A second nitrogen permease regulator in *Saccharomyces cerevisiae*

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Abstract **We describe a *Saccharomyces cerevisiae* mutant affected in its urea and proline transport capacities, and a gene coding for a protein complementing this mutation. This protein is not membrane-embedded and contains two PEST sequences, often found in regulatory factors. The mRNA is not down-regulated under nitrogen catabolite repression, and is induced by urea and proline. In the mutant, the *PUT4* mRNA encoding the proline permease is not affected, whereas the *DUR3* mRNA, involved in urea active transport, is strongly increased. Our data suggest that this protein is a post-transcriptional regulator of nitrogen permeases.**

Key words: Nitrogen source transport; Urea; Proline; NPR2; *Saccharomyces cerevisiae*

1. Introduction

Studies of the mechanisms of the trans-membrane urea transport and their regulation are of primary importance for the understanding of complex physiologic phenomenons, such as the urinary concentrating capacity of the mammalian kidney. Such a transport exists in a number of micro-organisms, including the yeast *Saccharomyces cerevisiae*, as demonstrated as early as 1975 [1].

Saccharomyces cerevisiae is able to use urea as sole nitrogen source by degrading it into CO_2 and NH_4^+ [2]. The urease responsible for this degradation consists of a bifunctional enzyme, DUR1-2, capable of transforming urea into allophanate in an ATP-dependent fashion, and then allophanate into CO_2 and NH_4^+ [3]. Urea might be the degradation product of other nitrogen sources, but it might also be incorporated from the environment. Two systems of saturable urea transport have been described [1]: one is a low-affinity facilitated transporter (K_m 1 mM), the other being a high affinity active transport system (K_m 14 μM). A mutant affected in this active transport [4] has enabled the cloning of a gene located on chromosome VIII, *DUR3*, coding for a putative trans-membrane protein which is necessary for a functional active transport to be present [5]. There is no direct demonstration of the DUR3 protein being the urea carrier, and authors agree that other proteins could be involved in the transport mechanisms, for instance in the coupling of the urea carrier with an energy source.

Proteins involved in the metabolism of poor nitrogen sources such as allantoin or proline are under the control of multiple transcriptional and post-transcriptional regulatory mechanisms. Nitrogen catabolite repression (see [6] for a review) is a regulatory phenomenon that strongly inhibits the transcription of the genes coding for these proteins when readily used nitrogen sources (ammonia, glutamine or asparagine) are present. The transcription factors and the DNA recognition sequences involved in nitrogen catabolite repression have been characterized, although a definitive scheme of this complex regulatory mechanism is difficult to draw [7]. The intermediate metabolite in urea degradation, allophanate, is the natural inducer of the majority of the proteins of this metabolic pathway [8]. It acts through a GATA-like transcription factor recognizing a 12 base pair region with the GATAA sequence at its core [9]. A post-transcriptional regulatory system involving two constitutive negative regulators, encoded by the *NPI1* and *NPI2* genes, has been described [10]. On media that promote permeases functions, this system is counteracted by the NPR1 protein [10,11]. The sequence of the *NPR1* gene is homologous to sequences of genes coding for protein kinases, suggesting that this system could work through phosphorylation/dephosphorylation of the relevant permeases [12].

In order to get new insights into the active transport of urea in eukaryotic cells, we have tried to identify other proteins involved in urea permeation in *Saccharomyces cerevisiae* by using a classical mutagenesis/complementation strategy. We describe here the identification and sequencing of a new gene, *NPR2*, that is necessary for a functional active transport of urea.

2. Experimental

2.1. Yeast strains, media, mutagenesis, and transformation

The yeast strain used for ethyl-methyl-sulfonate (EMS) mutagenesis was CMY375, a *ura3-52* derivative of the wild type S288C strain, and was kindly provided by C. Mann (Saclay, France). This strain was treated with EMS at 5% (v/v) for 1 h at 30°C under shaking, and plated on YPD medium (Difco, Detroit, MI). The plates were replica plated on Yeast Nitrogen Base w/o amino acids and w/o $(NH_4)_2SO_4$ (Difco, Detroit, MI) supplemented with 2% (w/v) glucose as carbon source and 0.1% (w/v) uracil (subsequently referred to as minimal medium or M), with 10 mM urea as sole nitrogen source. The 6 poorly growing strains selected with this protocol were called BUH1 to BUH6. The media used for maintenance of the yeast strains were either YPD or Yeast Nitrogen Base w/o amino acids supplemented with casamino acids (1 g/l) and glucose (2%) with or without uracil. For induction or analysis of gene expression, yeasts were cultivated in M plus 100 mM urea and 1 mg/ml proline (Mpro/u). For electroporation, cells were grown to mid-log phase in YPD, incubated for 10 min at 30°C with 25 mM mercaptoethanol, and washed twice in ice-cold pulsation buffer (Tris 1 mM pH 7.5, saccharose 250 mM, $MgCl_2$ 1 mM). Cells were resuspended at 10^9 cells/ml in pulsation buffer and electroporated with 20 to 50 ng of DNA at 870 V for 10 ms. After 15 min at 30°C in YPD, cells were washed and plated on appropriate selection medium. As urea rapidly degrades in solution, growth experiments were always performed with extemporaneously prepared urea stock solutions. Results of growth on solid media experiments were always determined after 3 days at 30°C.

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2.2. DNA manipulation and sequencing

Yeast plasmid DNA was extracted with a hot phenol method and directly transformed into competent XL1-Blue cells (Stratagene, La Jolla, CA). Plasmids amplified in XL1-Blue were recovered either by alkaline lysis miniprep extractions or by alkaline lysis/PEG-NaCl purification [13]. All restriction endonucleases were from New England Biolabs (Beverly, MA), and digestions and sub-cloning experiments were performed according to standard protocols [13]. A series of deletion mutants was constructed using the exonuclease III/mung bean nuclease protocol according to [13] with minor modifications. The DNA was sequenced on both strands using the Sanger dideoxy chain-termination method with the sequenase 2.0 T7 DNA polymerase (United States Biochemical, Cleveland, OH). Where needed, synthetic oligonucleotides were designed to serve as primers for the sequencing reaction. DNA and protein sequences were analyzed with the DNA Strider program [14]. Northern and Southern analysis were performed as described in [13].

2.3. Transport experiments

Cells were cultivated in the appropriate medium (Mpro/u unless otherwise indicated) until they reached mid-log phase (660 nm OD < 1). They were then washed 4 times in Mpro medium, and kept pelleted on ice until use. They were then adjusted to 20 OD per ml, equilibrated 5 min at 18°C, and mixed with the radioactive metabolite at a final concentration of 0.25 mM and 1 μCi/ml. At the indicated times, 500 μl of cells were added to 4 ml of ice-cold medium and immediately filtered through a GF/A membrane (Whatman, Paris, France). The filter was then rinsed with 5 ml of ice-cold medium, placed into a liquid scintillation counting vial with 4 ml of scintillation liquid (Ultima-Gold, Packard Instrument, Rungis, France) for at least 1 h at 18°C under vigorous shaking, and counted in a Tri-Carb 4000 counter (Packard Instrument, Rungis, France). The specific activity of the medium was determined by counting 5 μl of the solution in the same conditions. Backgrounds were calculated after immediate filtration and washout of the cell/metabolite mixture, and substracted from the data.

3. Results

3.1. Characterization of a mutant affected in urea transport

A wild type strain of *Saccharomyces cerevisiae*, carrying ony the ura3-52 marker, was mutagenized with EMS, and 6 clones poorly growing on 10 mM urea (BUH1 to BUH6) were selected. Among these 6 clones, only one (BUH3) was able to grow like the wild type when provided with 100 mM urea as sole nitrogen source, while showing no detectable growth at 0.25 mM urea, a phenotype potentially corresponding to the absence of the high affinity active urea transport.

We then measured the accumulation of ^{14}C-labelled urea inside cells grown on proline/urea medium. Under these conditions, the total radioactivity in the assay medium is rapidly vanishing, reflecting the degradation of urea into CO_2 and NH_4^+ by the dur1-2 enzyme (data not shown). This degradation appears to be as efficient in the mutant as in the wild type strain, and dramatically modifies the urea accumulation data. To bypass this problem, we have measured the accumulation of [^{14}C]thio-urea, a compound known to enter yeast cells through the urea transporter [15], and which is not degraded by dur1 [3]. The mutant strain appears to be affected in its urea transport capacities in terms of initial rate (1.26 nmol/min/10^8 cells vs. 5.31 nmol/min/10^8 cells for the wild type) as well as maximum accumulation (19.5 nmol in 20 min vs. 73.5 nmol in 35 min for the wild type) (Fig. 1A). The active transport of urea is confirmed by the dramatic effect of sodium azide on the entry of [^{14}C]thio-urea into the cells, as shown on Fig. 1B. The slight decrease in the intracellular thio-urea concentration after

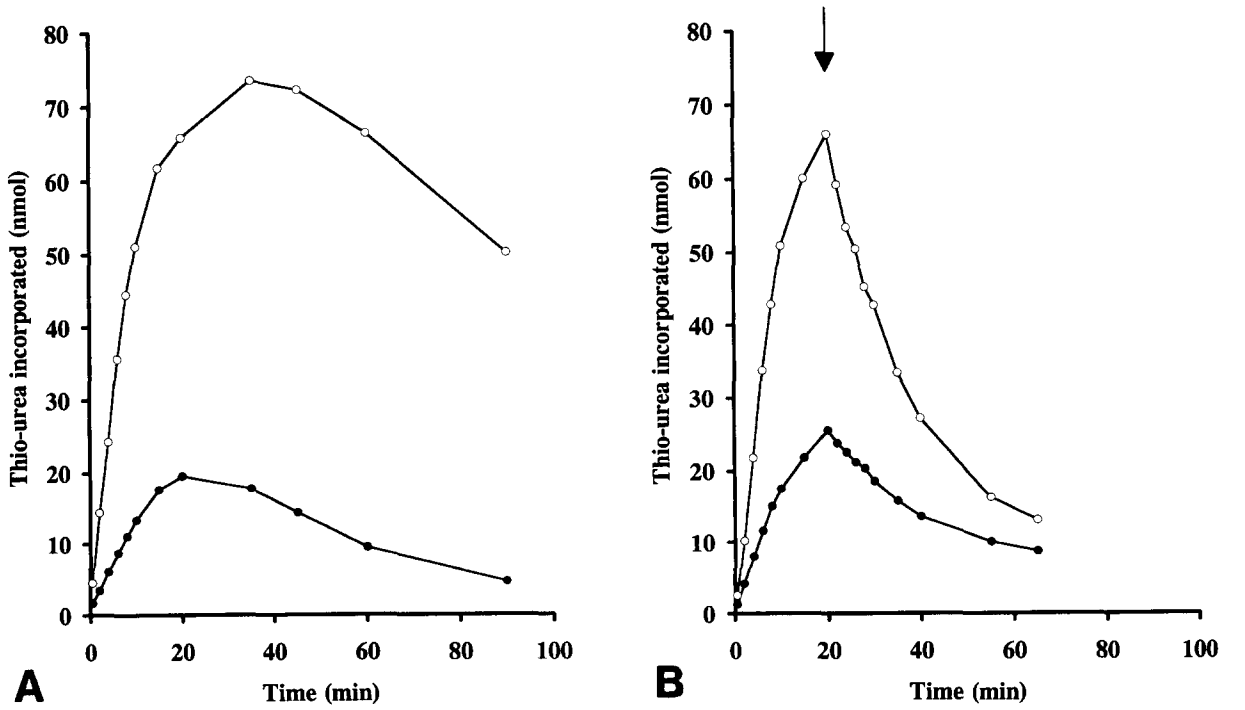

Fig. 1. Incorporation of ^{14}C-labelled thio-urea in BUH3 (●) and wild type (○) strains. (A) Time course of thio-urea incorporation; each time point representing 10 OD (10^8 cells); (B) effect of NaN_3 on thio-urea accumulation; at 20 min (black arrow), NaN_3 was added at a final concentration of 2 mM.

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-916                                                        CATtgctatgcctttt -901
-900 ttttttttgtttttacaggagttaagaagtctgaagaacttctgaaataaacttttcgatt -841
-840 gacgacagattaaaaactggatccaactataagtataatagtaacttatatatttctgtt -781
-780 ccagatatatacgtaaaaaaaagaaaaggataattgatgtttttgcaaaaagggattccc -721
-720 tgattcggtttactctgttcgcaaccactcttctttctgctaatttgtgctttatctcgt -661
-660 atcattctcttatctacttatcttaaccttatcgtcttcctcgaaatttttcattgataga -601
-600 gacaactgcaaaaaaagaaattcaaaaaagccgtatgactcacccggaaaccacacagaa -541
-540 accctagatttctatagggcaaatttcagggttatactaaaagatatattattgaccttg -481
-480 gttattcttcaagttcgccctattgaaaattcggacactttagggtttggaatctgttaa -421
-420 agatataagtaaatatgatgataaagaaataggtttaatttatacgcacttgccagtaag -361
-360 tgattgatattgcagaagtaacatgaatacggttctgtggcctttgctgtttgccgttat -301
-300 tttatttctttttttgttttttttctttttttcagcactatcaaggctaataagggaca -241
-240 agagttcttactcagtgtgaacgtgttctaaataagttcttgttctaattaattgtaact -181
-180 atttttatgcatttcttgtaagaggaactggagtaatcgtatctcggaaaatacatagagg -121
-120 tgcgtattcataatgggccctctatcacttgttgcctgtatctccgaataggactgataa -61
 -60 gtgataaaaaaaaaagaaaactgggttagcttgaagcgactttctttctctactaaaggga -1
   1 ATG GTC AGA TCA TCA GGC CAA CGG CAA ATG CTA AGC TAT TTC CAA  45
   1 M  V  R  S  S  G  Q  R  Q  M  L  S  Y  F  Q              15
  46 GGG TTT GTG CCC ATA CAC ACA ATA TTT TAC TCC GTG TTC CAT CCT  90
  16 G  F  V  P  I  H  T  I  F  Y  S  V  F  H  P              30
  91 ACA GAA GGT TCT AAG ATT AAA TAC GAG TTT CCG CCA AAT AAT TTG 135
  31 T  E  G  S  K  I  K  Y  E  F  P  P  N  N  L              45
 136 AAA AAT CAT GGT ATT AAT TTC AAC ACA TTT AAA AAC TAC ATC ATA 180
  46 K  N  H  G  I  N  F  N  T  F  K  N  Y  I  I              60
 181 CCG AAG CCT ATA TTA TGC CAC AAG TTG ATC ACT TTT AAG TAT GGT 225
  61 P  K  P  I  L  C  H  K  L  I  T  F  K  Y  G              75
 226 ACA TAT AGG ATT GTA TGT TAT CCT GTG ACA ATC AAT TCT CCA ATA 270
  76 T  Y  R  I  V  C  Y  P  V  T  I  N  S  P  I              90
 271 TAT GCT AGG AAT TTT TTT AGT TTT AAT TTT GTA TTT GTA TTC CCA 315
  91 Y  A  R  N  F  F  S  F  N  F  V  F  V  F  P             105
 316 TAT GAT TGT GAG ACG TCT CCC TAC GAA CCA GCT ATT ACT AGG CTG 360
 106 Y  D  C  E  T  S  P  Y  E  P  A  I  T  R  L             120
 361 GGC AAA ATG TTC AAA GTT CTT GAA GAA CAA AAT CAA TTG CTA TCC 405
 121 G  K  M  F  K  V  L  E  E  Q  N  Q  L  L  S             135
 406 AAA TCG GAA AGA GAT CCA GTT TTC TTC GAT TTG AAG GTG TTG GAA 450
 136 K  S  E  R  D  P  V  F  F  D  L  K  V  L  E             150
 451 AAC TCC ACA ACA ACA CCC TCC ACT GCA GGT CCT TCA TCC ACG CCA 495
 151 N  S  T  T  T  P  S  T  A  G  P  S  S  T  P             165
 496 AAT CCT AGT AGT AAC ACC ACA CCA ACT CAT CCT ACA TCC GAA AAG 540
 166 N  P  S  S  N  T  T  P  T  H  P  T  S  E  K             180
 541 GAT ACA AAG GAT ATG AGA AGC AGC AGA TAC AGT GAC CTT ATC AAG 585
 181 D  T  K  D  M  R  S  S  R  Y  S  D  L  I  K             195
 586 GAT TTG GGC CTT CCG CAA TCC GCA TTT TCC ATA CAA GAT TTA TTG 630
 196 D  L  G  L  P  Q  S  A  F  S  I  Q  D  L  L             210
 631 ATG AGA ATT TTC CAA GAC CTG AAT AAC TAT TCC GAA TGC CTC ATA 675
 211 M  R  I  F  Q  D  L  N  N  Y  S  E  C  L  I             225
 676 CCA ATT GAT GAG GGA AAT GCA GTA GAC ATA AAG ATT TTT CCG CTG 720
 226 P  I  D  E  G  N  A  V  D  I  K  I  F  P  L             240
 721 TTG AGA CCA CCG ACA ACT TGT GTC TCA TTA GAA GAT GTG CCC CTG 765
 241 L  R  P  P  T  T  C  V  S  L  E  D  V  P  L             255
 766 TCG TCT GTA AAT TTG AAG AAG ATT ATT GAT GTC AAC TGG GAT CCA 810
 256 S  S  V  N  L  K  K  I  I  D  V  N  W  D  P             270
 811 ACC ATG ATG AGT ATA GTC CCT TAC ATT GAT GGC TTA AAC AGC ATT 855
 271 T  M  M  S  I  V  P  Y  I  D  G  L  N  S  I             285
 856 GCC AAA ATT TCT AAA CTA AGC AAC AGT GAT CCT GGC CTA GTG ATA 900
 286 A  K  I  S  K  L  S  N  S  D  P  G  L  V  I             300
 901 GAG TGC ATA CGT CAC TTA ATA TAC TAT AAG TGT GTT ACA TTA TCA 945
 301 E  C  I  R  H  L  I  Y  Y  K  C  V  T  L  S             315
 946 GAT ATT TTC CAA TTT TCT AAC ATA TAC GCT CCT TCT TCA TTA ATT 990
 316 D  I  F  Q  F  S  N  I  Y  A  P  S  S  L  I             330
 991 AGG AAT TTT TTG ACT GAT CCA CTA ATG GCT AGT GAC TGT CAA TCT 1035
 331 R  N  F  L  T  D  P  L  M  A  S  D  C  Q  S             345
1036 TAT GTT ACG TTC CCA GAA GTG TCT AAA ATA TCG AAC TTG CCC TTA 1080
 346 Y  V  T  F  P  E  V  S  K  I  S  N  L  P  L             360
1081 AAC AAA AGT CTG GGT TCA GGT GAC CAA GAC TCA CCA TCA TTT TCT 1125
 361 N  K  S  L  G  S  G  D  Q  D  S  P  S  F  S             375
1126 GTA CGA AGA AAA TCC AAA TCA TCT AGC ATA CCG TCA AAC CCG GAT 1170
 376 V  R  R  K  S  K  S  S  S  I  P  S  N  P  D             390
1171 TCT AGA ACT ACA TCT TTT AGT TCT ACC AGT AGA GTT TCT CAA AAC 1215
 391 S  R  T  T  S  F  S  S  T  S  R  V  S  Q  N             405
1216 TCC TCC TTA AAT TCC TCT TTT TCA TCA ATT TAT AAA GAC TGG AGG 1260
 406 S  S  L  N  S  S  F  S  S  I  Y  K  D  W  R             420
1261 CAA TCA CAA ACC TCC TGC TCG AGT TCA AAT ATT CAT GTC ATC AAC 1305
 421 Q  S  Q  T  S  C  S  S  S  N  I  H  V  I  N             435
1306 AAT CGT AAC CGA TTC CTA CCA ACA AGA TCA TGC CTA TTT GAC CTT 1350
 436 N  R  N  R  F  L  P  T  R  S  C  L  F  D  L             450
1351 TAT AGG TCA CTT TCA CAA GGT CAA ACG CTG AAA ACA TGG TAT GAG 1395
 451 Y  R  S  L  S  Q  G  Q  T  L  K  T  W  Y  E             465
1396 TCG AAA TAT ATG ATT TTA AAA GAA AAT AAC ATT GAT ATA AGA AGG 1440
 466 S  K  Y  M  I  L  K  E  N  N  I  D  I  R  R             480
1441 TTC ATA ACG TTT GGC CTG GAA AAA CGT ATC ATT TAC AGG TGC TAC 1485
 481 F  I  T  F  G  L  E  K  R  I  I  Y  R  C  Y             495
1486 TCT TTT CCT GTA ATG ATA AAT GCA GGT TCA CGT GAA CCA AAA GAA 1530
 496 S  F  P  V  M  I  N  A  G  S  R  E  P  K  E             510
1531 ATG ACA CCA ATA ATA ACA AAA GAT TTA GTG AAT AAT GAT AAG TTA 1575
 511 M  T  P  I  I  T  K  D  L  V  N  N  D  K  L             525
1576 CTT GAA AAA AGA AAT CAC AAT CAT TTG CTT TCA GCC ACA GGG TCT 1620
 526 L  E  K  R  N  H  N  H  L  L  S  A  T  G  S             540
1621 AGA AAT ACG GCA CAA TCC GGC AAC TTG AAA CCA GAA AGA CCA TCA 1665
 541 R  N  T  A  Q  S  G  N  L  K  P  E  R  P  S             555
1666 AAG GTT TCA TTT GAA ATG CAA AGG GTA AGT TCG TTG GCA ACA GGC 1710
 556 K  V  S  F  E  M  Q  R  V  S  S  L  A  T  G             570
1711 AAA AGT ACT ATG CCG AAA CTC AGC GAC GAA GAA GAA GGT ATT CTT 1755
 571 K  S  T  M  P  K  L  S  D  E  E  E  G  I  L             585
1756 GAA GAG TCT ATT AGG AAT GCC GAA ACA TTT GAT AAA ATT TGC GTT 1800
 586 E  E  S  I  R  N  A  E  T  F  D  K  I  C  V             600
1801 TTA CTG AGC AAG CCT AAA TTA GAA GTC GAA AGC TAC CTA AAC GAA 1845
 601 L  L  S  K  P  K  L  E  V  E  S  Y  L  N  E             615
1846 TTA GGA GAA TTT AAG GTA ATT AAT AGC TGA gttaaaattagagaaattca 1895
 616 L  G  E  F  K  V  I  N  S  *                             625
1896 ttgtccatcaaaatgtttttttttttttttaatattacaacttatttccgtaaataaaga 1955
1956 tagtacacacgaatccaaacgtttatatagttagctctgtgaaaatttgatttaggttca 2015
2016 tctcttggtgtgtttggtagaggtggtttcacaccctctctcttctgtcttgccttctaat 2075
2076 gaataactgagtattctttacatactttgtttttattaaccactagtttgaatatatatt 2135
2136 cgactgaaaggcaatatcaaCTATTCAATCTTTAACATTTTTCTCGAGCCCTCATTGTCC 2195
2196 ACATTTTCACTGGATTT                                           2212
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30 min in the absence of NaN3 (Fig. 1A) could be explained by a progressive degradation of the active urea transport structures leading to a re-equilibration of the intra- and extra-cellular thio-urea concentrations. Indeed, we have shown that the active urea transport system has a short (<3 h) functional half-life in the absence of inducer (unpublished data).

3.2. Phenotypic characterization of the mutant

We have tested the growth of the mutant strain vs. the wild-type strain on solid minimum medium supplemented with uracil and different nitrogen sources. Although the BUH3 strain does not display any phenotypic change when provided with either casamino acids, arginine, or methionine as nitrogen sources, its growth is completely abolished in the presence of proline or glutamate (2.5 mM), and strongly affected in the presence of 2.5 mM ammonium sulfate. It is noteworthy that, in liquid medium, the mutant could not grow even in the presence of 100 mM proline, in contrast with what we observed for urea (data not shown). The incorporation of [^{14}C]proline appears to be affected in BUH3, showing about 30% of the wild-type incorporation after 5 or 30 min, suggesting that the PUT4 proline permease functions could also be affected in BUH3.

3.3. Molecular characterization of NPR2, a BUH3 complementing gene

A *Saccharomyces cerevisiae* genomic bank constructed in the YCP50 centromeric plasmid was used to complement the BUH3 mutant. Transformants were selected on uracil depleted medium, and then tested for their ability to grow on 2.5 mM urea. Four plasmids were isolated, rescued into *E. coli* and amplified. Among them, only one was able to repeatedly complement the BUH3 strain. This 16 kb fragment was sub-cloned and the complementing gene was localized to a 3.2 kb fragment, which was sequenced on both strands using exonuclease III/mung bean nuclease deletion mutants and synthetic oligonucleotides.

The sequence (Fig. 2) revealed one main ATG-headed open-reading frame of 1872 nucleotides, located between two previously sequenced genes, namely *CIN8* [16] and *CAN1* [17], in a reverse orientation compared with these two genes. Thus, the gene is located on the left arm of chromosome V. We could find a consensus *Saccharomyces cerevisiae* poly-adenylation signal (TATATA; [18]) on position 1979, i.e. 104 bp after the stop codon. Several putative TATA boxes were found upstream of the ATG start codon.

The deduced protein sequence is 624 amino acids long (Fig. 2), with a predicted MW of 70843 and a predicted pI of 8.8, and does not display significant homologies with known proteins (Swissprot release 27). The hydropathy profile of this protein (data not shown) is clearly not compatible with the protein being a trans-membrane carrier. Five potential *N*-glycosylation sites are present at positions 151, 219, 361, 405, and 409, and 3 potential tyrosine phosphorylation sites could be detected on

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Fig. 2. Nucleotide and deduced amino-acid sequence of *NPR2*. The *NPR2* non-coding strand is presented 5′–3′. The stop codon is indicated by a star. The *CAN1* ATG (−914/−916) and the end of the *CIN8* coding sequence (2212/2156) are indicated in small capitals. Putative TATA boxes are underlined, as well as the yeast consensus poly-A signal. PEST sequences are double-underlined, and the serines residues in the serine-rich region are underlined.

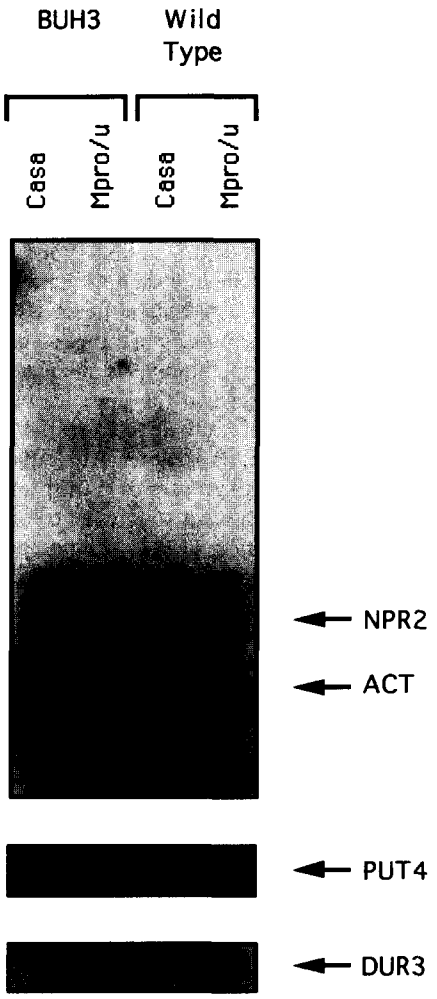

Fig. 3. Northern blot analysis of *NPR2*, *PUT4* and *DUR3* mRNAs. Total RNA from the BUH3 and wild type (WT) strains were prepared as described in section 2, after culture in YNB supplemented with glucose and casamino acids (Casa), or in proline/urea medium (Mpro/u). ACT indicates the signal obtained with an actin probe.

Tyr-220, -468, and -612. Two other features are worth noting: (i) the protein is rich in serine residues (about 14%), and presents a strong serine rich region (40% between residue 363 and 429); (ii) so-called PEST regions [19] are present between Lysine-147 and Histidine-175 (PEST-score: 16.2) and between Lysine-381 and Arginine-392 (PEST-score: 6.2); these scores are high compared to the scores of the PEST-sequences of the proteins provided with the PEST-find program (10.1, 5.7 and 4.4 for c-fos, and 2.3, 8.6 and 25.4 for c-myc).

Northern blot analysis of the gene transcription product revealed a unique 2.5 kb mRNA. This transcript is not subjected to nitrogen catabolite repression, as it can be easily detected in total RNA from mutant or wild type cells cultivated in Casa medium (Fig. 3). However, the amount of mRNA is significantly increased when the cells are cultivated with proline and urea as sole nitrogen sources (Fig. 3, compare NPR2 and actin as control).

We have also determined the total amount of the mRNAs coding for the PUT4 proline permease and the DUR3 urea active transport necessary protein in the BUH3 and the wild-type strains under different culture conditions (Fig. 3). Both genes are efficiently transcribed in the mutant under stimulatory conditions, and both are transcriptionally repressed under nitrogen catabolite repression. Surprisingly, the DUR3 mRNA is about 10 times more abundant in the BUH3 mutant than in the wild-type strain.

A strain containing a null allele of the *NPR2* gene was obtained by transforming a wild-type *ura3-52* haploid yeast strain with the *Pvu*II–*Pvu*II fragment of a pRS316 vector carrying the NPR2 DNA in which the *Aat*II–*Bst*EII fragment (nucleotides 327 to 1099 of the coding sequence) had been replaced by the *Nde*I–*Sma*I fragment of YCP50 carrying the *URA3* gene. The recombination events were checked by Southern blotting in clones growing on a selective ura$^-$ medium (data not shown). This confirmed that the *NPR2* gene is not essential for growth on complete medium. The phenotype of this disrupted strain is similar to that of the BUH3 mutant in terms of growth on different medium, demonstrating that NPR2 is essential for the growth on certain nitrogen sources.

4. Discussion

In this report, we describe a new *Saccharomyces cerevisiae* mutant strain, BUH3, that is affected in its nitrogen sources assimilation capacities. In particular, the BUH3 strain is not capable of synthesizing a fully functional active urea transport system, and appears to be strongly deficient in its proline transport capacities. We also report the isolation and sequencing of a low-copy number complementing gene for this mutant, *NPR2*. Although mutants affected in the post-transcriptional nitrogen permease regulator *NPR1* share a number of phenotypic characteristics with the BUH3 mutant, the sequences of the *NPR1* and *NPR2* genes are clearly not homologous. Two lines of evidence strongly suggest that *NPR2* is indeed the gene which is responsible for the BUH3 phenotype: (i) *NPR2* fully restores a wild-type phenotype in BUH3 when introduced on a low copy number plasmid; and (ii) a *npr2::URA3* strain and the BUH3 strain present the same growth defects.

The different phenotypic markers of BUH3 are complemented by the *NPR2* gene, although this gene has been isolated on its capacity to complement the urea growth deficiency. This confirms the notion that the permeases systems for multiple nitrogen sources are co-regulated. The NPR2 protein must be acting at a common step for all the affected permeases. On an other hand, the fact that the *NPR2* gene is not essential for growth on complete medium suggests that this step is specific for certain nitrogen sources.

The nucleotide sequence of the *NPR2* gene indicates that the protein is probably not highly expressed, with a codon bias index of 0.13. However, the *NPR2* mRNA is easily detected on Northern blots. It is noteworthy that the *NPR2* and the *CAN1* gene share a common 5′ untranscribed sequence, suggesting that they could also share functional regulatory sequences. Indeed, several copies of the *cis*-acting element UAS_{NTR}, mediating the allantoin metabolism *DAL* genes activation [9], are present upstream from the *CAN1* gene [7], and could be responsible for the transcriptional activation of the *NPR2* gene in proline/urea media.

The NPR2 protein is clearly not a trans-membrane carrier, as its hydropathy profile is not compatible with the formation of trans-membrane spanning helices. Several lines of evidence

suggest that it could be a nitrogen permeases regulatory protein, acting at a post-transcriptional level: (i) it is able to complement all the observed growth defects of the BUH3 mutant, which are highly probably a consequence of transmembrane transports defects, at least for urea and proline; (ii) it contains a characteristic PEST sequence, which are preferentially found in rapidly degraded regulatory proteins [19]; the active urea transport has indeed a short functional half-life, and PEST sequences are also found in the NPR1 protein; (iii) the amount of the *PUT4* mRNA coding for the proline permease and for the DUR3 active urea transport necessary protein are not decreased in the BUH3 mutant, showing that the defect is indeed post-transcriptional. Furthermore, there are two other analogies between NPR2 and NPR1: both are serine-rich proteins, and neither are under the transcriptional control of nitrogen catabolite repression. Thus, we propose to call this gene *NPR2*.

The fact that the level of the *DUR3* mRNA is strongly increased in the BUH3 mutant is puzzling. We cannot exclude that the over-expression of the *DUR3* gene could lead to a functional inactivation of the urea permease, suggesting that *NPR2* could in fact code for a transcriptional activator of *DUR3*. However, this hypothesis would not allow to explain the proline transport deficiencies of BUH3.

As there is no homology between NPR2 and other proteins, it is hard to speculate on its precise function. It could be involved in the putative NPR1-dependent phosphorylation of nitrogen permeases. It could also be implicated in the processing and targeting of the nitrogen permeases at the level of the endoplasmic reticulum, as demonstrated for the SHR3 protein [20].

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