Transduction of the Nitrogen Signal Activating Gln3-mediated Transcription Is Independent of Npr1 Kinase and Rsp5-Bul1/2 Ubiquitin Ligase in Saccharomyces cerevisiae*

Received for publication, June 9, 2006, and in revised form, July 13, 2006. Published, JBC Papers in Press, July 24, 2006, DOI 10.1074/jbc.M605551200

André Feller†, Mélanie Boeckstaens†, Anna Maria Marini†§, and Evelyne Dubois†§

From the †Institut de Recherches Microbiologiques J-M Wiame, Laboratoire de Microbiologie, Université Libre de Bruxelles, 1070 Brussels, Belgium, and the §Laboratoire de Physiologie Moléculaire de la Cellule, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 6041 Gosselies, Belgium

Nitrogen Catabolite Repression (NCR) allows the adaptation of yeast cells to the quality of nitrogen supply by inhibiting the transcription of genes encoding proteins involved in transport and degradation of nonpreferred nitrogen sources. In cells using ammonium or glutamine, the GATA transcription factor Gln3 is sequestered in the cytoplasm by Ure2 whereas it enters the nucleus after a shift to a nonpreferred nitrogen source like proline or upon addition of rapamycin, the TOR complex inhibitor. Recently, the Npr1 kinase and the Rsp5, Bul1/2 ubiquitin ligase complex were reported to have antagonistic roles in the nuclear import and Gln3-mediated activation. The Npr1 kinase controls the activity of various permeases including transporters for nitrogen sources that stimulate NCR such as the Mep ammonium transport systems. Combining data from growth tests, Northern blot analysis and Gln3 immunolocalization, we show that the Npr1 kinase is not a direct negative regulator of Gln3-dependent transcription. The derepression of Gln3-activated genes in ammonium-grown npr1 cells results from the reduced uptake of the nitrogen-repressing compound because NCR could be restored in npr1 cells by repairing ammonium-uptake defects through different means. Finally, we show that the impairment of the ubiquitin ligase complex does not prevent induction of NCR genes under nonpreferred nitrogen conditions. The apparent Rsp5-, Bul1/2-dependent Gln3 activation keeps to the cellular status, as it is only observed in cells having left the balanced phase of exponential growth.

Yeast cells have developed regulatory mechanisms of adaptation to the quality of nitrogen supply. During growth on a preferred nitrogen source, such as sufficient ammonium or glutamine, nitrogen catabolite repression (NCR) is exerted on catabolic enzymes and transport systems devoted to nonpreferred nitrogen sources such as proline utilization (reviewed in Ref. 1).

Under NCR conditions, the Gln3 GATA transcription factor bound to Ure2 is sequestered in the cytoplasm, thereby preventing Gln3-dependent activation of nitrogen-regulated genes (2). The nitrogen-regulated localization of Gln3 is thought to be under the control of the TOR (Target of Rapamycin) signaling pathway. For instance, upon treatment with rapamycin, condition which inactivates the TOR kinase complex, Gln3 is released from Ure2 and transferred to the nucleus in an active form (2). Similarly, upon a cell shift from NCR conditions to a nonpreferred nitrogen source, Gln3 enters the nucleus where transcription activation of nitrogen-regulated genes may occur.

The S/T kinase Npr1 has recently been described as a negative regulator of Gln3-dependent transcription because the absence of the kinase results in a nuclear localization of active Gln3 under ammonium supply (3). The Rsp5/Bul1/Bul2 ubiquitin ligase complex was further proposed to be required for Gln3 activation in the absence of Npr1 or upon a cell shift from the preferred to the nonpreferred nitrogen source, indicating that an ubiquitin-dependent signaling pathway controls Gln3-dependent transcription (3).

On the other hand, the Npr1 kinase is well known as a major element for the development of optimal activity for a variety of permeases (4–7), including transporters for nitrogen sources that stimulate NCR (8, 9). For instance, the kinase is required for the activity of the high affinity ammonium transport system Mep1 and for a lower affinity component (8).

Here, we show that derepression of Gln3-dependent genes in npr1 cells grown on ammonium results from the reduced uptake of the nitrogen repressing compound. We further unravel key components of the medium influencing the extent of nitrogen derepression in the absence of the Npr1 kinase. Finally, we show that in cells growing in the balanced phase of exponential growth, the impairment of the ubiquitin ligase complex does not prevent the induction of NCR gene expression under nonpreferred nitrogen conditions arguing against a direct involvement of the complex.

---

* This work was supported in part by the CoCoF (Commission de la Communauté Française) (to E. D.) and by FRSM Grant 3.4546.04 (to A. M. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† These authors contributed equally to this work.

‡ A recipient of a FRIA fellowship.

§ Research associate from FNRS.

§1,2 In the Laboratoire de Physiologie Moléculaire de la Cellule, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 6041 Gosselies, Belgium.

‡4 To whom correspondence should be addressed: Institut de Recherches Microbiologiques J-M Wiame, Laboratoire de Microbiologie, Université Libre de Bruxelles, avenue Emile Gryzon, 1, 1070 Brussels, Belgium.
**TABLE 1**

**Strains of *S. cerevisiae* used in this study**

| Strains          | Relevant genotype                        | Source                  |
|------------------|------------------------------------------|-------------------------|
| 23344c           | *ura3*                                   | André’s laboratory      |
| 21994b           | *nrp1* *ura3*                            | Vandenboul et al. (28)  |
| 27038a           | *ris5* *ura3*                            | Grenson (4, 5)          |
| 25126a           | *ris5* *nrp1* *ura3*                     | Hein et al. (29)        |
| 30170a           | *dow1* *nrp1*                            | Springael and André (30) |
| 30865b           | *bro1* *nrp1*                            | Springael et al. (18)   |
| 31034c           | *nrp1* *ana1* *ura3*                     | This study              |
| 12597a           | *ure2*                                   | Dabois’ laboratory      |
| 13830a           | *ure2* *ris5*                            | André’s laboratory      |
| 31019b           | *meg1A* *meg2A* *meg3A* *ura3*           | Marini et al. (11)      |
| OS27-1           | *bul1lox* *bul2lox* *kanMX* *ura3*       | Soesten et al. (7)      |
| OS29             | *bul1lox* *bul2lox* *ura3*               | This study              |
| FV049            | *URA3 GLN3-Myc13* ([KanMX])              | This study              |
| FV050            | *nrp1* *ura3* *GLN3-Myc13* ([KanMX])    | This study              |
| FV051            | *nrp1* *ana1* *ura3* *GLN3-Myc13* ([KanMX]) | This study              |
| FV052            | *ris5* *ura3* *GLN3-Myc13* ([KanMX])    | This study              |
| FV053            | *bul1lox* *bul2lox* *GLN3-Myc13* ([KanMX]) | This study              |

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The *Saccharomyces cerevisiae* strains used in this study are all isogenic to the Σ1278b strain except for the mutations listed in Table 1 (5, 7, 11, 18, 28–30). Strain OS29 was obtained from OS27–1 after KanMX removal using the Cre-lox P system (31). Strain 31034c was obtained by crossing was obtained from OS27–1 after KanMX removal using the mutations listed in Table 1 (5, 7, 18, 28–30). Strain OS29 was grown in minimal buffered medium (pH 6.1) supplemented in 500 μl of buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture tablet (Roche Diagnostics). Next, 300 mg of glass beads were added, and cells were disrupted three times for 30 s in a Bead-Beater. The broken cells were centrifuged for 2 min at 12,000 × g. The supernatant was added to an equal volume of gel loading buffer containing 4% (w/v) SDS, 125 mM Tris pH 6.8, b mercaptoethanol 10% (v/v), 20% (v/v) glycerol and blue bromophenol. Samples were heat-treated (100 °C, 10 min). After electrophoresis on NuPAGE 6% Tris-glycine/acetate gels, followed by transfer to Hybond-ECL membrane, the Gln3-Myc13 proteins were visualized using mouse monoclonal antibodies (working dilution, 1:5000) raised against the Myc epitope (Invitrogen). Western blots were performed according to a standard chemiluminescent protocol provided with the WesternBreeze Chemiluminescent kit-anti-mouse from Invitrogen. The Hybond-ECL membrane was analyzed using a chemiluminescence camera (Chemi-Smart from Vilbert-Lourmat).

**Indirect Immunofluorescence**—GlN3-Myc13 was visualized by indirect immunofluorescence on whole fixed cells. Immunofluorescence analysis was performed according to standard procedures (35). Cells were fixed for 30 min in formaldehyde (3.7%). They were washed and resuspended in sorbitol buffer (1.2 M sorbitol and 100 mM potassium phosphate, pH 7.5). Cell walls were digested for 50 min at 30 °C in sorbitol buffer supplemented with β-mercaptoethanol (40 mM final) and lyticase (80 units/ml) (92807 Fluka). Gln3-Myc13 was detected with monoclonal antibody c-Myc (9E10) (Santa Cruz Biotechnology) at a working dilution of 1:30. The secondary antibody was Alexa Fluor 488 goat anti-mouse immunoglobulin G (heavy plus light chains) (A-11029; Molecular Probes) (working dilution, 1:200). DNA was stained with DAPI (4,6’-diamidino-2-phenylindole). Cells were imaged using a Zeiss Axioskop2 Plus microscope equipped with a Plan Neofluar ×100/1.3 objective. Images were acquired with an AxiscamHkm camera and the native Axiosvision4 software from Zeiss (release 4.4). Images were formatted in Adobe Photoshop and Illustrator.

**RESULTS**

** Derepression of NCR Genes in *nrp1* Cells Results from Ammonium Uptake Limitation**—A direct negative role of the Npr1 kinase on the Gln3 transcription factor has been recently proposed because derepression of NCR genes was observed in *nrp1* cells grown on ammonium (3). We have previously shown that the *nrp1* mutation impaired ammonium uptake (8), indicating that the derepression of Gln3-dependent genes in *nrp1* cells could be a consequence of the reduced uptake rate of the repressing nitrogen compound (9). To evaluate both hypotheses, we analyzed the expression of NCR genes in conditions where, upon increasing ammonium uptake, wild-type and *nrp1* cells grew at similar rates.

Growth tests were performed in minimal medium (10) buffered at pH 6.1 and containing varying concentrations of ammonium as sole nitrogen source (hereafter cited as medium M.am). The growth defect of *nrp1* cells was similar.
Npr1 and Rsp5-independent NCR Gene Expression

To further sustain this hypothesis, we tested the consequence of expressing a heterologous ammonium transport system in the npr1 strain. The ectomycorrhizal HcAmt1 protein from the fungus *Hebeloma cylindrosporum* has previously been shown to rescue the growth ability of the yeast triple *mepΔ* strain even at 1 mM ammonium as the sole nitrogen source (12) and Fig. 1. HcAmt1 was not sensitive to the *npr1* mutation as the heterologous transporter restored growth on 1 mM ammonium upon expression in the yeast kinase mutant (Fig. 1). The latter transformed cells grew as the wild-type strain on 20 mM ammonium and displayed a very low level of *DAL5, DAL7, and MEP2* RNA on Northern blot (Fig. 2) confirming that derepression of NCR genes in *npr1* cells results from reduced ammonium uptake.

To extend these observations, we assessed Gln3 localization in wild-type, *npr1* and *npr1 amu1* cells growing on 20 or 80 mM ammonium. Strains bearing a chromosomal-tagged version of Gln3 (Gln3-Myc13) were constructed allowing visualization of Gln3-Myc13 by indirect immunofluorescence. The functionality of Gln3-Myc13 has been confirmed by Northern blot analysis (data not shown). In wild-type cells grown at 20 or 80 mM ammonium, i.e. NCR conditions, Gln3-Myc13 was mainly distributed in the cytoplasm but not uniformly (Fig. 3). This localization in foci has been previously described in glutamine-grown cells and has been proposed to reflect Gln3 association with intracellular membrane structures (13). In contrast, Gln3-Myc13 was mainly nuclear in *npr1* cells grown on 20 mM ammonium, in agreement with the induced expression of NCR genes (Fig. 3). In *npr1* cells at 80 mM ammonium as well as in *npr1 amu1* cells at 20 mM ammonium, Gln3-Myc13 was localized into the cytoplasm in keeping with the low expression of NCR genes (Fig. 3). Our data clearly demonstrate that in *npr1* cells the transduction of the nitrogen signal is restored as soon as ammonium uptake is no longer limiting, leading to cytosolic localization of Gln3 and to the repression of NCR genes.

**Restoration of Nitrogen Repression in npr1 Cells by Increasing the External Ammonium Concentration is pH- and Zinc-dependent**—Hall and co-workers (3) have proposed the Npr1 kinase to act downstream of the Sti4 phosphatase to repress Gln3 activity by preventing its entry into the nucleus. In strong contrast to our observations (Fig. 2), they found MEP2 to be highly expressed in *npr1* cells on YNB (Yeast Nitrogen Base) medium containing 76 mM ammonium (3). Because for at least part of the experiments the genetic background of strains is identical, the source of discrepancies could be linked to the composition of the growth medium. Growth tests simultaneously performed in our M.am laboratory buffered medium and in YNB medium containing 1, 20, or 80 mM ammonium were compared (Fig. 1 and Table 2). It is worth noting that *npr1* as well as triple *mepΔ* mutant cells presented a slow growth on YNB 20 mM ammonium compared with the growth on the buffered medium containing 20 mM ammonium (Fig. 1). In contrast, the *amu1* mutation or the expression of a heterologous ammonium transport system (HcAmt1) restored normal growth to the *npr1* strain on YNB 20 mM ammonium (Fig. 1).

Northern blot analysis was then performed using RNA extracted from cells growing on YNB medium. On this medium, raising the ammonium concentration from 20 to 80

| TABLE 2 | Generation doubling time of wild-type and mutated strains growing on buffered or unbuffered media containing different ammonium concentrations |
|---|---|---|---|
| | Media | Ammonium (23344c) | Wild type | npr1 (21994b) | npr1 amu1 (31034c) |
| | | mM ammonium | 1h05 | 2h19 | 2h07 |
| | M.am | 20 | 2h05 | 2h19 | 2h07 |
| | M.am | 80 | 2h08 | 2h11 | 2h06 |
| | YNB | 20 | 2h04 | 4h45 | 2h00 |
| | YNB | 80 | 2h07 | 2h32 | 1h57 |

**FIGURE 1. Growth efficiency on M.am and YNB media containing various ammonium concentrations as nitrogen source.** The buffered medium M.am is described under “Experimental Procedures.” The strains 23344c, 31019b (*mep1Δ mep2Δ mep3Δ ura3*), 21994b (*npr1 ura3*), 31034c (*npr1 amu1 ura3*) were transformed as indicated with the pFL38 (URA3) vector without insertion (−) or with pHcAMT1 (URA3 HcAMT1).
mM did not restore the repression of NCR genes in the npr1 strain whereas the amu1 mutation or the HcAmt1 expression were sufficient to recover repression on YNB 20 mM (Fig. 4). A major difference between both media is the presence of a buffer. For instance, when cells growing exponentially are harvested, the pH of the YNB medium was at a value of 3.6 whereas it was at 5.9 in the buffered M.am medium. The pH could have potential major effects on ammonium uptake and NH3 diffusion rate (see “Discussion”). Accordingly, upon buffering YNB medium (pH 6.1), npr1 cells displayed a significant increase of their growth rate at 20 mM ammonium (data not shown). However, Northern blot analysis showed that buffering the YNB medium was not sufficient to repress NCR genes in the npr1 mutant growing at 80 mM ammonium (Fig. 5). Progressively decreasing zinc concentration in the YNB buffered medium led to gradual restoration of NCR. Expression of the ZRT1 gene encoding a zinc transporter induced upon zinc limitation (14) was monitored as a control. Levels of ZRT1 mRNA illustrated that our M.am medium led to a zinc limitation whereas YNB medium did not (Fig. 5).

Buffer and low zinc concentration were also necessary to lead to the cytosolic localization of Gln3 in the kinase mutant strain at high ammonium concentration (80 mM) whereas Gln3 is clearly nuclear on nonbuffered YNB and on buffered YNB at high zinc concentration (Fig. 6). Interestingly, Gln3 is cytosolic in npr1 amu1 cells independently of the pH and of the zinc concentration in the growth medium.

The Npr1 Kinase Has No Major Influence on Gln3-Myc13 Phosphorylation—Differences in the phosphorylation state of Gln3 have been reported but a clear correlation with the subcellular localization of the transcriptional activator could not be demonstrated. Indeed methionine sulfoximine, the glutamine synthetase inhibitor and rapamycin, the Tor1,2 kinase inhibitor, although leading both to the nuclear localization of Gln3-
Npr1 and Rsp5-independent NCR Gene Expression

**FIGURE 4. Influence of the medium on NCR gene expression in npr1 cells.** A, the YNB medium prevents the restoration of nitrogen repression resulting from the increase of ammonium concentration in the medium. Total RNA was isolated from wild-type (23344c) and npr1 (21994b) cells grown on commercial YNB medium containing 20 or 80 mM ammonium. These experiments were repeated three times with RNA extracted from independent cultures. B, the YNB medium does not prevent the restoration of nitrogen repression when the ammonium flux is increased by the presence of the amu1 mutation or by expression of a heterologous ammonium transport system. Total RNA was isolated from wild-type (23344c), npr1 (21994b), npr1 amu1 (31034c), and npr1 cells, transformed with the plasmid expressing HcAmt1, grown on buffered minimal medium (M.am) or commercial YNB medium with 20 mM ammonium as a nitrogen source. These experiments were repeated twice with RNA extracted from independent cultures.

Myc13 and to NCR-sensitive transcription, generate opposite effects on Gln3-Myc13 phosphorylation (2, 15). We checked whether the Npr1 kinase and Amu1 might influence Gln3-Myc13 phosphorylation by comparing the electrophoretic mobility of Gln3-Myc13 in Western blot. Although Gln3-Myc13 is cytoplasmic in both conditions, we observed a significant decrease in the mobility of Gln3-Myc13 in wild-type cells upon raising the ammonium concentration from 20 to 80 mM (Fig. 7), in agreement with a previous report (15). The npr1 mutation had no major effect on this migration profile. However, it is worth noting that the Gln3-Myc13 signal detected in wild-type and npr1 cells grown on 80 mM ammonium was similar to that in npr1 amu1 cells on 20 mM ammonium (Fig. 7), indicating that the amu1 mutation, which restores Mep activity, mimics the effect of a high ammonium concentration on Gln3-Myc13 phosphorylation.

Our data are consistent with the lack of correlation between Gln3-Myc13 phosphorylation and intracellular localization in cells provided with various nitrogen sources (15, 16). Gln3-Myc13 phosphorylation/dephosphorylation is likely controlling other aspects than determining Gln3-Myc13 intracellular localization. However, simple analysis of electrophoretic mobility to study the phosphorylation status of a protein remains an approximate approach as a similar migration profile might correspond to the phosphorylation of different sites and the phosphorylation of some sites might have an inhibitory or activating action.

Npr1 and Amu1 Are Not Required for NCR Genes Activation in Response to Rapamycin Treatment and to Nitrogen Limitation or Starvation—Because we found that the transduction of the repressive nitrogen signal can be restored in npr1 cells, we next searched to determine whether Npr1 might influence the response to the activation signal induced by rapamycin treatment and by nitrogen limitation or starvation. The amounts of DAL5 mRNA were compared after rapamycin treatment, after a 1-h shift to proline medium and after a 1-h nitrogen starvation in wild-type and npr1 cells grown on 80 mM ammonium as well as in npr1 amu1 cells grown on 20 mM ammonium (Fig. 8). The induction of DAL5 expression was similar in the 3 strains indicating that the activation of NCR genes such as DAL5 does not require the integrity of Npr1, nor Amu1 (Fig. 8).

The Npi1/Rsp5 Ubiquitin Protein Ligase Is Not Required to Activate NCR Genes under Poor Nitrogen Supply—It has been reported that the kinase Npr1 and the ubiquitin ligase complex composed of Npi1/Rsp5 and Bul1/2 have antagonistic roles in the nuclear import and activation of NCR genes by Gln3 (3). These authors showed that derepression of NCR genes upon a cell shift from NCR growth conditions to proline requires the integrity of Rsp5 and Bul1/2 proteins and that rsp5 and bul1/2 mutations are epistatic to an npr1 mutation.

We compared the expression of NCR genes in ammonium-grown cells after rapamycin treatment, upon a 1-h shift to proline medium and after a 1-h nitrogen starvation. Unexpectedly, we observed a significant induction of DAL5 and MEP2 expression in all conditions tested (Fig. 9). Moreover, the rps5 mutation was not epistatic to the npr1 mutation as derepression on M.am medium was still observed in an npr1rps5 double mutant (Fig. 10). Similarly, the rbp5 mutation was not epistatic to the ure2 mutation, a condition where NCR is relieved (Fig. 10). Furthermore, mutations in two other proteins, Npi2/Doa4 and Npi3/Bru1, which are involved like Npi1/Rsp5 in ubiquitin-dependent control of permease trafficking (17–19), were not epistatic to an npr1 mutation (Fig. 10). The discrepancies between the present data and former results (3) did not result from the growth medium composition (data not shown). In contrast, we found an influence of the cellular growth status. The next experiments were performed with the YNB medium aiming at explaining the Hall group (Fig. 11). Cells were harvested at two different optical densities (0.5 × 10⁷ cells/ml and 1.2 × 10⁷...
cells/ml) on the growth curve. At the lower one, representing cells in the balanced phase of the exponential growth (20), Npi1/Rsp5 was clearly dispensable to transcriptional activation of NCR genes in proline-grown cells and to derepression in npr1 cells grown on 80 mM ammonium. Accordingly, immunofluorescent experiments revealed a nuclear localization of Gln3 in proline-grown rsp5 and bul1Δ bul2Δ cells as well as in the npr1, rsp5 cells growing on 20 mM ammonium showing that the polyubiquitination complex is not required for the transduction of the nitrogen signal (Fig. 12). At the higher optical density, representing cells in the late exponential phase, the presence of the rsp5 mutation led to a significant reduction of DAL5 and DAL7 mRNA levels in proline-grown cells and in ammonium-grown npr1 cells, indicating that the apparent Rsp5, Bul1/2 dependence of Gln3 is only observed when cells leave the balanced phase of exponential growth.

**DISCUSSION**

In this article, we show that the Npr1 kinase is not a direct negative regulator of Gln3-dependent transcription. Data from growth tests, Northern blot analysis and Gln3 immunolocalization reveal that the derepression of Gln3-activated genes in ammonium-grown npr1 cells results from the reduced uptake of the nitrogen repressing compound. It was known that the kinase is required for the activity of the high affinity ammonium transport system Mep1 and for a lower affinity component (8). Our present data show that the growth defect of npr1 cells on ammonium medium (M.am) is very similar to that of triple-mepΔ cells lacking the three endogenous Mep ammonium transport systems (11), and that the derepression of NCR genes is comparable in both strains. Accordingly, NCR was restored in npr1 cells by enhancing the ammonium entry through different means.

| FIGURE 5. **Restoration of nitrogen repression in npr1 cells by increasing the ammonium concentration in the medium is pH and zinc-dependent.** Total RNA was isolated from wild-type (23344c) and npr1 (21994b) cells grown on minimal medium containing concentrations of ammonium and ZnSO4 as indicated. M.am is a buffered medium (pH 6.1), YNB is not buffered. M.am- buf stands for M.am medium without buffer, YNB + buf stands for YNB medium with a buffer (pH 6.1). These experiments were repeated twice with RNA extracted from independent cultures.
| FIGURE 6. **The subcellular localization of Gln3 in npr1 cells is pH- and zinc-dependent.** Wild-type (FV049), npr1 (FV050), and npr1 amu1 (FV051) cells bearing a chromosomal version of Gln3 tagged with 13 copies of Myc epitope were grown on commercial YNB medium containing 20 or 80 mM ammonium as indicated or buffered YNB (pH 6.1) with 0.05 or 1.4 μM ZnSO4 as indicated.
| FIGURE 7. **Effect of npr1 and amu1 mutations on the electrophoretic mobility of Gln3-Myc13.** Wild-type (23344c), npr1 (21994b), and npr1 amu1 (31034c) cells were cultured on M.am 20 mM or 80 mM as indicated. Protein extracts from these cultures were prepared and the electrophoretic mobility of Gln-Myc13 was assayed by Western blot analysis as described under “Experimental Procedures.” These experiments were repeated twice with proteins extracted from independent cultures.
First, the ammonium entry in \( npr1 \) cells was increased and NCR restored by raising the ammonium concentration from 20 to 80 mM in the medium. Such an increase is able to repair the growth defect of a triple \( mep / H9004 \) strain (11), and we found it also to be true for \( npr1 \) cells. Second, the growth defect was suppressed and NCR restored in an \( npr1 \) strain further harboring an \( amu1 \) mutation which restores Mep-dependent ammonium uptake (8). Lastly, efficient ammonium uptake and NCR were restored in the yeast kinase mutant upon heterologous expression of an ectomycorrhizal ammonium transport system \( HcAmt1 \) (12), insensitive to the \( npr1 \) mutation.

Our data are in agreement with previous measurements of nitrogen catabolic enzymes and activity of several ammonium-sensitive permeases in \( npr1 \) cells, indicating that the derepression of enzymes is a consequence of the reduced uptake rate of the repressing nitrogen compounds (9). Also consistent are they with Northern blot data showing that an increase in ammonium concentration or the \( amu1 \) mutation both restored NCR to the Put4 proline permease gene in \( npr1 \) cells (21).

Key components of the medium influencing the extent of derepression in cells lacking the Npr1 kinase were unraveled. Particularly, restoration of NCR by increasing the external ammonium concentration was pH- and zinc-dependent. It was achieved by increasing pH to 6.1 and reducing zinc concentration to 0.05 \( \mu \)M. These observations shed light on the reasons of

---

\(^{6}\) A. M. Marini, unpublished results.

---

FIGURE 8. Activation of the DAL5 gene under nonpreferred nitrogen conditions in wild-type, \( npr1 \), \( npr1 amu1 \) cells. Total RNA was isolated from wild-type (23344c), \( npr1 \) (21994b) grown on M.am containing 80 mM ammonium and \( npr1 amu1 \) (31034c) cells grown on M.am 20 mM ammonium, M.am + rapamycin 200 ng/ml for 30 min (rap), after a 1-h shift from M.am to M.Pro (shift pro) and after a 1-h nitrogen starvation (shift-N).

FIGURE 9. Activation of NCR genes under nonpreferred nitrogen conditions is independent of the ubiquitin ligase complex. Total RNA was isolated from wild-type (23344c), \( rps5 \) (27038a), and \( bul1Δ bul2Δ \) (OS29) cells grown on M.am, M.am + rapamycin 200 ng/ml for 30 min (rap), after a 1-h shift from M.am to M.Pro (shift pro) and after a 1-h nitrogen starvation (shift-N). These experiments were repeated four times with RNA extracted from independent cultures.

FIGURE 10. Mutations in proteins involved in ubiquitin-dependent control of permease trafficking are not epistatic to an \( npr1 \) mutation. Total RNA was isolated from wild-type (23344c), \( npr1 \) (21994b), \( npr1 rps5 \) (25126a), \( npr1 doa4 \) (30170a), \( npr1 bro1 \) (30865b), \( ure2 \) (12597a), and \( ure2 rps5 \) (13830a) cells grown on M.am containing 20 mM ammonium. These experiments were repeated twice with RNA extracted from independent cultures.

FIGURE 11. The extent of NCR gene derepression in response to nitrogen limitation is dependent on the cellular growth status especially in an \( rps5 \) background. Total RNA was isolated from wild-type (23344c), \( rps5 \) (27038a), \( npr1 \) (21994b), and \( npr1 rps5 \) (25126a) cells grown on commercial YNB containing proline or 80 mM ammonium as indicated. Cells were harvested at two optical densities corresponding to 0.5 \( \times \) 10⁷ cells per ml (balanced phase of exponential growth) and 1.2 \( \times \) 10⁷ cells per ml (late phase of exponential growth). These experiments were repeated three times with RNA extracted from independent cultures.
The influence of zinc on NCR restoration in \textit{npr1} cells is less obvious. As a divalent cation, zinc might inhibit Nsc1 or yet unidentified ammonium transport systems, as described for a series of channels (25). However, the zinc concentrations tested in this study did not influence growth rate as revealed by growth tests and doubling time measurements (data not shown). This is consistent with the zinc effect being also present in wild-type cells but exacerbated in the \textit{npr1} strain. As a matter of fact, when wild-type cells are grown on 80 mM ammonium instead of 20 mM, a more efficient repression is observed on at least some NCR genes such as \textit{MEP2} and \textit{GAP1}, and this enhanced repression is also pH- and zinc-dependent although the growth rate with both ammonium concentrations is similar (data not shown).

It has been recently reported that an intact actin cytoskeleton is required for the nuclear transfer of Gln3 upon a shift from NCR conditions to a nonpreferred nitrogen source (26), consistent with Gln3 localization in foci, likely in association with membrane structures. With the actual knowledge and on the basis of Crespo and collaborators (3), an attracting model regarding the transduction of the NCR signal to Gln3 nuclear translocation was proposed (27). The actin cytoskeleton would play an essential role in nitrogen transduction to Gln3-Ure2 vesicular complex. Rsp5-Bul1/2-dependent polyubiquitination of a vesicular component associated with Gln3-Ure2 would result in Gln3 release and its subsequent transfer to the nucleus. According to this model, Npr1 would act as a ubiquitination antagonist, a role already described in other systems such as in the regulation of the general amino acid permease Gap1 (6, 7). However, our overall data do not support this model. The Rsp5 Bul1/2 ubiquitin ligase complex is not directly involved in the Gln3-dependent derepression upon a cell shift from the preferred nitrogen source to proline medium, to nitrogen-free medium, upon rapamycin treatment or in the absence of Npr1. The extent of derepression was similar in M.am and YNB media, but was dependent on the cellular status as reduced derepression at a late phase of exponential growth was more pronounced in \textit{rsp5} mutant cells. Furthermore, we have not been able to confirm a cytoskeleton dependence of Gln3 translocation (26) because latrunculin treatment to inhibit actin polymerization did not influence the derepression response upon a cell shift from NCR conditions to proline medium, nor the nuclear localization of Gln3 in proline-grown cells (data not shown).

Our overall data underline the importance of the medium composition, buffering and the cellular status as previously addressed by Wiame \textit{et al.} (20). Crucial components of NCR signaling remain to be characterized.

\textbf{Acknowledgments—}We thank Fabienne Vierendeels for excellent technical assistance. We thank Denis Lafontaine for helpful advice for immunofluorescence experiments, Bruno André for providing strains, and Isabelle Georis, Francine Messenguy and Bart Scheren for fruitful discussions.

\textbf{REFERENCES}

1. Magasanik, B., and Kaiser, C. A. (2002) \textit{Gene (Amst.)} 290, 1–18
2. Beck, T., and Hall, M. N. (1999) \textit{Nature} 402, 689 – 692
Npr1 and Rsp5-independent NCR Gene Expression

3. Crespo, J. L., Helliwell, S. B., Wiederkehr, C., Demougin, P., Fowler, B., Primig, M., and Hall, M. N. (2004) J. Biol. Chem. 279, 37512–37517
4. Grenson, M. (1983) Eur. J. Biochem. 133, 141–144
5. Grenson, M. (1983) Eur. J. Biochem. 133, 135–139
6. De Craene, J. O., Soetens, O., and Andre, B. (2001) J. Biol. Chem. 276, 43939–43948
7. Soetens, O., De Craene, J. O., and Andre, B. (2001) J. Biol. Chem. 276, 43949–43957
8. Dubois, E., and Grenson, M. (1979) Mol. Gen. Genet. 175, 67–76
9. Grenson, M., and Dubois, E. (1982) Eur. J. Biochem. 121, 643–647
10. Jacobs, P., Jauniaux, J. C., and Grenson, M. (1980) J. Mol. Biol. 139, 691–704
11. Marini, A. M., Soussi-Boudekou, S., Vissers, S., and Andre, B. (1997) Mol. Cell. Biol. 17, 4282–4293
12. Javelle, A., Andre, B., Marini, A. M., and Chalot, M. (2003) Trends Microbiol. 11, 53–55
13. Cox, K. H., Tate, J. J., and Cooper, T. G. (2002) J. Biol. Chem. 277, 37559–37566
14. Zhao, H., and Eide, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2454–2458
15. Tate, J. J., Rai, R., and Cooper, T. G. (2005) J. Biol. Chem. 280, 27195–27204
16. Cox, K. H., Kulkarni, A., Tate, J. I., and Cooper, T. G. (2004) J. Biol. Chem. 279, 10270–10278
17. Springael, J. Y., De Craene, J. O., and Andre, B. (1999) Biochem. Biophys. Res. Commun. 257, 561–566
18. Springael, J. Y., Nikko, E., Andre, B., and Marini, A. M. (2002) FEBS Lett. 517, 103–109
19. Nikko, E., Marini, A. M., and Andre, B. (2003) J. Biol. Chem. 278, 50732–50743
20. Wiame, J. M., Grenson, M., and Arst, H. N., Jr. (1985) Adv. Microb. Physiol. 26, 1–88
21. Jauniaux, J. C., Vandenbol, M., Vissers, S., Broman, K., and Grenson, M. (1987) Eur. J. Biochem. 164, 601–606
22. Bihler, H., Slayman, C. L., and Bertl, A. (1998) FEBS Lett. 432, 59–64
23. Bihler, H., Slayman, C. L., and Bertl, A. (2002) Biochim. Biophys. Acta 1558, 109–118
24. Roberts, S. K., Fischer, M., Dixon, G. K., and Sanders, D. (1999) J. Bacteriol. 181, 291–297
25. Mathie, A., Sutton, G. L., Clarke, C. E., and Veale, E. L. (2006) Pharmacol. Ther. 111, 567–583
26. Cox, K. H., Tate, J. J., and Cooper, T. G. (2004) J. Biol. Chem. 279, 19294–19301
27. Magasanik, B. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 16537–16538
28. Vandenbol, M., Jauniaux, J. C., Vissers, S., and Grenson, M. (1987) Eur. J. Biochem. 164, 607–612
29. Hein, C., Springael, J. Y., Volland, C., Haguenauer-Tsapis, R., and Andre, B. (1995) Mol. Microbiol. 18, 77–87
30. Springael, J. Y., and Andre, B. (1998) Mol. Biol. Cell 9, 1253–1263
31. Guldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996) Nucleic Acids Res. 24, 2519–2524
32. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Yeast 14, 953–961
33. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) Nucleic Acids Res. 18, 3091–3092
34. Foury, F., and Talibi, D. (2001) J. Biol. Chem. 276, 7762–7768
35. Pringle, J. R., Adams, A. E., Drubin, D. G., and Haarer, B. K. (1991) Methods Enzymol. 194, 565–602