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The Proline-Dependent Transcription Factor Put3 Regulates the Expression of the Riboflavin Transporter *MCH5* in *Saccharomyces cerevisiae*

Andrea Spitzner,[1] Angelika F. Perzlmaier,[2] Kerstin E. Geillinger,[3] Petra Reihl[4] and Jürgen Stolz[5]

Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Universitätsstraße 31, 93040 Regensburg, Germany

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ABSTRACT

Like most microorganisms, the yeast *Saccharomyces cerevisiae* is prototrophic for riboflavin (vitamin B_2). Riboflavin auxotrophic mutants with deletions in any of the *RIB* genes frequently segregate colonies with improved growth. We demonstrate by reporter assays and Western blots that these suppressor mutants overexpress the plasma-membrane riboflavin transporter *MCH5*. Frequently, this overexpression is mediated by the transcription factor Put3, which also regulates the proline catabolic genes *PUT1* and *PUT2*. The increased expression of *MCH5* may increase the concentrations of FAD, which is the coenzyme required for the activity of proline oxidase, encoded by *PUT1*. Thus, Put3 regulates proline oxidase activity by synchronizing the biosynthesis of the apoenzyme and the coenzyme FAD. Put3 is known to bind to the promoters of *PUT1* and *PUT2* constitutively, and we demonstrate by gel-shift assays that it also binds to the promoter of *MCH5*. Put3-mediated transcriptional activation requires proline as an inducer. We find that the increased activity of Put3 in one of the suppressor mutants is caused by increased intracellular levels of proline. Alternative *PUT3*-dependent and -independent mechanisms might operate in other suppressed strains.

MAMMALS depend on a dietary supply of riboflavin (vitamin B_2), which mostly derives from the flavoprotein cofactors FMN and FAD. These are deadenylated or dephosphorylated in the gut followed by the transport of free riboflavin across the mucosal membrane (Foraker *et al.* 2003). In contrast, although many microorganisms are dependent on various water-soluble vitamins, only few show a riboflavin auxotrophy (Koser 1968). This indicates that most microorganisms are capable of synthesizing riboflavin, a pathway, which starts with GTP and two molecules of ribulose-5-phosphate and is similar but not perfectly conserved in various species (Bacher *et al.* 2000). In the yeast *Saccharomyces cerevisiae*, which is an excellent dietary source of riboflavin (Bässler *et al.* 2002), the enzymes required for riboflavin synthesis are encoded by the genes *RIB1*, *RIB2*, *RIB3*, *RIB4*, *RIB5*, and *RIB7*. Both prokaryotic and eukaryotic microorganisms have been engineered to overproduce riboflavin and are used in industrial processes for riboflavin synthesis (Stahmann *et al.* 2000).

In addition to being able to synthesize riboflavin, single-celled organisms are also capable of taking up riboflavin from the culture medium. Because the riboflavin transport activities of most wild-type (wt) strains are low, most investigations were performed with riboflavin auxotrophic mutants. At least three different classes of riboflavin transporters exist in bacteria. These have been predicted by phylogenetic footprinting (Vitreschak *et al.* 2002) and functional data are now available for two proteins. RibU from *Lactococcus lactis* and *Bacillus subtilis* appear to work as very high-affinity transporters with five transmembrane domains (Cecchini *et al.* 1979; Burgess *et al.* 2006; Vogl *et al.* 2007). According to our analyses, RibU acts as an active riboflavin transporter in *B. subtilis*. Proteins of the RibM type are present in *Corynebacterium glutamicum* and *Streptomyces davawensis* (Grill *et al.* 2007; Vogl *et al.* 2007) and RibM from *C. glutamicum* acts as a facilitator when expressed in *Escherichia coli*. The third prototype bacterial riboflavin transporter, ImpX, has not been experimentally studied (Vitreschak *et al.* 2002). Yet another type of plasma-membrane riboflavin transporter is present in fungi. We used a multicopy suppressor screen of *S. cerevisiae* riboflavin auxotrophic strains to identify *MCH5*, the first known eukaryotic riboflavin transporter gene (Reihl and Stolz 2005). Riboflavin transport in yeast is not significantly stimulated by glucose or ethanol and not inhibited by proton ionophors, indicating that Mch5 acts as a facilitator.

[1]*Present address:* Institut für Biochemie, Universität Stuttgart, Pfaffenwaldring 55, 70569 Stuttgart, Germany.

[2]*Present address:* Lehrstuhl für Genetik, Universität Regensburg, Universitätsstraße 31, 93040 Regensburg, Germany.

[3]*Present address:* Lehrstuhl für Ernährungsphysiologie, Technische Universität München, Wissenschaftszentrum Weihenstephan, Am Forum 5, 85350 Freising-Weihenstephan, Germany.

[4]*Present address:* Landesbetrieb Hessisches Landeslabor, Standort Kassel, Druseltalstraße 67, 34131 Kassel, Germany.

[5]*Corresponding author:* Lehrstuhl für Ernährungsphysiologie, Technische Universität München, Wissenschaftszentrum Weihenstephan, Am Forum 5, 85350 Freising-Weihenstephan, Germany. E-mail: stolz@wzw.tum.de

Strains with a deletion of *MCH5* and of a *RIB* gene show synthetic growth defects and a reduced efficiency in catalyzing FAD-dependent cellular processes. Moreover, the expression of *MCH5* is regulated according to the riboflavin supply (Reihl and Stolz 2005). Most recently, a mammalian riboflavin transporter has been characterized, which again is not related to the previously known riboflavin transporters and was earlier mistaken as a G-protein coupled receptor (Yonezawa *et al.* 2008).

In the course of our experiments on the riboflavin transporter *MCH5* we noted that *rib* deletion strains segregate suppressor mutants with improved growth. A similar phenomenon was observed in a riboflavin auxotrophic strain of *Pichia guillermondii* (Boretsky *et al.* 2005). Here, we perform a detailed genetic and biochemical analysis of the *S. cerevisiae* mutants and find that the suppressor phenotype depends on the transcription factor *PUT3*. Put3 is hyperactive in the suppressor mutants, which results in increased expression of *MCH5* and other Put3 target genes on ammonium-containing media. Since Put3 also regulates the proline catabolic genes *PUT1* and *PUT2* (Brandriss and Magasanik 1979), this work establishes that riboflavin uptake is a part of the regulatory network that allows *S. cerevisiae* cells to use proline as the sole source of nitrogen.

MATERIALS AND METHODS

Yeast strains: Most experiments made use of the haploid strains BY4741, BY4742, or the diploid strain BY4743 (Brachmann *et al.* 1998). These and the haploid deletion strains *rib5*Δ, *put3*Δ, *mch1*Δ, *mch3*Δ, *mch4*Δ, and *mch5*Δ and the heterozygous diploid *rib3*Δ/*RIB3* and *rib7*Δ/*RIB7* strains were obtained from Euroscarf (Frankfurt/Main, Germany). Haploid *rib2*Δ, *rib3*Δ, and *rib7*Δ strains were generated from heterozygous diploids by sporulation and tetrad dissection on YPD plates with 200 mg/liter added riboflavin. *rib4*Δ strains were not included in the analysis because they are capable of riboflavin synthesis (Kis *et al.* 2001; Reihl and Stolz 2005). Alternatively, strains based on W303 (Thomas and Rothstein 1989) or CEN.PK113–13D (Makuc *et al.* 2001; Reihl and Stolz 2005) were used. The *PUT3*^{c} strain C74-6D (*Mat*a *his4-42 ura3-52 PUT3*^{c}*-683*) and the isogenic wild-type strain MB758-5B (*Mat*a *ura3-52*) were obtained from Marjorie C. Brandriss (Marczak and Brandriss 1989; Siddiqui and Brandriss 1989).

To generate double-deletion strains, *kanMX4* in *rib5*Δ was replaced with *his5*$^{+}$ from *Schizosaccharomyces pombe* using a restriction fragment from pFA6a-HIS3MX6 (Wach *et al.* 1994). The *rib5*Δ*::HIS3MX6* strain was mated to strains with the other desired deletion, followed by sporulation, tetrad dissection, and analysis of nutritional markers. *RIB1* was deleted in the BY strain background using a *rib1*Δ*::LEU2* disruption cassette, which inserted *LEU2* as a *Sca*I/*Bgl*II fragment into the natural *Eco*RV/*Bam*HI sites within *RIB1*, thus replacing 282 bp of the *RIB1* ORF. For one of the strains used in Figure 3E, *PUT3* was deleted in *rib5*Δ*::kanMX* using a *put3*Δ*::LEU2* deletion plasmid. In this construct, a 2602 bp *Hin*dIII/*Bam*HI fragment of *PUT3* was replaced with the *LEU2* marker gene. All genomic modifications were checked by PCR.

Yeast media: YPD medium (2% D-glucose, 2% bacto peptone and 1% yeast extract) and standard minimal medium (2% D-glucose, 0.67% yeast nitrogen base without amino acids, which contains 0.5% ammonium sulfate as nitrogen source) were prepared from compounds secured from Difco. According to flourimetric measurements (excitation wavelength 449 nm, emission wavelength 526 nm), YPD contains ~2 mg/liter riboflavin. For some experiments, YPD media were enriched by addition of riboflavin. The concentration of riboflavin in standard minimal medium is 0.2 mg/liter. To create minimal media with lower riboflavin concentrations, vitamin-free yeast nitrogen base without amino acids (BIO101) was supplemented with all vitamins except riboflavin, which was added from a 200 mg/liter stock solution. For media containing L-proline as the sole source of nitrogen, yeast nitrogen base without amino acids without ammonium sulfate (BIO 101) was supplemented with 0.85% L-proline. As a rule, only the required amino acids and nucleobases (adenine, histidin, methionine, tryptophan, and uracil: 20 mg/liter; lysine and leucine: 30 mg/liter) were added to minimal media. Media were solidified with 2% Difco bacto agar.

Isolation of *rib*Δ* suppressor mutants: To isolate *rib*Δ* suppressor mutants and to determine their frequency, 100 μl of a suspension of *S. cerevisiae* cells ($OD_{600} = 0.1$) were plated on YPD and the plate was incubated for 6 days at 30°. To determine the total amount of cells, the cell suspension was further diluted to $OD_{600} = 0.0001$ and 100 μl were plated on YPD containing 200 mg/liter riboflavin. The frequency of *rib*Δ* suppressor mutants was calculated as the number of colonies per 10^5 cells plated. *rib*Δ* mutants were purified by streaking on YPD followed by streaking on minimal medium containing 1 mg/liter riboflavin.

Plasmids: YCplac33 *PUT1-ZZ* was generated by PCR amplification of the *PUT1* ORF including 425 bp of promoter sequence, which included the Put3 binding site (−308 CGGCAATGGCTTTCCG −293) and lacked a stop codon. The product was digested with *Pst*I (partial digest to avoid cleavage of the internal site) and *Bam*HI and ligated into the same sites of the centromeric plasmid YCplac33-ZZ. This vector, based on YCplac33 (Gietz and Sugino 1988), extends the *PUT1* ORF with two copies of the IgG binding domain of *Staphylococcus aureus* protein A, which is followed by a stop codon and the *S. cerevisiae ADH1* terminator.

The plasmid to overexpress *PUT3* was based on pRS426 (Sikorski and Hieter 1989), into which a PCR fragment of genomic DNA including 236 bp of promoter and 275 bp of terminator sequence was ligated with blunt ends into the *Sma*I site.

To create a plasmid for the fluorimetric determination of the activity of the *MCH5* promoter, the entire promoter (−828 to −1 bp) was amplified from BY4741 genomic DNA, cut at the primer-encoded *Pst*I and *Bam*HI sites, and ligated into the multicopy plasmid YEplac195-GFP, which is a derivative of YEplac195 (Gietz and Sugino 1988). In this vector, the *MCH5* promoter drives the expression of a soluble form of GFP, whose translation is initiated at an ATG codon present in the primer to amplify the promoter. GFP starts with the amino acids MGSGRVGAGAGASKGEE (N-terminal extension underlined) and is followed by an *ADH1* terminator. The Put3 binding site within the promoter fragment (−501 CGGG GGTGGCTTCCCGA −486) was replaced by the sequence TAATTGAAGCTTCTTCT to produce plasmid YEplac195-MCH5prom mut-GFP.

Alternatively, we used a reporter plasmid that contained only the ORF-proximal Put3 binding site of *MCH5* in the UAS-less *MEL1* promoter from pMEL-β2 (Melcher *et al.* 2000). This plasmid was constructed in two steps. First, the *MEL1* minimal promoter containing a fragment of the *VHT1* pro-

moter (Pirner and Stolz 2006) was ligated into the *Pst*I and *Bam*HI sites of YEplac195-GFP. Next, the *VHT1* fragment was replaced with a PCR product containing the Put3 binding site (−501 to −486 bp relative to the start ATG of *MCH5*) along with 24 bp of upstream and 23 bp of downstream sequence. In the final product, the Put3 binding site lies in position −281 to −266 relative to the start ATG of GFP. A similar fragment amplified from YEplac195-MCH5prom mut-GFP was also used to replace the fragment containing *VHT1* sequences.

For sequencing, genomic DNA from *rib5*Δ*1* and *rib5*Δ*3* was used to amplify *PUT3* in two independent PCR reactions with high-fidelity Phusion DNA polymerase (New England Biolabs). The 3896-bp PCR products, which extended into the coding regions of the neighboring genes *ATP7* and *URB1*, were ligated with blunt ends into the *Sma*I site of pBluescript and sequenced.

Mch5 antiserum and Western blots: For immunization of rabbits, amino acids 3–106 from the hydrophilic N terminus of Mch5 were fused to the maltose-binding protein and produced as a soluble protein from vector pMAL-c2X in *E. coli* BL21(DE3) cells. The fusion protein was purified using amylose columns (New England Biolabs) and injected in rabbits in 2-week intervals until the serum detected specific signals in yeast cell extracts (Pineda Antikörper Service, Berlin). The serum was purified using a Mch5-GST fusion protein, which was produced from vector pGEX-2TK and contained the same N-terminal amino acids of Mch5. The Mch5-GST fusion was soluble after expression in *E. coli* and was affinity purified using glutathione sepharose 4B (GE Healthcare). Following purification it was immobilized on cyanogen bromide activated CH-sepharose 4B (GE Healthcare). Rabbit serum was diluted with PBS, loaded on the affinity column, and Mch5-specific antibodies were eluted with 200 mm glycine/HCl pH 2.9, followed by neutralization and dialysis against PBS.

Western blots were performed by transfer of gel-separated proteins to a nitrocellulose membrane, which was blocked and incubated with the primary and secondary antibodies as required. Put1-ZZ was detected in total cell extracts prepared by shaking 5 OD_{600} units of cells with glass beads in 100 μl SDS sample buffer in a FastPrep instrument (BIO 101). After heating the samples (2 min, 95°) and a brief centrifugation, 10 μl were loaded per lane. Mch5 was detected in total membrane preparations generated from 35 OD_{600} units of cells by centrifugation for 20 min at 20,000 × *g*. The membrane pellet was resuspended in 50 μl of 25 mm Tris/HCl, 5 mm EDTA, pH 7.5, diluted with SDS sample buffer, heated (2 min, 42°), and 10 μl were used per lane. After transfer, the nitrocellulose membrane was sequentially incubated with primary [rabbit polyclonal anti-Mch5 or rabbit polyclonal anti-HA (Santa Cruz, sc-805)] and peroxidase-linked secondary antibodies (Sigma A-6154) and developed with chemiluminescence reagents.

GFP as a reporter of promoter activity: *S. cerevisiae* cells containing reporter plasmids were grown in SD media containing 20 mg/liter riboflavin, diluted in media with 0.2 mg/liter riboflavin, and grown to log phase. The cells were washed twice with water and resuspended in water for $OD_{600} \approx 0.1$ in a 2.5 ml plastic cuvette. The fluorescence of the cells was measured in a Spex Industries FluoroMax-2 fluorescence spectrophotometer (excitation wavelength 488 nm, emission wavelength 512 nm) and corrected by subtracting the signal of cells lacking a GFP reporter plasmid. In parallel, the OD_{600} of the cell suspension was determined. Relative fluorescence units were calculated by dividing the corrected emission signal by the number of cells present in the cuvette assuming that a cell suspension of $OD_{600} = 0.1$ contains 10^6 cells/ml. Most measurements were performed in triplicate with three independent yeast cultures for calculation of means (represented by bars) and the standard deviation (represented by error bars). Values from single measurements are shown as columns lacking error bars.

Determination of proline: *S. cerevisiae* cells were grown to exponential phase, washed with water and 50 OD_{600} units of cells were frozen and stored at −80°. After thawing, the cells were suspended in 100 μl water and lysed with glass beads. The lysate was transferred to a fresh tube, the glass beads were washed with 2 × 100 μl water, and the fractions were pooled. After centrifugation to separate soluble from insoluble material, 40 μl of the supernatant were labeled with iTRAQ reagents (AA 45/32 kit, Applied Biosystems) as recommended by the manufacturer and analyzed on an Applied Biosystems 3200 Q TRAP LC/MS/MS system equipped with a RP-C18-column (150 mm length, 4.6 mm diameter, 5 μm particle size). The pellets containing insoluble cell components were dried at 60° and weighed. Proline concentrations are reported in micromole/gram insoluble cell material.

Gel-shift assays: Oligonucleotide Mch5-1 (ctcgagACAACG CACCTGTTATTATGATCTCGGGGGGTGGCTTCCCGAACAT CGTCGGTTGAATACTGCGgtcgag), putative Put3 binding site underlined, residues identical to the *MCH5* promoter [(nt −525 to nt −463) in uppercase] was purchased in a Cy5 labeled and unlabeled version and annealed to a complementary oligonucleotide. The annealed DNA was separated from single-stranded oligonucleotides by 12% polyacrylamide gel electrophoresis at 4°, excised from the gel, extracted in 10 mm EDTA 100 mm Tris/HCl, pH 7.5 overnight at 37°, and adjusted to 1–2 ng/μl with the same buffer. For protein extracts, 100 OD_{600} units of cells were resuspended in 25 mm Tris/HCl, pH 7.5, 5 mm EDTA, 1 mm PMSF and lysed with glass beads in a FastPrep instrument. Insoluble material was removed by centrifugation and the supernatant was stored in liquid nitrogen. Binding assays were performed by incubating 5 μl protein extract and 2 μl 5× gel binding buffer [20% glycerol, 5 mm $MgCl_2$, 2.5 mm EDTA, 2.5 mm DTT, 250 mm NaCl, 0.25 mg/mg poly(dI-dC), and 50 mm Tris/HCl (pH 7.5)] for 10 min on ice. Then, 1 μl of Cy5-labeled ds-DNA (1 ng/μl) and 2 μl H_2O were added and incubated for 20 min at 25°. Competition experiments contained 2 μl of unlabeled ds-DNA (2 ng/μl) for a fourfold excess instead of H_2O. Binding reactions were separated on a 5.3% acrylamide gel at 4°. The gel was scanned with a Typhoon Trio$^+$ imager (GE Healthcare) and data were loaded into Adobe Photoshop software for contrast enhancement and noise reduction.

RESULTS

Suppressor mutants do not bypass individual steps of riboflavin biosynthesis: *S. cerevisiae* strains with deletions that disable riboflavin biosynthesis (*rib*Δ strains) frequently segregate colonies with improved growth (Lesuisse *et al.* 2005; Reihl and Stolz 2005). These extragenic suppressor mutants, which we will refer to as *rib*Δ* mutants, appeared as colonies of variable sizes on YPD plates, where the majority of the cells were unable to grow (Figure 1A). These suppressed mutants occurred at an exceptionally high frequency in various strain backgrounds ($\sim 10^{-3}$–10^{-4}; Table 1).

To get the first idea of the molecular basis of this suppression, we analyzed a panel of different riboflavin biosynthetic mutants from the BY strain background. With the exception of *rib4*Δ, which is prototrophic for

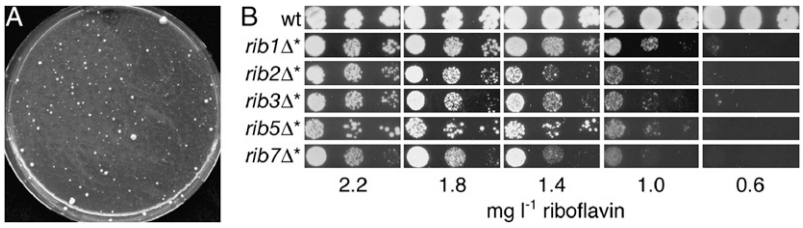

FIGURE 1.—Isolation and analysis of suppressor mutants. (A) Approximately 10^5 cells of the BY4742 *rib5*Δ deletion strain were plated on YPD and incubated for 6 days at 30°. Whereas the majority of the cells are unable to grow, some spontaneous suppressor mutants form colonies of various sizes. Similar analyses were performed for other strains and used to calculate the frequencies presented in Table 1. (B) Growth of purified *rib5*Δ* suppressor mutants on minimal medium containing the indicated concentrations of riboflavin. A riboflavin prototrophic wild-type strain (BY4742) was used as control. Nonsuppressed *rib5*Δ deletion strains require 20 mg/liter riboflavin (see Figure 3B).

riboflavin (KIS *et al.* 2001; REIHL and STOLZ 2005), all available *rib*Δ mutants were assayed. Most of the *rib*Δ strains segregated mutants with a frequency of 1–3 × 10^{-4} and the suppressors appeared with even higher frequency in *rib7*Δ (Table 1). This similar incidence of mutants in strains with different blocks in riboflavin biosynthesis made it unlikely that suppression was caused by reactions that bypass individual steps of riboflavin biosynthesis. This conclusion was supported by the analysis of double mutants that combined a deletion of *RIB5*, disabling the last step of riboflavin biosynthesis, with deletions abrogating earlier steps. The double knockouts segregated suppressed strains at similar rates as the single knockouts (Table 1). Taken together, a bypass of the riboflavin biosynthetic pathway is unlikely to account for the better growth of suppressed strains.

TABLE 1

Frequency of *rib*Δ* mutants in different strains

Relevant genotype	Suppressor mutants per 10^5 cells
BY4741 *rib1*Δ[a]	32 ± 6
BY4742 *rib1*Δ[a]	11 ± 4
*rib2*Δ	28 ± 9
*rib3*Δ	17 ± 7
BY4741 *rib5*Δ	22 ± 9
BY4742 *rib5*Δ	31 ± 4
*rib7*Δ	187 ± 75
*rib1*Δ *rib5*Δ	61 ± 16
*rib2*Δ *rib5*Δ	32 ± 5
*rib3*Δ *rib5*Δ	129 ± 16
*rib7*Δ *rib5*Δ	62 ± 23
*rib5*Δ/*rib5*Δ diploid	140 ± 26
W303-1A *rib5*Δ	102 ± 21
W303-1B *rib5*Δ	144 ± 33
BY4742 *rib5*Δ *mch1*Δ	16 ± 5
BY4742 *rib5*Δ *mch3*Δ	66 ± 7
BY4742 *rib5*Δ *mch4*Δ	173 ± 33
BY4742 *rib5*Δ *mch5*Δ	0[b]
CEN.PK *rib5*Δ	112 ± 21
CEN.PK *rib5*Δ *mch5*Δ	0[b]
CEN.PK *rib5*Δ *(mch1-5)*Δ	0[b]
BY4742 *rib5*Δ *put3*Δ	14 ± 8
BY4742 *rib5*Δ**1*/BY4741 *rib5*Δ diploid[c]	620 ± 72
BY4742 *rib5*Δ**3*/BY4741 *rib5*Δ diploid[c]	67 ± 11

A suspension containing ~500,000 *S. cerevisiae* cells of the given genotype were plated on five YPD plates without additional riboflavin. All colonies visible after 6 days at 30° were scored. Total cell counts were determined on YPD plates with added 200 mg/liter riboflavin. The values are means ± SD from five plates.

[a] Single or double mutants denoted BY4741 or BY4742 were derived from the respective parental strains by transformation with knockout cassettes. Double mutants lacking this designation were derived from BY4743 heterozygous diploids or from mating of deletion mutants in BY4741 and BY4742, followed by isolation of haploid progeny.

[b] The frequency of suppressor mutations is $<5 \times 10^{-5}$.

[c] Obtained by crossing.

We also analyzed the *rib*Δ* mutants for growth on minimal media containing defined amounts of riboflavin. Whereas wild-type cells grew on all plates, the suppressed mutants isolated from different *rib*Δ backgrounds showed no growth unless 1.0 mg/liter riboflavin were present (Figure 1B). Nonsuppressed *rib5*Δ cells required a riboflavin supplement of 20 mg/liter for growth (REIHL and STOLZ 2005), (also see Figure 3B). Thus, the *rib*Δ* mutants were riboflavin auxotrophs like their parents, but they possessed a reduced riboflavin requirement. We additionally found that suppressed strains were recovered with similar frequencies from haploid and homozygous diploid *rib5*Δ mutants (Table 1). At first sight, this indicated that suppression was caused by a dominant mutation. However, when we crossed two individual strains (*rib5*Δ**1* and *rib5*Δ**3*) to a nonsuppressed *rib5*Δ mutant, we found that the resulting diploids produced suppressed colonies at largely different rates (Table 1). In the diploid deriving from *rib5*Δ**3*, the frequency was similar to the spontaneous rate of suppressor mutations, indicating that *rib5*Δ**3* carried a recessive mutation. In the diploid deriving from *rib5*Δ**1*, the incidence of suppressed colonies was 10-fold higher but much lower than expected for a dominant mutation. The reason for this is not clear but it may indicate that the suppressor mutations in *rib5*Δ**1* and *rib5*Δ**3* are nonallelic. Thus, multiple genes may exist that when mutated give rise to the same phenotype and this may also account for the high frequency with which *rib*Δ* mutants occur.

Suppressor mutants overexpress the riboflavin transporter *MCH5*: The results presented above indicated that suppression of *rib*Δ mutants was not caused by a mechanism that created an alternative pathway for

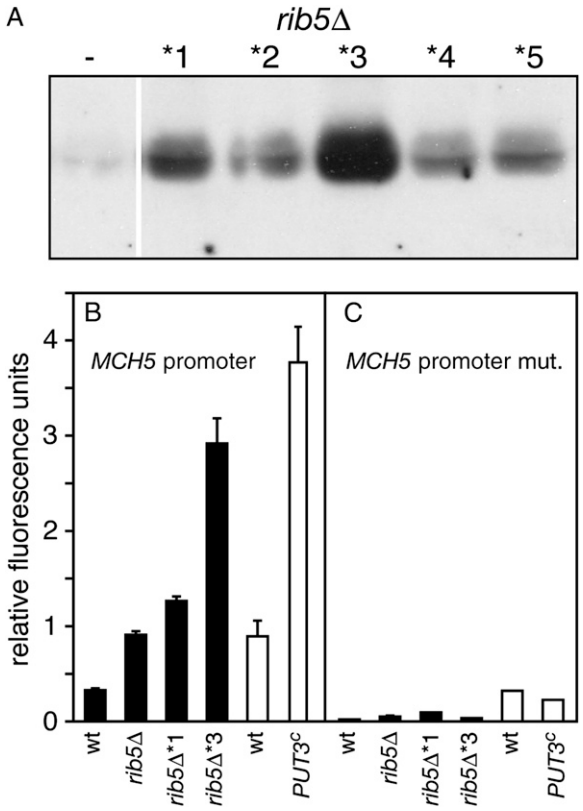

FIGURE 2.—Suppressor mutants overexpress *MCH5* and have increased amounts of Mch5. (A) Five independently isolated *rib*Δ* suppressor mutants were analyzed by Western blotting for the relative abundance of Mch5. A nonsuppressed *rib5*Δ strain was used as a control. (B) A reporter construct containing the full-length *MCH5* promoter fused to GFP was transformed into BY4742-based strains (solid bars) or into MB758-5B (wt) or an isogenic *PUT3*^{c} strain (open bars). Prior to the measurement, the cells were grown in media containing 0.2 mg/liter riboflavin for 6 hr and relative fluorescence units were determined as described in MATERIALS AND METHODS. (C) The same strains used in B carrying a similar plasmid lacking the Put3 binding site were subjected to GFP reporter assays.

riboflavin biosynthesis. We thus turned our attention to the plasma-membrane riboflavin transporter encoded by *MCH5*, which provides a second route to secure intracellular riboflavin. To analyze if the amount of Mch5 was changed, we prepared membrane protein extracts from *rib5*Δ and from five independently isolated suppressor mutants and analyzed them by Western blotting (Figure 2A). This showed that although the abundance of Mch5 was variable between individual *rib5*Δ* mutants, all had elevated levels of Mch5 when compared to a nonsuppressed *rib5*Δ strain (Figure 2A).

To analyze if this increased amount of Mch5 was caused by an increased expression of the *MCH5* gene, we generated a reporter plasmid in which the promoter of *MCH5* was fused to GFP. In intact cells carrying this plasmid, the activity of the *MCH5* promoter could be determined by fluorescence measurements. Using *lacZ* as a reporter we previously demonstrated that *MCH5* is more strongly expressed in *rib5*Δ cells (REIHL and STOLZ 2005). Measurements with a *rib5*Δ strain carrying the GFP reporter plasmid indicated a 2.8-fold induction relative to wild-type levels (Figure 2B), demonstrating that the GFP reporter is functional.

Fluorescence measurements were repeated with *rib5*Δ*1* and *rib5*Δ*3* that possessed the highest levels of Mch5 in the Western analysis (Figure 2A). Both mutants also showed increased levels of GFP relative to *rib5*Δ and, similar to the Western analysis, *rib5*Δ*1* had lower levels of activity than *rib5*Δ*3* (Figure 2B). Thus, the *rib5*Δ* mutants appear to overexpress *MCH5*. To check if *MCH5* is directly involved in the development of the suppressor phenotype, we analyzed if suppressor mutants can be derived from *rib5*Δ *mch5*Δ. Contrary to preliminary findings (REIHL and STOLZ 2005), we were not able to isolate suppressor mutants from this strain. These experiments were performed in the BY and CEN.PK genetic backgrounds (Table 1) but neither led to the identification of a single suppressed mutant. However, suppressed mutants appeared at normal rates when other *MCH* genes, that do not encode riboflavin transporters (REIHL and STOLZ 2005), were deleted in addition to *RIB5*. Moreover, a strain lacking all *MCH* genes and *RIB5*, similar to a strain lacking only *MCH5* and *RIB5*, did not segregate suppressed colonies (Table 1). Thus, *MCH5* is necessary for the development of the suppressor phenotype.

Proline induces the expression of *MCH5*: The promoter of *MCH5* contains two binding sites for the transcription factor Put3. Whereas the ORF-proximal site (−501 to −486) is well conserved in different Saccharomyces species (Figure 3A), the distant site (−1060 to −1045) is less conserved. A genomewide screen found Put3 bound to both sites *in vivo* (HARBISON *et al.* 2004). Put3 also binds to the promoters of *PUT1* and *PUT2*, the structural genes encoding proline oxidase and Δ^1-pyrroline-5-carboxylate dehydrogenase, respectively, and increases their expression when the inducer proline is present (DES ETAGES *et al.* 1996). Together, Put1 and Put2 convert proline to glutamate and are essential for growth when proline is the sole source of nitrogen. Because *PUT1* and *PUT2* are not sufficiently expressed in its absence, *PUT3* is also essential for growth when proline is the sole nitrogen source (BRANDRISS 1987).

To test if the Put3 binding site is functional in regulating *MCH5*, we made use of the fact that *rib5*Δ mutants show better growth on riboflavin-limited plates when *MCH5* is overexpressed (REIHL and STOLZ 2005). Indeed, growth of *rib5*Δ cells required 20 mg/liter riboflavin on ammonium plates but only 0.2 mg/liter riboflavin on proline (Figure 3B), indicating that the expression of *MCH5* is induced by proline. Similar to these observations, also *rib2*Δ, *rib3*Δ, *rib4*Δ, and *rib7*Δ mutants had a drastically reduced riboflavin requirement when the plates contained proline instead of

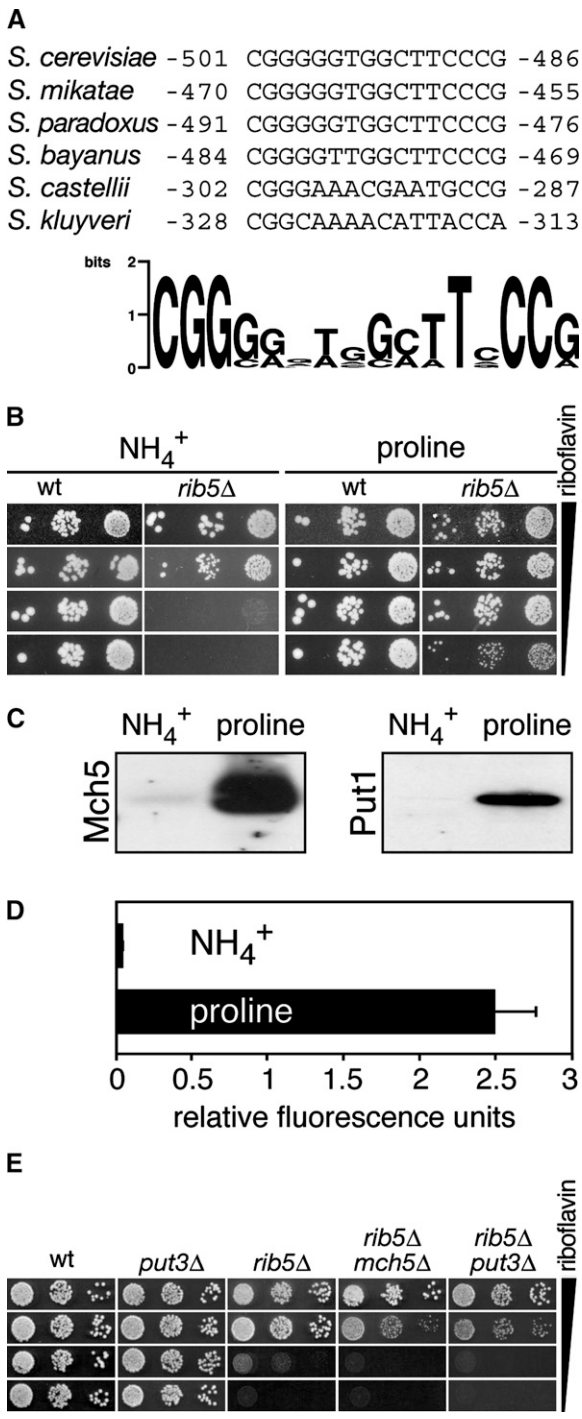

FIGURE 3.—Proline induces the expression of *MCH5*. (A) Alignment of the conserved Put3 binding sites from the *MCH5* promoters of various Saccharomyces species. The figure was generated using weblogo (http://weblogo.berkeley.edu/). (B) Growth of *rib5*Δ and a corresponding wild-type strain on minimal media containing proline or ammonium as sole nitrogen sources and various concentrations of riboflavin (from top to bottom: 200, 20, 2, or 0.2 mg/liter). Serial dilutions of cell suspensions were spotted and grown for 3 days at 30°. (C) Abundance of Mch5 and a Put1 after growth in proline or ammonium. Western blots were performed with the anti-Mch5 antibody or the anti-HA antibody (which binds to the ZZ-tag of Put1). *PUT1-ZZ* was expressed from a centromeric plasmid under control of its own promoter. Both strains were from the BY genetic background and prototrophic for riboflavin. (D) The Put3 binding site from the *MCH5*-promoter confers increased reporter activity in proline-grown cells. The GFP reporter construct containing the Put3 binding site from the *MCH5* promoter in a *MEL1* minimal promoter was transformed into BY4742 wild-type cells. The cells were continuously grown in media containing either ammonium or nitrogen as sole nitrogen source and GFP fluorescence was determined as described in MATERIALS AND METHODS. (E) *rib5*Δ *mch5*Δ and *rib5*Δ *put3*Δ double mutants show an increased requirement for riboflavin. Serial dilutions of cells of the indicated genotype were spotted on YPD plates to which various amounts of riboflavin were added. The additions were (from top to bottom) 200, 20, 2 mg/liter, or no riboflavin as indicated. The *rib5*Δ *mch5*Δ double mutant had the genotype *rib5*Δ*::HIS3MX6 mch5*Δ*::KanMX4*; the *rib5*Δ *put3*Δ double mutant had the genotype *rib5*Δ*::KanMX4 put3*Δ*::LEU2*.

ammonium (data not shown). These results were confirmed by Western blots that detected much more Mch5 in membrane protein extracts from proline-grown wild-type cells (Figure 3C). As expected, Put1 was also more abundant when cells were grown in proline rather than ammonium (Figure 3C).

Since *PUT3* is required for the growth on proline, we could not use *put3*Δ strains to demonstrate that Put3 is necessary for the proline-induced expression of *MCH5*. However, we constructed a plasmid containing the ORF-proximal Put3 binding site of the *MCH5* promoter to validate that the proline-induced expression of *MCH5* requires Put3. This construct is based on the minimal promoter of *MEL1* and is followed by the GFP reporter. Cells containing this plasmid had a 60-fold increased expression of GFP in proline relative to ammonium (Figure 3D). The strong induction of *MCH5* by proline is comparable to *PUT1*, which is induced 50-fold when proline is present (WANG and BRANDRISS 1987) and proves that the Put3 binding site mediates proline regulation of *MCH5*. These findings are also supported by transcriptome analyses in which increased expression of *MCH5* was found in proline-grown cells (BOER *et al.* 2007; GODARD *et al.* 2007).

To further support these data, we constructed strains with deletions of the *PUT3* gene and assayed their growth on YPD plates to which different amounts of riboflavin were added. On all plates, *put3*Δ was indistinguishable from wild type. *rib5*Δ possessed an increased requirement for riboflavin but showed full growth with riboflavin additions ≥20 mg/liter. In contrast, *rib5*Δ *put3*Δ was similar to *rib5*Δ *mch5*Δ. Both produced only small colonies with 20 mg/liter added riboflavin and required 200 mg/liter added riboflavin for full growth (Figure 3E). Together, these experiments confirm that Put3 is necessary for the expression of *MCH5* and that loss of either protein reduces growth on limiting riboflavin concentrations. In summary, the expression of the *MCH5* gene is strongly increased by proline and this depends on the transcription factor Put3.

Put3 binds to the promoter of *MCH5 in vitro* and the binding site is necessary and sufficient for the increased expression in *rib*Δ* mutants: We next addressed if the increased expression of *MCH5* in *rib*Δ* strains is mediated by Put3. To this end, we performed gel-shift assays with a fluorescently labeled DNA probe containing the Put3 binding site from the *MCH5* promoter. The DNA fragment was incubated with extracts from ammonium-grown wild-type cells, *put3*Δ mutants or from wild-type cells that carried *PUT3* on a multicopy plasmid. The extracts from *PUT3* overexpressing cells led to the formation of a protein-DNA complex with drastically reduced gel mobility that was undetectable in the other extracts (Figure 4A). Moreover, the intensity of this band decreased in the presence of a fourfold excess of the unlabeled probe (Figure 4B). Visualization of this binding event required neither cultivation of the cells in proline nor the addition of proline to the binding assay. However, overexpression of *PUT3* was required, indicating that wild-type cells contain only little free Put3. In conclusion, Put3 binds to a fragment of the *MCH5* promoter containing the conserved Put3 binding site *in vitro*.

To analyze the effect of Put3 binding on the transcriptional activity of *MCH5*, we deleted the Put3 binding site from the full-length *MCH5* promoter and repeated the GFP reporter assays. Upon deletion, the increased activity observed in *rib5*Δ and in the two *rib5*Δ* mutants was nullified (Figure 2C). Deletion of the Put3 binding site also reduced the promoter activity in wild-type cells. Thus, similar to *PUT1* and *PUT2* (Siddiqui and Brandriss 1989), Put3 appears to be necessary for the basal expression of *MCH5*. Similar results were obtained with the *MEL1* minimal promoter containing the conserved Put3 site from the *MCH5* promoter (Figure 4, C and D). In conclusion, Put3 binds to the conserved site in the *MCH5* promoter and this appears to be required for the improved growth of *rib*Δ* mutants.

The suppressor phenotype is caused by mutations that activate Put3: Consistent with the above results, we found that a *rib5*Δ *put3*Δ strain produced fewer suppressor mutants than any of the other *rib5*Δ strains analyzed except *rib5*Δ *mch5*Δ (Table 1). This demonstrates that while many suppressor mutations operate by activating Put3, other pathways exist that lead to suppression of *rib5*Δ.

Put3 is constitutively bound to DNA and becomes activated in the presence of proline (Axelrod *et al.* 1991; Sellick and Reece 2003). Binding of proline leads to a conformational change that is thought to increase the ability of Put3 to function as a transcriptional activator. Several dominant mutations in *PUT3* that cause constitutive activation of Put3 and increased expression of its target genes *PUT1* and *PUT2* in noninducing ammonium-containing media have been isolated and designated *PUT3*^{c} (Brandriss and Magasanik 1979; Marczak and Brandriss 1989, 1991). We deleted *RIB5*

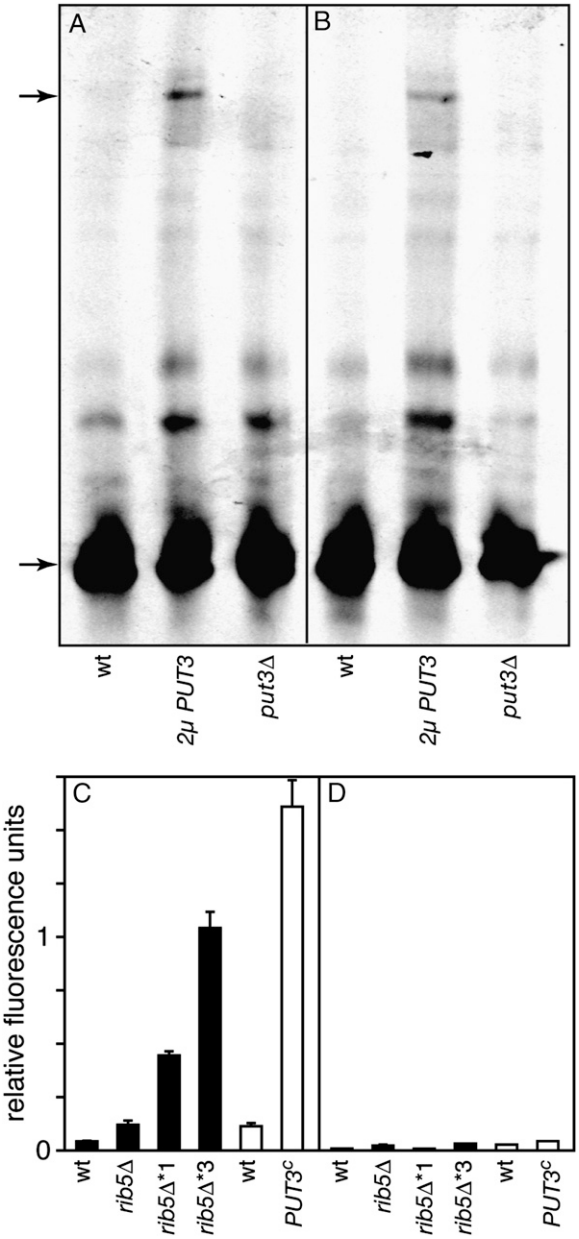

Figure 4.—Put3 binds to the promoter of *MCH5* and deletion of the binding site reduces its expression. (A and B) A double-stranded, Cy5-labeled DNA probe covering the Put3-binding site of the *MCH5* promoter was incubated with protein extracts from ammonium-grown cells. As indicated, these cells were either wild type, overexpressing *PUT3* from a multicopy plasmid (2μ *PUT3*), or *put3*Δ mutants. The binding reactions were analyzed by nondenaturing polyacrylamid gel electrophoresis, followed by fluorescence scanning. In B, a fourfold excess of the unlabeled probe was present during the binding assay. The positions of the free probes and of the bands appearing in cells overexpressing *PUT3* are marked with arrows. (C) A GFP reporter construct containing the Put3 binding site from the *MCH5* promoter in a *MEL1* minimal promoter was transformed into BY4742-based strains (solid bars), or into MB758-5B (wt), or an isogenic *PUT3*^{c} strain (open bars). The cells were grown in media containing 0.2 mg/liter riboflavin for 6 hr and GFP fluorescence was determined as described in materials and methods. (D) The same strains used in C carrying a similar plasmid lacking the Put3 binding site were subjected to GFP reporter assays.

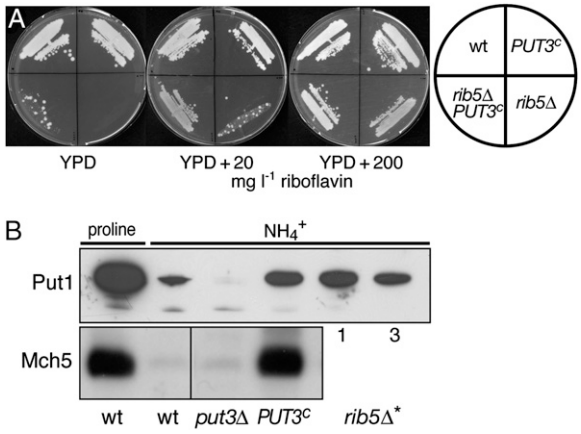

FIGURE 5.—Similarity of *PUT3*^{c} and *rib5*Δ* mutants. (A) Cells of the indicated genotype were streaked on YPD plates containing various amounts of added riboflavin. Growth was recorded after 5 days at 30°. (B) Cells of the indicated genotype were grown in media containing proline or ammonium as the sole nitrogen source. Put1 was detected in total cell extracts using the C-terminally fused ZZ tag and Mch5 was detected with the specific antiserum. For details, see legend to Figure 3C; for expression of *MCH5* in *rib5*Δ*1* and *rib5*Δ*3*, see Figure 2A.

in a *PUT3*^{c} strain to compare the growth properties of the double mutant to *rib5*Δ and *PUT3*^{c} single mutants. As expected, *PUT3*^{c} was similar to wild type and was able to grow without added riboflavin. The *rib5*Δ deletion strain was unable to grow on YPD, displayed numerous faster growing colonies (likely spontaneous suppressor mutants) on YPD plus 20 mg/liter riboflavin, and showed full growth when more riboflavin was present (Figure 5A). On all plates used, *rib5*Δ *PUT3*^{c} showed improved growth relative to *rib5*Δ cells. Thus, the growth properties of *rib5*Δ *PUT3*^{c} are similar to *rib5*Δ*.

When transformed with reporter plasmids containing either the full-length *MCH5* promoter (Figure 2B) or only the Put3 binding site from *MCH5* (Figure 4A), the *PUT3*^{c} strain displayed much higher levels of GFP fluorescence than an isogenic strain with a normal *PUT3* allele. For both types of reporter plasmids, the increased fluorescence in *PUT3*^{c} was abolished after mutation of the Put3 binding site (Figures 2C and 4B).

To find independent proof that the Put3 transcription factor is hyperactive in *rib5*Δ* mutants we analyzed the expression of the Put3 target *PUT1* in Western blots. Whereas no signal was obtained in *put3*Δ strains, *PUT1* was weakly expressed in wild-type strains grown in ammonium media and highly expressed when proline was the nitrogen source. The expression of *PUT1* in ammonium media was increased in the *PUT3*^{c} strain and a similar increase was also seen in *rib5*Δ*1* and *rib5*Δ*3* suppressor mutants (Figure 5B). Membrane protein extracts from the same cells were also analyzed for Mch5, whose abundance was increased in proline-grown wild-type cells and *PUT3*^{c} cells grown in ammonium (Figure 5B). We have shown before that *rib5*Δ*1* and *rib5*Δ*3* cells possessed increased levels of Mch5 relative to wild-type cells (Figure 2A). Taken together, *rib5*Δ* strains are similar to strains that carry a hyperactive allele of *PUT3*. Both overexpress *MCH5*, which leads to improved growth of riboflavin auxotrophic mutants.

TABLE 2

Intracellular proline concentrations in different strains

Genotype	Proline concentration (μmol/gram)	Proline concentration (%)
Wild type	22.4 ± 8.8	100
*rib5*Δ	14.7 ± 1.5	65.6
*rib5*Δ*3*	52.0 ± 8.8	232[a]

S. cerevisiae strains were grown in media containing 20 mg/liter riboflavin and 100 OD_{600} units of cells were used for the determination of the cellular proline concentrations. Values are reported as micromole proline per gram of insoluble cell material, which remained after lysis and centrifugation of the cells and mostly consists of cell walls.

[a] Significant ($P < 0.05$, *t*-test) increase relative to the proline content of *rib5*Δ cells.

Reasons for the increased activity of Put3: Many reasons could account for the increased activity of Put3 in *rib5*Δ* suppressor mutants. The phenotypic similarity of *rib5*Δ* suppressor mutants to *PUT3*^{c} mutants prompted us to first look for mutations in the *PUT3* gene from *rib5*Δ*1* and *rib5*Δ*3*. However, the sequence reads were fully identical with the published sequence of this region.

A priori, overproduction of Put3 alone could also lead to a suppressor phenotype. However, this explanation was unlikely because we did not find *PUT3* as a multicopy suppressor of *rib4*Δ and *rib5*Δ mutants in the genetic screen in which 14 *MCH5*-containing plasmids were isolated (REIHL and STOLZ 2005). Indeed, Western blots to detect Put3 expressed from a genomically integrated tagged allele (*PUT3*-3HA) demonstrated equal levels of Put3 in wild-type cells, *rib5*Δ, *rib5*Δ*1*, and *rib5*Δ*3* (data not shown).

Since the activity of Put3 requires proline as an inducer, we also analyzed if the cellular proline content was increased. This experiment was only performed with *rib5*Δ*3* because the *rib5*Δ*1* suppressor mutant used in the above experiments was genetically unstable and had lost some of its characteristics. While still able to grow on YPD without added riboflavin, it had lost the elevated expression of *MCH5* and of GFP reporter plasmids. This likely originated from the accumulation of additional unknown mutations. The phenotype of *rib5*Δ*3*, in contrast, was stable and we found that these cells contained more than twofold increased proline levels compared to wild-type cells. Relative to *rib5*Δ, the proline content was >3.5-fold higher in *rib5*Δ*3* (Table 2). Thus, the higher activity of Put3 in *rib5*Δ*3* was likely due to its higher proline content, which led to an

increased expression of *MCH5*. The finding that a proline-accumulating *S. cerevisiae* mutant (*put1-54 pro1-D154N*, a gift from Hiroshi Takagi) also displayed elevated levels of Mch5 in Western blots (data not shown) makes this hypothesis all the more appealing.

The analysis of suppressor mutants that spontaneously arise in yeast strains with defects in riboflavin biosynthesis thus led to the surprising finding that the expression of the riboflavin transporter gene *MCH5* is regulated by proline via the transcription factor Put3. Whereas *MCH5* is normally not highly expressed in ammonium-containing media, its expression is increased by unknown mutations that activate the transcription factor Put3. These mutations do not lie within *PUT3* but at least some of them seem to raise the intracellular concentration of proline, which is the inducer of Put3. Further experiments will be necessary to identify the mechanism of proline accumulation in *rib5Δ*3* and to resolve the basis of suppression in other mutants.

DISCUSSION

The *S. cerevisiae* Put3 protein is a dimeric Zn_2 Cys_6 transcription factor that is required both for the basal as well as the proline-induced transcription of *PUT1* and *PUT2* (Marczak and Brandriss 1991; Todd and Andrianopoulos 1997; Huang and Brandriss 2000; Sellick and Reece 2005). The N-terminal domain of Put3 binds to the DNA motif CGGNANGCNANNNC CGA *in vivo* and *in vitro* and independent of the presence or absence of proline (Todd and Andrianopoulos 1997). This domain is followed by a coiled-coil domain that mediates dimer formation and a C-terminal acidic domain that acts as a transcriptional transactivator (des Etages *et al.* 1996; Swaminathan *et al.* 1997; Walters *et al.* 1997). Binding of proline to Put3 leads to a conformational change that is thought to expose the transactivation domain (Axelrod *et al.* 1991; des Etages *et al.* 1996, 2001). Binding sites for Put3 are found in the promoters of *PUT1* and *PUT2*, encoding enzymes involved in proline degradation, but also in *MCH5*, the *S. cerevisiae* gene encoding the plasma-membrane transporter for riboflavin. Riboflavin-auxotrophic *S. cervisiae* mutants frequently segregate suppressed strains with improved growth on ammonium-containing media. The frequency of suppressor mutants is independent of the underlying defect in the riboflavin biosynthetic pathway, reduced in cells lacking *PUT3*, and further reduced in strains lacking *MCH5*. On ammonium-containing media, Mch5 and Put1 were more abundant in suppressed strains and in a mutant carrying a constitutively active (proline-independent) allele of *PUT3*. These assays were confirmed by reporter assays using the full-length *MCH5* promoter or fragments thereof harboring the Put3 binding site. Moreover, we demonstrate that Put3 binds to a fragment of the *MCH5* promoter *in vitro*. In summary, this led to the finding that the riboflavin transporter gene *MCH5* is regulated by Put3.

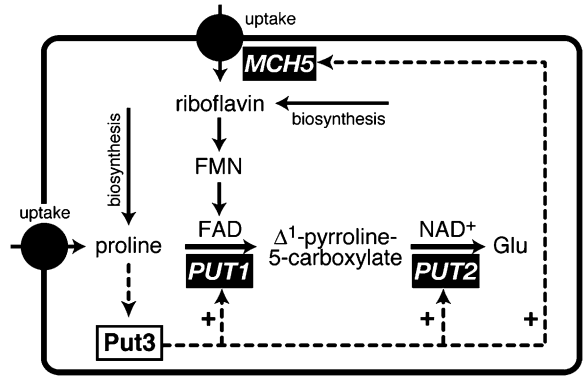

Figure 6.—Regulation of proline degradation in *Saccharomyces cerevisiae*. The scheme illustrates the pathway of proline catabolism in *S. cerevisiae*. Intracellular proline activates the transcription factor Put3, which acts as a positive regulator (dashed arrows) of the proline catabolic genes *PUT1* (encoding proline oxidase) and *PUT2* (encoding Δ^1-pyrroline-5-carboxylate dehydrogenase). This work demonstrates that Put3 also regulates *MCH5*, the plasma-membrane riboflavin transporter. Intracellular riboflavin is necessary for the generation of FAD, the catalytic cofactor required by Put1. For clarity, details of the subcellular compartmentation of these pathways were omitted, as were the pathways of proline and riboflavin biosynthesis. At least four amino acid permeases contribute to proline uptake in *S. cerevisiae* (Andreasson *et al.* 2004).

Most yeast media formulations contain ammonium ions as these are the preferred nitrogen source of *S. cerevisiae* (Godard *et al.* 2007). In their natural habitats (*e.g.*, on grapes), yeasts mostly encounter the amino acids arginine and proline, which are poorer sources of nitrogen (des Etages *et al.* 1996; Boer *et al.* 2007; Godard *et al.* 2007). We have demonstrated above that proline strongly increases the expression of the riboflavin transporter gene *MCH5* and that this effect is mediated by Put3. In batch cultures, no other genes besides *PUT1*, *PUT2*, and *MCH5* are significantly induced by proline, whereas in chemostat cultures, a group of 26 genes that also includes *MCH5* is induced (Boer *et al.* 2007; Godard *et al.* 2007). Why are riboflavin transport and proline degradation coregulated processes? *PUT1* encodes proline oxidase, an enzyme that creates Δ^1-pyrroline 5-carboxylate, which is further metabolized by Put2 to produce glutamate (Figure 6). Proline oxidase is localized to the inner mitochondrial membrane and contains the cofactor FAD, which derives from riboflavin. To increase proline oxidase activity, the cells have to increase the production of the Put1 apoprotein and also provide more FAD to generate the enzymatically active holoform of Put1. The data presented here suggest that the extra riboflavin required for the activity of Put1 derives from uptake via Mch5 and not from biosynthesis. Possibly, proline and riboflavin occur together in the natural habitats of yeast, making it highly

economical to use external sources of riboflavin to facilitate proline degradation (Figure 6). Thus, the increase in proline oxidase activity is coordinated by Put3, which synchronizes the formation of apo-proline oxidase with the formation of the coenzyme FAD. However, even when proline is used as the sole source of nitrogen, phenotypes associated with a lack of *MCH5* can only be observed in *rib* mutants. The likely reason for this is that, in laboratory growth conditions, intracellular riboflavin largely derives from biosynthesis. We speculate, however, that the Put3-mediated control of *MCH5*, *PUT1*, and *PUT2* may have biological significance for wild-type cells in natural habitats. The fact that regulation of *MCH5* by proline can be observed in wild-type cells (Figures 3C and 5B) and that the Put3 binding site in the *MCH5* promoter is evolutionary conserved (Figure 3A) suggests that it is more than an esoteric twist of nature.

In the absence of *MCH5*, no suppressed strains could be isolated. Thus, activation of *MCH5* appears to be the basis for the suppressor mutants that develop in riboflavin auxotrophic strains. The frequency of suppressor mutants was reduced in the absence of Put3, indicating that many suppression events were caused by the aberrant activity of Put3 in ammonium-containing media. For one of the suppressor mutants we found that the cells contained increased levels of proline, a known inducer of Put3. Accumulation of proline, for example, can be caused by dominant alleles of *PRO1*. This gene encodes the first enzyme in the proline biosynthetic pathway and the mutated form of the protein is insensitive to feedback inhibition by proline (Sekine *et al.* 2007). In other suppressed strains, however, other mutations may activate Put3. These mutations may cause proline-independent activity of Put3 (similar to *PUT3*^{c}), alter the phosphorylation of Put3, or lead to the accumulation of other metabolites that activate Put3 (Sellick and Reece 2003). Put3 is hyperphosphorylated in the presence of poor nitrogen sources and full activation requires both the presence of the inducer proline and the absence of good nitrogen sources (Huang and Brandriss 2000). Consistent with this, *PUT1* and *PUT2* are much higher expressed in nitrogen starvation (Godard *et al.* 2007), or in the presence of rapamycin (Saxena *et al.* 2003), conditions that do not affect the expression of *PUT3* (Axelrod *et al.* 1991; des Etages *et al.* 2001). The pathways that govern the phosphorylation of Put3 are not resolved, but mutations in these might lead to overexpression of *MCH5*.

Whatever the reason for the suppression in individual strains might be, further work with the suppressor mutants is difficult and hampered by their genetic instability. This fact, as well as the initial observation that suppressor mutants arise in all *rib*Δ strains with an extraordinary high frequency, might be related to an additional function served by the riboflavin biosynthetic pathway. It is known for *E. coli* that riboflavin biosynthesis contributes to the removal of 8-oxo-GTP, a spontaneous oxidation product of GTP, which is a mutagenic substrate for DNA synthesis, and that the expression level of *ribA* (encoding GTP cyclohydrolase II, the first enzyme of the riboflavin biosynthetic pathway), correlates with the rate of spontaneous mutations (Kobayashi *et al.* 1998). Although yeasts, like other organisms, have multiple pathways to sanitize oxidized nucleotides (Nunoshiba *et al.* 2004; Ishchenko *et al.* 2005) the high mutation rate in all *rib*Δ mutants suggests that riboflavin biosysnthesis also serves this purpose. The rare incidence of riboflavin auxotrophic microorganisms (Koser 1968) might also reflect this additional function of riboflavin biosynthesis, which in contrast to the generation of the flavin cofactors, cannot be satisfied by exogeneous riboflavin. Because the *rib5*Δ* suppressor mutants characterized here are still defective in riboflavin biosynthesis, they are also likely to have increased amounts of 8-oxo-GTP, which results in continued genetic instability. In support of this possible function of the riboflavin biosynthetic pathway, *P. guilliermondii rib1* mutants also display increased rates of spontaneous mutations and develop more canavanine and 5-fluoro orotic acid resistant clones than wild-type strains (Boretsky *et al.* 2005).

In summary, our analysis of spontaneous suppressor mutants arising in riboflavin auxotrophic strains has led to the provocative finding that the riboflavin transporter gene *MCH5* is regulated by proline via the transcription factor Put3. We provide strong evidence that the transcriptional activation of *MCH5* by Put3 forms the molecular basis of the suppression mechanism that improves the growth of *rib* mutants on ammonium-containing media.

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