Yeast GMP Kinase Mutants Constitutively Express AMP Biosynthesis Genes by Phenocopying a Hypoxanthine-Guanine Phosphoribosyltransferase Defect

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ABSTRACT

We have characterized a new locus, *BRA3*, leading to deregulation of the yeast purine synthesis genes (*ADE* genes). We show that *bra3* mutations are alleles of the *GUK1* gene, which encodes GMP kinase. The *bra3* mutants have a low GMP kinase activity, excrete purines in the medium, and show vegetative growth defects and resistance to purine base analogs. The *bra3* locus also corresponds to the previously described *pur5* locus. Several lines of evidence indicate that the decrease in GMP kinase activity in the *bra3* mutants results in GMP accumulation and feedback inhibition of hypoxanthine-guanine phosphoribosyltransferase (HGPRT), encoded by the *HPT1* gene. First, *guk1* and *hpt1* mutants share several phenotypes, such as adenine derepression, purine excretion, and 8-azaguanine resistance. Second, overexpression of *HPT1* allows suppression of the deregulated phenotype of the *guk1* mutants. Third, we show that purified yeast HGPRT is inhibited by GMP *in vitro*. Finally, incorporation of hypoxanthine into nucleotides is similarly diminished in *hpt1* and *guk1* mutants *in vivo*. We conclude that the decrease in GMP kinase activity in the *guk1* mutants results in deregulation of the *ADE* gene expression by phenocopying a defect in HGPRT. The possible occurrence of a similar phenomenon in humans is discussed.

MICROORGANISMS modify their metabolism in response to environmental changes. When metabolic precursors are present in the extracellular medium, yeast and bacteria generally use these precursors instead of synthesizing them *de novo*. Consequently, the synthesis of metabolic enzymes is regulated according to the presence of metabolites in the medium.

Such a regulatory mechanism exists for the purine biosynthesis pathway in *Saccharomyces cerevisiae*. Indeed, all the genes encoding enzymes required for *de novo* AMP biosynthesis are repressed at the transcriptional level by the presence of extracellular purines (adenine or hypoxanthine) (Mäntsälä and Zalkin 1984; Giani *et al.* 1991; Daignan-Fornier and Fink 1992; Denis *et al.* 1998). This regulation process requires two transcription factors, Bas1p and Bas2p (Daignan-Fornier and Fink 1992), and regulation by extracellular purines has been proposed to occur through interactions between these two factors (Zhang *et al.* 1997).

To gain an insight into the signal transduction pathway between extracellular adenine and the transcription factors, we have isolated mutants in which purine biosynthesis genes are no longer repressed by extracellular adenine (Guetsova *et al.* 1997). These mutants are termed *bra* for bypass of repression by adenine. The identification of some of these mutants has shown that the repression effect of adenine requires the purine base to enter the cell, *i.e.*, *bra7* mutants are alleles of the *FCY2* gene that codes for the purine cytosine permease (see Figure 1; Guetsova *et al.* 1997). Adenine itself is not the effector molecule since it has to be metabolized into AMP and then ADP to exert its regulatory effect. There are two possible routes for the synthesis of AMP from adenine: a direct one catalyzed by adenine phosphoribosyltransferase (APRT) and a more indirect one requiring four enzymatic steps via the formation of hypoxanthine and IMP (see Figure 1). The APRT route does not seem to play a major role in the repression process since disruption of *APT1*, the APRT encoding gene, had no effect on adenine repression (Guetsova *et al.* 1997). We found that mutations in the genes encoding hypoxanthine-guanine phosphoribosyltransferase (HGPRT), adenylosuccinate synthase (Ade12p), and adenylosuccinate lyase (Ade13p) abolished the repression signal (*BRA6*, *BRA9*, and *BRA1* are *HPT1*, *ADE12*, and *ADE13*, respectively; Guetsova *et al.* 1997; see Figure 1). The major route for the repression signal from adenine to AMP is thus via the formation of hypoxanthine and IMP. In addition, we have shown that AMP needs to be phosphorylated into ADP to exert its regulatory role (Guetsova *et al.* 1997).

The *bra* mutants define more than 10 complementation groups, indicating that the regulation process is complex and requires several proteins. To identify a new partner in the signal transduction pathway, we have now characterized the *bra3* complementation group. We show that *BRA3* is *GUK1*, an essential gene encoding

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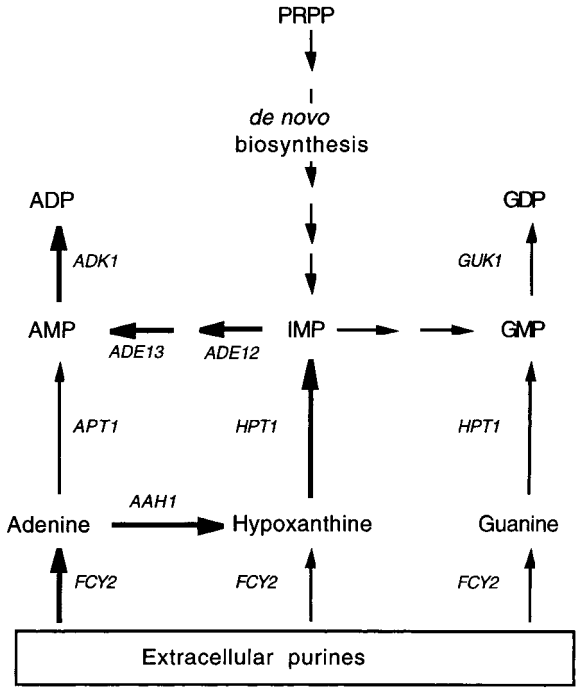

FIGURE 1.—Scheme of purine interconversion in yeast. The following abbreviations are used: PRPP, 5-phosphoribosyl-1-pyrophosphate; IMP, inosine 5′-monophosphate; GMP, guanosine 5′-monophosphate; GDP, guanosine 5′-diphosphate. Gene names are indicated in italic and encode the following enzymatic activities: *AAH1*, adenine deaminase; *ADE12*, adenylosuccinate synthetase; *ADE13*, adenylosuccinate lyase; *ADK1*, AMP kinase; *APT1*, adenine phosphoribosyltransferase; *FCY2*, purine cytosine permease; *GUK1*, GMP kinase; *HPT1*, hypoxanthine-guanine phosphoribosyltransferase. The thick arrows represent the major route for the repression signal by adenine. For the purpose of simplification, nucleosides are not represented.

GMP kinase. We document several new phenotypes associated with the GMP kinase defect and present evidence that the *guk1* mutations result in a phenocopy of *hpt1*. The possible implications for human diseases associated with purine overexpression and uric acid excretion are discussed.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains are listed in Table 1. Yeast media were prepared according to SHERMAN *et al.* (1986). Adenine and hypoxanthine were used at a final concentration of 0.15 mM. The XGal synthetic medium was prepared using the methods previously described (DANG *et al.* 1994). The base analog 8-azaguanine (8AG) was added to the medium at a final concentration of 0.2 mg/ml.

Plasmids: pCG3 (DEELEY 1992) is a YEp13 (BROACH *et al.* 1979) derivative carrying the *APT1* gene.

P556, the 2μ plasmid carrying the *HPT1* gene, was obtained by ligating the *Eco*RI-*Hin*dIII fragment from P385 that contains the *HPT1* gene (GUETSOVA *et al.* 1997) into the multicopy plasmid YEpLac181 (GIETZ and SUGINO 1988) linearized with *Hin*dIII and *Eco*RI.

P195, the plasmid carrying the *GUK1* wild-type gene, was constructed by insertion of the *Hin*dIII-*Bgl*II fragment from the pGUK1 plasmid (KONRAD 1992) into a centromeric *LEU2* vector named pRS315 (SIKORSKI and HIETER 1989) linearized with *Hin*dIII and *Bam*HI.

The P1718 plasmid expressing the Hpt1p-His_6 fusion in bacteria was constructed as follows: a 684-bp fragment carrying the *HPT1* coding sequence was amplified by PCR using the following synthetic oligonucleotides: HPT1Ca, 5′-GTGATG CATATGTCGGCAAACGATAAGC-3′ and HPT1Cb, 5′-CGAT GCTCGAGATTGCTTGTGTTCCTGCTC-3′. The PCR product was cut with *Nde*I and *Xho*I and introduced into a pJC20-HisC expression plasmid linearized with *Nde*I and *Xho*I. The resulting plasmid encodes a 26.7-kD Hpt1p-His_6 fusion protein. The pJC20-HisC vector was generated by introducing a double-stranded oligonucleotide linker into pJC20 (KONRAD 1993) restricted with *Bam*HI and *Apa*I: 5′-GATCC CAT CAC CAT CAC CAT CAC TGA GGGCC-3′ (sense) and 5′-C TCA GTG ATG GTG ATG GTG ATG G-3′ (antisense).

***LacZ* fusions and βGal assays:** The *lacZ* fusions used in this study have been previously described (DAIGNAN-FORNIER and FINK 1992; GUETSOVA *et al.* 1997). P115 is a plasmid carrying an *ADE1-lacZ* fusion in a 2μ *URA3* vector YEp356R (MYERS *et al.* 1986). P473 is a plasmid carrying an *ADE1-lacZ* fusion in a 2μ *LEU2* vector YEp367 (MYERS *et al.* 1986).

βGal assays were performed as described by KIPPERT (1995) on cells grown for 6 hr in the presence or absence of purine base. βGal units are defined as:

$$[OD_{420} \times 1000]/[OD_{600} \times t(\text{min}) \times \text{vol(ml)}] .$$

The repression factor is defined as the ratio of βGal units measured in the absence of purine to those measured in the presence of purine. In each experiment, at least two independent βGal assays were performed, and each assay was done on three independent transformants. The variation between assays in each experiment was <20%.

Integration of *LEU2* at the *GUK1* locus: A *Hin*dIII-*Xba*I fragment carrying the *GUK1* gene from plasmid P195 was cloned into the pRS305 integrative *LEU2* vector (SIKORSKI and HIETER 1989) linearized with *Hin*dIII and *Xba*I. The resulting plasmid named P1015 was linearized at the unique *Stu*I site in the *GUK1* coding region and used to transform the Y642 yeast strain. Correct integration of the plasmid at the *GUK1* locus was verified by Southern blot analysis of genomic DNA extracted from tranformants and cut with *Bgl*II (data not shown). One of these transformants named Y882 was used for linkage analysis.

AMP and GMP kinase enzymatic assays: AMP and GMP kinase activities in protein extracts were measured using a spectrophotometric assay in a coupled lactate dehydrogenase/pyruvate kinase system according to the method of AGARWAL *et al.* (1978). Briefly, yeast strains were grown in 20 ml of rich YPD medium to an OD_{600} of 0.75 ± 0.05. Cells were harvested, washed with water, and resuspended in 0.6 ml breaking buffer (20 mM TrisHCl pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol (DTT), 0.3 M ammonium sulfate, and 2 mM phenylmethylsulfonyl fluoride). The cells were then broken with glass beads by vortexing four times for 30 sec in the cold. After addition of 0.2 ml of breaking buffer, glass beads and unbroken cells were pelleted in a microfuge for 5 min and 0.5 to 10 μl of the supernatant was used for the enzymatic assay. The assay was done in 100 mM TrisHCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.25 mM NADH, 0.5 mM PEP, 2 mM ATP, 5 units lactate dehydrogenase (Sigma, St. Louis), and 4 units pyruvate kinase (Sigma) in a final volume of 1 ml. Finally, 1 mM AMP or GMP was added to the reaction mix, depending on which nucleotide kinase activity was tested. Conversion of AMP to ADP by AMP kinase or that of GMP to GDP by GMP kinase was monitored by the decrease of

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
PLY121[a]	*MAT*α *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52*	P. Lunjdall
129[a]	*MAT*α *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 bra3-1 (=guk1-1)*	B. Daignan-Fornier
130[a]	*MAT*α *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 bra3-2 (=guk1-2)*	B. Daignan-Fornier
PLY122[a]	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52*	P. Lunjdall
220[a]	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 bra3-3 (=guk1-3)*	B. Daignan-Fornier
239[a]	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 bra3-4 (=guk1-4)*	B. Daignan-Fornier
L4364[a]	*MAT***a** *ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52*	G. Fink
Y508[a]	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 hpt1::URA3*	B. Daignan-Fornier
Y642[a]	*MAT*α *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 trp1::hisG*	B. Daignan-Fornier
Y663[a]	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 trp1::hisG aah1::URA3*	B. Daignan-Fornier
Y882[a]	*MAT***a** *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 trp1::hisG GUK1::LEU2-GUK1*	This work
Y911[a]	*MAT***a** *ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52*	This work
Y912[a]	*MAT***a** *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 guk1-2*	This work
Y913[a]	*MAT*α *ade2 his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52 guk1-2*	This work
Y914[a]	*MAT*α *his3-Δ200 leu2-3,112 lys2-Δ201 ura3-52*	This work
Y915[a]	*MAT*α *leu2-3,112 lys2-Δ201 ura3-52 guk1-2*	This work
Y916[a]	*MAT*α *his3-Δ200 trp1::hisG leu2-3,112 lys2-Δ201 ura3-52 aah1::URA3*	This work
Y917[a]	*MAT***a** *his3-Δ200 trp1::hisG leu2-3,112 lys2-Δ201 ura3-52*	This work
Y918[a]	*MAT***a** *leu2-3,112 lys2-Δ201 ura3-52 guk1-2 aah1::URA3*	This work
pur5	*MAT***a** *pur5*	R. Woods
Y719	*MAT*α *leu2-3,112 ura3-52 pur5*	This work
L586	*MAT***a** *ade1*	G. Fink
L587	*MAT*α *ade1*	G. Fink

[a] Isogenic strains.

absorbance of NADH at 340 nm (extinction coefficient ε = 6.2 cm^2 μmol^{-1}). One unit of enzyme activity is defined as the consumption of 1 μmol of nucleoside triphosphate per minute. Protein concentration was determined using the Bio-Rad (Hercules, CA) Protein Micro Assay system with crystalline bovine serum albumin serving as the reference standard. GMP kinase activity values are the result of two independent enzymatic assays, each performed with three different protein extract concentrations.

Yeast HGPRT expression and purification: To produce yeast HGPRT, P1718 was expressed in the C41 (DE3) *Escherichia coli* strain (Miroux and Walker 1996). Yeast HGPRT was expressed and purified under native conditions using the QIAGEN (Chatsworth, CA) QIAexpressionist kit with the following modifications: in all buffers, NaH_2PO_4 was replaced by Tris, and elution buffer contained 350 mM imidazole.

Determination of yeast HGPRT kinetic parameters: HGPRT assay was done in a 50-μl mix containing [8-^{3}H]hypoxanthine (20 Ci/mmol, ICN Pharmaceuticals, Irvine, CA), 0.1 mM 5-phosphoribosyl-1-pyrophosphate, 100 mM Tris pH 8.0, 4 mM DTT, 10 mM $MgCl_2$, and 1.5 ng of purified yeast HGPRT. Initial rate measurements were performed at 30° and reactions were stopped after 90 sec. Hypoxanthine K_M value was determined using 1 to 200 μM hypoxanthine concentrations. Each reaction was stopped by adding 1 ml PRT stop buffer (50 mM Na-acetate and 2 mM Na_2HPO_4 pH 5.0) and lanthanium chloride (200 μl of 2.5 M $LaCl_3$), allowing precipitation of the nucleotide product. The samples were incubated on ice for 1 hr and the precipitate was collected on GF/C glass filters, washed 6 times with 1.5 ml cold water, and dried at 80° for 45 min. The filters were then placed in scintillation counting vials along with 5 ml Packard Filter Count scintillation liquid (complete LSC-cocktail for counting membrane filters, Packard, Meriden, CT) and counted on a TRI CARB 1500 Packard scintillation counter to determine the amount of [^{3}H]hypoxanthine converted into inosine 5′-monophosphate. To determine the K_i value for GMP, the apparent K_M ($K_{M\,app}$) for hypoxanthine in the presence of either 50 μM, 100 μM, or 200 μM GMP was assayed. The inhibition was determined to be competitive since there was no major variation of the yeast HGPRT V_{max} when increasing GMP concentration. The K_i was thus calculated using the equation

$$V = (V_{max} \times [\text{Hyp}])/(K_{M\,app} + [\text{Hyp}])$$

and

$$K_{M\,app} = K_M\,(1 + [\text{GMP}]/K_i).$$

***In vivo* [^{14}C]hypoxanthine incorporation:** Wild-type (Y350), *guk1* (220), and *hpt1* (Y508) strains were grown in 60 ml of minimal medium supplemented with 20 g/liter casamino acids, 0.2 mM uracil, and 0.2 mM tryptophan to an OD_{600} of 1.0. Cells were then harvested and resuspended in 6 ml of the same medium plus 20 μM hypoxanthine containing 1 μCi [8-^{14}C]hypoxanthine (50 mCi/mmol, ICN). Cells were allowed to grow for 15 or 90 min and then 1 ml of culture was used for extraction of the intracellular purine compounds according to Gonzalez *et al.* (1997). Separation of the purine compounds was then achieved by HPLC using a supelcosil LC-18 5-μm reversed phase column. Gradient was set up with A buffer (0.025 M K_2HPO_4) and B buffer (0.05 M K_2HPO_4, 25% methanol). The following proportions of A and B buffers, respectively (indicated in parentheses), were used at the indicated run time: 0 min (98/2), 5 min (96/4), 10 min (70/30), 20 min (20/80), 24 min (20/80), and 25 min (98/2) and the flow was 1.25 ml/min. Fractions were collected every 30 sec during the 5 first min of the run and then every minute. All fractions were adjusted with water to 1.25 ml, transferred to scintillation counting vials along with 5 ml PCS liquid scintillation cocktail (Amersham, Buckinghamshire, United King-

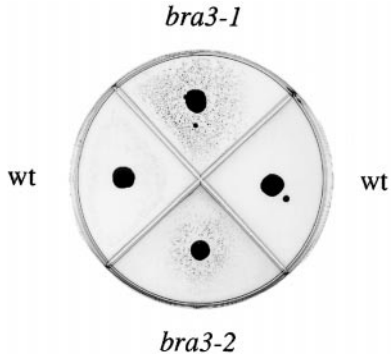

FIGURE 2.—Purine excretion by the *bra3-1* and *bra3-2* mutants. A lawn of *ade1* (L587) cells was plated on purine-free SC medium. A suspension of wild-type (PLY121) or *bra3* mutant (129 and 130) cells was dropped onto this lawn. Purine excretion was monitored after 5 days at 30°.

dom), and counted on a TRI CARB 2100TR Packard scintillation counter to determine the amount of ^{14}C radioactivity in each vial.

RESULTS

The *bra3* mutants are resistant to 8-azaguanine and excrete purines: In a previous report, we have shown that the *bra3* complementation group contains four members, two in each mating type (GUETSOVA *et al.* 1997). All four mutants in addition to their derepression phenotype showed resistance to base analogs such as 8-azaadenine (8AA) and 8-azaguanine (8AG). The *bra3-2* mutant was crossed with a wild-type strain and the meiotic products of this cross were scored for adenine repression and 8AG resistance. In 15 analyzed tetrads, we noticed a 2:2 segregation of the colony size on the germination plate (data not shown). Interestingly, all the spores forming smaller colonies were 8AG resistant and deregulated for expression of an *ADE1-lacZ* fusion (Bra^-), while all the large colonies were 8AG sensitive and Bra^+. Therefore, adenine derepression, resistance to 8AG, and a growth defect cosegregate in this cross. The *bra3-1* and *bra3-2* mutants and the isogenic PLY121 wild-type strain were grown in rich YPD medium at 30° and their generation time was measured. The *bra3-1*, *bra3-2*, and wild-type generation time during exponential growth was 148, 131, and 109 min, respectively, thus establishing that the *bra3* mutants have a slower vegetative growth. Finally, we found that the *bra3-1* and *bra3-2* mutants excrete purines in the medium, as shown by cross-feeding experiments, using a plate assay based on the rescue of an *ade1* mutant on purine-free medium. In this assay, the purine-excreting mutants are surrounded by a halo of growing *ade1* colonies. As shown in Figure 2, growth of *bra3-1* and *bra3-2* mutants on a purine-free medium sustained growth of an adenine auxotrophic strain. Apparently the *bra3-1* mutant was a more efficient purine excretor than *bra3-2*.

***BRA3* is *GUK1*:** The *BRA3* gene was cloned by complementation of the *bra3-3* derepression phenotype. This mutant was transformed with a genomic library carried on a centromeric vector. Candidates for complementation were isolated according to their ability to repress the expression of an *ADE1-lacZ* fusion in the presence of adenine. Such candidates were scored as pale blue among dark blue colonies on XGal medium plus adenine. The P131 plasmid, isolated as complementing the *bra3-3* mutant phenotype, was also shown to complement the derepression phenotype of the other three mutants of the complementation group. This plasmid was further analyzed and shown to contain a yeast DNA insert that hybridized to chromosome IV (data not shown). Sequence analysis of both termini of the yeast DNA insert revealed that this plasmid carried a 9.2-kb fragment from chromosome IV. This fragment contained five complete open reading frames (*YDR452w* to *YDR456w*), one of which (*YDR454c*) corresponded to the previously described *GUK1* gene that encodes GMP kinase, a purine metabolism enzyme catalyzing phosphorylation of GMP into GDP (KONRAD 1992). Because of its important role in purine metabolism, we then tested whether the *GUK1* gene alone could complement the derepression phenotype of *bra3-1* and *bra3-2* mutants.

A centromeric plasmid carrying a 919-bp *Hin*dIII-*Bgl*II fragment containing only the *GUK1* gene was constructed. This plasmid and a control plasmid were transformed in *bra3-1* and *bra3-2* mutants carrying an *ADE1-lacZ* fusion, and the repression by adenine of the *ADE1-lacZ* fusion expression was tested. The repression factor—defined as the ratio between expression of the *ADE1-lacZ* fusion under derepression (−ade) and repression (+ade) conditions—was calculated. Results in Table 2 show that whereas the repression factor in *bra3-1* and *bra3-2* mutants transformed with the control plasmid was very low (1.2 and 1.5, respectively), transformation of the two mutants with the plasmid carrying the *GUK1* gene re-

TABLE 2

Expression of an *ADE1-LacZ* fusion in the wild-type (PLY121) strain and in the *bra3-1* (129) and *bra3-2* (130) mutants transformed with a *CEN* plasmid carrying the *GUK1* gene

Relevant genotype	pRS315 insert	βGal units		
		−Ade	+Ade	R.F.[a]
Wild type	None	161	17	9.5
Wild type	*GUK1*	145	19	7.6
bra3-1	None	168	142	1.2
bra3-1	*GUK1*	154	19	8.1
bra3-2	None	186	125	1.5
bra3-2	*GUK1*	183	18	10.2

[a] R.F., repression factor.

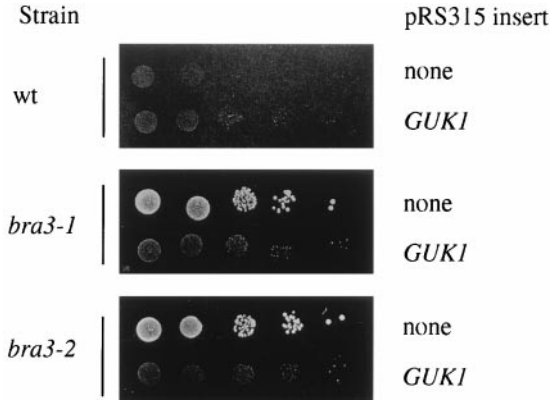

FIGURE 3.—Complementation of the *bra3* mutants' 8AG resistance by the *GUK1* gene. Strains were transformed with a control centromeric plasmid or a centromeric plasmid carrying the wild-type *GUK1* gene. A serial dilution of the different transformants was dropped onto SC medium containing 8AG and the growth on this medium was observed after 3 days at 30°.

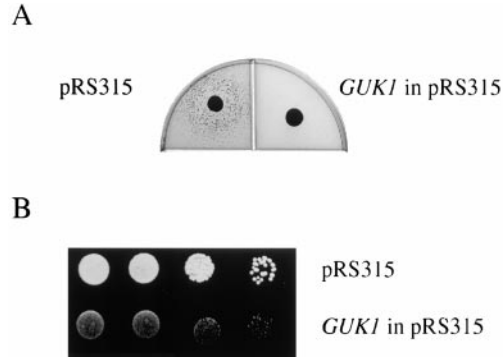

FIGURE 4.—The *pur5* phenotypes are complemented by *GUK1*. (A) A lawn of *ade1* (L587) cells was plated on purine-free SC medium. The *pur5* strain (Y719) transformed with the indicated plasmids was spotted onto the *ade1* lawn. Purine excretion was monitored after 4 days at 30°. (B) The *pur5* strain (Y719) was transformed with a control centromeric plasmid (pRS315) or a centromeric plasmid carrying the wild-type *GUK1* gene. A serial dilution of the *pur5* transformants was dropped onto SC medium containing 8AG and the growth on this medium was observed after 3 days at 30°.

stored a higher repression factor (8.1 and 10.2, respectively), similar to the wild-type level (7.6). This experiment thus clearly established that the 919-bp DNA *Hin*dIII-*Bgl*II fragment containing the *GUK1* gene was sufficient to restore adenine regulation when introduced into the *bra3-1* and *bra3-2* mutant strains. The *GUK1* gene alone was also able to fully complement the 8AG resistance of the *bra3* mutants (Figure 3).

We then tested the linkage between the *bra3* mutation and the *GUK1* gene. Since disruption of *GUK1* is lethal (KONRAD 1992), we could not use a *GUK1*-disrupted strain for linkage analysis. Therefore, we crossed the *bra3-2* mutant with a wild-type strain in which the *LEU2* marker was integrated at the *GUK1* locus (Y882, see MATERIALS AND METHODS). After sporulation of the diploid, 15 tetrads were dissected. In these tetrads, all the Leu^- spores were deregulated for expression of an *ADE1-lacZ* fusion and were 8AG resistant, while all the Leu^+ spores were wild type for these phenotypes.

These results strongly suggested that *bra3* mutants are alleles of *GUK1*, the GMP kinase encoding gene. This was further demonstrated by measuring GMP kinase activity in crude extracts from the *bra3-1* and *bra3-2* mutants. As expected, GMP kinase activity was found to be severely impaired in the *bra3* mutants compared to the isogenic wild-type strain (Table 3), while in the same experiment AMP kinase activity used as a control was not significantly affected in the *bra3* mutants. It is worth noting that the wild-type AMP and GMP kinase activity in the wild-type strain is in good agreement with those previously presented (KONRAD 1993).

TABLE 3

AMP and GMP kinase activity in crude extracts from the wild-type (PLY121) strain and the *bra3-1* (129) and *bra3-2* (130) mutants

Relevant genotype	Specific activity (units/mg protein)	
	AMP kinase[a]	GMP kinase[a]
Wild type	2.227 ± 0.270	0.369 ± 0.066
bra3-1	2.335 ± 0.445	0.018 ± 0.009
bra3-2	3.052 ± 0.755	0.032 ± 0.018

[a] Average ± standard deviation.

From all these data, we conclude that *BRA3* is *GUK1* and therefore renamed the *bra3-1*, *bra3-2*, *bra3-3*, and *bra3-4* alleles *guk1-1*, *guk1-2*, *guk1-3*, and *guk1-4*, respectively.

On the chromosome IV map, *GUK1* is located close to the previously characterized *pur5* locus (MORTIMER *et al.* 1991). This locus was initially described as associated with purine excretion, 8AA and 8AG resistance (ARMITT and WOODS 1970; WOODS *et al.* 1983). To test the possibility that *GUK1* and *PUR5* are the same gene, we transformed the *pur5* mutant strain with the wild-type *GUK1* gene and found that *GUK1* on a centromeric plasmid complemented the purine excretion (Figure 4A) and the 8AG-resistance phenotype of *pur5* (Figure 4B). Consistently, the *pur5* and *guk1-2* mutants did not complement for purine excretion and 8AG resistance (data not shown). Finally, we crossed the *pur5* mutant with the Y882 strain in which the *LEU2* marker is integrated at the *GUK1* locus. After sporulation of the diploid, 17 tetrads were dissected. In these tetrads, all the Leu^- spores were 8AG resistant, while the Leu^+ spores were 8AG sensitive (data not shown). Altogether these data show that *pur5* is an allele of the *GUK1* gene.

Utilization of adenine through the APRT route bypasses the deregulation phenotype of the *guk1* mutants: Why should a mutation in the *GUK1* gene induce a

TABLE 4

Bypass of *ADE1-LacZ* derepression in *guk1* mutants (A) by overexpression of *APT1* or (B) by combining the *guk1* mutation with an *aah1* mutation

Strain	Relevant genotype	Multicopy plasmid	βGal units		
			0[a]	+ Ade[a]	+ Hyp[a]
A.					
PLY121	Wild type	YEp13	210	44	57
PLY121	Wild type	*APT1* in YEp13	225	36	75
130	*guk1-2*	YEp13	253	142	281
130	*guk1-2*	*APT1* in YEp13	240	64	211
B.					
Y917	Wild type	—	448	97	118
Y915	*guk1-2*	—	337	235	355
Y916	*aah1*	—	413	98	99
Y918	*aah1 guk1-2*	—	317	88	310

[a] 0, +Ade, and +Hyp stand for no purine base, adenine, and hypoxanthine added to the growth medium, respectively.

deregulation of *ADE* gene expression in response to adenine? We have previously shown (Guetsova *et al.* 1997) that adenine needs to be metabolized into ADP to exert its regulatory role (see Introduction and Figure 1). Furthermore, the production of ADP from adenine preferentially occurs through the HPRT route (formation of hypoxanthine, IMP, and then AMP) rather than directly via APRT (see the Introduction section and Guetsova *et al.* 1997). We reasoned that if one of the reactions required for AMP synthesis from adenine is affected in the *guk1* mutants, overexpression of the *APT1* gene (encoding APRT) should suppress the adenine deregulation in these mutants. Indeed, the direct transformation of adenine into AMP should bypass the HPRT route (see Figure 1). Such a bypass was previously shown for a *hpt1* mutant (Guetsova *et al.* 1997). Results in Table 4A show that overexpression of *APT1* increased the regulation factor by adenine from 1.8 to 3.8 in the *guk1-2* strain. As an internal control, overexpression of *APT1* had no effect on regulation by hypoxanthine, a purine base that cannot be utilized through the APRT route (see Figure 1). This result suggests that the *guk1* mutation affects adenine regulation via the HPRT route (Aah1p, Hpt1p, Ade12p, Ade13p). Consistently, in the *guk1* mutant transformed with the control plasmid, hypoxanthine cannot repress *ADE1-lacZ* expression at all, while adenine has a weak repression effect.

This hypothesis was further tested in an independent approach using an *aah1* mutant. The *AAH1* gene, encoding adenine deaminase, participates in the transformation of adenine into AMP through the HPRT route but an *aah1* mutant is not deregulated. We hypothesized that in such a mutant, more adenine is available for Apt1p, which transforms it directly into AMP (Guetsova *et al.* 1997; see Figure 1). Thus, if the *guk1* mutation has an effect on AMP synthesis via Hpt1p, Ade12p, or Ade13p, an *aah1* mutation should suppress the adenine derepression phenotype in the *guk1* mutants. We tested this prediction by crossing an *aah1::URA3* disrupted strain (Y663) with the *guk1-2* mutant. Several tetrads were dissected from this cross and tetratypes carrying the four combinations of these two mutations were transformed with an *ADE1-lacZ* fusion to allow evaluation of the repression by adenine. Results presented in Table 4B show that both adenine and hypoxanthine efficiently repressed expression of the fusion in the wild-type and *aah1* spores. As expected, adenine and hypoxanthine, respectively, had little or no effect on expression of the *ADE1-lacZ* reporter gene in the *guk1-2* mutant. Finally, in the *aah1 guk1-2* double mutant, adenine but not hypoxanthine repressed expression of the reporter fusion. Therefore, repression by adenine is effective in the double mutant because adenine can be metabolized directly into AMP, whereas hypoxanthine, which must be transformed into AMP via the HPRT route, cannot repress the *ADE* genes. We conclude that the *guk1-2* mutation most probably affects the pathway between hypoxanthine and AMP.

***guk1* mutations phenocopy a HGPRT defect:** In the *guk1* mutants, the severe decrease in GMP kinase activity could lead to GMP accumulation, which could in turn inhibit activity of HGPRT, a key enzyme in the adenine repression process (*HPT1* is *BRA6*, Guetsova *et al.* 1997). Indeed, *guk1* and *hpt1* mutants share several phenotypes (adenine derepression, purine excretion, and 8-azaguanine resistance).

This hypothesis was tested by monitoring the effect of overexpression of *HPT1* on adenine regulation in the *guk1* mutants. Results (Figure 5A) show that overexpression of *HPT1* suppressed the deregulated phenotype of the *guk1* mutants. Consistently, overexpression of *HPT1* allowed suppression of the purine excretion phenotype of a *guk1* mutant (Figure 5B). The *guk1* mutation thus seems to affect Hpt1p activity and a simple

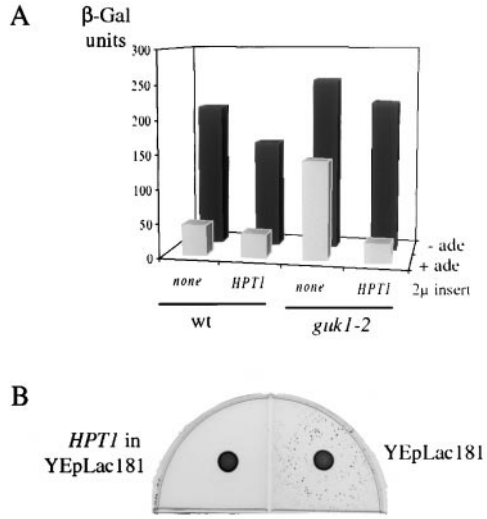

FIGURE 5.—*HPT1* overexpression suppresses the *pur5* phenotypes. (A) Expression of an *ADE1-lacZ* fusion was monitored by measuring βGal activity in the wild-type and *guk1-2* strains transformed with a control multicopy plasmid (YEpLac181) or a 2μ plasmid overexpressing *HPT1* (P556). Transformed strains were grown for 6 hr in the presence or absence of adenine as indicated. (B) A lawn of *ade1* (L586) cells was plated onto purine-free SC medium. The *pur5* strain (Y719), transformed with either a control plasmid (YEpLac181) or a plasmid overexpressing *HPT1* (P556), was spotted onto the *ade1* lawn. Purine excretion was monitored after 4 days at 30°.

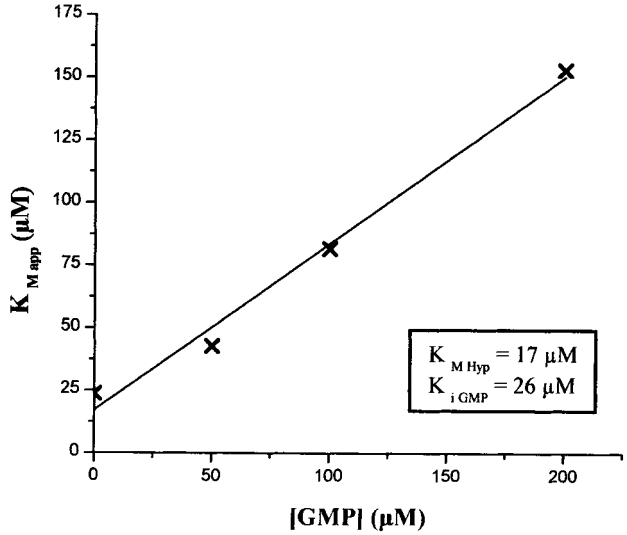

FIGURE 6.—Yeast HGPRT is feedback inhibited by GMP. A Hpt1p-His_6 fusion protein was purified from *E. coli* and assayed for hypoxanthine phosphoribosyltransferase activity. The K_M and $K_{M\ app}$ for hypoxanthine were determined in the absence of GMP and in the presence of either 50, 100, or 200 μM GMP. The K_i for GMP was then calculated as described in MATERIALS AND METHODS.

explanation would be that HGPRT activity could show feedback inhibition by GMP accumulated in the *guk1* mutants.

Feedback inhibition of HGPRT by GMP was assayed *in vitro* on a Hpt1p-His_6 protein. Hpt1p-His_6 was expressed and purified from *E. coli* and we then determined its kinetic parameters. The K_M for hypoxanthine was 17 μM, the k_{cat} was 5.2 sec^{-1}, and the estimated K_i for GMP was 26 μM (Figure 6). Therefore, GMP feedback inhibits the yeast HGPRT, further suggesting that *guk1* mutations could phenocopy a HGPRT defect. This was confirmed by assaying *in vivo* whether hypoxanthine utilization was affected in the *guk1* mutants.

First, we tested whether a double *ade2 guk1* mutant would behave like an *ade2 hpt1* mutant, *i.e.*, grow on medium supplemented with adenine but not hypoxanthine (GUETSOVA *et al.* 1997). The *guk1-2* mutant was crossed with an *ade2* isogenic strain (L4364). After sporulation of the diploid, the tetrads were dissected. Among these, a tetratype was characterized by monitoring the *ade2* mutation with adenine auxotrophy and the *guk1* mutation with 8AG resistance. Results (Figure 7A) show that a double *ade2 guk1-2* mutant could not utilize hypoxanthine as a purine source and that it required extracellular adenine for growth. This result confirms that a double *ade2 guk1-2* mutant has properties similar to an *ade2 hpt1* strain.

Second, we directly assayed whether a *guk1* mutant could incorporate radiolabeled hypoxanthine into nucleotides *in vivo*. Results presented in Figure 7B show that *guk1-3* and *hpt1* mutants accumulated radiolabeled hypoxanthine. Both mutants had difficulty converting hypoxanthine into nucleotides while the isogenic wild-type strain did not. As expected, this conversion appeared less affected in the partially inactive *guk1-3* mutant than in the *hpt1* knock-out. We conclude that the *guk1* mutations characterized in this report result in a phenocopy of a HGPRT defect.

DISCUSSION

Consequences of a GMP kinase defect in yeast: Mutants of the *bra3* complementation group were initially isolated for their ability to derepress the purine *de novo* pathway genes (GUETSOVA *et al.* 1997). Complementation and linkage data identified *BRA3* as *GUK1*, the GMP kinase gene. Here, we show that *pur5*, a previously described purine excretion mutant (ARMITT and WOODS 1970), is also allelic to *GUK1*. Mutations at the *guk1* locus lead to multiple phenotypes. Some of these are likely to be due to the impaired GDP synthesis whereas others are more probably due to the decrease in HGPRT activity resulting from GMP accumulation.

For example, the growth defect of the *guk1* mutants most probably results from the GDP starvation since *GUK1* is an essential gene (KONRAD 1992), while knock-out of *HPT1* does not lead to any vegetative growth defect (GUETSOVA *et al.* 1997). On the other hand, the purine excretion and deregulation phenotypes are likely to be due to the HGPRT defect phenocopy since both phenotypes have been reported for *hpt1* mutants

(Woods *et al.* 1983; Guetsova *et al.* 1997) and are suppressed by overexpression of *HPT1* (this work). We have found (K. Lecoq and B. Daignan-Fornier, unpublished results) that the *pur5* mutants excrete a mix of hypoxanthine, guanine, xanthine, inosine, and guanosine. Resistance of the *pur5* mutant to 8AA but not 8AG was previously shown to be abolished when purine excretion is suppressed by the *su-pur* mutation (Lomax and Woods 1973). This is interpreted as follows: excreted purines compete with 8AA for uptake and lead to an apparent resistance due to inefficient uptake of the analog. In contrast, 8AG resistance is likely to be due to the GMP kinase defect or to the HGPRT phenocopy that would impair transformation of 8AG into its toxic form.

Because guanine nucleotides are involved in multiple cellular processes, it is not surprising that GMP kinase mutants show a variety of phenotypes. Our *guk1* mutants could thus prove to be a useful tool to study the effect of GDP and GTP starvation in yeast. Interestingly, a mutation complemented by *GUK1* was recently shown to have a defect in glycosylation, most probably because of a lack in GDP-mannose (Shimma *et al.* 1998). We have found that our mutants mate and sporulate normally (data not shown) even under conditions where GDP is starting to be limiting for vegetative growth.

***guk1*: a *hpt1* phenocopy that leads to *ADE* gene derepression and purine excretion:** In this report, we show that mutations in the *GUK1* gene cause *ADE* gene derepression and purine excretion. We present several lines of evidence suggesting that the *guk1* mutations affect HGPRT, a key enzyme whose lack causes *ADE* gene derepression and purine excretion (Guetsova *et al.* 1997). We propose that *guk1* phenocopies *hpt1* as a result of HGPRT feedback inhibition by GMP, which is likely to accumulate in the GMP kinase-deficient mutants. Such a feedback inhibition is documented in this report and has already been reported for human HGPRT (Henderson *et al.* 1968).

Could a defect in human GMP kinase lead to purine overproduction and excretion as it does in yeast? It is well known that pathological purine overproduction and excretion lead to accumulation of extracellular hypoxanthine. This, in turn, is converted into uric acid, an insoluble compound that accumulates in joints and causes hyperuricemia and gout. A HGPRT defect in humans induces purine overproduction and excretion (Kelley *et al.* 1969) as it does in yeast (Woods *et al.* 1983; Guetsova *et al.* 1997). The finding that a defect in GMP kinase phenocopies a HGPRT defect may therefore have implications for human diseases. It is tempting to assume that a defect in human GMP kinase might be the primary cause of, or at least be implicated in, some cases of hyperuricemia. This assumption may have important consequences since all causes of hereditary gout are not yet identified and only two human genes encoding HGPRT and PRPP synthetase have been

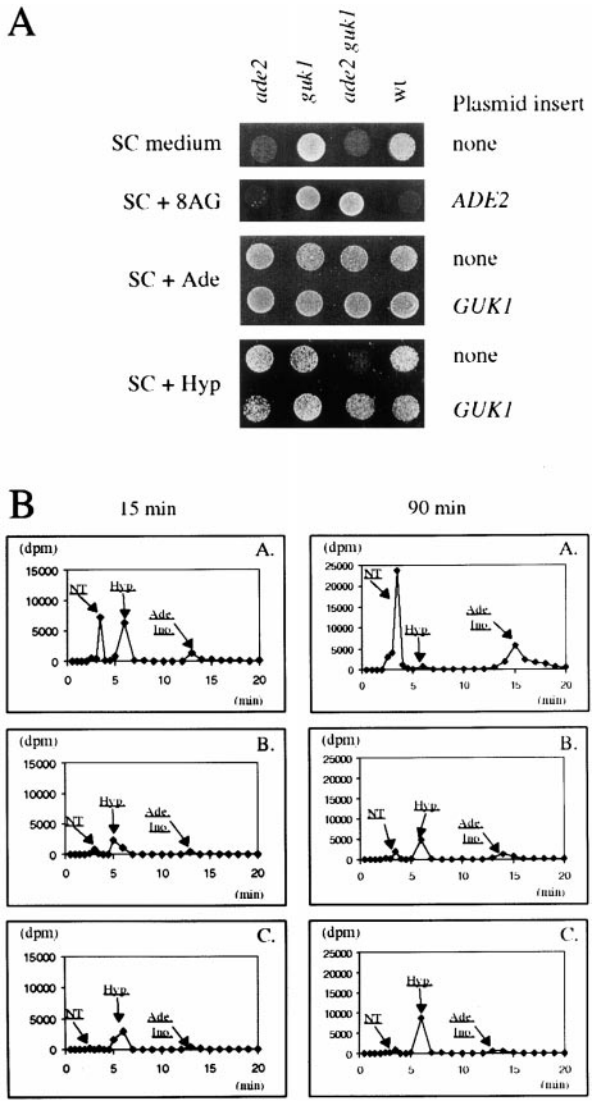

Figure 7.—*In vivo* hypoxanthine utilization is affected in the *guk1-2* mutant. (A) Growth of an *ade2 guk1-2* double mutant on various purine sources. The different strains were transformed with a *CEN* plasmid containing no insert (pRS315), the *ADE2* gene (pASZ11, Stotz and Linder 1990), or the *GUK1* gene (P195). Transformants were grown on SC medium or on the same medium supplemented with 8AG, adenine (Ade), or hypoxanthine (Hyp). Growth was monitored after 3 days. (B) [^{14}C]Hypoxanthine accumulation in the *hpt1* and *guk1* mutants. Isogenic strains that were either wild type (Y350, A), *guk1-3* (220, B), or *hpt1* (Y508, C) were first grown in minimal medium and then transferred into the same medium containing [^{14}C]hypoxanthine for either 15 min (left) or 90 min (right). Purine derivatives were extracted and separated by HPLC and each fraction was counted as described in materials and methods. Radioactivity detected in the fractions (expressed in disintegrations per minute) is presented as a function of retention time on the column. Arrows indicate the identified peaks with the following abbreviations: Ade, adenine; Hyp, hypoxanthine; Ino, inosine; NT, mix of all purine nucleotides (mono-, di-, and triphosphate).

shown to cause gout when mutated (Kelley *et al.* 1969; Becker *et al.* 1973). The hypothesis of a GMP kinase defect in families of patients suffering from hereditary gout could be tested simply by determining the level of GMP kinase activity.

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