C for 10 min. After being cooled to room temperature, 1 ml of Lowry reagent (2 ml of 0.5% $CuSO_4$-$^5H_2O$, 2 ml of 2% Na tartrate, 96 ml of 2% $Na_2CO_3$) was added, mixed, and incubated for 10 min. Then 100 μl of Folin reagent (Sigma, St. Louis, Mo.) diluted 1:1 with water was added during vortexing. After 40 min, the optical density at 670 nm ($OD_{670}$) was read. The protein standard was bovine serum albumin fraction V (Boehringer Mannheim, Indianapolis, Ind.). All assays were done in triplicate.

**Glutamine synthetase assays.** Total glutamine synthetase activity was measured by a transferase assay as previously described (32).

**Construction of a *GAP1-lacZ* translational fusion.** A 1.8-kb *Dra*I fragment which contains the entire upstream region and the first 165 bases of the open reading frame of *GAP1* was subcloned into the *Dra*I-*Ssp*I sites of the Amp$^r$ gene of pBR322. One of the plasmids which recreated the *Dra*I site in the open reading frame was designated pMS24. It was cut with *Dra*I and *Bam*HI 12-mer linkers (New England Biolabs) and ligated to the blunt ends, and then it was digested with *Bam*HI and religated; one of the resulting plasmids was chosen to be pMS25. A 1.7-kb *Asp*718-*Bam*HI fragment was liberated from pMS25 and cloned into the *Asp*718-*Bam*HI backbone of pSLFΔ178K (10), which resulted in pMS26, a 2μm *URA3 GAP1-lacZ* fusion plasmid. To create an *ARS-CEN URA3* version of this plasmid, pMS26 was digested with *Asp*718, filled in with Klenow fragment, and then digested with *Bam*HI and the 1.7-kb *Bam*HI-*Asp*718 fragment was subcloned into the *Sma*I-*Bam*HI backbone of pBL101 (24).

**TN10LUK insertional mutagenesis.** TN10LUK insertions were obtained in the Ycp50 shuttle vector clone of *GAP1* known as pMS1 by the method of Huisman et al. (19). Approximately 5,000 kanamycin-resistant colonies were pooled, plasmid DNA was purified from the pool, lambda-resistant *Escherichia coli* cells were transformed with plasmid DNA, and transformants were selected for ampicillin and kanamycin resistance. The resulting transformants were restriction mapped to determine the site of integration and the orientation of TN10LUK.

**Construction of a *GAP1* null allele.** Plasmid pMS16 was digested with *Bam*HI and *Eco*RV, and this fragment was replaced with a *Bgl*II linker, resulting in plasmid pMS18. Plasmid pMS18 was digested with *Eco*RI, which removes an 840-bp fragment that includes the first 210 bp of the promoter and roughly one-third of the open reading frame, and the *Eco*RI fragment was replaced with a *Bam*HI linker, resulting in plasmid pMS19. Disruption plasmid pMS20 was constructed by digesting pMS19 with *Bam*HI and inserting a 3.0-kb *Bgl*II fragment with the *LEU2* gene from Yep13. A *gap1::LEU2* disruption strain was created by transforming a 4.5-kb *Bgl*II fragment from pMS20 into strain MS138, selecting for *LEU2*, and testing for Gap1p activity. A *gap1 LEU2* clone was designated MS143.

**β-Galactosidase assays.** Fresh transformants were patched to minimal ammonia plates supplemented with 0.1% Casamino Acids (Difco Laboratories) and adenine for 1 or 2 days of growth, and these patches were used to inoculate 5-ml minimal cultures with various nitrogen sources for overnight growth. One milliliter of culture was harvested by centrifugation at an $OD_{600}$ of 0.8 to 1.1, washed once with assay buffer, and assayed by the method of Miller (28). Units were calculated as described by Miller (28) and normalized to $OD_{600}$. At least two independent transformants were assayed for each determination.

**Preparation of RNA and Northern (RNA) analysis.** Cultures were grown in minimal glucose medium to 80 to 100 Klett units (green filter). All cultures were supplemented with adenine, histidine, and uracil. Gap1p permease assays were performed on the same cultures. Total yeast RNA was isolated by the method described by Carlson and Botstein (3), and the yield was determined by $A_{260}$. Ten micrograms of total RNA per lane of a formaldehyde agarose gel was loaded and blotted, and Northern hybridization analysis was carried out as previously described (4). Probes for hybridization were produced by nick translation according to the directions of the supplier (Boehringer Mannheim). The *GAP1* probe used was a 1.8-kb *Bgl*II-*Xho*I fragment, and the *TUB1* probe was a 0.9-kb *Sal*I-*Asp*718 fragment.

**Western (immunoblot) hybridizations and immunoprecipitations.** The antibody used was the mouse monoclonal antibody 12CA5 raised against the HA1 epitope of the influenza virus hemagglutinin protein and was purchased from Babco (Berkeley, Calif.). Crude protein extracts were prepared from 3 ml of yeast culture at an $OD_{600}$ of 1.0 by the alkaline lysis (100 μl of 1.85 M NaOH–7% β-mercaptoethanol for 10 min on ice) method of Silve et al. (38). The crude lysate was trichloroacetic acid precipitated (100 μl of 50% trichloroacetic acid for 10 min on ice), rinsed with 0.5 ml of 1 M Tris base, and resuspended in 100 μl of 2× sample buffer (4% sodium dodecyl sulfate [SDS], 0.1 M Tris-HCl [pH 6.8], 4 mM EDTA, 20% glycerol, 2% β-mercaptoethanol, 0.02% bromophenol blue) (38). Ten microliters of protein extract in sample buffer per lane of a 7.5% acrylamide gel was loaded, blotted to a polyvinylidene difluoride membrane

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TABLE 2. Permease phenotypes of *gap1* mutants[a]

| Strain | Genotype | Nitrogen source | Gap1p units | Put4p units |
|---|---|---|---|---|
| JT113 | Wild type | Proline | 50 | 26 |
| BC14 | *gap1* | Proline | <0.5 | 124 |
| MS3 | *can1 gap1* | Proline | <0.5 | 28 |
| MS3 | *can1 gap1* | Urea | <0.5 | 29 |
| BC14/MS3 | *gap1/can1 gap1* | Proline | <0.5 | 142 |
| MS3/pMS1 | *can1 gap1*/2μm *GAP1* | Nitrogen starvation[b] | 175 | ND[c] |
| MS143/pSLFΔ178K | *gap1*::*LEU2*/2 μm | Urea | <0.5 | ND |
| MS143/pMS1 | *gap1*::*LEU2*/2 μm *GAP1* | Urea | 112 | ND |
| MS143/pPL247 | *gap1*::*LEU2*/2 μm *GAP1* | Urea | 107 | ND |

[a] Gap1p permease units of activity were computed as nanomoles of $^{14}$C-citrulline transported per minute per milligram of total protein as described in Materials and Methods. Put4p permease units of activity were computed as nanomoles of $^{14}$C-proline transported per minute per milligram of total protein. Values reported are the averages of determinations from two cultures.

[b] This culture was grown on urea to mid-log phase and then shifted to minimal glucose medium without a nitrogen source for 1 h.

[c] ND, not done.

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(Immobilon-P; Millipore), and hybridized as previously described by Haldi and Guarente (17). The secondary antibody employed was an affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G antibody from Cappel Laboratories (Durham, N.C.). Blots were developed with the chromogenic substrates 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitroblue tetrazolium according to the directions of the supplier (Bio-Rad, Richmond, Calif.) or with $^{125}$I-labelled protein A (NEN, Boston, Mass.) as outlined by Harlow and Lane (18).

Pulse labelling with [$^{35}$S]methionine was performed by the method of Silve et al. (38). Cells were grown in low-sulfate minimal medium (22) to an $OD_{600}$ of 1.0, and then 10 ml of culture was centrifuged and resuspended in fresh medium to an $OD_{600}$ of 10. [$^{35}$S]methionine was added to 200 μCi per ml, labelling was allowed to proceed for 3 min at 30°C, and then cells were pelleted by centrifugation and broken by alkaline lysis.

In vivo labelling with $^{32}P_i$ was done in low-phosphate minimal medium according to the protocol of Kolodziej and Young (22) on cells grown in 100 μM phosphate minimal medium. Cultures were grown to an $OD_{600}$ of 0.5, $^{32}P_i$ was added to 400 μCi/ml, cultures were incubated for another 6 h to reach an approximate $OD_{600}$ of 1.0, and then a 3-ml sample was processed by alkaline lysis.

Immunoprecipitations were performed as described by Silve et al. (38). After alkaline lysis, trichloroacetic acid precipitation, and a rinse with Tris base, the pellet was resuspended in 100 μl of SDS sample buffer and heated for 10 min at 37°C. The resuspension was diluted to 1 ml in immunoprecipitation buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors leupeptin [1 μg/ml], pepstatin [5 μg/ml], and aprotinin [5 μg/ml]) and was centrifuged for 20 min at 12,000 × *g* at 4°C. Phosphorylation samples were resuspended in immunoprecipitation buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 μg of leupeptin per ml, 5 μg of pepstatin per ml, 5 μg of aprotinin per ml, and phosphatase inhibitors sodium phosphate [50 mM] [pH 7.2], NaF [1 mM], and $Na_3VO_4$ [0.4 mM]). The supernatant was diluted to 3 ml with immunoprecipitation buffer, samples were counted, the counts per minute per sample were normalized before proceeding with immunoprecipitation, 15 μl of anti-hemagglutinin antibody 12CA5 was added, and the mixture was incubated overnight at 4°C. The immune complexes were adsorbed to 100 μl of protein A-Sepharose beads (Sigma) for 1 h at 4°C, the beads were centrifuged and washed with immunoprecipitation buffer three times, and proteins were eluted from the beads by the addition of 60 μl of sample buffer. Ten microliters of sample per lane of a 7.5% acrylamide gel was loaded; after electrophoresis, the gel was fixed and dried; the Gap1::Flu1p bands were visualized and counted on a phosphor screen by using version 3.1 of the ImageQuant program (Molecular Dynamics, Sunnyvale, Calif.).

## RESULTS

**Cloning of the *GAP1* gene.** The strategy used to clone the *GAP1* gene was to select a yeast strain which grew very poorly on a low concentration of an amino acid as the sole nitrogen source, to transform this strain with a yeast genomic library, and then to screen for transformants which grew faster on a low concentration of the same amino acid. Since most amino acids are transported in *S. cerevisiae* by a high-affinity, low-capacity specific permease and the low-affinity, high-capacity general amino acid permease, it was necessary to create a mutant strain which lacked both uptake systems (5). A double mutant was isolated in two steps by selection for resistance to L-canavanine, a toxic arginine analog which is transported into the cell by the arginine permease Can1p and by the general amino acid permease Gap1p (16). Spontaneous *can1* mutants in strain JT113 were selected by plating on minimal ammonia-canavanine medium, and then *can1 gap1* mutants were isolated in a second round of selection for canavanine resistance on minimal proline-canavanine medium. Strain MS3 (*can1 gap1*) had very low levels of Gap1p activity ($^{14}$C-citrulline uptake) and normal levels of the proline-specific permease Put4p ($^{14}$C-proline uptake) during growth on proline compared with those of JT113, which suggested that the mutant strain did not have a pleiotropic defect that prevented all amino acid permease activity (Table 2). MS3 did not complement a known *gap1* mutant, BC14, for Gap1p activity (Table 2).

The *ura3 can1 gap1* strain MS3 was transformed with either a low-copy-number or a high-copy-number *URA3* genomic plasmid library. Five clones were found by simultaneously selecting for *URA3* and screening for large, faster-growing colonies on 0.01% arginine. These clones were tested for sensitivity to canavanine and the ability to transport labelled arginine or citrulline during growth on proline. All five clones had regained sensitivity to canavanine during growth on minimal proline plates. Two clones restored only arginine uptake; they were not studied further because they were considered likely to be *CAN1* clones. The three remaining clones restored the uptake of both arginine and citrulline and had overlapping restriction digest patterns. Two were from a centromere library, and the other one was from a 2μm-based library. The phenotypes of canavanine sensitivity and arginine and/or citrulline transport were lost when strains were cured of the plasmid. The *Sal*I-*Bam*HI region of the putative *GAP1* clone was subcloned in the vector Yip5 to create pMS5. Strain MS3 was transformed with *Bgl*II-cut pMS5 to integrate the clone into its corresponding chromosomal locus. This strain was crossed to MS138 (*can1 GAP1*) and sporulated. Thirty tetrads were dissected; none gave rise to a canavanine-resistant *gap1* spore which could grow on proline-canavanine plates. Spores from six of these tetrads were also assayed for Gap1p activity and found to be *GAP1*. Therefore, the clones were *GAP1* and not suppressors of *gap1*.

The restriction map of the *GAP1* region is shown in Fig. 1. The open reading frame of *GAP1* was identified by subcloning and insertional mutagenesis with the TN10LUK system (Fig. 1). The gene was partially sequenced; all sequences matched the *GAP1* sequence published by Jauniaux and Grenson (21).

***GAP1* is transcriptionally controlled by two systems.** Physiological studies of Gap1p activity had shown that during

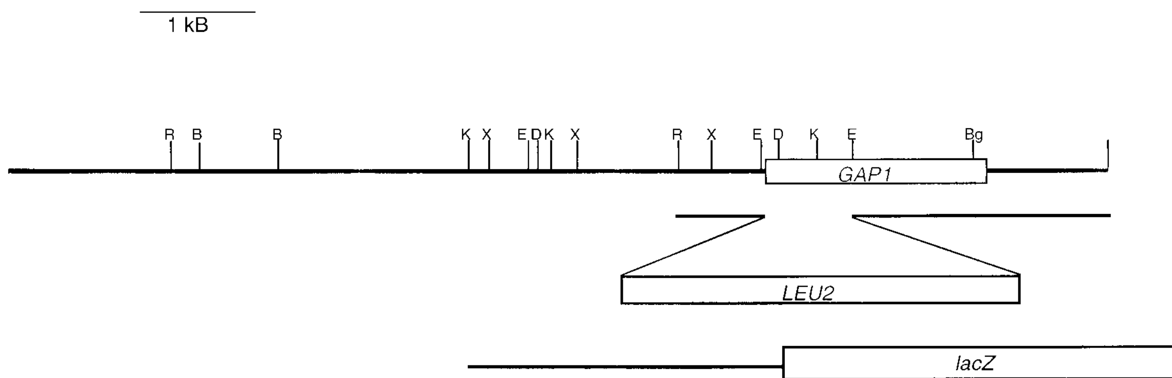


FIG. 1. Restriction map of the *GAP1* clone in pMS2 and schematics of a *gap1*::*LEU2* deletion construct and a *GAP1-lacZ* translational fusion. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; D, *Dra*I; E, *Eco*RI; K, *Kpn*I; R, *Eco*RV; X, *Xho*I.

growth on glutamate, there was a small amount of Gap1p activity in a wild-type strain and virtually no activity in a *gln3* strain, whereas Gap1p activity was very high in either strain grown on proline (8). In the wild-type strain, about 25-fold more Gap1p activity was present in proline-grown cells than in glutamate-grown cells. This suggested that independent systems were responsible for the production of Gap1p activity, in which one system (Ure2p/Gln3p) was active during growth on glutamate and a second system was active during growth on proline (7). Similar results were obtained when urea, rather than proline, served as the source of nitrogen.

Transcription of the *GAP1* gene was measured by assaying β-galactosidase activities in various strains that carried plasmid pMS29, an *ars-cen URA3* vector with the *GAP1* promoter and the first 53 amino acids of *GAP1* fused in frame to *lacZ*. In wild-type JT113, β-galactosidase activity was high during growth on glutamate or urea as the nitrogen source (Table 3). Growth on glutamine produced little β-galactosidase activity.

The *gln3* mutant P40-2c/pMS29 produced about 15- to 20-fold-less β-galactosidase activity than did the wild-type strain during growth on glutamate and about 2-fold less during growth on urea (Table 3). It appears that Gln3p is the primary transcriptional activator of *GAP1* during growth on glutamate and that Gln3p contributes to the activation of *GAP1* transcription during growth on urea in a wild-type strain, while an independent activator is responsible for about one-half of *GAP1* transcription in a wild-type strain grown on urea and for all *GAP1* transcription in a *gln3* strain grown on urea. We have named this independent activator Nil1p and have evidence that the product of the *NIL1* gene has a domain which is homologous to the DNA binding domains of GATA factors, including that of Gln3p (38a).

It has been established that Ure2p is a negative regulator of Gln3p (6, 9). Genes whose transcription is activated by Gln3p, such as *GLN1* and *GDH2*, are transcribed at high rates during growth on all nitrogen sources in *ure2 GLN3* strains. Very high β-galactosidase activities were produced on all nitrogen sources in the *ure2* deletion strain P40-3c with the *GAP1-lacZ* translational fusion plasmid pMS29 (Table 3). This confirms the role of Ure2p/Gln3p in *GAP1* transcription.

Northern blots of *GAP1* mRNA confirmed the results obtained with the *GAP1-lacZ* fusion (Fig. 2). There was strong expression of *GAP1* during growth on glutamate in the wild-type strain but only weak expression in the *gln3* strain. Expression was high in all strains grown on urea and was also high on all nitrogen sources in a *ure2* strain. There was poor expression in both glutamine-grown and glutamate-plus-ammonia-grown wild-type cells, which was expected since growth on glutamate plus ammonia is equivalent to growth on glutamine in wild-type strains (29). In contrast, this wild-type strain produced large amounts of *GAP1* transcript during growth on ammonia or glutamate alone. It is apparent that the mechanism which controls the transcriptional activation of nitrogen-regulated genes like *GAP1* does so by monitoring the intracellular concentration of a nitrogen metabolite such as glutamine. Transcription of the *GAP1* gene is tightly regulated in response to the nitrogen source by two independent systems, Ure2p/Gln3p and Nil1p. The nitrogen conditions which trigger maximal activation by these systems are different: growth on glutamate results in the activation of *GAP1* transcription by Gln3p,

TABLE 3. *GAP1* expression and Gap1p activity

| Strain | Nitrogen source | β-Galactosidase units | Gap1p activity (nmol/min/mg) |
|---|---|---|---|
| JT113/pMS29 | Glutamine | 7–20 | <0.5 |
| (wild type) | Glutamate | 299–410 | 1.2–1.7 |
| | Urea | 400–623 | 121–134 |
| P40-2c/pMS29 | Glutamine | 3–5 | <0.5 |
| (*gln3*::*LEU2*) | Glutamate | 19 | <0.5 |
| | Urea | 120–160 | 67–71 |
| P40-3c/pMS29 | Glutamine | 406–540 | <0.5 |
| (*ure2*::*LEU2*) | Glutamate | 520–530 | 1.3–3.6 |
| | Urea | 490 | 33–109 |

1 2 3 4 5 6 7 8 9 10 11

*ure2::LEU2* *gln3::LEU2* *wt*

gln glt urea gln glt urea gln glt+NH3 NH3 glt urea

0.7 0.6 29 0.5 0.8 56 0.3 0.1 37 3 150

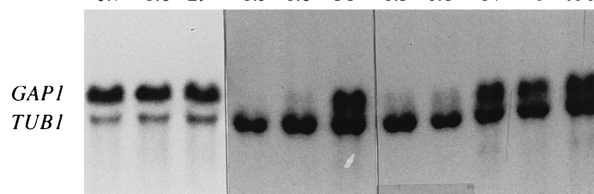


FIG. 2. A comparison of *GAP1* mRNA levels with Gap1p activity reveals that permease activity is determined posttranscriptionally. Minimal glucose cultures were grown with the indicated nitrogen source: glutamine (gln), glutamate (glt), ammonia ($NH_3$), or urea. The Northern blot was simultaneously probed with a *GAP1* probe and a *TUB1* probe for standardization. Gap1p activity (nanomoles of $^{14}$C-citrulline transported per minute per milligram of total protein) was determined for each culture and is indicated above each lane. wt, wild type.

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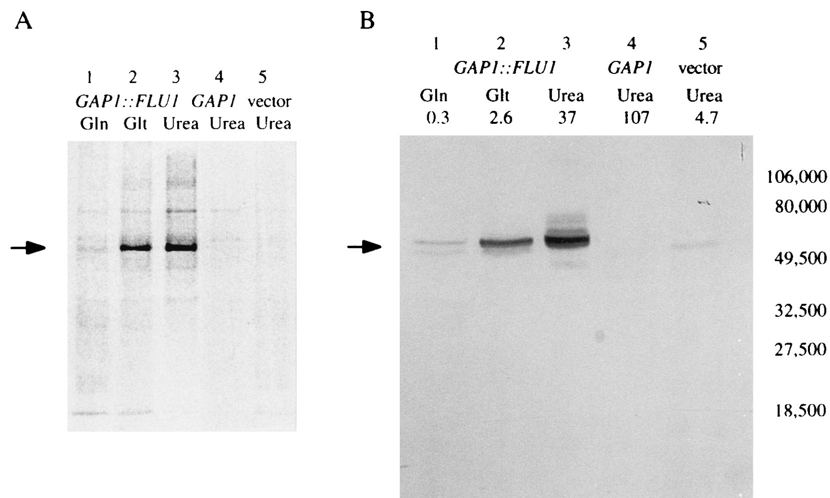


FIG. 3. Comparison of Gap1p activities, *GAP1* translation, and Gap1p levels during growth on various nitrogen sources. Experiments were performed in tandem on cells from the same cultures. Strain MS143 (*gap1*::*LEU2*) was transformed with either pPL257 (*GAP1*::*FLU1*), pPL247 (*GAP1*), or pRS316 (control vector). Lanes: 1, *GAP1*::*FLU1*, glutamine; 2, *GAP1*::*FLU1*, glutamate; 3, *GAP1*::*FLU1*, urea; 4, *GAP1*, urea; 5, control, urea. (A) Immunoprecipitations of lysates from [$^{35}$S]methionine-pulse-labelled cells. The relative amounts of labelled Gap1::Flu1p in the following lanes were determined by phosphor imager scanning of the area indicated by the arrow: 1, 26,000 cpm; 2, 141,000 cpm; 3, 226,000 cpm; 4, 12,000 cpm; 5, 11,000 cpm. (B) Immunoblot comparing the amounts of total Gap1::Flu1p protein produced by growth on different nitrogen sources. The blot was developed with mouse monoclonal 12CA5, goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase, and then the chromogenic alkaline phosphatase substrates 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitroblue tetrazolium. Gap1p activity for each culture is presented above the corresponding lane.

whereas growth on urea, proline, or ammonia results in the activation of *GAP1* transcription by Nil1p. However, while a high level of *GAP1* transcription is required for a high level of Gap1p activity, there are posttranscriptional controls which can limit Gap1p activity even when *GAP1* is highly transcribed.

**The prevailing control of permease activity is posttranscriptional.** Gap1p permease assays were performed in parallel with mRNA isolation from cultures grown on various nitrogen sources (Fig. 2). A high level of message resulted in a high level of permease activity only when the nitrogen source was urea or ammonia. This was especially apparent in glutamate-grown *GLN3* cells or in *ure2* cells grown on glutamine or glutamate. In each of these cases, there were very high levels of mRNA but quite low permease activities. The degree of posttranscriptional regulation of permease activity in glutamate-grown cultures was about 50-fold compared with that of urea-grown cultures of the same strain.

The same results were obtained when β-galactosidase assays and permease assays were performed in parallel (Table 3). Glutamate-grown wild-type cells had 70-fold-less Gap1p activity than urea-grown cells, whereas both cultures produced comparable amounts of β-galactosidase activity. The *ure2* strain had high levels of β-galactosidase activity on all nitrogen sources, yet the Gap1p activity was low when the nitrogen source was glutamine or glutamate.

Thus, growth on glutamate as the nitrogen source results in regulation of the general amino acid permease characterized by a high rate of *GAP1* transcription and low Gap1p activity. This allows the cell to exclude the high-capacity uptake of poor nitrogen sources and to quickly adapt to deteriorating nitrogen conditions by a rapid increase in Gap1p activity.

In wild-type cells of the Belgian strain background (Σ1278b), Gap1p function was rapidly inactivated when glutamine, glutamate, or ammonia was added to the medium (8). A mutant which retained the glutamine or glutamate inactivation response but lacked the ammonia inactivation response as a result of a mutation in the *PER1* gene was identified in that study. The *per1* strain produced a large amount of Gap1p activity during steady-state growth on ammonia, whereas the wild type produced very little. It is likely that the wild-type strain employed in this work, JT113, is also *per1* because it produces a high level of Gap1p activity during growth on ammonia (Fig. 2). This is a common phenotype for strains related to X2180-1A, the wild-type strain of the Yeast Genetic Stock Center (34).

It seemed likely that inactivation was solely responsible for the posttranscriptional regulation of the general amino acid permease, but in the absence of a strain that completely lacked the inactivation response, there was no direct way to test this hypothesis. In order to ascertain whether there were additional levels of posttranscriptional control, the relative rates of Gap1p synthesis and the levels of Gap1p protein were determined during growth on various nitrogen sources with the *GAP1*::*FLU1* construct, which encodes an epitope-tagged product (generously provided by Per Ljungdahl).

**Epitope-tagged Gap1::Flu1p is functional.** An oligonucleotide encoding a 9-amino-acid epitope of the influenza virus hemagglutinin protein HA1 was inserted in the *GAP1* sequence corresponding to the N-terminal hydrophilic domain between amino acids 62 and 63 (*GAP1*::*FLU1*) (25). This plasmid complemented the *gap1*::*LEU2* disruption strain MS143 for growth on minimal citrulline, washed agar plates. It was possible to see citrulline growth phenotypes for *GAP1* strains only on washed agar media. The Gap1p activity produced by N-terminal-tagged *GAP1*::*FLU1* was about 40% of that by untagged *GAP1* in the same *ars-CEN* plasmid vector (Fig. 3).

It was possible to visualize Gap1::Flu1p in Western blots of crude cell extracts made by quick alkaline lysis with a monoclonal antibody raised to the HA epitope (12CA5). Similarly, Gap1::Flu1p could be immunoprecipitated from crude alkaline extracts with this antibody.

**Posttranscriptional control of Gap1p activity is not translational.** It was not clear whether inactivation was solely responsible for the disparity between Gap1p activity and *GAP1* transcrip-

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tion during steady-state growth on a rich nitrogen source like glutamate. In order to determine whether all posttranscriptional control of Gap1p occurs at the level of inactivation, we compared the rates of Gap1::Flu1p synthesis and the steady-state levels of Gap1::Flu1p protein during growth on various nitrogen sources.

The relative rates of translation of *GAP1* mRNA during growth on glutamate and urea were established by pulse-labelling cells with [$^{35}$S]methionine for 3 min and immunoprecipitating Gap1::Flu1p from their crude alkaline lysates. Very little background was observed in control immunoprecipitations from cells without the *GAP1*::*FLU1* plasmid (Fig. 3A). Somewhat smaller amounts of Gap1::Flu1p were immunoprecipitated from glutamate-grown cells than from urea-grown cells that were pulse-labelled with [$^{35}$S]methionine. The approximately 2-fold difference in the amount of labelled Gap1::Flu1p in this experiment cannot account for the 14-fold difference in Gap1::Flu1p activity between glutamate-grown cells and urea-grown cells (Fig. 3B). In repeat trials, glutamate-grown cells produced as much (or more) labelled Gap1::Flu1p as urea-grown cells (data not shown). Therefore, it seems unlikely that Gap1p is regulated at the translational level.

The relative amounts of total Gap1::Flu1p protein in glutamate- and urea-grown cells were assessed to test the hypothesis that Gap1p activity was correlated with the total amount of Gap1p protein. Steady-state levels of Gap1::Flu1p protein were determined by Western blotting and compared with Gap1p activities in the same cultures (Fig. 3B). There was approximately 2- to 3-fold less Gap1::Flu1p protein in glutamate-grown cells than in urea-grown cells, whereas activity levels were about 14-fold different. This result indicates that both inactive and active forms of Gap1::Flu1p must be present in these cells.

A comparison of the data gathered for glutamate- and urea-grown cells shows that the largest difference between them was the amount of Gap1p activity and that this difference in activity was too large to be accounted for by the small differences in the transcription of *GAP1*, synthesis of Gap1::Flu1p, or the total amount of Gap1::Flu1p protein. There are two independent layers of control of Gap1p, transcriptional regulation and posttranslational regulation of activity. Posttranslational regulation of Gap1p activity can obviate a high degree of transcriptional activation of *GAP1* during growth on nitrogen sources which trigger permease inactivation, such as glutamate. The fact that most Gap1::Flu1p protein in glutamate-grown cells was inactive ruled out degradation as the immediate mechanism of inactivation, although it is possible that a faster rate of degradation is a consequence of inactivation. It did suggest that active Gap1p and inactive Gap1p were different as a result of either covalent modification or association with another factor. The presence of a secondary band just above the Gap1::Flu1p band in immunoprecipitates of lysates from labelled cells which carried Gap1::Flu1p and its absence in lysates from cells without this plasmid supported the possibility of covalent modification of Gap1p (Fig. 3A). In addition, multiple bands were seen just above Gap1::Flu1p in a Western blot of lysates from urea-grown cells that expressed *GAP1*::*FLU1*, but these bands were not seen in lysates from cells grown on glutamine or glutamate or in cells without the *GAP1*::*FLU1* plasmid (Fig. 3B). Since these secondary bands represented a small fraction of the total Gap1::Flu1p in urea-grown cells, they may be visualizable by immunoblot only in lysates which have a large amount of Gap1::Flu1p.

**Gap1p is phosphorylated.** Grenson and colleagues isolated a number of yeast mutants which lacked Gap1p activity but which were *GAP1*. One of these, *npr1*, was cloned, and the deduced protein sequence was found to be very homologous to those of protein kinases (42, 43). Phosphorylation thus seemed to be a candidate for covalent modification of Gap1p which could control Gap1p activity.

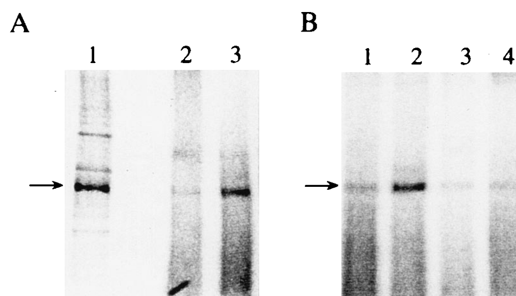


FIG. 4. Phosphor images of SDS-polyacrylamide gels of products immunoprecipitated with antibody 12CA5 from lysates of MS143/pPL257 (*GAP1*::*FLU1*) labelled with [$^{35}$S]methionine in low-sulfate minimal medium (lane A1) or with $^{32}$P$_i$ in low-phosphate minimal medium (lanes A2, A3, and B1 to B4) and grown with the following nitrogen source(s): urea (A1, A3, and B2); glutamate (A2 and B1); glutamate to mid-log phase, with glutamine added 45 min before harvest (B3); urea to mid-log phase, with glutamine added 45 min before harvest (B4). Arrows point to Gap1::Flu1p.

The phosphorylation state of Gap1::Flu1p was investigated by immunoprecipitation of lysates from $^{32}$P$_i$-labelled cells. MS143/pPL257 (*GAP1*::*FLU1*) was labelled by the addition of $^{32}$P$_i$ to low-phosphate minimal medium for 2 to 6 h of growth in mid-log phase. Extracts were prepared by alkaline lysis, Gap1::Flu1p was immunoprecipitated, proteins were resolved by SDS-polyacrylamide gel electrophoresis, and Gap1::Flu1p was quantitated on a phosphor imager. As can be seen in Fig. 4A, the major protein immunoprecipitated from $^{32}$P$_i$-labelled cells was the same size as Gap1::Flu1p immunoprecipitated from [$^{35}$S]methionine-pulse-labelled cells. Glutamate-grown cells had 10-fold-less phosphorylated Gap1::Flu1p than urea-grown cells. In another experiment, there was ninefold-more phosphorylated Gap1::Flu1p in immunoprecipitations of crude lysates from urea-grown cells than from glutamate-grown cells (Fig. 4B). There was 15-fold-more permease activity in urea-grown cells than in glutamate-grown cells. The addition of glutamine to these phosphorylated cultures for 45 min, which causes a rapid loss of Gap1p activity (8), also resulted in a drop in the amount of phosphorylated Gap1p in both cultures (Fig. 4B). There was only a small decrease in the amount of phosphorylated Gap1p in the glutamate-plus-glutamine culture (ca. 2-fold) but over a 12-fold drop in the urea-plus-glutamine culture. This suggested that the phosphorylation state of Gap1p may determine permease activity.

In order to correlate the kinetics of permease inactivation and Gap1p dephosphorylation, we measured the effects of the addition of glutamine to parallel subcultures of MS143/pPL257 grown in urea low-phosphate medium. The result of the experiment showed a strong correlation between the rates of dephosphorylation and inactivation of Gap1::Flu1p (Fig. 5).

The inactivation of Gap1::Flu1p permease activity and the loss of phosphorylated Gap1::Flu1p could have resulted from a rapid turnover of the protein. This possibility was tested by monitoring both Gap1::Flu1p permease activity and the level of Gap1::Flu1p protein after the addition of glutamine to a urea-grown culture of MS143/pPL257. As before, the loss of permease activity was quite rapid (Fig. 6). While there was a decrease in the level of Gap1::Flu1p protein, the rate of protein loss was considerably slower than the rate of permease inactivation. In the control subculture, which did not receive

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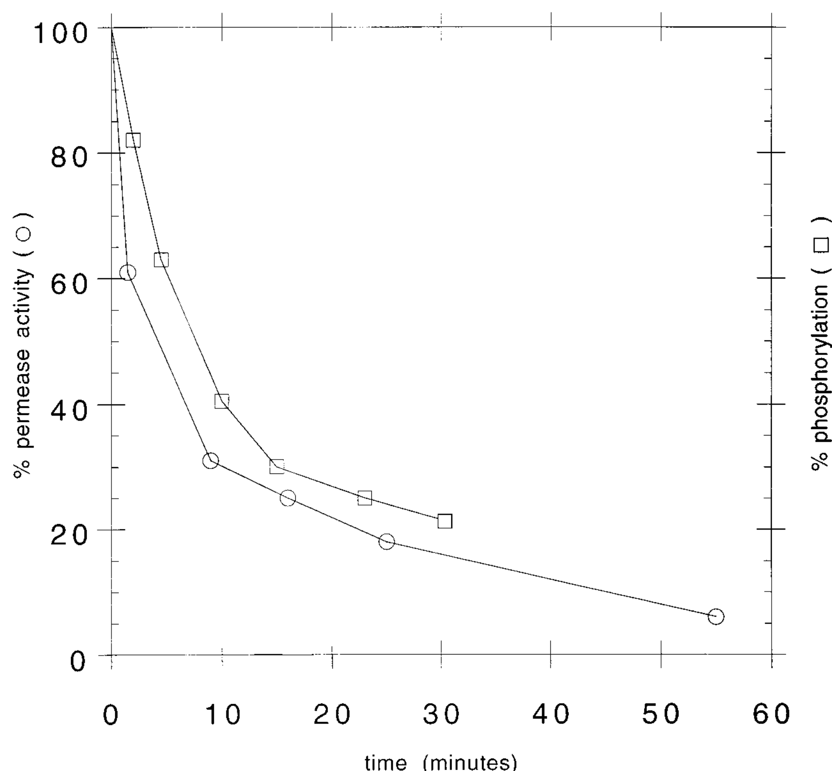


FIG. 5. Dephosphorylation of Gap1::Flu1p in response to the addition of glutamine is correlated with the inactivation of Gap1::Flu1p. The amounts of Gap1::Flu1p activity and phosphorylated Gap1::Flu1p were measured in parallel low-phosphate urea subcultures of MS143/pPL257 (*GAP1*::*FLU1*) after the addition of glutamine. Data are presented as percentages of initial activity or phosphorylation.

glutamine, permease activity and the level of Gap1::Flu1p protein declined slightly over the course of the experiment.

## DISCUSSION

Two separate systems regulate the transcription of the *GAP1* gene in response to nitrogen growth conditions. This has been demonstrated by assays of β-galactosidase activity of *GAP1-lacZ* fusions and by Northern analysis. The two transcriptional systems, Ure2p/Gln3p and Nil1p, had overlapping but different responses to growth on glutamate, urea, or proline as the nitrogen source but were both inactive during growth on glutamine. The activation of a *GAP1-lacZ* fusion during growth on glutamate was weaker in a *gln3* strain than that in the wild type, as was the synthesis of *GAP1* mRNA. It appears that Gln3p is the major activator of *GAP1* during growth on glutamate and that Nil1p is the major activator during growth on urea or proline in a *gln3* strain. More recent results with a *nil1* strain indicate that Gln3p as well as Nil1p activates transcription of *GAP1* during growth on urea or proline (unpublished data).

Northern analyses coupled with Gap1p activity assays revealed large differences between the level of transcription and the amount of uptake activity with glutamate as the nitrogen source, whereas when the nitrogen source was urea, proline, or ammonia, the levels of *GAP1* mRNA and Gap1p activity were correlated. Since urea- and glutamate-grown cells produced the same amounts of *GAP1* mRNA and β-galactosidase activity from a *GAP1-lacZ* translational fusion but there was 15-fold-more Gap1p activity in urea-grown cells than in glutamate-grown cells, the degree of posttranscriptional regulation of Gap1p activity was 15-fold. Experiments with an epitope-tagged version of Gap1p indicated that the principal mechanism of posttranscriptional regulation of the general amino acid permease was inactivation of functional permease. Permease inactivation may have secondary effects, such as a faster rate of degradation of inactive permease, which may explain why there was two- to threefold less Gap1p protein in Western blots of lysates from glutamate-grown cultures than from urea-grown cultures. It is clear that the mechanism of inactivation was not the destruction of the permease, since the rate of inactivation was far more rapid than the rate of degradation of the tagged permease.

In order to demonstrate the relationship of dephosphorylation to inactivation, experiments were performed to determine the amounts of Gap1p activity and phosphorylated Gap1p during glutamine inactivation. The kinetics of dephosphorylation and permease inactivation were parallel; therefore, we conclude that there is a strong correlation between the mechanisms of dephosphorylation and inactivation. Permease inactivation and dephosphorylation were very rapid (about 50% of permease activity and phosphorylation was lost in the first 5 min of inactivation), but after the initial drop-off, the rates of inactivation and dephosphorylation slowed considerably. The biphasic shape of these curves could be interpreted as evidence of two processes that result in lower Gap1p activity and phosphorylation. One process is obviously dephosphorylation, which is correlated with the inactivation of the permease, and the slower process is likely to be turnover of the permease, which could be triggered by inactivation.

It could be argued that dephosphorylation is a by-product of the true mechanism of inactivation, which perhaps causes a conformational change of Gap1p, allowing a phosphatase ac-

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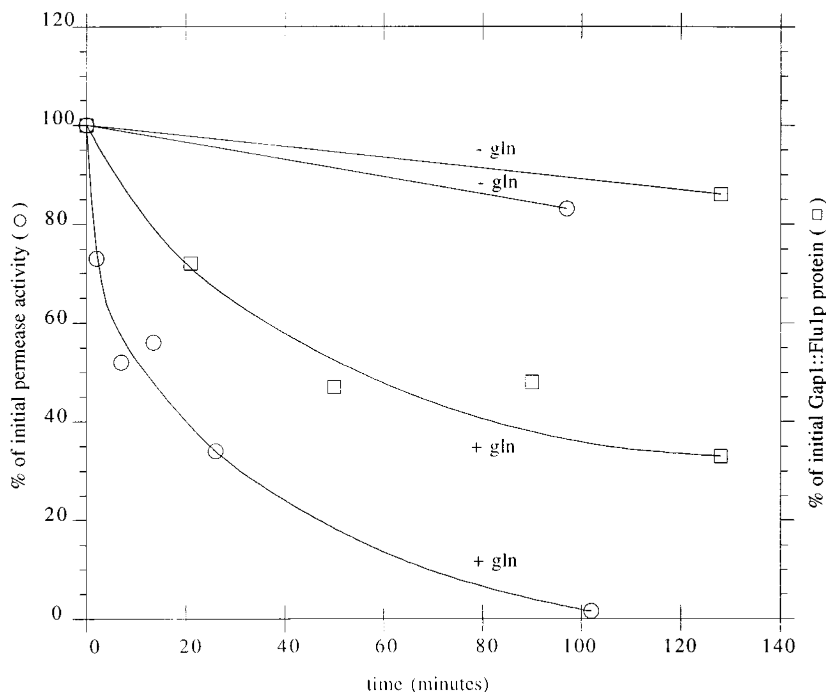


FIG. 6. Rate of Gap1::Flu1p inactivation is faster than the rate of loss of total Gap1::Flu1p protein. Glutamine was added to a urea-grown culture of MS143/pPL257 (*GAP1*::*FLU1*), and the amounts of Gap1::Flu1p activity and Gap1::Flu1p protein were determined over time. The relative amounts of Gap1::Flu1p protein were determined by phosphor image scanning of an immunoblot hybridized with the following (in order): antibody 12CA5, goat anti-mouse immunoglobulin G antibody, swine anti-goat immunoglobulin G antibody, and $^{125}$I-labelled protein A.

cess to phosphate groups. This would be similar to the case in which Gal4p undergoes a conformational change in the transition from an inactive state to an active state, allowing it to be fortuitously phosphorylated. The phosphorylation status of Gal4p was shown to be unimportant to the ability to become active by mutating the phosphorylation sites (35). This seems unlikely, however, in view of the homology of the *NPR1* product to protein kinases and the role of *NPR1* in maintaining Gap1p activity (42).

There are several overlapping layers of control of the general amino acid permease that are finely tuned to respond to the nitrogen environment of the yeast cell. In general, growth on nitrogen sources that do not result in the fastest doubling times increases the amount of Gap1p activity. Any change in nitrogen conditions that allows faster growth causes an immediate decrease in the amount of Gap1p activity by inactivation of the permease. While it isn't known whether there is a corresponding rapid change in the transcription of *GAP1* in response to a shift from limiting nitrogen sources to rich nitrogen sources, there is complex transcriptional control mediated by at least two systems that do not activate *GAP1* transcription on the best simple nitrogen source, glutamine. Control of Gap1p activity in turn regulates the synthesis of a host of catabolic enzymes by the mechanism of inducer exclusion (26). Well-studied examples of such genes include *PUT1* and *PUT2*, which encode enzymes required for the utilization of proline (2), and *CAR1*, which encodes arginase (40). Most of the specific amino acid permeases are high-affinity, low-capacity transporters that are produced constitutively or expressed to a lesser extent in the presence of rich nitrogen sources (5). They are ideally suited to relieve the cell of some of its amino acid biosynthetic chores and the synthesis of enzymes in these pathways, but they probably do not have sufficient capacity under normal growth conditions to trigger the production of catabolic enzymes. Thus, the complexity of control of the general amino acid permease is necessary because the import of large quantities of amino acids is a regulatory signal in itself.

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